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Screening of *Hydrilla verticillata* (L. F.) Royle (Hydrocharitaceae) crude leaf extracts for larvicidal efficacy against the filarial vector *Culex quinquefasciatus* say (Diptera: Culicidae)

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Abstract
Vector-borne diseases are one of the greatest contributors to human mortality and morbidity in the tropics and subtropics. Vector control remains the most effective measure and is often the only way to prevent disease outbreaks as there are no vaccines for many vector-borne diseases. Owing to their quick action, synthetic insecticides are the first line of action, but their continuous use led to the development of resistance and permanent residual effect on the bioenvironment which can be detrimental to animals including human. Therefore, biologically active plant materials have attracted considerable interest in mosquito control programs in the recent time. In the present study, the crude hexane, benzene, ethyl acetate, methanol and aqueous leaf extracts of *Hydrilla verticillata* were tested for the larvicidal efficacy against the early fourth instar larvae of *Culex quinquefasciatus* at concentrations of 62.5, 125, 250 and 500 mg/L. Mortality was recorded after 48 hours. Amongst the crude leaf extracts of *Hydrilla verticillata* tested, ethyl acetate extract was found to be effective. The LC_{50} value was 89.57 mg/L. Further investigations are needed to elucidate the larvicidal activity of *Hydrilla verticillata* crude ethyl acetate leaf extract against all stages of mosquito species and also the active ingredient(s) of the extract responsible for larvicidal activity should be identified.

Keywords: *Hydrilla verticillata*, crude leaf extracts, larvicidal efficacy, *Culex quinquefasciatus*

1. Introduction
Mosquitoes spread more diseases than any other group of arthropods and present an immense threat to millions of people, since they act as vectors for important parasites and pathogens, causing millions of death annually [1, 2] across the world, and especially in the Indian population every year [3]. Vector-borne diseases are one of the greatest contributors to human mortality and morbidity in the tropics and subtropics. Every year, more than one billion people are infected and more than one million people die from vector-borne diseases including malaria, dengue, yellow fever and lymphatic filariasis [4]. Vector control remains the most effective measure and is often the only way to prevent disease outbreaks because there are no vaccines for many vector-borne diseases and drug resistance is an increasing threat. One of the methods to manage these diseases is to control the vectors for bringing about interruption in disease transmission. The control of mosquitoes at larval stage is considered as an efficient way in the integrated vector management [5]. Therefore the ideal method for controlling mosquito infestation is the prevention of mosquito breeding by using appropriate larvicides. As stated by Amer and Mehlhorn [6], larval stages of the mosquitoes are attractive targets for pesticides to control mosquito populations. Hence, the advantage of targeting the larval stages are that mosquitoes are killed before they disperse to human habitations and that larvae, unlike adults, cannot change their behavior to avoid control activities [7] and also to reduce overall pesticide use in control of adult mosquitoes by aerial application of adulticidal chemicals [8]. Owing to their quick action, synthetic insecticides are the first line of action, but their continuous use led to the development of resistance and permanent residual effect on the bioenvironment which can be detrimental to animals including human [9, 10] and higher rate of biological magnification [11]. These factors have created a need for search of easily biodegradable alternative insecticides. Therefore, biologically active plant materials have attracted considerable interest in mosquito control programs in the recent time [12]. Current studies are focused to find out natural substances particularly from plants to control the disease transmitting vectors and a recent emphasis has been placed on plant material and various reports on the use of natural plant products against mosquito vectors have been documented [13-29].

Plant materials with insecticidal properties have been used traditionally for generations throughout the world [30]. Researchers are reconsidering botanicals containing active phytochemicals in their efforts to address some of these problems [31]. Consequently, one of the most effective alternative approaches under the biological control program is to explore the floral biodiversity and enter the field of using safer insecticides of botanical origin as a simple and sustainable method of mosquito control. The search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable, remains a top research issue for scientists associated with alternative vector control [32]. *Hydrilla verticillata* (L.F.) Royle (Hydrocharitaceae) commonly called tape grass or water thyme in English, amiranappaci, cikavalakam or cimpaka in Tamil and jhangi or kureli in Hindi is a non-native, aggressive, slender, submerged, aquatic weed found distributed throughout India, Sri Lanka, Malaysia, China, Australia, Europe and United States [33]. Various parts of this plant are being used in tribal medicines for neurological problems, gastrointestinal disturbances, malnutrition, cardiovascular disturbances, diabetes [34, 35], improved blood circulation, detoxification, control of blood sugar level, boosting immunity level and to slow ageing process [36]. It is also used in the treatment of abscesses, boils and wounds [37]. Phytochemical constituents include alkaloids, flavonoids, phenols, terpenoids and saponins [37]. The plant has antitumor, antibacterial [33, 38], antimicrobial [39] and wound healing [40] properties.

2. Materials and methods

2.1. Plant collection and extraction

Mature fresh plants of *Hydrilla verticillata* collected from Chembarambakkam lake, (13.01158° N 80.06063° E) Kanchipuram district, Tamil Nadu, India, were brought to the laboratory. Taxonomical identity of the plant was confirmed at the Department of Plant Biology and Plant Biotechnology, Women's Christian College, Chennai, Tamil Nadu, India. The fresh and healthy leaves were isolated from the plant, washed with dechlorinated tap water and shade dried at room temperature. Dried leaves of *Hydrilla verticillata* were powdered with the aid of an electric blender. The powdered leaves (1 Kg) was sequentially extracted with 3 L of both non-polar and polar solvents *viz*., hexane, benzene, ethyl acetate, methanol and distilled water using a Soxhlet apparatus [41]. The crude leaf extracts were filtered through a Buchner funnel with Whatman number 1 filter paper. The crude leaf extracts were then evaporated to dryness in a rotary vacuum evaporator. The hexane, benzene, ethyl acetate, methanol and aqueous leaf extracts of *Hydrilla verticillata* were thus obtained and one per cent stock solution prepared by adding adequate volume of acetone for each extract was refrigerated at 4°C until testing for bioassay.

2.2. Test mosquitoes

Culex immatures collected from various places in Chennai, Tamil Nadu, India were transported to the laboratory in plastic containers. In the laboratory, the immature mosquitoes were transferred to enamel larval trays until adult emergence. After emergence, the adult mosquitoes were identified upto species level and confirmed before rearing. Cyclic generations of *Culex quinquefasciatus* were maintained separately in two feet mosquito cages in an insectary with a mean room temperature of 27 ±2°C and a relative humidity of 70-80%. The adult mosquitoes were fed on 10% glucose solution. The eggs laid were then transferred to enamel larval trays maintained in the larval rearing chamber. The larvae were fed with larval food (dog biscuits and yeast in the ratio 3:1). The larvae on becoming pupae were collected, transferred to plastic bowls and kept inside a mosquito cage for adult emergence.

2.3. Larvicidal bioassay

Standard WHO [42] protocol with minor modifications was adopted for the study. The tests were conducted in glass beakers. *Culex quinquefasciatus* immatures particularly early fourth instar larvae from laboratory colonized mosquitoes of F_1 generation were used for the study. Larvicidal activity at test concentrations of 62.5, 125, 250 and 500 mg/L of the crude leaf extracts was assessed. The required test concentrations and quantity of test solution was prepared by serially diluting one per cent stock solution of each crude extract. Twenty healthy larvae were released into each 250 ml glass beaker containing 200 mL of water and test concentration. Mortality was observed 48 hours after treatment. A total of three trials with three replicates per trial for each concentration were carried out. Controls were run simultaneously. Treated control was prepared by the addition of acetone to distilled water. Distilled water served as untreated control. The larval per cent mortality was calculated and when larval control mortality ranged from 5-20% it was corrected using Abbott's formula [43].

$$\text{Larval per cent mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

2.4. Statistical analysis

Data from all replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated using SPSS software by probit analysis [44]. ANOVA was performed to determine the difference in larval mortality between concentrations. Results with $P<0.05$ level were considered to be statistically significant.

3. Results

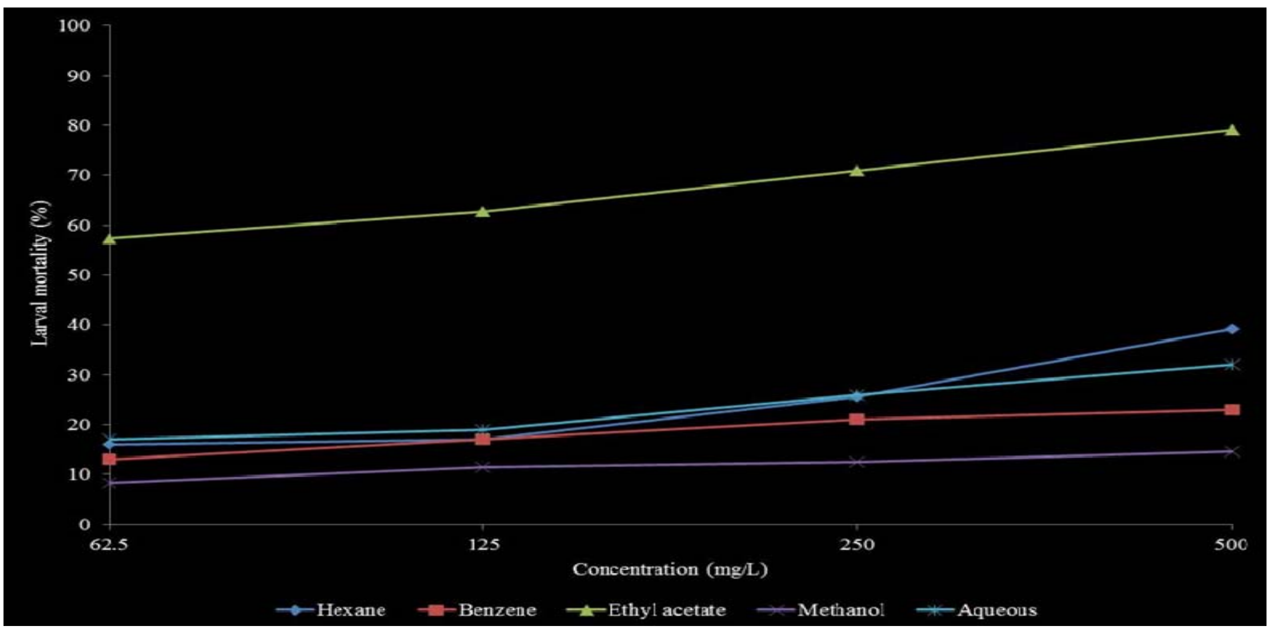
Results of the larvicidal effects of crude leaf extracts of *Hydrilla verticillata* against *Culex quinquefasciatus* are presented in Table 1 and Figure 1. No larval mortality was observed in treated and untreated control. The extracts showed a dose-dependent toxicity to *Culex quinquefasciatus* larvae. Amongst the extracts tested, the crude ethyl acetate extract of *Hydrilla verticillata* leaves was found to be effective with one hundred per cent mortality at 500 mg/L and LC_{50} value was 89.57 mg/L.

Table 1: Larvicidal activity of *Hydrilla verticillata* crude leaf extracts against *Culex quinquefasciatus*

		Hexane	Benzene	Ethyl acetate	Methanol	Aqueous
Concentration (mg/L)	62.5	3.20 ±1.55 ^{ab}	2.60 ±1.94 ^{ab}	11.48 ±5.24^{b}	1.68 ±1.75 ^{ab}	3.40 ±4.82 ^{ab}
		(16.00)	(13.00)	(57.40)	(8.40)	(17.00)
	125	3.40 ±1.78 ^{ab}	3.40 ±2.30^{b}	12.54 ±3.67^{b}	2.3 ±1.16^{ab}	3.80 ±3.27 ^{ab}
		(17.00)	(17.00)	(62.70)	(11.50)	(19.00)
	250	5.12 ±4.47^{b}	4.20 ±2.04^{b}	14.18 ±2.85^{b}	2.52 ±1.75 ^{ab}	5.20 ±4.32 ^{ab}
		(25.60)	(21.00)	(70.90)	(12.60)	(26.00)
	500	7.48 ±4.10^{b}	4.60 ±2.19^{b}	15.84 ±1.56^{b}	2.92 ±2.71^{b}	6.40 ±4.03^{b}
		(39.20)	(23.00)	(79.20)	(14.60)	(32.00)
LC_{50} (mg/L)		621.90	1318.11	89.57	1688.55	784.83
LC_{90} (mg/L)		1297.27	2895.52	650.86	3236.32	1758.23

Values are mean of three replicates of three trials ±standard deviation. Values in parenthesis denote per cent larval mortality. Different superscript alphabets indicate statistical significant difference in larval mortality between concentrations at $P<0.05$ level by one way ANOVA followed by Tukey's test. LC_{50}: Lethal concentration that kills 50% of the exposed larvae; LC_{90}: Lethal concentration that kills 90% of the exposed larvae.

Figure 1: Larvicidal activity of *Hydrilla verticillata* crude leaf extracts against *Culex quinquefasciatus* at 48 hours



4. Discussion

Reducing mosquito-borne diseases remains a big challenge even at the most advancement of modern sciences [45]. Mosquitoes in the larval stage are attractive targets for pesticides because they are confined to water and their habitat is easily treatable. Eliminating the larval stage is advantageous because the mosquitoes cannot disperse or acquire human pathogens [46]. Present scenario on the avoidance of synthetic insecticidal applications to combat mosquitoes, has resulted in development of resistance without eliminating the risk of vector-borne diseases outbreak [47, 48]. Thus, this has necessitated the exploration of natural products for the control of vector insects in general and mosquitoes in particular [49-51]. The results of the present study were comparable with earlier reports of larvicidal activity against *Culex quinquefasciatus*. Thangam and Kathiresan [52] screened seaweeds, seagrasses and mangroves for their larvicidal, skin repellent and smoke repellent activities against mosquitoes. In addition, Yu *et al.* [53] has provided an exhaustive review on the major bioactive components of seaweeds and their mosquitocidal potential. Coastal halophytes widespread in tropical and subtropical regions contain biologically active mosquito larvicidal compounds [54, 55]. The acetone and petroleum ether extracts of four mangrove plant species (*Avicennia marina*, *Avicennia officinalis*, *Rhizophora apiculata* and *Rhizophora mucronata*), were examined for their larvicidal activity against *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi*. Highest mortalities were recorded at 250 ppm for leaves of combination of *Avicennia marina* and *Avicennia officinalis* against *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* with LC_{50} values of 34.622, 87.681 and 206.047 µg/ml respectively [56]. Nazar *et al.* [57] screened one hundred Indian coastal plants for larvicidal activity against *Culex quinquefasciatus* and found *Cymbopogon citratus* (LC_{50} value 24.0 mg/L) and *Abrus precatorius* (LC_{50} value 30.0 mg/L) to show the highest larvicidal activity. Bream *et al.* [58] showed that the petroleum ether leaf and root extracts of the aquatic plant *Echinochloa stagninum* possessed larvicidal activity against *Culex pipiens* with LC_{50} values of 80.32 and 112.78 ppm respectively. Annie *et al.* [59] showed that crude hexane and methanolic leaf extracts of *Eichhornia crassipes* was found to be the most effective and LC_{50} values were 80.54 and 135.70 mg/L. Likewise, Jayanthi *et al*. [60] reported the ethanol fractionate of *Eichhornia crassipes* with LC_{50} values of 71.43, 94.68, 120.42, 152.15 and 173.35 ppm for I, II, III, IV instar larvae and pupae, respectively against *Culex quinquefasciatus*. Valentina *et al*. [61] reported the aqueous, acetone and ethanol extracts of *Turbinaria conoides* to exhibit larvicidal activity against *Aedes aegypti* (LC_{50} values of 18.74; 100.07; 64.27 mg/L), *Anopheles stephensi* (LC_{50} values of 66.62; 76.35; 88.18 mg/L) and *Culex quinquefasciatus* (LC_{50} values of 82.74; 62.12; 74.45 mg/L).

Bianco *et al*. [62] reported that at 10 ppm, the hexane extract of red seaweed *Laurencia dendroidea* exhibited the strongest larvicidal effect (100% mortality) against *Aedes aegypti* as compared to ethyl acetate (37% mortality), dichloromethane (70% mortality), and methanol (15% mortality) extracts. In general, extracts of the plants derived from specific solvents can influence the bioactivity, probably because of the active components present in large quantities [63]. It has been demonstrated that the extraction of active biochemical from plants confides upon the polarity of the solvents utilized. Polar solvent will extract polar molecules and non-polar solvents extract non-polar molecules. This was gained by using mainly eleven solvent systems ranging from hexane/ petroleum ether, the most non polar (polarity index of 0.1 that mainly extracts essential oil) to that of water, the most polar (polarity index of 10.2) that extracts biochemical with higher molecular weights such as proteins, glycans, etc. Ethyl acetate is a moderately polar solvent (polarity index of 4.4) that mainly extracts steroids, alkaloids, etc. Those biochemicals that were extracted using moderately polar solvents were seen to give good results as reported by a few bioassay. This corroborates with the findings of the present study. Thus, different solvent types can significantly affect the potency of extracted plant compounds and there is difference in the chemo-profile of the plant species [20].

Plants are store house of phytochemicals, which are widely used in the place of synthetic insecticides [64]. Plant extract might have some complex mixture of biocidal active compounds, including phenolics, terpenoides, flavonoids, steroids, saponins, tannins and alkaloids which may jointly or independently contribute to the mortality and delayed growth of larvae [65]. This may be the cause of larvicidal activity in the present study as earlier studies conducted on other macrophytes have reported that tannins and alkaloids in *Pistia stratiotes* and saponins and tannins in *Nymphaea lotus* to be responsible for larval toxicity of *Anopheles* mosquitoes [66]. The mode of action and site of effect for larvicidal phytochemicals has received little attention. Rey *et al*. [67] and David *et al*. [68] found that botanical derivatives primarily affect the midgut epithelium and secondarily affect the gastric caeca and the malpighian tubules in mosquito larvae. The ethyl acetate leaf extract of this plant may be fractioned in order to locate the particular bioactive toxic agent responsible for larval toxicity. Subsequently, for improving the potency and stability of the product, in depth investigation on the active compound of this plant is needed. Further investigations for the mode action of the phytoconstituents, effects on non-target organisms and field evaluation are necessary.

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