Int J Pharm Bio Sci 2015 July; 6(3): (B) 1347 - 1356

Research Article **Allied Sciences**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

LARVICIDAL ACTIVITIES OF EXTRACTS OF STEM BARK OF *Annona reticulata* AGAINST FILARIAL VECTOR *Culex quinquefasciatus*

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ABSTRACT

Crude and three different solvent viz. Petroleum ether, hexane, and ethyl acetate extracts of stem bark of *Annona reticulata* Linn. (*A. reticulata*), (Family Annonaceae) were taken for observation for mosquito larvicidal activity. Mortality percentages, Log probit analyses, regression equations, and R^2 values were determined for crude as well as different solvent extracts. Crude extract showed good larvicidal potency. LC_{50} values of Petroleum ether, hexane, and ethyl acetate extracts of stem bark of the plant were 15.2967, 14.0390, 1.0902 ppm respectively against 3rd instar larvae of *Culex. quinquefasciatus* Say, 1823 (*Cx. quinquefasciatus*) after 48 h of exposure. Ethyl acetate extract was applied again on 1st – 4th instars larvae of *Cx. quinquefasciatus* at different concentrations for larvicidal bioassay and was noticed 100% mortality with very low concentrations for all instars larvae. No mortality was noticed on control treatments. Qualitative phytochemical analyses revealed the presence of several secondary metabolites. No adverse effect was observed on non target organisms to ethyl acetate and crude extracts upto 72 h of post exposure.

KEYWORDS: ***Annona reticulata*, *Culex quinquefasciatus,* larvicide, non-target organisms, phytochemicals, stem bark extracts**

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INTRODUCTION

Among the biting dipterans, the most important group is the mosquitoes and act as vectors of many diseases such as filaria, malaria, dengue, Japanese encephalitis and yellow fever, mainly in tropical countries causing illness and death in broad scale. Out of 3000 species of mosquitoes, 100 species act as vectors of human diseases [1]. Lymphatic filariasis is transmitted by *Culex quinquefasciatus* Say, 1823 in many countries of the tropics and subtropics and about 120 million people are infected and 44 million people show chronic manifestations worldwide by this disease [2]. Among different control measures to eradicate the mosquitoes, the best one is the application of insecticides in their larval habitats as mosquito larvae are low mobile in nature in their breeding habitats[3]. Organophosphates like temiphos, fenthion etc as larvacide and diflubenzuron, methoprene etc as insect growth regulators are used in wide scale to reduce mosquito populations[1]. Use of chemical insecticides to control insect pests and vectors create many hazards in environment like biomagnifications through food chains, formation of resistant varieties of insects etc [4]. Insecticides of plant origin are biodegradable, less bio-accumulative and lack of toxicity to higher organisms [5, 6, 7]. Many researchers have reported many plants for their larvicidal properties against different species of mosquitoes[8-14]. Present study was done to unfold the larvicidal properties of stem bark of *A. reticulata* against larvae of *Cx. quinquefasciatus*. Common names of *A. reticulata are* custard apple, sugar apple, sweet apple in English [15]. Its native land is South America and West Indies [16]. A decoction of leaves is used as a vermifuge and decoction of bark is used as tonic, treatment of diarrhoea and dysentery. Fragments of root bark are used to relieve toothache and roots decoction is used as febrifuge [17]. Ethanol extract of its roots has an inhibitory effect against Hela, A- 549, K-562, and MDA- MB human cancer cell lines [18].

MATERIALS AND METHODS

2.1 Plant materials collection

After proper identification, fresh stems of about one to two years aged *A. reticulata* plants were collected during the month of September and October, 2014 from Burdwan town (23° 16´N, 87° 54´E), West Bengal, India and the plant (voucher specimen no. GCZSM-4) was kept as herberium to Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan, West Bengal, India.

2.2 Preparation of crude extract

After collection of stems, leaves were discarded and stems were cut into small pieces and washed with running tap water and thereafter rinsed with distilled water and soaked on paper towel. Then barks were seperated from stems. Barks were crushed on mechanical grinder and juice were filtered by muslin cloth and the filtrate was used as a stock solution for bioassay experiments. Required graded concentrations i.e. 0.01, 0.02, 0.03, 0.04, 0.05 % were prepared adding required volume of stock crude test solution with the required volume of distilled water.

2.3 Preparation of solvent extracts

Barks were cut into very small pieces and dried in shade about 13 -15 days with gentle blowing of air. 150 g shade dried small pieces of barks were packed in soxhlet apparatus and bark extracts were prepared using different solvents namely petroleum ether, hexane, and ethyl acetate (1500 ml each). The period of extraction with each solvent was 72 h with maximum 8 hours per day. For each solvent extraction fresh sample was packed. The extracts were filtered through a Buchner funnel with Whatman number 1 filter paper and filtrate was concentrated by rotary evaporator and semisolid extracts of fore said different solvents were obtained. Graded concentrations of 1, 2, 4 and 8 ppm were prepared for larvicidal bioassay experiments after initial trialing. 0.05 g semi solid of each solvent extract was

dissolved initially on 5 ml of ethanol and then added 95 ml of distilled water to make 100 ml of 5% ethanolic stock test solution of each extract. From stock solution, 1, 2, 4, and 8 ppm concentrations of each solvent extract was made adding required volume of distilled water. Stock solution of each solvent extract was made on the same day of bioassay experiments.

2.4 Test mosquito species

Larvae of *Cx. quinquefasciatus* were taken for bio assay experiments from a mosquito coloney of the Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan, West Bengal, India. Larvae of *Cx. quinquefasciatus* were well maintained in the laboratory. Larvae were maintained at relative humidity of 85% and 34 ± 2° C temperature. Mosquito larvae were fed with artificial food i.e. mixture of dog biscuits and dried yeast powder in the ratio of 3:1.

2.5 Larvicidal bioassay experiments

The bioassay experiments were done according to the standard protocal of WHO with slight modification [19]. Thirty larvae were put in 100 ml of test solutions of different concentrations of crude and different solvent extracts on plastic bowls (225 ml capacity and 9 cm in diameter). Each experiment was done three times excluding control treatments on seperate three days at room temperature 25-30° C and 80-90% relative humidity condition. Control treatments were done on 100 ml of tap water with 0.5 ml of ethanol (positive control) and 100 ml of tap water only (negative control). The larvae were identified dead when they did not move after touching with a fine brush in the siphon or cervical region of it. Larval mortalities were recorded after 24, 48 and 72 h of exposures cumulatively. Mortality percent was corrected by Abbott's formula whenever necessary [20].

2.6 Phytochemical analyses of stem bark of A. reticulata

Qualitative phytochemical analyses of aqueous and ethanol extracts of shade dried stem bark were done according to Trease and Evans et al. [21] with slight modification.

2.6.1 Test of presence of alkaloids (Mayer's test)

2 ml of ethanolic extract were taken in a clean test tube and added few drops of 2N HCL. Then added 1 or 2 drops of Mayer's reagent [1.36g of mercuric chloride dissolve in 60 ml of H_2O and then this solution was pour in potassium iodide solution (5 gm potasium iodide dissolve in 100ml of water)]. Appearence of precipitate of cream colour or pale yellow colour indicates the presence of alkaloids.

2.6.2 Test of terpenoids: (Salkowski test)

2 ml of ethanol extract was treated with 3 ml of chloroform in a test tube. A few drops of concentrated sulphuric acid were added carefully. Appearance of reddish brown color at the interface indicates the presence of terpenoids.

2.6.3 Test of flavanoids

2 ml of aqueous extract was treated with a few drops of NaOH solution. Formation of intence colour was formed which became colourless on addition of dilute HCL and indication of the presence of flavanoids.

2.6.4 Test for tannins and phenolic compounds (ferric chloride test)

2 ml of aqueous extract was taken in a test tube and few drops of ferric chloride were added. Appearence of blue green colour confirmed the presence of tannins and phenolic compounds in the sample.

2.6.5 Test of anthraquinones

2 ml of aqueous extract was added to 2 ml of 2N HCL and NH_3. The appearence of pink red was turned blue violet indicating the presence of anthrocyanines.

2.6.6 Test of saponins (frothing test)

10 ml of aqueous extract was taken in a test tube and shaken vigorously. Persistence of frothing indicate the presence of saponines.

2.6.7 Test of proteins (Ninhydrin test)

2 ml of ethanol extract was taken in a test tube and 2-3 drops of 0.1% ninhydrin reagent was added and boiled for one minute and then cooled. Appearence violet colour indicates the presence of proteins in the sample.

2.6.8 Test of steroids

2 ml of the ethanolic extract was taken in a test tube and added 5 ml of chloroform and then added 3 ml of concentrated sulphuric acid by the interior wall of the test tube. The upper layer showed red and sulphuric acid layer showed yellow with green florescence. This is the indication of the presence of steroids.

2.7 Effect on non target animals

There are some animals which live in the same habitat of *Cx. quinquefasciatus* larvae. Bioassay tests were done with LC_{50} values of 3rd instar larvae of *Cx. quinquefasciatus* for 24 h of crude and ethyl acetate extracts of stem bark of *A reticulata* on tadpol larva of toad, and *Chironomus circumdatus* larvae.

2.8 Statistical analysis

The computer software STAT PLUS 2009- trial version and MS EXCELL 2007 were used to calculate the LC_{50} and LC_{90} values (95% confidence level), regression equations (Y= mortality, X= concentration), coefficient of determination (R^2), ANOVA, mean mortality percent and standard error.

RESULTS

Crude extract of stem bark of *A. reticulata* showed 100% mortality only at 0.03% concentration for 1st and 2nd instars larvae of *Cx. quinquefasciatus* after 24 h of exposure and 3rd and 4th instars showed 100 and 86.66% mortality after 72 h of exposure respectively. In control treatments, no mortality was observed (Table 1). Table 2 depicts the mortality percent of different solvent extracts, *viz* petroleum ether, hexane, and ethyl acetate. Among three solvent extracts, ethylacetate extract showed excellent larvicidal activities at very low concentrations against 3rd instar larvae of *Cx. quinquefasciatus.* No larval mortalities were observed on control treatments. LC_{50} values (95% confidence level) of Petroleum ether, hexane and ethyl acetate extracts of stem bark of *A. reticulata* plant were 15.2967, 14.0390, 1.0902 ppm against 3rd instar larvae of *Cx. quinquefasciatus* after 48 h of exposure respectively. Among Petroleum ether, hexane, and ethyl acetate stem bark extracts of the plant, ethyl acetate extract was most potent than Petroleum ether, and hexane stem bark extract of the plant for its larvicidal activity. Table 3 presents the mortality percentages of different larval instars of *Cx. quinquefasciatus* exposed to different concentrations of ethyl acetate extract of the stem bark of *A. reticulata* upto 24, 48 and 72 h of exposures. No larval mortalities were observed on control treatments. Mortality percent increased with increase in concentrations and time of exposures. 100% mortalities were observed for 1st and 3rd instars larvae at 8 ppm concentration only after 24 h of exposure. LC_{50}, LC_{90} (95% confidence level), regression equations and R^2 values of crude and ethyl acetate stem bark extract of *A. reticulata* is presented in Table 4 and Table 5 respectively. LC_{50} and LC_{90} values (95% confidence level) gradually decreased with a period of exposures in different larval forms of *Cx. quinquefasciatus.* R^2 (co efficient of determination) values were close to 1 in allmost all cases which indicated the positive corelation between concentrations of the extract and mortality percentages. No adverse effects such as slugglishness, swiming activity and mortality were observed on non target organisms upto 72 h of the post exposure period with application of LC_{50} of crude and ethyl acetate solvent extracts of 24 h of exposure against *Cx. quinquefasciatus*. Qualitative phytochemical analyses of stem bark extracts (aqueous and ethanol) revealed the presence of several secondary metabolites such as alkaloids, terpenoids, steroids, tannins, phenols, flavanoids, anthraquinones and proteins but absence of saponins (Table 6). Three ways factorial ANOVA established statistical significance of larval mortality of *Cx. quinquefasciatus* ($p < 0.05$) in terms of instars, time and concentrations (Table 7).

Table 1

Mortality percent of different instars of Culex quinquefasciatus exposed to different concentrations of crude extract of of stem bark of Annona reticulata (Mean mortality percent ± Standard error)

Instars	Concentrations (%)	Mortality% at different exposure period		
		24h	48 h	72 h
1st	0.01	73.33±3.33	86.66±3.33	90±00
	0.02	90±5.78	96.66±3.33	96.66±3.33
	0.03	100±00	100±00	100±00
	0.04	100±00	100±00	100±00
	0.05	100±00	100±00	100±00
2nd	0.01	80±00	86.66±3.33	90±00
	0.02	90±5.78	90±5.78	96.66±3.33
	0.03	100±00	100±00	100±00
	0.04	100±00	100±00	100±00
	0.05	100±00	100±00	100±00
3rd	0.01	26.66±3.33	36.66±3.33	46.66±3.33
	0.02	43.33±3.33	53.33±3.33	63.33±3.33
	0.03	66.66±3.33	76.66±3.33	83.33±3.33
	0.04	76.66±3.33	83.33±3.33	93.33±3.33
	0.05	93.33±3.33	96.66±3.33	100±00
4th	0.01	20±00	23.33±3.33	36.66±3.33
	0.02	26.66±3.33	33.33±3.33	36.66±3.33
	0.03	46.66±3.33	43.33±3.33	46.66±3.33
	0.04	56.66±3.33	63.33±3.33	66.66±3.33
	0.05	73.33±3.33	80±5.78	86.66±6.66

Control No mortality (For all instars)

Table 2

Mortality percent of 3rd larval instars of Culex quinquefasciatus exposed to different concentrations of different solvent extracts of stem bark of Annona reticulate (Mean mortality percent ± Standard error)

Solven extracts	Instars	Conc. (ppm)	Mortality% at different exposure period		
			24h	48h	72h
Petroleum ether	3rd	1	00±00	00±00	20±5.77
		2	00±00	6.67±00	33.33±3.33
		4	00±00	20.67±5.77	46.67±3.33
		8	00±00	26.67±3.33	60±5.77
Hexane	3rd	1	00±00	6.66±3.33	43.33±3.33
		2	00±00	16.67±3.33	56.66±3.33
		4	00±00	26.67±3.33	66.67±3.33
		8	16.67±3.33	36.67±3.33	73.33±3.33
Ethyl acetate	3rd	1	16.67±8.81	43.33±6.67	66.67±3.33
		2	53.33±3.33	76.66±3.33	93.33±3.33
		4	70±00	100±00	100±00
		8	100±00	100±00	100±00

Control: no mortality

Table 3

Mortality percent of different instars of Culex quinquefasciatus exposed to different concentrations of ethyl acetate extract of stem bark of Annona reticulata (Mean mortality percent ± Standard error).

Instars	Conc.(ppm)	Mortality% at different exposure period		
		24h	48h	72h
1st	1	76.67±3.33	93.33±3.33	100±00
	2	86.67±3.33	100±00	100±00
	4	93.33±3.33	100±00	100±00
	8	100±00	100±00	100±00
2nd	1	60.00±00	66.67±3.33	73.33±3.33
	2	66.67±3.33	76.67±3.33	96.67±3.33
	4	80.00±00	100±00	100±00
	8	90±5.77	100±00	100±00
3rd	1	16.67±8.81	43.33±6.67	66.67±3.33
	2	53.33±3.33	76.66±3.33	93.33±3.33
	4	70±00	100±00	100±00
	8	100±00	100±00	100±00
4th	1	6.67±3.33	10±00	33.33±3.33
	2	16.67±3.33	50±00	50±5.577
	4	36.67±3.33	63.33±3.33	83.33±3.33
	8	76.67±3.33	83.33±3.33	100±00

Control No mortality

Table 4

Log probit and regression analyses of larvicidal activity of crude extract of stem bark of Annona reticulata against different larval instars of Culex quinquefasciatus

Instars	Period (h)	LC_{50} (ppm)	LC_{90}(ppm)	Regression equations	R^2- values
1st	24	0.0069	0.0163	Y=7.3667+63.3333X	0.6355
	48	0.0046	0.0116	Y=8.7667+30.0000X	0.5063
	72	0.0037	0.0104	Y=9.0333+23.3333X	0.5568
2nd	24	0.0056	0.0151	Y=7.9000+50.0000X	0.6465
	48	0.0039	0.0133	Y=8.4333+36.6667X	0.5215
	72	0.0036	0.0104	Y=9.0333+23.3333X	0.5568
3rd	24	0.0197	0.0599	Y=1.1333+166.6667X	0.9498
	48	0.0155	0.0501	Y=2.4333+150.0000X	0.9255
	72	0.0122	0.0360	Y=3.6333+136.6667X	0.9195
4th	24	0.0314	0.1326	Y=.3667+136.6667X	0.9380
	48	0.0273	0.1094	Y=.5667+143.3333X	0.9099
	72	0.0219	0.1124	Y=1.4333+136.6667X	0.8272

LC = Lethal concentration, R^2 = Co efficient of determination

Table 5

Log probit and regression analyses of larvicidal activity of ethyl acetate extract of stem bark of Annona reticulata against different larval instars of Culex quinquefasciatus

Instars	Period (h)	LC_{50} (ppm)	LC_{90}(ppm)	Regression equations	R^2-values
1st	24	0.3986	2.3208	Y=77.97+2.98X	0.86
	48	0.1401	0.6951	Y=95.94+0.64X	0.35
	72	-	-	-	
2nd	24	0.6830	9.3448	Y=58.41+4.20X	0.94
	48	0.7859	2.3800	Y=68.55+4.60X	0.72
	72	0.7108	1.444	Y=82.17+2.75X	0.66
3rd	24	2.0915	5.5692	Y=20.44+10.55X	0.88
	48	1.0902	2.4713	Y=54.34+6.84X	0.62
	72	0.7810	1.6779	Y=76.52+3.59X	0.49
4th	24	4.6777	15.0883	Y=-3.33+10X	1
	48	2.7008	9.9419	Y=18.41+8.87X	0.79
	72	1.6224	4.7481	Y=31.88+9.28X	0.89

LC = Lethal concentration, R^2 = Co efficient of determination

Table 6

Result of qualitative phytochemical analysis of aqueous and ethanol extracts of stem bark of Annona reticulate

Name of the plant	Plant parts	Alkaloids	Terpenoids	Steroids	Flavanoids	Tannins	Phenols	Anthraquinones	Saponines	Proteins
Annona reticulata	stem bark	+++	+++	+++	+++	+++	+++	+++	---	+++

+++ = present
--- = absent

Table 7

Completely randomized three way ANOVA analyses using instars (I) of Cx. quinquefasciatus, hour (H), and Concentration of ethyl acetate extract of stem bark (C) as three independent parameter.

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean of squares (MS)	F value	p-level
Instars(I)	392.69	3	130.90	496.03	0
Time(H)	129.17	2	64.58	244.74	0
Conc.(C)	354.52	3	118.17	447.82	0
I×H	19.17	6	3.19	12.11	0
I×C	116.40	9	12.93	49.01	0
H×C	21.67	6	3.61	13.68	0
I×H×C	26	18	1.44	5.47	0
Within groups	25.33	96	0.26	-	-
Total	1084.94	143	7.59	-	-

DISCUSSION

Recently Ghosh et al. reviewed many plants with mosquito larvicidal potency [22]. Present study indicates that crude as well as different solvent extracts stem bark of *A. reticulata* showed excellent larvicidal potency at very low concentrations against *Cx. quinquefasciatus* which is worthy mentioning. Many researchers worked with stem bark and other plant part of

many plants and reported their larvicidal activity. Rawani *et.al* (2010) worked with *Solanum nigrum* and showed 100% mortality at 50 ppm concentration by the application of a leaf ethyl acetate extract of *Solanum nigrum* against 3rd instar larva of *Cx. quinquefasciatus* having LC_{50} value 17.04 ppm after 24 h of exposure [23]**.** But in this study, ethyl acetate stem bark extract of *A. reticulata* showed 100% mortality only at 8 ppm concentration against 3rd instar larva of *Cx. quinquefasciatus* having LC_{50} value 2.0915 ppm after 24 h of exposure. Mwanaisha *et.al* (2013) worked with stem bark extract of *Commiphora swynnertoni* against late third instar larvae of *Anopheles gambiae*, *Cx. quinquefasciatus* and *Aedes aegypti* and showed LC_{50} values were 26.5528, 86.5375, and 238.3535 µg/ml respectively after 72 h of exposure [24]. But in this study, LC_{50} values of ethyl acetate stem bark extract of *A. reticulata* showed 2.0915 and 1.1579 ppm against 3rd instar larvae of *Cx. quinquefasciatus* after 24 and 48 h of exposures respectively. Kamraj *et.al* (2011) reported that LC_{50} values of bark methanol extract of *Annona squamosa*, leaf ethyl acetate extract of *Chrysanthemum indicum*, and leaf acetone extract of *Tridax procumbens* were 93.80, 39.98, and 51.57 mg/L against 4th instar larvae of *Anopheles subpictus* after 24 h of exposure. On the other hand, LC_{50} values of a bark methanol extract of *Annona squamosa,* leaf methanol extract of *Chrysanthemum indicum* and leaf ethyl acetate extract of *Tridax procumbens* against 4th instar larvae of *Culex tritaeniorhynchus* after 24 h of exposure were 104.94, 42.29, and 69.16 mg/L [25]. In the present study, 4th instar larvae of *Cx. quinquefasciatus* showed LC_{50} value 2.7008 ppm (mg/L) after 24 h of exposure. Arivoli and Tennyson (2012) reported the larvicidal activity of hexane, chloroform and ethyl acetate leaf extract of *Strychnos muxvomica* against 3rd instar larvae of *Cx. quinquefasciatus*. The ethyl acetate leaf extract was much effective with LC50 values of 222.28 and 146.99 ppm after 24 and 48 h respectively [26]. But present study Showed LC_{50} values 2.0915 and 1.1579 ppm against 3rd instar larvae of *Cx. quinquefasciatus* after 24 and 48 h respectively.

CONCLUSION

The present study revealed a great support to control *Cx. quinquefasciatus* mosquito at their larval stages as crude and ethyl acetate solvent extracts of the bark of stems work at very much low concentrations. Further study is needed to unfold their activities on other species of mosquitoes and to find out the active chemical compound (s) related to larvicidal activities. Nayak (2014) reported larvicidal efficacy of leaf methanol extract of *A. reticulata* [27], but no work with stem bark of *A. reticulata* relating to larvicidal activity of the plant has done. This is the first report of mosquito larvicidal efficacy of stem bark of *A. reticulata.*

ACKNOWLEDGEMENT

Authors are much gratefull to Professor Dr. A Mukhopadhyay, Department of Botany, The University of Burdwan, West Bengal, India for his kind help for identification of the plant species. We are also grateful to UGC DRS for providing financial supports.

CONFLICT OF INTEREST

We have no conflict of interest.

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