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Larvicidal efficacy of different extract of *Annona muricata* leaves against Dengue vector *Aedes aegypti* and filarial vector *Culex quinquefasciatus* Say

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Abstract
Background: Among the many existing vectors which cause serious threat to human life, mosquitoes are of primary importance. Malaria, Dengue, Chikungunya, Lymphatic filariasis, and Japanese Encephalitis are those which dominate the world with regard to diseases. Combating vector borne diseases are crucial to help eradicate diseases. The use of chemicals and synthetic insecticides although were effective in controlling the mosquito vectors initially, they now cause adverse effects to both human and environment. Secondary metabolites derived from plants are used to control mosquitoes. Against this background, *Annona muricata* leaf crude extracts were used to study the larvicidal efficacy against dengue vector *Aedes aegypti* and filarial vector *Culex quinquefasciatus*.
Results: *Annona muricata* leaves were investigated to evaluate the larvicidal effect against *Ae. Aegypti* and *Cx. quinquefasciatus* IV instar larvae using hexane, chloroform, ethyl acetate and methanolic crude extract. Most active extract was subjected to analysis of GC-MS method. The larvicidal efficacy was observed in 62.5, 125, 250, and 500 ppm after 24 h of exposure. Best activity was observed in chloroform extract with LC_{50} value of 227.853 ppm and 168.909 against *Ae. Aegypti* and *Cx. quinquefasciatus* respectively. The GC-MS chromatogram of the chloroform extract revealed the presence of 16 major bioactive compounds of which Minocycline, Heptadecane, 2,6,10,15- tetramethyl, Eicosane, Dodecane, 2,6,11-trimethyl, and Heptadecane, 2,6,10,15- tetramethyl are the dominant compounds.
Conclusion: From the results obtained, it can be concluded that *Annona muricata* leaves possess larvicidal property against mosquito larvae as it contains many bioactive compounds. The use of plant derived secondary metabolites will result in combating vector borne diseases without any harmful effect on humans and environment.

Keywords: Mosquito, vector borne diseases, *Aedes aegypti*, *Culex quinquefasciatus*, *Annona muricata*, secondary metabolites, mosquito larvicidal efficacy

Introduction

Mosquitoes are tiny hematophagous insects which cause highest number of human deaths every year. There are more than 3500 mosquito species identified so far (Harbach et al., 2007). Among the identified mosquitoes, 100 species are important vectors which cause serious threat to humans and animals as well. (Wegner et al., 2009, Paulraj et al., 2011). In the Indian-subcontinent, vector mosquitoes belong to genera *Aedes*, *Anopheles* and *Culex* genera (Rahuman et al., 2009, Borah et al., 2010, Raja et al., 2018). World Health Organization, 1996, has reported that there are more than one million deaths and 2500 million people infected by mosquito-borne diseases across the globe.

Mosquito vectors transmit pathogens like protozoans, viruses and nematodes which cause several diseases that makes the world standstill and human life immobile. *Aedes aegypti* transmit dengue and chikungunya. (Parthiban et al., 2020, Bagavan et al., 2011, Al-Salomi et al., 2021). Malaria, caused by the vector *Anopheles* species, is the most important killer disease that infects 300-500 million people globally. Nearly 1000 deaths and 2-3 million cases are recorded annually in India (Dev et al., 2003, Lal et al., 2010, Garcia et al., 2010). Among 450 *Anopheles* species identified, 100 species are malarial vectors and 40 species transmit plasmodium parasites (Bronner et al., 2009, Oaks et al., 1991). Among the Culex species *Cx. quinquefasciatus* is the primary vector that transmits *Wuchereria bancrofti* which is accountable for the spread of lymphatic filariasis. It is estimated that 90-100 million people are infected by filarial diseases (WHO, 1992, Govindarajan et al., 2008). 41% of the global lymphatic filariasis is contributed by India (Agarwal et al., 2006). *Culex tritaeniorhynchus*, *Culex vishnui* and *Culex pseudivishnui* transmit japanese encephalitis (Tiwari et al., 2012).

Controlling the spread of vector mosquitoes is essential to control the spread of diseases. Elimination of breeding sites, control of immature mosquito larvae and adults (Rodrigues et al., 2022, Rueda et al., 2018) through appropriate means are some of the methods that can be adopted to control the spread of mosquito borne diseases. Chemical insecticides like DDT, Temephos, Fenitrothion, Bendiocarb, Permethrin, deltamethrin, etofenprox Malathion, Synthetic organochlorine, organophosphates (Aparna N et al., 2021) etc were used, but however, they posed adverse effects on human health (Benelli et al., 2017, Komalamisra et al., 2005) along with increasing high pesticide - resistant capacity (Naqqash et al., 2016) among mosquitoes leading to pollution of the environment (Rodrigues et al., 2019). Hence, there emerged a great need to develop eco-friendly insecticides with the help of traditional medicinal plants. Medicinal plants are rich in bioactive compounds and produce secondary metabolites which can be used as toxins, repellents, deterrents, and growth regulators (Tyagi et al., 2016).

The plant *Annona muricata*, commonly known as soursop, belongs to the family Annonaceae (Ezemuoka et al., 2019).

It is an evergreen plant whose leaves are thick, dark green coloured with glossy upper surface. The fruit is green and heart-shaped containing white pulp with black seeds (Coria-Téllez et al., 2018, Moghadamtousi et al., 2015). It is spread in the areas like South and North America, also in parts of India, Malaysia, and Nigeria (Mishra et al., 2013, Adewole et al., 2006). Traditionally the leaves, fruit, root, bark and seed are used to treat neuralgic effects, diarrhoea, fever, malaria, dysentery, insomnia, headache, arthritis, cancer, astringent, pesticide, insecticide, cough and cold (Adewole et al., 2009, Amudha et al., 2017, Solis et al., 2020, Patel Sejal et al., 2015). Additionally, it also possesses anticancer, antimicrobial, antihelminthic, antimalarial, c, Antiplasmodial, Antitumour, Antihyperlipidemic, Antidiabetic, Antioxidant, cytotoxicity and insecticidal properties (Hajdu et al., 2012, Moghadamtousi et al., 2015). *Annona muricata* contains several compounds and secondary metabolites which include alkaloids, flavonoids, carotenoids, acetogenin, cyclopeptides, amides etc (Vijayameena et al., 2013, Coria et al., 2018, Nwonuma et al., 2023)

The primary aim of this research was to carry out and evaluate the potential larvicidal efficacy of the crude extracts of *Annona muricata* leaves against vector mosquitoes *Aedes aegypti* and *Culex quinquefasciatus*.

Methods

1. Plant Collection

A. muricata leaves (Fig. 1a) were collected from Kongampara, Palakkad district of Kerala, western Ghats (10.8101^0 N, 76.8198^0 E) in the month of February, 2022. The plant was identified by authenticated botanist and the voucher specimen of the same (ER-ENT-25) was submitted to the herbarium of ERI.

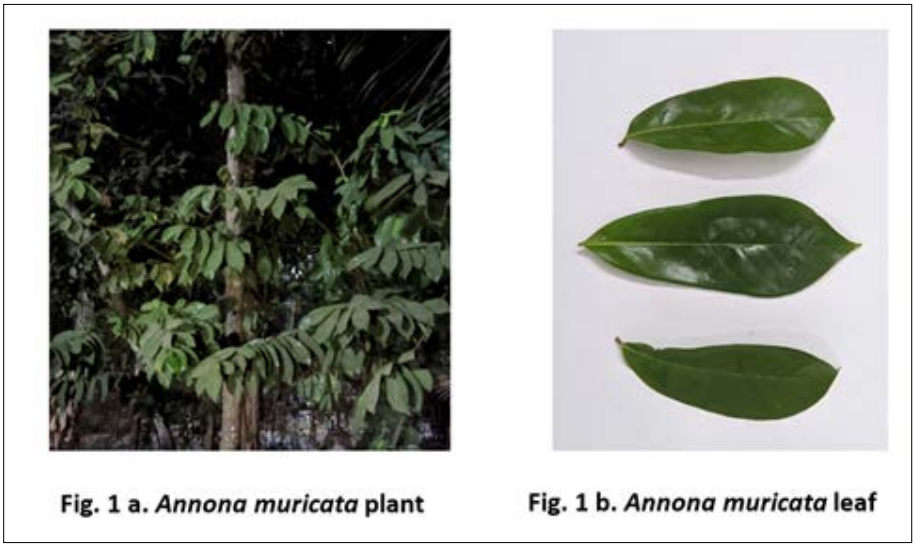

Fig. 1 a. *Annona muricata* plant Fig. 1 b. *Annona muricata* leaf

Fig 1: Annona muricata plant and leaf

2. Extract Preparation

The freshly collected *A. muricata* leaves (5 kg) were washed thoroughly in running water and was eventually subjected to shade dry for 3 weeks. The shade dried leaves were coarsely powdered using an electric blender and 1 kg of coarse powder was obtained. Sequential method was adopted for the extraction process. At first, the powdered plant (1 kg) was soaked in 3 litres of hexane for 72 h and periodically shaken. The content was then filtered using Whatman No 1 filter paper. Using rotary evaporator, the filtered solvent was concentrated and the hexane crude extract was obtained. In the like manner, the plant powder was subjected to soaking and extraction using chloroform, ethyl acetate and methanol (Fig. 2).

Fig 2: Schematic representation of plant extraction process

3. Test Organisms

The eggs of *A. aegypti* and *Cx. quinquefasciatus* were collected from St. Thomas Mount (13.0051^0 N, 80.1933^0 E), Chennai, Tamil Nadu, India. The collected eggs were maintained at Mosquito control laboratory. Within 3-4 days, the eggs hatched into larvae, and they were carefully transferred to the enamel tray containing water. *A. aegypti* and *C. quinquefasciatus* were cultured separately in a different enamel tray. The young larvae were fed with dog biscuit (pedigree) and Brewer's yeast (6:4). After 4-5 days, the larvae emerged into non-feeding stage pupae. The emerged pupae were carefully collected and transferred into a separate bowls containing water. The bowls were kept in the screened cages (23 × 23 × 32 cm in dimension). Adults emerged after 2-3 days. The male mosquitoes were fed with 10% sucrose (a piece of cotton soaked in 10% sucrose) placed separately in a container and 4-5 pieces of water-soaked raisins were kept separately in a container for female mosquitoes. After 3-4 days, the female mosquitoes were deprived of raisins. The female adults were fed with blood meal using a live chicken whose feathers were chopped off above the thigh region. The chicken kept in small cage within the mosquito screened cages were left undisturbed for 8-10 h during the night. After 3-4 days, an ovitrap was placed in the cage for the collection of freshly laid eggs. The collected eggs were cultured in similar fashion in separate cages and many generations of mosquitoes were obtained. F2 generation of this sample was used for the larvicidal activity. The laboratory bred mosquitoes were free from being exposed to pathogens. Temperature was maintained at 28 ± 1˚ C; relative humidity between 70–75%; and a photoperiod of 10:14 h (light: dark)

4. Larvicidal Bioassay

Bioassay against mosquito larvae using *A. muricata* leaf extracts were carried out according to WHO (2005) protocol. *A. aegypti* and *Cx. quinquefasciatus* were exposed to hexane, chloroform, ethyl acetate and methanolic extract of *A. muricata* at 62.5, 125, 250 and 500 ppm. For each concentration of the crude extract, five replicates were used. In each replicate 20 larvae were introduced. Acetone in water as solvent control and water control were used separately (99ml water and 1 ml acetone). After 24 h of exposure larval mortalities were observed. Mortality of the larvae were confirmed based on their non response to stimulus and their failure to come to the surface in the treatment. The lethal concentration of different crude extracts was calculated using EPA probit analysis. However, the percentage of larval mortality was calculated using the following formula

$$\text{Percentage of larval mortality} = \frac{\text{Number of dead organisms}}{\text{Number of organisms introduced}} \times 100$$

5. Gas Chromatography-Mass Spectrometry

The active extract of *A. muricata* was subjected to Gas Chromatography-Mass spectrometry on an Agilent (GC-MS-5975 MSD) to check the phytochemicals present (Ganesan et al., 2018).

Results

Larvicidal efficacies

The different crude extracts obtained from *A. muricata* leaves demonstrated potential larvicidal effect on *Ae. Aegypti* and *Cx. quinquefasciatus*. Chloroform, ethyl acetate and hexane showed remarkable larvicidal activity with LC_{50} values of 227.853, 257.971, 291.577 and 168.909, 180.594, 432.555 ppm against IV instar larvae of *Ae. Aegypti* and *Cx. quinquefasciatus* respectively. However, a relatively lower activity was observed in the methanolic extract (Tables 1-2).

Table 1: Lethal concentrations of different extracts of *Annona muricata* leaf against larvae of *Aedes aegyptii*

Ae. aegyptii	Treatment	LC_{50}	95% Confidence Limit		LC_{90}	95% Confidence Limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
Fourth Instar Larvae	Hexane	291.577	240.522	370.667	982.764	680.888	1817.330	0.9±0.8	2.4±0.3	2.1*
	Chloroform	227.853	190.430	277.894	723.034	530.818	1181.466	1.0±0.7	2.5±0.3	5.4*
	Ethyl Acetate	257.971	213.898	321.662	863.079	611.628	1518.002	0.8±0.7	2.4±0.3	3.5*
	Methanol	2581.210	896.965	50510 9.844	40242.090	4774.172	2758208 512.000	1.3±0.8	1.0±0.3	0.005*

LC50 lethal concentration that kills 50% of the exposed larvae, LC90 lethal concentration that kills 90% of the exposed larvae, LL lower limit (95% confidence limit), and UL upper limit (95% confidence limit).

*$P \leq 0.05$, level of significance of chi-square values

Table 2: Lethal concentrations of different extracts of *Annona muricata* leaf against larvae of *Culex quinquefasciatus*

Cx. quinquefascitaus	Treatment	LC_{50}	95% Confidence Limit		LC_{90}	95% Confidence Limit		Slope ± SE	Intercept ± SE	χ^2
			LL	UL		LL	UL			
Fourth Instar Larvae	Hexane	432.555	322.623	708.808	2304.985	1191.487	8932.608	0.3±0.7	1.7±0.3	0.2*
	Chloroform	168.909	139.034	204.416	574.825	426.069	924.238	0.3±0.7	2.4±0.3	5.5*
	Ethyl Acetate	180.594	149.598	218.384	598.685	444.472	957.985	0.5±0.7	2.4±0.3	3.8*
	Methanol	1451.440	704.583	15193.832	13458.653	3070.063	2227200.250	0.8±0.9	1.3±0.3	0.5*

LC50 lethal concentration that kills 50% of the exposed larvae, LC90 lethal concentration that kills 90% of the exposed larvae, LL lower limit (95% confidence limit), and UL upper limit (95% confidence limit).

* P ≤0.05, level of significance of chi-square value

GC-MS

GC-MS analysis for the active chloroform extract of *A. muricata* was carried out at IIT Madras (Fig. 3). The analysis revealed the presence of 16 major bioactive compounds Decane, 2,4,6-trimethyl-, Dodecane, 2,6,11-trimethyl, Pentadecane, Benzene, 1-(1,5- dimethyl-4-hexenyl)-4- methyl, Heptadecane, 2,6,10,15- tetramethyl, (R)-1-Methyl-4-(6- methylhept-5-en-2- yl)cyclohexa-1,4-diene, Heptadecane, 2,6,10,15- tetramethyl, Eicosane, Neophytadiene, Benzene, (1- methyldodecyl), Hexadecane, 2,6,11,15- tetramethyl, Eicosane, 2-methyl-, Eicosane, 7-hexyl-, Ipriflavone, Malvidin 3-O-galactoside cation and Minocycline along with their retention time, area percentage and probability percentage (Table 3).

Table 3: List of major compounds identified in the active chloroform extract of *Annona muricata* leaves through GC-MS

Sl. No	Compound Name	R.T.	Area	Probability %	CAS #	Area %
1	Decane, 2,4,6-trimethyl-	6.014	75758.920	16.19	62108-27-4	3.72
2	Dodecane, 2,6,11- trimethyl	10.099	174423.787	11.7	31295-56-4	8.56
3	Pentadecane	11.221	140682.406	6.26	629-62-9	6.91
4	Benzene, 1-(1,5- dimethyl-4-hexenyl)-4- methyl	15.054	117622.009	81.42	644-30-4	5.78
5	Heptadecane, 2,6,10,15- tetramethyl	15.318	211169.754	10.84	54833-48-6	10.37
6	(R)-1-Methyl-4-(6- methylhept-5-en-2- yl) cyclohexa-1,4-diene	15.735	162651.385	26.46	28976-67-2	7.99
7	Heptadecane, 2,6,10,15- tetramethyl	20.145	167468.800	9.05	54833-48-6	8.22
8	Eicosane	21.066	176669.521	5.67	112-95-8	8.68
9	Neophytadiene	22.991	68019.977	50.51	504-96-1	3.34
10	Benzene, (1- methyldodecyl)	24.917	104319.804	36.59	4534-53-6	5.12
11	Hexadecane, 2,6,11,15- tetramethyl	25.352	132920.033	7.39	504-44-9	6.53
12	Eicosane, 2-methyl-	31.428	79780.206	15.31	1560-84-5	3.92
13	Eicosane, 7-hexyl-	35.425	28671.354	5.91	55333-99-8	1.41
14	Ipriflavone	37.341	40141.593	80.85	35212-22-7	1.97
15	Malvidin 3-O-galactoside cation	39.893	20889.337	75.96	104880-34-4	1.03
16	Minocycline	49.982	335319.986	87.65	10118-90-8	16.47

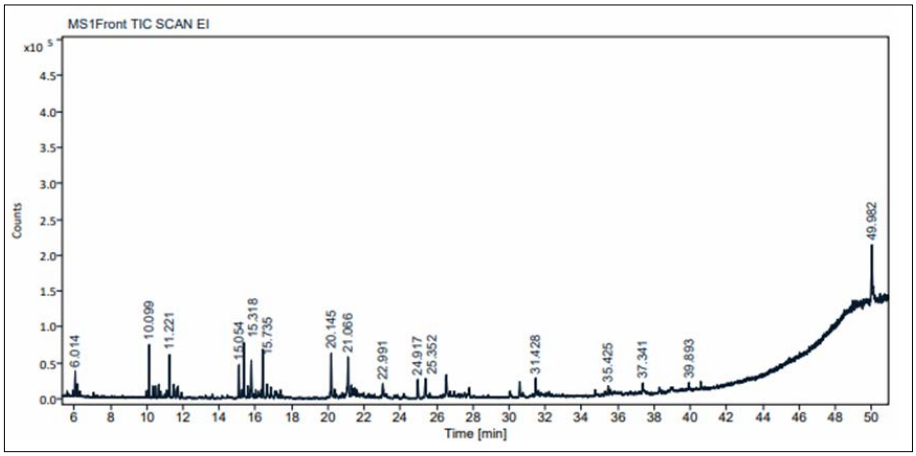

Fig 3: GCMS analysis of active chloroform extract of *Annona muricata* leaves

Discussion

Control of mosquito borne diseases can be achieved only through total eradication of mosquito population. However, as mosquitoes become highly resistant to chemicals and synthetic insecticides, it becomes all most impossible to arrive at a complete eradication. Besides, the chemicals and synthetic insecticides, lead to accumulation of toxic substances, biomagnification, and cause adverse impact on environment causing environmental pollution. The use of biological agents like the entomopathogenic fungi, protozoans, bacteria like *Bacillus thuringienis, Bacillus popilliae, Bacillus sphericus* in controlling vector mosquitoes are effective but expensive and costly (Vivekanandhan et al., 2020, Badii MH et al., 2006). Integrated Mosquito Management (IMM) stresses on the need to use alternative strategies to control mosquitoes (Gosh A et al., 2012). There is a great need to develop eco-friendly insecticides with the help of traditional medicinal plants. Plant-based insecticides can better serve to control mosquitoes as they are environmentally safe, easily available and cost effective.

In the present study, the larvicidal efficacy of different crude extracts of *Annona muricata* leaves showed great potential in controlling mosquito larvae. Highest activity with LC_{50} value of 227.853 and 168.909 ppm was seen in chloroform against *Aedes aegypti* and *Culex quinquefasciatus*, followed by ethyl acetate (257.971 and 180.594 ppm), and hexane (291.577 and 432.555 ppm). However, methanolic extract did showed very less activity. This result can be compared Darsana et al., 2019, who carried out the larvicidal activity of the aqueous extract of *A. Muricata* leaves against *Aedes albopictus* at 12h and 24h. They reported that Annona muricata leaves exhibited great larvicidal activity with percentage mortality rate of 40% and 100% respectively. Magadula et al., 2009, studied the bioefficacy of the ethanolic extract of *A. Muricata* leaves against *Cx. quinquefasciatus* III instar larvae with the LC_{50} value was 56.47 µg/ml.

Aparna et al., 2021, examined the potency of the leaf and seed extract of *A. muricata* and its trypsin inhibitors against the 2nd, 3rd and 4 instar larvae of *Ae. Aegypti*. *A. muricata* leaf led to 100, 96 and 66% mortality after 48 h exposure in II, III and IV instar larvae respectively. Protease activity in the gut of larvae exposed to leaf extract of *A. muricata* plant containing protease inhibitor recorded 52.4 ± 3.3%, while the seed recorded 11.1 ± 2.4% respectively. *A. muricata* leaf and seed resulted in decreasing the gut protease activity to 52%, 52% and 11%, for the 2nd, 3rd and 4th instar larvae respectively. Edwin et al., 2019, demonstrated the mosquitocidal activity of Leaf Extract and Fractions of *A. muricata* against *A. gambiae* larvae (III instar) at various concentrations of 0.15, 0.30, 0.45, 0.60 and 0.75%w/v at 24 h and 48 h exposure. The percentage mortality on ethanol leaf extract and aqueous fractions of *A. muricata* leaves at different concentrations 0.15 – 0.75%w/v at 24h and 48 h showed 0-0%, 0-0% and 0-0%, 0-0% respectively indicating no mortality rate. The percentage mortality of *A. gambiae* on n-hexane and ethyl acetate fractions of *A. muricata* leaves at different concentrations 0.15 – 0.75%w/v at 24h and 48 h showed 0-95%, 0-100% and 0-30%, 0-39% activity respectively.

Castillo et al., 2020, carried out the bioassay against the 3rd instar larvae of *Ae. aegypti* using the Ethanolic extract of leaves and seeds of *A. muricata* at various concentrations of 100, 750, 150, 3000, 5000 ppm and the mortality rate was observed at 8h, 16h, 24h, 32h, 40h, and 48h. 750 and 1500 ppm obtained by the Soxhlet method showed higher lethality. The larvicidal activity was due to the presence of bioactive compounds, and the effectiveness depends on the type of extraction method used. Amakiri et al., 2019, studied the toxicity efficacy of methanolic extract of *A. muricata* stem, bark and leaf against *Anopheles gambiae* larvae at various concentrations of 200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/mL. 100% and 70% mortality were recorded at the highest concentration of 200 mg/ml for stem bark and leaf extract respectively, while at lowest concentration of 25 mg/mL, the mortality rate was 30% and 20% respectively. EI- Inhibition of Emergence percentage were 70% and 100% for leaf extract and stem bark with LC_{50} and LC_{90} values of 67.03 and 570.96 mg/mL, 36.64 an d79.82 mg/mL respectively.

Maia et al., 2022, studied the larvicidal property of the various extracts and fractions of *A. muricata* leaves against *Cx. quinquefasciatus.* The hydroethanolic extract recorded LC_{50} value of 210.17, while recorded Hexane fraction recorded 223.24, Chloroform fraction recorded 102.75, Ethyl acetate fraction and Butanol fraction were Inactive. Ezemuoka et al., 2019, carried out the larvicidal and toxicity activity against the 4th instar larvae of *Ae. Aegypti* using the aqueous extract of *A. muricata* leaves and stem bark at various concentrations of 12.5, 25.0, 50.0, 100.0 and 200.0 mg/ml at a time interval of 3, 6,9,12, and 24 h. The mortality percentage of *A. muricata* leaf aqueous extract recorded at various concentrations of 12.5, 25.0, 50.0, 100.0 and 200.0 at 24 h were 19.4, 29.0, 35.5, 54.0, and 65.0% respectively, while the percentage mortality of *A. muricata* stem-bark aqueous extract recorded 38.7, 54.8, 45.2, 77.0 and 87.0% respectively. Bobadilla et al., 2005, illustrated the larvicidal efficacy of ethanolic extract of seeds, flowers, leaves, stem bark and root bark of *A. muricata* against the 4th instar larvae of *Ae. Aegypti*. The LC_{50} and LC_{90} values of seed, flower, leaves, and stem bark were 0.22 and 0.11 mg/mL, 3.33 and 12.16 mg/mL, 8.25 and 26.87 mg/mL, 97.23mg/mL respectively. At 0.05 mg/mL, seed extract recorded 100 % (highest) mortality at 24 h.

These activities carried out earlier suggests that plant derived secondary metabolites possess great potentiality to control mosquito larvae without affecting the non-target organisms. These findings support our study and further validates the importance of secondary metabolites of plant origin in controlling mosquito population.

Conclusion

The present study establishes that the extracts of *A. muricata* leaves, an evergreen tropical medicinal plant, displays great mosquito larvicidal activity against the early stages of *Ae. Aegypti* and *Cx. quinquefasciatus*. Chloroform extract which showed the highest larvicidal activity was subjected to GC-MS analysis and it showed the presence of many secondary metabolites which are of great importance in controlling mosquitoes. An extensive study of the plant and isolation of mosquitocidal compound from *Annona muricata* has already been carried out which is highly effective. However, further investigation and study of the plant would help in the preparation of mosquitocidal formulation and thereby reduce the toxicity and enhance the environment.

List of abbreviation

GC-MS: Gas Chromatography-Mass spectrometry
LC50: Lethal Concentration 50
IMM: Integrated Mosquito Management

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