

Isolation And Biological Properties Of Neurotoxin From Sea Anemone (*Stichodactyla mertensii*, *S. haddoni*)

A Veeruraj, M Arumugam, T Ajithkumar, T Balasubramanian

Citation

A Veeruraj, M Arumugam, T Ajithkumar, T Balasubramanian. *Isolation And Biological Properties Of Neurotoxin From Sea Anemone (Stichodactyla mertensii, S. haddoni)*. The Internet Journal of Toxicology. 2007 Volume 5 Number 2.

Abstract

Neurotoxic proteins obtained from sea anemones *Stichodactyla mertensii* and *S. haddoni* were assessed for neurotoxic activity of the isolated venom and several bioassays were carried out, and the effect of toxin was determined in vivo using sea shore crabs (*Ocyropsis macrocera*) the dose causing 50% lethality (LD₅₀). The LD₅₀ of these two crude extracts was obtained as 0.47 mg/kg and 0.71 mg/kg of crude toxic protein was used. The Brine shrimp lethality (LC₅₀) bioassay was performed with *Artemia salina* nauplii. The LC₅₀ of the *S. mertensii* showed higher cytotoxicity (LC₅₀ = 0.625 mg/ml) than that of *S. haddoni* (LC₅₀ = 0.852 mg/ml). The protein patterns of isolated toxin were also analyzed by SDS-PAGE with an apparent molecular weight of 216 kDa (SmNt1), 193 kDa (SmNt2), 95 kDa (SmNt3), 60 kDa (SmNt4) & 46 kDa (SmNt5) and 103 kDa (ShNt1), 82 kDa (ShNt2) & 63 kDa (ShNt3). Neurotoxic activity was estimated by injecting a small amount of the crude toxic protein into the third walking leg of a crab and observed the typical convulsions, paralysis, color changes and death provoked by neurotoxins. Hemolytic activity was also estimated as 13 mg/ml, 7 mg/ml in *S. mertensii*, *S. haddoni* respectively. The hemolytic values were estimated as 2.46, 4.92, 27.38 & 9.85 HT/mg and 2.29, 4.57, 18.29 and 9.14 HT/mg respectively in chicken, cow, goat and human erythrocytes.

INTRODUCTION

Sea anemones possess toxins which are poisonous substances which are produced by nematocyst. Cnidarians produce polypeptide toxins which function as Neurotoxin or Cytotoxin. Sea anemones are rich sources of pore forming toxins, which are used for prey acquisition or may act as chemical signals by repelling predators. Cytolytic toxins are secreted by intact contracting sea anemones on mechanical stimulation and four major groups of cytolytic toxins from a number of sea anemones have been characterized extensively.

The most widely studied cytotoxic and cytolytic protein from sea anemone is equinatoxin II (EqT II), a basic protein from *Actinia equina*. It is toxic when administered intravenously to rats and causes lysis of blood cells in vitro [1, 2]. Therefore, it is expected that some biologically potent activities reported in other species of sea anemones are present in extract from *Stichodactyla mertensii* and *S. haddoni*. Hence the present study was aimed at determining the biological properties of neurotoxin from two species of sea anemones *Stichodactyla mertensii* and *S. haddoni*.

MATERIALS AND METHODS

SAMPLE COLLECTION

The specimens were collected from Coral reefs at Mandapam near Kurusadai island (78° 11' to 79° 15' E longitude and from 8° 49' to 9° 15' N latitude) in the Gulf of Mannar, Tamilnadu, India. The animals were brought to the laboratory as live and immediately washed, kept in sterile clean saline water and stored at -20 °C until further use.

PREPARATION OF THE CRUDE EXTRACT

The crude extract was collected according to the standard methods [3]. Briefly 100 g of the freeze-dried animal pieces was homogenized in an electrically driven tissue grinder/blender for 5 min, the homogenate was dissolved in 100 ml normal saline (0.9% NaCl) and this was centrifuged at 10,000g for 10 min. Finally, the supernatant was collected into sterile test tubes. The desired test concentrations of the extract were always prepared from this stock by serial dilution.

ESTIMATION OF THE PROTEIN CONTENT OF THE CRUDE EXTRACT

Amount of protein in the samples were estimated according to the method of [4] with bovine serum albumin was used as a standard.

SDS-PAGE ELECTROPHORESIS

Molecular weights of the partially purified active fractions of crude extracts were analyzed by SDS-PAGE [5]. Running gels contained 10.0% acrylamide, and electrophoresis was conducted at 50 V for 3 h in Tris-glycine buffer, maintained the pH 8.3. After run, gel was stained with 0.3% Coomassie blue(R-250) and their molecular weights were determined in gel documentation system (Lark, India) by comparison with protein Standards (GeNei™) Myosin, Rabbit Muscle 205 kDa; Phosphorylase b 97 kDa; Bovine Serum Albumin 66 kDa; Ovalbumin 43 kDa; Carbonic anhydrase 29 kDa.

TOXICITY TEST

The acute toxicity study was carried out for the isolated toxin extract using adult sea shore crabs *Ocypode macrocera* (10–20 g total body weight). This assay was carried out by the injection of 0.1 ml of the crude extract into the third walking leg of the shore crabs. To determine the LD50, ten crabs were injected with dilutions of the extract (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) and observed for 2 hrs. Triplicates of each concentration were used. The LD50 was obtained by the Lehman method [6]. The crude extract, which displayed activity at the sodium-channel, can be already detected by this test. Positive reactions were observed as tetanic contractions of the extremities of the shore crab. The LD50 of these extracts were calculated graphically using standard method [7].

CYTOTOXICITY TEST

The cytotoxicity assay was evaluated using the standard method of [8] with *Artemia salina* nauplii (24 h post hatching). Fifty organisms were used for each concentration of crude extract, which were 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/ml subjected to 24 h exposures. Triplicates of each concentration were used for assessed the toxicity [9].

HEMOLYTIC ACTIVITY

The hemolytic activity of the crude toxin was assessed by the micro hemolytic method [10]. Chicken, Cow, Goat and Human blood (A1+ve) was collected from slaughter house and Government hospital, Parangipettai, Tamilnadu, with EDTA solution (2.7g/ 100 ml) as anticoagulant and brought to the laboratory. The blood was centrifuged at 5,000 rpm for 5 minutes; the supernatant are discarded, the pellet was suspended in normal saline (pH 7.4). The mixture was further centrifuged at 5,000 rpm for 5 min, the supernatant was discarded and pellet resuspended in normal saline (pH 7.4). This procedure is repeated thrice. From these, 1%

erythrocyte suspension was prepared by adding 99 ml normal saline to 1ml of packed RBC.

The micro hemolytic test was performed in 96 well 'U' bottom microtitre plates. Different rows were selected for these different blood samples. Serial dilution of the crude toxin were made in 100 µl then also discarded. 100µl of 1% RBC was added into all the wells with appropriate controls were included in the test. 1% RBC suspension, 100 µl of distilled water was added, which served as a positive control and 100 µl of normal saline, which served as, negative control. The plate was gently shaken and allowed to stand for three hour at room temperature and the results were recorded. Uniform red color suspension in the wells considered as positive hemolysis and a button formation in the bottom of the wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude extract showing the hemolytic pattern was taken as one Hemolytic Unit (HU).

RESULTS

ESTIMATION OF THE PROTEIN CONTENT OF THE CRUDE EXTRACT

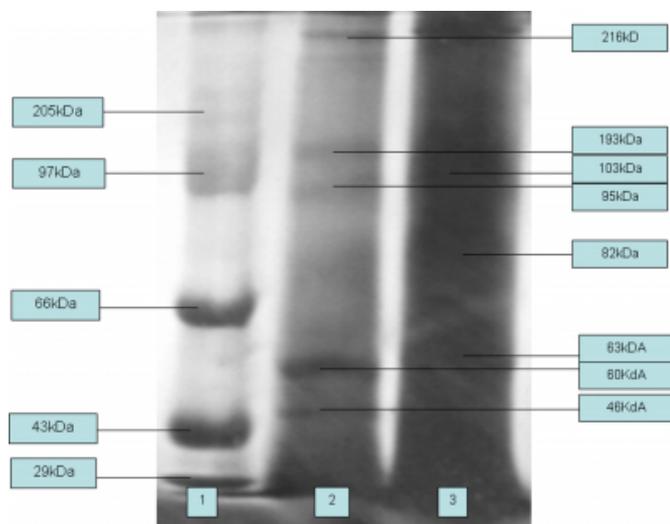
A total of 30 g of crude extracts were obtained from the samples. The protein concentration of crude was 130 µg/mg and 70 µg/mg respectively.

SDS-PAGE ELECTROPHORESIS

The protein patterns and molecular weight of the crude extracts of *S. mertensii* and *S. haddoni* were analyzed. The molecular weight was estimated as 216 kDa (SmNt1), 193 kDa (SmNt2), 95 kDa (SmNt3), 60 kDa (SmNt4) & 46 kDa (SmNt5) and 103 kDa (ShNt1), 82 kDa (ShNt2) & 63 kDa (ShNt3) (Fig.1) respectively.

Figure 1

Figure 1: SDS-PAGE analysis of crude venom from *S. mertensii* and *S. haddoni*. Purified Protein Standards (GeNei) fractions. A 10% polyacrylamide mini-slab gel was prepared, and 10 µl of each sample was injected onto each well. Lanes: 1. Protein Standards (GeNei); 2. *S. mertensii*; 3. *S. haddoni*.



TOXICITY TEST

The results showed that crude extract of both species (*S. mertensii* and *S. haddoni*) produced an evident biological activity on LD 50 test used crabs (*Ocypode macrocera*). After injection of crude extract (1 mg/ml) into their third walking leg, strong concentration of the walking appendages was observed followed by intense spasmodic movement. The legs turned tremble with involuntary lateral movements, shivering and stiffness appendages, change in carapace color, complete loss of control and paralysis. Mortality was observed in 30 sec., at 0.8 mg/ml, the crabs died within 5 min. After injections with 0.6mg/ml crabs died within 15 min. and 0.4 mg/ml crabs died within 25 min respectively. Over dose range of 0.4-1.0 mg/ml all crabs showed spasmodic movements. The LD 50 for the two crude extract was obtained with 0.47 mg/kg and 0.71 mg/kg of toxin protein was used.

CYTOTOXIC TEST

The bioassay results (*Artemia salina* nauplii) were represented in fig.2 and 3. Both sea anemone extracts displayed toxicity, with LC50 <1mg/ml and they showed significant cytotoxicity (LC 50 = 0.65mg/ml) and (LC 50 = 0.90mg/ml).

Figure 2

Figure 2: Shown the bioassay of crude toxin of brine shrimp lethality

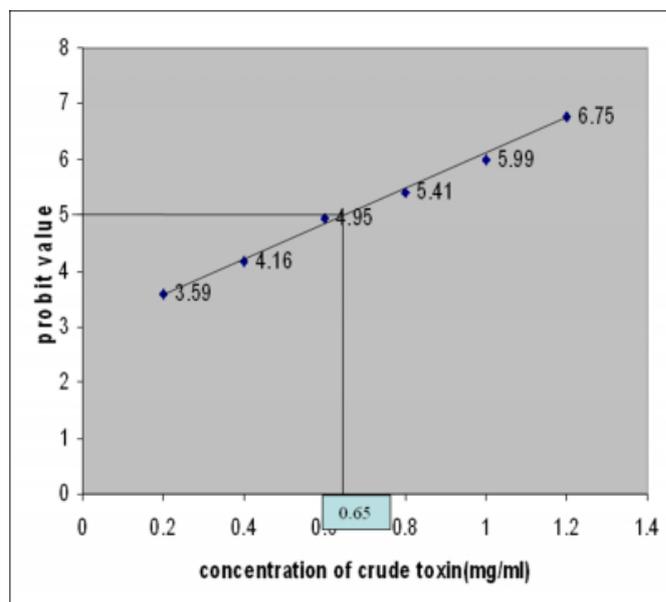
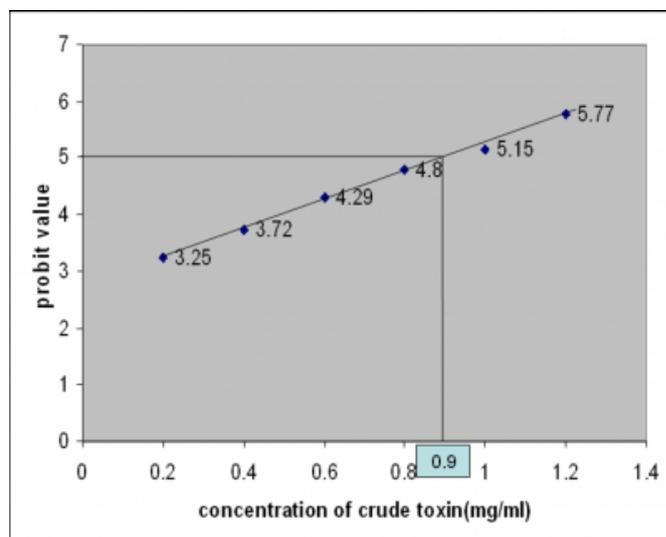


Figure 3

Figure 3: Shown the bioassay of crude toxin of brine shrimp lethality



HEMOLYTIC ACTIVITY

The hemolytic assay of sea anemones was represented in fig.4 and 5. The *S. mertensii* shown the maximum hemolytic activity in goat erythrocyte (10^{-8}), the minimum hemolytic activity in chicken erythrocyte (10^{-5}). The medium hemolytic activity was observed in cow and human erythrocytes (10^{-6} & 10^{-7}). The *S. haddoni* shown the maximum hemolytic activity in goat erythrocyte (10^{-7}) and minimum hemolytic activity was observed in chicken

erythrocyte (10^{-4}). The medium hemolytic activity was found in cow and human erythrocytes (10^{-5} & 10^{-6}).

Figure 4

Figure 4: Shown the hemolytic activity of .

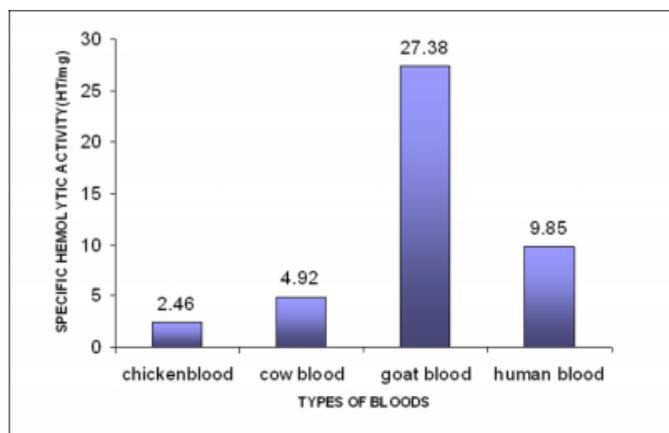
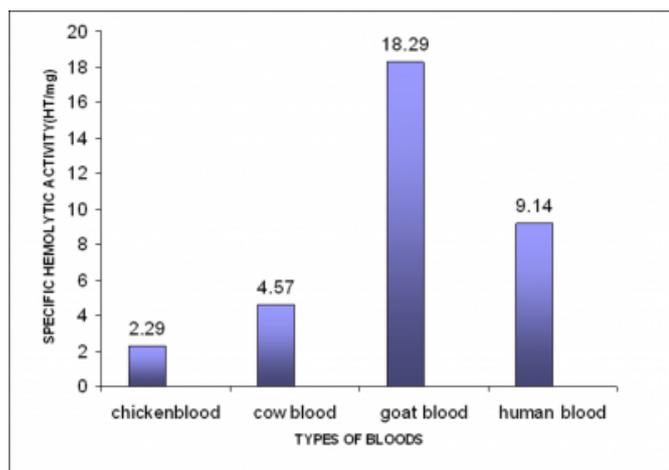


Figure 5

Figure 5: Shown the hemolytic activity of



DISCUSSION

In the last two decade, the importance of the biologically active compounds present in the venom of different marine organisms has become evident. The study of these compounds has permitted an improved understanding in the fields of pharmaceutical, neural, and biological sciences, and has allowed for the development of novel drugs.

It is known that the toxic compounds in sea anemones are proteins and it is possible to determine their structural properties. Previously, a toxic protein from the sea anemone *Radianthus macrodactylus* with the molecular weight of 20 kDa [11]; further two neurotoxins isolated from the sea anemone *Lebrunia danae* with molecular weights of 62.5 and

58 kDa [12]. In the case of *S. mertensii* and *S. haddoni* having three types of neurotoxins with the molecular weight of 216 kDa for (SmNt1), 193 kDa (SmNt2), 95 kDa (SmNt3), 60 kDa (SmNt4) & 46 kDa (SmNt5) and 103 kDa (ShNt1), 82 kDa (ShNt2) & 63 kDa (ShNt3). The presence toxins *S. mertensii* and *S. haddoni* having higher molecular weight than that of *R. macrodactylus* and *L. danae* toxins. This type of action mechanism in ion channels will be examined in the future.

In earlier demonstrated that the three different kinds of nematocysts such as Microbasic amastigophores, Macrobasic amastigophores and Spirocysts [13]. Nematocysts can be discharged the toxin using chemical or mechanical stimuli. The nematocyst immediately contact with a victim in order to cause pain, urtications, paralysis or death [14].

The hemolytic activities of these sea anemones are comparatively significant with earlier findings [15]; Hemolytic activities of *Heteractis magnifica* [16, 17] are indicating the cytolytic activity of venom through pore-formation onto biological membranes and this has amply been demonstrated. In the present study the extensive level of membrane breakdown in the various tissues. The *S. mertensii* and *S. haddoni* toxins shown the hemolytic activity was observed in all erythrocytes in the goat, human, cow and chicken (27.38, 9.85, 4.92 and 2.46 HT/mg) & (18.29, 9.14, 4.57 and 2.29 HT/mg) respectively. Hemolytic factors are probable among the active agent present in these sea anemone extract.

The acute toxicity study shows a dose- mortality relationship which was apparently sigmoidal condition. A Probit values (% mortality) of extract gave a straight line from which the LD50 was extrapolated. In the present investigation of neurotoxicity test for crude extract from *S. mertensii* and *S. haddoni* of sea shore crabs (*O. macrocera*) show the LD50 value was about 0.47 mg/kg and 0.71 mg/kg of protein. This assay was documented of first time by who used this assay as a standard method to characterize biological activity and physiological effects of this neurotoxin produced in the organism [18]. The neurotoxic effects are convulsions, paralysis and death. We have been isolated and purified a neurotoxin and it was extremely active. These neurotoxic polypeptides affect the muscular cells producing contractions on the crab's legs. Before that the jellyfish classified as highly toxic are *Physalia*, LD50 0.7mg/kg, mice [19] and *Stomolophus meleagris* 0.85 mg/kg, mice [20].

The present results indicate that the *S. mertensii* and *S. haddoni* possesses cytotoxic and cytolytic activities which is responsible for various protein pattern like, (SmNt1), (SmNt2), (SmNt3), (SmNt4) & (SmNt5) and (ShNt1), (ShNt2) & (ShNt3) isolated from this extract is a potent cytolytic with moderate cytotoxic activity. The LC 50 for *S. mertensii* and *S. haddoni* crude toxin was showed strong cytotoxicity (LC 50 = 0.65 mg/ml) and (LC 50 = 0.90 mg/ml). So compared with previous report on LC 50 for *L. danae* crude extract was 2.82 mg/ml and whereas for *Anthopleura elegantissima* the LC 50 was 5.78 mg/ml, therefore, *S. mertensii* and *S. haddoni* has more potent cytotoxic compounds than that of *L. danae* and *Anthopleura elegantissima*. Sea anemones are known to contain mainly polypeptides and proteins which account for most of their pharmacological properties [21, 22, 23, 24]. Crude extracts and partially or fully characterized toxins have been tested for in vitro and in vivo antitumour and cytotoxic activities [25, 26, 27]. The cytotoxic effects may difference due to cell selective or toxin spatial selectivity (28).

For the forgoing account, the tropical sea anemone *S. mertensii* and *S. haddoni* have an abundant source of biological active substances, which may be used as tool to study molecular organization of biological, cytotoxic, neurotoxic, cytolytic activities and model membranes as well as of sodium and potassium channels.

References

1. Komatsu S., Furukawa K., Abe K., Hirano H., Ueda M., 1992, Isolation and characterization of equinatoxins from the sea anemone *Actinia equina* L. *Chem Pharm Bull.* 40, 2873-2875.
2. Sket D., Drašlar K., Ferlan I., Lebez D., Equinatoxin, 1974, A lethal protein from *Actinia equina*. II. Pathophysiological action. *Toxicon.* 12, 53-68.
3. Walker M. J.A., 1977, Pharmacological and biochemical properties of a toxin-containing material from the jellyfish, *Cyanea caillata*. *Toxicon.* 15, 3-14.
4. Lowry O.H., Rosebrough A.L., Farr A.L., and Randall R.J., 1951, *J. Biol. Chem.* 193, 265-275.
5. Laemmli UK., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
6. Lehman A.L., 1980, Dosis Letal o Efectiva Media: Calculo manual facily rapido. *Arch Far Toxicol* 6, 161-178.
7. Miller L.C. and Tainter M.L., 1944, Estimation of the LD50 and its error by means of logarithmic probit graph paper 24, 839-840
8. Meyer B.N., Ferrigni N.R., Putnam J.E., Jacobsen L.B., Nichols D.E., McLaughlin J.L., 1982, Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med.* May, 45(5), 31-34.
9. Wah, S.T., 1993, Toxicity testing using the brine shrimp: *Artemia salina*. In: Colegate SM, Molyneux RJ (Eds) *Bioactive natural products, detection, isolation and structural determination.* CRC London, pp. 441-456.
10. Paniprasad K., Venkateshwaran K., 1997, Micro hemolytic assay, p.41-42. in: training manual on advanced techniques in marine biotechnology, K.Venkateshwaran, and K. Paniprasad, Ed's. cas in fishery science central institute of fisheries education. India: 73 p.
11. Monastyrnaya M.M., Zykova T.A., Apalikova O.V., Shwets T.V., Kozlovskaya E.P., 2002, Biological active polypeptides from the tropical sea anemone *Radianthus macrodactylus*. *Toxicon.* 40, 1197-1217.
12. Sanchez-Rodriguez J., Karina Cruz-Vazquez., 2006, Isolation and biological characterization of neurotoxic compounds from the sea anemone *Lebrunia danae* (Duchassaing and Michelotti, 1860): *Arch Toxicol.* 80, 436-441
13. Kem W.R., Parten B., Pennington M.W., Dunn B.M., Price D., 1989, Isolation and characterization, and amino acid sequence of a polypeptide neurotoxin occurring in the sea anemone *Stichodactyla helianthus*. *Biochemistry.* 28, 3483-3489.
14. Rottini G.D., Dobrina A., Forgiarini O., Nardon E., Almirante G., Patriarca P., 1990, Purification and properties of a cytolytic toxin in venom of the jellyfish *Carybdea marsupialis*. *Toxicon.* 33, 315-326
15. Elyakov G.B., Kozlovskaya E.P., 1995, Sea anemone neurotoxins investigative tools and therapeutics. Abstracted in *Toxicon.* 33(6), 715
16. Mebs D., Yangisawa M., Samejima Y., 1995, Amino acids sequence of two cytolytic sea anemone toxins. Abstracted in *Toxicon.* 33(3), 293
17. Karthikayalu S., Dharani G., Kirubakaran R., Venkatesan R., Yogeewaran G., Ravindran M., 2004, Isolation, Purification and Characterization of Haemolytic toxin from *Heteractis magnifica*. Proceeding of MBR 2004 National Seminar on New Frontiers in Marine Bioscience Research, pp 17-24
18. Beress L., Zwick J., 1980, Purification of two crab-paralyzing polypeptides from the sea anemone *Bolocera tuediae*. *Marine Chem.* 8, 333-338
19. Garriott J. C., Lane C. E., 1969, some autonomic effects of physalia toxin. *Toxicon.* 6, 281-285.
20. Toom, P.M. and Chan, D.S. (1972) preliminary studies of nematocysts from the jellyfish- *Stomolophus meleagris*. *Toxicon* 10, 605-612
21. Hessinger, D.A., Lenhoff, H.M. and Kahan, L.B. (1973) Hemolytic, phospholipase A and nerve affecting activities of sea anemone nematocyst venom. *Nature New Biol.* 241, 125-127.
22. Khoo K.S., Kam W.K., Khoo H.E., Gopalakrishnakone P., Chung M.C.M., 1993, Purification and partial characterization of two cytolytic toxins from a tropical sea anemone *Heteractis magnifica*. *Toxicon.* 31, 1567-1579.
23. Macek P., 1992, Polypeptide cytolytic toxins from sea anemones (Actinaria). *FEMS Microbiol Immunol.* 105, 121-130.
24. Turk T., 1991, Cytolytic toxins from sea anemones. *J Toxicol Toxin Rev.* 10, 223-262.
25. Mahnir V.M., Shumilov U.N., Kovalevskaya A.M., 1993, Evidence for several types of biologically active substances in North Pacific sea anemones. *Comp Biochem Physiol.* 106C: 661-665.
26. Quinn R.J., Kashiwagi M., Norton T.R., Shibata S., Kuchi M., Moore R.E., 1974, Antitumour activity and cardiac stimulatory effects of constituents of *Anthopleura elegantissima*. *J Pharm Sci.* 63, 1798-1800.

27. Norton T.R., Kashiwagi M., 1972, Purification of a potent antitumour agent from a Tahitan sea anemone and methods of administration studies with Ehrlich as cite tumor

in mice. J Pharm Sci., 61, 1814-17.

28. Batista U., Macek P., Sedmak B., 1986, The influence of equinatoxin II on V-9-379 A cell line. Period Biol. 88, 97-98.

Author Information

Anguchamy Veeruraj

Centre of Advanced Study in Marine Biology, Annamalai University

Muthuvel Arumugam

Centre of Advanced Study in Marine Biology, Annamalai University

Thangappan Ajithkumar

Centre of Advanced Study in Marine Biology, Annamalai University

Thangavel Balasubramanian

Centre of Advanced Study in Marine Biology, Annamalai University