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Larvicidal and knockdown activity of *Citrus limetta* Risso oil against dengue virus vector

S Prakash Rao[1], Parag Jain[2*], Premlata Rathore[1] and Vijay Kumar Singh[1]

[1]Department of Phytochemistry and Pharmacognosy, Columbia Institute of Pharmacy, Raipur-493111, India
[2]Department of Pharmacology, SLT Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Koni, Bilaspur-495009, India

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The aim of the present study was to evaluate larvicidal and knockdown effects of *Citrus limetta* Risso (CL) against dengue virus vector. The study was carried out to evaluate the efficacy of CL fixed oil against larvae and mosquitoes of *Aedes* species. CL fixed oil was obtained from the fresh CL seeds using solvent n-hexane. Three different concentrations of CL oil i.e. 7.5, 10, and 15 % were employed to explore the bioefficacy of fixed oil. *O*-tetramethyl *O*,-thiodi-p-phenylene bis-phosphorothioate (Temephos) and *N, N*-Diethyl-meta-toluamide (DEET) were employed as standard for larvicidal and knockdown activities, respectively. CL oil was found to be effective against mosquitoes and larvae. The 15 % CL oil exhibited highest percentage of mortality equal to 82.20 % of the mosquito larvae and 70.00 % knockdown. Prevention is better than cure, so, the mosquito knockdown and larvicidal action of CL oil may be an effective step to prevent dengue virus transmission. Commercial utility by including CL oil in herbal formulations to prevent dengue virus infection needs to be explored.

Keywords: *Citrus limetta* Risso, Dengue, Larvicidal effect, Mosquito knockdown, Peet grady chamber.

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Introduction

Dengue fever is an infectious tropical disease caused by dengue virus. *Aedes* species play a major role in transmission of dengue fever[1]. It is transmitted by several species of mosquito within the genus *Aedes*, principally *Aedes aegypti*. Mosquitoes are important arthropods transmitting diseases like dengue, malaria, filariasis, and Japanese encephalitis[2] and have the potential to feed on more than one individual during a single gonotropic cycle[3]. Proper control of mosquitoes lies in personal protection, whereas public awareness is the most economical method in eradicating breeding sites and controlling these through environment friendly larvicides[4]. Dengue fever is also known as 'breakbone fever' based on its symptoms such as body ache, headache, muscle pain, and skin rashes. Repellency is known to play an important role in preventing the vector borne diseases by reducing man-vector contact. Synthetic chemicals and insecticides used for control of vectors are causing irreversible damage to the eco-system, as some of them are non-degradable in nature[5]. Temephos, an organophosphate insecticide, is recommended as a larvicide by World Health Organization to control mosquitoes, midge, blackfly, and other insects[6]. Studies have shown temephos to be effective in controlling *Ae. aegypti* in several parts of India[7-8]. Majority of the commercial repellents are prepared by using chemicals like allethrin, N-N-diethyl-meta-toluamide (DEET), dimethyl phthalate and N, N-diethyl mendelic acid amide[9-10]. *Ae. albopictus*, which is an important disease vector[11], including dengue fever[12], has spread with the growth of towns and poor sanitation[13].

Citrus limetta Risso (CL), commonly known as sweet lime is a flowering plant of family Rutaceae. It grows in tropical and subtropical climates and is believed to have originated in the part of Southeast Asia bordered by Northeast India, Burma (Myanmar) and the Yunnan province of China. Citrus fruit has been cultivated in an ever-widening area since ancient times; the best-known examples are the oranges, lemons, grapefruit, and limes[14]. A total of 46 components were found in the essence of lime, among

*Correspondent author
Email- paragjain1510@gmail.com
Ph- 07755-260027

which the highest concentration of compounds present were aldehydes such as limonene. Linalool, sabinene, and bergamol were more abundant[15]. CL fruits and leaves are used for common cold, decreasing cholesterol level, fever regulation, regulating inflammation, digestive disorders as well as modulating blood pressure and brain trouble. The rind is anthelmintic; seeds are used as astringent to the bowels, cooling, strengthens the gums and the teeth, arrest vomiting and retching[16].

Materials and Methods

Collection, authentication, extraction, and isolation of oil

The seeds of CL were collected from fresh citrus fruits, which were authenticated by Dr. S Prakash Rao, Department of Phytochemistry and Pharmacognosy, Columbia Institute of Pharmacy, Raipur and a voucher specimen (No. 0135) was deposited in the herbarium of the institute. After being exposed to direct sunlight for a few days, the seed coats were removed and the seed kernels were ground. Dried seeds (1 kg) were fed into a Soxhlet extractor for 6 h, fitted using h-hexane as solvent. The solvent was evaporated with a rotary evaporator (Buchi type, Sigma-Aldrich, USA) under reduced pressure and controlled temperature (37-40 °C). The residue from the evaporator was poured into an evaporator dish and then placed in a hotwater bath at 50 °C until the remaining solvent was completely removed. The final product obtained from this process was the 'fixed oil'. The oil was centrifuged for 12 min at 3000 rpm, then left with anhydrous sodium sulfate for 5 min, and finally stored in a polyethylene terephthalate bottle under refrigeration at 4 °C until analyses[17]. Yield of the fixed oil obtained during the study was 26.8 %. The fixed oil was further used to evaluate mosquito knockdown and larvicidal activity.

Phytochemical investigation of CL oil

Seed oil may contain some significant photochemical that can be detected using best solvent and extraction methods. CL oil was subjected to screening for various phytochemicals employing standard protocol[18].

Test for fixed oils

Few drops of 0.5 N alcoholic potassium hydroxide was added to a small quantity of CL oil with a drop of phenolphthalein separately and heated on a water bath for 1-2 h. The formation of soap or partial neutralization of alkali indicated the presence of fixed oils.

Test for steroids

Five drops of concentrated H_2S0_4 was added to 1 mL of CL oil. A red coloration indicated the presence of steroids.

Test for saponins

To 2 mL of CL oil, 5 mL of distilled water was added and the solution was shaken vigorously for 30 seconds. Stable persistent frothing indicated the presence of saponins.

Test for proteins and amino acids

Million's reagent (2 mL) was added to 1 mL of CL oil. A white precipitate indicated the presence of proteins.

Collection of mosquito larvae

Mosquito larvae were collected from the stagnant water present in the abandoned cooler bodies and then placed in a small room enclosed with a mosquito net.

Insect culture

The mosquitoes and larvae were reared under controlled temperature condition of 28–30 °C and relative humidity of 60–80 % in the vector control laboratory. They were fed on dog biscuits and yeast powder in the 3:1 ratio. Adults were provided with 10 % sucrose solution and one week old chick for blood meal with a photo period of 14 h light, 10 h dark.

Larvicidal bioassay

The efficacy of the CL oil for larvacidal activity was evaluated in accordance with the guideline of WHO[19]. Batches of larvae were placed in a small transparent glass container with 50 mL de-chlorinated water and placed in the netted area in the college laboratory room at 30-32 °C. For the control group, mosquito larvae were exposed to CL oil. Temephos, 1 % (sand granules) was employed for larvicidal activity[20]. Test group received CL oil in different concentration i.e. 7.5, 10, and 15 %. All the treatment groups were monitored by carefully counting the number of dead larvae after 12 and 24 h. The percentage of larval mortality was calculated using following formula:

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Preparation of stick

Stick was prepared using husk, lemon grass, jiggit, gum acacia, and *Guggul* as per the standard procedure and mixed uniformly with the CL oil in different

concentration (15, 10, and 7.5 %). These impregnated sticks were used for bioefficacy against mosquitoes in Peet-Grady chamber[21]. Initially, CL oil was evaluated at 15 % concentration for its bioefficacy. Then, the lower concentrations of CL oil were further selected and evaluated. Finally, CL oil mixed with above ingredients required for stick preparation was evaluated. The stick was dried in the oven at 70 °C for 6 h, and then kept in the room for half an hour for drying. CL oil with suitable diluents were mixed (w/w) on the stick by using a spray pump. Finally these sticks were packed in a suitable container and stored for 2–3 days.

Knockdown effect

Test arena was designed as Peet-Grady chamber of 5.8 m^3, made up of glass from 6 sides with controlled temperature (28–30 °C) and humidity (60-80 %) conditions as per the protocol requirement. Mosquitoes (2-3 days old) were collected from the rearing cage and released in the handling bottles. Before testing each product, the mosquitoes from the handling bottles were released into the Peet-Grady chamber and were kept in it for about 30 min. If the mortality exceeded 5 % in the chamber, then the chamber was cleaned and the above procedure was repeated. If mortality was below 5 %, the test was continued by replenishing only the dead insects. The stick was lit outside the chamber and then pushed inside up to the center. The stick was allowed to smolder continuously for 120 min (observation period). The knockdown count was taken after every 5 min till 120 min, using a hand-counter. The knockdown mosquitoes were collected and kept for 24 h for mortality observation in a jar with sucrose solution in it[22-24]. The percentage of mosquito knockdown was calculated using the following formula:

$$\text{Percentage knockdown} = \frac{\text{Number of mosquitoes knockdown}}{\text{Number of mosquitoes introduced}} \times 100$$

Statistical analysis

The results were analyzed statistically using two way analysis of variance (ANOVA) followed by Bonferroni post-tests to calculate the level of significance. All values were expressed as Mean ± SEM (Number of mosquitoes, n=30).

Results

Phytochemical screening

Phytochemical screening of CL seed oil revealed the presence of fixed oils, saponins, steroids, and proteins.

Larvicidal activity of CL oil

The larvicidal activity of the CL oil against the larvae was determined through mosquito larval bioassay. The mortality percentage was highest (82.20 %) for 15 % concentration of CL oil as seen from Table 1. However, the lower concentrations of CL oil were also effective for causing larval death with mortality percentage of 45.53 and 67.76 % for 7.5 and 10 % concentrations of CL oil, respectively. Temephos (1 %) was consumed as standard larvicidal agent whose percentage mortality was found to be 97.76 %.

Knockdown activity of CL oil

This activity was performed in a Peet-Grady chamber to observe the efficacy of CL oil against *Aedes* mosquitoes. The knockdown percentage of mosquitoes was noted in 15, 10, and 7.5 % concentrations of the CL oil after 24 h of exposure. Figure 1 show the knockdown effect of seed oil, which was observed to be concentration depended, as knockdown of mosquitoes increased with increasing concentration of CL oil. Highest effect (70 %) was observed with 15 % concentration of CL oil. DEET (12 %) was employed as standard whose percentage knockdown (98.86 %) was greater than CL oil.

Table 1-Larvicidal activity of *Citrus limetta* oil

S. No.	CL oil Concentration (%)	Groups				
		Control	Standard		Test	
			Dead larvae	Mortality (%)	Dead larvae	Mortality (%)
1	7.5	1.66 ± 0.33	28.66 ± 0.33***	95.53	13.66 ± 0.27***	45.53
2	10	1.00 ± 0.57	28.33 ± 0.33***	94.43	20.33 ± 0.72***	67.76
3	15	1.33 ± 0.33	29.33 ± 0.33***	97.76	24.66 ± 0.27***	82.20

Values are expressed as mean ± SEM (number of mosquitoes, 30) using two way ANOVA followed by Bonferroni post-test, significant different at **P<0.05, **P<0.01, ***P<0.001, when compared with control group. Control group (untreated), Standard group (Temephos, 1 %), Test group (CL oil)

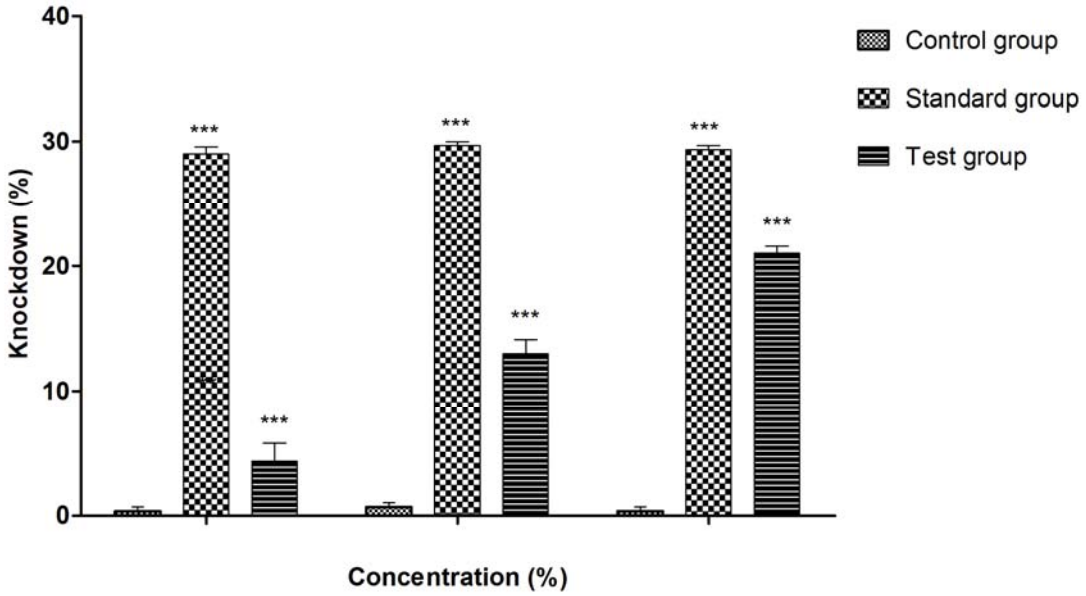

Fig. 1—Mosquito knockdown activity of *Citrus limetta* oil. Values are expressed as mean ± SEM (number of mosquitoes, 30) using two way ANOVA followed by Bonferroni post-test, significant different at **P<0.05, **P<0.01, ***P<0.001, when compared with control group. Control group (untreated), Standard group (DEET, 12 %), Test group (CL oil).

Discussion

The present study investigated the larvicidal and knockdown effect of CL oil against *Aedes* species to control dengue infection. Using CL seed oil to control mosquitoes is a better and environmentally safe option than the use of synthetic chemical pesticides. The percentage yield of CL seed oil was comparable with seed oil of other species of the citrus family namely *C. sinensis*, *C. paradisi*, *C. aurantium*, *C. reticulate*, and *C. aurantifolia* obtained from Pakistan and Nigerian countries[17,25]. In this study, the standards (Temephos and DEET) have also been found to be effective. However, there was a little significant difference between the larvicidal and knockdown activities of standards (Temephos and DEET, respectively) and the test samples. Temephos and DEET both exhibited more than 95 % larvicidal and knockdown activities, respectively at the end of 24 h, while the CL oil competes favorably with the standard at the end of 24 h. For instance, at the end of exposure time, the CL oil was able to knockdown mosquitoes and kill the larvae up to 70 and 82.20 %, respectively in its highest concentration.

Anwar *et al.* [17] carried out proximate analysis of the seeds of four citrus plants i.e. *C. limetta*, *C. paradisi*, *C. sinensis*, and *C. reticulata* and found 3.9–9.6 % protein, 5.0–8.5 % fiber, and 4.6–5.6 % ash contents. Another study[26] observed that linoleic acid is the main acid in citrus seed oil (36.1-39.8 %) and the other key fatty acids were palmitic acid (25.8-32.2 %), oleic acid (21.9-24.1 %), linolenic acid (3.4-4.4 %), and stearic acid (2.8-4.4 %). Manimaran *et al.*[27] observed that essential oils are also effective in killing mosquitoes and have knockdown effect. In short, through the larvicidal and knockdown effects, the present study clearly demonstrated that CL oil had high potential to control *Aedes* species of vector mosquitoes.

Conclusion

Mosquito knockdown and larvicidal action of CL oil against dengue vector mosquitoes may be a better option in the present scenario to prevent dengue virus transmission. CL seed oil can be used to develop herbal formulations with larvicidal and knockdown effects against the vector mosquitoes. In future, the CL oil may be commercially used in the production of formulations in an economic and harmless manner to prevent dengue virus infection.

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