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Isolation of aliphatic-antibiotic compounds from marine invertebrate, *Heteractis magnifica* 'Quoy & Gaimard, 1833' against captive marine ornamental fish pathogens

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Present study is aimed to screen and isolate the antibiotic compounds from the mucus of Sea-anemone *Heteractis magnifica.* Mucus was extracted and characterized using gel chromatography, Reverse Phase High Performance Liquid Chromatography and spectroscopic methods. A molecular weight of purified protein was found to be 17KDa on SDS-PAGE. Protein content of mucus recorded was 0.62mg/ml - Mucus extract exhibited significant hemolytic activity in human, sheep, chicken and fish erythrocytes. Median lethal concentration was determined using *Artemia* nauplii. Antimicrobial activity screening was performed against the ten isolated bacterial pathogens and well inhibits the growth of *Aeromonas hydrophila, Flavobacterium* sp*., Pseudomonas fluorescens, Micrococcus* sp*.,* and *Streptococcus* sp. The aliphatic nature of the mucus was determined by using FTIR spectroscopic analysis. This is the first report on the aliphatic-antibiotic compounds derived from *Heteractis magnifica* mucus which is used as marine ornamental fish disease controlling agent.

[**Keywords:** *Heteractis magnifica, Artemia* nauplii, Hemolytic activity, Fish pathogen, Antimicrobial activity]

Introduction

The ornamental fish sector is a widespread global component of fisheries and aquaculture. Scope of this sector and the impact on human and aquatic communities are often inaccurately known and unappreciated. According to FAO the world export value of ornamental fish in 1998 was US$174 million, with imports valued at US$257 million[1]. Recent research outputs have proven that the organic compounds obtained from marine organisms have interesting biological activities. Marine environment provides a number of novel substances to control the pathogenic microbes. Sea-anemone shows a very good symbiotic partnership with the marine ornamental fishes, especially with clowns. *Heteractis magnifica* is an important sea-anemone species host's number of clown fishes. Some of these fish species possess considerable resistance to the sea-anemone, but appear to be mainly protected by a mucus coat which prevents discharge of the nematocytes[2]. Substances extracted from about 15 species of sea-anemones exhibit a wide diversity of biological activities such as haemolysis, cytotoxicity, cardio tropic activity, membrane depolarization and block of potassium channels[3,4]. Highly active toxin has been isolated from the mucus secretion and body homogenate of the sea-anemone, *Heteractis magnifica*[2]. Toxins isolated from other sea-anemones, *Actinia tenebrosa* (Tenebrosin-C, TN-C), *Actinia equina* (Equinatoxin, EqT) and *Stichodactyla helianthus* exhibit pore-forming, hemolytic, cytotoxic and heart stimulatory activities[5-7]. To overcome the difficulties in captive breeding and aquarium keeping of marine ornamental organisms, the present study has been design to develop antibacterial agents for clown fishes from its symbiotic partner. *Heteractis magnifica* is a common anemone found in the reef regions of India, so that it is chosen for the screening and characterization of antibacterial compounds, which prevent the bacterial born diseases in ornamental fishes.

Materials and Methods

Sea-anemone, *Heteractis magnifica* 'Quoy & Gaimard, 1833' was collected from Mandapam (9º17′N and 79º7′E) coast, South east coast of India. Immediately after collection from the wild, they were transported to the laboratory using sterilized polythene bags with minimum seawater. Sea-anemone was identified by using the standard literature of Indo-Pacific coral reef field guide[8]. Secreted mucus was removed and extracted adopting the method of Goudet *et al.*[9] Lyophilized mucus was

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purified according to the modified method of Andrej *et al*.[10] Mucus was subjected to gel filtration chromatography performed on G-50 column (1.5 × 60 cm) pre-equilibrated with 10 m*M* Tris-HCl; *p*H 7.0 at 4°C temperature by a flow rate of 40 mL/hour. Reverse-phase high performance liquid chromatography with the concentration of acetonitrile in 0.1% trifluoroacetic acid for 60 min, was fractioned by C_{18} column (10 × 250 mm^2, Shimadzu LC 10 ATVP, USA). Flow rate was 0.5 mL/min following the method of Andre *et al*[11]. Determination of molecular weight of partially purified protein of mucus toxin extract was carried out using SDS-PAGE (15% (W/V)), according to the method of Laemmli[12]. Protein concentration of the anemone mucus was determined, following the method of Lowry *et al*[13], with the bovine serum albumin (BSA) as a standard. Hemolytic activity was measured by means of turbid metric method as described by Macek and Lebez[14]. Typically, 25 mL of different parborlysin solutions were added to 175 mL of washed erythrocytes suspended in a physiological saline consisting of 140 m*M* NaCl and 20 m*M* Tris-HCl buffer, *p*H 7.4. Erythrocyte suspension was around 0.5% and the initial turbidity (apparent absorbance) reading at 650 nm was 0.1 ± 0.02. Apparent decrease in absorbance was recorded for 30 min at 650 nm using a microplate reader where different erythrocytes (human, sheep, chicken and fish) were used. HC_{50} (mg/ml) was defined as the amount of the toxin that cause 50% of lysis in 2.5 min. The LC_{50} of mucus toxin was investigated using the Brine Shrimp, *Artemia* nauplii the method developed by Meyer *et al*.[15]. Triplicate of each concentration was used for assessing the toxicity (Mearns - Spragg et al., 1998)[16]. Percentage lethality was determined by comparing the mean survival of *Artemia* nauplii of the test and control. LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality.

Antimicrobial activity of the mucus toxin, extracted fraction was carried out using a standard paper disc assay[16] against the marine ornamental fish pathogens isolated from marine ornamental fishes. Ten fish pathogens namely *Aeromonas hydrophila, Enterobacter aerogenes, Flavobacterium* sp., *Micrococcus* sp., *Pseudomonas fluorescens, Streptococcus* sp., *Vibrio parahaemolyticus, Vibrio alginolyticus, Edwardsiella tarda* and *Proteus* sp., obtained from the Microbiology laboratory of the Marine ornamental fish hatchery of Annamalai University were used for the assays[17]. IR spectra was recorded using Bio RAD FTIR (Fourier-transform infrared)-40 model spectrometer. Toxin sample (10 mg) was dissolved in KBr (100 mg), and compressed to prepare a salt disc (10 mm diameter) and the recordings were documented. The absorbance was determined by the OMNIC 6.0a analysis programme.

Statistical analysis was done with the SPSS statistical and Origin 6.1 software and significance level at $P<0.05$ was used. One-way ANOVA was performed with different variables of this study of crude mucus against captive marine ornamental fish pathogens.

Results

Sea-anemone possesses a variety of defence mechanisms to cope up with competitors. In the present study a total yield of 8 g/kg crude mucus and lyophilized was purification. Crude extract of mucus was fractionated by gel filtration using Sephadex G-50 (Fig. 1) and lyophilized. Subsequently, the resulting chromatographic purification procedure incorporating hydroxyapatite and RP-HPLC gave an

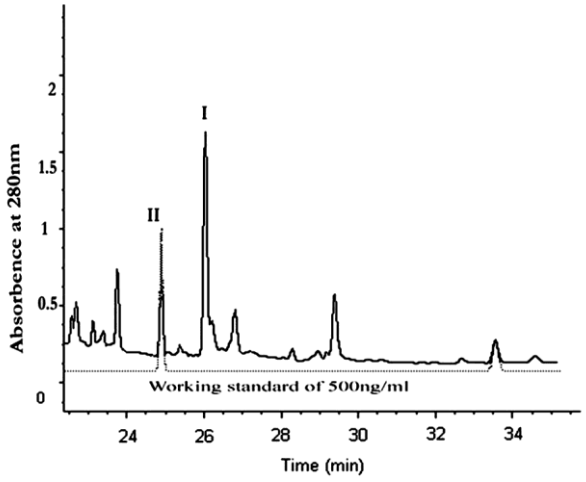

Fig. 1 — Gel Chromatograpm of mucus extract of *Heteractis magnifica*

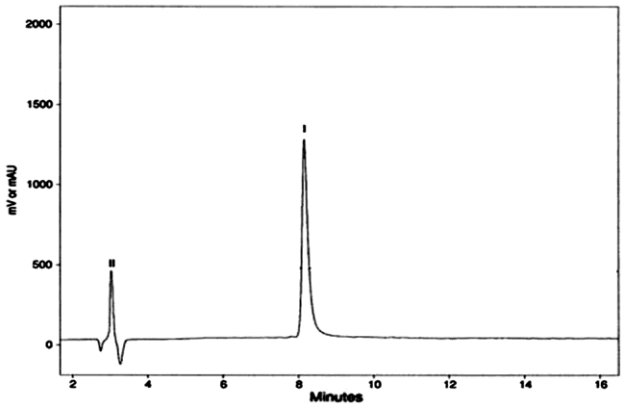

Fig. 2 — RP-HPLC chromatogram of *Heteractis magnifica* mucus extract, eluted with acetonitrile

apparently homogenous peak as shown in the Fig. 2. Molecular weight was 17 KDa as evidenced by SDS - PAGE (Fig. 3) and its protein content of the purified mucus extract was found to be 0.62 mg/mL. The

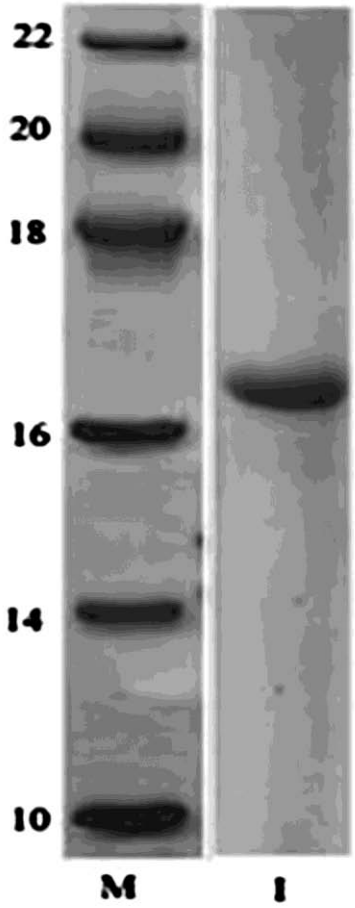

Fig. 3 — SDS-PAGE analysis of HMT. M: molecular mass standards. Lane 1 sample collected from RP-HPLC fraction

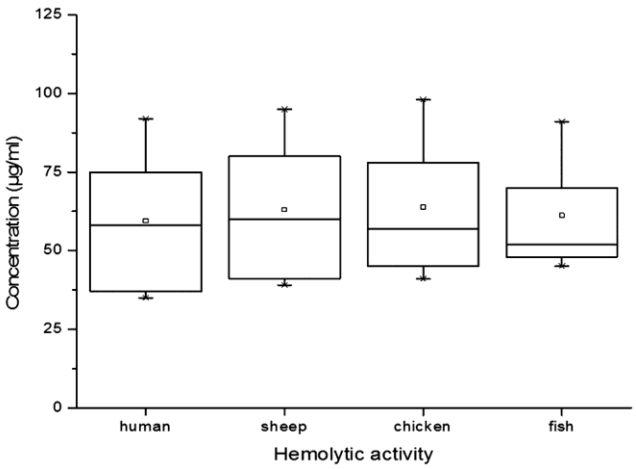

Fig. 4 — Hemolytic activity of crude mucus on human, sheep, chicken and fish erythrocytes

hemolytic activity of mucus protein had a threshold concentration value, below which there was no hemolysis in the time scale of the experiment. Lyses of 50% erythrocytes occurred at the concentration of 1.25 ± 0.03 µg/mL mucus protein (Fig. 4). Brine shrimp lethality was detected at 12 and 24 hours. The LC_{50} value of toxic protein against Brine shrimps was estimated to be 3.5 µg/mL (Fig. 5). Mucus showed a concentration-dependent antibacterial activity against selected strains of *Aeromonas hydrophila, Flavo-bacterium* sp., *P. flurescens, Micrococcus* sp., *Streptococcus* sp. Control (10 m*M* sodium phosphate buffer without mucus) did not show any antibacterial activity (Fig. 6). The LD_{50} value of anemone toxins, *S. mertensii* and *S. haddoni* as 0.65 and 0.90 mg/mL

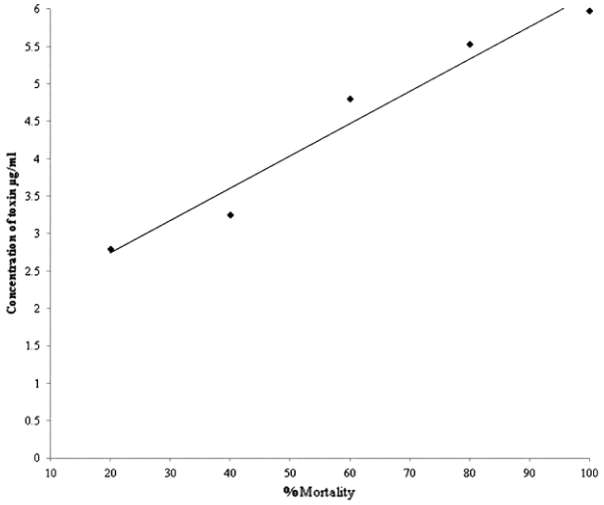

Fig. 5 — Biological screening assay using *Artemia* sp.

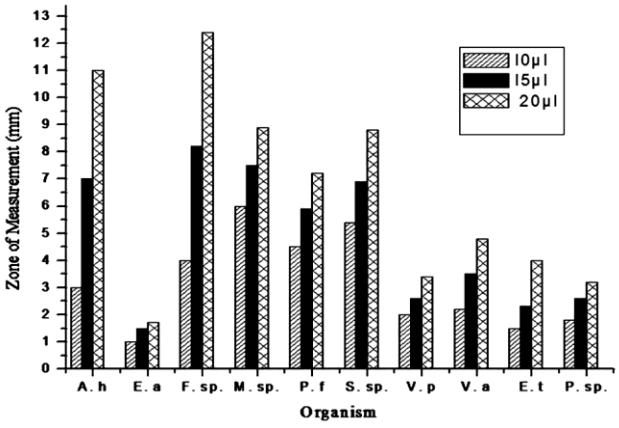

Fig. 6 — Antibacterial activity of mucus extract in various concentrations against marine ornamental fish pathogens: A. h - *Aeromonas hydrophila*, E. a - *Enterobacter aerogene,* F. sp - *Flavobacterium* sp., M. sp. - M*icrococcus* sp., P. f - *P. flurescens, S.* sp. - *Streptococcus sp., V. p - Vibrio parahaemolyticus, V. a - V. alginolyticus, E. t - Edwardsiella tarda, P.* sp. - *Proteus* sp.

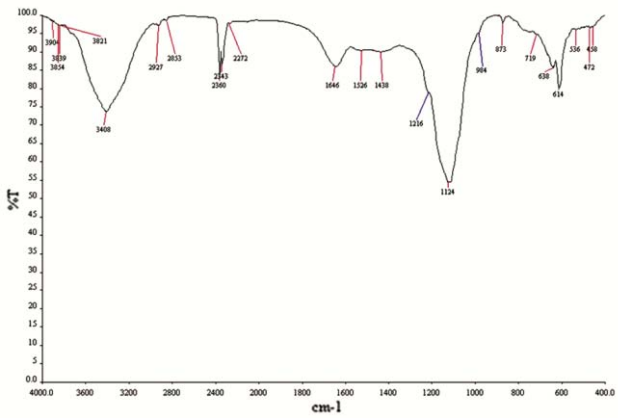

Fig. 7 — IR spectra of anemone mucus sample (10 mg) was using a Bio red FTIR - 40 model spectrometer

respectively[30] and LC_{50} value of *H. magnifica* was 0.062 mg/L in the present study. Further, we analyzed highest peak elution by FTIR spectroscopy and found a highly similar absorbance pattern with seven major bands (Fig. 7) respectively, 1630-1870 cm^{-1} show a strong C=O stretching band. It indicates the protein structure, 3904-3408 cm^{-1} shows the O-H band stretching, that is a hydroxyl groups. 2927 and 614 cm^{-1} peaks indicates the C-H and 1000-1260 cm^{-1} shows C-O band then 873 cm^{-1} indicates the Po_4 groups. Finally the 2927 cm^{-1} specified the CH_2 symmetric and asymmetric stretching of methyling groups. All of these seven major bonds designated the aliphatic compounds were present in the purified mucus of the Sea-anemone.

Discussion

In the last three decades, biologically active polypeptides from sea-anemones have been widely studied[18-21]. Most common toxins belong to the actinoporin family and have been so far isolated from at least 20 sea-anemone species and the toxins share many similar features. They are basically proteins with molecular weights of around 20 kDa[22]. Yawen Wang *et al*.[23] have proved *H. magnifica* toxins have a major protein band of approximately 19 kDa which is in agreement with the present study. Mucus crude of *H. magnifica* was also observed effective in producing toxicity in fish erythrocytes, as indicated by haemolysis. The haemolytic response is a very sensitive test to assess and characterize toxins from sea-anemones[24]. This finding is consistent with earlier reports[25] on hemolytic activity of sea-anemone *Paracondylactis indicus.* Enden[26]; Toom *et al.*[27] and Walker[28] reported the hemolytic activity of other species of sea-anemones. Similar hemolytic activities with the hemolysin Pstx20 has been reported from a sea-anemone *P. semoni*[29]. Present study proved antibacterial activity of *H. magnifica* mucus toxin and prepared in aqueous extract against *A. hydrophila, Flavobacterium* sp. *P. fluorescens, Micrococcus* sp., *Streptococcus* sp. Lee seong wei *et al*.[31] have reported that methanol extract of *Radianthus ritteri,* inhibits the growth of fish pathogenic bacteria such as *Edwardsiella tarda, Citrobacter freundii, Aeromonas hydrophila, Pseudomnas aeruginosa, Streptococcus agalactiae* and *Yersinia enterocolitica.* A similar report has also been made that, ethyl acetate extract of the anemone *Stichodactyla haddoni* shows antimicrobial activity against *Aspergillus* sp. (22 mm dia.)[32].

Conclusion

The crude mucus of the sea-anemone *H. magnifica* contained haemolytic protein with 17 kDa molecular weight and this mucus was proved promising to develop antimicrobials to protect marine ornamental fish from diseases. These aliphatic antibiotic compounds will be used in the drug development, for the disease free marine ornamental fish culture system.

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References

1 Devin Bartley, Manual of Fisheries and Aquaculture topics Ornamental fish Topics Fact Sheets.FAO Man. Fish. Sci. (2005) pp. 165.

2 Mebs D, Claus I, Schroter A, Takeya H, Iwanaga S, Gopalakrishnakone P & Tan C K, *Recent Advances in Toxicology Research*, (Venom and Toxin Research Group, National University of Singapore, Singapore) 1992.

3 Turk T, Cytolytic toxins from sea anemones. *J. Toxicol. Toxin Rev.* 10 (1991) 223-262.

4 Macek P, Polypeptide cytolytic toxins from sea anemones (Actiniaria). *FEMS Microbiol. Immunol.* 5 (1992) 121-129.

5 Blumenthal K M & Kem W R, Primary structure of *Stoichactis helianthus* cytolysin III. *J. Biol. Chem.* 258 (1983) 5574-5581.

6 Batista U, Macek P & Sedmak B Y, The cytotoxic and cytolytic activity of equinatoxin II from the sea anemone *Actinia equine*. *Cell Biol. Int. Rep.* 14 (1990) 1013-1024.

7 Simpson R J, Reid G E & Moritz R L, Complete amino acid sequence of tenebrosin-C, a cardiac stimulatory and haemolytic protein from the sea anemone *Actinia tenebrosa. Eur. J. Biochem.* 190 (1990) 319-328.

8 Allen G R, Steene R & Steene R C, *Indo-Pacific coral reef field guide*. (Odyssey, California, USA) 1994].

9 Goudet C & Tania Ferrer Loipa Gala, Characterization of two *Bunodosoma granulifera* toxins active on cardiac sodium channels. *British J. Pharma.* 134 (2001) 1195-1206.

10 Andrej R, Igor K, William R & Kem, A new cytolytic protein from the sea anemone *Urticina crassicornis* that binds to cholesterol- and sphingomyelin-rich membranes. *Toxicon,* 53 (2009) 762-769.

11 Andre J Z, Wilson A F J & Oliveiraa J S, Revisiting cangitoxin, a sea anemone peptide: Purification and characterization of cangitoxins II and III from the venom of *Bunodosoma cangicum*. *Toxicon,* 51 (2008) 1303-1307.

12 Laemmli U K, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227 (1970) 680-682.

13 Lowry O H, Rosebrough N J & Fearr A L, Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193 (1951) 265-275.

14 Macek P & Lebez D, Kinetics of hemolysis induced by equinatoxin, a cytolytic toxin from the sea anemone *Actinia equine,* Effects of some ions and *p*H. *Toxicon* 19 (1981) 233-244.

15 Wah S T, Toxicity testing using the brine shrimp: *Artemia salina*. In: Colegate S M Molyneux R J ed. *Bioactive natural products, detection, isolation and structural determination*. (CRC London pp.130-141) 1993.

16 Mearns-Spragg A, Bregu M & Boyd K G, Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett. Appl. Microbiol.* 27 (1998) 142-146.

17 Dhayanithi N B, Ajith Kumar T T & Kathiresan K, Effect of neem extract against the bacteria isolated from marine fish. *J. Environ. Biol.* 31(2010) 409-412.

18 Shiomi K, Tanaka E & Yamanaka H, Isolation and characterization of a lethal hemolysin in the sea anemone *Parasicyonis actinostoloides*. *Toxicon,* 23 (1985) 865-874.

19 Kem W R, Sea anemone toxin: structure and action. In: Hessinger DA, Lenhoff HM (eds) *The Biology of Nematocysts* (Academic Press, New York) 1988].

20 Harvey H L, Cytolytic toxins In: Shier WT, Mebs D (eds) *Handbook of Toxicology*. (Marcel Dekker, New York) 1990].

21 Bernheimer A W, Cytolytic peptides of sea anemones In: Hall S Strichartz G (ed) *Marine Toxins Origin Structure and Molecular Pharmacology*, (ATS Symposium Series-418, American Chemical Society, Washington) 1990].

22 Anderluh G & Macek P, Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). *Toxicon,* 40 (2002) 111-124.

23 Yawen Wang Kim, Lee Chua, Hoon Eng & Khoo, A new cytolysin from the sea anemone, *Heteractis magnifica*: isolation, cDNA cloning and functional expression. *Biochimica. Biophysica. Acta,* 1478 (2000) 9-18.

24 Macek P, Belmonte G, Pederzolli C & Menestrina G, Mechanism of action of equinatoxin II, a cytolysin from the sea anemone *Actinia equina* L. belonging to the family of actinoporins. *Toxicol.* 87 (2000) 205-227.

25 Dipan Adhikari Samir K & Samanta Arnab Dutta, In vitro hemolysis and lipid peroxidation-inducing activity of the tentacle extract of the sea anemone *Paracondylactis indicus* Dave in rat erythrocytes. *Ind. J. Pharmacol.* 39(3) (2007) 155-159.

26 Enden R & Noble M, Toxic material from the tentacles of the *cubomdusan Chironex cheri. Toxicon,* 9 (1971) 255-264.

27 Toom P M, Larsen J B & Chan D S, Cardiac effects of *Stomolophus meleagris* (cabbage head jelly fish) toxin. *Toxicon,* 13 (1975) 159-64.

28 Walker M J, Pharmacological and biochemical properties of a toxin containing material from the jelly fish *Cyanea capillata*. *Toxicon,* 15 (1977) 3-14.

29 Nagai H, Oshiro N & Takuwa-Kuroda K, A new polypeptide toxin from the nematocyst venom of an Okinawan sea anemone *Phyllodiscus semoni* (Japanese name 'unbachi-isoginchaku'). *Biosci*. *Biotechnol. Biochem.* 66 (2002) 2621-2625.

30 Veeruraj A, Arumugam M & Ajith Kumar T T, Isolation and biological properties of neurotoxin from Sea anemone (*Stichodactyla mertensii, S. haddoni*). *The Int. J. Toxicol.* (2008) 1556-3916.

31 Lee Seong, Wei Najiah Musa, Wendy Wee & Nadirah Musa, Antimicrobial property of 12 spices and Methanol extract of ornamental sea anemone *Radianthus ritteri* against Edwarsiellosis agent and other bacteria. *Advan*. *Biologic*. *Res*., 1 (2007) 164-166.

32 Prakash Williams G, Babu S & Ravikumar S, Antimicrobial activity of tissue and associated bacteria from benthic sea anemone *Stichodactyla haddoni* against microbial pathogens. *J. Environ. Biol.* 28(4) (2007) 789-793.