Chromatographic separation of bioactive compounds of *Leucas aspera* (L) and larvicidal activity of *Anopheles stephensi* larvae

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Abstract

Malaria is a life-threatening disease caused by plasmodium parasites that are transmitted to people through the bites of infected female mosquito Anopheles stephensi. Medicinal plant Leucas aspera was selected to determine the larvicidal activity due to its potent pharmacological activity. The present study evaluates the bioactive components and larvicidal potential of leaf extracts of Leucas aspera against Anopheles stephensi larvae. The bioactive components were identified using thin layer chromatography (TLC). Larvicidal efficacy was studied with different concentration of Leucas aspera leaf extracts against Anopheles stephensi. The yield of leaf extract was 45%, 21.25%, 37.2% for ethanol, distilled water and hexane solvents respectively.

The presence of phytochemicals was higher in ethanolic leaf extract rather than water and hexane extract. The phytochemical screening showed the presence of tannin, phlobatanins, steroids, saponin, terpenoids, phenol, glycosides, mentione, terpene alcohol, sterol and quercertin. The LC50 and LC90 for the Leucas aspera ethanolic extract against the 4th instar larvae after 24hrs of incubation were 24.08 ppm and 168.96 ppm respectively and after 48hrs of exposure 51.67 ppm and 189.46 ppm respectively. It is clearly evident from the study that the ethanolic leaf extract of Leucas aspera will probably function as an eco-friendly vector controlling agent.

Keywords: *Leucas aspera,* Phytochemicals, Larvicidal activity, *Anopheles stephensi,* Eco-friendly larvicides.

Introduction

Mosquitoes are the vectors for numerous infectious diseases. World Health Organisation (WHO) has declared mosquitoes as public health pest throughout the world; they are responsible for the transmission of various dreadful disease-causing pathogens. *Anopheles, Aedes, Culex* species are the major vectors of transmitting mosquito borne diseases. Malaria is a life-threatening disease caused by *plasmodium* parasites that are transmitted to people through the bites of infected female *Anopheles stephensi* Liston in 1901. In 2018, WHO estimated 228 million new infections and 405000 case deaths resulted from malaria worldwide[60]. The mosquito borne diseases can be controlled by the interruption of the disease transmission, either by preventing mosquitoes bite using repellents or by causing larval mortality using larvicides[15]. Synthetic chemicals such as organophosphates, insect growth regulators (e.g. diflubenzuron, methoprene) are used as larvicides[14]. The mosquito larvae have developed resistance against chemical insecticides. This has become a significant trouble in vector control. Larvicidal activity is the easiest and best way to control mosquitoes in their breeding places before they emerge into adults. The overuse of synthetic insecticides and larvicides has resulted in environmental hazards and synthesis of non-bio-degradable toxic components[19].

Plants are natural source of bioactive compounds (secondary metabolites) containing significant larvicidal property[14]. Some phytochemicals act as general toxicants against both the adult as well as larval stages of mosquitoes. They inhibit the growth and development and metamorphosis of larvae[9,44]. Plant origin larvicides do not cause toxicity to human and domestic animals and are easily biodegradable. *Leucas aspera* (Thumbai) is a small, herbaceous, erect plant and commonly used as an antipyretic herb in India[2]. The juice from the leaves is used as an external applicant for psoriasis and painful swellings. The leaves are useful for the treatment of chronic rheumatism and the bites of serpents, poisonous insects and scorpion stings[35.]

Thus, *Leucas aspera* is a source of medicinally active compounds having various pharmacological effects[44]. The smoke of leaves of *Vitex negundo* and *Leucas aspera* is more toxic to the filarial vector mosquitos.[2,58] Hence, the present study focuses on extraction of essential oils from *Leucas aspera* by solvent extraction and to determine its larvicidal activity against the *Anopheles stephensi*.

Material and Methods

Collection of plant material: The leaves of *Leucas aspera* were collected from the Maruthamalai hill region of Coimbatore[28]. The plant was authenticated in Tamil Nadu Agricultural University, Coimbatore. Leaves were washed thoroughly in water to remove dust and shade dried for two days at room temperature. Dried leaves were grinded and sieved to get homogeneous leaf powder.

Extraction: The solvent extraction method was adopted to extract the phytochemicals using Soxhlet apparatus[28]. About 20g of dry powdered sample of plant leaves of *Leucas aspera* was filled in thimble and extracted using three solvents which includes 70% ethanol, 70% hexane and distilled water. Extraction of each solvent was carried out

separately. The boiling point of each solvent was maintained throughout the extraction process for 10 hours.

After 10 hours of extraction, the various secondary metabolites in the leaves of *Leucas aspera* were extracted by the solvent and collected in round bottom flask along with the solvent[7]. The rotary vacuum evaporator embedded in this unit was utilised to removes the solvents and to concentrate the extract. The extract was preserved in 5°C in airtight bottle until further use. The yield of leaf extract was calculated as:[43]

Yield % = (weight of concentrated extract (after removal of solvent) / weight of dry plant) x 100

Phytochemical analysis: The plant extracts were analysed for the presence of phytochemicals using thin layer chromatography. Silica plates were prepared and leaf extracts of *Leucas aspera* were loaded on 3 silica plates for the separation of phytochemicals. The mobile phase and the visualizing agents are summarized in table 1[48]. The movement of the analytes is expressed by its retardation factor R_f:

R_f = Distance moved by analytes from origin / Distance moved by solvent front from origin.

Collection of *Anopheles stephensi* larvae: The larval sampling was done by the standard dipping method as recommended by WHO[61]. The larvae of *Anopheles stephensi* were collected from the stagnant clean water around Government College of Technology, Coimbatore, Tamilnadu, India. The collected larvae were kept in the tray containing water in which the larvae had grown (culture medium) at laboratory condition[4] (29°C). All the instar larvae were collected and the 4th instar larvae were used in this study (Figure 7)[22]. Each sample larva was individually mounted in Berlese's medium on a microscope slide and identified to species extent by the morphological characters[10].

Larvicidal Bioassay: Larvicidal activity was carried out with various concentrations (ppm) of ethanolic extract. 10mg of concentrated extract was added to 1ml acetone and this solution was made up to 50ml by adding distilled water. 30 larvae per plate were taken for the test. The larvae were treated with the plant extracts of 50ppm, 100ppm, 200ppm concentrations. A corresponding control was maintained. The larval mortality of fourth instar of *Anopheles stephensi* was observed. The number of larvae surviving at the end of 24 and 48 hours were recorded. The percentage of mortality was calculated as:[36]

(No. of larva dead /Total No. of larvae) *100

Based on the percent mortality values, LC_{50} and LC_{90} values of plant extract of *Leucas aspera* against *Anopheles stephensi* were calculated by the regression line employing probit analysis[18].

Results and Discussion

Solvent extraction: The leaves of the plant *Leucas aspera* (Figure 1) was collected and shade dried and homogenised (Figure 2) to obtain as fine granules.

The percentage yields of leaf extract obtained from ethanol, distilled water and hexane extracts were 45%, 21.25% and 37.2% respectively by Soxhlet method (Figure 3).

Table 1
TLC analysis

Component	Stationary phase	Mobile phase	Visualizing agent
Phenol, flavonoid	Silica plate	Acetone: ethyl acetate (1:1)	Chloroform: methanol (9:1)
Tannin	Silica plate	Chloroform: water (6:4)	Ferric chloride spray (1%)

Figure 1: *Leucas aspera*

Figure 2: Homogenised leaf powder of *Leucas aspera*

Thin layer chromatographic separation (TLC): TLC profiling was performed to analyse the compounds present in the extract. When subjected to TLC, the ethanol extract showed the presence of more phytochemical compounds compared to the other solvent extracts. Thus, the ethanolic extract on TLC plate (Figure 4) showed 7 bands with Rf values of 0.94, 0.73, 0.66, 0.625, 0.58, 0.5 and 0.32 which correspond to the following compounds such as triterpenoids and steroids, phenolic compounds, flavonoids, mentione, saponin, terpene alcohol, sterols. The TLC profiling for aqueous extract showed 3 bonds (Figure 5) with Rf values of 0.91, 0.68, 0.36 which corresponds to the compound such as steroids and triterpenoids, flavonoids and glycosides, sterols.

Similarly, TLC profiling of hexane extract (Figure 6) showed 3 bonds with Rf values of 0.73, 0.62 and 0.3 which correspond to the compounds such as phenolic compound, flavonoid and sterol. Thus, the ethanolic extract shows the presence of many secondary metabolites when compared to aqueous and hexane solvent extracts (Table 2).

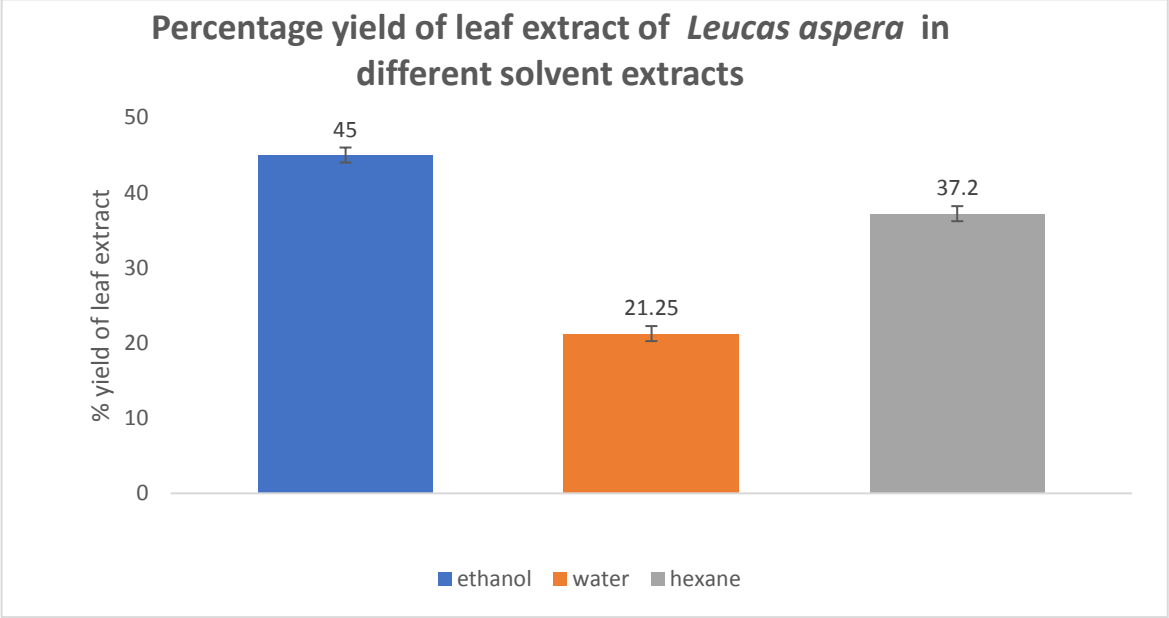

Figure 3: The percentage yield from the ethanol, water and hexane extracts.

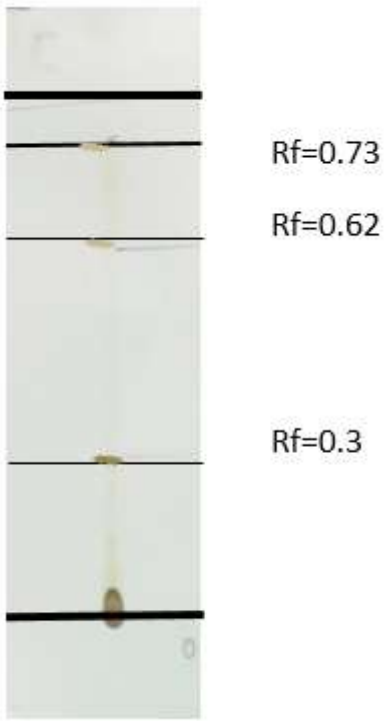

Figure 4

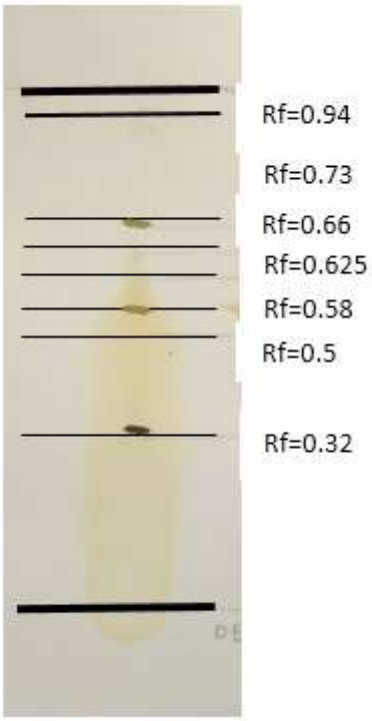

Figure 5

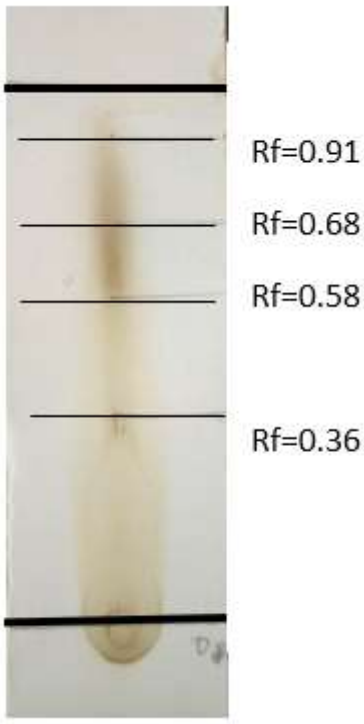

Figure 6

Figure 4,5,6: TLC separation of phytochemicals from the hexane, ethanol and water extracts of *Leucas aspera*

Larvicidal activity: The percentage of mortality after the exposure of ethanolic leaf extract on the larvae of *Anopheles stephensi* in 24hrs and 48hrs interval was determined (Figure 8). Percentage of mortality of larvae was understood from the count of dead larvae count. At 50 ppm concentration of the ethanolic extract, the percentage of mortality was 50% and 60% for 24 and 48 hours of incubation. Similarly, the mortality % of larvae were 63% (24 hrs) and 66% (48 hrs) at 100 ppm and increased to 93% (24 hours) and 100% (hours) when the concentration of ethanolic leaf extract increased to 200ppm. The LC50 and LC90 for the ethanolic extract of the *Leucas aspera* against the 4th instar larvae after 24hrs of incubation were 24.08 ppm and 168.96 ppm respectively and after 48hrs of exposure 51.67 ppm and 189.46 ppm respectively (Figure 9).

Table 2
Phytochemical analysis

S.N.	Solvent	Water Extract	Ethanol Extract	Hexane Extract
1	Tannin	+	+	-
2	Phlobatannin	+	-	-
3	Steriod	+	+	+
4	Saponin	+	+	+
5	Terpenoids	+	+	+
6	Phenol	+	+	+
7	Glycosides	+	+	+
8	Mentione	-	+	-
9	Terpene alcohol	-	+	-
10	Flavonoid	+	+	+
11	Sterols	-	+	+
12	Quercertin	-	+	-

Figure 7: the different instar larvae of *Anopheles stephensi*

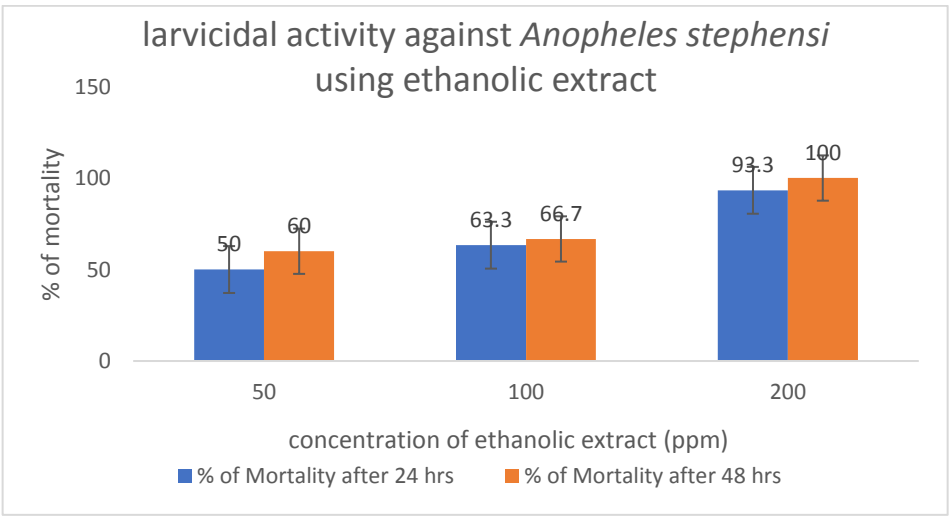

Figure 8: the larvicidal activity against *Anopheles stephensi* was tested after 24 hr and 48 hr with different percentage of concentration

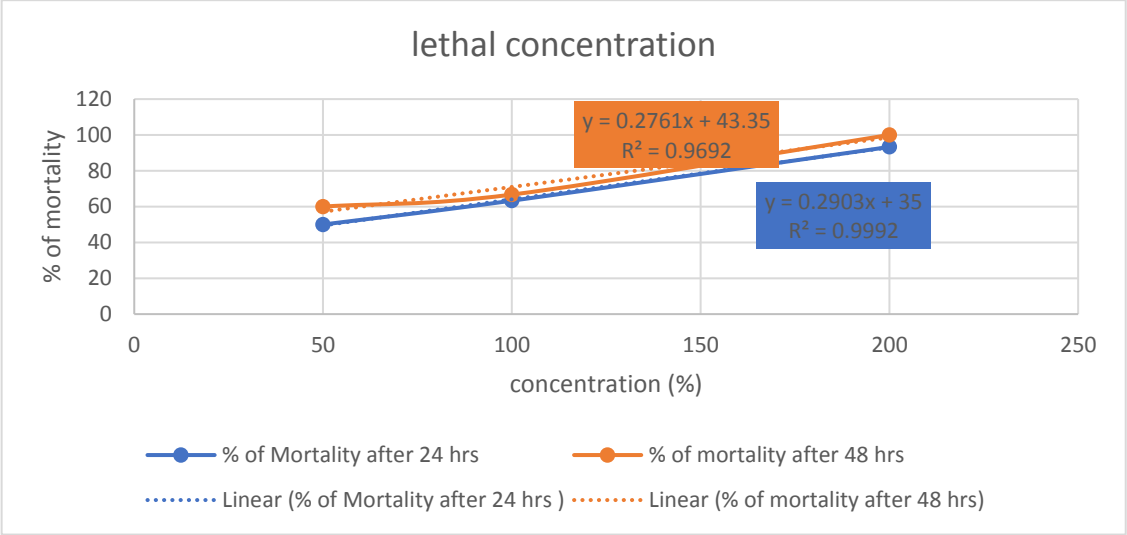

Figure 9: LC50 and LC90 of ethanolic extract of *Leucas aspera* against *Anopheles stephensi*

Anopheles stephensi is the primary urban vector of malaria in India[15]. India contributes nearly 77% of the total malaria in Southeast Asia[5]. Mosquito control is a difficult task and the development of insecticide resistance of the vector has made the controlling process even more complicated[52]. One of the promising strategies to control the vector borne disease is controlling the growth of larvae with the aid of larvicides. Application of chemical larvicides may harm the environment and an alternate larvicide which is eco-friendly is the need of the hour. The present study focussed on the use of plant derived phytochemicals as a potential eco-friendly larvicide. A significant number of plant extracts have been reported to have larvicidal or repellent activities against the mosquito vectors but only few plant products have been shown to have practical utility for mosquito control [15].

Leucas aspera is an annual, branched, herb of height 15-60 cm with stout and hispid acutely quadrangular stem and branches. The leaves are sub-sessile or shortly petiolate, linear or linearly lanceolate, obtuse, pubescent up to 8.0 cm long and 1.25 cm broad, with entire or crenate margin, petiole 2.5-6 mm long[9,35].

Rahman and Islam[39] have reported that *Leucas aspera* is rich in antibacterial, antifungal, larvicidal, antihyperglycemic, hepatoprotective and antihyperglycemic activity. Hence, *Leucas aspera* plant was selected to determine the larvicidal activity. Water, ethanol and hexane solvents were used to extract the phytochemicals from the leaves of *Leucas aspera* using Soxhlet method. TLC profiling of all the three extract was carried out and among them, the ethanolic extract showed increased presence of phytochemicals. The phytochemical analysis of the ethanolic extract of *Leucas aspera* showed the presence of Tannins, Phlobatannin, Steroid, Saponin, Terpenoids, Phenol, Glucosides, Mentione, Terpene alcohol, Flavanoids, Sterols and Quercertins. The result was in good agreement with Manda[37] who reported that polar solvent extracted higher quantity of secondary metabolites rather than non-polar solvents. Ethanol is less dangerous compared when compared to other solvent such as methanol and hexane and also requires less volume by which less time is required for rotary evaporation[54]. Satla et al[50] and Gupta et al[23] have summarized the chemical constituents of the leaves of *Leucas aspera* which include flavonoids, alkaloids, steroids, resins, saponins and proteins. The results of phytochemical analysis of leaf extract of *Leucas aspera* are similar with the reports of Mahadeva et al[35].

The larvicidal activity of the ethanolic extract of the leaves of *Leucas aspera* was evaluated. The percentage of mortality of larvae of *Anopheles stephensi* increased with increased concentration of the ethanolic extract. 100% mortality was attained at 200 ppm concentration. The LC50 and LC90 values for the ethanolic extract of the *Leucas aspera* against the 4th instar larvae after 24hrs of incubation were 24.08 ppm and 168.96 ppm respectively and after 48hrs of exposure 51.67 ppm and 189.46 ppm respectively.

The present findings are in good correlation with Elumalai et al[15]. They evaluated the larvicidal activity of the methanol whole plant extract of *Leucas aspera* on the fourth-in star larvae of *Aedes aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* and reported that *Aedes aegypti* was found to be most susceptible and the methanol extracts of *Leucas aspera* had pronounced larvicidal activity. The LC50 and LC90 values of *Leucas aspera* 4th instar larvae of *Anopheles stephesi* was 35.624 ppm and 64.260 ppm for 24 hrs incubation and after 48hrs incubation the LC50 LC90 values were 20.867 and 60.096 ppm respectively.

Kovendan et al[30] studied the larvicidal effect and pupicidal activity of *Leucas aspera* against *Anopheles stephensi* and showed that the ethanol extract of *Leucas aspera* and *B. sphaericus* exhibited an excellent control over the malarial vector *Anopheles stephensi*. Maheswaran[36] has reported that the hexane extract of *Leucas aspera* showed highest larvicidal activity. The LC50 values of *Leucas aspera* against fourth instar larvae of *Culex quinquefasciatus* were

230.71ppm and against *Aedes aegypti* 257.17 ppm respectively. Sakthivadivel and Daniel[49] have demonstrated 90% mortality of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* when exposed to 4% of *Leucas aspera* extract.

Several studies have focused on natural products for the control of *Anopheles stephensi* mosquitoes and larvae with various results. Suganya et al[52] have evaluated the larvicidal potential of the silver nanoparticles synthesized from *Leucas aspera* leaf extract against dengue vector *Aedes aegypti* and found that the silver nanoparticles have a higher larvicidal potential as compared to crude solvent extracts. Similar study was carried out by Elumalai et al[13]. They used silver nano particles synthesised from *Leucas aspera* against *Anopheles stephensi* larvae. Elumalai et al[14] have reported LC50 and LC90 values of *Tridax procumbens* against 4th instar larvae of *Anopheles stephesi* were 74.418 ppm and 207.398 ppm for 24hrs incubation and after 48hrs incubation were 65.168 and 199.047 ppm respectively.

Velu et al[57] have studied the histopathological changes in the fourth instar of *Aedes aegypti* when exposed to methanol peel extract of *A. hypogaea.* They have reported that the larval mortality can be attributed to the presence of alkaloids, phenols, flavanoids and terpenoids in an insecticidal compound [21].

Raymond[47] reported that reduction in the rate of mortality could be due to biodegradation of terpene in water as its resistance in water is reduced by its volatilization. Elumalai et al[15] have reported that the polyphenolic compound catechin has high potential to collapse the midgut of larvae. Larvae consists of a unicellular epithelial layer resting upon a basement membrane. The phenolic compound targets the midgut of the larvae and cause swelling, elongation, enlargement of nucleus and separation from the basement membrane.

In the present study, 100% mortality obtained at 200 ppm of the ethanol leaf extract can be attributed to the presence of phenolic compounds, tannins, terpenoids and flavonoids in the ethanolic extract of the leaf extract of *Leucas aspera.* These phytochemicals inhibit the metamorphosis of larvae. It is clearly evident from the present analysis that the ethanol leaf extract of *Leucas aspera* has a promising larvicidal efficacy. The leaf extract of the plant *Leucas aspera* may serve as suitable alternative to synthetic insecticides in future as they are relatively safe, inexpensive and are readily available throughout the year.

Conclusion

Leucas aspera is rich in secondary metabolites. The ethanol extract of the leaves of this plant can be an ecofriendly biodegradable larvicide. The stagnant water bodies are the main breeding places for mosquitoes which act as vectors for many dreadful infectious diseases. Such eco-friendly larvicides can be sprayed on the breeding places to control mosquito densities. This could be a cost-effective mosquito larvicide.

Presence of alcohols, ketones and carboxylic ester (Terpenoids) leads to high mortality rate. Bioactive compounds like flavonoids, tannins and saponin inhibit the metamorphosis of larvae.

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