Original Article

Journal of Phytology 2015, 7: 26-31
http://scienceflora.org/journals/index.php/jp/
doi: 10.19071/jp.2015.v7.2898

Effect of *Gliricidia sepium* leaves extracts on *Aedes aegypti*: Larvicidal activity

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Received: 15.06.2015
Accepted: 20.10.2015
Published: 05.12.2015

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ABSTRACT

Mosquitoes are the single most important group of insects in terms of the public health significance and causing diseases. They are the vectors for the transmission of many viral pathogens and pose serious threat to human health. Chemical insecticides are widely used, but they are non-selective and harmful to beneficial organisms. In the present study, *Gliricidia sepium* leaves extracts were used to analyze its effect against *Aedes aegypti*, to compare the mortality rate while using different solvent extracts of the leaf, to identify the solvent extract which give a high rate of larval mortality, and to characterize the compounds present in the corresponding leaf extract using phytochemical analysis. The ethanolic extract of *G. sepium* leaves constitutes flavonoids, steroids, glycoside, carbohydrate, and saponins compound. Thus, it was found to have an inhibitory effect on the growth of larvae than other solvent extracts. In the statistical analysis, the highest significant difference was observed between 1.5 and 2.0 g/l concentration of ethanolic extract and other extracts. However, no significant difference was observed in other concentrations. The larvicidal activity of the plant extract may be attributed to the presence of active compounds such as terpenoids, saponins, and steroids.

KEY WORDS: *Aedes aegypti, Gliricidia sepium,* larvicidal, mortality rate, phytochemical

INTRODUCTION

Mosquitoes are the single most important group of insects in terms of public health significance and causing diseases such as malaria, dengue fever, Japanese encephalitis, and other fevers (Kamaraj *et al.*, 2011). India is endemic to mosquito-borne diseases due to the favorable ecological conditions. They are the vectors for the transmission of many viral pathogens and pose serious threat to human health (Arthi and Murugan, 2012). It is very difficult to control these vectors due to their remarkable ability to adapt to various environments, their close contact with humans, and their reproductive biology. The rich organic content, stagnant water, low illumination, and small orifice of the coconut shells in rubber plantations favor intense breeding (Honorio *et al.*, 2006).

Dengue fever continues to be a major public health problem in the countries of Western Pacific and Southeast Asia. The increasing trend of dengue outbreaks accompanied by DHF is posing a problem of outmost importance to the public health of India (Fulmali *et al.*, 2008). There has been an outbreak of chikungunya and dengue all over India from 2006 to 2009 (Arunachalam *et al.*, 2008).

The mosquito *Aedes aegypti,* an important vector of arboviruses such as a dengue fever, urban yellow fever, and chikungunya is a holometabolous insect processing a life cycle with four instar stages: Egg, four larval instars, pupa, and adult (Kaushik and Saini, 2008). Being fundamentally aquatic, this mosquito reaches the terrestrial environment only as an adult. Its preference for humans as a host is an important factor for transmission. Thus, environmental assessment at the household level is necessary for dengue control.

Insecticide applications that were highly effective against the vector control are facing a threat due to the development of resistance to chemical insecticides resulting in rebounding vectorial capacity. However, they are non-selective and potentially harmful to other beneficial organisms. Concerning *A. aegypti* control, in addition to, the recommended mechanical elimination of breeding sites, those permanent recipients that cannot be discarded are generally treated with chemical insecticides (Srivastava *et al.*, 2008). Most of these insect repellents and bug sprays used are poisonous and cause health problems especially for the children (suffocation).

The larvicidal activity of extracts from leaves, flowers, and roots of many plants were reported in many studies. The phytochemical screening of those plants for saponins, flavonoids, terpenoids, tannins, cardiac glycosides, and steroids are also carried out (Joji Reddy and Beena, 2010). *Gliricidia sepium* often simply referred to as *Gliricidia* is a medium size leguminous tree belonging to the family *Fabaceae*. The active medical compounds present in *Gliricidia* are afrormosin, medicarpin, tannin, and some isoflavins. Tannin is reported to have antidiarrheic, antidysentric, antimutagenic, antinephritic, antiviral, bacterial, cancer preventive, hepato-protective, pesticide, psycotrophic, and viricide activities (Akharay *et al.*, 2012). The leaves of *Gliricidia* are used in south India as a mosquito repellent, and they have antifungal and antibacterial activity. Various phytochemicals such as flavonoids, triterpenoid, coumarin, coumaric acid, melilotic acid, and stigmastanol glucoside have been identified and isolated from various parts of this plant. 42 known compounds are found in the leaves and flowers of *G. sepium* (Joji Reddy and Beena, 2010).

In the present study, we sought to determine the effect of *G. sepium* leaves extract can be used for mosquito control. Different solvent extracts of the leaves of *Gliricidia* are compared for their ability to make the desired mortality rate in the *A. aegypti*. Many researchers are proceeding to use natural insecticides for the control of mosquitoes and may thus contribute for the control of vector transmitted diseases such as malaria, dengue fever, and many others.

MATERIALS AND METHODS

Collection of Sample

The leaves of *G. sepium* were collected from the Kerala Agricultural University, Mannuthy, Kerala, India. The first instar larvae of *A. aegypti* were collected from the National Institute for Communicable Diseases, Mettupalayam, Tamil Nadu, India.

Preparation of Extract

The leaves of *G. sepium* were dried under shade and made into fine powder. To prepare the leaf extract, 30 g of leaf powder was immersed in 200 ml of respective solvent (ethanol, acetone, toluene, and iso-propanol) by the cold extraction method. The solvents were selected based on the order of polarity. This mixture was kept in dark for 24 h at room temperature.

Maintenance of Mosquito Larvae

The larvae of *A. aegypti* were maintained at room temperature as reported earlier (Arthi and Murugan, 2012). The breeding cups were covered with wire mesh to avoid contact with foreign mosquitoes. The larvae were observed for the different instar stages, and the third instar larvae were used throughout the experiment.

Phytochemical Analysis

Test for flavonoids

To 1 ml of aqueous extract, add 1 ml of 10% lead acetate (Zoran *et al.*, 2004). Formation of yellow precipitate indicated the presence of flavonoid.

Test for steroids

2 ml of organic extract was dissolved in 2 ml of chloroform and was treated with sulfuric acid and acetic acid. The appearance of green confirmed the presence of steroids (Gowthami and Tamilselvi, 2012).

Test for phlobatannins

About 2 ml of aqueous extract was added to 2 ml of 1% hydrochloric acid, and the mixture was dried (Mehta *et al.*, 2013). Deposition of red precipitate was taken as evidence for the presence of phlobatannins.

Test for glycosides

About 2 ml of organic extract was mixed with 2 ml of chloroform to which 2 ml of acetic acid was added carefully. A color change from violet to blue to green indicated the presence of a steroidal nucleus that is aglycone portion of glycoside (Akharay *et al.*, 2012).

Test for carbohydrates

To 3 ml of aqueous extract, 1 ml of iodine solution was added (Balasubramanian, 2012). A purple coloration at the interface indicated the presence of carbohydrates.

Test for Saponins

5 ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube. The mixture was warmed for few minutes (Meena and Jolly, 2011). The formation of stable foam, honeycomb in shape was taken as the evidence for the presence of saponins.

Larvicidal Activity of Leaf Extracts

From the10% stock solution of respective solvent leaf extract, 100-400 µl were added in 20 ml of distilled water in the Petri plates (Abdelouaheb *et al.*, 2009). Ten numbers of third instar larvae of *A. aegypti* were added to the above Petri plates. The control was also maintained by adding respective solvents in 20 ml of water in a Petri plate. The larvae were fed with food which was a

mixture of dog biscuit and yeast granules. The number of dead larvae was observed after 24 h. This experiment was repeated three times, and the triplet values were recorded.

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100$$

$$\text{Mortality}(\%) = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Column Chromatography

Column chromatography was done for the ethanolic extract of *G. sepium*. The silica 60-120 mesh was used as the stationary phase (600 mm × 30 mm), and ethanol was used as mobile phase (Fang *et al.*, 2002). 3 ml fractions were collected and used to perform thin layer chromatography (TLC).

TLC

The TLC plate was prepared manually in which chloroform, acetone, and methanol in the ratio 7:1:1 was used as mobile phase. The sample to be analyzed was spotted onto the plate and allowed to run via capillary action. It was then allowed to air dry. The sample spots were developed under iodine chamber and were located from which Rf value was calculated.

$$\text{Rf value (cm)} = \frac{\text{Distance moved by analyte}}{\text{Distance moved by solvent}}$$

RESULTS AND DISCUSSION

Phytochemical Screening of Ethanolic and Aqueous Extract of *G. sepium*

The medicinal property variations among plants are due to the variation of family and genus in which they belong and the gene expression of bioactive compounds. Thus, the analysis of phytochemical compounds in the experimental plant should give more information about the plants. In the present study (Table 1), the phytochemical analysis of the ethanolic extract of *G. sepium* showed the presence of flavonoids, steroids, glycosides, carbohydrates, and saponins as reported earlier (Akharay *et al.*, 2012). The presence of these phytochemicals in the leaf extract evaluates them strongly for the antibacterial activity of the plant (Ajaieoba, 2002).

Larvicidal Activity of Different Solvent Extracts

The phytochemical constituents of the *G. sepium* have been fractionated by four different polar solvents. These solvent extracts were checked for larvicidal activity and the positive control (mosquito in distilled water) and the negative control (mosquito insolvent) were maintained for all the solvents. The ethanol, iso-propanol, and acetone extracts were found to be active against *A. aegypti,* whereas toluene extract showed a negative result. The number of dead larvae was found to be more in a higher concentration of all extracts.

From the Table 2, the ethanol extract was found to be more effective than all other extracts. The plants extracts of *Annona squamosa, Canarium indicum, and Tridax procumbens* showed moderate effects against larvae of *Anopheles subpictus* and *Culex tritaeniorhynchus* after 24 h of exposure. The highest toxic effects were observed in *A. squamosa* bark methanol extract, *C. indicum* leaf ethyl acetate and methanol extract, and *T. procumbens* leaf acetone and ethyl acetate extract against the larvae of *A. subpictus* and *C. Tritaeniorhynchus* (Kamaraj *et al.*, 2011).

The mortality percentage was studied at different instar stages of *Culex quinquefasciatus, A. aegypti, Anopheles stephensi* larvae using *Millingtonia hortensis* acetone leaf extract and the mortality rate of larvae was higher at initial stage than other stages of instar larvae (Kaushik and

Table 1: Qualitative phytochemical screening of *G. sepium*

Test	Observation	Result
Flavonoids	Yellow precipitate	+
Steroids	Greenish	+
Phlobatannins	No red precipitate	-
Glycoside	Color change from violet to blue to green	+
Carbohydrate	Purple in the inter-phase	+
Saponins	Stable foam, honeycomb in shape	+

-: Absence, +: Presence, *G. sepium: Gliricidia sepium*

Table 2: Larvicidal activity of different solvent extracts of *G. sepium*

Solvent extract	Volume (µl)	Concentration (mg/l)	Mortality (%)
Ethanol	100	500	26.6
	200	1000	46.6
	300	1500	70
	400	2000	90
Acetone	100	500	3.3
	200	1000	20
	300	1500	50
	400	2000	33.3
Iso-propanol	100	500	13.3
	200	1000	33.3
	300	1500	63.3
	400	2000	46.6

G. sepium: Gliricidia sepium

Saini, 2008). Karthikeyan *et al*. (2012) reported that the second and third instars larvae of *C. quinquefasciatus* have low mortality by the *Leucas aspera* extract whereas *Vitex negundo* and *Eucalyptus* ethyl acetate and ethanol extracts have more larvicidal activity against *C. quinquefasciatus* (Karthikeyan *et al.*, 2012). From the initial study of this work *A. aegypti* have a high mortality rate in ethanol extract, and Iso-propanol showed the least mortality rate at 2g/l concentration. Therefore, the experiment was focused in the range of 1.5-2.0 g of ethanol extract. The mortality percentage was increased by plant extract in a dosage-dependent manner (Srivastava *et al.,* 2003; Choochote *et al.,* 2004; Singh *et al.,* 2006).

Statistical Analysis

The bioassays were performed by taking 10 larvae in each plate.

According to the bar diagram (Figure 1), ethanolic extract showed highest mortality rate in the concentration of 2 g/l with 91%. The lowest mortality rate was 69% observed at 1.5 g/l concentration with ethanolic extract. The larvicidal bioassay values for *A. squamosa* bark methanol extract, *C. indicum* leaf ethyl acetate extract, and *T. procumbens* leaf acetone extract (LC50 = 93.80, 39.98, and 51.57 mg/l; LC90 = 524.90, 145.70, and 226.56 mg/l) against the larvae of *A. Subpictus,* whereas for *A. squamosa* bark methanol extract, *C. indicum* leaf methanol extract, and *T. procumbens* leaf ethyl acetate against the larvae of *C. tritaeniorhynchus* were calculated to be LC50 = 104.94, 42.29, and 69.16 mg/l; LC90 = 443.79, 172.34, and 287.21 mg/l, respectively. The obtained bioassay data were analyzed using Chi-squared test with an established significance level at $P < 0.05$ (Kamaraj et al., 2011). The leaf extract of *M. hortensis* has the highest sensitivity to II instar larvae of *C. quinquefasciatus* with their lowest LC values (LC50 83.18 and LC90 190.5 ppm), whereas lower susceptibility was shown by IV instar larvae of *A. stephensi* (LC50 223.9 and LC90 426.6 ppm) (Kaushik and Saini, 2011).

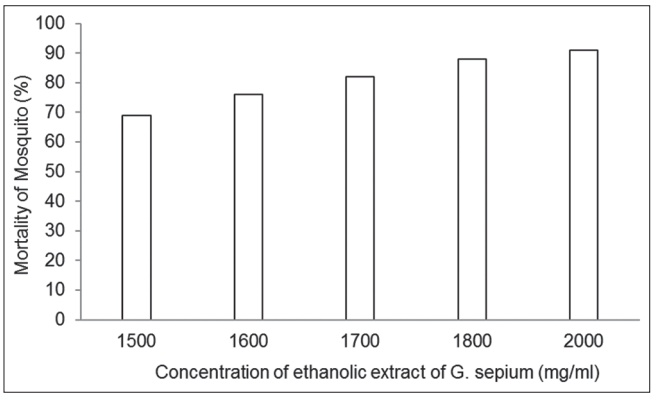

Figure 1: Larvicidal bioassay using ethanolic extract of *Gliricidia sepium* on *Aedes aegypti*

Analysis of Variance (ANOVA)

Triplet values from the mortality data were subjected to one-way ANOVA from which the Standard error and critical difference were calculated. From the analysis result, it was observed that the highest significant difference obtained was between 1.5 and 2 g/l concentration of all extracts. However, no significant difference was observed between other concentrations of extracts (Table 3). It was concluded that among the three solvent extracts taken, ethanolic extract was the one with highest larvicidal activity. From the above experiment, a significant difference was observed between 1.5 and 2 g/l concentration of *G. sepium* ethanolic extract. Thus, it was concluded that 2 g/l concentration of the ethanolic extract showed high larvicidal activity against the larvae of *A. aegypti* (Table 4). The petroleum ether extract of *Eucalyptus* has no significance different at 0.125% and 0.500% concentration, whereas $P > 0.05$ level for 0.250 and 1% concentration (Karthikeyan *et al.*, 2012).

TLC

150 fractions were collected and subsequently checked for analytes. Only 50 fractions were found to show some spots or smear on the plates, and 10 fractions were found to show visible spot in TLC. The compounds were identified based on the retention factor (R_f) values as well as the colors they exhibited.

From the Table 5, R_f values 0.59 cm, 0.27 cm, 0.61 cm, 0.25 cm, 0.88 cm, 0.64 cm, 0.78 cm, 0.46 cm, 0.53 cm,

Table 3: ANOVA single factor-larvicidal bioassay of *G. sepium* with different solvent extracts

	Mean of mortality			
Concentration (mg/l)	500	1000	1500	2000
Ethanol	2.66	4.66	7	9
Acetone	0.33	2	4.33	3.33
Iso-propanol	1.33	3.33	6.33	4.66
SE±	0.47	0.9	0.9	1.3
CD at P=0.05	2.42	4.64	2.04	4.02

SE: Standard error, CD: Critical difference, *G. sepium: Gliricidia sepium*

Table 4: ANOVA single factor-larvicidal bioassay of *G. sepium* with ethanolic extract

Concentration (mg/l)	1500	1600	1700	1800	2000	SE±	CD at P=0.05
Mean of mortality	5	7.33	7	8	8.66	-	-

SE: Standard error, CD: Critical difference, *G. sepium: Gliricidia sepium*, ANOVA: Analysis of variance

Table 5: Analysis of different TLC plates

Fractions	Distance traveled by analyte (cm)	Distance traveled by solvent (cm)	Rf value (cm)
F 1	3.7	6.2	0.59
F 2	1.7	6.5	0.27
F 3	3.5	5.9	0.61
F 4	1.6	6.7	0.25
F 5	6	6.8	0.88
F 6	4.1	6.4	0.64
F 7	4.4	5.7	0.78
F 8	2.4	5.9	0.46
F 9	3.2	6.0	0.53
F 10	4.2	6.2	0.67

TLC: Thin layer chromatography

and 0.67 cm with brown, light brown, blackish green, yellowish brown, blue, violet, and green, respectively, confirmed the presence of saponins, terpenoids, tannins, triterpenoids, alkaloids, and flavonoids. The larvicidal activity of the plant extract may be attributed to the presence of active compounds like terpenoids, saponins, and steroids. The potency of *G. sepium* was due to the presence of saponins, phenolic compounds, essential oils, and flavonoids (Akharay *et al*., 2012).

CONCLUSION

In the present study, the ethanolic extract of *G. sepium* leaves was found to have an inhibitory effect on the growth of larvae of *A. aegypti*. It was concluded that the ethanolic extract of *G. sepium* was the most effective when compared to other solvent extracts by the mortality rate of *A. aegypti*. In the statistical analysis, the highest significant difference was observed between 1500 and 2000 mg/l concentration of ethanolic extract and other extracts. However, no significant difference was observed in other concentrations. The larvicidal activity of the plant extract may be attributed to the presence of active compounds such as terpenoids, saponins, and steroids which were confirmed by TLC studies and phytochemical analysis.

Phytochemical analysis of aqueous and ethanolic extract confirmed the presence of flavonoids, steroids, saponins, phlobatannins, glycoside, and carbohydrates. Thus, the larvicidal activity of leaf extract of *G. sepium* may be due to the presence of compounds such as saponins, flavonoids, terpenoids, triterpenoids, and alkaloids. The direct and indirect contribution of such compounds in the efficiency of killing larvae and fitness need to be properly understood to guide the use of botanical insecticide for the management of *A. aegypti*. Natural insecticides may play an important role in future regarding the control of mosquitoes and may thus contribute for the control of vector transmitted diseases such as malaria, dengue fever, and many others. Thus, this work is contributing evidence for the potentiality of botanicals in the public health integrated management.

ACKNOWLEDGMENT

The authors hearty acknowledge to The Management, Principal and Head of the Department, Department of Biotechnology, K. S. Rangasamy College of Technology, Tiruchengode, for provided support to carry out this work. The authors are thankful to Mr. Pradeep Kumar, for the help rendered toward English correction and aligning of the manuscript.

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