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REVIEW ARTICLE

TOXICITY OF *GRACILARIA VERRUCOSA* METHONAL EXTRACT AGAINST SELECTED MEDICALLY IMPORTANT VECTOR MOSQUITOES (DIPTERA: CULICIDAE) OF PUDUCHERRY, INDIA

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ABSTRACT

Objective: To evaluate the larvicidal, ovicidal and repellent activities of methanol extract of *Sargassum ilicifolium (Phaeophyta), Caulerpa sertularoides (Chlorophyta) and Gracilaria verrucosa (Rhodophyta)* against Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus. **Methods:** The selected seaweed extracts were dissolved in DMSO to prepare graded concentrations. Larvicidal efficacy of selected seaweed were tested at various concentrations against the early third instar larvae of *Ae.aegypti, An.stephensi* and *Cx.quinquefasciatus*. Bioassay test was carried out as per the guidelines of WHO (2005). The 24h $LC5_0$ values of the selected seaweed extracts were determined by probit analysis. For ovicidal activity modified method of Su and Mulla was used. Ovicidal activity was determined against selected mosquitoes at various concentrations ranging from 25-200 ppm under laboratory conditions. The hatch rates were assessed 48h post treatment. The *Gracilaria verrucosa* repellent efficiency was determined against selected mosquitoes at three concentrations such as 1.0, 2.0 and 3.0 mg/cm^2 under laboratory conditions. **Results:** The $LC_{50\ and}$ LC_{90} values of methanol seaweed extract of *Gracilaria verrucosa* against early third instar larvae of *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* were 0.125, 0.131, 0.135; ppm, respectively. The crude extract of seaweed exerted 100% egg mortality (zero hatchability) at 240, 300 and 360 ppm for *Ae. aegypti, An.* stephensi and *Cx. quinquefasciatus*. Similarly, a higher concentration of 8 mg/cm^2 provide 100% protection up to 210, 180 and 150 min against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus*. **Conclusions:** The present results suggest that the *Gracilaria verrucosa* methanol seaweed extract provides an excellent potential for controlling selected medically important vector mosquitoes.

INTRODUCTION

Mosquitoes serve as a vector of several diseases, causing serious health problems to human; they transmit diseases such as yellow fever, human lymphatic filariasis, malaria and other several disease which are today among the greatest health problems in the world (Abdel – Hameed, *et al*., 1994). The present resurgence of these diseases is due to higher number of breeding places in today's throwaway society. Further the indiscriminate use of synthetic insecticides is creating multifarious problems such as environmental pollution, insecticide resistance and toxic hazardous to human beings. Synthetic insecticides such as organochlorine, organophosphourous, carbamates, pyrethrins and pyrethroids are commonly used for controlling the ever increasing population of vectors (Shaalan, 2012; Bilal *et al*., 2012). All these restrictions on the usage of synthetic pesticides have stimulated investigations for an environmentally safe, degradable and target specific insecticides against mosquitoes. Natural products of plant origin with insecticidal properties have been tried in the recent past for the control of variety of insect pests and vectors. Plants are considered as a rich source of bioactive chemicals (Kamaraj *et al*., 2008) and they may be an alternative source of mosquito control agents. Natural products are generally preferred because of their less harmful nature to non – target organisms and due to their innate biodegradability.

Plant products have been used traditionally by the human communities in different parts of the world against the vectors and species of insects. The phyto – chemicals derived from plant sources can act as larvicides and insect growth regulators and have deterrent activities observed by many researches. Marine halophytes are the specialized group of plants adopted for high saline conditions which include seaweed, mangrove and seagrass. The biodiversity of marine ecosystem provides important sources of chemical compounds, which have many therapeutic applications such as antiviral, antibacterial, antifungal, antifertility and anticancer activities (Kamaraj *et al*., 2011; Ravikumar *et al.*, 2010). They have been proven to have a rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential. The secondary metabolites synthesized by seaweed demonstrate a broad spectrum of bioactivity varying from neurologically active in humans to

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nematicidal and insecticidal in lower form of animals (Chen, 2010; Bazes *et al*., 2009). In this background, the present study was made as an attempt to find out the mosquito larvicidal efficacy of methanolic extracts of seaweed against *Aedes aegypti (Ae. aegypti), Culex quinquefasciatus (Cx.quinquefasciatus)* and *Anopheles stephensi (An. stephensi)* vectors.

MATERIALS AND METHODS

Plant Materials

Fresh sample of seaweed viz., *Gracilaria verrucosa (G. verrucosa)* were collected from South east coast of India (Latitude 12°15' N and longitude 79°36' E) Puducherry. The identified seaweed were authenticated by Central Marine Fisheries Research Institute (CMFRI), Chennai Research Station (Chennai). Voucher specimens were deposited in the herbarium. All the collected samples were washed thrice with tap water and twice with distilled water to remove the adhering salts and other associated animals.

Extract Preparation

Shade dried seaweed samples were subjected for percolation by soaking in methanol and water mixture (3:1). After 21days of dark incubation, the filtrate was concentrated separately by rotary vaccum evaporation (>45°c) and then freeze-dried (-80°c) to obtain solid residue. The percentage of extraction was calculated by using the following formula,

$$\% \text{ Extraction} = \frac{\text{Weight of extract}}{\text{Weight of the plant material}} \times 100\ \%$$

The extracts of seaweed were screened for the presence of phytochemical constituents by following the method of Sofowora (1982) and Kepam (1986).

Mosquito larval culture

To satisfy the enormous number of mosquitoes need for the day to day bioassays, a colony is essential. The eggs and egg rafts of *Cx. quinquefasciatus* were procured from Vector Control Research Centre, Puducherry. Filter paper attached with eggs was dipped into a plastic tray containing 500 ml of de-chlorinated water for 30-40 min, time enough to allow for eggs to hatch into larvae. They were reared indoors at (28±2)°C temperature and 14:10 light and dark period cycle. The larvae were fed with powdered mixture of dog biscuits and yeast powder in 3:1 ratio. After five days emergence, female mosquitoes were moved into a mosquito cage where the emergent adults were fed with a 100 g/l sucrose solution and allowed for blood feed using white albino for 2.3h. A few days after having a blood meal, the gravid mosquito laid their eggs.

Larvicidal activity

The test for the larvicidal effect of methanolic extract derived from seaweed against mosquitoes larvae *Cx. quinquefasciatus* was conducted in accordance with the WHO standard method (WHO, 2005). Each seaweed extract was dissolved in DMSO to prepare a graded series of concentration. Batches of 25 early 4th instar larvae of three mosquitoes (*Cx. quinquefasciatus*) were transferred to 250ml enamel bowl containing 199ml of distilled water and 1ml of *Gracilaria verrucosa* extracts (10-100µg). Each experiment was conducted with three replicates and a concurrent control group. A control group consisted of 1ml of DMSO and 199 ml of distilled water only. After treatment, symptoms in treated larvae were observed and recorded immediately with at time intervals and no food was offered to the larvae. At the end of 24 h, the larvae were considered dead, they showed no sign of swimming movements even after gentle touching with a glass rod, as described in the WHO'S technical report series. Subsequently, the lower concentration of crude extract that had successfully produced more than 50% larval mortality rate was used in a toxicity test on a non-target organism. The percentage of mortality was calculated using Abbott's formula (Abbott, 1987).

$$\%\text{Mortality} = \frac{\%\text{ Test mortality- \% control mortality}}{100 - \%\text{ control mortality}} \times 100\ \%$$

Ovicidal activity

The method of Su and Mulla, (1998) was slightly modified and used to test the ovicidal activity. The various concentrations as stated in the previous experiments were prepared from the stock solution. Before treatment, the eggs of selected mosquitoes were counted individually with the help of hand lens. Freshly hatched eggs (100) were exposed to each concentration of seaweed extract until they hatched or died. Eggs exposed to DMSO in water served as control. After treatment, the eggs from each concentration were individually transferred to distilled water cups for hatching assessment after counting the eggs under a microscope. Each test was replicated five times. The hatchability was assessed at 48h of post treatment.

Repellent activity

The repellent study was following the methods of WHO (WHO,1996). 3-4 days old blood-starved female selected mosquitoes (100) were kept in a net cage (45cmx 45cmx 40cm). The volunteer had no contact with lotions, perfumes or perfumed soaps on the day of the assay. The arms of the test person were cleaned with isopropanol. After air drying the arm only 25 cm^2 of the dorsal side of the skin on each arm was exposed, the remaining area being covered by rubber gloves. The seaweed extract was dissolved in isopropanol and this alcohol served as control. The seaweed leaf extract at 1.5, 3.0 and 6.0 mg/cm^2 concentration was applied. The control and treated arms were introduced simultaneously into the cage. The numbers of bites were counted over 5 min every 30 min. The experiment was conducted five times. It was observed that there was no skin irritation from the seaweed extract. The percentage protection was calculated by using the following formula.

$$\% \text{ Repellency} = [(Ta-Tb)/ Ta] \times 100$$

Where Ta is the number of mosquitoes in the control group and Tb is the number of mosquitoes in the treated group.

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating $Lc_{50,}$ Lc_{90} and other statistics at 95% confidence limits of upper confidence limit and lower confidence limit and lower confidence limit and chi-square value were calculated using the SPSS software package 12.0. Results with $p<0.05$ were considered to be statically significant.

RESULTS

The percentage yields of extracts of *G. verrucosa* was found to the 1.43 from a weight of 82g. The toxicity of methanol seaweed extract of *Gracilaria verrucosa* was tested against larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*. The data were recorded and statistical data ranging $LC_{50,}$ LC_{50}, LCL, UCL and chi – square value were calculated.

Table 1. Larvicidal activity of crude methanol extract of *Gracilaria verrucosa* against *Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus.*

Mosquitoes	Concentration ppm	24th mortality (%)	95% Confidence Limits (ppm) LC_{50} (LCL-UCL)	LC_{90}	R^2	P - Value
	60	52.0±1.2[b]				
	120	72.0±1.3[c]	0.44±0.010			
Ae. aegypti	180	85.4±1.2[d]	(0.023-0.104) 0.055	0.098	7.761	0.032
	240	98.9±1.7[e]				
	Control	0.0±0.0[a]				
	60	41.7±1.5[b]				
	120	63.2±1.2[c]	0.091±0.013 (0.071-0.112)			
An. stephensi	180	73.5±1.3[d]	0.074	0.132	9.156	0.728
	240	96.0±1.2[e]				
	Control	0.0±0.0[a]				
	60	37.4±1.4[b]				
	120	57.6±1.2[c]	0.066±0.134 (0.023-0.010)			
Cx. quinquefasciatus	180	73.0±1.2[d]	0.087	0.154	6.234	2.178
	240	93.8±1.7[e]				
	Control	0.0±0.0[a]				

Each value mean ±SD represents mean of six values. Values in a column with a different superscript alphabet are *significantly different at $p<0.05$ (MANOVA; LSD-Tukey's test). LCL- Lower confidence limit; UCL- Upper confidence limit.

Table 2. Ovicidal activity *of Gracilaria verrucosa* extracts against *Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus.*

Mosquitoes	Percentage of egg hatch ability, concentration (ppm)						
	Control	60	120	180	240	300	360
Ae. aegypti	100.0±0.0	51.2±2.1	35.4±1.7	17.6±1.2	NH	NH	NH
An. stephensi	100.0±0.0	79.8±1.8	51.4±1.5	38.6±1.4	20.2±1.2	NH	NH
Cx. quinquefasciatus.	100.0±0.0	87.3±1.6	63.7±1.3	44.7±1.2	38.7±1.3	15.4±1.1	NH

Each value mean ±SD represents mean of six values. NH – No hatchability (100 % mortality)

Table 3. Repellent activity of crude methanol extract of *Gracilaria verrucosa* against *Aedes aegypti, Anopheles stephensi and Culex quinquefasciatus.*

Mosquitoes	Concentration ($mg/cm^{2)}$	% of repellency, Time post application of repellent(min)							
		30	60	90	120	150	180	210	240
Ae. aegypti	1.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	80.2±1.4	70.0±1.1	60.1±1.3
	1.5	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	86.5±1.0	65.3±1.6
	3.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	90.1±1.4
An. stephensi	1.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	92.1±1.5	74.3±1.0	60.4±1.2	44.2±1.7
	1.5	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	90.1±1.1	73.2±1.6	60.1±1.3
	3.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	90.7±1.2	86.1±1.6
Cx. quinquefasciatus	1.0	100.0±0.0	100.0±0.0	100.0±0.0	90.2±1.6	82.2±1.2	100.0±0.0	49.8±1.3	35.1±1.1
	1.5	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	90.1±1.0	75.0±1.3	60.7±1.4	42.1±1.5
	3.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	91.0±1.2	82.2±1.6	74.1±1.2

Each value mean ±SD represents mean of six values.

Results on the larvicidal, ovicidal and repellent effects of seaweed extract were reported. The present study confirm their potential for control of the mosquito populations (Table. 1 – 2). The LC_{50} and LC_{90} values of methanol seaweed extract of *Gracilaria verrucosa* against early fourth instar larvae of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* were 0.055, 0.074 and 0.087ppm respectively. The crude extract of *Gracilaria verrucosa* exerted of 100% egg mortality (zero hatchability) at 240, 300 and 360ppm of *Ae. aegypti*, *An stephensi* and *Cx. quinquefasciatus*. Similarly, a high concentration of 6.0/cm^2 provide 100% production up to 210, 180 and 150 min. against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*. This study show that *Gracilaria verrucosa* extract would be a potent source of natural larvicidal, ovicidal and repellent activities against selected medically important mosquito species.

DISCUSSION

The present results showed that, crude extract of seaweed *Gracilaria verrucosa* have significant larivicidal, ovicidal and repellent activities against selected medically important vector mosquito species. The results are comparable with an earlier report by Poonghuzali, (2011); Ravikumar (2010) and Syed Ali (2013) the LC_{90} values of ulva lactuca root extract for *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* were 132.55, 27.82 and 11.68ppm, respectively whereas those of *Anacardium occidentale* leaf extract were 56.81, 912 and 11.68ppm, respectively. Screening of natural products for mosquito larvicidal activity against three major mosquito vectors *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* resulted in the identification of eight potential seaweed extracts from viz., *Ulva lactuca*, *C. racemosa*, *S. myriocystum*, *C. scalpelliformis*, *G.corticata*, *T. decurrens*, *C. toxifolia*, *T. conoides* for mosquito larval control (Syed Ali, 2013), and the studies on larvicidal activities with seaweed extracts are too restricted (Subhash *et al.*, 2011; Aziz *et al*., 2011); hence, the present study was investigated with *Gracilaria verrucosa* seaweed extracts for mosquito larval control.

Result of present investigation indicate that *Gracilaria verrucosa* was maximum larvicidal activity against *Aedes aegypti*, *Anopheles stephensi and Culex quinquefasciatuas*. Similarly, Tawatsin *et al.*, (2001) have reported the repellent and larvicidal activity against *Aedes aegypti*, *Anopheles stephensi and Culex quinquefasciatuas* which is due to 5% vanillin which has been added to the essential oil of *Curcuma longa* (Tawatsin *et al*., 2001). Sosan *et al.*, (2001) reported that larvicidal activities of essential oils of *Gracilaria verrucosa* (against *Ae. aegypti* and achieved 100% mortality at 120, 200, and 300 mg/l concentrations respectively. Similarly, antifungal (Ravikumar *et al.*, 2009; Sotomon, 2008; Patra *et al.*, 2008), antiviral (Wang *et al*., 2008; Vallim *et al*., 2010), anti – inflammatory (Tan *et al*., 2000; Margret, 2000), cytotoxic (Jimenez *et al*., 2010; Manilal *et al*., 2009), nematicidal (Manilal *et al*., 2009; Rizvi, 2006), antifeedant and larvicidal (Manilal *et al.*, 2009), phytotoxic (Jimenez *et al.*, 2010) and anticoagulant activities (Ganesh *et al*., 2009) have been reported. The biological activity of this marine seaweed extracts might be due to various compounds, including phenolic, terpenoides, flavonoids, saponins and alkaloids existing in seaweed. These compounds may jointly or independently contribute to produce larvicidal activity against both species of mosquitoes (Ravikumar *et al*., 2010).

It is concluded from the present study that the seaweeds, which were collected from coastal area of Puducherry, reneable enormous resources to find out the new marine product with mosquito larvicidal activities. Further studies on synergistic combinations and isolation of bioactive fraction/constituent may provide futuristic lead products for field application of mosquito control.

Conflict of interest statement

We declare that we have no conflict of interest.

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