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Larvicidal, repellent and ovicidal activity of *Calotropis gigantea* against *Culex gelidus, Culex tritaeniorhynchus* (Diptera: Culicidae)

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In this study, aqueous of *Calotropis gigantea* leaves were screened for larvicidal activity, mosquito repellent activity and ovicidal activity against *Culex gelidus* and *C. tritaeniorhynchus* mosquitoes. The extract doesn't exhibit 100 % mortality against both the mosquito larvae. However, the extract showed dose dependent larvicidal activity with an motility rate of 86±1.42% (LC_{50}=137.90) against *C. gelidus* and 94±1.31% (LC_{50}=110.05) against *C. tritaeniorhynchus.* At 125, 250, 500 and 1000 ppm dose *C. gigantea* leaves extract exhibits complete protection from mosquito bite for 60, 90, 90 and 240 minutes against *C. gelidus* and 60, 90, 90 and 120 minutes respectively against *C. tritaeniorhynchus. C. gigantea* showed moderate ovicidal at 1000 ppm concentration against both mosquitoes eggs. Phytochemical analysis of the extract exhibited the presence of phenolic compounds, flavonoids, alkaloids, tannins, saponins, glycosides and phytosterols as major phytochemical groups. These results suggest that the leaves of *C. gigantea* have the potential to be used as a natural source for the development of new, safe, potential and eco-friendly insecticide for the control of *C. gelidus* and *C. tritaeniorhynchus* mosquitoes.

Key words: *Calotropis gigantea,* larvicidal activity, mosquito repellent activity, ovicidal activity*, Culex. tritaeniorhynchus*, *C. gelidus*

Introduction

Japanese encephalitis virus is one of the major causes of encephalitis worldwide. First case of Japanese encephalitis was reported in Japan in 1870,

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since then Japanese encephalitis have been reported in many countries including Cambodia, China, India, Japan, Korea, Malaysia, Myanmar, Nepal, Russia, Taiwan, Thailand and Vietnam etc. (Okuno, 1978; Endy and Nisalak, 2002). Annually about 50,000 cases and 15,000 deaths are reported worldwide because of Japanese encephalitis (Solomon *et al.* 1998). Japanese encephalitis virus is one of the major causes of viral encephalitis in most Asian countries.

Japanese encephalitis was first recognized in India in 1955 (Work and Shah, 1956) followed by several outbreaks in many parts of the country including Andhra Pradesh, Assam, Tamil Nadu, Karnataka, Kerala, Bihar, Uttar Pradesh, Pondicherry, Goa and Haryana (Reuben and Gajanana, 1997). Japanese encephalitis virus is transmitted through the mosquito (*Culex* sp.) from one host to other. Japanese encephalitis virus has been isolated from several mosquito species; however *C. tritaeniorhynchus* and *C. gelidus* are the major one. *C. tritaeniorhynchus* and *C. gelidus* serve as primary and secondary vector for Japanese encephalitis virus. The habitat of these mosquitoes are ponds, wells, small rivers, canals, puddles, marshes, swamps, rice fields and cattle farming regions (Colless, 1957). Adult mosquito attack humans in large number during night time and transmit the Japanese encephalitis virus to human.

Mosquito control represents an important strategy for prevention of disease transmission and epidemic outbreaks. In the present time, over use of chemical insecticides leads mosquitoes to develop the resistance towards chemical insecticides. To overcome this problem scientists have initiated the search for alternative control measures. Thus, research is focused on finding newer insecticides of plant origin with high potency, safety and easy availability at low cost (Brogdon and McAllister, 1998; Hemingway and Ranson, 2000). Secondary metabolites produced by plants protect them against pathogenic microorganisms and insects. These metabolites are present in plant as key candidate with insecticidal properties and can be explored to develop the natural compounds to control mosquito population. Numerous plants have been reported to possess mosquito controlling properties against a variety of mosquitoes such as *Euphorbia tirucalli* (Yadav *et al.* 2002), *Melia azedarach* (Maciel *et al.* 2006), *Cymbopogan citrates* (Pushpanathan *et al.* 2006), *Centratherum anthelminticum* (Srivastava *et al.* 2008), *Swertia chirata* Buch. (Balaraju *et al.* 2009), *Croton bonplandianum* Baill. (Jeeshna *et al.* 2010), *Cymbopogon citratus* (DC), *Croton macrostachyus* Del. (Karunamoorthi and Ilango, 2010) and *Adhatoda vasica* L. (Al-shaibani *et al.* 2008).

In this study *Calotropis gigantea* was screened for its mosquito controlling potential. *C. gigantea* belongs to the family Asclepiadaceae, which includes the latex bearing plants. *C. gigantea* is a common wasteland weed

native to India, Indonesia, Malaysia, Philippines, Thailand, Sri Lanka and China. It is a large shrub growing to 4 m tall and commonly known as giant milk weed. It has clusters of waxy flowers of white colour. The plant has oval, light green leaves and milky stem. *C. gigantea* is frequently available in India and used for several meditational purposes. Traditionally, *C. gigantea* is used for the treatment of paralysis, swellings, intermittent fevers, asthma, catarrh, anorexia, helmintic infections, inflammations, fever, intestinal worms, cough, bronchitis and dyspepsia (Kumar, *et al*. 2011). Recently, flowers of the *C. gigantea* are reported for analgesic activity (Pathak and Argal, 2007). Roots are reported to contain anti-pyretic activity (Chitme, *et al*. 2005), cytotoxic activity (Wang *et al.* 2008), antimicrobial activity (Alam *et al.* 2008), insecticidal activity (Alam *et al.* 2009), CNS activity (Argal and Pathak, 2006) and pregnancy interceptive properties (Srivastava *et al.* 2007). Leaves and areal parts of the plant are used in the treatment of external swellings and diarrhoea (Chitme *et al.,* 2004) and reported for anti-Candida activity (Kumar *et al.* 2010a) and antibacterial activity (Kumar *et al.,* 2010b). Latex of the plant is reported to contain purgative properties, procoagulant activity (Rajesh *et al.* 2005) and wound healing activity. *C. gigantea* also uses to cure toothache, earache, sprain, anxiety, pain, epilepsy and mental disorders.

The aim of this work was to investigate the larvicidal, ovicidal, repellent activity of aqueous extract of *C. gigantea* Linn leaves against *C. gelidus* and *C. tritaeniorhynchus*.

Materials and methods

Plant material

C. gigantea plants material was collected from the natural population growing in Vellore district, TN, India (12°54′40″N 79°8′10″E) in the month of December, 2008. Plants were brought to the Molecular and Microbiology Research Laboratory, VIT University, Vellore. Voucher specimens were maintained in our laboratory for further references (Accession number: VIT/SBST/MMRL/CG/10.1.2009/101).

Processing of the plant sample

Mature and healthy leaves of the *C. gigantea* were collected and washed properly under running tap water followed by distilled water. Leaves were shade dried and powdered using a mechanical grinder. Pulverized leave material was extracted with distilled water. These extracts were concentrated

with a rotary evaporator and dried using lyophilizer. Dried extract was collected in air tight container and stored at 4°C up for further use.

Insect rearing

C. gelidus and *C. tritaeniorhynchus* larvae were collected from rice field and stagnant water area of Melvisharam (12°56'23"N, 79°14'23"E) and identified in Zonal Entomological Research Centre, Vellore (12°55′48″N, 79°7′48″E), Tamil Nadu, India. To start the colony larvae were kept in plastic and enamel trays containing tap water. All the experiments were carried out, at 27±2°C and 75–85% relative humidity under 14:10 light and dark cycles. Larvae were fed a diet containing brewer's yeast, dog biscuits, and algae collected from ponds in a ratio of 3:1:1, respectively. Pupae were transferred from the trays to a cup containing tap water and were maintained in our insectary (45×45×40 cm), where adults emerged. Adults were maintained in glass cages and were continuously provided with 10% sucrose solution in a jar with a cotton wick. On day five, the adults were given a blood meal from a pigeon placed in resting cages overnight for blood feeding by females. Glass petri dishes with 50 ml of tap water lined with filter paper was kept inside the cage for oviposition (Kamaraj *et al.* 2008).

Larvicidal activity

During preliminary screening with the laboratory trial, 0.1 gm of crude extracts were dissolved in 10 ml of distilled water (stock solution). From the stock solution, 1,000 ppm solution was prepared with dechlorinated tap water. Polysorbate 80 was used as an emulsifier at the concentration of 0.05% in the final test solution. The larvicidal activity was assessed by the procedure of WHO (1996) with some modifications and as per the method of Rahuman *et al.* (2000). For larvicidal bioassay, larvae were taken in five batches of 20 in 249 ml of water and 1.0 ml of the plant extract (1,000, 500, 250, 125, 62.5, 31.25 ppm concentration). The control was set up with distilled water and polysorbate 80. The numbers of dead larvae were counted after 24 h of exposure, and the percentage mortality was reported from the average of five replicates.

Mosquito repellent activity

The stock solutions of the extracts were diluted with acetone, polysorbate 80 and distilled water to obtain test solutions of 31.25, 62.50, 125.00, 250.00, 500.00, and 1,000 ppm. For repellent experiment, 50 laboratory reared blood-starved adult female mosquitoes (3 to 10 days old) were placed into separate

laboratory cages (45×45×40 cm). Before each test, the forearm and hand of a human subject were washed with unscented neutral soap, thoroughly rinsed, and allowed to dry 10 min before extracts application. The plant extract was applied from the elbow to the fingertips. The arm was left undisturbed. An arm treated with acetone and polysorbate 80 served as control. The control and treated arms were introduced simultaneously into the cage. The numbers of bites were counted over 5 min, every 30 min, from 18:00 h to 06:00 h.

Protection time was recorded as the time elapsed between repellent application and the observation period immediately preceding that in which a confirmed bite was obtained. If no bites were confirmed at 240 min, tests were discontinued and protection time was recorded as 240 min. An attempt of the mosquito to insert its stylets was considered as a bite. The experiments were conducted five times in separate cages and in each replicate different volunteer were used to nullify any effect of skin differences on repellency. The percentage protection was calculated by using the following formula (Venkatachalam and Jebanesan, 2001; Fradin and Day, 2002).

$$\% \text{ Protection} = \frac{(\text{No. of bites received by control arm}) - (\text{No. of bites received by treated arm})}{(\text{No. of bites received by control arm})} \times 100$$

Ovicidal activity

For ovicidal activity, the freshly laid eggs were collected by providing ovitraps in mosquito cages. Ovitraps were kept in the cages 2 days after the female mosquitoes were given a blood meal. The eggs were laid on filter paper lining provided in the ovitrap. After scoring, 100 gravids were placed in a screen cage where ten oviposition cups were introduced for oviposition, 30 min before the start of the dusk period. Out of these ten cups, nine were filled with test solution of 31.25, 62.50, 125, 250, 500 and 1000 ppm concentration of plant extracts, and one was filled with 100 ml of distilled water respective solvent and used as a control. A minimum of 100 eggs was used for each treatment, and the experiment was replicated five times. After treatment, the eggs were sieved through muslin cloth, thoroughly rinsed with tap water, and left in plastic cubs filled with dechlorinated water for hatching assessment after counting the eggs under microscope (Su and Mulla, 1998). The percent egg mortality was calculated on the basis of nonhatchability of eggs with unopened opercula (Chenniappan and Kadarkarai, 2008). The hatching rate of eggs was assessed after 98 h post treatment (Rajkumar and Jebanesan, 2009).

Phytochemical screening

Phytochemical screening of the leaves of *C. gigantea* was carried out by using the standard protocols (Harborne, 1973) for the presence of carbohydrates, proteins, phenolic compounds, saponins, flavonoids, alkaloids, tannins, glycosides, phytosterols, oil and fats.

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating LC50 and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit, and chi-square values were calculated using the software developed by Reddy *et al*. (1992). Results with $p<0.05$ were considered to be statistically significant.

Results and discussions

Classically, chemical insecticidal are extensively used to control mosquito growth, over use of theses chemical insecticide results in to insecticide resistant in mosquitoes. The cases of insecticide resistant in mosquitoes have been reported worldwide (Davidson and Jackson, 1961; Brown, 1986). Chemical insecticides also cause several health hazards in humans and animals as well as toxic to environment. To overcome these problems, it is important to discover some new and potent insecticides from alternative sources such as plants and microorganisms (Sharma, 2001).

Plants are extensively reported to possess toxic effect on the mosquitoes and can be utilizes as a potent source of mosquito control. Earlier, some plants such as *Annona squamosa* L., *Gloriosa superba* L., *Millingtonia hortensis, Abuta grandifolia*, *Minthostachys setose*, *Azadirachta indica*, *Ocimum gratissimum and Hyptis suaveolens* etc have been reported to control mosquito population (Ciccia, 2000; Kaushik and Saini, 2008; Bagavan *et al*. 2009; Okigbo *et al*. 2010). This study is completely focused on the biological control of *C. tritaeniorhynchus* and *C. gelidus* (primary and secondary vector for Japanese encephalitis, respectively) using the aqueous extract of *C. gigantea* leaves. Selection of *C. gigantea* for the present study was based on its pharmaceutical use in traditional medicinal system to cure several diseases.

In this study, larvicidal activity of aqueous extract of *C. gigantea* leaves was examined against two mosquito larvae included *C. gelidus* and *C. tritaeniorhynchus*. The extract exhibited 86±1.42% (LC_{50}=137.90) larvicidal

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activity against *C. gelidus* and 94±1.31% (LC_{50}=110.05) against *C. tritaeniorhynchus* at 1000 ppm dose. Data was reported in terms of percentage mortality and expressed in terms of mean±SD (n=5), results are reported in Table 1.

Table 1. Larvicidal activity of aqueous extract of *Calotropis gigantea* leaves against fourth instar larvae of *C. gelidus* and *C. tritaeniorhynchus*

Mosquitoes species	Concentrations (ppm)	Percent mortality[a] ± SD	LC_{50} (ppm) (LCL–UCL)	r^2	Slope	χ^2 (df=4)
C. gelidus	1000	86±1.42	137.90	0.837	48	8.787
	500	75±2.19	(103.24-184.20)			
	250	62±1.84				
	125	48±1.21				
	62.5	34±1.40				
C. tritaeniorhynchus	1000	94±1.31	110.05	0.848	52	6.399
	500	82±0.78	(79.17-152.96)			
	250	68±1.62				
	125	52±1.24				
	62.5	39±2.10				

Control -Nil mortality. Significant at P<0.05 level, LC_{50} lethal concentration that kills 50% of the exposed larvae, UCL upper confidence limit, LCL lower confidence limit, r^2 regression coefficient χ^2 chi-square, df degree of freedom, [a] Mean value of three replicates.

Mosquito repellent activity of aqueous extract of *C. gigantea* leaves was performed against two adult mosquitoes included *C. gelidus* and *C. tritaeniorhynchus*. Data is reported in terms of percentage protection and represented as mean±SD (n=5). The results exhibit that at high dose (1000 ppm) *C. gigantea* leaf extract provided complete protection from mosquito bite for 240 and 120 minutes for *C. gelidus* and *C. tritaeniorhynchus* respectively. At moderate dose (125, 250 ppm) extract provided complete protection against mosquito bite for 60 to 90 minutes, however at very low concentration (31.25) extract did not showed complete protection (Table 2). The result showed dose dependency and time dependency, as dose increased, percentage protection was increased and as time increases, percentage protection decreased.

Table 2. Mosquito repellent activity of aqueous extract of *Calotropis gigantea* leaves against *C. gelidus* and *C. tritaeniorhynchus*

Mosquito species	Extract dose (ppm)	percentage of repellency				
		Time post application of repellent (min)				
		30 min	60 min	90 min	120 min	240 min
C. gelidus	31.25	84±2.34	78±1.14	71±1.13	54±1.08	12±3.00
	62.5	91±2.11	89±1.09	81±1.89	71±1.72	30±2.16
	125	100±0.0	100±0.0	94±2.14	85±1.64	68±1.74
	250	100±0.0	100±0.0	100±0.0	92±2.34	76±1.82
	500	100±0.0	100±0.0	100±0.0	96±2.74	84±2.10
	1000	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0
C. tritaeniorhynchus	31.25	91±2.34	79±1.14	74±1.16	53±1.18	38±2.41
	62.5	83±2.25	83±1.09	81±1.85	74±1..32	46±1..18
	125	100±0.0	100±0.0	91±1.14	83±1.61	70±1.18
	250	100±0.0	100±0.0	100±0.0	91±2.31	78±1.73
	500	100±0.0	100±0.0	100±0.0	94±2.44	90±1.22
	1000	100±0.0	100±0.0	100±0.0	100±0.0	94±1.43

The values of percentage repellency are represented as mean±standard deviation of five replicates

Ovicidal activity of aqueous extract of *C. gigantea* leaves was performed against the eggs of two mosquitoes included *C. gelidus, C. tritaeniorhynchus*. Aqueous extract of *C. gigantea* leaves exhibited moderate ovicidal activity against both mosquito eggs. Extract treatment resulted in 22±1.35% (78% ovicidal activity) and 12±1.22 (88% ovicidal activity) hatching of the eggs. Results are reported in terms of percentage egg hatching and expressed as mean±SD (n=5), results are summarized in Table 3.

Table 3. Ovicidal activity of aqueous extract of *Calotropis gigantea* leaves against eggs of *C. gelidus* and *C. Tritaeniorhynchus*

Mosquitoes species	Concentrations (ppm)	Percentage of egg hatching
C. gelidus	1000	22±1.35
	500	36±1.22
	250	48±1.12
	125	64±1.25
	62.5	76±1.26
	31.25	96±2.68
C. tritaeniorhynchus	1000	12±1.22
	500	24±1.21
	250	40±1.14
	125	55±1.25
	62.5	65±1.12
	31.25	86±2.11

The values of percentage of egg hatching are represented as mean±standard deviation of five replicates.

Earlier study, Alam *et al.* (2009) reported that the methanol extract *C. gigantea* root bark and its chloroform and petroleum ether fractions were evaluated for residual film toxicity, fumigant toxicity and repellent effect of against several instar of larvae and adult of *Tribolium castaneum*. Methanol extract showed high insecticidal activity against *T. castaneum* followed by petroleum ether fraction and chloroform fraction. None of the sample showed fumigant toxicity.

The crude aqueous extract of the *C. gigantea* leaves was screened for the presence of major phytochemical groups. The Preliminary phytochemical screening revealed the presence of phenoic compounds, flavonoids, alkaloids, tannins, saponins, glycosides and phytosterols, whereas, carbohydrates, proteins, oil and fats were not present in the extract (Table 4). These phytochemical compounds are the key candidates in the medicinal value of this plant. In previous studies, *C. gigantea* was reported to possess cardenolides, flavonoids, terpenes, pregnanes, nonprotein amino acid and cardiac glycoside (Wang *et al.* 2008).

Table 4. Phytochemical composition of *Calotropis gigantea* leaves

Phytochemicals	***Calotropis gigantea***
Phenoic compounds	Present
Flavonoids	Present
Alkaloids	Present
Tannins	Present
Saponins	Present
Carbohydrates	Absent
Proteins	Absent
Oil and fats	Absent
Glycosides	Present
Phytosterols	Present

In conclusion, the present work is a preliminary study for the screening of larvicidal, mosquito repellent and ovicidal activity of aqueous extract of *C. gigantea*. The presented report indicates mosquito controlling potential of *C. gigantea*, In future these plants can be used as an alternative source for the development of new mosquito controlling agents. Further studies can be made to investigate the active principle of the insecticidal compound present in the tested plants.

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