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Combined effect of seaweed (*Sargassum wightii*) and *Bacillus thuringiensis* var. *israelensis* on the coastal mosquito, *Anopheles sundaicus*, in Tamil Nadu, India

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ABSTRACT: Laboratory studies were made to determine the effectiveness of seaweed (*Sargassum wightii*) extract combined with *Bacillus thuringiensis* var. *israelensis* for the control of *Anopheles sundaicus* Liston, a malaria vector that occurs in the coastal areas of peninsular India. The results revealed that the different concentrations of crude extract of *S. wightii* resulted in considerable mortality and the LC_{50} value for I Instar larvae at 1.0 mg/l was 0.88, for II Instar 0.73, for III Instar 1.34, for IV Instar 1.56, and for pupa 1.71. The LC_{90} values of I, II, III, and IV Instar, and pupa were 2.73, 2.43, 3.03, 3.21, and 3.23 mg/ml, respectively. Among the larval instars, instar II was the most susceptible. A considerable repellency was noted; a 10 mg/l concentration of *S. wightii* showed a repellency of 89%. Sea weed extract and *B. thuringiensis* toxins affected the larval duration and adult emergence. A synergistic factor was also found for the effect of seaweed extract against larvae and pupae of mosquito. The synergistic factor showed at I Instar was 1.74, II Instar was 1.93, III Instar was 1.37, IV Instar was 1.27, and pupa was 1.24, respectively. The result revealed that the seaweed extract of *S. wightii* in combination with microbial toxins has interfered in the gut system and resulted in mortality as well as growth inhibitory effects on mosquitoes.

KEYWORDS: larvicidal activity, repellency, vector control, malarial vector

INTRODUCTION

Mosquitoes are important blood sucking insects. They transmit disease agents that cause malaria, dengue, yellow fever, encephalitis, and filariasis. Human malaria occurs mainly in tropical and subtropical regions of the world and is caused by infection with *Plasmodium falciparum*, *P. vivax*, *P. ovale*, or *P. malariae*[1], which are transmitted to humans only by *Anopheles* mosquitoes.

Mosquito control is critical for managing the spread of disease agents they transmit and is based primarily on the use of conventional synthetic chemical insecticides. The effectiveness of these chemicals for vector control is diminished when mosquitoes develop resistance to the insecticide(s)[2]. Additionally, the use of synthetic chemical insecticides presents the potential for environmental pollution and some evidence suggests these materials act as immunosuppressants[3].

Interest in alternatives to synthetic chemical insecticides for mosquito control has resulted in the evaluation of plant extracts with insecticidal activity. Advantages to the use of botanical insecticides include safety to humans and animals, rapid breakdown of the toxic molecules in the environment, and comparatively few adverse effects to nontarget organisms.

A number of extracts from terrestrial plants have been studied for toxicity to larvae of *Anopheles* mosquitoes, including those from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus* (Acanthaceae), *Solanum suratense* (Solanaceae), *Samadera indica* (Simaroubaceae), and *Myriophyllum spicatum* (Haloragaceae)[4,5]. *Sargassum wightii* Greville (Sargassaceae) is an abundant marine brown alga commonly found in the shorelines of India. It is a macroscopic, multicellular, photosynthetic, non vascular, pelagic marine species[6] rich in sulphated polysaccharides that manifest potent free radical scavenging[7] and antioxidant[8] effects. These properties justify *S. wightii* investigations for searching biologically active compounds that may be useful in mosquito control and as alternatives to conventional synthetic

insecticides.

Bacillus thuringiensis var. *israelensis* (*Bti*) is a naturally occurring bacterium that kills certain arthropod species. When ingested by a mosquito larva, the *Bti* parasporal body dissolves in the alkaline gut juices where midgut proteases cleave the protoxin yielding active δ-endotoxin proteins. These endotoxins bind to specific receptors, disrupt the activity of midgut epithelial cell membranes, and damage the gut wall leading to rapid death from starvation[9]. This study aimed to determine the combined effect of *Sargassum wightii* extract and *Bacillus thuringiensis* var. *israelensis* as a mosquito larvicide and/or pupicide, as a regulator of growth in immature mosquitoes, and as a repellent to host seeking female *Anopheles sundaicus* Liston.

MATERIALS AND METHODS

Collection of mosquito eggs, mosquito rearing, and blood feeding

The eggs of *A. sundaicus* were collected (using an 'O' type brush) from drinking water containers located in coastal areas of Velankanni (79.8° E, 10.7° N), Nagapattinam (79.8° E, 10.7° N), and Cuddalore (79.4° E, 11.4° N), Tamil Nadu, India. In the laboratory, eggs were transferred to 18 cm L × 13 cm W × 4 cm H enamel trays containing 500 ml of tap water. Larvae were reared at 27 ± 2 °C, 75–85% RH, in a 14:10 (light:dark) photoperiod and fed ground dog biscuit and brewers yeast in a 3:1 ratio until pupation.

Pupae were collected from the rearing trays using a pipette and transferred to plastic containers (12 cm H × 12 cm D) containing 500 ml of water. Plastic containers with pupae were placed inside 90 cm H × 90 cm L × 90 cm W cages prior to emergence of the adult mosquitoes. Each cage consisted of a wooden frame covered with polythene on the sides, back, and top. Adults were allowed access to 10% sucrose solution ad libitum via cotton wick. Female mosquitoes were fed stored human blood using methods described[10].

Collection and extraction of *S. wightii*

S. wightii was collected at the Gulf of Mannar Biosphere Reserve (GoMBR) on the SE coast of Tamil Nadu, India from an area between 8° 49′ N and 9° 15′ N and 78° 11′ E and 79° 15′ E. Each specimen was washed with water and dried at room temperature. Dried material was ground into powder (100–150 μm) using a blender. One hundred grams of the powder was extracted with 300 ml of methanol for 8 h in a Soxhlet apparatus and the extract was dried in a rotary vacuum evaporator to yield *S. wightii* extract residue. One gram of the residue was dissolved in 100 ml of acetone to make a 1% stock solution. Five different concentrations of the extract (2, 4, 6, 8, and 10 mg/l) were prepared from the stock solution for testing.

Preparation of *B. thuringiensis* var. *israelensis*

The *Bti* was obtained from T-Stanes & Company Pvt. Limited, Coimbatore, India. Five concentrations, i.e., 0.25, 0.50, 1.0, 1.5, and 2.0%, were prepared by diluting the original material with distilled water.

Larval toxicity test of *S. wightii*

Twenty-five I Instar, II Instar, III Instar, and IV Instar instars were placed in 249 ml of dechlorinated water in separate 500 ml glass beakers followed by the additional of 1 ml of the desired concentration of *S. wightii* extract. Food (see before) was provided for larvae in each test. At five tested concentrations, 3 trials were made and each trial consisted of three replicates. In each test, the control comprised of 1 ml of acetone mixed with 249 ml of de-chlorinated water. Correction for control mortality was made using Abbott's formula[11]: Mortality = (Number of dead larvae)/(Number of larvae introduced).

LC_{50} and LC_{90} were calculated from toxicity data by using probit analysis[12].

Pupal toxicity test of *S. wightii*

A laboratory colony of mosquito pupae were used for pupicidal bioassay. Twenty freshly emerged pupae were kept in 500 ml glass beaker containing 249 ml of de-chlorinated water and 1 ml of desired *S. wightii* concentration was added separately. Three replicates were set up for the five tested concentration and a control set up by mixing 1 ml of de-chlorinated water. The control mortality was corrected by Abbot's formula[11]: Mortality = (Number of dead pupae)/(Number of pupae introduced).

LC_{50} and LC_{90} were calculated from toxicity data by using probit analysis[12].

Test for growth regulatory activity

Methanolic extracts of *S. wightii* and *Bti* were tested for larval, pupal, and adult development activity against recently hatched I Instar *A. sundaicus*[13]. The test of *S. wightii* for development activity was drawn at five different concentrations ranging from 1–10 mg/l, *Bti* from 0.25–2 mg/l, and the desired concentration of the test solution achieved by adding 1.0 ml of an appropriate stock solution to 249 ml of dechlorinated water. Three replicates for each concentration were set up. All larvae were monitored to adult emergence

Table 1 Combined effect of methanolic extract of *Sargassum wightii* Greville and microbial insecticide, *Bacillus thuringiensis* var. *israelensis* at various concentrations on larvicidal and pupicidal activity against coastal malarial vector, *Anopheles sundaicus*.

Larval & pupal stages	Larval and pupal mortality (%) Combined concentration of *S. wightii* and *Bti* (mg/l) 2.00+0.25	2.00+0.50	2.00+1.00	2.00+1.50	2.00+2.00	Value of LC_{50} (%) (LCL–UCL)	Value of LC_{90} (%) (LCL–UCL)	Chi-square value (χ^2)
I	29[b]	43[ab]	58[ab]	62[ab]	79[b]	0.88 (0.69–1.04)	2.73 (2.34–3.37)	3.137
II	34[a]	45[a]	60[a]	69[a]	84[a]	0.73 (0.54–0.89)	2.43 (2.11–2.94)	0.933
III	20[c]	28[b]	39[b]	54[b]	70[c]	1.34 (1.18–1.52)	3.03 (2.63–3.68)	0.261
IV	14[d]	22[c]	34[c]	46[d]	64[d]	1.56 (1.40–1.78)	3.21 (2.78–3.90)	0.523
Pupa	9[c]	17[c]	28[d]	39[e]	59[e]	1.71 (1.52–1.99)	3.23 (2.78–3.94)	0.612

Within column means followed by the same letter(s) are not significantly different at 5% level by DMRT.

and were provided with larval food. Observations were made at 24 h intervals and the dead larvae and pupae were removed daily and counted. The development stages of larvae, pupae, and adults were monitored. The percentage of total emergence at different concentration was recorded. The emergence inhibition concentrations (EI_{50} and EI_{90}) were derived from the experimental data through probit analysis[12].

Combined activity

The methanolic activity of *S. wightii* was studied for the combined effect with *Bti* at five different concentrations ranging from 0.25–2 mg/l (0.25, 0.50, 1.00, 1.50, 2.00). A control was set up (in each test) with 1 ml of acetone and 249 ml of dechlorinated water. The synergistic factor was calculated from LC_{50} value of microbial insecticide alone divided by the LC_{50} value of the algae extract. A synergistic factor (SF) greater than one is considered to be synergism, an SF value less than one is considered to be antagonism: Synergistic Factor = (LC value of microbial insecticide)/(LC value of plant extract with insecticide).

Repellent activity of *S. wightii*

Repellent activity of plant compounds tested with human volunteers. For the repellent activity of *S. wightii* percentage protection in relation to dose method was adopted[14]. Three to four day old blood starved female of adult mosquitoes (100) were kept in a net cage. The arms of the test person was cleaned with disinfectant isopropanol. After air-drying the arm only 25 cm^2 of the dorsal side of the skin on each lower arm was exposed, the remaining area being covered by rubber gloves. The *S. wightii* was dissolved in isopropanol and this alcohol served as a control. The extracts *S. wightii* at 2, 4, 6, 8, 10 mg/l concentrations was applied. The control and treated arms were introduced separately into the cage, the number of bites counted over 5 min every 60 min, from 5.00–10.00 h. The experiment was conducted five times. The percentage of protection (post treatment) was calculated by using the following formula[4]: Protection = (Number of bites received by control arm − Number of bites received by treated arm)/(Number of bites received by control arm).

STATISTICAL ANALYSIS

The data from the bioassay were subject to statistical analysis. The SPSS software package was used for computing all the data including probit analysis, correlation co-efficient, and mean of the sample.

RESULTS

Table 1 illustrates the combined effect of methanolic extracts of *S. wightii* and microbial insecticide, *B. thuringiensis* against all the larval instars and pupae of *A. sundaicus*. A *S. wightii* weed extract concentration 2 mg/l was constantly added with different *Bti* concentrations in the range of 0.25–2 mg/l. A 79% mortality was observed at 2 mg/l in I Instar larva, whereas II, III, IV Instar larvae, and pupae showed 84, 70, 64, and 59% mortality after the same treatment. The LC_{50} value of I, II, II, IV Instar, and pupa were 0.88%, 0.73%, 1.34%, 1.56%, and 1.71%, respectively. The LC_{90} value of I, II, III, IV Instar, and pupa were 2.73%, 2.43%, 3.03%, 3.21%, and 3.23%,

Table 2 Combined effect of methanolic extract of *Sargassum wightii* Greville and microbial insecticide, *Bacillus thuringiensis* var. *israelensis* at various concentrations on the growth and development against Coastal Malarial vector, *Anopheles sundaicus*.

Concentration of *S. wightii* + *Bti* (mg/l)	Mean duration in each instars (days)†					Total number of days	Total mortality (%)	Total emergence (%)
	L_1–L_2‡	L_2–L_3‡	L_3–L_4‡	Pupae	Adult			
2.00+0.25	1.0 ± 0.4^{c}	3.0 ± 0.6^{b}	5.0 ± 1.2^{b}	2.0 ± 1.6^{a}	2.0 ± 1.0^{a}	13.0 ± 0.3^{d}	6^{e}	94^{ab}
2.00+0.50	2.0 ± 0.4^{b}	3.0 ± 0.4^{b}	5.0 ± 2.0^{b}	2.0 ± 0.8^{a}	2.0 ± 1.0^{a}	14.0 ± 1.0^{c}	11^{d}	89^{b}
2.00+1.00	2.0 ± 0.8^{b}	3.0 ± 0.6^{b}	5.0 ± 1.2^{b}	2.0 ± 1.2^{a}	2.0 ± 1.2^{a}	14.0 ± 1.0^{c}	23^{c}	77^{c}
2.00+1.50	3.2 ± 0.8^{a}	4.0 ± 0.8^{a}	6.0 ± 1.2^{a}	2.0 ± 1.6^{a}	2.0 ± 1.0^{a}	17.2 ± 0.4^{a}	32^{b}	68^{d}
2.00+2.00	2.00 ± 0.18^{b}	4.0 ± 1.8^{a}	5.0 ± 1.0^{b}	2.0 ± 2.0^{a}	2.0 ± 0.6^{a}	11.5 ± 0.4^{e}	46^{a}	54^{e}
Control	1.0 ± 0.4^{c}	3.0 ± 0.6^{b}	4.0 ± 1.2^{c}	2.0 ± 1.2^{a}	1.5 ± 0.6^{b}	11.5 ± 0.4^{e}	5^{e}	95^{a}

Within column means followed by the same letter(s) are not significantly different at 5% level by DMRT.
† Values ± SE
‡ L_1, L_2, L_3, L_4: Larval stages

respectively. Among the different larval and pupal stages, the II Instar larvae were more susceptible than the other larval or pupal stages.

Table 2 provides the combined effect of methanolic extract of *S. wightii* and microbial insecticide, *Bti* at various concentrations on the growth and development of *A. sundaicus* larvae. The *S. wightii* weed extract concentration 2 mg/l is constantly added with different *Bti* concentrations ranging from 0.25–2 mg/l. Days of development were 11.5 ± 0.4 and the percentage of emergence was 95% at control. At 0.25 mg/l and 0.5 mg/l *Bti* concentration, days of development were 13.0 ± 0.3 and 14.0 ± 1.0 and the rates of emergence were 94% and 89%, respectively. At the concentrations of 1.00 mg/l and 1.50 mg/l *Bti* the total days of development were 14.0 ± 1.0 and 17.2 ± 0.4 and the rate of total emergence was 77% and 68%, respectively. The EI_{50} and EI_{90} values were 2.6 and 4.4%. Among the different concentrations 2.0 mg/l was more effective than the other concentrations in the total emergence of adults.

Table 3 illustrates repellent activity *S. wightii* on malarial vector, *A. sundaicus*. At 2 mg/l concentration the repellent activity was 26% and at 10 mg/l concentration the percentage of repellency was 89%.

The synergistic factor of methanolic extracts of marine sea weed *S. wightii* with microbial insecticide *Bti* for I, II, III, IV Instar larva, and pupa were: 1.74, 1.93, 1.37, 1.27, and 1.24, respectively. The higher synergistic value was on II Instar larvae. This may be due to the action of plant compounds from *S. wightii* (dioctyl phthalate) might have interacted with Bt cry toxins from the *B. thuringiensis* and brought out such a toxicity against different larval and pupal populations of *A. sundaicus*.

Table 3 Repellent activity *Sargassum wightii* Greville on malarial vector, *Anopheles sundaicus* Liston.

Repellent activity observation (h)	Number of mosquitoes fed					
	Control	Concentration of *S. wightii* (mg/l)				
		2	4	6	8	10
5.00–6.00 pm	25	20	16	12	7	3
6.00–7.00 pm	20	17	12	9	5	2
7.00–8.00 pm	16	11	10	7	4	2
8.00–9.00 pm	13	8	7	5	3	1
9.00–10.00 pm	10	6	5	3	2	1
Fed mosquitoes	84	62	41	30	17	9
Unfed mosquitoes	16	44	50	64	79	91
Protection (%)		26	40	57	71	89

DISCUSSION

Most of the plant based products are not as effective as their synthetic counterparts, and to use mosquito control in a large scale programme under epidemic conditions may not be acceptable. However, the use of indigenous plant based products by individuals and communities can provide prophylactic measures for protection against various mosquito- borne diseases. In the present study, after the treatment of seaweed extract had considerable mortality against different larval instars of *A. sundaicus*. The plant chemicals might have dissolved in the water media and brought out such mortality to the larvae.

Earlier, several authors[5,15,16] made an attempt to use plant extracts (*Azadirachta indica*, *Ocimum sanctum*, *Albizzia amara*, and *Toddalia asiatica*) against different *An. stephensi* and *Aedes aegypti*. After the exposure of plant extracts the percentage of repellency was increased and it may have been due to the volatile compounds.

There were numerous reports on mosquito lar-

vicidal activity of terrestrial plants. We report here the first study on mosquito larvicidal and repellent activity of marine plants subsequently the mosquito larvicidal activity of seaweeds, *Plocamium telfairiae* and *Laurencia nipponica*[17–21]. Laboratory evaluation of traditionally used plant-based insect repellents against the malaria vector, *Anopheles arabiensis* Patton (Diptera: Culicidae). Ref. 22 reported that the alkaloid derived from the tropical vine *Triphyophyllum peltatum* was found to have larvicidal activity against the malarial vector, *A. stephensi*. Ref. 23 reported that the different age of eggs of *A. stephensi* treated with different concentrations of leaf extracts caused ovicidal activity resulting in failure to hatch the eggs. Ref. 15 reported the larvae hatched from the treated eggs showed much higher levels of mortality in all the treatments. Furthermore, plant-based repellent products are inexpensive, easily available, locally known, and culturally acceptable[24,25], this finding would be useful in the field of mosquito control without polluting the environment. However, Ref. 26 showed that the skin repellent test at concentrations of 1.0, 2.5, and 5.0 mg/cm^2 of *C. citratus* essential oil against the filarial mosquito *Culex quinquefasciatus* gave 100% protection up to 3.00, 4.00, and 5.00 h, respectively.

The total percentage of protection of this essential oil was 50% at 1.0 mg/cm^2, 62% at 2.5 mg/cm^2, and 74% at 5.0 mg/cm^2 for 12 h. In the present study, *C. citratus* extract established higher repellent activity against *A. arabiensis* even at the lower concentration. Effective repellent compounds, like dimethyl phthalate, available in the market are very costly and moreover they can give protection only for a short period of 1–2 h[27].

The synergistic factor has been worked out and higher synergism was found to be on fourth instar larvae rather than other larval instars. Earlier investigations used the seaweeds *Caulerpa scalpelliformis* and *Dictyota dichotoma* and mangrove *Rhizophora apiculata* extracted in acetone, combined with synthetic insecticides (DDT, BHC, HCH, and malathion), and evaluated for activity against fourth instar larvae of *Aedes aegypti* and the higher synergistic activity with all three insecticides, especially HCH[28].

In the present study, *S. wightii* frond extract treatment resulted in higher larval and pupal mortality which might be due to the multiple actions of dioctyl phthalate and other bioactive compounds present in the weed. A similar study reported the evaluation of the use of *Parthenium hysterphourus* against mosquito[29] and combined effect of other phenolic acids such a caffeic acid, vanillic acid, ansic acid, *p*-ansic acid, chlorogenic acid and parahydroxy benzoic acid may possess larvicidal and pupicidal property on *A. aegypti* and *C. quinquefasciatus*. In the present study, the exposure to plant extracts had a repellency effect against adult mosquito, and the presence of volatile compounds may be responsible for such effect[2]. Vineetha and Murugan[16] had also worked out the repellent effect of *Toddalia asiatica* against the dengue vector, *A. aegypti*. It is now well understood that a mixture of functionally diverse toxins proves more effective than a single one and also causes delay in the development of resistance in targeted insects[1,5].

The applications of *Bti* combined with plant extracts produced a high mortality of the target organism. Spore forming bacteria are able to be mass produced, stored, easily transported and applied. Their larvicidal activity is due to large amounts of crystal proteins produced during sporulation and transformed into toxins under specific conditions after ingestion by larvae of certain insect species. Their selectivity is determined by both the structure of the proteins produced by the bacterium strain and the presence of proteolytic enzymes and receptor in the host larvae midgut. Moreover, the active compounds in the *S. wightii* might interact with Bt toxins crystals and increased the toxicity against mosquito larvae. In view of the above, the synergistic interaction of plant compounds from *S. wightii* and microbial cry toxins from *B. thuringiensis* showed toxicity and biological effects on the larvae and adults of malarial vector, *A. sundaicus*. Biopesticides are not only used as mosquito vector control program, but also permit the maintenance of traditional knowledge for the benefit of communities. Hence the exploitation of plant chemicals and microbial pesticides are not only used as mosquito vector control program, but it can also be used for the control of mosquito-transmitted diseases. Moreover, adopting this kind of strategy would enable us to use pesticides that are safe for the environment in the future.

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