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Research Article

INSECTICIDAL AND ARTEMIA TOXICITY OF SEA ANEMONES STICHODACTYLA HADONII AND ANTHOPLEURA ELEGANTISSIMA COLLECTED IN KANYAKUMARI COASTAL WATERS, GULF OF MANNAR.

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ABSTRACT

This study examined the cytotoxic potential of two anemones found in the coastal waters of Kanyakumari. The crude extracts were tested for cytotoxicity using the brine shrimp and rice weevil Sitophilus oryzae .The crude Diethylether extract of anemone indicated the highest activity with LC50 value of (183.28µg/ml) by Anthopleura elegantissima crude extract and LC50 value of 164.99 µg/ml by Stichodactyla hadonii extracts. Brine shrimp lethality assay showed that the diethyl ether crude extracts of Anthopleura elegantissima produced 50% mortality at 100 mg concentration at 5th hour of the experiment. The 100 mg concentrations of diethyl ether and butanol extracts were also good enough to bring the 50% mortality The 80:20 chloroform: acetone column purified fractions of anthopleura elegantissim at a concentration of 500mg caused 100% mortality. Also the 100% acetone fractions of S. hadonii showed a prominent effect on the tested Artemia nauplii. Further studies are necessary for a better characterization of the active principles of these extracts and a possible elucidation of the mechanisms of action.

Keywords: Sea anemones-Stichodactyla hadonii, Anthopleura elegantissima, crude extracts, cytotoxic assay, Insecticidal activity.

INTRODUCTION

Marine invertebrates especially sedentary sea anemones are evolved with rich sources of bioactive metabolites, which could be used for novel animicrobial drugs. Among the marine organisms, anthozoans are ecologically important animals, which need to protect themselves against the lethal or debilitating consequences of microbial or parasitic invasion (Grigg and Dollar, 1990; Ramalingam and Ramarani, 2006). Sea anemones produce toxins which are used for prey acquisition or as chemical signals for repelling predators. Toxicity of sea anemone toxin is comparable with most toxic organophosphate chemical warfare agents (Munro *et al*., 1994). Recently, studies have also suggested that some bioactive compounds isolated from marine organisms have been shown to exhibit anti-cancer, anti-microbial, anti-fungal or anti-inflammatory and other pharmacological activities (Venkateswara Rao *et al*., 1998; Venkateswarlu *et al*., 2001; Proksch *et al.,* 2002; Donia and Hamann, 2003; Haefner, 2003; Jha and Xu, 2004; Gul and Hamann, 2005; Mayer and Hamann, 2005). The current thrust of the investigations involves identifying newer drugs and other pharmaceuticals from marine origin, where as comparatively little attention has been paid with respect to the discovery of pesticide molecules. The repeated use of pesticides for the control of pests has raised the problem of contamination of pesticides in the soil, atmosphere, water etc. The contamination of pesticides can be quantified either by bioassay or chemo-assay. The term bioassay means; the measurement of intensity of any stimulus (physical, chemical and biological) in the living organisms. For the bioassay of insecticides, mostly insects are used as a tool to estimate the small quantities of insecticides and insecticidal residues although organisms like protozoans, crustaceans and fishes are also used. In the field of insecticides and pesticides, the bioassay technique is used to evaluate toxicity of insecticide and insecticide residues and these breakdown products. Since these chemicals are toxic and biologically active, their use for insect control has become a serious concern. *Artemia salina* plays an important role in the energy flow of the food chain (Lewan *et al*., 1992) and it can be used in laboratory bioassay to determine toxicity (LC50). The shrimp lethality assay was proposed and later developed by (Vanhaecke, *et al*., 1981 and Sleet and Brendel, 1983). It is based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp. The assay is considered as a useful tool for preliminary assessment of toxicity (Solis, *et al*., 1993), and it has been used for the detection of fungal toxins. Toxicity of sea anemones like *S.hadonii*, *P.sinensis* and *H.magnifica,* collected from reef areas were also studied by Vinoth (2005) using albino rats as animal model and produced prominent results indicating the toxic nature of the sea anemone extracts. The Secondary metabolites isolated from marine sea anemones may be an alternative source for vector control agents to replace existing and highly toxic synthetic insecticides and will play an important role in future insecticide development programme. Following the codal formalities of the Institute's Ethical Committee, in the present study an attempt has been made to find out the effect of the crude and column purified extracts of *A.elegantissima* and *S.hadonii* on the insecticidal and toxicity against *S.oryzae* and *Artemia salina* nauplii.

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MATERIALLS AND METHODS

Description of the study area

The sea anemones *Anthopleura elegantissima* and *Stichodactyla hadonii* for the present study were collected from Kanyakumari coastal area. The Kanyakumari coast (Lat. N08º04'463 Long. E77º31' 270 – Lat. N08º04'403 Long. E77º33'075) is located at the southern tip of Tamil Nadu state, India and is the meeting point of Arabian Sea, Bay of Bengal and the Indian Ocean.

Fig.1. Study area map

Specimen collection and extract preparation

Live animals were collected in the inter-tidal rocky areas of the Kanyakumari coastal waters with the help of SCUBA divers and were immediately brought to the laboratory in an aerated container. The animals were washed thoroughly with sea water and fresh water to remove the debris, salt and air dried. The whole body of the samples (50g) were cut in to small pieces and air-dried for 24 hours at room temperature before extraction with solvents. Then the tissues were used for extraction in different solvents, such as acetone, diethyl ether, dimethyl sulphoxide, chloroform, hexane, butanol, ethyl acetate and methanol. 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 for initial insecticidal and artemia toxicity assay.

Partial purification of the active crude extract:

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 5mg of each fraction was dissolved in 50µl of DMSO (Dimethyl Sulfoxide) and was again tested for toxicity assay.

Insecticidal activity

The insecticidal activity of acetone, diethyl ether, dimethyl sulphoxide, chloroform, hexane, butanol, ethyl acetate and methanol crude extracts of *Anthopleura elegantissima* and *Stichodactyla hadonii* were tested using contact bioassay by the modified method of (Broussalis *et al*., 1999). The extract showing activity obtained from Diethyl Ether was fractionated using normal phase silica gel and 1g of each of the dried extracts was dissolved in 1ml of their respective solvents and from these 750, 500, 250, 100, 50, 25 and 10 mg/ml were poured in separate Petri plates in triplicates and allowed to evaporate overnight to get the same concentration in mg/ml of extract. Controls with solvents alone were taken in separate petri plates and allowed to evaporate overnight. Ten healthy adults of *Sitophilus oryzae* were introduced into each petri dish and sufficient food was provided to the test organisms, so that the view of death due to starvation could be ruled out. After 24 hours, the numbers of dead insects were counted and percentage of mortality was noted. The efficiency of the extracts obtained with different solvents at varied concentrations in killing the insects was determined. The LC_{50} values of the crude fractions were determined by the probit analysis (Finney, 1971).

Fig. 2. Rice weevil insect- Sitophilus oryza

Brine shrimp assay

Bioactivity of the extract was monitored by the brine shrimp lethality test (Meyer *et al*., 1982). Samples were dissolved in 10% dimethyl sulphoxide (DMSO) (v/v) and diluted with artificial seawater. Two ml of seawater was placed in all bottles. A two-fold dilution was carried out to obtain the concentration from 10 mg/ml to 0.15 mg/ml. The last bottle was left with sea salt water and 10% DMSO (v/v) only, serving as the drug free control. Brine shrimp (*Artemia salina*) eggs were hatched in a beaker filled with filtered sea water under constant aeration. After 48h, the phototrophic nauplii were collected by pipette. The nauplii were counted macroscopically in the stem of the pipette against a lighted background. Ten shrimp were transferred to Hundred micro liters of suspension containing about 10 -15 larvae. The plates were maintained under illumination. Survivors were counted after 24 h of incubation and the percentage of deaths at each dose and controls (seawater) were determined bottle and incubated for 24 h. Each concentration was tested (750, 500, 250, 100, 50, 25 and 10 mg/ml) in triplicates and controls were maintained each time. The bottles were then examined and the number of dead larvae in each bottle was counted. The mean percentage mortality was plotted against the logarithm of concentrations. The concentration (LC_{50}), at which 50% of the larvae were killed, was determined from the graph. The LC50 values of brine shrimp were obtained from counts using the probit analysis method .Finney, 1971.

RESULTS

Out of the 10 different solvents used for the extraction of sea anemones, Diethyl Ether crude extract was found to be the highest on both the animals *Anthopleura elegantissima* and *Stichodactyla hadonii.* Crude extract of 1 gram for *A. elegantissima* and 1.253 g for *S. hadonii* was obtained. However, crude extract yield of acetone was found to be substantial as 0.750 mg in *A.legantissima* and 0.843 mg in *S.hadonii*. The crude extract yield of DMSO and water was found to be the lowest. Preliminary toxicity test against storage pest; *S. oryzae*, and *artemia nauplii* revealed that the acetone hexane, Diethyl ether, Butanol, chloroform and Ethyl acetate of crude extracts showed strong insecticidal activity (1mg/ml caused 100% mortality to subjected pests). Therefore, Diethyl ether, extracts were subjected for

fractionation. The common chromatography methods were used for fractionation and final separation at this level.

Insecticidal potential of the crude extracts of sea anemones

The insecticidal activity of the crude extracts of sea anemones *Anthopleura elegantissima* obtained using different solvents was tested against *Sitophilus oryzae* and the percentage mortality is given in (Table 1).

Table 1. Insecticidal activity of *A.elegantisima* extracts

Concentration (mg/ml)	Solvents used							
	A	DEE	B	CH	H	EA	DMSO	
750	8±1	10±0	5.7±0.58	7±0.58	8.6±0.57	6±1.15	6±1.7	
500	6±0.58	8.3±0.57	4±0.47	6±0.47	5±0.94	5±0.58	4.6±0.58	LC 50 183.28
250	4±0.58	5±0.8	3.7±0.94	4.6±0.58	4±0.58	4±1	4±1	
100	3±0.47	4±1	3±1	3.6±0.58	2.6±1.15	3±0.58	2.6±0.58	
50	2±1	3±0.58	2±1	1.6±1.15	1.6±0.58	3±1	1.3±0.58	
25	0±0	0±0	0±0	0±0	0±0	0±0	0±0	

A - Acetone; DEE - Diethylether; B - Butanol; CH - Chloroform; H - Hexane; EA - Ethylactate; DMSO - Di-Methyl Sulfoxide;

The LC 50 value was found to be (183.28). All the extracts were found to cause mortality, 100% (10±0) mortality was observed only for the insects exposed to the extracts of diethyl ether at a highest concentration of 750 mg after 24hrs, the extracts of acetone and hexane at the same concentration caused 80.0% (8±1) and 86% (8.6±0.57) mortality.50% mortality was observed at 500mg concentration of A.elegantissima extract, (6±0.58) by diethyl ether extract followed by chloroform (6±0.47), hexane (5±0.94) and ethyl acetate (5±0.58). The extracts of Stichodactyla hadonii also showed similar type of insecticidal activity as shown by the A.elegantissima extracts. Diethyl ether extracts caused 100% mortality at a concentration of 750mg; however ethyl acetate, acetone hexane and butanol were observed to produce 87, 86, 80 and 77.0% mortality at the same concentration (Table 2) and the LC 50 value of 164.99 was obtained for the Stichodactyla hadonii extracts.100mg concentration of Stichodactyla hadonii extract produced 50% mortality, while diethyl ether, 5±0.58; Butanol, 5±0.58, and hexane, 5.7±0.58 mortalities.

Table 2. Insecticidal activity of *S.hadonii* extracts

Concentration (mg/ml)	Solvents used							
	A	DEE	B	CH	H	EA	DMSO	
750	9±1	10±0	9±1	7.67±0.58	7.33±0.58	7.33±0.94	7.33±0.58	
500	8±0.58	9±1	7.33±0.58	6.33±0.58	6.33±0.58	6	6±1	
250	6±0.58	8±0.58	6.33±0.58	5.67±0.58	5.67±0.58	4	5.33±0.58	LC 50 164.99
100	4±0.58	6±0.58	5.67±0.58	3.33±0.58	3.33±0.58	3.33±0.47	3.33±0.58	
50	3±1.15	4±0.58	3.33±0.58	2	2.67±0.58	2.33±0.47	1.67±0.58	
25	0±0	0±0	0±0	0±0	0±0	1.33±0.47	1±1	

Different solvent crude extracts of sea anemone *Anthopleura elegantissima* and *Stichodactyla hadonii* were also investigated for brine shrimp toxicity (*Artemia salina*). The diethyl ether extract of *Anthopleura elegantissima* showed 100% mortality against the *Artemia naupli* at 750mg concentration, whereas the butanol, acetone, ethyl acetate, chloroform, hexane extracts showed 90, 86, 83% inhibition at the same concentration (Table3). The extract with LC 50 value of 36.0176 showed significant activities against the tested animal. The diethyl ether crude extracts of *Anthopleura elegantissima* produced 50% mortality at 100 mg concentration at 5th hour of the experiment. The 100 mg concentrations of diethyl ether and butanol extracts were also good enough to bring the 50% mortality (6.33±0.58, 5.67±0.58), followed by ethyl acetate, hexane, chloroform and dimethylsulphoxide extracts (5.33±0.58, 5.33±0.58, 6.00±1.00, 5.00±1.00) at the 9th hour of the experiment.(Tables 4).
The extract of *Stichodactyla hadonii* also showed 100% mortality at the highest concentration. In this case, 250 mg concentration of the diethylether extract was good enough to bring 50% mortality but at the 6th hour of the experiment, whereas the butanol, hexane, acetone, ethyl acetate, chloroform and dimethylsulphoxide extracts at 750mg level produced 50% mortality. 25 mg concentration of the diethyl ether extract was totally absent from inhibiting the survival ratio of the *Artemia naupli*.

Table 3: Artemia Toxicity of *Anthopleura elegantissima* extracts

Concentration (mg/ml)	Solvents used						
	DEE	B	A	EA	H	CH	DMSO
750	10.00±00	9.00±1.00	9.00±1.00	8.67±0.58	8.33±0.58	8.67±1.15	7.67±0.58
500	9.00±0	8.67±0.58	8.33±0.58	7.67±0.58	7.33±0.58	8.00±1.00	7.33±0.58
250	7.67±0.58	7.67±0.58	7.33±0.58	6.67±0.58	6.33±0.58	7.33±0.58	6.33±0.58
100	6.33±0.58	6.33±0.58	5.67±0.58	5.33±0.58	5.33±0.58	6.00±1.00	5.00±1.00
50	4.99±0.58	4.49±0.58	4.16±0.58	3.99±0.58	3.86±1.00	4.63±1.00	4.00±1.53
25	0±0	0.0±0.00	0.0±0.00	0.00±0.00	1.67±0.58	0.00±0.00	0.00±0.00

Table 4: Artemia Toxicity of *S.hadonii* extracts

Concentration (mg/ml)	Solvents used						
	DEE	B	A	EA	H	CH	DMSO
750	10.00±0.00	8.33±0.58	8.00±100	8.00±0.00	8.33±0.58	8.67±0.58	8.00±1.00
500	8.25±0.58	7.11±0.58	6.75±1.00	6.75±0.58	6.99±0.58	7.16±0.58	7.00±0.58
250	7.53±0.58	6.58±1.00	6.21±0.58	6.18±0.58	6.27±1.00	6.23±0.58	6.12±0.58
100	5.94±0.58	6.12±1.15	5.70±1.00	5.62±0.58	5.71±1.15	5.65±1.15	5.4±6 1.00
50	4.37±0.58	3.59±0.58	3.18±0.58	4.10±0.58	3.20±1.53	2.16±1.53	1.95±1.00
25	1.69±1.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Insecticidal and toxicity assay using column purified fractions

The 80:20 chloroform: acetone column purified fractions of *Anthopleura elegantissim* at a concentration of 500mg caused 100% mortality. Also the 100% acetone fractions of *S.hadonii* showed aprominent effect on the tested *Artemia nauplii.*

LC_{50} values for the crude extracts of the sea anemones used in Toxicity assay.

The LC_{50} values for the extracts like diethyl ether, acetone, chloroform, dimethyl sulphoxide, and methanol of *A.elegantissima* were found to be 38.016, 42.16, 50.03, 57.18 and 70.03 mg/ml respectively. The LC_{50} value of diethyl extract of *A.elegantissima* was found to be lower (38.016) than the other four solvents. Also the LC_{50} value for the extract of *S.hadonii* was noted to be lower for the diethyl ether (29.68 mg) than the other solvents like ethyl acetate (47.28 mg), chloroform (66.16), dimethyl sulphoxidee (67.256 mg) and methanol (78.76 mg) (Table 5).

Table 5. LC_{50} values of the crude extracts of the sea anemones possessing Cytotoxicity against *Brine shrimp*

Sea anemones	DEE	A	CH	DMSO	M
	LC_{50} values (mg)				
A.elegantissima	36.016	42.16	50.03	57.18	70.03
S.hadonii	29.64	47.28	66.16	67.256	78.76

DISCUSSION

The use of marine natural products is an alternative pest control method, which helps to minimize the usage of toxic pesticides and their deleterious effects on non target insect species, livestock, wildlife and on the environment (Fatope *et al*., 1993). Rice weevil, *Sitophilus oryzae* is a common pest, which destroys stored rice, wheat and sorghum grains and produces a rise in humidity, which in turn quickens the propagation of molds and bacteria. A single larva (*S.oryzae*) is reported to consume 10mg of grain during its development. Insects, weeds and phytopathogenic microorganisms cause great damage to agriculture. Where pests and disease are not systematically controlled, an estimated one-third of a typical crop is lost (Melnikov, 1992). Much of the increase in agricultural productivity over the past half century has been due to the control of these pests with synthetic chemical pesticides (SCPs) (Duke *et al*., 1993). In the present study a high lethality was found in the diethyl ether extracts of the *Anthopleura elegantissima* showing 100% insecticidal activity (10±0) followed by the acetone and butanol extracts (90%), the results of the present study coincides with the results of Jose Luis Carballo *et al*., (2002) who found that the invertebrate extracts exhibited prominent lethality as indicated below, gorgonians *Pacifigorgia adamsii* (68%), *Muricea* sp. (83%), the tunicate *Polyclinum laxum* (96%) and the echinoderms *Toxopneustes roseus* (96%), *Isostichopus fuscus* (96%) and *Pharia pyramidata* (93%). Also the extracts of acetone and hexane caused 80.0% (8±1) and (8.6±0.57) mortality respectively at 750 mg concentration. As per the report of Vinoth (2005) toxicity exhibited by the purified fractions of *S.hadonii, H.magnifica* and *P.sinensis* against the mice was the highest at a concentration of 500µg and the minimal lethal dose was least for the *P.sinensis* extracts, the results are in par with the findings of the present study that the *S.hadonii* extracts exhibited 100% mortality at 750 mg concentration and a moderate type of inhibition was observed for the 500 and 250 mg concentration of the extracts and 25mg concentration of the *S.hadonii* extracts did not produced any cidal activity indicating that the crude extracts of sea anemone extracts at higher concentration was able to produce good insecticidal activity. Conclusively the extracts obtained from *S.hadonii* and *A.elegantissima* at lesser concentration have showed great activity when compared with that of RTX toxins isolated from *R.macrodactylus* anemones reported to be toxic to mice at 3000,1650,25,40 and 350 µg/kg concentration (Zykova *et al*., 1988a; Odinokov *et al*.,1989). Also the present result coincides with the result of (Gnanambal *et al*., 2006 and Chellaram, 2007) whose extracts like sea grass and mollusk were also showed insecticidal activity at increased concentration. Venkateswara Rao, (2007) recorded bio-active sponge crude extracts, which possess larvicidal and insecticidal activities As a conclusion, the crude fractions of the sea anemones, *Anthopleura elegantissima* and *Stichodactyla hadonii* possess assured insecticidal ethics with lower LC_{50} value and it might be assumed that extracts contain insecticidal compounds. Plants, insects and other organisms have co-existed for more than three hundred million years. During this time, plants have been under a continuous selection pressure from predators and numerous environmental factors. From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumoral compounds in terrestrial plant extracts (Solis *et al*., 1993; Mayer *et al.,* 1982; Mackeen *et al*., 2000). Brine Shrimp Test (BST) is known as a low-cost test indicative of antibacterial, cytotoxic, pesticidal, and insecticidal activity and could be used as a simple method for screening antibacterial products. The very low hatching rate detected after the 12h treatment was probably due to an alteration in the development of *Artemia* embryos. In the present study the diethyl ether extracts of *A.elegantissima* exhibited 100% mortality at 750mg concentration (10±0) followed by butanol (9.00±1.00), acetone (9.00±1.000, ethyl acetate (8.67±0.58) and DMSO extract exhibited (7.67±0.58) mortality. 50 % mortality was shown by the 100mg concentration of the diethyl ether, butanol and acetone extracts at 24 hours of incubation. Thus the 100 mg concentration of the sea anemone extract was enough to carry out the mortality of the insects and pests. However, most of the invertebrates presented toxicity in some of the bioassays at 1000 µg/ml in a way that was consistent with the cytotoxicity results. It has also been shown that *Artemia* is highly vulnerable to toxins at the early developmental stages (Sorgeloos *et al*.,1978; Sleet and Brendel,1985). In contrast, in the brine shrimp lethality test, maximum sensibility was reached after 48 h of exposure (the oldest age class tested by us) Sanchez-Fortun *et al*., (1996). At this stage in their life cycle the nauplii have reached their second and third in star and exhibit their greatest sensitivity to test compounds. The present results may be useful as blue prints to isolate the active principles from these active crude extracts.

CONCLUSIONS

In conclusion, these results indicated that crude (acetone, diethylether, butanol and hexane) extracts of *Anthopleura elegantissima* and *Stichodactyla hadonii* screened for Artemia toxicity and cytotoxic activity against the tested insects, diethyl ether extracts may possess some biologically active compounds. The activity of the butanol and ethyl acetate were also found to be prominent in comparison to the diethyl ether extracts. The column purified 80:20 chloroform: acetone extracts and 100% acetone extracts of *Anthopleura elegantissima* and *Stichodactyla hadonii* produced promising results at 500mg concentration. So the diethyl ether extracts from *A. elegantissima* and *S.hadonii* undeniably provide as a tool for the development of novel toxic compounds. Use of these invented novel products in mosquito control instead of synthetic insecticides and toxicants could reduce the environmental pollution. Further studies on identification of active compounds, toxicity and field trials are needed to recommend the active fraction of these anemone extracts for development of eco-friendly chemicals for the control of insect vectors.

Conflict of Interest declaration

The author declares no conflict of interest.

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