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Larvicidal, ovicidal and repellent activities of marine sponge *Cliona celata* (Grant) extracts against *Anopheles stephensi* Liston (Diptera: Culicidae)

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ABSTRACT

Objective: To evaluate the larvicidal, ovicidal and repellent properties of solvent extracts of marine sponge *Cliona celata* (*C. celata*) (Grant) against the malarial vector *Anopheles stephensi* (*An. stephensi*) Liston. **Methods:** Marine sponge *C. celata* was thoroughly washed with distilled water and shade dried for 48 h. Then the sponges were homogenized and extracted sequentially with hexane, ethyl acetate and methanol. Larvicidal and ovicidal activities were tested at four different concentrations *viz*., 62.5, 125.0, 250.0 and 500.0 ppm. For repellent study extracts were taken in three different concentrations *viz*., 5.0, 2.5, 1.0 mg/cm^2 at. **Results:** Among the three solvent extracts of *C. celata*, methanol extract showed the highest larvicidal activity at 500 ppm against the fourth instar larvae of *An. stephensi*. The LC_{50} and LC_{90} values of *C. celata* methanol extract were recorded as 80.61 and 220.81 ppm against *An. stephensi* larvae respectively. High ovicidal activity of 91.2% was recorded at 500 ppm concentration of methanol extract. The haxane extract was found to be the most effective protectant against the adult female mosquitoes of *An. stephensi*. The mean protection time recorded in hexane extract was up to 245 min at 5 mg/cm^2 dosage against *An. stephensi* adults. **Conclusions:** The screening results suggest that the hexane and methanol extracts of *C. celata* are promising in mosquito control. Considering these bioactivities, *C. celata* could be probed further to obtain some novel pesticidal molecules.

1. Introduction

Mosquitoes are medically important arthropod vectors and most dangerous human health pests. Malaria remains the most important vector-borne parasitic disease in the world[1]. *Anopheles stephensi* (*An. stephensi*) Liston (Diptera) is widespread in tropical and subtropical regions; it is the primary vector of malaria in India and other West Asian countries[2]. Each year, more than 90% of malaria deaths occur in Africa, primarily among young children. The number of cases outside tropical Africa may be as high as 20 million, with about 80% being found in Asia where severe drug resistance has developed both in parasite and vector[3]. Therefore, improved and effective methods are urgently needed to control vector mosquitoes[4]. Natural insecticides are generally pest specific, biodegradable, usually non-allergic to human as well as non-target organisms[5], and may possess novel compounds with a wide range of activities[6,7]. In recent years, plant products and phytochemicals have been studied for the control of mosquitoes[8-13]. Likewise, some investigators have reported that the secondary metabolites of marine sponges possessed insecticidal activities[14-17].

Sponges (Phylum: Porifera) are the oldest metazoan organisms and have been recognized as rich source of biologically active compounds that are of potential interest to mankind[18,19] and are good alternatives for synthetic pesticides[20]. They are a potential source of novel antimicrobial agents[21]. Till date nearly 8 000 species of sponges have been described throughout the world and 108 species of sponges have been identified in Gulf of Mannar, India[22]. Till date, very few species of sponges have been

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studied for their mosquito larvicidal activity[23-26]. However, studies on mosquitocidal properties of marine sponges from Indian waters are limited and the larvicidal, ovicidal and repellent efficacy of the marine sponge *Cliona celata* (*C. celata*) (Porifera: Hadromeridae: Clionidae) has not been studied previously against *An. stephensi*. Hence the present study was undertaken to screen the crude extracts of marine sponge *C. celata* from the Gulf of Mannar for their larvicidal, ovicidal and repellent activities against the malarial vector mosquito *An. stephensi*.

2. Materials and methods

2.1. Collection of sponges

Marine sponge *C. celata* was collected from the Gulf of Mannar (between 8°47′ to 9°05′ N Latitude and 78°12′ to 79°07′ E Longitude, India) during March 2012 at depths varying from 15-25 feet by scuba-diving. Sponges were gently removed from the substratum and transferred to laboratory within 48 h; then the specimen was identified and deposited (deposition number: MBRC/ZSI-S.225) in National Zoological Collection of Marine Bio Resource Centre, Zoological Survey of India (13°04′ N Latitude and 80°17′ E Longitude, India).

2.2. Preparation of crude extracts

The Marine sponge *C. celata* was thoroughly washed with distilled water to remove the sand particles and cut into small pieces before shade drying for 48 h. Then the sponges were homogenized and extracted sequentially with hexane, ethyl acetate and methanol. Initially the powder of the *C. celata* (500 g) was soaked in 1 L hexane for 72 h and filtered through filter paper. The residue was dried and then extracted sequentially with ethyl acetate and methanol solvents after 72 h of soaking in each solvent separately. The extracts were condensed separately under reduced pressure by using vacuum evaporator and the solvent free crude extracts were collected in glass vials and stored in 4 ℃ until use. Stock solutions (10% in acetone) of all the three solvent extracts were prepared and then subjected to bioassay screening.

2.3. Test insect

Eggs, larvae and adults of *An. stephensi* were obtained from the stock culture maintained at Entomology Research Institute, which were free of exposure to pathogens, insecticides or repellents. Laboratory rearing was done at a temperature of (27±2) ℃, 75%-85% relative humidity and a photoperiod of (11.0±0.5) h.

2.4. Larvicidal bioassay

Larvicidal activity was evaluated by following the methods of World Health Organization[27]. Twenty numbers of early fourth instar larvae of *An. stephensi* were introduced into the test containers. The extracts taken in four concentrations were 500.0, 250.0, 125.0 and 62.5 ppm. Acetone in water was used as solvent control. Mortality and survival rate were registered after 24 h exposure period. The moribund and dead larvae were collected, and larval mortality was calculated for each concentration. The bioassays were performed at a room temperature of (27±1) ℃ with five replicates for each concentration. Mortality was converted into percent mortality (a) and corrected mortality was calculated using Abbot's formula (b)[28].

$$\text{Percentage of mortality (a):} \quad \frac{\text{no. of dead larvae}}{\text{no. of larvae introduced}} \times 100$$

$$\text{Corrected percentage of mortality (b):} \quad \frac{\text{1-n in T after treatment}}{\text{N in C after treatment}} \times 100$$

Where, n is the number of larvae, T-treated and C is the control.

The corrected percentage mortality value of each concentration was considered to estimate LC_{50} and LC_{90} values using SPSS Probit analysis statistical pack, version 11.5.

2.5. Ovicidal bioassay

Ovicidal activity was evaluated by following the method of Elango *et al*[29] with slight modification. Twenty five freshly laid eggs of *An. stephensi* were treated separately with *C. celata* extracts at 62.5, 125.0, 250.0 and 500.0 ppm concentrations. Acetone in water was served as control. Each treatment was replicated five times. The ovicidal activity was assessed up to 120 h post treatment and thereafter control and treated eggs were observed under the microscope and photographed using stereo zooming microscope (Wild M7S TYP 308700, Switzerland). The non-hatched eggs with unopened opercula were counted in each treatment and the percent mortality was calculated using the following formula and analysed in Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, CA, U.S.A.

$$\%\ \text{of egg mortality} = \frac{\text{No. of unhatched eggs}}{\text{Total No. of eggs}} \times 100$$

2.6. Repellent bioassay

For repellent experiment, 3 and 6 days old hundred laboratory reared blood-starved adult female mosquitoes were introduced into separate laboratory cages (45 cm×45 cm×40 cm). Experiments with *An. stephensi* were conducted in the night time between 19.00 pm to 24.00 pm. Before each test, the forearms of a human subject were washed with unscented neutral soap, thoroughly rinsed, and allowed to dry before the application of the extract at 5.0, 2.5, 1.0 mg/cm^2 concentration. The *C. celata* extracts being tested were applied on the right upper forearm and remaining regions were covered with gloves. The arm was left undisturbed. The left arm served as control. N-N Diethyl benzamide (12%, w/w) was used as negative control. The mosquito bites were observed for three full minute of every fifteen minutes. Protection time was recorded as the time elapsed between extract application and the observation period immediately preceding that in which a confirmed bite was obtained. The experiments were replicated five times in separate cages and in each replicate different volunteer were used to nullify any effect of skin differences on repellence. The protection time of each extract was calculated using previously established methods[30,31].

2.7. Statistical analysis

The results were presented as mean±SD. Statistical analyses of all the data obtained in larvicidal activity were evaluated using SPSS (version 11.5). The differences were considered as significant at $P<0.05$. Percent ovicidal activity was analysed in Graph Pad Prism version 3.0.

3. Results

3.1. Larvicidal activity and lethal doses

All the three extracts of the sponge *C. celata* showed larvicidal activity. The larvicidal activity varied between the solvent extracts. Table 1 shows the results on effective lethal concentration (LC_{50} and LC_{90}) values of hexane, ethyl acetate and methanol extracts of *C. celata* after 24 h treatment period. It was clear from the results that the methanol extract recorded the maximum larvicidal activity against *An. stephensi*. The LC_{50} and LC_{90} values of methanol extract were 80.61 and 220.81 ppm respectively. Significant *chi*-square values were recorded in all the extracts (Table 1). In control all the larvae were active and exhibited normal movement. But in the treated, restless movement was observed. After 1 h tremor and convulsion were observed in all the treated larvae and dead larvae settled down as reported earlier[12].

3.2. Ovicidal activity

The ovicidal activities of *C. celata* extracts on *An. stephensi* eggs are given in Figure 1. Methanol extract was found to be highly lethal to the eggs of *An. stephensi*. Methanol extract presented 91.2% ovicidal activity at 500 ppm concentration in 120 h post treatment period. The lowest concentration (62.5 ppm) of methanol extract caused 18.4% egg mortality against the eggs of *An. stephensi*. The ovicidal effect of all the three extracts was directly proportional to the concentration. In methanol extract treated *An. stephensi* eggs, most did not hatch and some hatched in an abnormal way at 500 ppm and the larvae died before completion of eclosion.

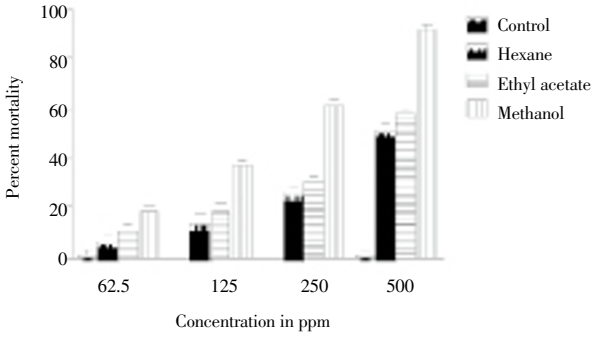

Figure 1. Ovicidal activity of marine sponge *C. celata* extracts against the eggs of *An. stephensi*.

3.3. Repellent activity

The complete protection times for all the three extracts of *C. celata* against *An. stephensi* mosquitoes were recorded by standard skin repellence experiments and the results are given in Tables 2. The repellence was directly proportional to the dose and protection time (min) for each extract showed

Table 1
Lethal concentrations (in ppm) of *C. celata* extracts against *An. stephensi*.

Mosquito species	Treatment	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
An. stephensi	Hexane	264.55	145.15	435.79	948.19	533.50	7 664.57	2.3 ± 0.7	−0.6 ± 1.7	0.08*
	Ethyl acetate	225.81	147.20	335.16	769.08	469.87	2 754.61	2.4 ± 0.6	0.6 ± 1.4	0.40*
	Methanol	80.61	46.59	109.42	220.81	158.60	445.74	2.9 ± 0.7	−0.5 ± 1.5	0.50*

LC_{50} lethal concentration that kills 50% of the exposed larvae, LC_{90} lethal concentration that kills 90% of the exposed larvae, LL lower limit (95% confidence limit), UL upper limit (95% confidence limit). *$P<0.05$, level of significance of *chi*-square values.

variations against *An. stephensi* mosquitoes. In general hexane extract gave maximum protection time against *An. stephensi* compared to ethyl acetate and methanol extracts. Furthermore, it was noted that all the three extracts were found to be more effective against *An. stephensi* mosquitoes. Hexane extract gave a maximum protection time of 245 min against *An. stephensi* at a dose 5 mg/cm^2 (Table 2). These results were comparable with negative control (N-N Diethyl benzamide 12%, w/w), which showed maximum of 282 min protection at 5 mg/cm^2 dosage against *An. stephensi* mosquitoes.

Table 2
Complete protection time of three solvent extracts of *C. celata* against *An. stephensi*.

Extract	Concentration (mg/cm^2)	Complete protection time (min)	
		Control	Treated
Hexane	1.0	1.80 ± 0.44	95.00 ± 3.16
	2.5	2.40 ± 0.89	140.00 ± 2.91
	5.0	2.20 ± 1.09	245.00 ± 2.82
Ethyl acetate	1.0	2.00 ± 0.70	42.00 ± 2.23
	2.5	1.50 ± 0.44	78.00 ± 1.87
	5.0	1.60 ± 0.54	146.00 ± 2.91
Methanol	1.0	2.20 ± 0.30	24.00 ± 2.44
	2.5	2.00 ± 1.41	65.00 ± 1.41
	5.0	2.20 ± 0.44	103.00 ± 2.34
N-N Diethyl	1.0	1.20 ± 0.44	73.00 ± 1.58
benzamide 12%	2.5	2.00 ± 0.70	151.00 ± 2.00
	5.0	1.60 ± 0.89	282.00 ± 1.58

Each value represents mean of five replicates ± SD.

4. Discussion

Searching for eco-friendly pesticide molecules from natural sources has become an important research these days. Nature is providing innumerable bioactive molecules for the well-being of mankind. Plant kingdom and marine organisms provide majority of the beneficial biomolecules or products that are used by people in their daily life. Marine sponges are known to produce toxins and other compounds to repel and deter predators[32,33]. These compounds of marine sponges are reported to be bioactive compounds which can be used in the treatment of many diseases[34-38].

In recent years, researchers are concentrating on marine organisms to study their biological activities; especially marine sponges (Porifera) have attracted significant attention from various scientific disciplines[39]. The literature also indicates that the marine products possess maximum percentage of bioactive substances with novel biological properties than the molecules originating from terrestrial origin[40,25]. Recently, marine sponge extracts and their compounds have been screened for anti-microbial[41,42], anti-plasmodicidal[43], anti-filarial[44,45] and anti-helminthic activities[46]. Marine sponge extracts have also been screened against agricultural pests. Edrada *et al*[47] have reported an insecticidal and growth regulating compound from the philippine marine sponge *Xestospongia ashmorica* against *Spodoptera littoralis*, an important polyphagous pest. Supriyano *et al*[48] have reported that two guanidine alkaloids namely hymenialdisine and debromohymenialdisine from the tropical marine sponge *Axinella carteri* which exhibited insecticidal activity with LD_{50} value of 88 and 125 ppm respectively, against the neonate larvae of *S. littoralis* in feeding bioassay experiments.

In this regard, very few researchers have studied the mosquito larvicidal potential of marine sponge extracts. Venkateswara Rao *et al*[24] have screened the methanol-dichloromethane (1:1) extracts of 18 different sponges collected from Palk bay and Gulf of Mannar waters against the larvae of *Aedes aegypti* (*Ae. aegypti*) and houseflies (*Musca domestica*). They found that *Psammaplysilla purpurea* (LC_{50}=25.9 ppm) and *Haliclona cribricutis* (LC_{50}=31.46 ppm) were more effective against *Ae. aegypti* mosquito larvae. In our study we observed that the methanol extract of *C. celata* was the most effective as larvicide and the LC_{50} of methanol extract against *An. stephensi* was found to be higher (80.61 ppm). An interesting finding in this study was that the low polar solvent extract, *ie*. hexane extract showed repellent activity and the high polar solvent (methanol) extract showed larvicidal and ovicidal activities. This result was comparable with the earlier report of Sonia and Lipton[26] who had screened the methanol extracts of five marine sponges namely *Acanthella elongata, Echinodictyum gorgonoides, Axinella donnani, Callyspongia subarmigera* and *Callyspongia diffusa* for larvicidal activity against *Culex* sp. They found that methanol extract of *Acanthella elongata* was the most effective with a LC_{50} value of 0.066 mg/mL than other extracts.

In the present study all the three solvent extract treatments were not equally effective against *An. stephensi* larvae, eggs and adults. Aquatic life stages of eggs and larvae of *An. stephensi* were found to be more susceptible to methanol extract treatments and adults of *An. stephensi* were found to be more susceptible to hexane extract treatments. A similar result was reported by Martínez *et al*[23] who had screened the ethanol extracts of five marine sponges namely *Amphimedon compressa, Topsentia ophiraphidites, Svenzea zeai, Ircinia campana* and *Agelas sventres*, against fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. They found that *Ircinia campana* extract was the most effective against the larvae of two mosquitoes and the activity was higher in *Ae. aegypti* than in *Cx. quinquefasciatus*.

In conclusion, the present study reports for the first time

the repellent, larvicidal and ovicidal activity of marine sponge *C. celata* against *An. stephensi*. The screening results suggest that the hexane and methanol extracts of *C. celata* are promising in mosquito control. The isolation and identification of active principles present in this marine sponge species will be useful for developing an ecofriendly mosquito control product.

Conflict of interest statement

We declare that we have no conflict of interest.

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