International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2017; 4(4): 05-11

Received: 02-05-2017
Accepted: 03-06-2017

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Evaluation of ovicidal and developmental effects of *Callistemon citrinus* (Myrtaceae) methanolic leaf extract against human dengue vector *Aedes aegypti* (Diptera: Culicidae)

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Abstract
Mosquitoes transmit serious human diseases, causing millions of death every year. Vector control is facing a threat due to the emergence of resistance to synthetic insecticides. Insecticides of plant origin may serve as suitable alternative to synthetic insecticides in the future. Therefore, the present investigation was undertaken to evaluate ovicidal and developmental effects of methanolic leaf extract of *Callistemon citrinus* against human dengue vector mosquito *Aedes aegypti*. Effect of methanolic leaf extract of *C. citrinus* on the hatchability of *Ae. aegypti* eggs were determined, hatching rate was calculated on the basis of non-hatchability of eggs. The hatchability of *Ae. aegypti* eggs was decreased when placed in media of methanolic leaf extract. The reduction in percent hatch was inversely proportional to the concentration of methanolic leaf extract used. Percent hatch of eggs placed in control medium was 88 % where as in 0.1, 0.10, 0.20 and 0.30% concentrations it was 72, 63, 45, and 20. 0.40% dose completely arrested hatching eggs. Extension in larval development time was in correlation to concentrations of methanolic leaf extract used. The study revealed that interference of bio – active compounds with the normal hormonal activity of the larvae. It can be concluded that plant leaf extract have a great potential to control *Ae. aegypti* and could be used as a part of vector management program.

Keywords: *C. citrinus*, leaf extract, *Aedes aegypti*, ovicidal, lengthening larval duration

1. Introduction
Mosquitoes are the most important single group of insects in terms of public health importance, which are responsible for spreading a number of serious diseases, such as malaria, flariasis, dengue, Japanese encephalitis, etc. and generate huge economic loss; both in terms of healthcare cost and less productivity [1]. *Ae. aegypti* is a medium- sized blackish mosquito easily recognized by a silvery- white Iyre- shaped pattern of scales on its scutum. The colouration of both males and females is similar. *Ae. aegypti* preferentially breeds and develops in artificial or domestic containers such as cisterns, flasks, bottles, earthen pots, flower vases, tin cans, jars, over-head tanks, discarded automobiles tyres, unused water closets, rain barrels, sagging roof gutters and in natural sites such as coconut shells, snail shells, leaf axils and treeholes [2]. *Ae. aegypti* is a vector of dengue viral infection, dengue hemorrhagic fever, dengue shock syndrome, chikungunya and zika virus (ZIKV).At present, no effective vaccine is available for these diseases; therefore, mosquito control is the only way of reducing the incidence of such diseases [3].
Targeting mosquito larvae and egg is more desirable than controlling adults because they are concerntrated in a relatively small area [4]. The frequent use of insecticidal sprays and other chemical compounds to kill mosquitoes has lead to a destabilization of the ecosystem and has affected the natural environment. Insecticides resistance is also increasingly becoming a challenge in many vector control activities [5]. To overcome from these problems there is an insistent need for search and development of new insecticides. The plant products or plant-derived compounds are promising alternatives to synthetic insecticides in controlling insect pests of medical importance as these are environmentally safe, biodegradable, of low cost and may be produced using indigenous methods, for vector control [6-10] and can be used with minimum care by individual and communities [11].

Previous workers used plants belonging to the families Asteraceae, Labiatae, Myrtaceae, Meliaceae, Rutaceae, Piperaceae, etc. for extracting potential toxic compounds from them and applied against household insects like mosquitoes [12].

The phytochemicals derived from plant sources possess a complex of chemicals with unique biological activity. The phytochemicals derived from plant resources can act as larvicidal, ovicidal, oviposition deterrence, growth and reproduction inhibitors, repellents, growth regulation, fecundity suppression, male sterility and smoke toxicity [13, 14]. Some of the plant leaves extracts are tested for their diverse insecticidal properties on the medically important mosquitoes [15-35].

As far as our literature survey is concerned that there was no information available on ovicidal, developmental effects of the methanolic leaf extract of the *C. citrinus*. The present study was therefore carried out to evaluate the ovicidal, developmental effects of *C. citrinus* methanolic leaf extract against the vector mosquito, *Ae. aegypti*.

Callistemon citrinus (Linn.) (Myrtaceae) is a slow-growing ornamental shurb that grows to a height of around 10 meters and comprises over 30 species that has medicinal importance [36]. *C.citrinus* is a native to Australia, but is also widely distributed in Asian countries. It is commonly known as crimson bottle brush, red bottle brush or lemon bottle brush and in Tamil it is called as palasu tree because of its spiky inflorescence that resembles a bottle brush. The inflorescence is crimson in colour and cylindrical and flowers are borne in spring and summer. Leaves are lanceolate shaped broadly up to 7.5 cm long with prominent veins. The plant has been used by tribal communities of India for the treatment of gastrointestinal disorders, pain, infectious diseases and a local community of lower Himalaya region used it for genitourinary, kidney infection, bleedingulcer, arthritis, gout, cough, bronchitis, tuberculosis and insecticide effects [37]. Over the years, *C. citrinus* have been extensively analysed scientifically and reported to possess anti-cholinesterase activity [38], hepatoprotective activity [39], cardioprotective activity [40], anti-inflammatory activity [41], antidiabetic activity, hypolipidemic activity, antioxidant activity, nematicidal, larvicidal, and pupicidal activites [42]. *C. citrinus* also used as weed control [43] and as bioindicators for environmental management [44]. The leaves were used to cure respiratory tract infections. The leaf of the plant is used as a tea substitute and it has a refreshing flavor too. The oil from the leaves of *C. citrinus* has been proved to have anti-nociceptive and anti-inflammatory effects in experimental animals [45]. Several flavonoids, saponins, quinones, steroids, terpenoids, tannins, phenolic compounds have been isolated from its leaves [46].

The aim of the present study is therefore to find out:

- Ovicidal activity of the methanolic leaf extract of *C. citrinus* on *Ae. aegypti* eggs,
- Evaluation of methanolic leaf extract of *C. citrinus* on the larval duration of *Ae. aegypti*.

2. Materials and Methods

2.1 Colonization of *Ae. aegypti*

The eggs of *Ae. aegypti* were collected from National Institute for Communicable Disease (NICD), Mettupalayam, Coimbatore (Dt), Tamil Nadu, India without exposure to any insecticide. The eggs were then brought to the laboratory and transferred to enamel trays containing water and kept for larval hatching. They were hatched, reared and have been still maintained for many generations in the laboratory. The eggs and larvae obtained from this stock were used for different experiment. The larvae were reared in plastic cups. They were daily provided with commercial fish food *ad libitum* [47]. The human arm used for feeding female mosquitoes [48, 49]. Both females and males were provided with 10% glucose solution on cotton wicks [50]. The cotton was always kept moist with the solution and changed every day. An egg trap (cup) lined with filter paper containing pure water was always placed at a corner of the cage. This arrangement made the collection of eggs easier.

2.2 Collection of plant materials

C. citrinus leaves were collected from Gandhipuram, Coimbatore District, Tamil Nadu, India. The identification of the plant was authentified at BSI (Botanical Survey of India), Coimbatore.

2.3 Preparation of plant extract

The collected leaves brought to the laboratory. The plant leaves were observed carefully for any kind of diseases or infection and if found any, those parts were separated and not used for the experiment. The selected leaves washed with distilled water in order to clean dust or any particle stuck to them. Then the leaves kept for drying under shade at room temperature (27± 2 ºC) for about 2 weeks till they dried completely. The leaves were finely powdered using electric blender. Powdered plant material (100g) was soaked in methanol (1000 ml) in airtight wide mouth bottle and kept for 4 days with periodic shaking. After that, the extract was filtered using Whatman No.1filter paper and kept in Petri dishes for drying at room temperature [51]. Dried extract was used for the preparation of stock solution. 1 g of the concentrated extract of *C. citrinus* leaves was dissolved in 100ml water and kept as stock solution. This stock solution was used to prepare the desired concentrations (form 0.1 to 0.40%) of the extract for exposure of the mosquito larvae.

2.4 Ovicidal assay

Effect of methanolic leaf extract of *C. citrinus* on the hatchability of *Ae. aegypti* eggs were determined and hatching rate was calculated on the basis of non-hatchability of eggs [52]. Five replications were conducted at each concentration (0.1, 0.10, 0.20, 0.30 and 0.40%) of test compound. The data were statistically examined using Student's *t*-test.

2.5 Laboratory assay for larval duration

To determine the effect of methanolic leaf extract of *C. citrinus* on the length (duration) of the larval stage (larva-pupation) of different concentrations (0.10, 0.15, 0.20, 0.25 and 0.30%) test solutions were prepared. 50 first instar larvae were placed in the extract allowed to develop further. In parallel, the duration of larval stage was calculated for the larvae reared in control for comparison [53]. The data were statistically examined using Student's *t*-test.

3. Results

3.1 Effect of methanolic leaf extract of *C. citrinus* on hatching of *Ae. aegypti* eggs

Freshly laid eggs obtained from the general stock of

mosquitoes were tested for their hatching ability in relation to the different concentrations of methanolic leaf extract of *C. citrinus*. Percent hatch of eggs placed in control medium was 88 % where as in 0.1, 0.10, 0.20 and 0.30% concentrations it was 72, 63, 45, and 20. 0.40% dose completely arrested hatching eggs (Fig. 1). The decrease in hatchability was found to be dose dependent.

3.2 Effect of methanolic leaf extract of *C. citrinus* on total larval duration of *Ae. aegypti*

The methanolic leaf extract of *C. citrinus* at 0.10, 0.15, 0.20, 0.25 and 0.30% tested against the *Ae. aegypti* was found to prolong larval and pupal period. In the control it took 8 days for all the larvae to become pupae, whereas the methanolic leaf extract at 0.10, 0.15, 0.20, and 0.25% took 10, 12, 15 and 19 days respectively. In 0.30% the larvae required 27 days to become pupae (Fig. 2).

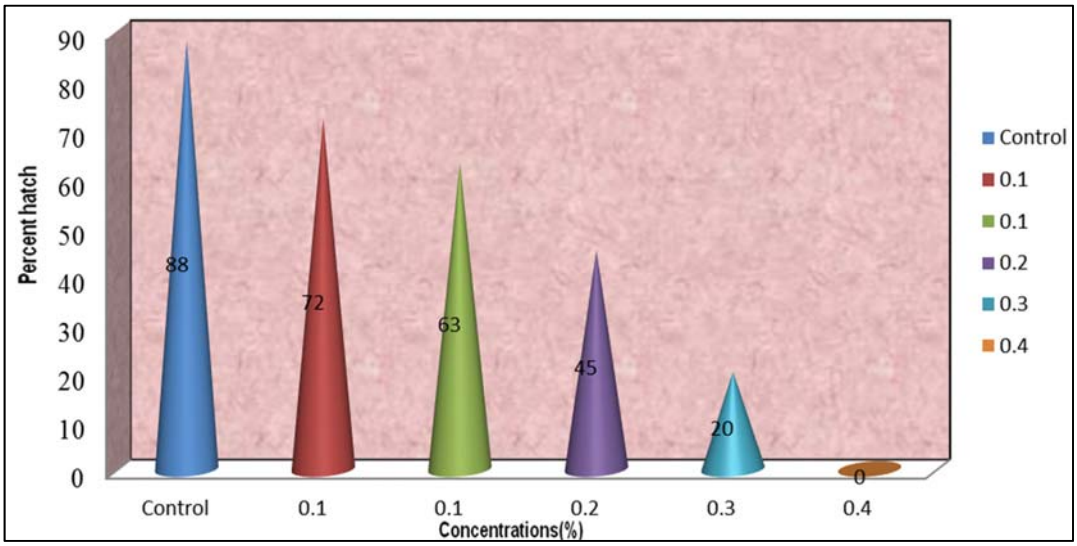

Fig 1: Changes in the hatchability (percent hatch) of *Aedes aegypti* eggs exposed to different concentrations of methanolic leaf extract of *C. citrinus* and control.

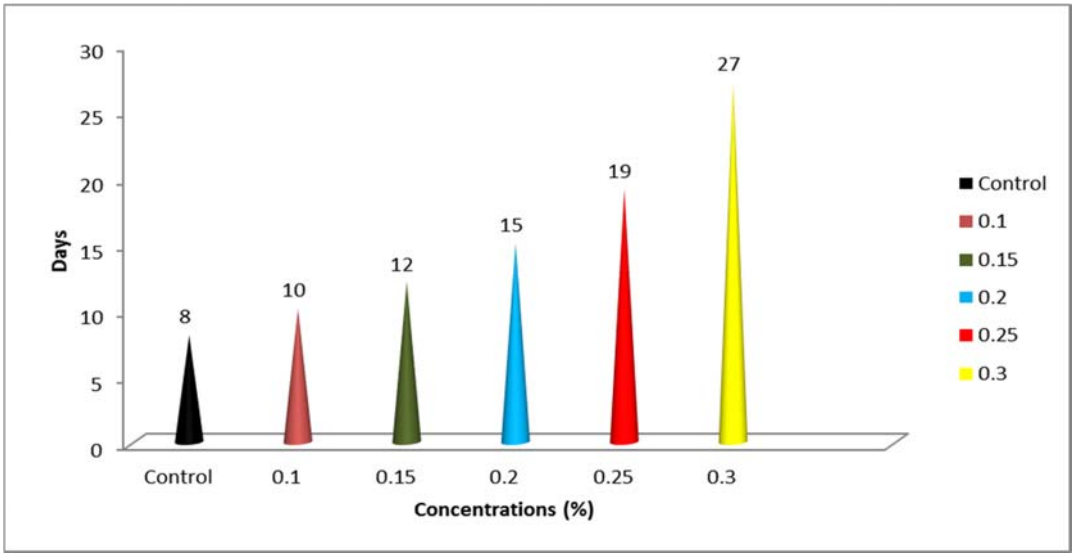

Fig 2: Changes in development time (days) of *Aedes aegypti* larvae reared in different concentrations of the methanolic leaf extract of *C. citrinus* and control.

4. Discussion

Mosquito risk has become more acute in recent time and the death of millions of people every year due to mosquito-borne diseases has resulted in the loss of socioeconomic wealth in many countries. The control of mosquito by chemical substance is not safe at present because of insecticide resistance by vectors and environmental imbalance. Application of chemical or synthetic insecticides leads to deleterious effects in the long term; hence it does not provide absolute results. That is why alternative mosquito control method is needed [54]. The secondary compounds of plants are vast repository of compounds with a wide range of biological activities. The extract which is obtained from plant parts like leaves, root, flower, bark, seed and fruits in their crude extracts has been used as conventional larvicide [55].

The crude methanol and benzene leaf extracts of

Cardiospermum halicacabum exerted 100% reduction of egg hatching at 300 ppm against *Cx. quinquefasciatus* and in *Ae. aegypti* 100% reduction of egg hatching at 400 ppm [56]; aqueous leaf extract of *Calotropis procera* treatment at 1000 ppm *Cx. tritaeniorhynchus* and *Cx. gelidus* eggs resulted in to 100% ovicidal activity [57]; hundred percent ovicidal activities were observed at 350 ppm and 450 ppm of methanol, benzene, acetone extracts of *Pemphis acidula* leaves against *Ae. aegypti* [58]; aqueous extract of *Leucas aspera* leaves was found to be ovicidal against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* with hatchability values of 39.4 and 21.2; 42.4 and 27.8; 50.6 and 30.2 percent at 500 and 1000 ppm respectively [59]; ovicidal activity with ethyl acetate, aqueous solution, ethanol leaf extracts of *Nerium oleander* against *An. stephensi* at 100, 150, 200, 250 and 300 ppm were calculated. With each extract at a concentration of 100 ppm, the percentage of hatchability was very high and nil hatchability was recorded when the concentration of extract was increased to 300 ppm in the case of aqueous and ethanolic extracts [60]; at 300 ppm of ethanolic leaf extract of *Celosia argentea*, *Anthocephalus cadamba, Gnetum ula, Solena amplexicaulis* and *Srermacoce hispida* showed 100% ovicidal activity against *An. stephensi, Ae. aegypti* and *Cx. Tritaeniorhynchus* [61]; percent hatch of eggs placed in control medium was 80% where as in 0.1%, 0.2%,0.4% and 0.6% concentrations of aqueous leaf extract of *Spathodea campanulata* against *Ae. aegypti* was 65, 46, 40 and 20%. 0.8% dose completely arrested hatching eggs [16]; the methanol extract of sea weed leaf exerted 100% egg mortality (zero hatchability) at 240, 300 and 360 ppm for *Ae. aegypti*, *An. stephensi* and *Cx. Quinquefasciatus* [62]; maximum percentage of *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* eggs was killed when they were exposed to 500 ppm concentration of *Clausena excavate* leaves of the methanolic extract [63]; the diethyl extract of *Coleus aromaticus* leaves exerted 100% ovicidal activity against *Ae. aegypti* at 200 ppm [64]; the higher concentration of *Polygala arvensis* leaf methanolic extract possesses strong ovicidal activity at 200 ppm against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus*, no egg hatchability was recorded [65]; the methanolic extract of *Ageratum conyzoides* leaves exerted 100% egg mortality at 250 ppm against *An. Stephensi* [66]; the acetone leaf extract of *Argemone mexicana* was found to exhibit the highest ovicidal potential against *Ae. aegypti* with a significantly reduced egg hatch of 27.32% when exposed to 400 ppm extract as compared to control treatment; this extract causing the lowest percent hatch of 18.35% at 1000 ppm [67]; *Breynia vitis-idaea* ethyl acetate leaf extract possesses strong ovicidal activity at 250 ppm concentration against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus,* no egg hatchability was recorded [68]; at the highest concentration (2000ppm), the methanolic fraction of *Boswellia dalzielii* leaves reduced percent of *An. gambiae* eggs that hatched from 100% in the control to 9.67% [69].

In the case of ovicidal activity, exposure of freshly laid eggs was more effective than that of the older eggs [70].

The methanolic leaf extract of *C. citrinus* treated eggs exhibited an allayed hatchability and this may be due to the action of phytochemicals present in the extract. The extract may inhibit the hatchability of the eggs by interfering with their chorion [71]. Eggs and egg shells treated with plant extracts become damaged probably due to endosmosis. After the initial phase of swelling, eggs become desiccated, followed by shrinkage and death of larvae trapped within [72]. The treated eggs contained developed embryos the eclosion of the egg was incomplete. Any compound that can cause permeability or a disruption to the chorionic layers in order to effectively deliver compounds that can terminate embryogenesis can be considered for development of effective ovicides [70]. The eggs were directly exposed to high concentrations of the compounds, more chemicals entered the egg shell, which affected the embryogenesis; similarly, longer exposure periods also facilitated the increased penetration of the compounds into the shells, thus increasing their effectiveness [73].

The findings of the present investigation were comparable with other ovicidal studies and revealed that the methanolic *C. citrinus* leaf extract possesses strong ovicidal activity against the eggs of *Ae. aegypti.*

The results of present study are comparable with earlier reports. Prevention of pupation upto day 7 at dosage of 1000 ppm in the immature of *Ae. aegypti* in ethanolic extract of *Cassia holosericea*; [74] *Leucas aspera* leaf (500, 1000 ppm) showed prolonged larval and pupal periods among *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi*. It took 8 days for all the extracts to become pupae; whereas in the aqueous extract it took 11 days for *Ae. aegypti* and 9 days in the case of *An. stephensi* and *Cx. quinquefasciatus* [75]; exposure of *An. stephensi* larvae to sub–lethal doses of neem leaves in the laboratory prolonged larval development [76]. In the present study, lengthening of larval and pupal periods indicates the interference of the bio–active compounds of methanolic leaf extract of *C. citrinus* with the normal hormonal activity coordination of the metabolic process of the developing stages. Prolongation of development period of mosquito larvae treated with plant extract were generally attributed to interference of the active ingredients of the extract with the endocrine system [77].

5. Conclusion

The present investigation revealed that methanolic leaf extract of *C. citrinus* possesses remarkable ovicidal and developmental effects against dengue vector mosquitoes. These results could encourage the search for new active natural compounds offering an alternative to synthetic insecticides from other medicinal plants. These extract are inexpensive, easy to handle and safer products for the control of mosquito in immature stage.

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