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Efficacy of ethyl acetate extract of *Alangium salviifolium* fruit pericarp against *Culex quinquefasciatus* larvae

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Abstract

Mosquitoes transmit major human diseases, resulting in millions of fatalities each year and the development of chemical insecticide resistance, leading to a rebound in vectorial capacity. Plants could be used as a mosquito repellent alternative. The purpose of this study was to evaluate the biocontrol potentiality of ethyl acetate extract of fruit pericarp of *Alangium salviifolium* against *Culex quinquefasciatus* larvae. 100% larval mortality was recorded after 72 h of exposure with 50, 40 ppm, and 30 ppm concentrations of ethyl acetate extract against 3rd instar mosquito larvae. The bioactive compound responsible for larval mortality was isolated by TLC (R_f value of 0.33). 3rd instar larvae were found to be the most susceptible (LC_{50} = 3.60 ppm) among all the instars and corresponding LC_{50} values were 4.45 ppm and 4.52 ppm for 2nd and 4th instars larvae respectively after 72 h of exposure. The mortality rate of all larval instars was directly proportional to the concentration of bioactive compounds. Three-way ANOVA analysis revealed that larval instars, the concentration of bioactive compound, and time of exposure had a significant effect on larval mortality. In the bioactive TLC fraction of *A. salviifolium* (Rf value of 0.33), FT-IR spectroscopy analysis revealed the presence of numerous functional groups. GC-MS analysis revealed the presence of Benzoyl bromide and 3-Amino -5 (2-Furyl) Pyrazole in the extract. The compounds were also studied on non-target organisms such as *Anisops sardea*, 4th instar larvae of *Chironomus* sp. and *Diplonychus annulatum,* and in all the cases no abnormalities were recorded.

Keywords: bioactive compound; *Culex;* mortality; pericarp; pyrazole; solvent

Introduction

In tropical countries, vector-borne diseases are very common because of the diversity of different mosquito species and the widespread breeding habitat of mosquitoes. Mosquitoes create a serious threat to modern civilization in terms of mortality, morbidity, and economic loss. The mosquito plays an important role in transmitting life-threatening diseases like protozoan disease malaria, viral disease Yellow Fever, Dengue Fever, Chikungunya fever, West Nile fever, Japanese Encephalitis, and helminthic disease like Filariasis (Mondal *et al.*, 2014; Inziani *et al.*, 2020).

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In tropical and subtropical countries, lymphatic filariasis is the most common mosquito-borne disease caused by infections of three species, *Wuchereria bancrofti, Brugia Timori* and *Brugia malayi* (WHO, 2016). Lymphatic filariasis is also very common in West Bengal, India (Hati *et al.*, 1989; De and Chandra, 1994; Chandra, 2008). Approximately one billion people from 72 countries are at risk for lymphatic filariasis infection (Fang and Zhang, 2019). *Cx. quinquefasciatus* also transmits Reticuloendotheliosis virus (Holder, 1999), Japanese Encephalitis Virus, and Ross River Virus (Reuben *et al.*, 1994). It also acts as a vector of bird pox and the avian malaria-causing protozoa (Derraik and Slaney, 2005).

The reduction of the mosquito population is one of the important ways to reduce the frequency of mosquito-borne diseases. Usually, various inorganic insecticides are commonly used for mosquito control. But excessive use of synthetic insecticide creates various hazards like biomagnifications through the food chain, toxic effects on human health, and non-target organisms. To overcome the problems, insecticides of botanical origin are now widely used in the mosquito control programme as plants contain an array of bioactive phytochemical compounds having mosquito larvicidal potentialities. Furthermore, possible mosquito biocontrol options include the utilization of natural mosquito predators such as fish (Ghosh *et al.*, 2005; Bhattacharjee *et al.*, 2009), habitat modifications (Chandra *et al.*, 2006) etc. However, in this study, we focused on mosquitocidal phytochemicals derived from plants, as plants have been used for decades due to their extraordinary potentialities as bioactive agents with antibacterial (Bhattacharjee et al., 2011), anthelminthic (Hossain *et al.*, 2012), and mosquitocidal properties (Singha *et al.*, 2011). Botanically derived chemicals act as a general toxicants, repellents, growth and reproductive inhibitors, and oviposition deterrent against mosquitoes (Ghosh *et al.*, 2012; Ghosh *et al.*, 2016).

Alangium salviifolium is well known medicinal plant in India, China, and Phillipines (Zahan *et al.*, 2013). It is a deciduous shrub or tall thorny tree and is commonly known as Sage Leaved *Alangium*, stone mango, hill sack tree, and Ancolah (Figure 1). In India, different parts of the *A. salviifolium* plant are commonly used in traditional medicine. Its root, bark, leaves, seeds, and fruits are used in traditional therapeutic uses against diabetes, inflammation, hypertension, cancer, epilepsy, and ulcer. The size of this plant is usually from 3 feet to 12 feet and leaves are alternate, oblong, unequal, acute or rounded at the base, lanceolate or oval, globous above, and pubescent on veins beneath acuminate and obtuse at the apex with 3-6 pairs of oblique veins. Fruits are usually smaller in size, globular in shape (Figure 2) and change into purplish-red colour when ripen, and are encircled with white pulp rich in mucilage (Panara *et al.*, 2016). The larvicidal, pupicidal, and repellence activities of leaf extract of *A. salviifolium* was previously reported against *Culex vishnui* group of mosquitoes (Ghosh *et al.,* 2016).

Figure 1. *Alangium salviifolium* plant

Figure 2. *Alangium salviifolium* fruits

The objective of the present study was to isolate bioactive principles (if any) from ethyl acetate extract of fruit pericarp of *A. salviifolium* plant extract through fractionation; purification by Column Chromatography and Thin Layer Chromatography and characterization by FT-IR and GC-MS techniques. Evaluation of the toxic effect of isolated bioactive compounds was also done on the target organism (*Cx. quinquefasciatus*) as well as on the non-target organisms such as *Anisops sardea*, *Chironomus* larvae, and *Diplonychus annulatum*.

Materials and Methods

Collection of larvae

An egg raft of *Culex quinquefasciatus* was collected from the stagnant water body and drains surrounding Bankura Sammilani College campus, Bankura (23.14°N and 87.07°E), West Bengal, India with the help of the standard scooping and dipping method (Robert *et al.*, 2002). After collection, the larvae were kept in a Plastic tray containing tap water. Larvae were fed with a mixed diet of yeast, dog biscuits, and algae in a ratio of 3:1:1 respectively (Kamaraj *et al.*, 2011). Pupae were transferred to a beaker containing tap water and kept inside a mosquito cage for adult emergence. 10% glucose solution was used for glucose meal of adult mosquitoes and allowed to periodically blood-fed on immobilized pigeon. Eggs laid were reared and larvae of the same age were obtained and used for bioassay and control experiments.

Preparation of solvent extract

Unripe fruits of *A. salviifolium* were harvested randomly from plants growing outskirts of Bankura Municipality, Bankura during the summer season. Fruits were cut into pieces and seeds were removed. The pericarp of fruits was collected and air-dried for 10-12 days under shade at room temperature. The dried pericarp of fruit was crushed by a Bajaj mixer grinder and 200 g finely ground fruit pericarp was taken in Soxhlet apparatus. Extraction was done with 2 L ethyl acetate. The total extraction period was 72 h at a temperature of 40 °C. After 72 hours of extraction, the solvent extract was collected, filtered through Whatman No.1 filter paper, and kept in glass beakers covered with a fine cloth. The semisolid or dried extract was obtained after 30 days and stored in a refrigerator for further use.

Mosquito larvicidal bioassay with solvent extract

From stock solutions, required test concentrations (10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm) were prepared by the addition of distilled water. These graded concentrations were added with the suitable amount of distilled water to make the total volume of 100 ml in different plastic bowls. With the help of a glass dropper, twenty-five 3rd instar *Cx. quinquefasciatus* larvae were added to each bowl and bioassay experiments

were conducted. At the same time, a control set of experiments was conducted with only distilled water. Dead larvae were counted after 24 h, 48 h, and 72 h exposer with solvent extract in each bowl. Dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region. Percentage mortality was calculated from the mortality data.

Column chromatography analysis

The glass column tube was cleaned by washing with absolute alcohol and then dried with an air drier. The column tube was filled with 100 ml petroleum ether (PET) solvent and the slurry was prepared by mixing 50 g dry silica gel and 100 ml of petroleum ether (PET) was quickly poured into the column tube. The solvent was allowed to pass down into the beaker with a flow rate of 2 ml per minute by opening the screw clamp. This process was repeated 5 times so all silica gel gets packed within the tube. When the packing was completed, 2.5 gm semisolid ethyl acetate solvent extract mixed thoroughly with 10 ml ethyl acetate extract was added above the top of the column solvent. Then the column was eluted with single and mixtures of organic solvents with an increased polarity like petroleum ether, petroleum ether: benzene (1:1 v/v), benzene, benzene: chloroform (1:1 v/v), chloroform, chloroform: methanol (1:1 v/v), methanol, methanol: acetone (1:1 v/v), acetone, acetone: absolute alcohol and absolute alcohol. Several fractions of chemicals were collected in separate test tubes.

Thin-layer chromatography (TLC) analysis

Bioactive compounds were separated by thin-layer chromatography on silica gel "G" (Merck, India) coated (0.5 mm thickness) plates using different ratios of solvent systems as mobile phase. After one hour of running of solvent in a closed chamber, the plates were air-dried. Different bands with similar R_f values were determined under the UV light chamber. Plates were then removed from the UV light chamber and the bands with similar R_f values were scratched and collected together and stored for further analysis. The distance of the run of the developing solvent from the bottom of the plate was measured and the run of the sample spot was also measured. The R_f value was then calculated using the formula:

R_f = Distance of spot centre from the start point/Distance of mobile solvent run from the start point

Bioassay with active ingredients

From each TLC plate, bands with similar R_f value ((R_f value of 0.33) were scrapped and added in 25 ml of absolute alcohol. The active compounds were dissolved in alcohol and separated from silica gel. Now, dissolved chemicals were collected and allowed to evaporate in a water bath under temperature regulations of 45-50° C for 5 minutes. Alcohol was evaporated and solid mass was scrapped by a spatula from the flask and weighed. With the help of a micropipette, different concentrations (10 ppm, 20 ppm, and 30 ppm) of bioactive compounds were prepared by adding distilled water. For the bioassay experiment, twenty-five different instars of *Cx. quinquefasciatus* larvae were introduced in a plastic bowl and allowed to expose to different graded concentrations of bioactive principles. The larval mortality rate was recorded after 24 h, 48 h, and 72 h of exposure (World Health Organization, 2005). These bioassay experiments were repeated three times on three different days for each larval instar (n=9) at 25-28 °C temperature and 70-80% relative humidity. Simultaneously on each day of the experiment, a control set of experiments was also carried out by using distilled water only against each larval instar.

FT-IR and GC–MS analyses of bioactive principle

During FT-IR analyses, the sample was taken in vacuum desiccators over KOH pellets for 48 h, and infrared spectral analysis was carried out in an FT-IR spectrometer (Model No. Spectrum RX 1; Holland) using potassium bromide (KBr) pellets. The scanning range used was 450 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.TLC

purified fractions that exhibit maximum larval mortality were analysed directly by Gas chromatography (GC) on TRACE GC ULTRA (THERMO SCIENTIFIC) fitted with a column DB-5MS instrument. The oven temperature-programmed initially at 40 °C with 2-minute hold. The rise in temperature rate was 20 °C/minute and going to 130 °C with a 2-minute hold, 12 °C/minute and going to 180 °C with a 2-minute hold and lastly 3 °C/minute and going to 290 °C with a 10-minute hold. The sample was introduced at 240 °C. Carrier gas was Helium and the rate of flow was 1ml/minute. In the case of Mass spectrometry (MS), the ion source temperature was 50 °C. Solvent delay time was 3 minute and the mass range was 40 a.m.u to 700 a.m.u. MS Transfer line temperature was 290 °C.

Toxicity test on non-target organism

Toxic effect of bioactive compound isolated from fruit pericarp of *A. salviifolium* was tested against non-target organisms like *Anisops sardea*, 4th instar larvae of *Chironomus* sp. and *Diplonychus annulatum* because all of them share common habitat as shared by larvae of *Cx. quinquefasciatus* mosquito. Non-target organisms were exposed to the sub-lethal dose, LC_{50} value (at 24 h for 3rd instar larvae of *Cx. quinquefasciatus*) of the obtained bioactive principle using the procedure recommended by Halder *et al.* (2013). Percent mortality of non-target organisms were recorded after 24 h, 48 h, and 72 h of exposure.

Statistical analysis

The percent mortality observed was corrected by Abbott's formula (Abbott, 1925) during the observation of mosquito larvicidal activity against solvent extract as well as against active principles of this plant extract. Statistical analysis of the experimental data was performed using the computer software's "STAT PLUS 2007 (Trial version)" and "MS EXCEL 2007" to find out the regression equations (Y = mortality; X = concentrations) and regression coefficient values. Probit analysis was done by Stat plus 2007 software to find out LC_{50} and LC_{90} values. A completely randomized three-way factorial ANOVA was also conducted.

Results

Laboratory bioassay showed 100% larval mortality of the 3rd instar larvae of *Cx. quinquefasciatus* after 72 h of exposure with 50 ppm test concentration. 100% larval mortality was also recorded at 30 ppm and 40 ppm test concentrations after 72 h of the exposure period. In the control set of experiments, no larval mortality was noticed (Figure 3). In the bioassay experiment, the eluted bioactive materials of chloroform: methanol (5:5) fraction from column chromatography exhibited maximum larval mortality. After 72 h of exposure with 30 ppm concentration, 77.33% mortality was observed against the 3rd instar followed by 58.66% mortality after 48 h and 53.33% mortality after 24 h of exposure (Figure 4).

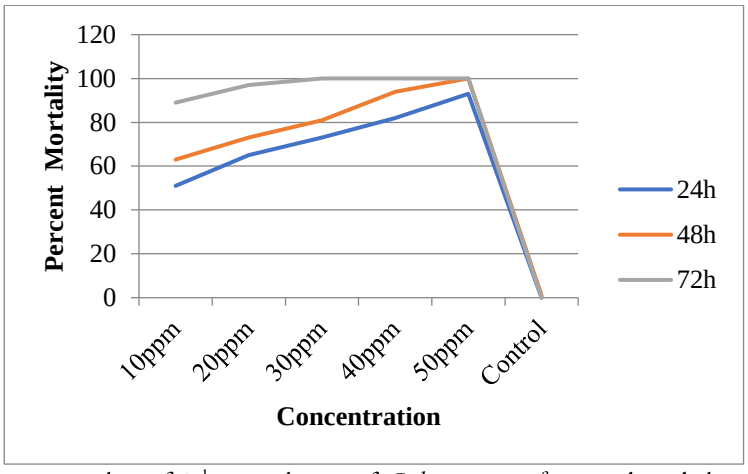

Figure 3. Percent mortality of 3rd instar larvae of *Culex quinquefasciatus* by ethyl acetate extract of *Alangium salviifolium* fruit pericarp

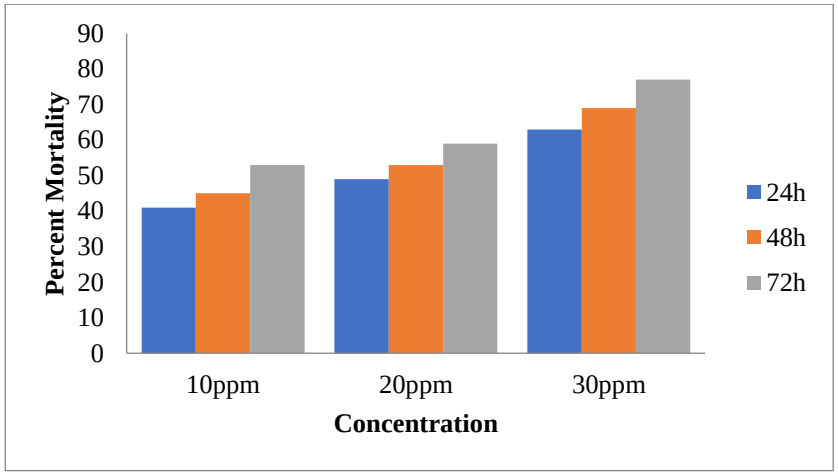

Figure 4. Percent mortality of 3rd instar larvae of *Culex quinquefasciatus* by bioactive compound (column fraction) obtained from ethyl acetate extract of *Alangium salviifolium* fruit pericarp

In TLC, two spots were detected, one with an R_f value of 0.33 and an upper band with an R_f value of 0.85. From scrapped material; 10 ppm, 20 ppm, and 30 ppm concentrations of TLC separated bioactive compounds were prepared and tested against *Cx. quinquefasciatus* larvae. Compounds present in the TLC fraction having an R_f value of 0.85 are ineffective in the bioassay experiment done on *Cx. quinquefasciatus* larvae. However, the bioactive compounds present in the TLC fraction having an R_f value of 0.33 exhibit good larvicidal activity. 97.33% of larval mortality was observed at 30 ppm of concentration against 3rd instar larvae whereas 81.33% mortality was observed against 1st instar larvae after 72 h of exposure. 3rd instar larvae were found to be more susceptible (LC_{50} = 3.60 ppm) and corresponding LC_{50} values were 4.45 ppm, and 4.52 ppm for 2nd and 4th instars larvae respectively after 72 h of exposure. 1st instar larvae exhibited less death (LC_{50} = 6.28 ppm) among all instars after 72 h of exposure. From the regression equation, it was found that in all larval stages, the mortality rate(Y) was positively correlated to its corresponding dose (X) and in all cases, R^2 values were very close to 1 which indicates that the mortality rate linearly increased with the concentration of increasing bioactive compounds (Table 1).

Table 1. Probit and regression analyses of the bioactive compound of *Alangium salviifolium* fruit pericarp against *Culex quinquefasciatus* larvae

Instars	Time	LC_{50} (ppm)	LC_{90} (ppm)	Regression equations	R^2 value
1st	24h	19.02	578.16	Y=0.80x+35.55	0.96
	48h	8.55	232.73	Y=0.86x+45.77	0.95
	72h	6.28	71.78	Y=1.00x+52.00	0.98
2nd	24h	11.91	409.65	Y=0.66x+42.22	0.94
	48h	7.33	255.76	Y=0.86x+47.55	0.95
	72h	4.45	92.70	Y=0.80x+57.77	0.99
3rd	24h	4.45	92.70	Y=0.73x+59.55	0.99
	48h	3.41	35.27	Y=0.73x+68.44	0.93
	72h	3.60	17.09	Y=0.73x+76.44	0.93
4th	24h	14.47	162.79	Y=1.26x+31.55	0.97
	48h	6.28	71.78	Y=1.00x+52.00	0.98
	72h	4.52	27.34	Y=0.93x+65.77	0.99

Three-way ANOVA analysis revealed that larval instars, the concentration of bioactive compound and time of exposure had a significant effect on larval mortality of *Cx. quinquefasciatus*. During complex interactions with the three parameters, a significant difference in larval mortalities were observed between the interaction of instars and hour but no significant difference observed between instars and concentration, hour and concentration and between all the three factors jointly (Table 2).

Table 2. Three-way factorial ANOVA of percent mortality of four larval instars of *Culex quinquefasciatus* against isolated bioactive compound from *Alangium salviifolium* fruit pericarp

Source of Variation	Sum of square	d.f.	Mean square	F value	P-value
INSTARS (I)	415.00	3	138.33	97.64	0*.
HOUR (H)	470.68	2	235.34	166.12	0*.
CONC.(C)	352.79	2	176.39	124.51	0*.
I X H	23.61	6	3.93	2.77	0.017*
I X C	10.16	6	1.69	1.19	0.318 (N.S)
H X C	2.09	4	0.52	0.36	0.82*(N.S)
I X H X C	6.05	12	0.50	0.35	0.97*(N.S)
Residuals	1020.00	72	1.41		
Total	1,382.40	107	12.91		

From the FT-IR spectroscopy analysis (Figure 5), many functional groups were detected which were presented in Table 3. The results of the GC–MS analysis showed that at least 2 compounds were present in ethyl acetate extract of fruit pericarp of *A. salviifolium* (Figure 6). These identified compounds were Benzoyl bromide (retention time 6.69) (Figure 7) and 3-Amino -5 (2-Furyl) Pyrazole (retention time 17.69) (Figure 8) in the TLC fraction of *A. salviifolium* fruit pericarp. Isolated compounds from fruit compounds can be considered a potent mosquito larvicidal agent, which was effective against *Cx. quinquefasciatus* mosquito.

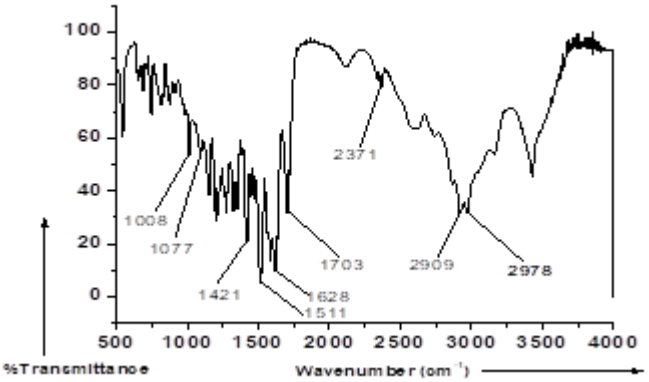

Figure 5. FT-IR spectrum of the bioactive compound (Rf value of 0.33) isolated from TLC fraction of *Alangium salviifolium*

Table 3. Functional groups revealed from absorbance peaks of the pure bioactive compound from TLC fraction of *Alangium salviifolium*

Absorption Spectra(cm^{-1})	Probable function groups present
2978	C=CH stretching
2909	Alkane (C-H) stretching
2371	Nitrile (CN) stretching
1703	RCOOH stretching
1628	C=C stretching
1511	Weak aromatic stretching
1421	Assigned Ca-O stretching symmetry
1077	-Si-O / C=O network vibrational stretching

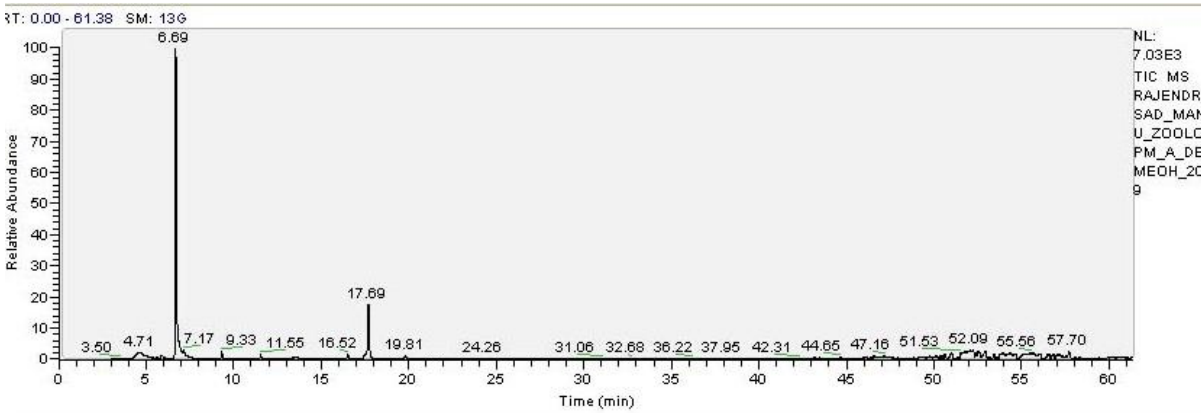

Figure 6. GC- MS total Chromatogram of TLC fraction of *Alangium salviifolium* fruit pericarp

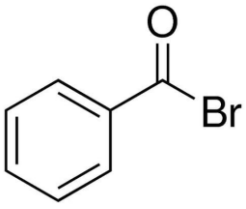

Figure 7. Structure of Benzoyl bromide

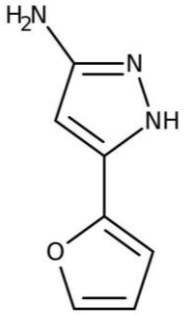

Figure 8. Structure of 3-Amino -5 (2- Furyl) Pyrazole

Discussion

In tropical countries, more than two billion people are at risk of various mosquito-borne diseases. Vector-borne diseases create huge medical expenses, financial loss, and social burdens in low and middle-income countries like India (Sathe *et al*., 2002). So, effective management of the mosquito population is necessary to reduce the disease burden associated with mosquitoes, according to World Health Organization (WHO, 1982). For vector control programmes, the use of synthetic insecticides creates many environmental hazards as well as human health hazards. Mosquitoes also develop resistance against these synthetic, inorganic insecticides (Severini *et al*., 1993). To overcome this situation, alternative eco-friendly natural plant products are applied to control mosquitoes because of their easy availability, low cost, innate biodegradability, and uncomplicated use (Sakthivadivel and Thilagavathy, 2003). Secondary metabolites which are present in plants show a wide range of medicinal as well as insecticidal activity (Jacobson, 1975; Halder *et al*., 2011; Rawani *et al*., 2012). Roark (1947) documented approximately 1,200 plant species and Sukumar *et al.* (1991) listed about 344 plant species that have mosquitocidal activity. Tennyson *et al.* (2015) and Kamaraj *et al.* (2011) also revealed mosquito larvicidal properties of many plant derivatives. Many researchers throughout the globe evaluated the larvicidal properties of different plant parts against various vector mosquitoes. Ethyl acetate extract of leaf of *S. mahagoni* exhibited significant mortality against *Anopheles stephensi* with LC_{50} values of 51.45, 45.65, and 40.55 ppm at 24h, 48h, and 72h respectively (Adhikari and Chandra, 2014). Ethyl acetate extract of leaf of *Ocimum sanctum* showed the LC_{50} values of 425.94 and 592.60 ppm respectively against *Aedes aegypti* and *Cx. quinquefasciatus* (Anees, 2008). LC_{50} values of ethyl acetate extract of *Aegle marmelos* leaves were 167.00 and 99.03 ppm against *An. subpictus* and *Cx. tritaeniorhynchus* mosquito respectively (Elango *et al.* 2009). LC_{50} value of ethyl acetate extract of leaves of *Solanum nigrum* was 17.04 ppm against 4^{th} instar larvae of *Cx. quinquefasciatus* at 24 h exposure (Rawani *et al.* 2010). In the present study, we have shown for the first time that ethyl acetate extract of fruit pericarp of *A. salviifolium* plant exhibited a good larvicidal effect and its LC_{50} value was much lower than previously established other plant extracts. Prakash *et al.* (2013) revealed that chloroform and methanol extract of leaves of *A. salviifolium,* showed 100 % mortality at 0.25ml/10ml v/v concentration against aquatic crustacean *Artemia salina.* Various characteristic functional groups of different phytoconstituents were identified by FT–IR spectrum analysis of the TLC fraction of *A. salviifolium* fruit pericarp. GC-MS analysis revealed the presence of two phytoconstituents, viz. Benzoyl bromide and 3-Amino -5 (2-Furyl) Pyrazole in *A. salvifolium* fruit pericarp. Among These two compounds, one containing Pyrazoles compounds among the azole family exhibited good larvicidal properties against all larval instars of *Cx. quinquefasciatus* mosquito. Many pyrazole derivatives like furametpyr, fenpyroximate, cyantraniliprole, and cyenopyrafen were successfully applied as insecticides, herbicides, acaricides, and fungicides (Kim *et al*. 2006; Selby *et al.,* 2013; Yu *et al*., 2012). Finkelstein *et al.* (1999) also evaluated the insecticidal properties of novel

pyrazole methanesulfonates, a pyrazole derivative against *Nilaparvata lugens*, *Diabrotica undecimpunctata Howardi*, *Nephotettix cincticeps*. So, either Benzoyl bromide or 3-Amino -5 (2-Furyl) Pyrazole or maybe the synergistic activity of both compounds is responsible for larval mortality in the bioassay experiments. The isolated bioactive compound may penetrate through the integument and accumulate in the different parts of the larval body of *Cx. quinquefasciatus* causing larval death. However, further studies are required to know the mode of action and formulations of active ingredients for improving the larvicidal potentiality.

Conclusions

Conclusively, the bioactive compound isolated from the ethyl acetate extract of fruit pericarp of *A. salviifolium* showed remarkable mortality against the larval form of *Cx. quinquefasciatus* mosquitoes. If the compound(s) are commercially formulated, they can be effectively used in field conditions as a potential biocontrol agent.

Authors' Contributions

RPM conducted the experiments, analysed the data and drafted the manuscript. GC designed the study, revised the manuscript and supervised the research done. AG participated in study design, statistical analyses and manuscript preparation. SB participated in TLC analyses, data acquisition, and revision of the manuscript. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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