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Original Article

Isolation and characterisation of mosquitolarvicidal compound from *Gliricidia sepium*[Jacq.]

Jiji Thomas[1,*], Shonima Govindan M [1], Muraleedhara Kurup G [2]

[1] School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India.
[2] Dept. of Biochemistry, Kariavattom, Kerala University, Trivandrum, Kerala, India.

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ABSTRACT

Objective: Isolation and identification of larvicidal phytochemical from the plant *Gliricidia sepium*, which is commonly used for smouldering to repel mosquitoes. Methods: Larvicidal activity of petroleum ether, hexane, acetone, methanol and water extracts of *Gliricidia sepium* leaves were assayed for toxicity against 4th instar larvae of *Culex quinquefasciatus*. The larval mortality was observed after 24 h exposure. The crude petroleum ether extract was further purified by column chromatography and eluates were tested for larvicidal activity. Selected one was identified by spectral analysis. Result: In the present study, bioassay- guided fractionation of *G.sepium* leaf extract led to the separation and identification of 8,11,14- eicosatrienoic acid as a potential new mosquitolarvicidal compound with LC_{50} value 0.011 mg/ml and LC_{90} as 0.060 mg/ml against 4th instar larvae of *Culex quinquefasciatus*. GC-MS, FTIR, ^{1}H NMR and ^{13}C NMR spectral analysis confirmed the identification of active compound. Conclusion: This work could grab success in extricating ourselves by emanating a safe eco friendly solution from the plant *Gliricidia sepium*. As the source plant is ubiquitous in Kerala zone and the method of extraction is not any way cumbrous it may be easily manufactured and launched into the market for the effective application.

Key words: Gliricidia sepium, Culex quinquefasciatus, eicosatrienoic acid, Larvicidal activity

1. INTRODUCTION

The Mosquitoes, commonly called “flying syringes”, as they are sanguivorous vectors, cause more sufferings to the humans than any other organism. It is not time to forget the recent rampage of Chikungunya and Dengue fever all over the state, pushing the people into death or

Corresponding author *
Jiji Thomas, School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala, India.

at least to permanent ill health. Due to inadequate management of land and water resources, and failure to solve problems of waste management, more productive habitats for mosquito continue to grow and, cause diseases and intolerable annoyance. Culex mosquitoes are vectors for Japanese Encephalitis, Lymphatic Filariasis, West Nile Fever, St. Louis Encephalitis, Avian Malaria etc. They are painful and persistent biters and also attack in dusk and dark. An obvious method for the control of mosquito borne diseases is the use of insecticides, and many synthetic agents have been developed and employed in the field with considerable success The botanicals might be used as an alternative to other insecticides for the control of mosquito and thus mosquito borne diseases, and hence, such studies would be helpful in developing plant-based anti-mosquito agents. [1] The use of botanicals for mosquito management is gaining importance in the recent years in view of their selective properties of low cost and safety to ecosystem.

Gliricidia, belonging to the legume family Fabaceae, is a medium sized tree known in different vernacular names, *Cacao* in Honduras, *Kakawate* in Philippines, *Konnai* in South India and *Seemakonnai* in Kerala. *Gliricidia* is well known as a small semi deciduous tree, in the tropics as well as sub tropics, where the climate is seasonally dry and the soil is deep and well drained. Planters grow this plant for affording shade, to shade-loving crops like Coffee, Cacao and also as hedge plants or fence posts along the boundaries. It prefers vegetative propagation by stem cuttings to the sexual reproduction by seeds.

2. MATERIALS AND METHODS

2.1 Culturing and maintenance of mosquito

Culex quinquefasciatus mosquitoes collected from field were used for raising the colony. After oviposition, eggs were collected in filter paper and kept separately at 27±2 °C. The adult mosquitoes were provided with water soaked raisins and cotton swabs dipped in 5 % glucose solution. The female mosquitoes were fed on blood meal. Plastic cups of 6 cm height and 8 cm diameter lined with filter paper and half filled with water were introduced into the cages for oviposition. After the eggs were laid, the ovitraps were taken out of the cages and fresh ones were placed in the cages for subsequent ovipositions. After hatching, the first instar larvae were transferred to an enamel tray of $30\times25\times5$ cm^3 containing well water. The larvae were fed on a diet of finely powdered biscuits and yeast in the ratio 3:1. The water in the tray was changed every day and dead larvae were removed.[2]

2.2 Collection and extraction of Plant materials

Leaves of *Gliricidia sepium* were collected from Vagamon, located 1100 m above sea level at Idukki district, in Kerala state. Fresh mature and healthy leaves were chopped into small pieces, spread out and dried under shade until they could be broken easily by hand. Dried leaves were ground in an electric mixer, and were used for soxhlet extraction using petroleum ether, hexane, acetone, methanol and water as solvents. The petroleum ether leaf extract which exhibited high larvicidal activity [3], was extracted with various solvents like chloroform, diethyl ether, acetonitrile, acetone and methanol. Since acetonitrile fraction of the crude extract showed maximum larvicidal activity, it was selected for column chromatographic purification.

2.3 Purification of larvicidal compound by column chromatography

For the extraction, glass column of 1 cm diameter and 20 cm length was used. 300 mg of Silica gel 230-400 mesh size was taken in a beaker and prepared slurry by pouring, 3 ml of chloroform. This mixture was carefully transferred into the column. 10 mg extract mixed with 1 ml chloroform was applied on the column. 10 ml of chloroform was added and eluate was collected in a beaker at the rate of not more than 1

ml/min and marked as 'A'. After draining the first solvent in the column, 15 ml of acetone: methanol (9:1) was added and the eluates were collected in a rate not more than 0.5 ml / min. It was named as 'B'. After draining the second solvent, 10 ml methanol was added and collected the extract, which was named as 'C'. The three fractions were evaporated, dissolved in a small volume of chloroform: methanol (2:1), and were stored in refrigerator for further experiments.

The compound 'B' which shows maximum larvicidal activity was selected for further studies. Isolation and separation of the active ingredient in 'B', was done by column chromatography using silica gel column and acetone: methanol (9:1) solvents as described above. Compounds, separated based on colour and named as AM_1 to AM_7, were subjected for larvicidal assay.

2.4 Spectral analysis

The GC-MS analysis of the phytochemical was carried out by using a Shimadzu GC-17A with QP5050 with the following specifications. An apolar 30 m DB-5 column (0.25 mm i.d.and 0.25 µm film thicknesses) and helium as carrier gas were used (Agilent techniques, USA). Injector temperature was 250 °C; interface heating was 300 °C; ion source heating: 200 °C, EI mode; scan range was 40-600 amu. For compound identifications NIST library spectra as well as reference MS –spectra were used. FTIR spectrometer, Shimadzu FTIR- 8400S was used to investigate the functional group molecules and polar bonds.^{1}H NMR, ^{13}C NMR spectra of phytochemical was recorded using Bruker DRX-500 NMR spectrometer.

2.5 Bioassay on larvae and pupae

For bioassay tests of all the crude extracts, larvae were taken in four batches of twenty five and experimented with 1000 ppm solution. After 24 h, number of dead larvae was counted. The experimental media, in which 100 % mortality of larvae occurs, alone were selected for isolation and purification of larvicidal compound.

The compound AM_7 was subjected to dose response bioassay to determine lethal concentrations at which larvae and pupae showed 50 % (LC_{50}) and 90 % (LC_{90}) mortality level by following the procedure of WHO [4] with slight modification. [5]The larvicidal phytochemical extracted from *G.sepium*, was prepared in concentrations ranging from 0.01 to 0.10 mg/ml using 0.1 % of Tween 20 as emulsifier. Sample in each concentration was in replicates of four and, a control containing only Tween 20 (0.1 %) was run for comparison. [6] Twenty five numbers of 4th instar larvae were used for the experiments. The larvae were fed dry yeast powder. [7] The number of dead larvae at the end of 24 h was recorded and percentage of mortality was calculated.

2.6 Statistical analysis

The larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, at 95 % confidence limits.

3. RESULTS

The acetonitrile fraction of petroleum ether extract of *Gliricidia sepium* leaf powder showed highest larvicidal activity among all other fractions. In column chromatographic separation, as the compound 'B' exhibited high mortality rate against larvae, it was further purified. Among the seven compounds obtained, spectral analysis of AM_7 was carried out, since the compound exhibited high larvicidal activity among all the effluents. The yield of AM_7 was found to be 0.58 g /100g of leaf powder. GC-MS spectrum indicated that the compound AM_7 shows the structure of 8, 11, 14-eicosatrienoic acid (Dihomo-gamma-linolenic acid). It is with molecular formula $C_{20}H_{34}O_2$ and molecular weight 306.48 g/mol. It is a 20-carbon-chain omega-6 fatty acid, unsaturated at positions 8, 11, and 14. FTIR and NMR spectra also confirmed the

identity of the compound 8, 11, 14-eicosatrienoic acid. In ^{13}C NMR spectrum, the signals from 127.63-130.43 ppm revealed the presence of C=O. Signals appeared in 76.68-77.32 showed the presence of C=C carbons and the signals of alkane carbon atoms were appeared in the region of -0.02-31.52. Spectrum clearly exhibits the picture of 8, 11, 14-eicosatrienoic acid. ^{1}H NMR spectrum exhibits peaks due to H nuclei at different environments. Peak at 2.3 ppm is due to proton at C_2, where the electron withdrawing $^{-}COOH$ group decreases the electron density around the protons. Ethylenic protons at C_8, C_9, C11, C_{12}, C14 and C_{15} absorbs at higher ppm value (5.4) due to anisotropic deshielding. Peak at 2.1 ppm is attributed to allylic protons at C_7 and C_{16}. Protons at C_{10} and C_{13} absorb at still higher ppm values (2.8). End $–CH_3$ protons (at C_{20}) resonate at lower frequencies (0.97 ppm). Peaks at 1.3 ppm and 1.7 ppm are due to $^{-}CH_2$ groups at C_3, C4, C5, C6, C17, C18 and C_{19}. In FTIR spectrum the signal at 3011.51 cm^{-1} is due to OH stretching vibration. Signal at 2927.01^{-1} is due to stretching vibration of C-H and 1709.19^{-1} is due to stretching vibration of C=O. Other bands assigned at 1267.89, 1458.10 and1046.49 correspond to the vibrations of C-C, C=C and C-O groups. Studies using GC-MS, ^{13}C NMR ,^{1}H NMR and FTIR spectra clearly showed that compound AM_7 isolated from *G.sepium* is 8,11,14-eicosatrienoic acid.

In bioassay test it was found that the compound Am_7 (8,11,14-eicosatrienoic acid) was highly effective against the 4th instar larvae of *C.quinquefasciatus* with LC_{50}, 11 ppm (0.010 LL-0.013 UL) and LC_{90}, 60 ppm(0.327 LL-0.017 UL) at 95 % confidence limits. Tween 20 did not exert any toxic effect as the control showed no mortality.

4. DISCUSSION

Pipercide, a constituent of *Piper nigrum* have LC_{50} value 0.004 mg/l against *C. Pipiens* larvae. [8] The oil resins from the *Copaifera reticulate* showed LC_{50} value for the 4th instar larvae of *C. quinquefasciatus* as 80 ppm. [9] The combination of Neem and Karanja oil exhibited an LC_{50} of 0.06, 0.038 and 0.048 % against the larvae of *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* respectively. [10]

8, 11, 14-eicosatrienoic acid is a fatty acid which is an intermediary in the synthesis of prostaglandin. Though prostaglandins are present in plants [11], no report seems to be available regarding the toxic activity of prostaglandins against mosquitoes. Similarly, though many fatty acids were reported to have the mosquitocidal activity [12], this is the first report establishing the toxicity of 8, 11, 14-eicosatrienoic acid.

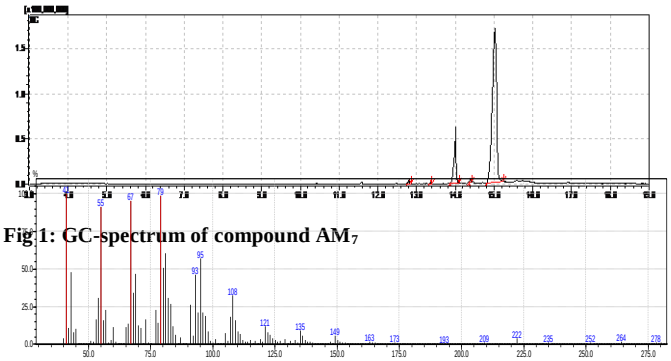

Fig 1: GC-spectrum of compound AM_7

Fig 2: MS spectrum of the compound AM_7

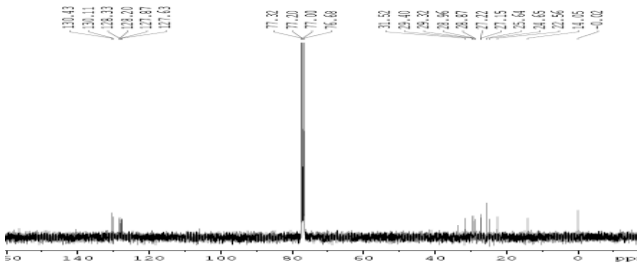

Fig 3: ^{13}C NMR spectrum of AM_7 isolated from *G.sepium*

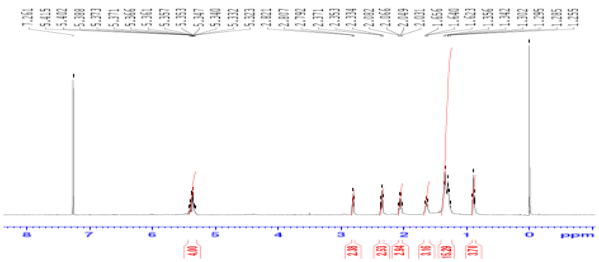

Fig 4: ^{1}H NMR spectrum of AM_7 isolated from *G.sepium*

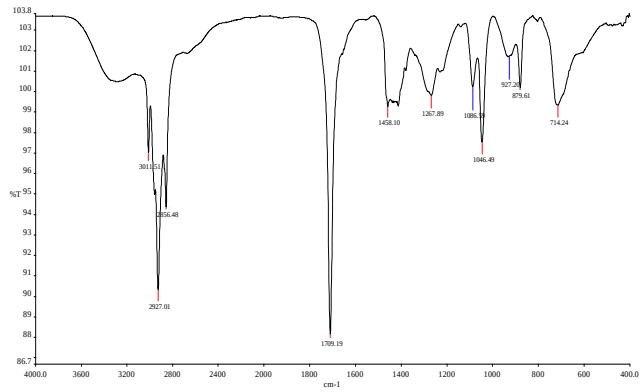

Fig 5: FTIR spectrum of AM_7

5. CONCLUSION

In the present paper we report the larvicidal activity of the compound 8, 11, 14-eicosatrienoic acid extracted from leaves of *Gliricidia sepium* against *Culex quinquefasciatus.* The results of the present study would be useful in promoting research aiming at the development of new agent for mosquito control based on bioactive compounds from indigenous plant source. There is no report of 8, 11, 14- eicosatrienoic acid in the genus *Gliricidia* and, their larvicidal activity is being evaluated for the first time. Results of this study show that the petroleum ether extract of *G. sepium* may be considered as a potent source and 8, 11, 14-eicosatrienoic acid as a new natural mosquitocidal agent.

6. ACKNOWLEDGEMENTS

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Conflict of interest statement

We declare that we have no conflict of interest.