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Larvicidal and irritant activities of hexane leaf extracts of *Citrus sinensis* against dengue vector *Aedes aegypti* L.

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ABSTRACT

Objective: To assess the larvicidal and irritant activities of the hexane extracts of leaves of *Citrus sinensis* (*C. sinensis*) against the early fourth instars and female adults of *Aedes aegypti* (*Ae. aegypti*). **Methods:** The larvicidal potential of the prepared leaf extract was evaluated against early fourth instar larvae of *Ae. aegypti* using WHO protocol. The mortality counts were made after 24 h and LC_{50} and LC_{90} values were calculated. The efficacy of extract as mosquito irritant was assessed by contact irritancy assays. Extract-impregnated paper was placed on a glass plate over which a perspex funnel with a hole on the top was kept inverted. Single female adult, 3-day old unfed/blood-fed, was released inside the funnel. After 3 min of acclimatization time, the time taken for the first take-off and total number of flights undertaken during 15 min were scored. **Results:** The citrus leaf extracts from hexane possessed moderate larvicidal efficiency against dengue vector. The bioassays resulted in an LC_{50} and LC_{90} value of 446.84 and 1 370.96 ppm, respectively after 24 h of exposure. However, the extracts were proved to be remarkable irritant against adults *Ae. aegypti*, more pronounced effects being observed on blood-fed females than unfed females. The extract-impregnated paper was thus proved to be 7-11 times more irritable as compared with the control paper. **Conclusions:** The hexane extracts from *C. sinensis* leaves are proved to be reasonably larvicidal but remarkably irritant against dengue vector. Further studies are needed to identify the possible role of extract as adulticide, oviposition deterrent and ovicidal agent. The isolation of active ingredient from the extract could help in formulating strategies for mosquito control.

1. Introduction

Mosquitoes being vector for many tropical and subtropical diseases are the most important single group of insects well-known for their public health importance. Despite progress in vaccine development, no effective and acceptable multi-valent vaccines are currently available against mosquito-borne diseases. The approach to combat these diseases largely relies on interruption of the disease transmission cycle by either destruction of the aquatic stages or by killing the adult mosquitoes using chemical insecticides[1,2].

The drastic effects of chemical insecticides-based intervention measures for the control of disease vectors have received wide public apprehension and have caused many problems like insecticide resistance, resurgence of pest species, environmental pollution, toxic hazards to humans and other non-target organisms[3]. To alleviate these problems, major emphasis has been on the use of natural plant-based products as larvicides which can provide an alternate to synthetic insecticides[4]. Plants are rich sources of bioactive compounds that can be used to develop environmentally safe vector and pest-managing agents. A number of plants have been reported as selective with little or no harmful effect on non-target organisms and the environment. The botanical extracts from the plant leaves, roots, seeds, flowers and bark in their crude form have been used as conventional insecticides for centuries[5]. Plant products are important natural alternatives to insecticides, as phytochemicals extracted from the whole plant or specific part of the plant using different solvents may also act against mosquitoes as toxicant, growth regulators, repellents and ovipositional deterrent[6-8].

The preliminary screening is a good mean of evaluation of the potential larvicidal activity of plants popularly used for this purpose. *Citrus* plants have been known for their use as food, food-flavoring agents, and also for their mosquitocidal components. Different parts of the *Citrus* plant *i.e.* fruits, seeds, roots and leaves, have been tested

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for their use as mosquitocidal components[9,10]. Amusan *et al*[5] have tested the ethanolic extracts of the orange peel of *Citrus sinensis* (*C. sinensis*) for the toxicity effect on the larvae of the yellow fever mosquito *Aedes aegypti* (*Ae. aegypti*) and found them quite effective. Lee[11] had tested essential oil of *Citrus bergamia* against larvae of *Ae. aegypti* and *Culx pipiens*, while Amer and Mehlhorn[6] investigated essential oil from *Citrus limon* against *Ae. aegypti* and *Culex quinquefasciatus*, and *Anopheles stephensi*. The essential oils contained in the rind of the fruit and the leaves of bergamot from Greece (*C. aurantium* subsp. *bergamia*) have also been studied[12]. All these reports suggest the toxic potential of *Citrus* plant against mosquitoes. Keeping in view this and that not much studies have been carried out on the *Citrus* leaves for the possible potential for dengue vector control, present investigations were performed to study the larvicidal and irritability effects of the hexane extract of *C. sinensis* leaves against dengue fever mosquito, *Ae. aegypti*. The *Citrus* leaves being commonly and widely available may prove to be an excellent alternative for mosquito control because of non-toxicity to environment and non-target organisms.

2. Materials and methods

2.1. Preparation of leaf extract

The fresh and tender leaves of *C. sinensis* were collected from the surrounding areas. These were thoroughly washed with tap water and kept under shade at room temperature [(27±2) ℃] for about 20 days till they dried completely. The dried leaves were mechanically grinded and sieved to get fine powder. The 200 g of powdered leaves was soaked in 1 L of hexane. The soaked material was left undisturbed for five days and the crude extract, thus formed, was concentrated using a vacuum evaporator at 45 ℃ under low pressure. After complete evaporation of the solvent the concentrated extract was collected and stored in a refrigerator at 4 ℃ as the stock solution of 1 000 ppm for further use.

2.2. Mosquitoes

The present investigations employed the dengue fever mosquito, *Ae. aegypti* originated from fields of Delhi and surrounding areas. The colony was maintained in an insectary at (28±1) ℃, (80±5)% relative humidity and 14/10 light:dark photoperiod[13].

2.3. Larvicidal bioassays

The larvicidal bioassay was performed at (28±1) ℃ on the *Ae. aegypti* larvae in accordance with the procedure described by WHO with slight modifications[14]. For experimental treatment, the graded series of the leaf extract was prepared using 5 parts of hexane and 95 parts of acetone as the diluting solvent. The early fourth instar larvae of *Ae. aegypti*, in batches of 25, were taken in plastic bowls containing 99 mL of distilled water and transferred to glass jar containing 150 mL of distilled water and 1 mL of the particular concentration of the leaf extract. Four replicates were carried out simultaneously for each dilution.

Controls were exposed to the 1 mL of the diluting solvent, *i.e*. 5 parts of hexane and 95 parts of acetone. During the treatment period, the larvae were not provided with any food. The dead and moribund larvae were recorded after 24 h as larval mortality.

2.4. Statistical analysis of data

The tests with more than 20% mortality in controls and pupae formed were discarded and repeated again. If the control mortality ranged between 5%-20%, it was corrected using Abbott's formula (Abbott 1925).

The data were subjected to regression analysis using computerized SPSS 11.5 Programme. The LC_{50} and LC_{90} values with 95% fiducial limits were calculated in each bioassay to measure difference between the test samples. The results obtained with different extracts were analyzed using Student's *t*-test with statistical significance considered for $P \leqslant 0.05$.

2.5. Contact irritancy assays

Whatman filter paper circles were impregnated with 100 ppm hexane leaf extracts, separately. These papers were completely dried and used afresh for contact irritancy assays. Each paper was placed on a glass plate and a perspex funnel with a hole on the top was kept inverted over them. Single female adult, 3-day old unfed, was released inside the funnel on the paper. The hole on the top of funnel was plugged with cotton. The adult was allowed to settle for 3 min after which the time taken for the first take-off was recorded. The experiment was continued for 15 min during which the total number of flights undertaken by the mosquito was scored.

Parallel control tests were performed with acetone-impregnated papers. Similar experiments were also carried out with freshly blood-fed females to compare the repellency effects of the *Citrus* leaves. Each treatment had 3 replicates. Data were analyzed and the relative irritability of the extract in each case was calculated with respect to control.

3. Results

The results of the larvicidal bioassay performed on the early fourth instars and contact irritancy carried out on the female adults of *Ae. aegypti* with the hexane leaf extract of *C. sinensis* were presented in Table 1 and Table 2. The results obtained showed the moderate larvicidal efficacy of the hexane leaf extract, but the extracts proved to be remarkably significant irritant against the dengue vector. The larvicidal bioassay resulted in the LC_{50} value of 446.84 ppm and LC_{90} value of 1 370.96 ppm (Table 1). The larvae remained alive developed into normal pupae and then adults within (60-72 h). No morphological alterations or the delayed mortality was noticed during the development negating the possibility of delayed toxicity of leaf extracts.

When the female adults of *Ae. aegypti* were subjected to contact irritancy assays, a significant behavioural response was observed. The most remarkable response on exposure to leaf extract was observed in blood-fed mosquitoes with

first flight only after 4 sec and an average total of 54.33 take-offs as against only 5.00 take-offs against control (Table 2). Against unfed females though the extract was relatively less effective yet caused a significant number of 34.33 take-offs in just 15 min. The extract-impregnated paper was thus proved to be 7-11 times more irritable as compared with the control paper.

Table 1
Larvicidal activity (in ppm) of hexane extract prepared from the leaves of *C. sinensis* against early fourth instars of *Ae. aegypti*.

Parameters	LC_{30}	LC_{50}	LC_{70}	LC_{90}	S.E.	χ^2 (*df*)	Regression coeffient
Value	282.44	446.84	706.92	1 370.96	1.40	4.94	2.63
Upper FL	99.31	269.22	508.75	863.92	–	(4)	–
Lower FL	403.65	639.03	1 451.36	7 002.14	–	–	–

FL indicates the 95% lower and upper fiducial limits.
S.E. indicates standard error and *df* indicates degrees of freedom.

Table 2
Response of 3-day old adult females (blood-fed and non blood-fed) of *Ae. aegypti* to papers impregnated with hexane extracts of *C. sinensis* leaves in the contact irritancy assays (Mean±SEM).

Test	Mean time lapse before first take-off (in min)	Mean number of take-offs for females (in 15 min)	Relative irritability
Control	0.00 ± 0.00^{a} (0.00)	5.00 ± 2.00^{c}	1.00 (1.15)
Non blood-fed	3.30 ± 0.00^{a} (1.00)	34.33 ± 8.69^{d}	6.86 (15.04)
Blood-fed	4.00 ± 0.58^{b} (1.00)	54.33 ± 21.60^{d}	10.86 (37.42)

Figures in parentheses indicate standard deviation value.
Figures in each column followed by the same letter are not significantly different at P=0.05 (Students' paired t-test).

4. Discussion

Natural pesticides derived from plants are a promising tool especially for targeting mosquitoes in the larval stage[6]. Different parts of the *Citrus* plant *i.e.* fruits, seeds, roots and leaves have been tested for their use as mosquitocidal components[9,10]. A number of studies have also been carried out on the larvicidal potential of essential oil extracted from the *Citrus* leaves and peels[11,12]. However, very few reports are available regarding the larvicidal effects of crude extracts of *Citrus* leaves against *Ae. aegypti* whereas no work reports about their irritancy effects against the female adults.

Our investigations showed that 24 h exposure to early fourth instars of *Ae. aegypti* with hexane extracts of the leaves of *C. sinensis* resulted in 50% mortality at 446.84 ppm. This value is much higher than that obtained by Cavalcanti *et al*[15] who reported LC_{50} value of 69 ppm but with essential oil extracted from *Cymbopogon citratus* against early fourth instars of *Ae. aegypti*. Bagavan *et al*[16] tested the crude extracts of *Citrus* peels against mosquito larvae and obtained the LC_{50} value of 58.25 ppm and LC_{90} value of 298.31 ppm with the chloroform extract of *C. sinensis* peels against *Anopheles subpictus* larvae; and the LC_{50} value of 38.15 ppm and LC_{90} value of 184.67 ppm with the peel methanolic extract against the larvae of *Culex tritaeniorhynchus*. Traboulsi *et al*[9] have accounted the insecticidal activity of essential oil extracts from leaves, flowers and roots of *C. sinensis* Osbeck against fourth instar larvae of the mosquito *Culex pipiens molestus* Forskal as 60.0 mg/L.

It has been reported that behavioural modifications of the mosquitoes through chemical actions by contact irritancy, are expected to have population effects that may ultimately reduce disease transmission[17]. Several reports are available regarding the repellent properties of essential oils against adult mosquitoes[18-20]; however very few works have been reported proving the irritability potential of leaf extracts and practically no work with *Citrus* leaf extracts. Our studies showed the significant irritancy potential of hexane extracts of *Citrus* leaves against the adults of *Ae. aegypti* as it caused 54.33 flights in blood-fed females and 34.33 flights in unfed females when exposed for just 15 min. The effect was, thus more pronounced in blood-fed females (10.86 irritability ratio) than unfed females (6.86 irritability ratio). This result may prove significant as the extract could be used as a repellent on the human skin surface to keep away the mosquitoes from biting. Similar significant repellency behaviour was reported in *Ae. aegypti* when the female adults were exposed to various extracts prepared from the leaves of *Parthenium hysterophorus*[13] and to the crude leaf extracts of *Cassia fistula* prepared in acetone[21]. The application of *Mentha piperita* oil on human skin had resulted in 100% protection till 150 min after which only 1-2 bites were reported for next 30 min[22]. Essential oils from some Verbenaceae plants have shown repellent and/or insecticidal effects against mosquitoes[7,23]. The five most effective oils reported were those of litsea (*Litsea cubeba*), cajeput (*Melaleuca leucadendron*), niaouli (*Melaleuca quinquenervia*), violet (*Viola odorata*), and catnip (*Nepeta cataria*), which induced a 100% repellency against *Ae. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*[7]. Petroleum ether extracts of *Vitex negundo* leaves offered 8 h of bite protection (2 mg/cm^2) by different mosquito species in the field[24,25]. A patented extract of the seeds of *Vitex agnus castus* was shown effective against mosquitoes resulting in more than 6 h protection against *Culex quinquefasciatus* and *Anopheles stephensi*, and 3-4 h against *Ae. aegypti* [26]. Nour *et al*[27] suggested the use of the essential oils of *Ocimum basilicum* as promising new natural repellents at 0.1% concentration against *Anopheles* mosquito.

Mosquito control is vital for many countries and is still in a state of evolution. During the last decades, it depended upon synthetic organic insecticides, many of which have been removed from the arsenal of weapons[28] and botanicals are the new weapons of mosquito control under exploration. The activity of crude plant extracts is often attributed to the complex mixture of active compounds. Natural pesticides derived from plants are a promising tool especially for targeting mosquitoes in the larval stage[6], but there are some factors that need more investigation in order to extract useful conclusions: light and storing conditions[29], enantioselectivity of major and minor ingredients[30], harvesting time and plant material[12], *etc*. Further studies are needed to identify the possible role of extract as adulticide, oviposition deterrent and ovicidal agent. The isolation of active ingredient from the extract could help in formulating strategies for mosquito control.

Conflict of interest statement

We declare that we have no conflict of interest.

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