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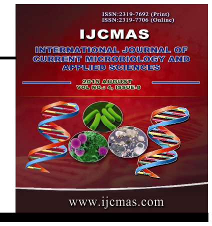

Original Research Article

Mosquito Larvicidal Efficacy of the Leaf Extracts of *Annona reticulata* Against *Aedes aegypti*

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A B S T R A C T

Vector borne diseases are the major concern in the developing countries. All the regions experiencing reoccurrence of deadly diseases. *Aedes aegypti* is the vector responsible for the transmission of dengue fever, and it is a cosmotropical species that proliferates in the water stagnant areas near the houses. Chemically synthesized compounds and the larvicidal proteins from the microbes were used to control the mosquitoes, it is necessary to find an ecofriendly and economically viable alternative for the synthetic chemicals. *Annona reticulata* has been studied for its larvicidal efficacy in the present investigations. The Fourier Transform Infra Red spectroscopy (FTIR) results reveal the presence of various functional groups in the extracts with 100% larvicidal activity. The LC50 values were 9.96 and 6.918 mg/L with reference to ethanol and methanol extracts. The larvicidal activity is attributed by the mixture of compounds like saponin, terpenoids and alkaloids. However, it is required to optimize the concentration as well as to identify the novel compound with larvicidal efficacy.

Keywords

Larvicidal, Mosquito, *Annona reticulate*, FTIR, *Aedes aegypti*

Introduction

Insect-transmitted disease remains a major source of illness and death. Mosquitoes are responsible for the transmission of many medically important pathogens and parasites such as viruses, bacteria, protozoans and nematodes, which cause serious diseases such as malaria, dengue, yellow fever, Chikungunya fever and filariasis (Kettle, 1995).

All tropical regions of the world are experiencing the resurgence and reoccurrence of one of the world's most deadly diseases, i.e., malaria, filariasis, dengue and Chikungunya in world and India is no exception. *Aedes aegypti* is generally responsible for the transmission of dengue fever, yellow fever and it also act as a vector for Zika fever. However, Dengue fever has become an important public health problem as the number of reported cases continues to increase, especially with more severe forms of the disease, dengue hemorrhagic fever, and dengue shock syndrome, or with unusual manifestations such as central nervous system involvement (Pancharoen *et al.*, 2002). *A. aegypti* is a cosmotropical species that proliferates in water containers in the residential areas. (Muir and Kay, 1998; Harrington *et al.,* 2005; Murugan *et al.,* 2011).

Thus, various species of mosquitoes impose a serious health problem and increasing mortality both in human and live stocks leads to the devastation of both health and economic problems in developing and under developed countries which is of higher priority. The common mosquito larvicides are organophosphates, temephos, methopere, and the protein extracted from the *Bacillus thuringiensis* and *B. sphaerius.* However, the high amount of chemical larvicides could lead to long term residual effect to the environment while the use of bacterial protein requires high cost and complex purification process, significantly the increase in the resistance to larvicides of mosquito have been reported. Mosquitoes develop genetic resistance to synthetic insecticides and even to biopesticides such as *Bacillus sphaerius* (Wattal *et al.,* 1981; Tabashnik, 1994)

Irrespective of the less harmful and eco-friendly methods, suggested and used in the control programmes, there is still a need to depend upon the chemical control methods in situations of epidemic outbreak and sudden increase of adult mosquitoes. Hence it is necessary find alternatives to synthetic insecticides which is more potent at low cost. Plants constitute a rich source of bioactive compound which might act deadly on the insect physiological system and kill them (Daoubi *et al.,* 2005; Kim *et al.,* 2005). Insecticidal properties of chemical derived from plants are active against specific target species and are biodegradable and potentially suitable for use in integrated management program (Markouk *et al.,* 2000).

Several plant extracts and isolated compounds from different plant families have been evaluated for their promising larvicidal activities. About 2000 species of terrestrial plants have been reported for their insecticidal properties. The bioactive chemical may act as insecticides, antifedants, moulting hormones, oviposition deterrents, repellents, juvenile hormone mimics, growth inhibitors, antimoulting hormones as well as attractants. The use of different parts of locally available plants and their various products in the control of mosquitoes has been well established globally by numerous researchers. The larvicidal properties of indigenous plants have also been documented in many parts of India along with the repellent and anti-juvenile hormones activities (Singh and Bansal, 2003). In all perspectives the plants were used to control insects by the presence of phytochemicals that were predominantly secondary compounds produced by plants to protect themselves against herbivorous insects (Shaalan *et al.,* 2005).

Plant based pesticides does not have any hazardous effect on the ecosystem, and the secondary metabolites and the synthetic derivatives provide an alternative for the control of mosquitoes. Thus, innovative vector control strategies like use of phytochemicals as alternate source of insecticidal and larvicidal agents in the fight against the vector borne diseases has become inevitable. Several indigenous plants in India and subtropical parts of Asia, such as *Ocimum basilicum, Ocimum santum, Azadirachta indica, Lantana camera, Vitex negundo, Cleome viscose, Leucas aspera,* were studied for their larvicidal action on various stages of larvae of *A. aegypti* and *A. stephensi* (Senthil Nathan *et al.,* 2006; Kalyanasundaram and Dos, 1985; Chavan 1984; Schmutterer 1990; Murugan and Jeyabalan, 1999).

Larval treatment is much more effective for managing this notorious insect because in this stage larvae are localized and restricted to a small space due to the low mobility (Howard *et al.,* 2007). Plant extracts are safer for non-target organisms including man, therefore, plant based Formulations would be more feasible from environmental perspective than synthetic mosquitocides (Bhat and Kempraj, 2009). *Annona reticulata* is a small deciduous or semi-evergreen tree in the plant family Annonaceae. It is best known for its fruit, called custard apple. Different parts of the plant has been used as febrifuge, relieve toothache, for diarrhea and dysentery as a vermifuge, for boils, abscesses and ulcers. The review of literature reveals that there is paucity of information on the larvicidal activity of *Annona reticulata*, hence the present investigation is attempted to study the larvicidal activity of different leaf extracts of the plant on *Aedes aegypti.*

Materials and Methods

Collection of plants

Healthy fresh leaves of *Annona reticulata* were collected from the campus of Queen Mary's College and the plants were authenticated by the Post graduate and research department of Botany of Queen Mary's college, Chennai, Tamil Nadu India. The plant materials were cleaned, air-dried at room temperature for two weeks and coarsely powdered.

Extract preparation

Powdered plant materials were extracted successively by using different solvents of increasing polarity (aqueous, ethanol and methanol) in soxhlet apparatus for minimum of ten to twelve cycles. Then the extracts were concentrated using rotary evaporator and the dried extracts were refrigerated until further use.

Aedes aegypti mosquito was evaluated in accordance with the guidelines of World Health Organization. Larvicidal activity was studied based on the method of Karthikeyan *et al.* (2014) in accordance with the guidelines of World Health Organization (2005). Lab reared third instar larvae of the *Aedes aegypti* mosquitoes were used for studying larvicidal activity. In the present investigation five replicates were done with 20 larvae per replicate. Various concentrations of aqueous, ethanol and methanol mediated solvent extracts were used to study the larvicidal efficacy against the third instar larvae of *Aedes aegypti*. The control was set with plain water. The numbers of dead larvae were counted after 24 hrs and 48hrs of exposure to various extracts.

Dose -response bioassay

From the stock solution, different concentrations ranging from 50 to 250 mg/l were prepared. Based on the preliminary screening result 100% mortality of larvae extracts were tested at different concentrations. The leaf extract prepared from *A. reticulata* were subjected to does response bioassay against *Aedes aegypti* the numbers of dead mosquito larvae were counted after 24 hrs and 48 hrs of exposure and the percentage mortality was reported from average of five replicate.

Fourier Transform Infrared Spectrophotometer (FTIR)

To identify the functional groups (Chemical bonds) present in the compounds Fourier Transform Infrared Spectrophotometer (FTIR) analysis was performed. Dried powder of the various solvent extracts was used for the analysis. FTIR studies were carried out in the FIST –sponsored Lab at Queen Mary's College, Chennai, India.

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating LC50, LC90,and other statistics at 95 percent confidential limits of upper confidence limits and lower confidence limits were calculated by using the SPSS software *p<0.05* was considered to be statistically significant.

Results and Discussion

Mosquitoes in the larval stage are attractive targets for pesticides because mosquitoes breed in water, which makes it easy to deal with them in this habitat. The use of conventional pesticides in the water sources, however, introduces many risks to people and the environment. Vector control is facing a serious threat due to the emergence of resistance in vector mosquitoes to conventional synthetic insecticides or development of newer insecticides. However, due to the continuous increase in resistance of mosquitoes to familiar synthetic insecticides, better alternative means are sought. Tikar *et al.* (2008) has reported that the development of insecticide resistance in populations of *Aedes aegypti* indicates the need for the search of safe and effective alternative measures. Natural pesticides, especially those derived from plants, are more promising in this aspect.

Results presented in table 1 reveals the presence of various phytoconstituents of aqueous, ethanol and methanol crude leaf extracts of *Annona reticulata*. Presence of tannins, saponins, phenols and alkaloids were observed in aqueous extract. Steroids, terpenoids, tannins, saponins, phenols, and flavonoids were identified in ethanol and methanol leaf extracts.

FTIR analysis of the methanolic crude leaf extract of *Annona reticulata* was given in figure 1A. Analysis of the graph reveals the presence of characteristic absorption bond of O-H stretch at 3384.5 cm^{-1} with the functional group representing phenols. Two bands were absorbed at 2923.55 and 2853.54 cm^{-1} characteristic of C-H stretch representing alkanes. C=O saturated aliphatic groups at 1734 cm^{-1}. Characteristic peak at 1488.7 cm^{-1} and 1318cm^{-1} representing aromatic and carboxylic acids respectively.

Infrared spectrum of crude ethanolic leaf extract (Figure 1B) reveals the presence of characteristic peak at 2923.5 and 2853.7 cm^{-1} representing the medium bond C-H with a functional group of alkanes. Presence of saturated aliphatic group with strong C=O stretch at 1735.5 cm^{-1}. A medium bond C-C stretch (in ring) represents aromatics were absorbed at 1458.00 cm^{-1}. Presence of aliphatic amines were identified with the presence of bond C-N stretch were identified with 1203 and 1176 cm^{-1}. The infrared spectrum of the aqueous extract of *Annona reticulata* shown in figure 1C showed the characteristic absorption bond at 3330.96 cm^{-1} with a bonding of strong O-H stretch representing the functional groups with alcohols and phenolic compounds followed by 1928.49 cm^{-1} –representing alkyl substituted ketones aromatics with C-C stretch.

The activity of crude plant extracts is often attributed to the complex mixture of active compounds. In the preliminary screening, potential larvicidal activity of the different solvents crude extracts of *Annona reticulata* plants was noted. Nowadays, the control of mosquitoes at larval stage is focused with plant extracts. The advantage of targeting mosquito at the larval stage is that they cannot escape from their breeding sites until the adult emergences and also to reduce the overall pesticide use to control of adults by aerial application of adulticidal chemicals.

Bioactive crude extracts or isolated phyto-constituents from the plant could be used as alternative to the currently used synthetic insecticides. The biological activity of plant extracts might be due to various compounds, including phenolics, terpenoids, and alkaloids present in plants (Rafael *et al.*, 2009). The present investigation reveals the presence phenols and terpenoids in methanol and ethanol extracts were the maximum larvicidal activity was observed.

The findings presented in table 2 and 3 revealed that all the extracts tested for larvicidal activity showed a significant effect on the tested organism. Patil *et al.* (2011) evaluated larvicidal activity of extracts of medicinal plants *Plumbago zeylanica* and *Cestrum nocturnum* against *A. aegypti* the LC50 values of both the plants were less than 50 ppm. The present investigations are in agreement with the previous findings as the LC50 values after 48 hours amounts less than 50ppm mg/L in ethanol and methanol extracts and a maximum LC50 value was observed with aqueous extract is about 43 mg/L.

Govindarajan *et al.* (2008) has reported that the methanol extract of *Cassia fistula* exhibited LC50 value of 17.97 and 20.57mg/L against *Anopheles stephensi* and *Aedes aegypti*, respectively. It is evident from the present results that the ethanol and methanol extracts of *Annona reticulata* the LC50 value was about9.996 mg/L and 6.9184 mg/L with reference to ethanol and methanol respectively.

The change in the larvicidal activity of the extracts may be due to the presence of secondary metabolite constituents of the extracts. No death was observed with control. The larval mortality was observed after the treatment of leafs extracts of *Annona reticulata*, after 24hrs exposure LC50 values were 161.447, 132.636, 162.156mg\L and the LC90 values were 411.225, 390.731, 511.771mg/L with reference to aqueous, ethanol and methanol extracts respectively.

48 hours after the exposure to the extracts the LC50 and LC90 values of *Annona reticulate* leaf extracts were impressive as the LC50 values of aqueous, ethanol, methanol were 43.081, 9,996, 6.9184 while the LC90 value amounts to 157.438, 113.214; 80.022 mg/L respectively.

The percentage mortality of third instar larvae of *Aedes aegypti* after the exposure to aqueous extracts of *A. reticulata* were 33.60±0.8124 and 67.20 ±0.374 with reference to 50ppm and 250ppm respectively after 24 hours of exposure to the aqueous extract. The same trend has been observed with reference to methanol, ethanol extracts tested against the larvae. 48hours after the exposure to the aqueous extract of *A. reticulata* the percentage mortality amounts to 59.60 ± 0.040 and 98.80 ± 0.374 with reference to 50ppm and 250 ppm. However, the maximum larvicidal potential of the extracts were observed (99.20%) with ethanol and methanol extracts after 48 hours of exposure. It is evident from the results presented in table 2 and 3 that a dose dependant increase in the percentage mortality of *A. aegypti* after the exposure of all the three extracts studied.

Nayak (2014) reported the larvicidal activity of *A. reticulata* leaf crude extract at different concentration shows 100 % mortality rate of larvae was observed at 5, 10, 25, 50, 100 and 200 ppm concentrations of crude extract. Present results are in agreement with these findings as nearly 100% mortality was observed after 48 hours of treatment. Identifying plant based insecticides that are efficient as well as suitable and adaptive to local ecological conditions, biodegradable and have the widespread insecticidal property will obviously work as a new weapon in the arsenal of insecticides and in the future may act as a suitable alternative product to fight against mosquito-borne diseases (Rawani *et al.,* 2009). The biological activity of plant extracts might be due to various compounds, including phenolics, terpenoids and alkaloids present in plants (Rafeal *et al.*, 2008; Pavela, 2008). The present investigation is in agreement with the above findings as the varying presence of phytoconstituents such as terpenoids, phenols, saponins and alkaloids were evident in all the four leaf extracts of the plant may play the impact on the tested organisms. The larvicidal activity depends on the presence of several bioactive chemicals in different parts of the plant (Gutierrez *et al.,* 2014). The presence of cytotoxic compound saponin was observed and the presence of saponin along with other phytoconstituents may be the reason for the 100 percent mortality observed with reference to the extracts of the tested plant. The current investigation reveal that the crude extracts of leaves *Annona reticulata* possesses remarkable larvicidal activity against *Aedes aegypti*. In conclusion *Annona reticulata* extracts were used as better alternative to eradicate the mosquito population in the larval stage, further studies are required to identify mode of action of the compounds.

Table.1 Phytoconstituents of the leaf extracts of *Annona reticulate*

Phytoconstituents	**Aqueous**	***Ethanol***	***Methanol***
Steroid	-	+++	+++
Terpenoids	-	++	+++
Tannin	++	++	+
Saponin	++	+	++
Anthocyanin	-	-	-
Leuco anthocyanin	-	-	-
Coumarins	-	-	-
Phenol	+++	+++	+++
Alkaloids	++	+	+
Flavonoids	-	++	++
Cardiac glycosides	-	-	-
Protein	-	-	-
Carbohydrate	-	-	-

Table.2 Dose-response larvicidal bioassay (24hours) of different solvent of leaf extracts of *Annona reticulata* against third instar larvae of *Aedes aegypti*

	LC50	LOWER BOUND	UPPER BOUND	LC90	LOWER BOUND	UPPER BOUND
AQUEOUS	161.447 ± 0.7600	140.718 ± 0.4092	187.098 ± 0.4414	411.225 ± 0.2975	347.156 ± 0.4810	526.660 ± 2.4126
ETHANOL	132.636 ± 0.5319	113.764 ± 0.2918	159.194 ± 0.2878	390.731 ± 0.7235	331.323 ± 0.2860	492.951 ± 2.609
METHANOL	162.156± 1.0772	131.863± 1.1101	195.765± 1.5691	511.771± 2.9198	406.970± 2.0130	749.432± 2.4910

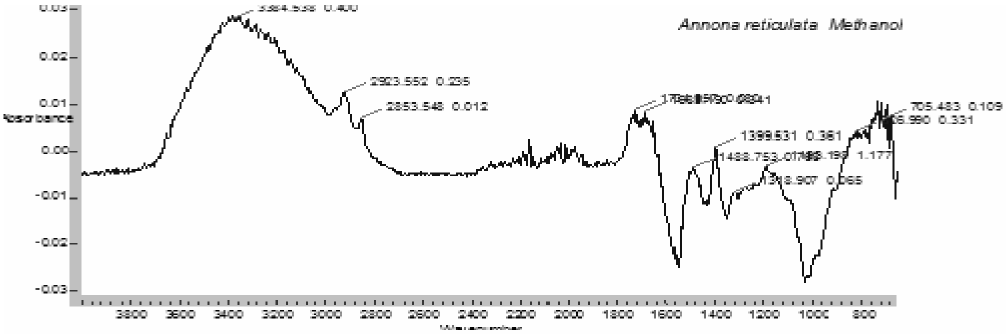

Fig1A FTIR Spectrum of Methanol extract of A. reticulata

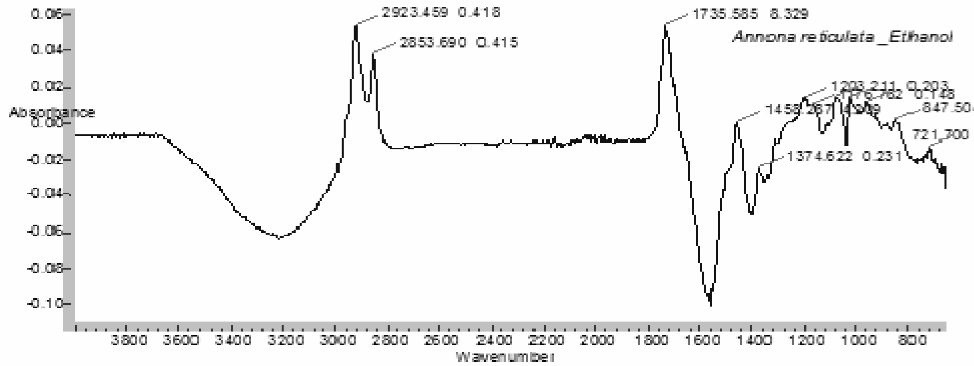

Fig 1B FTIR Spectrum of Ethanol extract of A.reticulata

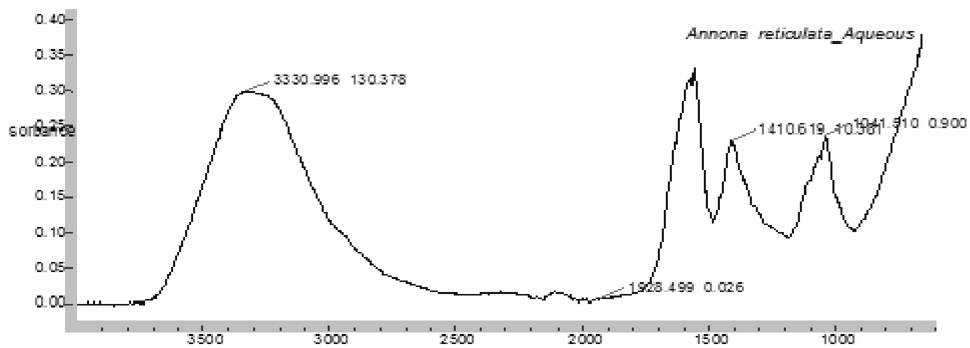

Fig 1C FTIR Spectrum of Aqueous extract of *A. reticulata*

Table.3 Dose–response larvicidal bioassay (48 hours) of different solvent leaf extracts of *Annona reticulate* against 3rd instar larvae of *Aedes aegypti*

	LC50	Lower bound	Upper bound	LC90	Lower bound	Upper bound
Aqueous	43.081 ±1.172	27.262 ± 0.929	60.751 ± 1.395	157.438 ± 4.283	114.451 ± 3.393	228.932 ± 6.280
Ethanol	9.996 ± 0.3462	0.794 ± 0.624	25.514 ±0.789	113.214 ± 2.736	53.198 ± 1.996	234.010 ± 6.546
Methanol	6.918 0.1986	0.191 ± 0.0477	20.332± 1.0657	80.022 ± 1,7855	23.194 ± 3.1599	164.593 ± 6.960

Table.4 Larvicidal activity (percentage Mortality after 24 hours) of different concentrations of various leaf extracts of *Annona reticulata* on *Aedes aegypti*

Dosage	Aqueous	Ethanol	Methanol
50 PPM	33.60 ± 0.8124	38.00 ± 0.3162	38.40 ± 0.4000
100 PPM	42.80 ± 0.3742	44.00 ± 0.4472	44.40 ± 0.509
150 PPM	48.00 ± -.3162	54.60 ± 0.6782	47.60 ± 0.5099
200 PPM	57.20 ± 0.5831	62.20 ± 0.7348	56.80 ± 0.3742
250 PPM	67.20 ± 0.3742	67.60 ± 0.5099	59.60 ± 0.5099

Table.5 Larvicidal activity (percentage Mortality after 48 hours) of different concentrations of various leaf extracts of *Annona reticulata* on *Aedes aegypti*

Dosage	Aqueous	Ethanol	Methanol
50 PPM	59.60 ± 0.400	84.40± 0.600	86.60±0.509
100 PPM	75.60±0.400	86.20±0.583	92.00±0.316
150 PPM	89.00±0.547	91.00±0.447	94.80±0.374
200 PPM	93.20±0.489	94.80±0.374	97.20±0.374
250 PPM	98.80±0.374	99.20±0.3741	99.20±0.3741

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