Bioactive Potential of Seagrass Extracts Against Dengue Fever Mosquito

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Received 11 April 2014; Accepted 22 May 2014; Published 22 May 2014

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Abstract

Objective: To identify the larvicidal activity of the seagrass extracts against Aedes aegypti L.

Methods: Seagrass extracts, Halodule pinifolia (Milki) Hartog (H. pinifolia), Cymodocea serrulata (R.Br.) Asch & Mangnus (C. serrulata) and Thalasia testudinum Banks ex Koenig (T. testudinum) were dissolved in DMSO to prepare a graded series of concentration. Batches of 25 early 4th instars larvae of Aedes aegypti (Ae. aegypti) were transferred to 250 mL enamel bowl containing 199 mL of distilled water and 1 mL of plant extracts (0.01 mg – 0.1 mg). After 24 h the mortality rate was identified. Each experiment was conducted with three replicates and a concurrent control group. A control group consisted of 1 mL of DMSO and 199 mL of distilled water only. The extract fractions was eluted with the ethanol and subjected to FTIR.

Results: The root extract of H. pinifolia showed maximum larvicidal activity with minimum concentration of extract of LC50= 22.0±5.2µL/mL and LC90=54.2±2.5 µL/mL followed by leaf extract of T.testudinum LC 50 (44.8±1.6µL/mL) and LC 90(81.2±3.8µL/mL), C.serrulata leaf extract of LC 50 (42.9±2.4µL/mL). The regression equation of H. pinifolia for 4 th instar larvae of Ae. aegypti were Y=2.8+3.6x (R²=1.130) and analysis of variation was significant at P<0.05 level. The result of the preliminary phytochemical constituents shows the presence of saponin, steroids, terpenoid, phenols, protein and sugars. FTIR analysis revealed that presence of phycocollides substances at different ranges.

Conclusions: From the present study the ethanolic extracts of seagrass of H.pinifolia possesses lead compound for development of larvicidal activity.

Keywords: Cymodocea serrulata, Dengue fever, FTIR, Halodule pinifolia, Larvicides, Thalasia testudinum,
1. Introduction

Sea grasses are the marine flowering plants. They are the only angiosperms that successfully grow in tidal and subtidal marine environment. Sea grass belongs to the families Hydrocharitaceae and Potamogetonaceae and they are in no way related to the terrestrial grass of Poaceae [1]. There are 13 genera and 58 species available all over the world of these six genera (Amphibolis, Heterozostera, Phyllospadi, Posidonia, Pseudalthenia and Zostera) are mostly restricted to temperate seas and the remaining seven genera (Cymodocea, Enhalus, Halodula, Halophila, Syringodium, Thalasia and Thalassodendeon) are distributed in tropical seas. Several species of sea grasses have obligate microbial populations inhabiting their roots, leaves and rhizomes. A variety of medicines and chemical are also prepared from sea grass and their associates [2]. New trends in drug discovery from sea grass emphasize on investigation of the marine ecosystem to explore numerous complex and novel chemical entities. These entities are the source of new lead for treatment of many diseases such as cancer, AIDS, inflammatory condition, arthritis, malaria and large variety of viral, bacterial, fungal diseases [3 and 4].

Mosquito borne diseases have an economic impact, including loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates. However, no part of the world is free from vector-borne diseases [5]. Mosquitoes are the most important single group of insects in terms of public health importance, which transmit a number of diseases, such as malaria, filariasis, dengue, Japanese encephalitis, etc., causing millions of deaths every year. Ae aegypti, a vector of dengue is widely distributed in the tropical and subtropical zones. Dengue fever incidence has increased fourfold since 1970 and nearly half the world’s population is now at risk. In 1990, almost 30% of the world population, 1.5 billion people, lived in regions where the estimated risk of dengue transmission was greater than 50% [6].

One can speculate that people controlled and killed mosquitoes and other domestic insect pests by physically removing them or by using plant parts and plant derivatives before the advent of synthetic chemicals. In all probability, some plants containing insecticidal photochemicals that were predominantly secondary compounds were used to protect themselves against herbivorous insects. However, there is a little other than anecdotal, traditional or cultural evidence on this topic [7]. The control of mosquito larvae worldwide depends primarily on continued applications of organophosphates such as temephos, fenthion and insect growth regulators such as Bacillus formulations, diflubenzuron and methoprene [8]. Effective, repeated use of these controlling agents has fostered several environmental and health concerns, including disruption of natural biological control systems, outbreaks of other insect species, widespread development of resistance and undesirable effects on non-target organisms [9]. The objective of the present study was to evaluate larvicidal effect of ethnic extract of sea grasses against the 4th instars larva of Ae. aegypti mosquito.

2. Materials and Methods

2.1. Sample collection and extract preparation

Live and healthy samples of the sea grasses like Halodule pinifolia, Cymodocea serullata and Thalasia testudinum (leaves and root) were collected from Tuticorin coast (Lat. 8048’N and Long. 78011’E) of Gulf of Mannar. These samples were thoroughly washed with seawater to remove all epiphytes, shells etc, and again washed with fresh water to remove the surface salts, sand particles if any and allowed to dry in the shady place for 3 to 4 days. The collected samples were identified by using standard books and manuals [10, 11 and 12]. Shade dried sea grasses were subjected to percolation by soaking in ethanol and water mixture (3:1). After 21 days of dark incubation, the filtrate was concentrated separately by rotary vacuum evaporation (>45 °C) and then freeze-dried at -80 °C to obtain solid residue. The percentage of extraction was calculated by using the following formula: % of extraction= Weight of the extract /Weight of the plant material ×100. The extracts of sea grasses were further tested for the presence of phytochemical constituents by following the methods of Ravikumar et al [13].

2.2. Mosquito larval culture

To satisfy the enormous number of mosquitoes need for the day to day bioassays, a colony is essential. The eggs of Ae. aegypti were procured from Vector
Control Research Centre, Puducherry, India. Filter paper with attached eggs was dipped into a plastic tray containing 500 mL of dechlorinated water for 30 – 40 min, time enough to allow for eggs to hatch into larvae. They were reared indoors at (28 ± 2) °C 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. Five days after emergence, female mosquitoes were moved into a mosquito cage where the emergent adults were fed with a 10% sucrose solution and allowed to blood feed from white mice for 2–3 h. A few days after having a blood meal, the gravid mosquito laid their eggs.

2.3. Larvicidal activity

The larvicidal effect of ethanolic crude extract of three sea grasses viz., *H. pinifolia*, *C. serrulata* and *T. testudinum* against *Ae. aegypti* was conducted in accordance with the WHO standard method [14]. Sea grass extracts were dissolved in DMSO to prepare a graded series of concentration. Batches of 25 early 4th instar larvae of *Ae. aegypti* were transferred to 250 mL enamel bowl containing 199 mL of distilled water and 1mL of different concentration of plant extracts (0.01 mg – 0.1 mg). After treatment, symptoms in treated larvae were observed and recorded immediately at different time intervals and no food was offered to the larvae at this time. The larvae were considered dead if, at the end of 24 h, showed no sign of swimming movements even after gentle touching with a glass rod, as described in the World Health Organization’s technical report series. Each experiment was conducted with three replicates and a concurrent control group. A control group consisted of 1 mL of DMSO and 199 mL of distilled water. 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 mortality was calculated with Abbott's formula: 

\[
\text{[(% of test mortality - % of control mortality) / (100 - % of control mortality)]} \times 100
\]

2.4. FTIR analysis

The lyophilized samples of sea grasses (*H. pinifolia*, *C. serrulata* and *T. testudinum* each 10 mg) were mixed with 100 mg of dried potassium bromide (kbr) and compressed to prepare as a salt disc. The disc was then read spectro photometerically (Bio-Rad FTIR-40 model, USA). The frequencies of different components present in each sample were analyzed.

2.5. Statistical analysis

The average larval mortality data were subjected to probit analysis to calculate LC50, LC90 and 95% fiducial limits of upper confidence limit (UCL) and lower confidence limit (LCL), regression equation, Chi- square. Analysis variations were assessed using Stat Plus 2009 software. Results with *P*<0.05 were considered to be statistically significant.

3. Results

The present studies have tested with sea grasses against with the vector borne disease causing mosquito like *Ae. aegypti* . The percentage yields from 2.49 to 6.27 and the values are presented in table 1. It revealed that, *H. pinifolia* root extract (6.27%) showed maximum yield followed by *C. serrulata* root extract (3.54%). The LC50 and LC 90 values of the sea grass extract *Ae. aegypti* are listed in table 2. The root extract of *H. pinifolia* showed maximum larvicidal activity with minimum concentration of the extract of LC50 at dose of (22.0±5.2)µL/mL and LC 90 (54.2±2.5µL/mL) followed by leaf extract of *T. testudinum* LC 50 (44.8±1.6µL/mL) and LC90 (81.2±3.8µL/mL), *C. serrulata* leaf extract of LC50 (42.9±2.4µL/mL). The regression equation of *H. pinifolia* for 4 th instar larvae of *Ae. aegypti* were \(Y=2.8+3.6x\) (R²=1.130) and analysis of variation was significant at *P*<0.05 level. The preliminary phytochemical study reveals that the extracts from sea grasses have variety of phytochemical constituents, such as saponin, steroids, terpenoid, phenols, protein and sugars (Table 3). The second derivative, IR spectrum in the mid-infrared region (400-4 000 cm -1 ) was used for discriminating and identifying various function groups present in, *H. pinifolia*, *C. serrulata*, and *T. testudinum*. The variation in spectral features of the IR band suggestions of the functional groups were given in the table 4 (Fig 1 to 3).
Table 1: Extractive value of chosen seagrass species.

<table>
<thead>
<tr>
<th>Seagrass species</th>
<th>Plant parts</th>
<th>Weight of plant part (g)</th>
<th>Yield [g (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pinifolia</em></td>
<td>leaf</td>
<td>52.00 ± 2.01</td>
<td>1.29 (2.49)</td>
</tr>
<tr>
<td></td>
<td>root</td>
<td>100.00 ± 1.59</td>
<td>6.00 (6.27)</td>
</tr>
<tr>
<td><em>C. serrulata</em></td>
<td>leaf</td>
<td>68.00 ± 2.45</td>
<td>1.98 (3.24)</td>
</tr>
<tr>
<td></td>
<td>root</td>
<td>86.00 ± 2.01</td>
<td>2.61 (3.54)</td>
</tr>
<tr>
<td><em>T. testudinum</em></td>
<td>leaf</td>
<td>74.00 ± 2.80</td>
<td>1.85 (2.95)</td>
</tr>
</tbody>
</table>

Table 2: Larvicidal activity of ethnolic extracts of seagrass against Ae. aegypti

<table>
<thead>
<tr>
<th>Name of the seagrass</th>
<th>Plant part</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (µL/mL) (Mean±SD) (LCL-UCL)</th>
<th>LC&lt;sub&gt;90&lt;/sub&gt; (µL/mL) (Mean±SD) (LCL-UCL)</th>
<th>Regression equation</th>
<th>R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pinifolia</em></td>
<td>Leaf</td>
<td>32.4±1.6 (30.8-33.8)</td>
<td>62.4±0.90 (57.43-63.80)</td>
<td>Y=3.47+4.1x</td>
<td>2.040</td>
<td>1.260</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>22.0±5.2 (21.4-23.6)</td>
<td>54.2±2.5 (53.3-55.2)</td>
<td>Y=2.8+3.6x</td>
<td>1.130</td>
<td>0.850*</td>
</tr>
<tr>
<td><em>C. serrulata</em></td>
<td>Leaf</td>
<td>42.9±2.4 (40.8-43.7)</td>
<td>76.30±1.43 (75.20-77.16)</td>
<td>Y=2.65=3.4</td>
<td>1.960</td>
<td>0.960</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>No mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. testudinum</em></td>
<td>Leaf</td>
<td>44.8±1.6 (43.6-45.8)</td>
<td>81.2±3.8 (80.2-82.3)</td>
<td>Y=3.2+0.84</td>
<td>2.180</td>
<td>1.360</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>No mortality</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05; LCL- Lower confidence level; UCL- Upper confidence level
Table 3 Phytochemical constituents in chosen seagrass species.

<table>
<thead>
<tr>
<th>Phytochemical constituents</th>
<th>H. pinifolia</th>
<th>C. serrulata</th>
<th>T. testudinum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
<td>Root</td>
<td>Leaf</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carboxylic acid</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Coumarins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Quinones</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenols</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Xanthoproteins</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteins</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sugars</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

-; Absent, +; Medium, ++; High

4. Discussion

Insect-transmitted diseases are major health problems in tropical regions. *Ae aegypti* (Culicidae) occurs in Asia, Africa and Central and South America. It transmits virus of *Flavivirus* genus, etiologic agents of human diseases like dengue and yellow fever [15]. The bioactivity of phytochemicals against mosquito larvae can vary significantly depending on plant species, plant parts, age of plant parts, solvent used in extraction and mosquito species [16]. Most studies on phytochemicals focus on herbs and other medicinal plants. This is because historical experiential knowledge and some scientific studies have shown them to be particularly active against certain organisms. Several studies have focused specifically on medicinal plants in different geographical regions. Commonly a connection is extrapolated between plant activity against disease agents based on traditional experience and insecticidal activity against mosquitoes. A wide selection of trees and shrubs has been found to contain phytochemicals that may be of use in the control of mosquitoes. Some trees and shrubs have also been tested more frequently due to observations indicating a degree of resistance to pests such as termites or herbivorous insects [17].
Table 4 Identification of functional groups through FTIR analysis.

<table>
<thead>
<tr>
<th>Name of the species</th>
<th>Frequency (cm⁻¹)</th>
<th>Bond</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Halimeda macroloba</strong></td>
<td>3 416.48 (s, n)</td>
<td>O-H stretch, H-bonded</td>
<td>Alcohols, phenols</td>
</tr>
<tr>
<td></td>
<td>2 974.45 (m, sh)</td>
<td>C-H stretch</td>
<td>Aromatics</td>
</tr>
<tr>
<td></td>
<td>1 599.12 (s, n)</td>
<td>N-H bend</td>
<td>t¹ amines</td>
</tr>
<tr>
<td></td>
<td>054.68 (s, sh)</td>
<td>C-O stretch</td>
<td>Alcohols, Carboxylic acids</td>
</tr>
<tr>
<td><strong>Cymodocea serrulata</strong></td>
<td>3 415.52 (s, b)</td>
<td>O-H stretch, H-bonded</td>
<td>Alcohols, phenols</td>
</tr>
<tr>
<td></td>
<td>2 954.55 (m, sh)</td>
<td>C-H stretch</td>
<td>Aromatics</td>
</tr>
<tr>
<td></td>
<td>1 649.39 (m, sh)</td>
<td>N-H bend</td>
<td>t¹ amines</td>
</tr>
<tr>
<td></td>
<td>1 408.18 (m, b)</td>
<td>C-C stretch (in-ring)</td>
<td>Aromatics</td>
</tr>
<tr>
<td></td>
<td>1 025.48 (s, sh)</td>
<td>C-O stretch</td>
<td>Alcohols, Carboxylic acids, esters &amp; ethers</td>
</tr>
<tr>
<td></td>
<td>659.64 (s, b)</td>
<td>-C=H: C-H bends</td>
<td>Alkynes</td>
</tr>
<tr>
<td><strong>Thalasia pestudinum</strong></td>
<td>3 492.97 (s, b)</td>
<td>O-H stretch, H-bonded</td>
<td>Alcohols, phenols</td>
</tr>
<tr>
<td></td>
<td>2 968.35 (m, b)</td>
<td>O-H stretch</td>
<td>Carboxylic acid</td>
</tr>
<tr>
<td></td>
<td>1 637.99 (m, n)</td>
<td>N-H bend</td>
<td>t¹ amines</td>
</tr>
<tr>
<td></td>
<td>1 058.94 (s, n)</td>
<td>C-O stretch</td>
<td>Alcohols, Carboxylic acids, Esters &amp; ethers</td>
</tr>
<tr>
<td></td>
<td>880.81 (m, sh)</td>
<td>C-H “opp”</td>
<td>Aromatics</td>
</tr>
<tr>
<td></td>
<td>660.92 (m, b)</td>
<td>C-Br stretch</td>
<td>Alkyl halides</td>
</tr>
<tr>
<td></td>
<td>1 394.32 (m, b)</td>
<td>C-H rock</td>
<td>Alkynes</td>
</tr>
</tbody>
</table>

Figure 1. FTIR Spectrum of the bioactive fractions of Halodule pinifolia.
Martine plants are considered as a rich source of bioactive chemicals [14] and they may be an alternative source of mosquito control agents. Bioactive marine natural products play an important role in chemotherapy. The evidence for the use of marine flora to be precise in treatment of human ailments is extensive. In Asian maritime areas, sea grass are used as curative agents for various maladies such as anti malarial [18, 19, and 20], antibacterial [21 and 22], antihehminitic, cough, antipyretic, wound healing, treatment of gallstone and goiter [23-31]. The studies on seaweed extracts with larvicidal activities are too restricted. Hence, the present study was carried out to find out the mosquito larvicidal effect of sea grass extract. The sea grass root extract of H. pinifolia showed maximum larvicidal activity with minimum concentration of the extract of LC 50 at dose of (22.0±5.2)µL/mL and LC 90 (54.2±2.5µL/mL) when compared with other sea grass species involved in this study. This might be due to the flavonoid sulfate which inhibits the mosquito larvae alterations in the spiracular valves of the siphon and anal papillae [32 and 33]. The presence of phenols and reducing sugars are proved to have potential mosquito larvicidal activity [34]. Phenolic groups are highly hydroxylated which includes flavonols, hydroxycoumarins,
hydroxycinnamate derivatives, flavanols, flavanones, anthocyanins, proanthocyanidins, hydroxystilbene, aurones etc. Ravikumar et al.[18] reported that, the antibacterial activity of root extracts of C. serrulata against the poultry pathogen might be due to the presence of major chemical classes such as alkaloid and tannins. It was evident that, all the extracts showed moderate and low larvicidal effects; however, the highest larval mortality was found in ethanolic root extract of H. pinifolia (22.0±5.2) µL/mL. It is concluded from present findings that, the root extract of S. isoetifolium can be used as potential larvicidal agent against Ae. aegypti mosquito larvae.

The study of carrageenans by FTIR and FT-Raman spectroscopy shows the presence of very strong absorption bands in the 1 210-1 260 cm-1 region (S-O of sulfate esters) and in 1 010-1 080 cm-1 region (glicosidic linkage) in all carrageenan types. The other chemical groups are characteristics of a given carrageenan type: 3,6-anhydro-D-galactose at 925/935 cm-1, D-galactose- 4-sulfate at 840-850 cm-1, D-galactose-2-sulfate at 820-830 cm-1, D-galactose-6-sulfate at 810-820 cm-1, and 3,6-anhydro-D-galactose-2-sulfate at 800-805 cm-1[35]. In the FTIR spectra, both k and i-carrageenan present the 845-850 cm-1 band, but 800-805 cm-1 band is characteristic and distinctive of i-carrageenan. The relative shape of the 820-830 cm-1 band allows us to distinguish the l (broad band) and j-variant (sharp band) [36]. In comparative studies of carrageenan types, the FTIR spectra provide enough information. However, FT-Raman is a more easily applied method and the correspondent spectra have a clear resolution. Discrimination between k- and i-carrageenan is based in the 805 cm-1 peak, which has a stronger signal in FTRaman spectra than in FTIR one. FT-Raman spectra have an 815-900 cm-1 band with additional information to distinguish the -family carrageenan variants when compared with FTIR spectra. The -variant spectrum shows the 825 and 900 cm-1 peak and j-variant spectrum shows the 815, 850 and 900 cm-1 peaks. This may be an advantage of FTRaman spectroscopy when compared with FTIR [37 and 38].

In the present study FTIR analysis reveals the presence of phycocollides compounds at different ranges. The research shows that the medicinal values of the seagrass may be high quality of potential larvicidal activity.

This report demonstrating the mosquito larvicidal activity of the H. pinifolia is an encouraging trend unraveling the potential of the Indian coastline as a source of marine organisms worthy of further investigation. These organisms are currently being investigated in detail with the objective of isolating biologically active molecules which could be lead chemicals for bio-insecticides.

**Acknowledgement:**

The authors are thankful to the UGC Major Research project, New Delhi, India (MRP R.No: 41-472/2012(SR)) for providing financial assistance for this project. We specially express our thanks to the management of A.V.V.M. Sri Pushpam College (Autonomous), Poondi, for providing them necessary facilities and support to carry out this work.

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