



## TOXICITY OF *SARGASSUM WIGHTII* TO THE MALARIA VECTOR *ANOPHELES STEPHENSI* LISTON (DIPTERA: CULICIDAE)

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### ABSTRACT

Seaweed based pesticides exhibit mosquitocidal properties and are found to be effective, biodegradable and non-toxic to non-target organisms. *Sargassum wightii* Greville butanol and ethanol extracts were tested for its toxicity against the second and third instar larvae of *Anopheles stephensi* Liston in the present study. One hundred percent larval mortality was observed in the second instar of *An. stephensi* by the ethanol extract at the highest concentration of 500 mg/l after 24 hours of exposure. The  $LC_{50}$  values of *S. wightii* butanol and ethanol extracts were 107.03 and 89.67 mg/l, against the second instar of *An. stephensi*; and 132.56 and 92.00 mg/l against the third instar of *An. stephensi* respectively. Phytochemical analysis of *S. wightii* extracts revealed presence of alkaloids, carbohydrates, fatty acids, flavonoids, phenols, proteins, tannins and terpenes. Larval mortality of second and third instars of *An. stephensi* can be attributed to the major compounds present in *S. wightii* revealed by GC-MS analysis, viz., ethyl salicylate, methyl salicylate, ethyl palmitate, palmitic acid, oleic acid, phytol and diethyl phthalate. The present study accentuates *S. wightii* butanol and ethanol extracts to cause lethal effects to the second and third instar larvae of *An. stephensi*.

**Key words:** *Sargassum wightii*, brown seaweed, phytochemical constituents, *Anopheles stephensi*, larvicide

Malaria continues to be a significant public health problem, and the recent reports of WHO state that nearly 219 million people are living in malaria risk areas (WHO, 2019), and nearly half of the world's population are exposed to malaria (WHO, 2021). *Anopheles stephensi* is the major malaria vector known to transmit human malaria parasites *Plasmodium falciparum* and *P. vivax* in India (Das et al., 1990; Korgaonkar et al., 2012). Vector control is the most important public health objective, and the successful method of reducing the occurrence of vector-borne diseases is through the control of the disease transmitting vector mosquitoes (Manikandan et al., 2022). Chemical insecticides to control mosquitoes cause harmful side effects on non-target organisms, humans and to environment as well (Kaushik et al., 2019; Siam et al., 2022). Further, problems associated with extensive usage of synthetic compounds have increased substantially which makes mosquito/vector control more difficult over the years (Demirak and Canpolat, 2022). Hence, an outcry is exhibited against the use of pesticides due to their hazardous effects. In this situation, ecofriendly alternatives are important for safer mosquito management. Traditionally, plant-based products have constituted an important source of insecticides against

*Anopheles* mosquitoes (Muhammed et al., 2022). Current research community is paying more attention towards marine natural products (Yasmeen et al., 2018). Bioinsecticides from marine algal extracts represent safe, alternatives for synthetic pesticides for mosquito management, which negatively affect the environment and health (Elbanna and Hegazi, 2011). The field of seaweed based pesticides steadily progresses as they offer a natural source of compounds to the development of insecticides from marine algae which has become more rigorous in recent years with calls for more standardization, especially against mosquitoes as they display their mosquitocidal properties (Thangam and Kathiresan, 1991a,b; Yu et al., 2014, 2015; Deepak et al., 2019; Suganya et al., 2019; Haleem et al., 2022), and are found to be effective, biodegradable and non-toxic to non-target organisms. In addition to the direct use of seaweed extracts for mosquito control, biosynthesized nanoparticles using seaweeds has gained momentum too (Murugan et al., 2015, 2018). Nevertheless, crude seaweed extracts still dominate in combating mosquitoes. Extracts of the brown seaweed, *Sargassum wightii* has been reported for larvicidal activity against *Aedes aegypti*, *An. stephensi*, *An. sudaicus*, *Culex quinquefasciatus* and *Cx. tritaeniorhynchus* (Manilal

et al., 2011; Kumar et al., 2012; Poonguzhali and Nisha, 2012; Murugan et al., 2018; Suganya et al., 2019). However, few reports present the larvicidal activity of its ethanol extracts (Suganya et al., 2019), and no report has been published on its butanol extract. Hence, the toxicity of *S. wightii* butanol and ethanol extracts were assessed for its toxicity against the second and third instar of *An. stephensi* larvae in this study.

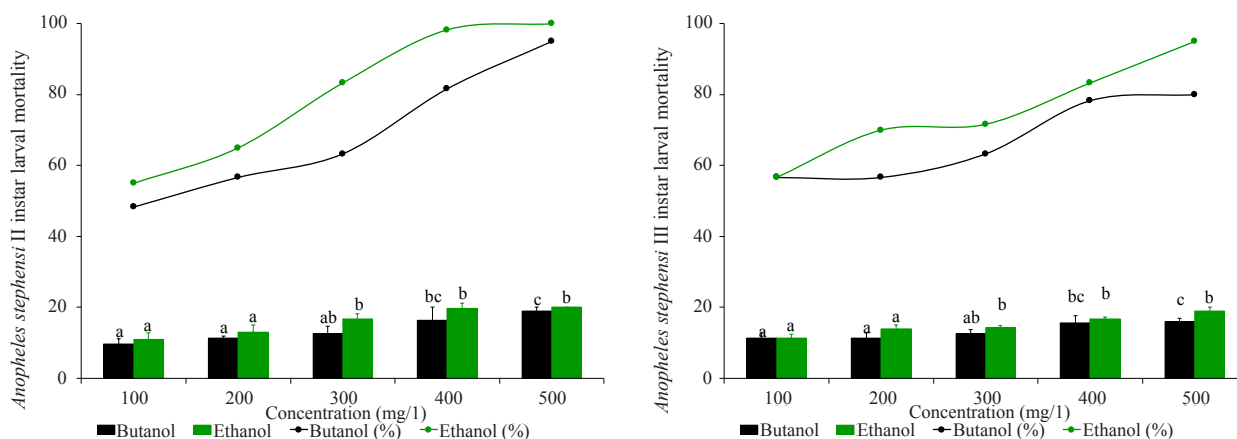
## MATERIALS AND METHODS

*S. wightii* (Fig. 1) collected by hand picking from the intertidal zone of Rameswaram, Tamil Nadu, India ( $8^{\circ} 46' N$ ,  $78^{\circ} 9' E$  and  $9^{\circ} 14' N$ ,  $79^{\circ} 14' E$ ), was immediately rinsed in water to remove all kinds of epiphytes and other particles (sand, molluscs, and sea grasses), and kept in sterilized ziplock bags, and transferred to laboratory for further studies. Taxonomical identification and confirmation of the collected seaweed was done at the Marine Algal Research Station, Mandapam, Tamil Nadu, India with the aid of morphological key characters and identification manual (Dhargalkar and Kavlekar, 2004; Rao, 2012). The cleaned macroalgae was shade dried at room temperature, and powdered with the aid of a mixer grinder. The powdered sample (250g) was suspended in butanol and ethanol (750 mL) each separately, and transferred to soxhlet apparatus for eight hours for extraction (Vogel, 1978). Thereafter, the extracted sample was filtered using Whatman No.1 filter paper, and the filtered sample was centrifuged at 5000rpm for 10 minutes at  $4^{\circ}C$ , and the supernatant was collected in a separate flask. The butanol and ethanol extract each was then concentrated using a rotary vacuum evaporator (Puchi RII, Switzerland).

The final concentrated butanol and ethanol crude extract was stored in sterile air tight amber coloured bottle, and kept in a refrigerator until further use. Prior to this, the percentage of yield of extraction of these two crude extracts were calculated. The phytochemical profile of *S. wightii* extracts was performed to identify the plant chemicals (Harborne, 1978), followed by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The spectrum of components obtained were compared with the database of spectrum of known components stored in the GC-MS-National Institute for Standards and Technology library.

*Anopheles* adults collected from cattle sheds of Madurai, Tamil Nadu, India with an aid of an aspirator were transferred to an one feet mosquito cage, and transported to laboratory. The adults on emergence were identified and species confirmed prior to rearing (WHO, 2020). Subsequently, their cyclical generations were provided a blood meal, and was maintained in two feet mosquito cages with an average room temperature of  $27 \pm 2^{\circ}C$  and a relative humidity of 70-80% inside an insectary. Ovitrap inside the mosquito cages collected the oviposited eggs which were shifted to the larval rearing room in enamel larval trays, and the larvae on hatching were provided with larval food (yeast and dog biscuits of ratio 1:3). The larvae on turning into pupae were moved to another mosquito cage in enamel bowls for adult emergence.

World Health Organization (WHO) protocol was adopted for the study with minor modifications (WHO, 2005). Serial dilution of 1.0% stock solution of the crude seaweed extracts yielded requisite test concentrations



Data in bar represent larval mortality as Mean±Standard deviation; and data in line denotes percent larval mortality. Different superscript alphabets on the bar indicate statistical significant difference in larval mortality between concentrations at  $P < 0.05$  level by One way ANOVA followed by Tukey's test performed. Similarity in superscript alphabets on the bar indicate no significant variation.

Fig. 1. Larval mortality of *An. stephensi* on exposure to *S. wightii* extracts

(100, 200, 300, 400, and 500 mg/L) and amount of test solution. Early second and third instar larvae obtained from the laboratory colonized  $F_1$  generation formed the choice as the test instar. The early second and third instars numbering twenty five each were added separately into glass beakers (250 mL) holding distilled water and test concentration for each replicate apiece trial. Distilled water (250 mL), and Tween 80 (1.0 mL) dissolved in distilled water (249 mL) maintained separately and run simultaneously served as positive and negative control, respectively. Larval mortality after 24 hrs was confirmed when the moribund larvae showed no signs of movement when prodded by a needle on their respiratory siphon, and were scored dead. A total of five replicates and a negative and positive control were run concurrently for every trial, and overall five trials were run. Larval mortality (%) and corrected mortality where control mortality ranged from 5-20% (Abbott, 1925) was calculated. Statistical analysis of all mortality data were subjected to probit analysis. Chi-square and regression analysis were performed. One-way analysis of variance and Tukey's honestly significant difference post hoc tests were used to determine if the mortality in treated bioassays significantly differed from that of controls, and at which dosages in specific. The differences were considered as significant at  $P \leq 0.05$  level. All statistical analysis of data were carried in IBM SPSS Statistics v22 with significance set at 95% confidence (SPSS, 2010).

## RESULTS AND DISCUSSION

The percentage yield of *S. wightii* butanol and ethanol extract was 0.98% and 2.45%, respectively, and its phytochemical screening revealed presence of alkaloids, carbohydrates, fatty acids, flavonoids, phenols, proteins, tannins and terpenes. This was correlated with the results of Begum and Hemalatha (2017). The major phytochemical compounds present in butanol and ethanol extracts revealed by GC-MS analysis were ethyl salicylate, methyl salicylate,

ethyl palmitate, palmitic acid, oleic acid, phytol and diethyl phthalate. The mortality of second and third instar larvae of *An. stephensi* exposed to various concentrations of *S. wightii* butanol and ethanol extracts are presented in Fig. 1. No larval mortality was observed in both controls. One hundred percent mortality was observed in *An. stephensi* second instar larvae at the highest concentration on exposure to ethanol extract. *S. wightii* butanol and ethanol extracts reported  $LC_{50}$  values of 107.03 and 89.67mg/L; and 132.56 and 92.00mg/L against the second and third instar larvae of *An. stephensi* respectively. One Way ANOVA, comparing treated and control group, with a significance level established at  $P < 0.05$  showed that *S. wightii* concentrations significantly influenced the mortality of *An. stephensi* larvae (Table 1). Overall results indicated the ethanolic extract of *S. wightii* to be slightly more toxic on both the tested larval instars of *An. stephensi*, and amongst the two instar stages tested, the second instar was found to be more susceptible than the other. Thangam and Kathiresan (1996) classified seaweeds with larvicidal activity less than  $LC_{50}$  100 mg/L as effective larvicide,  $LC_{50}$  between 100 and 200mg/L as less effective larvicide, and  $LC_{50}$  more than 200mg/L as ineffective larvicide. The results of the present study revealed that *S. wightii* ethanol and butanol extracts were effective and less effective larvicides based on their  $LC_{50}$  values and classification of Thangam and Kathiresan (1996). The susceptibility of mosquito larvae to seaweed insecticides depends in general on the seaweed used, the solvent used for extraction, and the mosquito species tested. A thorough knowledge on the phytochemical profile of the seaweed used should be drawn, prior to selection of solvents, in order to get a potent extract as there exists a relationship between the extract effectiveness and solvent polarity. The bioactivity of a seaweed compound is related to the compound's chemical structure and chemical reactions (Barbosa et al., 2012). Marine algal extracts possess mosquito larvicidal activity since they contain bioactive

Table 1. Probit analysis and other associated statistical inferences for *S. wightii* extracts

Solvent	$LC_{50}$ (mg/L)	$LC_{90}$ (mg/L)	Chi-square	Regression equation	$R^2$	F	P
<i>An. stephensi</i> II instar							
Butanol	107.03	327.62	18.38*	$Y=2.71+0.04x$	0.86	5.19	<0.04*
Ethanol	89.67	573.90	8.01*	$Y=3.02+0.03x$	0.82	4.07	<0.04*
<i>An. stephensi</i> III instar							
Butanol	132.56	647.64	24.17*	$Y=2.09+0.03x$	0.87	4.30	<0.05*
Ethanol	92.00	748.08	9.21*	$Y=2.90+0.03x$	0.77	4.28	<0.05*

\*Values significant at  $p < 0.05$  level

compounds (Manilal et al., 2009; Ravikumar et al., 2011), rich in polyphenolic and triterpene compounds, causing larval mortality, as they inhibit the protein responsible for the cholesterol transportation during the larval development (Blunt et al., 2011; Bibi et al., 2020). Extracts of brown, green and red seaweeds have strong larvicidal activity against mosquito larvae (Ahmad et al., 2016), and brown algae under which *Sargassum* genus is housed are rich in alkaloids, glycosides, quinones, saponins, tannins and terpenoids (Suganya et al., 2019).

Secondly, the choice of solvent is influenced by what is intended with the extract, as it targets the compounds to be extracted (Shaalán et al., 2005; Ghosh et al., 2012). Variation of the larvicidal potential of *S. wightii* changed with different solvents used and tested against different species of mosquito larvae, and the results of the present study were comparable with the earlier reports of *S. wightii* against mosquito larvae in view of the aforementioned factors. *S. wightii* methanol extracts showed LD<sub>50</sub> values of 87.09 µg/ mL and 107.15 µg/ mL against *Cx. quinquefasciatus* and 84.82 µg/ mL and 97.28 µg/ mL against *Ae. aegypti* second and third instars, respectively, which was due to presence of tannins (Manilal et al., 2011); LC<sub>50</sub> values of 0.88%, 0.73%, 1.34% and 1.56% against first, second, third and fourth instar of *An. sundaicus*, respectively due to the action of dioctyl phthalate (Kumar et al., 2012); LC<sub>50</sub> values of 424.57 mg/ L (methanol extract), 363.14 mg/ L (acetone extract) and 435.24 mg/ L (benzene extract) against *An. stephensi* (Poonguzhali and Nisha, 2012). Rare reports have been documented on the butanol extracts for its toxicity to the second and third instars of *An. quadrimaculatus*, *Ae. aegypti*, *Cx. pipiens* and *Cx. quinquefasciatus* larvae (Spielman and Lemma, 1973; Kumar et al., 2014; Raveen et al., 2017), and on the other hand, Samuel et al. (2018) provided an exhaustive review on the list of ethanolic extracts reported for mosquito larvicidal property. *S. wightii* extracts has been reported for its toxicity against *An. stephensi* second and third instar larvae in this study. Mosquito larvicidal effectiveness of the ethanolic extracts from *S. wightii* and *Halimeda gracilis* against *Ae. aegypti*, *An. stephensi*, and *Cx. tritaeniorhynchus* have been reported (Suganya et al., 2019). Ali et al. (2012) reported larvicidal activities of ethanolic extract of seagrasses, *Syringodium isoetifolium* and *Cymodocea serrulata* against *Ae. aegypti*. Butanol and ethanol has the property to extract alkaloids, flavonoids, phenols, saponins, sterols, tannins, terpenes and terpenoids. All

these phytochemicals are toxic to immature stages of mosquito (Shaalán et al., 2005).

Suganya et al. (2019) reported that *S. wightii* ethanolic extract caused shrinkage and disintegration of midgut epithelial cells, and outer cuticle leading to larval death in *Ae. aegypti*. This would have been the cause of larval death too in the present study. Rey et al. (1999) reported the larvicidal mode of action by phytochemicals on mosquito larvae affects the midgut epithelium, gastric caeca and the malpighian tubules. Hexadecanoic acid methyl ester from leaf extracts of *Heliotropium indicum* and *Mukia maderaspatana* have been reported for its larvicidal property against *Ae. aegypti* (Krishnaveni and Ramamurthy, 2014). *Sargassum* species possess oleic, linoleic, linolenic, palmitic, and stearic acids and their respective methyl esters, were found to affect the metabolism and morphology of midgut along with the fat body of the fourth instar of larvae of *Cx. quinquefasciatus* (de Melo et al., 2018). The same can be corroborated to the present study, wherein, mortality to *An. stephensi* larvae can be attributed to the presence of its ethyl salicylate, methyl salicylate (phenols), ethyl palmitate (fatty acid ester), palmitic acid (sterol), oleic acid (fatty acid), phytol (terpene) and diethyl phthalate in *S. wightii* extracts, for the reason that phytochemicals work as stomach poison in mosquito larvae, as they enter the larvae body through the digestive tract, and lowers surface tension of the mucosa membrane in the digestive tract, making it easier to be damaged, and functions as a stomach toxin that disrupts digestion, nutrition absorption, ion transport, and osmoregulation (Sina and Shukri, 2016). Further, the abnormal behaviour of *An. stephensi* larvae in the present study can be correlated to the toxicity of this seaweed in affecting the nervous system and motor coordination. Nonetheless, further investigations are needed to confirm the neurological effect of seaweed extract that act as a nerve poison. Finally, selection of mosquito species for testing is also of fundamental importance since great variations exist in responses between the genera and species. The susceptibility of different mosquito species toward seaweed treatment varies (Ali et al., 2013). Manilal et al. (2011) reported *Ae. Aegypti* larvae to be more susceptible (with lower LC<sub>50</sub> values) towards treatment of seaweed extracts. However, in the present study Anophelines larvae too were susceptible when exposed seaweed extracts which exhibited low LC<sub>50</sub> values.

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#### AUTHOR CONTRIBUTION STATEMENT

Vaithiyanathan Selvi prepared the extracts, performed the bioassay experiments, and carried out GC-MS analysis. Subramanian Arivoli contributed to literature review and manuscript drafting. Samuel Tennyson wrote the manuscript and statistically analysed the data. All authors read and approved the final manuscript.

#### CONFLICT OF INTEREST

No conflict of interest.

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