The Bioscan 12(1): 149-153, 2017
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

BIOACTIVITY OF INTERTIDAL BLOOD CALM *CARDITA ANTIQUATA* (LAM.) FROM NORTH WEST COAST OF INDIA

PAWAN KUMAR[1]*, SATENDRA KUMAR[2] AND S. K. NAYAK[3]
[1]ICAR - Central Institute of Fisheries Education (Deemed University),
Off Yari Road, Panch Marg, Versova, Mumbai - 400 061, Maharashtra, INDIA
[2]Krishi Vigyan Kendra, Badgaon, Pala, Balaghat - 481115, Madhya Pradesh, INDIA
[3]ICAR-Central Institute for Women in Agriculture,
Bharatpur Square, Nandan Kanan, Khandagiri Road, Baramumda, Bhubaneswar - 751 003, Orissa, INDIA
e-mail: pawanfrm@gmail.com

KEYWORDS
ATPase
AChE activity
bioactive
Cardita antiquata
marine molluscs

Received on :
16.04.2015

Accepted on :
22.12.2016

***Corresponding author**

ABSTRACT
The current study is to identifiy the bioactivities of the whole body extracts from marine molluscs *Cardita antiquata*, occurring in the Mumbai coast. The crude extract from Methanol, Chloroform:Methanol (2:1) and Chloroform extract checked for different biological activity. The extracted bioactive compound was subjected to partial purification using column chromatography. The protein content of the bioactive compound and their fractions was determined using Peterson's method found that the highest protein content was in Chloroform extract. The species used here did show significant hemolytic activity on the human blood cell and moderate activity with Chloroform in chicken blood turn out to be a good pharmaceutical importance. The extracts from *C. antiquata* gave some antibacterial activity show broad spectrum activity against human pathogenic bacteria. *In-vitro* effect of neuromodulatory activity on brain of mice was found significant effective on the Na^+K^+ ATPase, Mg^{++}ATPase and AChE. Its has known for a long time that marine organisms have a whole lot of pharmacological potential to be wonderful therapeutic agents.

INTRODUCTION

Nature has been a source of medicinal agents for natural products for thousands of years. The ocean and its manifold living and non-living resources have long attracted the close attention of mankind. The rapid development of the pharmaceutical market has brought about a boom of information regarding various toxins native to the mollusks Studies on the antibacterial activity of the toxins, which would lead to the development of extremely useful pharmaceutical compounds. The marine environment is an exceptional reservoir of bioactive natural products, many of which include chemical / structural features not found in terrestrial natural product. Marine organisms have evolved biochemical and physiological mechanisms that include production of bioactive compound for such purpose as reproduction, communication, protection against prediction, infection and competition (Halvorson, 1998). Protein and polypeptides may have a greater importance in such studies. The number of natural products isolated from marine organisms increases rapidly and now exceeds 18,000 (Datta *et al.,* 2015). The phylum Mollusca is probably the third most important animal group after the arthropods and vertebrates, forming a major part of the world fauna. Although most natural medicines are derived from plants, marine invertebrate phyla, including the Mollusca, are of increasing interest as a source of novel bioactive compounds (Coates and Nairn 2014; Dolashka *et al.,* 2015; Dang *et al.*, 2015). Marine clam *Anadara granosa* showed the identification of bioactive compounds responsible for antimicrobial and anticancer activities (Ramasamy and Balasubramanian, 2012). Narayankar *et al.* (2002) reported that the physiological studies on an intertidal blood clam *Cardita antiquata* (Lam) from Mumbai coast. The extracellular hemoglobin of the false cockle (*Cardita antiquata*) was found to be considerably more resistant than the intracellular hemoglobin of the blood cockle, *Anadara granosa* (Patel and Patel, 1971; Dallas, 2013). The major work of marine toxicologists has been the search of potential pharmaceutical products. Hence, in the present study the crude extract from Methanolic, Chloroform:Methanol (2:1) and Chloroform of *Cardita antiquata* was investigated for their pharmaceutical potential for development of drugs.

MATERIALS AND METHODS

Specimen collection

The molluscs *Cardita antiquata* were handpicked from the Khardanda in Mumbai during lowest low tide. They were brought live to the laboratory, and frozen at -20°C for further use.

Extraction of crude toxin

Methanolic, Chloroform:Methanol (2:1) and Chloroform were the extracting solution that was used. The Methanolic, Chloroform:Methanol (2:1) and chloroform extract of *Cardita*

antiquata yielded a total amount of 0.185g, 0.127g and 0.112g of lyophilized crude extract respectively from approximately 25g of whole body tissue.

Extraction of crude bioactive compound

Crude toxin was extracted following the method of Braekman et al. (1989) with certain modifications. For extraction, the shells of the molluscs were broken with a hammer. About 25g of *C. antiquata* was weighed for each extraction solvent. The extraction solvent used here were Methanol, Chloroform:Methanol (2:1) and Chloroform. The samples were kept in this solvent for 3 days for the extraction process to take place. The solvent was evaporated at low pressure by using a Buchi Rotavapor R-200 at a 30ºC. The resultant compound was finally dried in a vacuum desiccator and stored at 4ºC in a refrigerator in phosphate buffer for further use as crude extracts.

Partial purification

The crude protein from *Cardita antiquata* was extracted and fractionated by method Stempein *et al*. (1970) in a Diethylaminoethyl (DEAE) Cellulose Anion Exchange Chromatography Coloum.

Protein estimation

Protein estimation of the lyophilized toxin sample was done as described by Peterson (1977) using Bovine Serum Albumin (BSA) at the rate of 1 mg/ 1ml as the of the standard ranging from 0.1 to 1 mg/1ml. The absorbance of standard and sample were read after 3 minute in a spectrophotometer at 650 nm.

Hemolytic activity

Hemolytic activity of the extracts was assayed on human erythrocyte (A, B, AB and O) Blood groups, following Micro Hemolytic Method (Pani Prasad and Venkateshvaran, 1997).

Antibacterial activity

Seven species of human pathogenic bacteria namely *Bacillus Subtilis, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Pseudomonas valgaris, Acinatobacter baumanii* and *Enterobacter aerogenes* were used to screen the antibacterial activity (Soma Roy *et al*., 2011) The antimicrobial activity of the crude extracts was assayed on selected bacterial culture using the standard disc diffusion method (Becerro *et al*., 1994, Slattery *et al*., 1995, Murugan and Ramasamy, 2003, Ram *et al*., 2012). The human pathogenic bacterial strains were grown in Nutrient broth (HiMedia) for 24 hrs before seeding the Muller Hinton agar plates. The Whatman No 1 sterilized disc of 6mm diameter, impregnated with 100 μg/ disc of the crude extracts and air dried, were placed on the Muller Hinton agar (HiMedia) plates and were incubated for 24hrs at 30ºC. The zone of inhibition was measured from the edge of the disc to the clear zone in millimeter. The antibacterial activity was assessed in triplicates.

Neuromodulatory activity

Homogenate preparation

Partial purification fraction (mitochondrial nerve endings) from male mouse (20 ± 2 gm) brain was prepared by the method of Green *et al*. (1957). Brain isolated from the male albino mouse weighing 20 ± 2 gm was homogenized in ice cold sucrose solution (0.32 M) and centrifuged (Sorvall super T – 20 Refrigerated centrifuge) at 2,500 rpm for 15 minutes at temperature 4ºC. Again, the supernatant was centrifuged at 15,000 rpm for 20 min at 4ºC temperature. Then the supernatant was discarded and the pellet dissolved in sucrose solution and again re-spun at 15,000 rpm for 20 minutes. It was washed once again in the same fashion and the resultant pellet was dissolved in the sucrose solution depending upon the pellet size and kept in deep freezer as enzyme source.

Estimation of Na^+ K^+ATPase assay

The ATPase assay was measured as described by Lowry and Lopez (1946) was followed. For total ATPase reaction mixture, 0.8 ml of Imidazole buffer (0.135 mM) with 100 mM NaCl, 20 mM KCl and 5 mM MgCl, were taken in each test tube and 0.1 ml enzyme (this quantity depends on the protein mg/hr of enzyme source) was added and stirred. For Mg^{++} ATPase reaction mixture, 0.07ml Digoxin was added as inhibitor for Na^+ K^+ ATPase in addition to the above mixture. 0.1 ml of triple distilled water was added to the total ATPase reaction mixture and 0.030 ml of triple distilled water was added to Mg^{++}ATP ase mixture to bring the reaction mixture to a total volume of 1.05 mL.

The reaction was started by adding 50 ml of ATP substrate (4.5 mM) in each tube. All the tubes were gently shaken and incubated at 37ºC for 30 minutes in a water bath. By adding 0.5 ml of 10% TCA the reaction was stopped and the content of all tubes were centrifuged and the supernatants were taken. To this supernatant, 0.3 ml of 0.1 N sodium acetate solution followed by 0.4 ml of Ammonium molybdate (1%) and H_2SO_4 (0.05 N) solution were added to each tube. The color developed was read at 800 nm in a spectrophotometer after 15 minute. Control experiments were also run simultaneously with 100 ml of triple distilled water instead of toxins.

Estimation of acetyl cholinesterase (AChE) activity

In-vitro evaluation of the toxins effect on the mouse brain AChE enzyme activity described by method Ellman *et al*. (1961). Brain isolated from the male albino mouse weighing 20 ± 2gm was homogenized 0.25 M ice cold sucrose solution and 2% (w/v) tissue homogenate was prepared in the same sucrose solution and stored in the freezer as enzyme source.

Statistical analysis

Results were expressed as mean value ± standard error (SE). All data were analyzed using analysis of variance (ANOVA) followed by student's t-test, taking p value < 0.05 was regarded as significant. All the statistical analyses were performed using MS Excel Microsoft Office 2010.

RESULTS

Protein estimation

The protein estimated in crude samples of Methanolic, Chloroform: Methanol (2:1) and Chloroform extract of *C. antiquata* was 0.135mg/ml, 0.083mg/mL and 0.242mg/mL respectively (Fig. 1). The protein content was ranged from 0.0008 mg/ml to 0.096 mg/mL in fractions of Methanolic extract, 0.0292 mg/mL to 0.0436 mg/mL in the fractions of Chloroform:Methanol (2:1) extract and 0.0084 mg/ml to 0.0391 mg/ml in the fractions of Chloroform extract (Table 1).

Table 1: Protein concentration (mg/ml) content in various fractions of the Methanol, chloroform:Methanol (2:1) and chloroform extract of *Cardita antiquata*

Molarity of solution	Methanol	Chloroform: Methanol (2:1)	Chloroform
0.1M	0.0008	0.0292	ND
0.2M	0.0020	0.0405	ND
0.3M	0.0042	0.0436	0.0084
0.4M	0.0058	ND	0.0161
0.5M	0.0096	ND	ND
0.6M	ND	ND	0.0364
0.7M	ND	ND	0.0223
0.8M	ND	ND	0.039
0.9M	ND	ND	0.0293
1.0M	ND	ND	0.0322

Table 2: Hemolytic activity of *Cardita antiquata* on chicken and human erythrocytes

Extract	Protein Esti mation (mg/ml)	Hemolytic Titer Chicken Blood	Human Blood Group A+	Human Blood Group B+	Human Blood Group AB+	Human Blood Group O+
Methanol	0.135	0	0	0	0	0
Chloroform: Methanol (2:1)	0.083	39.36	14.36	0	0	0
Chloroform	0.242	0	3.815	0	3.815	0

Table 3: Antibacterial activity of crude extracts of marine Molluscs *Cardita antiquate* by disc-diffusion method (NI: Not Indicated)

Type of extract	Zone of Inhibition (mm) B. subtilus	E. coli	S.typhi	P. aeruginosa	P. valgaris	A. baumanii	E. aerogenes
Methanol	NI	NI	NI	0.5	1.2	0.8	NI
Chloroform: Methanol (2:1)	NI	NI	0.7	1.2	1.4	0.8	0.8
Chloroform	NI	NI	1.0	0.6	1.2	0.6	NI

Table 4: *In-vitro* evaluation of the effect of the toxins from *C. antiquata* on mouse brain ATPase enzymes $Na^{2+}K^{+}$ATPase, Mg^{2+}ATPase and AChE enzyme (μM/mg protein/hour) activity

Type of extract	Na^{2+} K^{+} ATPase Activity	%Activity	Mg^{2+} ATPase activity	%Activity	AChE Activity	%Activity
Control	0.005369	-	0.00142	-	0.03260	-
Methanol	0.004126	- 44.0	0.00158	0	0.02100	76.66
Chloroform: Methanol (2:1)	0.005236	-42.8	0.00402	150	0.01236	65.05
Chloroform	0.00502	-35.48	-0.00176	-21	0.01238	56.20

Hemolytic activity

The crude was tested for the cytotoxic effect on human as well as chicken blood. The *C. antiquata* extracted in Chloroform: Methanol (2:1) showed some cytotoxic effect on chicken blood as well as human blood. As far as the human blood is concern extract were effective though positive indication was given in Chloroform:Methanol (2:1) and Chloroform extract (Table 2).

Antibacterial activity

The crude extracts at concentrations of 1mg/ml were tested against seven species human pathogenic antibacterial activities by disc diffusion assay. The Methanol extract was found effective on *P. valgaris*, *A. baumanii* and *P. aeruginosa*. The Chloroform: Methanol (2:1) extract was found effective against *S. typhi, P. aeruginosa, P. valgaris, A. baumanii* and *E. aerogenes*. Similarly the Chloroform extract was *S. typhi, P. aeruginosa*, *P. valgaris, A.* and *baumanii* (Table 3).

Neuromodulatory activity

The present results on *in-vitro* effect on $Na^{+}K^{+}$ATPase activity impact of the toxic extract on mouse brain activity did not show any remarkable activity in Methanol, Chloroform: Methanol (2:1) and Chloroform extract. Effect on Mg^{++}ATPase % activity shown by crude extract in Chloroform:Methanol showed remarkable activity**,** while other extracted did not show any activity. In case of AChEase % activity was found all type of extract showed approximately >50% AChEase activity (Table 4).

DISCUSSION

Protein estimation

The present investigation found that the highest protein content was in Chloroform extract. The protein estimated in crude samples of Methanolic, Chloroform: Methanol (2:1) and chloroform extract has more protein concentration was 0.0135mg/mL, 0.0083 mg/mL and 0.242mg/mL respectively**.** Kumar *et al.* (2014) have showed protein content 432.5 μg/mL from the *Conus lentiginosus*.Thangaraj and Brgadeeswaran (2012) reported protein content of extracted sea anemone

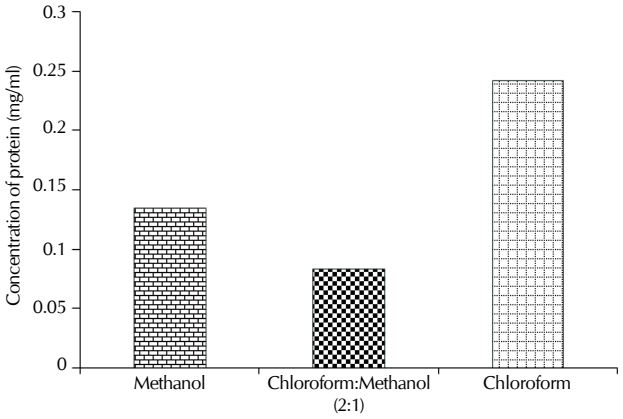

Figure 1: Protein content in crude extracts of *Cardita antiquate*

Stichodactyla mertensii was 2.10 μg/mL and *S. giganta* was 1.87 μg/mL.

Hemolytic activity

The advantage of use red blood cells in the study of hemolysis is that the loss of heamoglobin can be easily detected (Shier, 1988). The species used here did show significant hemolytic activity on the human blood cell though *C. antiquata* showed moderate activity with Chloroform in chicken blood. The hemolytic activity is reported to be the most common biological activity and the modes of action of these heamolysin have been described by (Long and Burnett, 1989). The potent hemolytic activity was studied in *Conus monile,* belonging to Gastropoda class (Nallathambi, 1993; Ramu, 1993; Sakthivel, 1999). There were no earlier reports of any hemolytic activity done on *Cardita* species. Since, molluscs sample show no hemolytic or cytolytic activity on human blood groups, if turn out to be a good pharmaceutical importance, then these samples are safer to be directly injected intravenously.

Antibacterial activity

The extracts from *C. antiquata* gave some antibacterial activity but that was not very significant when compared to the antibacterial activity of the commercially available Streptomycin. The whole body extract of *Cerithidea cingulata* and *Hemifusus pugilinus* showed a range of up to 1.5mm against 9 pathogenic bacteria (Rajaganapathi, 1996). More or less similar results are obtained in present work. The methanolic: water (1:1) extract of whole body extracts of *Nerita albicilla and Nerita oryzarum* showed broad spectrum activity against 93% and 95% of the biofilm bacteria (Santhana and Muruganas, 2005). Previous reports indicated broad spectrum activity against the human pathogenic bacteria, the extracts of bivalves seem to be ineffective or inhibit fewer biofilm bacteria. Gastropoda showed better activity when compared to bivalves and cephalopoda (Santhana and Muruganas, 2005). The result of this work also agrees with the earlier work on molluscs species *i.e.* they show broad spectrum activity against human pathogenic bacteria. *C. antiquata* showed better activity.

Neuromodulatory activity

All the three extract of *Cardita antiquata* had either a stimulatory or inhibitory effect on the neural system of mice. The chloroform : methanol extract shows the highest percentage of Mg^{++}ATPase activity and $Na^{+}K^{+}$ ATPase activity did not show any activity in all the extract. In this study observed that AChE activity in mice brain tissue has significant variation. Malarvannan (2002) revealed that crude toxin of *Protonibea diacanthus, Otolithoides biauritus* and *Muraesox talabonoides* is reported to elevate the Mg^{++} ATPase activity. It is also reported that the horseshoe crab extracts enhanced the mouse brain Mg^{++} ATPase (Wankhede, 1996). Present findings showed that the toxins affected the neural system of mice whereas, $Na^{+}K^{+}$ ATPase activity has been reported to be inhibited by paramyosin from the squid *Todarodes pacificus* (Konno et al. 1988) and by the cell extracts of the brain shrimp *Artemia salina* (Morohashi, 1991). Tetrodotxins inhibitory effects on Na^{+} K^{+} ATPase activity is well documented (Elancheran 1994). The observed result indicate the biopharmaceutical potential of the extracts which may have implication in the treatment of CNS disorders such as epilepsy and paralysis.

REFERENCES

Becerro, M. A., Lopez, N. I., Turon, X. and Uniz, M. J. 1994. Antimicrobial activity and surface bacterial film in marine sponges. *J. Exp. Mar. Biol. Ecol.* **179:** 195-205.

Braekman, J. C., Daloze, D., Moussiaux, B., Stoller, C. and Deneubourg, F. 1989. Sponge secondary metabolites: New results. *Pure Appl. Chem.* **61:** 509-512.

Coates, C. J. and Nairn, J. 2014. Diverse immune functions of hemocyanins. *Devel. and Compar. Immun.* **45(1):** 43-55.

Dallas, L. J. 2013. An ecotoxicological assessment of the impacts of chronic exposure to metals and radionuclides on marine mussels: relating genotoxicity to molecular and organism-level effects. *Ph.D thesis. Plymouth University.* p. 367.

Dang, V. T., Benkendorff, K., Green, T. and Speck, P. 2015. Marine snails and slugs: a great place to look for antiviral drugs. *J. Virol.* **89(16):** 8114-8118.

Datta, D., Talapatra S. N. and Swarnakar, S. 2015. Bioactive compounds from marine invertebrates for potential medicines - An overview. *Int. Letters of Natural Sci.* **7:** 42-61.

Dolashka, P., Dolashki, A., Velkova, L., Stevanovic, S., Molin, L., Traldi, P., Velikova, R. and Voelter, W. 2015. Bioactive compounds isolated from garden snails. *J. BioSci. Biotechnol.* pp. 147-155.

Elancheran, P. 1994. Studies on toxins of certain marine fishes. *Ph. D. Thesis, Central Institute of Fisheries Education,* Mumbai, p. 55.

Ellman, G. L, Courtney, K. D., Andres, V. Jr. and Feather-Stone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* **7:** 88-95.

Green, D. E., Lester, R. L. and Ziegler, D. M. 1957. Studies on the mechanism of oxidative phosphorylation. Preparation and properties of a phosphorylating electron transfer particle from beef heart mitochondria. *Biochem. Biophy. Acta.* **23(3):** 516-524.

Halvorson, H. O. 1998. Aquaculture, marine sciences and oceanography: A confluence connection. *New England's J. Hig. Edu. Eco. Develop.* **13:** 28-42.

Konno, K., Matsuura, M., Fujita, M., Miyagishima, Y. and Arai, K. 1988. Inhibitory effect of squid (*Todarodes pacificus*) paramyosin on actomyosin ATPase and on superprecipitation. *Comp. Biochem. Physiol. B.* **90(4):** 795-801.

Kumar, P., Venkateshvaran, K., Srivastava, P. P., Nayak, S. K., Shivaprakash, S. M. and Chakraborty, S. K. 2014. Pharmacological studies on the venom of the marine snail *Conus lentiginosus* Reeve,

1844. *Int. J. Fish. Aqu. Stu.* **1(3):** 79-85.

Long, K. O. and Burnett, J. 1989. Isolation characterization and comparison of hemolytic peptide in nematocyst venom of two species of jellyfish (*C. quinquecrrha* and *C. capillata*). *Com. Biochem. Hyiol.* **948(4):** 641-646.

Lowry, O. H. and Lopez, J . A. 1946. The determination of inorganic phosphate in the presence of labile phosphate esters. *J. Biol. Chem.* **162:** 421-428.

Malarvannan, G. 2002. Ichthyootoxins from marine carnivorous fishes and their biomedical appications. *Ph.D. Thesis. Annamalai University. India.*

Morohashi, M., Tsuchiya, K., Mita, T. and Kawamura, M. 1991. Identification of (Na,K) ATPase inhibitor in brine shrimp, *Artemia salina*, as long-chain fatty acids. *J. Comp. Physio.* B. **161(1):** 69-72.

Murugan, A. and Ramasamy, M. S. 2003. Biofouling deterrent natural product from the ascidian Distaplia nathensis. *Indian J. Mar. Sci.* **32:** 162-164.

Nallathambi, T. 1993. Studies on the venom of *Conus betulinus* Linnnaeus (Mollusca: Gastropoda) from the Southeast coast of India. Published Ph. *D. Thesis. Annamalai University,* India. **31:** 1124-1132.

Narayankar, M., Kulkarni, B. G. and A. Jaiswar, 2002. Physiological studies on an intertidal blood clam *Cardita antiquata* (Lam) of Mumbai coast. *In proceedings of The National Seminar on Creeks, Estuaries and Mangroves-Pollution and Conservation.* pp. 162-166.

Pani Prasad, K. and Venkateshvaran, K. 1997. Microhaemolytic Assay, In: Training manual on Advance techniques in marine biotoxinology, *Central Institute of Fisheries Education,* India. pp. 41-42.

Patel, S. and Patel, B. 1971. Effect of ionizing radiation on haemoglobin of marine lamellibranchs. *Mar. Biol.* **10(3):** 272-279.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al., which is more generally applicable. *Ana. Biochem.* **83:** 346-356.

Rajaganapathi, J. 1996. Studies on antibacterial activity of marine molluscs. *M.Sc Thesis Annamalai University, Parangipettai.* India. p.43.

Ram, G. D., Verma, D. K. and Yadav, I. 2012. Evaluation of the antimicrobial activity of crude herbal extracts against different microorganisms. *The Bioscan.* **7(2):** 263-266.

Ramasamy, M. and Balasubramanian, U. 2012. Identification of bioactive compounds and antimicrobial activity of marine calm *Anadara granosa* (Linn). *Int. J. Science and Nature*. **3(2):** 263-266

Ramu, Y. D. 1993. Investigations on the Biology, Biochemical and Pharamacological Properties of the Venomous Marine Snail *Conus amadis* Gmelin (Mollusca: Gastropoda) from the South East Coast of India. *Published Ph.D. Thesis, Annamalai University,* India.

Sakthivel, A. 1999. Biomedicinal activity of *Conus lentiginosus* and *Conus mutabilis* from Mumbai coast. *Published M.F.Sc. Dissertation, Central Institute of Fisheries Education,* Mumbai, India.

Santhana, R. M. and Muruganas, A. 2005. Potential antimicrobial activity of marine molluscs from tuticorin, southeast coast of India against 40 biofilm bacteria. *J. Shell Fish. Res.* **24(1):** 243-252.

Shier, W. T. 1988. Cytotoxic Effect of Marine Toxins and venoms.in: Handbook of Natural Toxins. Vol. 3. Marine Toxins and Venoms, Anthony T. Tu. (ed.), Marcel and Dekker Inc, Newyork, U.S.A. pp. 477-486.

Slattery, M., Mc Clintock, J. B. and Heine, J. N. 1995. Chemical defenses in antarctic soft corals: Evidence for antifouling compounds. *J. Exp. Mar. Bio. Ecol.* **190:** 61-77.

Soma Roy, Sudipto Roy, Sunita Dutta and Abhijit Dutta. 2011. Bacteriological analysis of post monsoon water samples from selected areas of Ranchi (Jharkhand). *The Bioscan.* **6(1):** 107-109.

Stempein, M. F., Ruggieri, G. D., Negrelli, R. F. and Cecil, J. T. 1970. In "food drugs from the sea, proceeding 1969" (H.W.Youngken, jr., ed), *Marine technology Society*, Washington, D.C., p. 295.

Thangaraj, S. and Bragdeeswaran, S. 2012. Assessment of biomedical and pharmacological activities of sea anemones *Stichodactyla mertensii* and *Stichodactyla gigantea* from Gulf of Mannar Biosphere Reserve, southeast coast of India. *The J. Ven. Ani. Toxins Trop. Dis.* **18(1):** 53-61.

Wankhede, M. 1996. Neuroinhabitory activity of fishes bile and ovarian extracts of the horseshoe crab, M.F.Sc Dissertation. *Central Institute of Fisheries Education*, Mumbai. India. p. 58.