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Larvicidal, pupicidal and repellent activities of *Gaultheria* oil (Plantae: Ericaceae) against the filarial vector, *Culex quinquefasciatus* (Insecta: Diptera: Culicidae)

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Abstract
Over the world, more than 50% of persons with filariasis receive their infections from the bites of mosquitoes, particularly *C. quinquefasciatus.* Some photochemical comprise toxic compounds that can be exploited to control mosquito larvae. *Gaultheria* oil or wintergreen oil which contains methyl salicylate as a major component and has been used in the present study to investigate the larvicidal, pupicidal and repellent activities against the different instar (I, II, III and IV) larvae and pupae of *C. quinquefasciatus* at different concentrations (100, 200, 300 and 400 ppm). The LC_{50} values of *Gaultheria* oil against different instar larvae were 166.40, 214.95, 303.47, 386.63, respectively and the LC_{90} values were 613.91, 669.40, 755.80, 883.46, and 866.12, respectively. LC_{50} and LC_{90} values of pupae were 435.82 and 866.12, respectively. *Gaultheria* oil showed 100% repellency in 250 min and showed complete protection in 90 min at 450 ppm against *C. quinquefasciatus*. The results obtained show that this plant material exhibited significant activity and could be considered as potent natural larvicidal, pupicidal and repellent agent against *C. quinquefasciatus*.

Keywords: *Culex quinquefasciatus*, *Gaultheria* oil, larvicidal, pupicidal, repellent activity.

1. Introduction

Mosquitoes are the vectors for the dreadful diseases of mankind [30]. The mosquito *Culex quinquefasciatus* plays a major role, as a vector of lymphatic filariasis. This species also serves as vectors of important diseases, such as West Nile virus, filariasis, Japanese encephalitis, St. Louis encephalitis and avian malaria. *C. quinquefasciatus*, as a vector of lymphatic filariasis, is widely distributed in tropical zones with around 120 million people infected worldwide and 44 million people having common chronic manifestation [4]. Most of the mosquito control programs target the larval stage in their breeding sites with larvicides [12] because the adulticides may only reduce the adult population temporarily [6]. Therefore, a more efficient way to reduce mosquito population is to target the larvae. Organophosphates and different insecticides are widely used for mosquito control. A drawback with the use of chemical insecticides is that they are non-selective and could be more harmful to other non-target organisms and be a pollutant to environment. Moreover, after few years the mosquitoes develop resistance against the insecticides due to frequent use of them [22]. An alternative and recent approach for mosquito control is the use of natural products of plant origin, botanical derivatives. Plant essential oils in general have been recognized as important natural resources of insecticides and used as anti-larvicidal and pupicidal agent. Bio-pesticide with plant origins have been given new importance in recent years for their use against several insect species Essential oils (EOs) are defined as any volatile oil(s) that have strong aromatic components. The aromatic characteristics of Essential oil act as strong repellent agent against insects and that give distinctive odour, flavour or scent to a plant [31].

In the search for alternatives to conventional insecticides, EOs extracted from aromatic plants have for decades received much interest as potential bioactive agents [20]. The essential oil of *Gaultheria procumbens* belongs to the family *Ericaceae* (oil of wintergreen) is a colorless to yellowish liquid with a characteristic 'wintergreen' odour, slightly soluble in water, soluble in organic solvents and oils. It is marketed for the external treatment of neuralgia, malign, arthralgia and other pains [29].

According to Ribnicky DM *et al*., [19] wintergreen contains 10 mg/g of total salicylate –the highest level observed in plants. The salicylate was present predominantly as a derived form of methyl salicylate called gaultherin which was found to be an effective agent in mosquito control program. Transmission of mosquito-borne diseases is mainly due to its bite, therefore personal protective measures, including repellents, by minimizing the contact between humans and vectors can prevent the spread of deadly therefore personal protective measures, including repellents, by minimizing the contact between humans and vectors can prevent the spread of deadly diseases [16]. Essential oil in turn acts as key product for efficient repellent [13]. A wide variety of Essential oils have been used as toxicants with ovicidal, larvicidal, pupicidal and adulticidal activities to sub-lethal effects including repellent action [3, 10, 25] and [5]. Efficacy of 25 essential oils was screened against filarial vector, *C. quinquefasciatus* [14]. Larvicidal and repellent properties of some essential oils shows good result against *C. tritaeniorhynchus Giles* and *Anopheles subpictus Grassi* (Diptera: Culicidae) [9]. Hence, in the present investigation an attempt has been made to evaluate the larvicidal, pupicidal and repellent effect of *Gaultheria* oil against lymphatic filarial vector, *C. quinquefasciatus.*

2. Materials and Methods

2.1 Collection of egg and maintenance of larvae

The eggs of *C. quinquefasciatus* were collected from National Centre for Disease Control (NCDC) field station of Mettupalayam, Tamil Nadu, India, using an 'O' type brush. These eggs were brought to the laboratory and transferred to 18 X 13 X 4 cm size enamel trays containing 500 ml of water for larval hatching. The mosquito larvae were fed with pedigree dog biscuits and yeast in 3:1 ratio. The feeding was continued until the larvae transformed into the pupal stage.

2.2 Maintenance of pupa and adult

The pupae were collected from the culture trays and transferred to plastic containers (12×12 cm) containing 500 ml of water with the help of a dipper. The plastic jars were kept in a 90 X 90X 90 cm size mosquito cage for adult emergence. Mosquito larvae were maintained at 27 ± 2 ºC, 75-85 % relative humidity, under a photoperiod of 14:10 (light/dark). A 10% sugar solution was provided for a period of 3 days before blood feeding.

2.3 Maintenance of blood feeding, adult mosquito vectors.

The mosquito cage was made up of wooden frames and covered with polythene sheets on four sides (two laterals, one back and other one upper) and the front part was covered with a muslin cloth. The bottom of the cage was fitted with 10% sugar solution for a period of three days before they were provided with a host for blood feeding. The adult female mosquitoes were allowed to feed on the blood of a rabbit (exposed on the dorsal side) for two days to ensure adequate blood feeding for 5 days. After feeding enamel trays with water from the culture trays were placed in the cage for the adults to lay eggs.

2.4 Preparation of *Gaultheria* oil concentrations

Gaultheria oil was bought from T. Stanes & Company Ltd, Coimbatore, Tamil Nadu, India. Required quantity of *Gaultheria* oil was thoroughly mixed with DMSO to prepare various concentrations, ranging from 100 to 400 ppm.

2.5 Larval and pupal toxicity test

Laboratory colonies of mosquito larvae/ pupae were used to study larvicidal/ pupicidal activity. Twenty-five numbers larval instars (each instar) and pupae were introduced into 500-ml glass beaker containing 249 ml of dechlorinated water, and 1ml of desired concentration of *Gaultheria* oil. Larval food was given for the test larvae. At each tested concentration two to five trials were made and each trial consisted of five replicates. The control was setup by mixing 1 mL of acetone with 249 mL of dechlorinated water. The mortalities were calculated using Abbott's formula [1].

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

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

The LC_{50} and LC_{90} values were calculated from toxicity data by using probit analysis [7].

2.6 Repellent bioassay

Repellent activity of oil was tested with the human volunteers. For the repellent activity of *Gaultheria* oil percentage protection in relation to dose method was used as adopted by WHO [30]. The test solutions of 50, 100, 150, 200 and 250 ppm were prepared by dissolving the *Gaultheria* oil in isopropanol. Isopropanol along with water acted as a control. For repellent experiment, 50 laboratory-reared blood-starved adult female mosquitoes that were between 3 and 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 different oil extracts being tested were applied from the elbow to the fingertips. The arms were left undisturbed. An arm treated with isopropanol served as a control. The control and treated arms were introduced simultaneously into the cage. The number of bites was counted over 5 min, every 30 min, from 1800 hours to 0600 hours. Protection time was recorded as the time elapsed between repellent applications and the observation period immediately preceding that in which a confirmed bite was obtained. If no bite were confirmed at 180 min, tests were discontinued, and protection time was recorded as 180 min. An attempt of the mosquito to insert its stylets was considered a bite. If no mosquito attempted to bite the control arm during the observation period, that trial was discarded, and the test was repeated with a new batch of mosquitoes. 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. It was observed that there was no skin difference on repellency. It was observed that no skin irritation from the oil. The percentage protection was calculated

by using the formula [7, 8, 26, 27].

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

2.7 Statistical Analysis
The data so obtained were subjected to probit analysis for calculating LC_{50}, LC_{90} and other statistics at 95 % fiducial limits of upper fiducidal limit and lower fiducidal limit, and Chi-square values were calculated using the SPSS Statistical software package 16.0 version. Results with $P<0.05$ were considered to be statistically significant.

3. Results
Larvicidal and pupicidal activity of *Gaultheria* oil at various concentrations against the filarial vector, *C. quinquefasciatus* is given in Table - I. Considerable mortality was evident in all treatments in a concentration dependent manner. At 100 ppm concentration the mortality rate of first instar larva was 42%; whereas at 400 ppm, it was increased to 76%. The pupae mortality also increased with increase in concentration, i.e. 16% at 100 and 47% at 400 ppm. LC_{50} values for first, second, third and fourth instars were found to be 90.96, 157.70, 259.10 and 328.63 ppm, respectively. Similarly the LC_{90} values for the respective instars were found to be 613.91, 669.40, 755.80 and 883.46 ppm, respectively. The LC_{50} and LC_{90} values for pupa were calculated as 435.822 and 866.12 ppm, respectively. The 95% confidence limits LC_{50} and LC_{90} (LFL-UFL) were also calculated and results expressed in Table 1.

Table 1: Larval and Pupal toxicity effect of *Gaultheria* oil against filarial vector *C. quinquefasciatus*

Larval and Pupal stage	% of Larval and Pupal mortality± SD Concentration of *Gaultheria* oil (ppm)				LC_{50} (LC_{90})	95% Confidence limit		X^2 (df-4)
	100	200	300	400		LC_{50} LFL-UFL	LC_{90} LFL-UFL	
First instar	42±1.41	56±1.89	62±2.82	76±1.67	166.40 (613.91)	90.96 (212.29)	501.63 (867.54)	0.64*
Second instar	37±1.78	49±1.41	59±2.00	70±2.44	214.95 (669.40)	157.70 (259.63)	542.67 (958.90)	0.32*
Third instar	30±2.28	36±2.82	49±1.67	62±1.54	303.63 (755.80)	259.10 (366.97)	607.25 (1096.65)	0.49*
Fourth instar	24±1.41	30±0.89	41±2.00	52±1.67	386.63 (883.46)	328.16 (509.14)	686.07 (1392.28)	0.18*
Pupa	16±1.26	25±0.63	32±0.89	47±1.09	435.82 (866.12)	373.40 (568.08)	688.98 (1278.19)	0.33*

Control: Nil mortality, LFL**:** lower fiducidal limit, **UFL**: upper fiducidal limit, **χ2**: chi- square value, **df:** degrees of freedom * P<0.05 level, mean values of five replicates.

The results for the skin repellent activity of *Gaultheria* oil against the blood starved adult female of *C. quinquefasciatus* are given in the Table 2. *Gaultheria* oil showed significant repellency against *C. quinquefasciatus* and gave protection against mosquito bites without any allergic reaction to the test person. The repellent activity was found to be dependent on the strength of the oil applied. The highest concentration 450ppm provided over 120, 150 and 180 min protection against *C. quinquefasciatus*.

Table 2: Repellent activity of *Gaultheria* oil against filarial vector *C. quinquefasciatus*

Mosquito species	Concentration (ppm)	% of Repellency ± SD					
		Time post application of repellent (min)					
		30	60	90	120	150	180
C. quinquefasciatus	**50**	90.2±0.8	87.6±1.8	72.2±1.3	79.2±1.1	61.8±1.7	69.2±0.4
	150	98.6±1.0	93.6±0.7	83.8±1.7	76.6±2.0	73.4±1.6	61.4±1.0
	250	100±0.0	99.2±0..9	91.4±1.0	81.8±1.6	79.2±0.7	68.4±0.8
	350	100±0..0	100±0.0	96.2±0.7	85.8±1.7	81.8±1.1	71.8±0.9
	450	100±0.0	100±0.0	100±0.0	97.4±1.0	92.2±1.6	83.2±1.3

4. Discussion
Essential-oil extracted from plants act as promising alternative natural Products for the control of many insect pests especially mosquitoes [18]. Oils are widely used as an anti-larvicidal and anti-pupicidal agent against mosquitoes. Many of the research findings show that the oil is obtained from hydro-distillation method is widely used for larvicidal and pupicidal agent [28].

In our present studies commercially available *Gaultheria* oil was treated against the filarial vector *C. quinquefasciatus* to evaluate the larvicidal and pupicidal activity. Senthilkumar *et al*., [21] noticed

the preliminary screening of commercially available 22 plant essential oil against the fourth instar larvae of *C. quinquefasciatus*. Out of these, eight essential oil *viz*., aniseed, calamus, cinnamon, clove, lemon, orange, thyme, and tulsi oils gave promising results on larvicidal activity. The mortality was recorded at different exposure periods *viz*., 1, 3, 6, 12 and 24 h, at 500 ppm concentration. The *clove* oil gave 100 percent mortality at all exposure periods. *Vetiver* oil recorded 36.2, 61.2, 76.2, 87.5 and 100% mortality. *Lemongrass* oil after 24 h exposure shows the lowest mortality of 28.7%. In *Gaultheria fragrantissima* at different exposure periods moderate amount of larval mortality was found to be ranging from 5.0 ± 0.4, 21.2 ± 0.4, 33.7 ± 0.7, 58.75 ± 0.6, 73.7 ± 1.1, respectively. Similarly in our present studied the oil obtained from *Gaultheria procumbence* exhibit larval mortality of 52 ±1.6 for fourth instar larvae at 24h of exposure at 400ppm concentration against *C. quinquefasciatus*. Ramar *et al*., [17] reported the larvicidal activity of *Acorus calamus (A. calamus*) rhizome essential oil against the filarial vector mosquito, *C. quinquefasciatus*. The larvicidal activity of the essential oil at different concentrations, ranging from 12.5, 25.0, 50.0, 100.0 and 200.0 mL/L was treated against the 4th instar larvae. Promising larvicidal activity was noticed and the LC_{50} value of 63.43 mL/L (95% confidence limit of 33.02-101.84 mL/L) and LC_{90} value of 14.95 mL/L (95% confidence limit of 105.82-283.11 mL/L). The mortality of *C. quinquefasciatus* was (11 ±2.9) %, (31± 3.0) %, (52± 3.6) %, (71± 3.1) % and (97± 2.3) % respectively.

The third instar larvae of *C. quinquefasciatus* when treated with oil of *Gaultheria procumbence* shows the LC_{50} value of 303.63 ppm and LC_{90} value of 755.80 and this is compared with that of [11] in which the larvicidal activity of *J. procera* essential oil against late third instar larvae of *An. arabiensis* shows the LC_{50} and LC_{90} values were 14.42 and 24.65 mg/L, respectively under laboratory condition. The larvicidal efficacy of *J. procera* essential oil against late third instar wild-collected *anopheline* larvae under the semi-field conditions the highest larvicidal activity with LC_{50}=24.518 and LC_{90}=34.212 mg/L. Thus the mortality rate depends on the dosage, different instar stage of the larvae and environmental condition.

Mandal *et al*., [23] reported the repellent activity of Eucalyptus oil and *A. indica* seed oil against *C. quinquefasciatus.* The *A. indica* seed oil provides 90.26% and 88.83% protection and the Eucalyptus oil provided 93.37% and 92, 04% at the concentration of 50% and 100%. No bite was observed after applying *A. indica* seed oil and Eucalyptus oil within 180 min and 120 min respectively. Thus 100% protection was achieved. Our present repellency activity is similar that at highest concentration of 450 ppm provide protection over 120, 150 and 180 min against *C. quinquefasciatus*.

Nikolic *et al*., [15] have already reported the chemical analysis of GC-MS of *G. procumbens* essential oil having 8 essential components, out these the most dominant one is volatile esters of salicylic acid, methyl salicylate (96.90%). Thus in *G. procumbens* salicylic acid is considered as a major constituent it exhibit significant pupal mortality of 16% at the lowest concentration of 100 ppm, whereas it considerably increased to 47% at 400 ppm and the LC_{50}-LC_{90} values were (435.82-866.12 ppm). This result are compared to the earlier report of gaultheria oil against black cut worm *Agrotis ipsilon* whereas at 2% concentration maximum insecticidal activity was recorded in *Gaultheria* oil (89.92%) [2]. Investigation reported that the commercially available oil (Gaultheria oil) contains methyl salicylic acid, which may act as an anti-larvicidal and pupicidal agent against *C. quinquefasciatus*.

Hence this study clearly reveals that the wintergreen oil obtained from *Gaultheria procumbens* has potency to control the larvae and pupae of the mosquito *C. quinquefasciatus* also acting as repellent. Thus these natural products prove to be an effective approach in eco-friendly mosquito management and control programmes.

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