Int. J. Adv. Res. Biol.Sci. 2(6): (2015): 27–35

International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Antimicrobial activity of sea anemone *Stichyodactyla hadonii* and *Anthopleura elegantissima* extracts against human pathogens

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Abstract

In the present study ten different solvents were used for the extraction of sea anemones *Anthopleura elegantissima* and *Stichodactyla hadonii*, of these diethyl ether crude extract was found to be the highest concentration on both organisms. Diethyl ether extract of *A. elegantissima* was found to produce a pronounced inhibition of 9 mm against *V. cholerae* and *S. hadonii* inhibit *P. mirabilis* with a maximum zone of 6 mm. The column purified extracts of *A. elegantissima* maximum inhibition of 6mm against *S. pneumoniae* was shown by the 80:20 chloroform: acetone at a concentration of 5mg, 100% acetone fractions of *S. hadonii* inhibited *K. pneumoniae* with a maximum zone of 6 mm. The MIC of different column purified values of *A. elegantisima* found to be lower for 80:20 chloroform:acetone fractions for the pathogens *E. coli* and *S. typhi* (0.05mg) and *P. aeruginosa* (0.1mg). In the case of *S.hadonii*, the MIC was found to be lower for *S. epidermidis, K. pneumoniae* (0.1mg) and *V. cholerae, B. cereus* (0.2mg) for the same fractions. The present study revealed that sea anemones may also contain some biologically active agents which have potential activity against pathogenic microorganisms.

Keywords: Sea anemones, *Anthopleura elegantissima* and *Stichodactyla hadonii*, antibacterial activity, human pathogens.

Introduction

Marine organisms are a rich source of structurally novel and biologically active metabolites. The biodiversity of marine ecosystem provides an important source of chemical compounds which have many therapeutic applications such as antiviral, antibacterial, antifungal and anticancer activities (Caccamese and Azzolina, 1979; Perez *et al*., 1990; Harada and Kamei, 1997; Siddhanta *et al*., 1997; Pereira *et al*., 2004). Sea anemones, like other coelenterates, produce many biologically active polypeptides and proteins, including neurotoxins, pore - forming toxins (or) cytolysins, phospholipases and proteinase inhibitors. Anemone neurotoxins (polypeptides with relative low molecular weight 3000–5000) are very important tools in neuro physiological and pharmacological research as blockers and modulators of Kþ and Naþ channels. Their structure and function relationships were studied and discussed (Schweitz *et al*., 1985; Kem, 1988; Kelman *et al*., 1998; Kem *et al*., 1999; Rauer *et al*., 1999). As a consequence of increasing demand for the biodiversity in the screening programs seeking therapeutic drugs from natural products, there is now a greater interest in marine organism. There is a copious number of works pertaining to the antibacterial agents from marine bacteria, micro algae, Seaweeds, sponges, mollusks and ascidians. The sea anemones belong to the class Anthozoa, phylum Cnidaria have got much

attention in recent years. The members of this class are solitary and ocean dwelling. More than 32 sea anemones have been reported to produce cytolytic peptides and the proteins exhibits a wide diversity of biological activities such as haemolysis, cytotoxicity, cardio tropic activity and membrane depolarization (Kendour *et al*., 2010; Naga Deepthi *et al*., 2010 and Anderluh and Macek, 2000). In the present study, two different sea anemone species such as *Stichyodactyla hadonii* and *Anthopleura elegantissima*, which are commonly occurring along the Kanyakumari coastal areas, were chosen in an attempt to test the antibacterial activity of the crude and column purified extracts. In addition, the minimal inhibitory concentrations of the column purified fractions were also tested.

Materials and Methods

2.1 Specimen collection and identification

Two species of sea anemones, *Stichyodactyla hadonii* and *Anthopleura elegantissima* were collected from Kanyakumari coastal area (Lat. N08^{o} 04’463 Long. E77^{o}31’ 270 – Lat. N08^{o}04’403 Long. E77^{o}33’075), Southeast coast of India by SCUBA diving at the depth ranging from 3 to 5m. The samples were thoroughly washed with sea water and removed sand, mud and overgrowing organisms at the site of collection, and the sample was packaged with air in thin cover to the laboratory and maintain in culture tank. Collected specimens were identified by following the standard literature of Indo-Pacific coral reef field guide (Geraled and Steene, 1998).

2.2 Preparation of crude extract

The entire body of two different sea anemones *Stichyodactyla hadonii* and *Anthopleura elegantissima* were washed with cleaned sea water and later for extraction; 1 kg of each sea anemones were cut into small pieces and approximately 200 g of sea anemone pieces were immersed with ten different solvents in separately. The solvents were Butanol, Hexane, Methanol, Diethyl ether, Dichloromethane, Chloroform, Acetone, Toluene, Dimethyl sulphoxide and water. The extracts were cold steeped overnight at -18^{0}C, filtered with Whatman No.1 filter paper. The filtrate was poured in previously weighed petri plate, evaporated to dryness in rotary evaporator (Becerro *et al*., 1994; Riguera, 1997 and Wright, 1998) and the dried crude extracts were used for antibacterial assay against human pathogens.

2.3 Pathogenic microorganisms

To test the antibacterial effect of the extracts obtained from different solvents, 10 human pathogens such as *Bacillus cereus* (ATCC10876), *Vibrio cholerae* (ATCC15748), *Staphylococcus aureus* (ATCC 29737), *Enterobacter aerugenes* (ATCC13048), *Bacillus subtilis* (ATCC6633), *Staphylococcus epidermidis* (ATCC12228), *Streptococus pneumoniae* (ATCC6301), *Pseudomonas aeruogenosa* (ATCC29336), *Escherichia coli* (ATCC25922) and *Vibrio cholerae* (ATCC15748) were used as test strains. The strains were obtained from Christian Medical College, Vellore. All the test organisms were cultured in Tryptone Soya broth (TSB) and the 18 - 24 hours old broth was used for the experiments.

2.4 Antibacterial assay

The antibacterial activity of the crude extracts was assayed by the standard Nathan's Agar well Diffusion (NAWD) technique (Nathans *et al.*, 1978) against the test strains on Tryptone Soya Agar (TSA) in Petri dishes with drilled wells of 6 mm diameter, 25 mg of the dried extract in 50µl Dimethyl Sulphoxide (DMSO) was loaded on to each wells. The wells at the centre served as control (without the extract). After 22 - 24 hours of incubation at room temperature, the susceptibility of the test strain was determined by measuring the radius of zone of inhibition around each well which is the distance between the border of the well and the edge to which the test strains are completely inhibited.

2.5 Partial purification of the Active crude extracts:

Partial purification of the active crude extract was carried out following the method of Wright, (1998). After initial screening, the extract showing activity obtained from Diethyl Ether was fractionated using normal phase silica gel (200-400 mesh LOBA CHEMIE Mumbai) column chromatography employing a step gradient solvent system from low to high polarity. The step gradient protocol used was 100% hexane, 80% hexane: 20% chloroform, 60% hexane: 40% chloroform, 40% hexane: 60% chloroform, 20% hexane: 80% chloroform, 100% chloroform, 80% chloroform: 20% acetone, 60% chloroform: 40% acetone, 40% chloroform: 60% acetone, 20% chloroform: 80% acetone and 100% acetone. The fractions thus obtained were evaporated in previously weighed Petri plates and concentrated 5 mg of each fraction was dissolved in 50µl of DMSO (Dimethyl Sulfoxide) and was again tested for antibacterial activity. After 24 hours of incubation at room temperature, the susceptibility of the test organisms was determined by measuring the radius of the zone of inhibition around each well.

Results

The ten different solvents were used for the extraction of sea anemones, of these Diethyl Ether crude extract was found to be the highest on both the animals *Anthopleura elegantissima* and *Stichodactyla hadonii.* The concentration of Crude extract obtained from *A. elegantissima* (1mg) and of *S. hadonii* (1.253mg) (Table 1). However, crude extract yield of acetone was found to be substantial as 0.750 mg in *A. elegantissima* and 0.843 mg in *S. hadonii*. The crude extract yield of DMSO and water was found to be the lowest.

Table 1. Crude extract yield from sea anemones using different solvents

Sl. No	Solvents	Raw samples (g)	Crude extract yields (mg)	
			Anthopleura elegantissima	***Stichodactyla hadonii***
1	Di ethyl ether	50	1.0	1.25
2	Methanol	50	0.825	0.950
3	Acetone	50	0.750	0.843
4	Ethyl Acetate	50	0.750	0.843

3.1 Antibacterial activity against human pathogens

Out of the 10 solvents used for the extraction of the sea anemones, the diethyl ether extract of *A. elegantissima* was found to produce a pronounced inhibition of 9 mm against *V. cholerae* and *S. pneumoniae*, 8mm against *P. aeruginosa*, *P. mirabilis* and *S. typhi* and 7 mm against *E.coli*, *Enterobacter aerugenosa* and 6mm against *Bacillus cereus* at a concentration of 25 mg. However, the acetone extract was able to produce a zone of 8 mm against *E. coli* followed by hexane; however the butanol and ethanol extract was also able to produce a zone of 6 mm against *K. pneumoniae* and *P. mirabilis*. The Diethyl ether extract of *S. hadonii* at the same concentration was able to inhibit *P. mirabilis* with a maximum zone of 6 mm followed by *P. aeruginosa, V. cholerae, S. pneumoniae, S. epidermidis* 5 mm and *E.coli* and *S.typhi* with zones of inhibition of 4 mm against *K. pneumoniae, E.coli* and *V.cholerae*. On the other hand, the ethyl acetate extract was able to produce a zone of 6 mm against *V.cholerae* and 5 mm against *K. pneumonia* (Table 2). However, only slight activity was shown by the Ethyl acetate extract of *A. elegantissima*. The water, methanol, DMSO extracts of both sea anemones tested was able to show moderate inhibition.

Table 2. Antibacterial activity of extracts of *Anthopleura elegantissima* and *Stichodactyla hadonii* against human pathogens

Sl. No	Pathogens	*Anthopleura elegantissima* Radius of the Zone of Inhibition (mm)				*Stichodactyla hadonii* Radius of the Zone of Inhibition (mm)			
		E	A	DEE	B	E	A	DEE	B
1	*Escherichia coli*	3	7	7	3	2	4	4	3
2	*Staphylococcus epidermidis*	4	8	4	1	2	T	5	2
3	*Pseudomonas mirabilis*	3	4	8	3	7	7	6	1
4	*Klebsiella pneumonia*	6	3	5	6	5	4	5	3
5	*Pseudomonas aerogenosa*	3	4	8	3	2	2	5	3
6	*Salmonella typhi*	1	3	8	2	3	4	4	4
7	*Vibrio cholerae*	3	4	9	3	6	4	5	2
8	*Streptococcus pneumonia*	4	3	9	4	2	3	5	3
9	*Bacillus cereus*	T	1	6	1	1	1	3	1
10	*Enterobacter aerogenes*	T	T	7	T	2	1	4	1

A-Acetone, E- Ethanol, DEE- Diethyl Ether, B- Butanol

3.2 Antibacterial activity of column purified extract against human pathogens

The effect of the column purified extracts of *A. elegantissima* against human pathogens. A maximum inhibition of 6mm against *S. pneumoniae* was shown by the 80:20 chloroform: acetone column purified fractions at a concentration of 5mg, the fraction was also able to inhibit *K. pneumoniae* to 5mm and *S. pneumoniae* to 4mm. 80:20 hexane: chloroform fractions were able to inhibit *E. aerugenes* and *S. pneumoniae* by 5 mm and 4 mm against *E.coli*. 100% hexane, 80:20 hexane:chloroform, 60:40 hexane:chloroform, 100% acetone, 100% chloroform fractions exhibited only trace inhibitions. 100% acetone fractions of *S. hadonii* inhibited *K. pneumoniae* with a zone of 6 mm and an inhibition of 4mm against *S. typhi*. 80:20 hexane:chloroform fractions and 20:80 chloroform:acetone fractions inhibited *K. pneumoniae* and *P. mirabilis* with a zone of 4 mm. 100% hexane, 80:20 hexane:chloroform, 60:40 hexane:chloroform and 100% chloroform fractions of *S. hadonii* were able to inhibit all the ten pathogens used for the present study (Table 3). Also the 100% acetone fractions of *S. hadonii* showed a prominent effect on the tested pathogens.

Table.3. Antibacterial activity of the column purified fractions of *Anthopleura elegantissima* and *Stichodactyla hadonii* against human pathogens

Sl. No	Pathogens	Radius of the Zone of Inhibition (mm) *Anthopleura elegantissima*											Radius of the Zone of Inhibition (mm) *Stichodactyla hadonii*										
		H	80 : 20	60 : 40	40 : 60	20 : 80	C	80 : 20	60 : 40	40 : 60	20 : 80	A	H	80 : 20	60 : 40	40 : 60	20 : 80	C	80 : 20	60 : 40	40 : 60	20 : 80	A
1	*Escherichia coli*	-	2	T	1	4	-	2	2	3	2	T	1	3	2	T	T	-	3	2	2	2	3
2	*Staphylococcus epidermidis*	-	T	T	2	-	-	4	1	2	4	-	3	2	T	-	T	-	T	4	2	4	T
3	*Pseudomonas mirabilis*	-	2	2	3	T	-	5	T	T	T	T	-	T	2	-	2	-	4	3	T	4	5
4	*Klebsiella pneumoniae*	-	T	2	4	3		6	2	T	5	-	2	3	2	T	3		5	2	T	2	6
5	*Pseudomonas aerugenosa*	-	T	T	5	T	-	4	T	-	T	T	-	2	T	T	3	-	T	3	-	2	T
6	*Salmonella typhi*	-	3	T	6	T	T	2	4	T	3	2	2	T	T	T	T	T	3	2	T	3	4
7	*Vibrio cholerae*	-	T	T	7	5	-	3	T	2	2	T	2	T	T	T	T	-	5	2	2	2	4
8	*Streptococcus pneumoniae*	-	T	T	8	-	T	4	T	T	6	T	-	3	T	2	3	3	2	2	T	4	T
9	*Bacillus cereus*	-	T	3	9	T	-	3	2	T	2	T	-	T	3	T	2	-	2	2	T	2	T
10	*Enterobacter aerogenes*	-	T	T	10	5	T	1	T	2	T	T	-	T	T	T	2	T	3	2	2	T	T

H-Hexane; A-Acetone; ME-Methanol

3.3 Minimal Inhibitory Concentration (MIC)

The Minimal inhibitory values of the column purified fractions of the sea anemone *Anthopleura elegantissima* for the human bacterial pathogens tested. The MIC of different column purified values of *A. elegantisima* found to be lower for 80:20 chloroform:acetone fractions for the pathogens *E. coli* and *S. typhi* (0.05mg) and *P. aeruginosa* (0.1mg). In the case of *S.hadonii*, the MIC was found to be lower for *S. epidermidis, K. pneumoniae* (0.1mg) and *V. cholerae, B. cereus* (0.2mg) for the same fractions (Table. 4). MIC values of 0.1 mg and 0.2 mg were obtained for the pathogens *V. cholerae* and *S. typhi* respectively. Similar MIC values were obtained for the same fractions of *S. hadonii* for *E.coli* (0.1mg) and *S. pneumoniae* (1.5mg).

Discussion

In the present study the crude extract yield of the sea anemones was found to be maximum in diethyl ether extract of *Anthopleura elegantissima* (1mg), followed by methanol (0.825 mg), acetone (0.750 mg) and ethyl acetate. In case of *Stichodactyla hadonii* too the extracts ranged in the similar way as diethyl ether showed the maximum of 1.253 mg followed by methanol (0.950 mg), acetone (0.853 mg) and ethyl acetate extracts . Higher extract yield from diethyl ether shows that this particular solvent has the ability to dissolve much of the compounds from the sea anemone. The values of the present study are comparable to the value of crude protein of *H. magnifica* (980µg/ml), followed by *S.hadonii* (820 µg/ml) and *P. sinensis* (600 µg/ml) (Vinoth, 2007). During the present investigation, it was observed that the content of crude extracts has decreased considerably on column purification. Similar reduction in the protein content on purification through different columns has been reported earlier by Lin *et al*., (1996) and Grotendorst and Hessinger (1999), in the case of the toxin of *Aiptasia pallid*a.

Among the two anemone species, pronounced inhibition was conferred by the diethyl ether extract of *A. elegantissima* against the bacterial pathogens *S. pneumoniae, V. cholerae P. mirabilis* and *S. typhi* with zones of 9 mm and 8 mm respectively, the same way acetone extract of *A. elegantissima* was found to inhibit the *S. epidermidis* with a maximum zone of 8 mm, like wise the extract of *S.hadonii* obtained from the diethyl ether solvent possessed higher antibacterial properties against most of the test strains. Only moderate activity was conferred by the acetone extract of *S.hadonii* with maximal activity of 4mm against *K.pneumoniae* and *E.coli*. The crude extract of diethyl ether showed good activity against Gram positive and Gram negative bacteria (Ravikumar *et al.*, 2002). Of the human pathogens tested, *Klebsiella pneumonia* is highly inhibited (20 mm) by the tissue extract of sea anemone by using chloroform and methanol extract and 24 mm with the hexane tissue extract against fish pathogen reported by Prakash-Williams *et al.*, (2007). Bhosale *et al.*, (2002) have reported antimicrobial property of marine organisms against biofilm bacteria isolated from test panels. As an earlier report has been made, the crude extract of *Stichodactyla haddoni* showed good activity against Gram-negative bacteria (Sureshkumar *et al.*, 2002). Hutton & Smith, (1996) has reported the amoebocytes from the sea anemone *Actinia equine* showed considerable inhibitory activity against Gram-negative bacteria within 3 h.

The purified fractions were also found to inhibit some of the tested pathogens; the Diethyl Ether Extract (DEE) which was found to possess higher antibacterial activity was hence chosen to localize the active component through column purification. In the present study, 80:20 chloroform: acetone column purified *A. elegantisimma* fraction was found to possess utmost antibacterial activity of 6mm against *S. pneumoniae* and the same fraction was also able to inhibit *K. pneumoniae* by 5 mm. In the case of *S.hadonii*, a clear zone of 6 mm was shown by the 100% acetone column purified fraction against *S. pneumoniae* at a concentration of 5mg. Even the same fraction was also able to produce a clear zone of 4mm against *S. typhi*. The present work coincides with the result of Dovi Kelman *et al.*, (1998) who found that bioassay-guided fractionation of the extract of the soft coral *Parerythropodium fulvum* produced broad spectrum antimicrobial activity against the marine bacteria but it was confirmed that the antimicrobial activity was due to the presence of a variety of secondary compounds of different polarities. As in the present work, instances of toxicity of purified toxins from three sea anemones *H. magnifica, S. hadonii* and *P. sinensis* have been established by Vinoth (2007). Chellaram *et al*., (2004) have reported that acetone fraction of *Pteria chinensis* exhibited broad spectral antibacterial activity that substantiates the present work. The hypobranchial glands of *Chicoreus virgineus* and egg capsules of *Rapana rapiformis* extracted with polar solvents like ethanol and methanol also have been reported to show wide spectral antibacterial activities (Anand *et al*., 1997). *Cypraea errones* was reported to have antibacterial activity at the non-polar fractions

Table.4. Minimal Inhibitory Concentration (MIC) of the column purified fractions of *Anthopleura elegantissima* and *Stichodactyla hadonii* against human pathogens

Sl. No	Pathogens	Radius of the Zone of Inhibition (mm) *Anthopleura elegantissima*											Radius of the Zone of Inhibition (mm) *Stichodactyla hadonii*										
		H	80 : 20	60 : 40	40 : 60	20 : 80	C	80 : 20	60 : 40	40 : 60	20 : 80	A	H	80 : 20	60 : 40	40 : 60	20 : 80	C	80 : 20	60 : 40	40 : 60	20 : 80	A
1	*Escherichia coli*	-	+	+	++	+	+++	+	++	++	++	-	-	+	++	+	++	+++	+	+	+	++	-
2	*Staphylococcus epidermidis*	-	+	+	+	+	+++	++	++	+++	++	-	-	+	+	+	+++	+++	-	++	+++	++	-
3	*Pseudomonas mirabilis*	-	-	-	+	+	++	++	+	T	++	+	-	-	-	+	-	-	+	-	-	++	_
4	*Klebsiella pneumoniae*	-	-	T	+	T	++	+	++	T	++	-	-	-	T	+	T	-	-	-	-	+	-
5	*Pseudomonas aerugenosa*	-	-	-	+	++	+++	T	+	T	T	T	-	-	-	++	-	+++	-	-	+	+	-
6	*Salmonella typhi*	-	-	T	+	+	+	+++	+	T	+	-	-	-	T	++	-	++	++	-	-	+	-
7	*Vibrio cholerae*	-	-	T	+	+	+++	++	++	T	+	-	-	-	T	-	-	+	-	-	-	-	-
8	*Streptococcus pneumoniae*	-	-	+	+	T	+++	T	+	T	+	-	-	-	+	-	T	+++	-	-	-	-	-
9	*Bacillus cereus*	-	-	T	+	+	+	+	T	++	+	T	-	-	T	-	-	-	-	T	++	-	T
10	*Enterobacter aerogenes*	-	+	+	+	+	+	+	T	++	+	++	-	+	+	+	-	-	-	T	-	-	+

+++ - Minimal inhibitory concentration ranging from 0.5-1mg, ++ - 1 to 2mg concentration, + More than 2 mg concentration, - - No inhibition, T - Trace

(Anand and Edward, 2002) lesser degree of inhibition by the column fractionated extracts in comparison to the crude could be opined that the active compound may have degraded or modified during the fractionation process.

Also, the Minimal Inhibitory Concentration (MIC) was found to be lower for the 80: 20% chloroform: acetone phases of *A.elegantissima* (0.05 mg) for *E. coli* and *S. typhi* at the same time a little higher for *P.aeruginosa* (0.1 mg). However, MIC values of 20µg/mL were recorded for the metabolites of soft corals, *Caldiella* sp, and *Sinularia* sp. for human pathogenic bacteria (Radhika *et al*., 2004). Horikawa *et al*., (1999) revealed that the marine algal extracts exhibited the antibacterial activity with MIC of 0.7 and 0.69 mg/ml respectively against MRSA which slightly coincides with the results of the present study. Another study by Kelman *et al*., (2001) in determining the MIC values of the pure compounds obtained from the sponge, *Amphimedon viridis* against marine bacteria have estimated that the values are greater than 250 µg, Dovi Kelman, (1998) also reported a bit higher MIC values of 1.25 mg/ml by the crude extract of soft coral *Parerythropodium fulvum fulvum* against the *Vibrio* sp.

From the above discussion it could be concluded that the crude extracts of the two sea anemones viz., *A.elegantissima* and *S.hadonii* revealed the fact that they have higher potential to produce broad spectral antibacterial activity with minimal concentration against a wide range of human pathogens. The purified compounds also showed pronounced inhibition against the tested pathogens, it has a higher potential to be evaluated as compounds with clinical significance. Further purification of the active compounds is necessary in order to clinically evaluate the potential of the newly derived compounds as novel drugs. The structural characterization of these molecules promote the utilization of this active principle as a lead in case of drug discovery from animal based formulations to control the emerging infectious drug resistant pathogenic microorganisms.

Acknowledgments

The authors gratefully thank to Prof. K.R. Venkatesan, Principal, Sri Sankara Arts and Science College (affiliated to Madras University), Enathur, Kanchipuram for his valuable support.

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