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LARVICIDAL ACTIVITY OF SEAGRASS CYMODOCEA SERRULATA (R.BROWN) AGAINST THE DENGUE VECTOR (AEDES AEGYPTI)

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ABSTRACT

The larvicidal effects of seagrass cymodocea serrulata extracts were tested on the larvae of the dengue-vector, Aedes aegypti. A. *aegypti* mosquito late third and early fourth instars larva were exposed to a wide range of concentration of seagrass extract i.e. 10, 5, 2.5, 1.25 and 0.625 µg/ml and control to find out activity. Batches of 25 healthy late third and early fourth instars larvae were transferred to the 250ml water containing chambers and different concentrations of polar *C. serrulata* extract were added to assess the desired target dosage. Three replicates were performed for each concentrations and equal number of controls were also setup with tap water. The activity of extract showed maximum mortality effect on larvae of *A. aegypti* (L.). The LC_{50} and LC_{90} values of *C. serrulata* (*A. aegypti* (L.)) and Bleaching powder µg/ml values were: 2.34 and 15.49 & 2.73 and 19.31 at 24h.

KEYWORDS: Larvicidal activity, *Cymodocea serrulata*, Aedes aegypti, DNA.

INTRODUCTION

Aedes 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 of 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%.[1] *Culex quinquefasciatus* is a vector for an assortment of diseases that can be potentially fatal to humans. Although the *Culex* mosquito is not a primary vector for prevalent mosquito-borne diseases such as malaria, dengue and yellow fever, it can transmit a number of other ill effects that can pose serious health problems to human beings. It was known to contribute the spreading of the West Nile Virus, filariasis, and encephalitis. *Anopheles stephensi* is the major malaria vector in India. With an annual incidence of 300-500 million clinically manifest cases and a death of 1.1-2.7 million people. In all probability, some plants containing insecticidal phytochemicals that were predominantly secondary compounds were used to protect themselves against herbivorous insects.[2]

Fig. 1: Morphological aspects of *Aedes aegypti*. The left panel shows control larvae, pupa and adults.

The control of mosquito larvae worldwide depends primarily on continued applications of organophosphates such as temephos, fenthion and insect growth regulators such as diflubenzuron and methoprene.[3] Effective repeated use of these controlling agents have been adopted by several environmental and health agencies, including the disruption of natural biological control systems, other insect species, widespread development of resistance and undesirable effects on non-target organisms.[4] These problems have highlighted the need for new strategies for the control of mosquito larvae. Researchers are now looking for natural insecticides which do not have any ill effects on non-target population and are easily degradable.

DNA Isolation

DNA extraction protocols comprise of the basic steps of disruption of the cell wall, cell membrane and nuclear membrane to release the DNA into solution followed by precipitation of DNA while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites. This is brought about by disruption of the tissue in a mortar and pestle aided by liquid nitrogen and the various components of the homogenization or extraction buffer followed the precipitating and purification methods employed. Since DNA can be extracted from various types of tissues such as seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus, roots etc., the tissue type along with the concentration of DNA finally required determine the methodology of DNA extraction to be followed by the experimenter.[5,6]

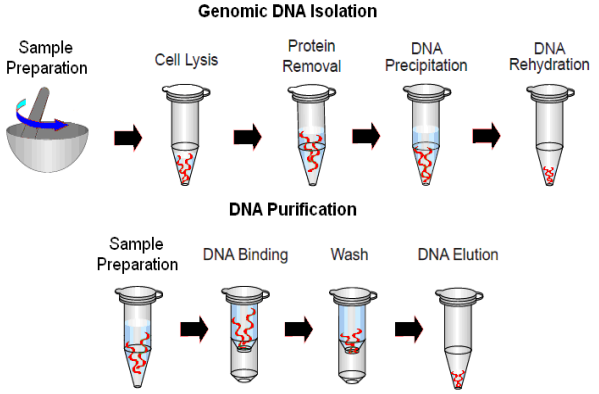


Fig. 2: DNA isolation of larva.

MATERIALS AND METHODS

Larvicidal Activity

Test organisms

The larvae of the mosquito species, *Aedes Aegypti* were collected from the aquatic environments of Chennai, Kanchipuram Districts. The larva were kept at 25 ± 2ºC and proper photoperiod was given for their growth. Late third and early fourth instars larva were (4-5 mm in length) used for larval bioassay purpose. The morphological and anatomical characteristics of the collected larva were observed and identified through microscopic analysis and by comparing to the standard keys.[7]

Bioassay

Larvicidal bioassay was performed based on WHO protocol.[8] *A. aegypti* mosquito late third and early fourth instars larva were exposed to a wide range of concentration of seagrass extract i.e. 10, 5, 2.5, 1.25 and 0.625 µg/ml and control to find out activity. Batches of 25 healthy late third and early fourth instars larvae were transferred to the 250ml water containing chambers and different concentrations of polar *C. serrulata* extract were added to assess the desired target dosage. Three replicates were performed for each concentrations and equal number of controls were also setup with tap water. Larval mortality was observed after 24 h. Mortality percentage was calculated using Abbott's formula.[9] LC_{50} and LC_{90} values were calculated using Probit analysis.[5,4]

$$\text{Mortality}(\%)=\frac{X-Y}{X}\times 100$$

Where, X= survival in the untreated control. Y= survival in treated sample.

Dose-response bioassay

Based on the preliminary screening results, crude extract were subjected to dose-response bioassay for larvicidal activity against the late third and early fourth instars larva of *A. aegypti*. The number of dead larvae was counted after 24 h and the samples turned out to be equal in their toxic potential.

Statistical Analysis

The larvicidal activity of seagrass extract was expressed in terms of lethal concentrations (LC_{50} and LC_{90}) of the average of larval mortality data that were subjected to Probit analysis.[10] For calculating LC_{50}, LC_{90} and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit and chi-square values were calculated using the software type, SPSS SOFTWARE 2007. Results with $p<0.05$ were considered to be statistically significant.

Isolation of Genomic DNA (DNA Fragmentation)

General instructions

- Wipe the work place with 30% hydrogen peroxide.
- Gloves and mask are must while performing the experiment.
- Perform the experiment on ice**.**

Materials required

Late third and early fourth instars Mosquito larva, 10%SDS, 1X TE buffer (pH 8), 5M Sodium acetate (pH 5.2), Phenol-chloroform-isoamyl alcohol (25:24:1), 70% Ethanol, isopropanol, Proteinase K (10mg/ml), distilled water, ice packs, 10µl tips, 200µl tips, 1000µl tips, 0.2ml eppis, 1.5ml eppis and 2ml eppis.

Equipments required

Cooling centrifuge (4°C), spectrophotometer, vortexer or cyclomixer, variable volume pipettes and Electrophoresis unit.

Reagent preparation

1X TE Buffer - Stock

1M Tris base: Add 1.214g in 10ml of distilled water. Adjust to pH 8 with HCl.

0.5M EDTA: Add 1.861g in 10ml of distilled water. Adjust to pH 8 with NaOH.

Note: EDTA is insoluble until it reaches pH 8. Use vigorous stirring or moderate heat (if desired).

Working solution (10mM Tris, 1mM EDTA)
1M Tris (pH 8) - 5ml
0.5M EDTA (pH 8) - 1ml
Make up to 500ml with distilled water. Store at 4°C.

1. 10% SDS
Dissolve 10g of SDS in 100 ml autoclaved distilled water Store at room temperature.

2. Proteinase K
Dissolve 10mg of Proteinase K in 1ml autoclaved distilled water. Store at 4°C.

3. 5M Sodium acetate
Dissolve 41g of sodium acetate in 100ml distilled water and adjust pH with acetic acid (pH 5.2). Store at 4°C.

4. 70% Ethanol
To 70ml of absolute ethanol add 30ml of distilled water Stored at room temperature.

Procedure
1. 2 ml culture was taken and the cells were harvested by centrifugation for 10 minutes.
2. 875 µl of TE buffer was added to the cell pellet and the cells were resuspended in the buffer by gentle mixing.
3. 100 µl of 10% SDS and 5 µl of Proteinase K were added to the cells.
4. The above mixture was mixed well and incubated at 37º C for an hour in an incubator.
5. 1 ml of phenol-chloroform mixture was added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes.
6. The contents were centrifuged at 10,000 rpm for 10 minutes at 4º C.
7. The highly viscous jelly like supernatant was collected using cut tips and is transferred to a fresh tube.
8. The process was repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.
9. 100 µl of 5M sodium acetate was added to the contents and is mixed gently.
10. 2 ml of isopropanol was added and mixed gently by inversion till white strands of DNA precipitates out.
11. The contents were centrifuged at 5,000 rpm for 10 minutes.
12. The supernatant was removed and 1ml 70% ethanol was added.
13. The above contents were centrifuged at 5,000 rpm for 10 minutes.
14. After air drying for 5 minutes, 200 µl of TE buffer or distilled water was added.
15. 10 µl of DNA sample was diluted to 1 or 2 ml with distilled water.
16. The concentration of DNA was determined using a spectrophotometer at 260/280 nm.
17. The remaining samples were stored for further experiments.

Agarose Gel Electrophoresis
General Instructions
- Wear gloves while performing the experiment.
- Preferably use a nitrile glove while handling EtBr.
- Stay quite away from UV light while observing the bands.

Materials required
Tris base, glacial acetic acid, EDTA, agarose, distilled water, ethidium bromide (EtBr), 6X gel loading dye.

Equipments required
UV trans-illuminator, electrophoresis unit (gel casting tray, comb and tank), power pack with electrodes, hot plate, weighing balance.

Reagent preparation
Stock: 50X TAE Buffer (pH 8) – 100ml
- ✓ Tris base 24.2g
- ✓ Glacial acetic acid 5.7ml
- ✓ EDTA 1.86g

Dissolve the above contents in 50ml of distilled water and adjust the pH to 8. Mix well and make up the volume to 100ml with distilled water and store at 4°C.

Working concentration: 0.5X TAE buffer (Tank buffer)
- ✓ 50X TAE 5ml
- ✓ Distilled water 495ml

Mix well and store at room temperature.

1% Agarose

Dissolve 1g of agarose in 100ml of 0.5X TAE buffer.

Ethidium bromide (EtBr)
Stock (10mg/ml): Dissolve 10mg of EtBr in 1ml of sterile distilled water and store at 4°C.

Working concentration: 0.5µg/ml

Protocol
Preparation of agarose gel
- Tape the ends of the casting tray and place the comb in its port.
- Prepare 1% agarose (1g in 100ml 0.5X TAE buffer).
- Heat to dissolve the contents on a hot plate or microwave oven until the solution becomes clear (Care should be taken while using microwave as it may overflow).
- Bring down the solution to luke warm condition by swirling periodically.
- Add 1µl of EtBr solution to the gel and mix well.
- Transfer the contents to the casting tray set up and allow it to solidify.
- After solidification, pull out the combs and the tape carefully.

- Place the tray in the electrophoresis chamber and add enough 0.5X TAE buffer till the gel submerges.

Loading the gel

- To 5µl of the DNA sample, add 1µl of 6X gel loading dye. (Final concentration should be 1X).
- Note down the order in which the samples were loaded.
- Carefully load the samples into the well (The samples should be loaded in the negative (black) terminal and run towards positive (red) as DNA is negatively charged).
- Also, load the DNA ladder (100bp) in one of the wells.

Running the gel

- Cover the chamber with the lid.
- Connect the electrodes to the power pack.
- Turn on the power supply. Set the voltage initially at 50V and look for bubble formation from the electrodes through the buffer to ensure that the set up works.
- Then shift the voltage to 100V. Maximum voltage can be varied depending on the size of the electrophoresis chamber.
- Let the samples run till it reaches the dye front (1cm above from the end of the gel, at the positive terminal).
- Turn off the power supply, disconnect the electrodes and remove the lid from the chamber.
- Carefully, remove the gel along with tray from the chamber.
- Place the gel alone in the UV trans-illuminator to visualize the bands.

Note

- Gel casting buffer and the tank buffer should be of same concentration.
- EtBr is a potent mutagen (may cause genetic damage) and moderately toxic after an acute exposure. It can be absorbed through skin, so it is important to avoid any direct contact with the chemical.
- The volume of gel can be varied depending on the size of the tray and the number of samples.

RESULTS AND DISCUSSION

Larvicidal Activity

Identification of mosquito larvae

Larvae of a mosquito can be identified from any other aquatic insects since it has a combination of two characters, they have no legs and the thorax is wider than the head or abdomen. The three divisions of the body part of mosquito larvae are head, thorax and abdomen. The structure of three body regions serves as the basis for identifying the mosquito larvae. The mosquito larva was identified using a compound microscope. A small amount of water with a mosquito larvae was drop in a slide to be able to view the specimen in the compound microscope.

The target mosquito larva in this study was the late third and early fourth instars larva of dengue carrying mosquito *Aedes aegypti*. *Aedes aegypti* larvae can be distinguished from any other mosquito larvae since it normally has a single hair, a three branch hair tufts on each side of the air tube. When the hair tuft has two or more branches all branches arise from the same socket. Other species have two or more hairs, branches and hair tufts on each side of the air tube or siphon. Identified *Aedes aegypti* mosquito larvae were separated from the other mosquito species and were placed in a water- filled plastic molder.

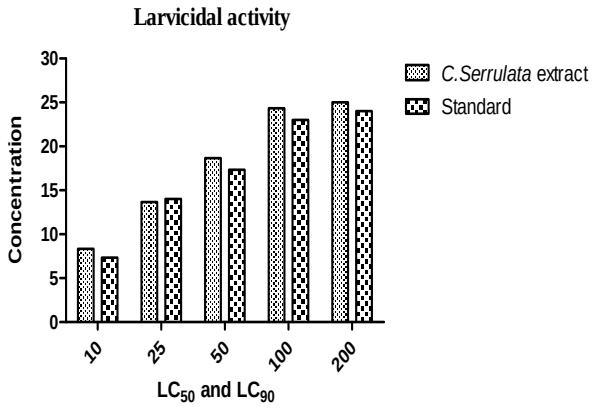
Larvicidal Assay

The larvicidal activity of the extract *C. serrulata* acid are tested on late third and early fourth instars larvae of *A. aegypti* (L.). The recorded percentage of mortality and the lethal concentrations (LC_{50} and LC_{90}) of the compounds are presented. The activity of extract showed maximum mortality effect on larvae of *A. aegypti* (L.). The LC_{50} and LC_{90} values of *C. serrulata* (*A. aegypti* (L.)) and Bleaching powder µg/ml values were: 2.34 and 15.49 & 2.73 and 19.31 at 24h.

Table 1: Larvicidal activity of *Cymodocea serrulata* extracts against *A. aegypti*.

Test larvae	Extracts	Mortality (%)	Conc. (µg/ml)	LC_{50} 95%confidence (LCL-UCL)	LC_{90} 95%confidence (LCL-UCL)	χ^2 Value
A. aegypti	Aqueous	- 23 31 49 64 89	Control 10 25 50 100 200	2.34 (1.90-2.88)	15.49 (10.58-27.37)	6.14
	Standard (Bleaching powder)	- 17 29 47 67 80	10 25 50 100 200	2.73 (2.21-3.40)	19.31 (12.75-36.20)	3.18

Where, LC_{50} & LC_{90} - lethal concentration at 50 % and 90 % of samples, respectively; LCL- lower confidential limit; UCL- upper confidential limit; χ^2 value – chi square value at $p > 0.05$ significant level.



Graph 1: Larvicidal Activity Curve.

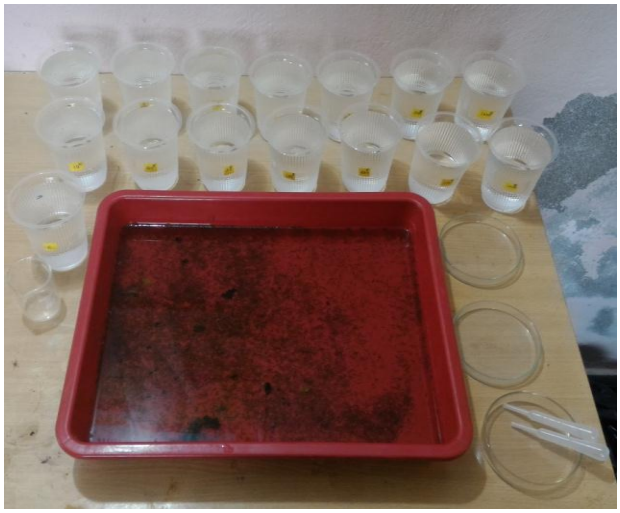


Fig. 3: Untreated larva.

Fig 4: Microscopic observation of live larva.

Fig 5: Treated larva.

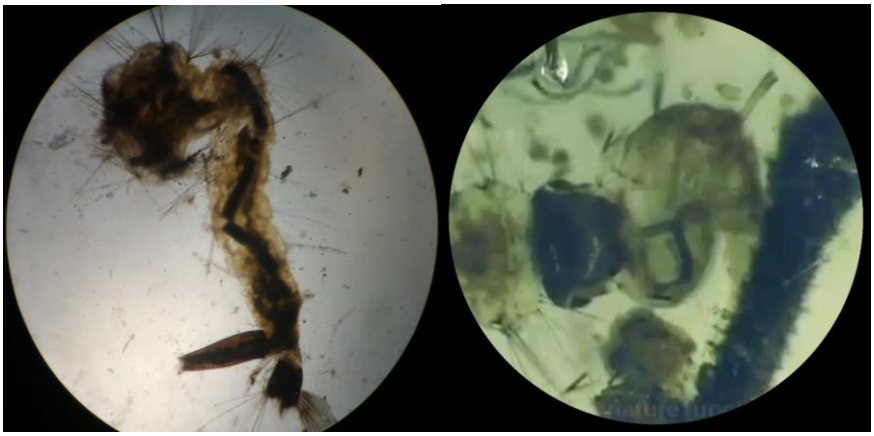


Fig 6: Microscopic observation of dead larva

Isolation of Genomic DNA

The genomic DNA was isolated and it was confirmed on agarose gel with DNA marker The concentration and purity of the DNA was measured the sample at 260/280nm by UV visible spectrophotometer and Photograph showed genomic DNA of larva. (Lane marker, 1, 2 & 3 – Genomic DNA of mosquito larva).

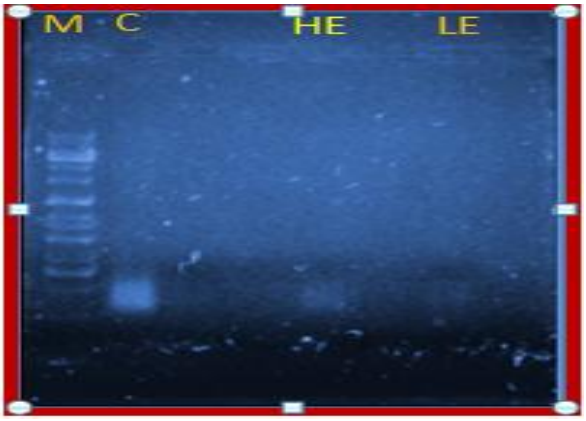


Fig 7: Agarose Gel Electrophoresis.

M - Marker
C - Control
HE - Higher Extract Concentration
LE - Lower Extract Concentration

DISCUSSION

The objective of the present study was the scientific assessment on the usefulness of seagrass specifically their potent antilarvicidal metabolites for dengue-drug development. The arthropoda group, mosquitoes (Diptera) is the vectors for a large number of human pathogens that cause a number of diseases including dengue.

The earlier researchers have also demonstrated that Indian marine plant extracts possesses potential larvicidal activity also reported the presence of diverse secondary metabolites in several seagrass that showed significant larvicidal activity.[11,12] Mosquito-larvicidal activity of leaf extract of *Cymodocea serrulata* against late third and early fourth instars larvae of *Ae. aegypti*have showed the LC_{50} and LC_{90}value: 0.0 780 and 0.1 675. The biological activity of this marine plant extractsmight be due to various compounds, including phenolic, terpenoides, flavonoids, saponins and alkaloids existingin plant. These compounds may jointly or independentlycontribute to produce larvicidal activity against mosquitoes.[13] The presence of phenols and reducing sugarsare proved to have potential mosquito larvicidal activity.[14]

Isolation of Genomic DNA

The genomic DNA was isolated and it was confirmed on agarose gel with DNA marker. The concentration and purity of the DNA was measured in the sample at 260/280nm by UV visible spectrophotometer.

DNA fragmentation

Initial morphology showed that the isolate was larva DNA.Futher, the PCR amplification of 16S rDNA gene revealed a single band of amplified DNA product of ~1500-bp, indicating efficient amplification. The reason for different sensitivity between larvae could be ascribed to the morphological differences between these larva. Furthermore, larva DNA damage using pant extract by strengthening natural host defenses that acts as a guardian against invading pathogens by creating a barrier against infection. Since, DNA isolate showed interesting larvicidal properties, it would be also useful to investigate their probiotic properties as it have been comparatively less studied as a pathogenic studies. The obtained results are considered to be sufficient for the further studies and ongoing studies deals with the process of isolation and identification of the active principles. These the first report to isolated mosquito larva DNA damage.

Therefore these findings could stimulate the development of new larvicidal agent. Further, investigations are progress to isolate the active compounds from the seagrass thatwere responsible for the larvicidal activity.

SUMMARY AND CONCLUTION

The larvicidal assay of crude extracts of seagrass species on late third and early fourth instars larvae of the mosquito, *Aedes aegypti*, showed maximum activity when compared to commercial using bleaching powder respectively. The isolated genomic mosquito damage DNA was identified and analyzed by agarose gel electrophoresis. The seagrass, *C. serrulata* extracts showed could form potential new source for the development of antimalarial drugs.

From the present findings, it is concluded that the larvicidal activity of seagrass extract against mosquito larvae, *A. aegypti* (L.). The larva DNA damage identification is being reported for the first time and these seagrass could be potential source of mosquito control agents. The GC-MS analysis methyl and phenols compounds were identified by seagrass extracts. Based on these premises that active compounds maybe responsible for larvicidal killing agents. It is clear that seagrass species are strongly suggested, when compared to the bleaching powder particularly regarding their action in the prevention of vector born diseases. Based on these premises, it is clear that this extract and seagrass species are strongly recommended, particularly regarding their action in the prevention of various diseases. The presently available antidengue drugs have been derived from natural products or their analogues and currently some-more such drugs are under clinical development. But these present investigation has been aimed to assess the mosquito larvicidal activities of seagrass and DNA fragmentation were identified and may lead to development of new drug with larvicidal activity.

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