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CHEMICAL COMPOSITION AND LARVICIDAL PROPERTIES OF *CLAUSENA ANISATA* (WILLD.) HOOK. F. EX BENTH (RUTACEAE) ESSENTIAL OIL AGAINST *ANOPHELES SUBPICTUS* AND *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE)

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A R T I C L E I N F O

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A B S T R A C T

Objective: To determine the mosquito larvicidal activity of leaf essential oil and their chemical constituents from *Clausena anisata* (Willd.) Hook. f. ex Benth. against *Anopheles subpictus* and *Aedes albopictus*. In addition, the chemical composition of the leaf essential oil was analyzed using gas chromatography–mass spectrometry.
Methods: Twenty late III instar larvae of two mosquito species were exposed to based on the wide range and narrow range tests, essential oil was tested at 50, 100, 150, 200 and 250 ppm and each compound was tested at various concentration (5-75 ppm) and were assayed in the laboratory by using the protocol of WHO 2005; the 24 h LC_{50} values of the *Clausena anisata* leaf essential oil and their major compounds were determined by Probit analysis.
Results: The oil contained mainly β-pinene (32.8%), sabinene (28.3%), germacrene-D (12.7%), estragole (6.4%) and linalool (5.9%). The essential oil from the leaves of *Clausena anisata* exhibited significant larvicidal activity, with 24 h LC_{50} values of 122.57and 134.08 ppm, respectively. The five pure constituents extracted from the *Clausena anisata* leaf essential oil were also tested individually against two mosquito larvae. The LC_{50} values of β-pinene , sabinene , germacrene-D, estragole and linalool appeared to be most effective against *Anopheles subpictus* (LC_{50} – 24.68, 20.93, 17.16, 12.25, 36.26 ppm) followed by *Aedes albopictus* (LC_{50} – 28.10, 22.66, 19.51, 13.01, 39.99ppm).
Conclusions: The present findings suggest that the leaf essential oil of *Clausena anisata* and its five major compounds might be considered as a potent source for the production of natural larvicides.

INTRODUCTION

The medical importance of mosquitoes as vectors for the transmission of serious diseases that cause morbidity, mortality, economic loss and social disruption such as malaria, lymphatic filariasis and viral diseases is well documented [1]. *Aedes albopictus (Ae. albopictus)*, a vector of dengue, is widely distributed in tropical and subtropical zones. Dengue fever incidence has increased fourfold since 1970 and nearly half the world's population is now at risk. In 1990, almost 30% of the world population, 1.5 billion people, lived in regions where the estimated risk of dengue transmission was greater than 50% [2]. An outbreak of chikungunya virus infection emerged in the southwest Indian Ocean islands in 2005, spread out to India, and resulted in an ongoing outbreak that has involved >1.5 million patients, including travelers who have visited these areas [3]. *Anopheles subpictus* (*An. subpictus*) is known to transmit malaria and filariasis, in an isolated study of multiple host-feeding in field populations, and its specific role in transmitting malaria in Sri Lanka revealed that multiple blood feeding within the same gonotrophic cycle was attributed to a local "frequent feeding strategy" in this primarily zoophagic and endophilic malaria vector. On the contrary, in Indonesia, *An. subpictus* is a potential vector of bancroftian filariasis and fed on microfilaraemia carriers that harbored *Wuchereria bancrofti* larvae [4]. *An. subpictus* breeds profusely in rainwater accumulations and fallow rice fields, waste water disposal systems, and irrigated sites, and is also associated with floating and submerged aquatic vegetation in the vicinity of rice plants [5]. Night time human biting collection in Rajasthan, India, showed two

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feeding peaks for *An. subpictus*, one early in the night and the other just before dawn [6].

Synthetic pesticides have been extensively used for the control of mosquitoes in different ways as adulticides, repellents, and deterrents or as larvicides at the breeding sites of the vectors [7]. Although effective, their repeated use has disrupted natural biological control systems resulting in the widespread development of resistance. The accumulation and adverse effects of synthetic pesticides imposed on the environment and non-target organisms have demanded the necessity for developing alternative strategies. It has been shown that the use of botanicals as mosquito control agents can be effectual in minimizing these adverse impacts due to their eco-safety, target specificity, negligible resistance, reduced number of applications, higher acceptability, and suitability for rural areas [8]. Many researchers have reported that extracts from various plants can be used as effective and advantageous alternatives to synthetic insecticides or along with other insecticides under integrated vector control programs for the control of mosquitoes [9].

Essential oils are natural volatile substances obtained from a variety of plants. Commercially, essential oils have many uses such as pharmaceuticals, flavor in many food products, and odorants in fragrances. Also, particular emphasis has been laid on their antibacterial, antifungal, and insecticidal activities. These have received much attention as potentially useful bioactive compounds against insects in terms of their growth regulation, fecundity suppression, male sterility, larvicidal, ovicidal, and oviposition activity mostly as deterrence because of their low mammalian toxicity and rapid degradation in the environment [10]. Thus, many researchers were intrigued to exploit essential oils as a potential source for the identification of novel natural pest control agents, with a strong focal point on mosquito control [11]. Previous literature findings have indicated that the essential oils of the Rutaceae family plants constitute efficient natural mosquito control agents [12]. Essential oils provide a rich source of biologically active monoterpenes and are well documented for bioactivities against insect pests. Some of the essential oils with promising mosquito control potential are plant from genus *Tagetes* spp. [13], *Ocimum* spp. [14], *Cymbopogon* spp. [15], and *Mentha* spp. [16] etc. Further, essential oils of cassia, camphor, wintergreen, pine, and eucalyptus are already being used in several commercial products for mosquito control [17]. The essential oils are generally considered nontoxic to human beings [18] apart from their uses in flavoring, pharmaceuticals, and confectionary industries. In this study, *Clausena anisata* (*C. anisata*) leaf essential oil and its major compounds were tested as larvicides against *An. subpictus* and *Ae. albopictus* larvae.

MATERIALS AND METHODS

Plant material and extraction of essential oil

The leaves of *C. anisata* were collected from the Sirumalai hills, Dindugal District, India. It was authenticated by a plant taxonomist from the Department of Botany, Annamalai University. A voucher specimen is deposited at the herbarium of plant phytochemistry division, Department of Zoology, Annamalai University. Essential oil was obtained by the hydro- distillation of 3 kg fresh leaves in a Clevenger apparatus for 4 h. The oil layer was separated from the aqueous phase using a separating funnel. The resulting essential oil was dried over anhydrous sodium sulphate and stored in an amber-coloured bottle at 8 °C for analysis.

Gas chromatography analysis

Analysis was carried on a varian-gas chromatograph equipped with a flame ionization detector and a BPI (100 % dimethyl polysiloxane) capillary column. Helium at a flow rate of 1.0 ml min^{-1} and 8 psi inlet pressure was employed as a carrier gas. Temperature was programmed from 60 to 220^{o}c at 5 ^{o}c min^{-1} with a final hold time of 6 min .The injector and detector temperatures were maintained at 250 and 300^{o}c, respectively. The sample (0.2 µl) was injected with 1:20 split ratio.

Gas chromatography –mass spectrometry analysis

Gas chromatography –mass spectrometry (GC-MS) analysis was performed on an Agilent 6890 GC equipped with 5973 N mass selective detector and an HP-5(5% phynyl methylpolysiloxane) capillary column. The oven temperature was programmed from 50 to 280^{o}c at the rate of 4^{o}c min-1and held at this temperature for 5 min. The inlet and interface temperatures were 250 and 280^{o}c, respectively. The carrier gas was helium at a flow rate of 1.0 ml min^{-1} (constant flow).The sample (0.2µl) was injected with a split of 20:1. Electron impact mass spectrometry was carried out at 70 eV. Ion source and quadrupole temperatures ware maintained at 230 and 150 ^{o}c respectively.

Mosquitoes

The mosquitoes, *An. subpictus* and *Ae. albopictus* were reared in the vector control laboratory, Department of zoology, Annamalai University. The larvae 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. Mosquitoes were held at 28 ± 2^{0}, 70 - 85 % relative humidity (RH), with a photo period of 12 h light, 12 h dark.

Larvicidal activity

Larvicidal activity of the essential oil and its five major compounds (β-pinene (32.8%), sabinene (28.3%), germacrene-D (12.7%), estragole (6.4%) and linalool (5.9%)) isolated from *C. anisata* leaves were evaluated according to WHO protocol [19]. Based on the wide range and narrow range tests, essential oil was tested at 50, 100, 150, 200 and 250 ppm and each compound was tested at various concentrations (5-75 ppm). Essential oil or/and individual compounds were dissolved in 1 ml DMSO, then diluted in 249 ml of filtered tap water to obtain each of the desired concentrations. The control was prepared using 1 ml of DMSO in 249 ml of water. Twenty late third instar larvae were then introduced into each solution. For each concentration, five replicates were performed, for a total of 100 larvae. Larval mortality was recorded at 24 h after exposure, during which no food was given to the larvae.

The lethal concentrations (LC_{50} and LC_{90}) were calculated by probit analysis [20]. 1, 3 and 2

Statistical analysis

The average larval mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90} and other statistics at 95% confidence limits of upper confidence limit and lower confidence limit, and chi-square values were calculated using the SPSS12.0 (Statistical Package of Social Sciences) software. Results with $p<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The yield of *C. anisata* leaf essential oil was 4.8 ml/kg fresh weight. The oil was a pale yellow colour. Table 1

Table 1 Chemical composition of leaf essential oil from *Clausena anisata*.

Peak	Compounds	Retention time (Kovats index)	Concentration (%)
1	α-Pinene	940	1.2
2	Sabinene	974	28.3
3	β- Pinene	977	32.8
4	Myrcene	992	1.6
5	α- Phellandrene	1004	0.2
6	ρ-Cymene	1026	0.2
7	Limonene	1029	1.2
8	1,8-cineole	1031	0.8
9	γ -Terpinene	1067	0.3
10	Linalool	1100	5.9
11	Estragole	1192	6.4
12	β- Elemene	1395	0.4
13	β-Caryophyllene	1425	1.3
14	α-Caryophyllene	1459	1.5
15	α- Humulene	1458	1.8
16	Germacrene- D	1487	12.7
17	Germacrene- A	1517	0.9
18	Caryophyllene oxide	1587	0.7

shows the constituents of the essential oil, their percentage composition and their Kovats Index (KI) values listed in order of elution. A total of 18 compounds representing 98.2% of the essential oil were identified. The major constituents of this oil were β-pinene (32.8%), sabinene (28.3%), germacrene-D (12.7%), estragole (6.4%) and linalool (5.9%). The percentage compositions of remaining thirteen compounds ranged from 0.2% to 1.8%. The 24 h larvicidal results of essential oil and its five major compounds against *An. subpictus* and *Ae. albopictus* larvae are presented in Tables 2 and 3.

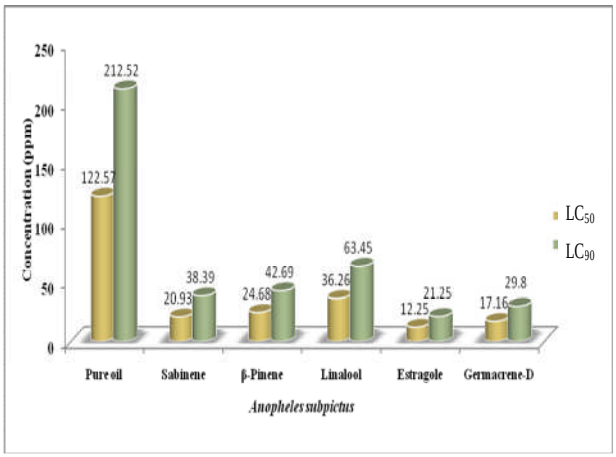

Fig. 1 LC_{50} and LC_{90} values of *Anopheles subpictus* at different concentrations of pure oil and five major active compounds

Table 2 Larvicidal activity of essential oil from *Clausena anisata* against *Anopheles subpictus* and *Aedes albopictus.*

Mosquito	Concentration	24 h mortality	LC_{50} (ppm)	95% Confidence Limits (ppm)		LC_{90} (ppm)	χ^2
				Lower	Upper		
An. subpictus	Control	0.0±0.0	122.57	97.16	147.81	212.52	14.352*
	50	22.8±0.8					
	100	40.2±0.6					
	150	59.8±1.0					
	200	82.5±1.4					
	250	99.4±1.2					
Ae. albopictus	Control	0.0±0.0	134.08	109.81	159.13	230.29	12.599*
	50	19.8±1.0					
	100	36.4±0.8					
	150	52.1±1.2					
	200	77.2±1.4					
	250	96.9±0.6					

*Significant at P<0.05 level.

Table 3 Larvicidal activity of five major compounds based on the concentration (%) from essential oil of *Clausena anisata* against *Anopheles subpictus* and *Aedes albopictus* larvae.

Compounds	Mosquitoes	LC_{50} (ppm)	95% Confidence Limits (ppm)		LC_{90} (ppm)	χ^2
			LCL	UCL		
Sabinene	*An. subpictus*	20.93	15.04	26.37	38.39	17.633*
	Ae. albopictus	22.66	17.72	27.41	40.38	13.408*
β- Pinene	*An. subpictus*	24.68	20.41	28.91	42.69	10.487*
	Ae. albopictus	28.10	23.13	33.38	47.63	13.128*
Linalool	*An. subpictus*	36.26	29.37	43.04	63.45	11.799*
	Ae. albopictus	39.99	32.77	47.42	68.36	12.639*
Estragole	*An. subpictus*	12.25	9.71	14.78	21.25	14.352*
	Ae. albopictus	13.01	10.54	15.50	23.36	13.179*
Germacrene- D	*An. subpictus*	17.16	13.62	20.68	29.80	14.194*
	Ae. albopictus	19.51	15.91	23.32	33.29	13.763*

*Significant at P<0.05 level.

The essential oil from the leaves of *C. anisata* exhibited significant larvicidal activity, with 24 h LC_{50} values of 122.57and 134.08 ppm, respectively. The five pure constituents extracted from the *C. anisata* leaf essential oil were also tested individually against three mosquito larvae. The LC_{50} values of β-pinene , sabinene , germacrene-D, estragole and linalool appeared to be most effective against *Anopheles subpictus* (LC_{50} – 24.68, 20.93, 17.16, 12.25, 36.26 ppm) followed by *Aedes albopictus* (LC_{50} – 28.10, 22.66, 19.51, 13.01, 39.99ppm). Chi-square values of the essential oil and its five compounds show significant larvicidal activity.

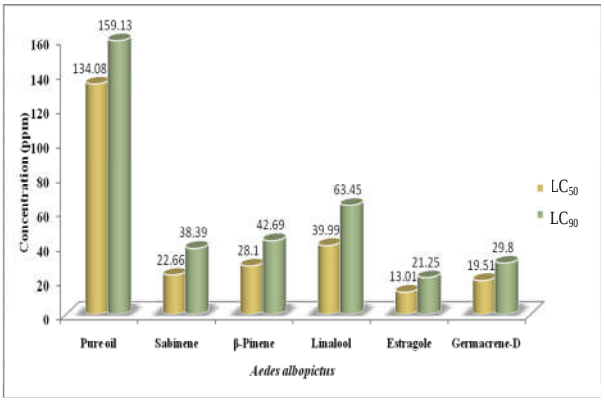

Fig. 2 LC_{50} and LC_{90} values of *Ae. albopictus* at different concentrations of pure oil and five major active compounds

Phytochemicals may serve as suitable alternatives to synthetic insecticides in future as they are relatively safe, inexpensive, and are readily available in many areas of the world. In the search for an ecofriendly pesticide, researchers have considered pesticides of biological origin, and the replacement of chemical pesticides with biopesticides as a generally acceptable one. Different parts of plants contain a complex of chemicals with unique biological activity which is thought to be due to toxins and secondary metabolites, which act as attractants or deterrents [21]. In my result showed that essential oil of the leaf of *C. anisata* have significant larvicidal activity. This result is also comparable to earlier reports of Singh *et al.* [22] who observed the larvicidal activity of *Ocimum canum* oil against vector mosquitoes namely, *Ae. aegypti* and *Cx. quinquefasciatus* (LC_{50} 301 ppm) and *An. stephensi* (LC_{50} 234 ppm). The larvicidal, growth inhibitor and repellent actions of *D. sissoo* oil against *Anopheles stephensi, Aedes aegypti* and *Culex quinquefasciatus* under laboratory conditions. 100 % mortality of *Cx. quinquefasciatus* immatures was observed within 24 h at 4 ml/m2, followed by *Ae. aegypti* (90%) and *An. stephensi* (60%), and pupation was totally inhibited [23]. Essential oil from *Ipomoea cairica* Alba against *Culex, Aedes*, and *Anopheles* larvae ranged between 15 and 162ppm [24]. Traboulsi *et al.* [25] reported that the larvicidal activity of essential oils of *Citrus sinensis*, *Eucalyptus* spp., *Ferrula hermonis, Laurus nobilis*, and *Pinus pinea* against *Cx. pipiens*.LC_{50} values were 60.0, 120.0, 44.0, 117.0, and 75.0 ppm, respectively.

Carvalho *et al.* [26] demonstrated that these alkylated phenol derivatives, carvacrol and thymol, which were the major components of *Lippia sidoides* Cham essential oil, showed divergent toxicity on *Ae. aegypti* larvae. Thymol was much more potent than carvacrol causing 100% mortality at a concentration of 0.017% (w/v) within 1.5 h after treatment. Cavalcanti *et al.* [27] reported that the larvicidal activity of essential oils from Brazilian plants with LC_{50} values ranging from 60 to 69 µg /ml against *Ae. aegypti* larvae. Rahuman *et al.* [28] also found that n-hexadecanoic acid in *Feronia limonia* dried leaves was effective against fourth-instar larvae of *Cx. quinquefasciatus*, *An.stephensi* and *Ae. aegypti* with LC_{50} values of 129.24, 79.58, and 57.23 µg /ml, respectively. While the essential oils of *Abuta grandifolia* and *Minthostachys setosa* demonstrated lower LC_{50} values, 2.6 and 9.2ppm, respectively, against the larvae of *Ae. aegyti* [29]. Traboulsi *et al.* [30], who analyzed the main constituents of *Mentha*, *Myrtus* and *Origanum* spp., reported that carvacrol, thymol, and alpha- pinene, with LC_{50} values of 36–49 ppm, were more toxic than linalool (LC_{50}=155ppm) when tested against *C. pipiens* larvae. Moreover, the LC_{50} values of carvacrol, thymol, and linalool were similar to those we found for the same compounds against *Oc. caspius.* Compared with earlier reports, our results revealed that the experimental plant essential oil were effective to control *An. subpictus* and *Ae. albopictus*. This study reveals that the essential oil of *C. anisata* has remarkable larvicidal properties. The flora of India has rich aromatic plant diversity with potential for development of natural insecticides for control of mosquito and other pests. In brief, our findings suggested that the essential oil from *C. anisata* leaves and its effective constituents may be explored as a potential environmental-benign larvicide. Further investigations for the mode of the constituents' actions, effects on non-target organisms and field evaluation are necessary. These results obtained are useful in search of more selective, biodegradable and naturally produced larvicidal compounds.

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