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Phytochemical composition, mosquito larvicidal, ovicidal and repellent activity of *Calotropis procera* against *Culex tritaeniorhynchus* and *Culex gelidus*

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Abstract

Focus of this study was to determine the phytochemical composition and mosquito controlling potential of aqueous extract of *Calotropis procera* (Ait.) R.Br. leaves using *in vitro* methods. Preliminary phytochemical analysis of the extract showed the presence of phenolic compounds, flavonoids, alkaloids, tannins, saponins, glycosides and phytosterols as major phytochemical groups. Aqueous extract of *C. procera* leaves (1,000 ppm) exhibited 100% larvicidal activity against fourth instar larvae of *Culex tritaeniorhynchus* and *Cx. gelidus*. Extract treatment (1,000 ppm) of both mosquitoes' eggs resulted in to 100% ovicidal activity. At 1,000 ppm, extract provided complete protection from mosquito bite for 240 min against both mosquitoes; however at lower doses the protection time was less. The findings of the current study emphasise the potentiality of *C. procera* leaves for controlling the mosquito population and their possible way in the developing the natural insecticide for the control of *Cx. tritaeniorhynchus* and *Cx. Gelidus* mosquitoes.

Introduction

Japanese encephalitis (JE) is a virus born disease of humans and animals, caused by JE virus (Flaviviridae). JE is transmitted by the bite of *Culex* sp. mosquitoes such as *Cx. tritaeniorhynchus* (Prominent vector), *Cx. gelidus*, *Cx. vishnui* and *Cx. fuscocephala* (Self et al., 1973). JE is most predominant in Asia and parts of the western Pacific resulting in to about 30,000-50,000 cases and 10,000-15,000 deaths worldwide annually (CDC, 2010).

Mosquito control is an important strategy to control the outbreaks of vector born diseases. In past, a variety of synthetic insecticides have been used to control mosquito population, however, their toxicity towards environment and increase in the incident of insecticide resistant opened up the opportunity for the natural insecticides (Jaga and Brosius, 1999; Eskenazi et al., 2008; Raghavendra et al., 2010; Kumar et al., 2011). Plants are the rich source of medicinally important bioactive compounds and well recognised as a potential source of insecticidal agents. Plants based insecticides possess several advantages over the synthetic chemical insecticides as they are cost effective, highly potent, less toxic and biodegradable (Isman, 2008; Govindarajan et al., 2011).

Calotropis procera (Asclepiadaceae) is a wild evergreen shrub, commonly known as milk weed. Plant is native to Asia, Africa, and Northeast of Brazil. Parts of *C. procera* is used in traditional medicinal system to cure several diseases such as skin diseases, intestinal worms,

cough, asthma, elephantiasis, toothache, ascites and anasarca. It also used to improve digestion, increase appetite and enlargements of abdominal viscera. Latex of the plant was reported to possess analgesic activity (Dewan et al., 2000), anti-inflammatory activity (Singh et al., 2000), anti-oxidant activity (Roy et al., 2005), anti-diarrhoeal activity (Kumar et al., 2001), antinociceptive activity (Soares et al., 2005) and antimalarial activity (Sharma and Sharma, 2000). Roots are reported for anti-fertility activity (Kamath and Rana, 2002), antitumor activity (Mathur et al., 2009) and anticonvulsant activity (Jalalpure et al., 2009). Seeds and stem are reported to possess antimicrobial activity (Kuta, 2008; Bhaskar and Ajay, 2009). Flowers are reported for antihelmintic (Zafar et al., 2005) and hepatoprotective activity (Ramachandra Setty et al., 2007).

The aim of this work was to investigate the phytochemical composition, mosquito larvicidal, ovicidal and repellent activity of aqueous extract of *C. procera* leaves against *Cx. gelidus* and *Cx. tritaeniorhynchus*.

Materials and Methods

Plant material

Fresh and mature leaves of *C. procera* were collected from the wasteland of Vellore district, TN, India (12°54′ 40″N 79°8′10″E) in the month of December, 2008. Plant material was brought to the Molecular and Microbiology Research Laboratory, VIT University, Vellore. The taxonomic identification of the plants was made by Prof. V Palanichamy, Plant Biotechnology Division, VIT University, Vellore, Tamil Nadu, India. Voucher specimen (CP/VIT/MMRL/15.12.2008-9) is maintained in our laboratory for future references.

Processing of the plant sample

Fresh and mature leaves of the *C. procera* were collected and washed properly under running tap water followed by distilled water. Leaves were dried in hot air oven at a temperature of 40°C. Dried leaves were powdered using a mechanical grinder. Pulverized leave material was extracted with distilled water using a Soxhlet extractor. These extracts were concentrated at 40°C under reduced pressure (72 mbar) with a rotary evaporator and dried using lyophilizer. Dried extract was collected in air tight container and stored at 4°C for further use.

Phytochemical screening

Phytochemical screening of the leaves of *C. procera* was carried out by using the standard protocols for the presence of carbohydrates, proteins, phenolic compounds, saponins, flavonoids, alkaloids, tannins, glycosides, phytosterols, oil and fats (Harborne, 1998; Raaman, 2006).

Insect rearing

Cx. gelidus and *Cx. 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 g 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 World Health Organization (WHO) with some modifications (WHO, 1996; 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 ppm concentration). The control was set up with distilled water and polysorbate 80. The numbers of dead larvae were counted after 24 hours of exposure, and the percentage mortality was reported from the average of five replicates. The extract in which 100% mortality of larvae occurs were selected for the dose–response bioassay.

Dose response bioassay

From the stock solution, different concentrations ranging from 62.5 to 1,000 ppm were prepared. Based on the preliminary screening results, crude extract were subjected to dose- response bioassay for larvicidal activity against the larvae of *Cx. gelidus* and *Cx. tritaeniorhynchus*. The numbers of dead larvae were counted after 24 hours of exposure, and the percentage mortality was reported from the average of five replicates.

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 31.2, 62.5, 125, 250, 500 and 1000 ppm concentration of plant extracts, and one was filled with 100 mL of distilled water. Respective solvent was used as control. A minimum of 100 eggs was used for each treatment, and the experiment was repeated five times. After treatment, the eggs were sieved through muslin cloth, thoroughly rinsed with tap water, and left in plastic cups 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 non-hatchability of eggs with unopened opercula (Chenniappan and Kadarkarai, 2008). The hatching rate of eggs was assessed after 98 hours post treatment (Rajkumar and Jebanesan, 2009).

Mosquito repellent activity

The stock solutions of the extracts were diluted with acetone, polysorbate 80 and distilled water to obtain test solutions of 31.2, 62.5, 125.0, 250.0, 500.0, 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. 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 a bite. The experiments were conducted five times in separate cages and in each replicate different volunteers 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).

$\% \text{ Protection} = [(N_c - N_t)/N_c] \times 100$ Where,

N_c: No. of bites received by control arm; N_t: No. of bites received by treated arm.

Statistical analysis

The values of larvicidal, ovicidal and mosquito repellent activity of the aqueous extract of *C. procera* leaves are expressed as mean ± standard deviation of the response of five replicates determinations per sample. The average parasite and larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, and other statistics at 95% fiducial limits of upper confidence limit and lower confidence limit, and Chi-square values were calculated by using the software developed by Reddy et al. (1992). Results with $p<0.05$ were considered to be statistically significant.

Results

In this study, aqueous extract of the *C. procera* leaves was screened for the presence of major phytochemical groups. The preliminary phytochemical screening revealed the presence of phenolic compounds, flavonoids, alkaloids, tannins, saponins, glycosides, protein and phytosterols, whereas, carbohydrates, oil and fats were not present in the extract. These phytochemical compounds are the key candidates in the medicinal value of this plant.

Aqueous extract of *C. procera* leaves was examined for larvicidal activity, repellent and ovicidal activity against *Cx. tritaeniorhynchus* and *Cx. gelidus* mosquitoes. Extract exhibited high larvicidal activity against fourth instar larvae of *Cx. tritaeniorhynchus* and *Cx. gelidus* after 24 hours exposure. At a dose of 1000 ppm, extract resulted in 100% larvicidal activity against both the mosquito larvae. In dose dependent larvicidal bioassay the extract exhibited increase in larvicidal activity with increase in dose (Table I). The extract exhibited larvicidal activity with an LC_{50}= 125.7 ± 10.4 ppm (UCL –LCL: 146.0-105.4) and LC_{90}= 926.5 ± 142.2 ppm (UCL – LCL: 1205.2-647.7) against *Cx. tritaeniorhynchus*, whereas, LC_{50}= 125.4 ± 8.7 ppm (UCL –LCL: 142.5-108.3) and LC_{90}= 575.1 ± 67.1 ppm (UCL –LCL: 706.7-443.5) against *Cx. gelidus*. Data was found significant by Chi-square test at $p<0.05$ level.

Ovicidal activity of aqueous extract of *C. procera* leaves was performed against the eggs of *Cx. tritaeniorhynchus* and *Cx. gelidus*. *C. procera* resulted in 100% ovicidal activity (no hatching) against both mosquito eggs at the concentration of 1000 ppm, at low concentrations extract did not show 100% ovicidal activity (Table I). The extract showed dose dependent ovicidal activity against both the mosquito eggs.

Mosquito repellent activity of aqueous extract of *C. procera* leaves was performed against two adult mosquitoes included *Cx. tritaeniorhynchus* and *Cx. gelidus*. At the higher dose (1,000 ppm) *C. procera* leaf extract

Table I

Dose dependent larvicidal and ovicidal activity of aqueous extract of *Calotropis procera* leaves *Cx. Tritaeniorhynchus* and *Cx. gelidus*

Mosquitoes species	Concentrations (ppm)	Percent larvicidal activitya	Percentage of egg hatchinga
Cx. tritaeniorhynchus	1,000	100 ± 0.0	NH
	500	77 ± 0.1	29 ± 1.1
	250	57 ± 0.8	35 ± 2.1
	125	33 ± 0.1	55 ± 1.0
	62.5	15 ± 0.1	71 ± 1.1
Cx. gelidus	1,000	100 ± 0.0	NH
	500	89 ± 0.1	26 ± 1.1
	250	70 ± 0.3	35 ± 2.1
	125	56 ± 0.6	48 ± 1.0
	62.5	44 ± 1.2	68 ± 1.1

aMean ± SD value of five replicates; NH- No hatchability (100% mortality)

Table II

Dose dependent mosquito repellent activity of aqueous extract of *Calotropis procera* leaves against *Cx. tritaeniorhynchus* and *Cx. gelidus*

Mosquito species	Extract dose (ppm)	Percentage of repellency				
		Time post application of repellent (min)				
		30 min	60 min	90 min	120 min	240 min
Cx. tritaeniorhynchus	31.2	98 ± 1.1	82 ± 1.4	71 ± 1.6	53 ± 1.6	26 ± 2.4
	62.5	100 ± 0.0	72 ± 3.1	70 ± 3.1	60 ± 1.9	38 ± 1.9
	125	100 ± 0.0	100 ± 0.0	100 ± 0.0	82 ± 1.4	62 ± 1.0
	250	100 ± 0.0	100 ± 0.0	100 ± 0.0	92 ± 2.7	71 ± 2.1
	500	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	80 ± 2.0
	1000	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Cx. gelidus	31.2	98 ± 1.1	94 ± 2.3	82 ± 1.1	55 ± 1.4	22 ± 2.4
	62.5	100 ± 0.0	100 ± 0.0	79 ± 3.1	63 ± 1.9	36 ± 1.1
	125	100 ± 0.0	100 ± 0.0	100 ± 0.0	87 ± 1.4	65 ± 1.0
	250	100 ± 0.0	100 ± 0.0	100 ± 0.0	95 ± 2.3	72 ± 2.1
	500	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	84 ± 2.3
	1000	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

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

provides complete protection from mosquito bite up to 240 min against both mosquitoes. At lower dose (62.5 ppm) protection time was quite low and varies from 30 to 60 min for *Cx. tritaeniorhynchus* and *Cx. gelidus* respectively. However, at very low concentration (31.2 ppm) extract didn't showed complete protection against both mosquitoes (Table II). The result showed dose dependency and time dependency, as dose increased, percentage protection was increased and as time increases, percentage protection decreased.

Discussion

Insecticide resistance in mosquitoes is a biggest hurdle in the strategy of mosquito control to limit the vector born diseases; therefore, there is an emerging need for developing better and safer insecticide. Synthetic insecticides provide rapid control on vector population, however they possess several safety issues and many of them have been banned (Sharma, 2001). Plants synthesize powerful secondary metabolites which protect them from the environmental stress factors, microbial infections and predator insects, some of these metabolites possess mosquito controlling properties and could be used as alternative source of mosquito controlling agents. Phytochemical based insecticides exhibits a variety of toxic effect in various phases of mosquito life cycle such as adulticidal, repellent, ovipositional, ovicidal, larvicidal, pupicidal, growth and reproduction

inhibition (Das and Chandra, 2012; Govindarajan et al., 2008; Govindarajan et al., 2011; Chenniappan and Kadarkarai, 2008). These metabolites significantly attribute to the other bioactive properties as well and determine the medicinal potential of the respective plants. In this study, aqueous extract of *C. procera* is reported to possess several classes of phytochemicals. These results are in agreement with the previous studies where *C. procera* leaves extracts have been reported to possess glycosides, tannins, phenolics, terpenoids, cardenolides, flavonoids and saponins as major phytochemicals (Murti et al., 2010; Moustafa et al., 2010).

We focused on the biological control of *Cx. Tritaeniorhynchus* and *Cx. gelidus,* (major vector for JE) using aqueous extract of *C. procera*. The results of this study exhibits that the low concentrations of the *C. procera* leaves extract can effectively control the population of *Cx. tritaeniorhynchus* and *Cx. gelidus* by destroying the developmental stages (egg and larva stage) of mosquito life-cycle. Results of this study emphasize that aqueous extract of *C. procera* leaves possesses very high larvicidal and ovicidal properties. Our observations are strongly supported by other studies conducted in different parts of the world; these studies have demonstrated the larvicidal and ovicidal potential of *C. procera* against several mosquitoes of *Anopheles* sp., *Aedes* sp. and *Culex* sp. The cardenolide extract isolated from the *C. procera* showed the ovipositinal and larvicidal activity against *Cx. pipiens* (Al-Rajhy et al., 2000). Fresh leaf extract of *C. procera* was reported to show larvicidal properties against mosquito larvae of *Anopheles stephensi*, *Cx. quinquefasciatus* and *Aedes aegypti*. Methanol extract of the same plant were, however, more effective as larvicide (Singh et al., 2005). Latex of *C. procera* was reported for its toxic effects against egg hatching and larval development of *A. aegypti*. The crude latex showed 100% mortality of 3rd instars within 5 min. Fractions of latex prevent egg hatching and most of individuals growing under experimental conditions died before reaching second instars or stayed in first instars (Ramos et al., 2006). Different solvent extracts of *C. procera* were reported to exhibit moderate larvicidal activity against second and fourth instars larvae of *Cx. quinquefasciatus* (Rahuman et al., 2009). Aqueous extract of *C. procera* leaves exhibited larvicidal (2, 3, 4 instar larva), adult emergence inhibition and oviposition deterrent activity *A. arabiensis* and *Cx. quinquefasciatus* (Elimam et al., 2009). Fresh latex and methanolic extracts of *C. procera* leaves was repoted to possess larvicidal activity against *Cx. quinquefasciatus* and *A. stephensi* (Shahi et al., 2010).

Mosquito repellents are the compounds that applied on skin, clothing or surface to prevent the mosquito to reach the target. N-diethyl-3- methylbenzamide (DEET) is a synthetic repellent that is been widely used in the repellent products, provides 2, 4 and 5 hours protection from mosquito bite at 6.7, 20 and 23.8% concentrations, respectively (Fradin and Day, 2002). In this study, protection time of the extract was very competitive to DEET; extract (1,000 ppm) protect the host from the mosquito bites for quite a time (4 hours or more), thus indicates the control of virus transmission caused by the bite of infected mosquito (Table II). However, at lower doses percentage of protection and duration of protection was lesser than that of at higher dose. Although there are no reports available on repellent activity of *C. procera* against *Cx. tritaeniorhynchus* and *Cx. gelidus* mosquitoes, there are few studies reported the repellent activity of other plant extracts and essential oils, examples includes, *Corymbia citriodora*, *Eucalyptus camaldulensis*, *Lantana camara*, *Azadirachta indica*, *Citrus hystrix* and *Curcuma longa* (Maia and Moore, 2011).

These observations establish aqueous extract of *C. procera* leaves as a potent source of mosquito controlling agent and its possible use for mosquito control. However, mosquito controlling potential of *C. procera* may vary according to the parts of the plant used, solvent choice, season of plant collection, geographical location where the plants were grown, resistant level of mosquito and the application method.

Conclusion

The present work infers mosquito controlling potential of *C. procera* and its possible application to develop effective and safer formulations in future.

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