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TOXICITY OF MYRCENE RICH ESSENTIAL OILS OF *CURCUMA LONGA* (VAR. CH-66) LEAF AGAINST MOSQUITOES, *AEDES AEGYPTI* AND *ANOPHELES STEPHENSI*

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ABSTRACT

Phytochemicals derived from plants can act as larvicides, insect growth regulators and repellents. They can play an important role in the interruption of the transmission of mosquito borne diseases. Essential oils are rich source of bioactive monoterpenoids against insects. *Curcuma longa* L. (Zingiberaceae) is well known for its medicinal values and contain pungent and odoriferous oils. Among the various genotypes of *C. longa* maintained at this institute, 25 genotypes were screened for insecticidal activities against two mosquito species and genotype CH-66 was found to be active. Therefore, leaf essential oil of this genotype was studied for toxic effects against eggs and larvae of two mosquito species, *Anopheles stephensi* and *Aedes aegypti*. The leaf essential oil was obtained by hydrodistillation and analyzed by gas chromatography. The leaf oil rich in compound myrcene (40.19%) gave 100% larvicidal activity at 0.25 and 0.30 mg/ml against *A. stephensi* and *A. aegypti* with LC_{50} of 0.12 and 0.14 mg/ml, respectively. However, chlorpyrifos taken as positive control gave LC_{50} of 0.0013 and 0.0014 mg/ml against *A. stephensi* and *A. aegypti*, respectively The effective concentration (EC_{50}) to inhibit 50% egg viability was observed to be 0.27 and 0.29 mg/ml for *A. stephensi* and *A. aegypti*, respectively. Since only rhizome part of *C. longa* is currently used, bioactivities of the leaf may find use as leads in controlling vectors spreading dreadful diseases like malaria and dengue fever.
***Keywords*: *Curcuma longa*, larvicidal myrcene, *Aedes aegypti*, *Anopheles stephensi*.**

Introduction

Mosquitoes act as vector for many tropical and subtropical diseases such as dengue fever, malaria, filariasis and encephalitis (Govindarajan *et al*., 2012). Major vectors for spreading such diseases are *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* in India. Interruption of mosquito life cycle and targeting either larval stage or adults are the approaches to combat vector borne diseases. Larval control largely depends on the use of synthetic chemical insecticides like temephos and fenthion (Organophosphates), but their repeated use has resulted in the wide spread development of resistance (Ranson *et al*., 2001; Asgarian *et al*., 2023). An alternative approach for mosquito control is the use of botanicals because they contain a range of bioactive chemicals (Hedlin *et al.,* 1987; Demirak and Canpolat, 2022). Efforts have focused on phytochemicals as potential source of mosquito control agents, especially essential oils, which are rich source of bioactive monoterpinoids against insects (Cheng *et al*., 2003). Studies of essential oils from the plants, *Cymbopogon* species (Poaceae) (Sukumar *et al.,* 1991; Kumar *et al*., 2018), *Mentha* species (Lamiaceae) (Ansari *et al*., 2000; Dung & Tuyet, 2020), *Tagetes* species (Asteraceae) (Dharmagadda *et al*., 2005; Jayaraman *et al*., 2015), *Hyptis* species (Lamiaceae) (Araujo *et al*., 2003; John *et al*., 2022), *Lippia* species (Verbenaceae) (Gleiser & Zygadalo, 2007; Mahanta *et al*., 2019), *Travhyspermum ammi* (Pandey *et al.,* 2009; Seo *et al.,* 2012) and many other plants (Traboulsi *et al*., 2005; Ghosh *et al.,* 2012) have demonstrated promising larvicidal activities against mosquitoes.

Curcuma longa L. (Zingiberaceae) rhizome, commonly known as turmeric, is well known for its medicinal values in the Indian traditional system of medicine and has been a recipe for several ailments (Srimal, 1997; Soleimani *et al*., 2018). Turmeric contains pungent, odoriferous oils and oleoresins and the rhizomes have been reported to possess many kinds of biological activities (Rath *et al*., 1998; Giordano & Tommonaro, 2019).

A number of anti-insect properties of turmeric have been documented in the literature. The insect repellent components in turmeric are turmerones (Su *et al.,* 1982). Aqueous extract of *C. longa* rhizome reduced larval population of *Anopheles stephensi* Liston (Murugan & Jayablam, 1998) and *A. gambiae* (Ajaiyeoba *et al*., 2008). Essential oil of turmeric is reported to possess antifungal, antibacterial and insect repellent activities (Saju *et al.,* 1998; Rath *et al*., 1999; Zhu *et al*., 2008; Lacoma *et al*., 2015; Li *et al*., 2020; Dong *et al*., 2021). It has been shown to possess feeding-deterrence against the lesser grain borer, *Rhyzopertha dominica* (Jilani & Saxena, 1990; Tripathi *etal*., 2002) and repellent to *T. castaneum* and *R. dominica* (Mohiuddin *et al*., 1993).

Essential oil of turmeric extracted from rhizome contains turmerones whereas leaf oil contains phellandrene (18.2%), 1,8-cineole (14.2%) and *p*-cymene (13.3%) as major constituents (Sharma *et al*., 1997). The fresh leaf oil of *C. longa* contains 82.9% monoterpenes whereas fresh rhizome oil has only 16.3% monoterpenes (McCarron *et al*., 1995). Monoterpenes in general have been well documented to be active as fumigants, repellents, or insecticides towards stored grain insects (Obeng-Ofori *et al.,* 1997; Tsao *et al.*, 1995; Tripathi *et al.*, 2002; Reis *et al.*, 2016; Fauad *et al.*, 2021). However, work on potential of essential oil of *C. longa* leaf is negligible. In the present paper, we report the larvicidal and ovicidal activities of the myrcene rich essential oil extracted from the leaf of *C. longa* (var. CH-66) against two species of mosquito vectors, *Aedes aegypti* and *Anopheles stephensi*. Findings of the present study would be useful in the development of new agents for mosquito control based on bioactive compounds from indigenous plant sources.

Materials and Methods

Collection of plant material

Mature and fresh leaves of *C. longa* (var. CH-66) were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow in the month of November, 2022. Plant materials were identified by Botany and Pharmacogonosy Division of CIMAP, Lucknow and voucher specimen deposited.

Essential oil extraction

Collected leaves of *C. longa* (var. CH-66) dried and essential oil was obtained by using a Clevenger type apparatus in accordance with Guenther (1955). The essential oil was dried over sodium sulfate.

Analysis of the essential oil

Analysis of the essential oil by gas chromatography was conducted using a model CX-3400, Varian Gas Chromatogram under the following conditions: carrier gas hydrogen, injector temperatures, 220ºC and 225ºC respectively, using a capillary column (Supelcowax –10, 30 m x 0.32 mm, film thickness 0.20 mili micron). The oven was programmed from 80ºC - 150ºC at 5ºC/min, then 215ºC at 7ºC/min. The area percentage was obtained on a Varian 440 integrator. The identity of the component was assigned by comparing their retention time with those of authentic samples and by comparison of their mass spectral fragmentation patterns (Martins *et al*., 2001). The density of the oil was also determined and expressed as g/ml.

Test organism

Egg, larval and adult stages *A. aegypti* and *Anopheles stephensi* were obtained from culture maintained at 27 ± 2ºC, 70-85 % relative humidity with a photoperiod (14 h light and 10 h dark). The larvae were fed on dog biscuits and yeast powder in 3:1 ratio. Adults were provided with 10% multivitamin syrup solution and periodically fed with rabbit blood. Five days after blood feeding, the gravid mosquitoes were used for obtaining different age groups of eggs in 3 h intervals. All the bioassay experiments were conducted at the above temperature and humidity conditions.

Bioassay

Larvicidal assay

The larvicidal activity of turmeric leaf oil was assayed against 3rd instar larvae of *A. aegypti* and *A. stephensi* as per method (WHO 2005). Stock solution of the oil was prepared at 1 mg/ml with ethanol. Oil was evaluated at the level of 0.30, 0.25, 0.20, 0.15, 0.10 and 0.05 mg/ml in tap water. Tween-80 (Polyoxyethylene sorbitan monooleate) was used as emulsifier at a concentration of 0.001%. Tap water mixed with Tween-80 and ethanol was used as control. Chlorpyrifos was taken as positive control and tested at 0.008, 0.004, 0.002, 0.001, 0.0005, and 0.00025 mg/ml concentrations. Fifty larvae were introduced to each test concentration as well as control. For each concentration, ten replicates were used. Observation on larval mortality was recorded after 24 h of exposure, during which no food was given to the larvae. Larvae were considered dead, when they did not react to touching with a needle. The percentage of mortality was calculated using Abbott (1925) formula and data were subjected to Probit Analysis to analyze lethal doses (LD_{50}).

Ovicidal assay

Stock solution of the *C. longa* leaf oil was prepared at 1 mg/ml with ethanol. The oil was tested at various concentrations ranging from 0.05 - 0.30 mg/ml. Freshly laid eggs (50) were counted under a microscope and exposed to each concentration of the oil. Eggs exposed to tap water mixed with Tween - 80 (0.001%) and ethanol served as control. Chlorpyrifos was taken as positive control and tested at 0.008, 0.004, 0.002, 0.001, 0.0005, and 0.00025 mg/ml concentrations. All the treatments were replicated ten times. After treatment, the eggs from each concentration were individually transferred to distilled water for hatching assessment. Percentage egg viability was calculated by dividing the number of larvae that emerged from the eggs 5 days after treatment (Su & Mulla 1999). Data were analyzed statistically for effective concentration to inhibit egg viability (EC_{50}).

Statistical analysis

Dose-response relationship and regression analysis was performed (Finney, 1971; SPSS, 1999). Mortality data were corrected using Abbott's formula (Abbott, 1925).

Results

Oil extraction

The percentage yield of essential oil in the leaf of *C. longa* (var. CH-66) was 0.61% w/w. The main constituent profile of the oil is given in Table 1. The oil contains myrcene (40.19%), p-cymene (23.05%) and 1,8-cineole (13.16%) as the major compounds. The minor compounds like turmerones which are reported in literature as insect repellent or insecticidal are also present in higher concentration as compared to other cultivars of *C. longa* grown at this institute. The density of the oil was 0.928 ± 0.005 g/ml.

Larvicidal activities

The leaf oil demonstrated toxicities against the third instar larvae of both the mosquito species tested. Larvae of *A. stephensi* were more susceptible (LC_{50} = 0.12 mg/ml) as compared to the larvae of *A. aegypti* (LC_{50} = 0.14 mg/ml) when exposed to *C. longa* leaf oil (Table 2). Linear regression analysis performed on the data showed significant toxicity towards larvae of *A. stephensi* (F = 141.81 df = 6, P<7.36) and *A. aegypti* (F = 275.59 df = 6, P<1.45) (Fig. 1) when exposed to *C. longa* (var. CH-66) leaf oil. The oil showed only 40.0 and 26.0 per cent larval mortality against *A. stephensi* and *A. aegypti* larvae, respectively at the dose of 0.10 mg/ml. Chlorpyrifos taken as standard gave LC_{50} of 0.0013 and 0.0014 mg/ml against *A. stephensi* and *A. aegypti*, respectively.

Ovicidal activities

C. longa leaf oil was found to be less effective in inhibiting the egg viability of both the mosquito species as compared to larvicidal activities. Below 0.15 mg/ml concentration, oil could not show any ovicidal effect towards both the mosquito species. However, at higher concentrations, oil was more effective against both the mosquito species *A. stephensi* (EC_{50} = 0.27 mg/ml) and *A. aegypti* (EC_{50} = 0.29 mg/ml) (Table 2). Dose-response relationship showed concentration dependent ovicidal activity and regression analysis of the data showed significant toxicity toward eggs of *A. stephensi* (F = 31.10, df = 6, P<0.002) and *A. aegypti* (F = 19.95, df = 6, P<0.006) exposed to the oil (Fig.2).

Discussion

With the culminating problem associated with the development of resistance (Curtis & Pasteur, 1981) in mosquitoes and health problem associated with views of conventional pesticide, natural products (Arnason *et al.*, 1989) especially essential oil, may provide useful future alternative means of mosquito control. Quantitative analysis of *C. longa* (var. CH-66) leaf oil showed low concentrations of turmerones with high monoterpenoid content which is comparable to that of previous reports (Bansal *et al.*, 2002; Sakuntala *et al.*, 2002). This study has shown larvicidal potential of *C. longa* leaf oil against the two mosquito species with varied activities. In the present study, GC data (Table 1) revealed that besides myrcene, there are many oxygenated monoterpenes and related compounds present in the oil. Cheng *et al.* (2009) reported larvicidal activity of myrcene against *A. aegypti* and *A. albopictus* with LC_{50} value of 0.36 mg/ml and 0.27 mg/ml, respectively, whereas *C. longa* (var. CH-66) leaf oil showed LC_{50} value of 0.12 - 0.14 mg/ml. Thus activities in this oil may be attributed to synergistic effect of myrcene with other constituents present in the oil. Bioactive fraction of *Cymbopogon nardus* rich in myrcene (8.73%) have been reported for larvicidal activity against *Culex quinquefasciatus* with LC_{50} value of 1.7 mg/l and compound myrcene with LC_{50} value of 0.3 mg/l (Ranaweera, 1996). Similarly, Cheng *et al.* (2013) reported larvicidal activity of myrcene rich oils extracted from leaves and wood of *Cunninghamia konishii,* Hayata against *A. aegypti* (LC_{50} = 35.8 ug/ml) and *A. albopictus* (LC_{50} = 27 ug/ml). Recently, Hung *et al.* (2019) extracted 59.3% and 26.1% myrcene rich essential oils from leaf and stem of *Crassocephalum crepidioide,* respectively and reported 24 hrs larvicidal activities against *Culex quinquefasciatus* (LC_{50} =18.4 ug/ml), *A. aegypti* (LC_{50} = 4.95 ug/ml) and *A. albopictus* (LC_{50} = 14.3 ug/ml). They suggested high concentration of myrcene in essential oils causes mortality in mosquito larvae. In present study the oil contain 43.0% myrcene, therefore activity correlates with this report.

Ajaiyeoba *et al.* (2008) evaluated turmerone rich essential oils of both rhizome and leaf of *C. longa* and found more toxicity in rhizome oil (LC_{50} = 0.017 mg/ml) than the leaf oil (LC_{50} = 0.029 mg/ml) against malarial vector, *Anopheles gambiae*. However, Park *et al.* (2003) did not find contact toxicity by myrcene against adults of *Callosobruchus chinensis*.

Thus activity of the oil against mosquito larvae may be attributed to the additive or synergistic effect of the constituents of the oil. Such an effect has been previously observed with some essential oils where the activity was due to the combination of the major constituents and not by individual compounds (Papachristos *et al.*, 2004). However, we have not tested pure compound myrcene, but the activities displayed by *C. longa* (var. CH - 66) leaf oil may be attributed to the presence of myrcene as well as combination effects of other oxygenated monoterpenes.

The findings of the present study, therefore suggest the use of the whole oil as a local source in controlling mosquito larvae. Only rhizome part of *C. longa* is utilized, whereas leaf part has no use, therefore bioactivities in the leaf oil may have additional advantage. This particular genotype of *C. longa* may be utilized by local people for controlling mosquito larvae in rural and sub urban dwellings.

Conclusions

Leaves of *C. longa* remains untilized and in view of this, essential oils of 25 genotypes of *C. longa* leaves were evaluated against two mosquito species, *A. stephensi* and *A. aegypi*. Genotype CH - 66 was found to be better larvicidal with LC_{50} of 0.12 and 0.14 mg/ml against *A. stephensi* and *A. aegypi*, respectively than ovicidal with EC_{50} of 0.27 and 0.29 mg/ml for *A. stephensi* and *A. aegypti*, respectively. Therefore, bioactivities of the leaf of this particular genotype may find use as leads in controlling vectors spreading dreadful diseases like malaria and dengue fever.

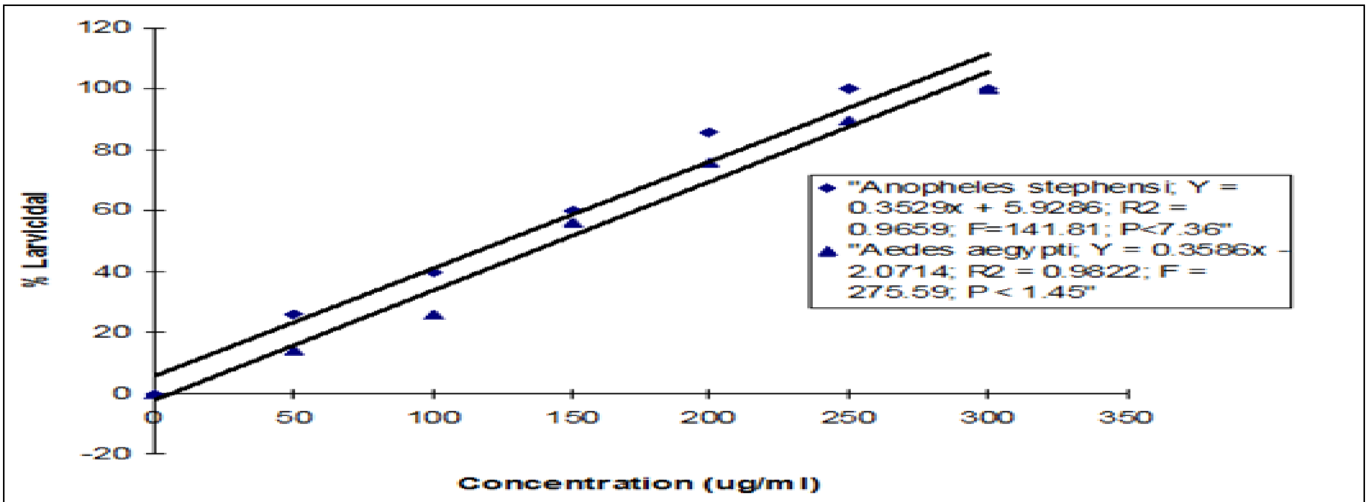

Fig. 1 : Larvicidal activities of myrcene rich essential oil of *C. Longa* (var. CH-66) towards 3rd instar larvae of mosquito species

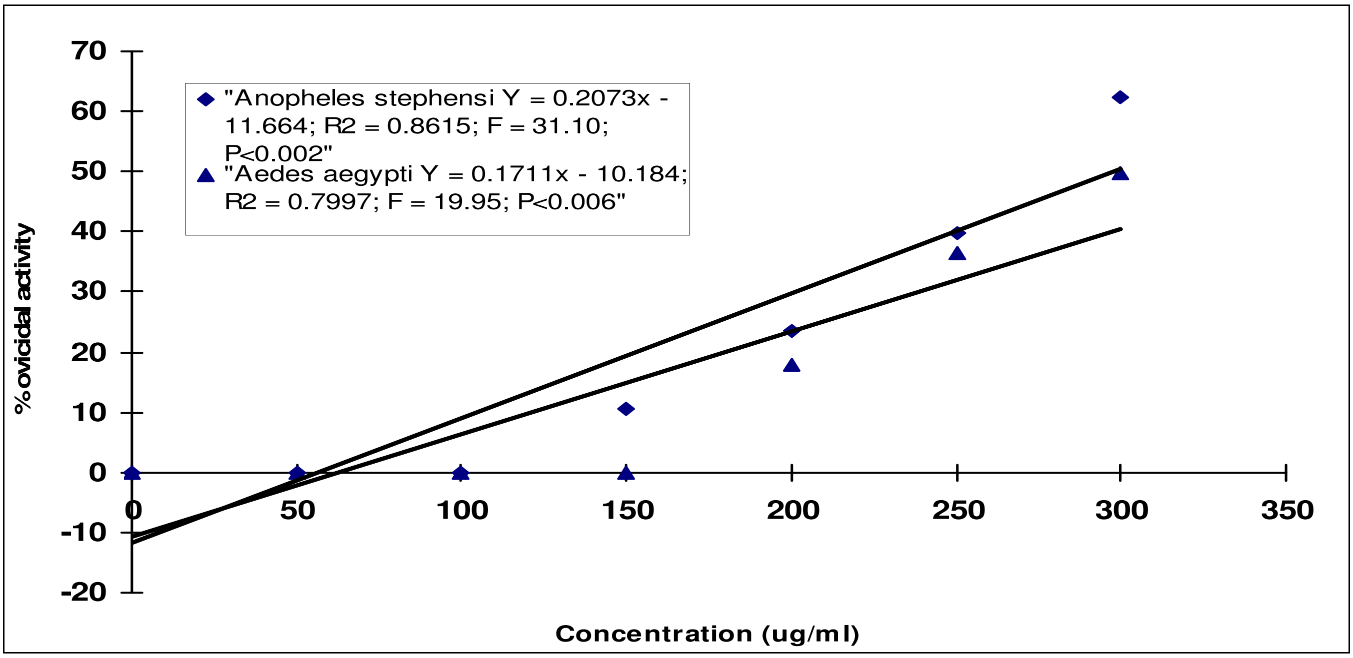

Fig. 2 : Ovicidal activities of myrcene rich essential oil of *C. Longa* (var. CH-66) against eggs of mosquito species

Table 1 : Main constituents of the leaf essential oil of *C. Longa* (Var. CH-66)

Compound name	% content in oil
α-Pinene	1.83
β-Pinene	3.42
Myrcene	40.19
1,8-Cineole	13.16
γ-Terpinene	2.03
p-Cymene	23.05
Terpenolene	0.11
Linalool	1.37
p-Cymene	0.17
Myrtenal	0.53
ar-Turmerone	0.71
α-Turmerone	3.0
β-Turmerone	1.06

Table 2 : Lethal concentrations of *C. longa* (var. CH-66) leaf essential oil causing 50% mortality in larvae and eggs of *A. stephensi* and *A. aegypti*

Substance	**Mosquitospecies**			
	A. stephensi		*A. aegypti*	
C. longa oil	Larvicidal (LC_{50}) (mg/ml)	Ovicidal (EC_{50}) (mg/ml)	Larvicidal (LC_{50}) (mg/ml)	Ovicidal (EC_{50}) (mg/ml)
LC_{50}/EC_{50}	0.12	0.27	0.14	0.29
LCL	0.094	0.251	0.135	0.251
UCL	0.141	0.283	0.153	0.275
Chlorpyrifos				
LC_{50}	0.0013	-	0.0014	-
LCL	0.0009	-	0.001	-
UCL	0.002	-	0.004	-

LC_{50}: Lethal concentration to kill 50% larvae, EC_{50}: Lethal concentration to inhibit 50% egg viability, LCL: Lower Confidence Limit, UCL: Upper Confidence Limit

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Conflicts of interest

There are no apparent conflicts of interest.

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