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Toxicity of essential oils on *Aedes aegypti*: A vector of chikungunya and dengue fever

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Abstract
Vector-borne diseases such as chikungunya, dengue, encephalitis, filariasis, and malaria pose an enormous menace to humans. Use of larvicidal strategies acting against the immature stages of mosquitoes might be useful in combating these diseases. The available synthetic insecticides have serious side effects, are toxic to the environment and the insects probably have higher chances of developing resistance against them. As compared to synthetic pesticides, essential oils (EOs) are ecologically safe, have no mammalian toxicity or the chances of development of resistance are reasonable and highly popular with the organic growers. In the present investigation, we have studied the larvicidal properties of four different concentrations (10, 50, 100 and 500 mg/l) of 3 essential oils (EOs), namely, citronella (*Cympobogon nardus*), eucalyptus (*Eucalyptus globulus*), and patchouli (*Pogostemon cablin*) against the larval stage of *Aedes aegypti* mosquitoes. Biochemical and histopathological changes in the *Aedes aegypti* larvae after exposure to essential oils were studied. Essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS) for chemical compositions. The highest larvicidal activity was observed in the EOs from patchouli oil against the larvae of *Aedes aegypti* with LC_{50} (lethal concentration required to kill 50% of the population) value 25.14 mg/l respectively. LC_{50} values for citronella, eucalyptus and patchouli oils were found to be 38.37, 51.93, 25.14 mg/l respectively. Glucose, total protein and urea levels were misbalanced in the larvae after oil exposure. A serious damage in brush border, digestive cells, basal membrane, epithelium, and peritrophic membrane in the mid gut of the essential oil treated larvae were observed under histopathological study. The major aromatic compounds identified by the GC-MS were linalool, citronellol, citronellal and β-Citral in citronella oil; eucalyptol, d-pinene, and o-cymene in eucalyptus oil; patchouli alcohol and seychellene in patchouli oil respectively. A concentration-dependent larvicidal effect was shown by the EOs against *Aedes aegypti* larva. The aromatic compounds identified in the EOs triggered significant responses in mortality. EOs can be applied as green pesticide which could be effective to control the vector borne diseases.

Keywords: *Aedes aegypti*, essential oils, GC-MS, mosquitoes

Introduction
Mosquitoes comprising the genera *Aedes*, *Anopheles* and *Culex* are common vectors for the pathogens of different diseases like chikungunya, dengue, polyarthritis, filariasis, Japanese encephalitis, malaria, and yellow fever [1, 2, 3]. Female mosquitoes are the major vector to transmit diseases causing serious health problems among people living in developing countries of the tropical and subtropical zones [4].

Most common approach to vector-borne disease is by chemical control, mostly through the use of insecticides [5]. Larviciding is an effective tool to decrease mosquito populations before they emerge as adults [6, 7]. The chemical method is an effective weapon for quick and easy use because such chemicals, even in smaller quantities, are sufficient for the control of a large population of mosquitoes. Although synthetic organic insecticides are very effective, they are ecologically unsound and have many bad impacts, resulting in ecological hazards [8]. This would be highly dangerous for the environment and there are higher chances of developing resistance in insects and affecting the nontarget organisms [9, 10]. Deltamethrin is widely used for mosquito larvicidal and adulticidal purpose. Due to the involvement of hydrolytic esterases, mono-oxygenases, or a knockdown resistance based mechanism, different authors have recently reported the development of resistance in mosquitoes against deltamethrin [11].

It is, therefore, pertinent, to explore the mosquito larvicidal and adulticidal activity of natural products, mainly essential oils (EOs) [12, 13].

Several researchers suggested that EOs are composed of various potent bioactive compounds that are having larvicidal activities against various pests and mosquitoes [14, 15, 16]. A study report [17] indicated camphor, clove and eucalyptus oil to exhibit larvicidal potential against the larvae of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* respectively. EOs may be an alternative to synthetic insecticides by virtue of their eco-friendly, inexpensive, easily biodegradable effectiveness [18, 19]. Such plant-based EOs contains phytochemicals that are biodegradable, safe for the non-target organism and have been also used as pesticides for many years to manage the agricultural pests [20]. These phytochemicals have previously shown different properties such as antiviral, bactericidal, fungicidal, insecticidal, insect growth inhibitor [21, 22] and are known to cause toxic effects on various insects. The aim of this present investigation was to evaluate the toxicity of 3 promising EOs against *Aedes aegypti* mosquitoes.

Materials and Methods

Chemicals and reagents

Citronella and eucalyptus oil were purchased from Surajbala Exports Pvt. Ltd, Gurgaon, India. Patchouli oil was received as gift sample from the Brahmaputra valley aromatic oil industry, Assam, India. Deltamethrin (99.6%) was purchased from Sigma-Aldrich Laborchemikalien, Germany. Acetone and n-hexane were purchased from Merck Specialities Pvt. Ltd., Mumbai, India.

Rearing of Mosquitoes

Wooden cages (750 x 600 x 600 mm) were used in the Medical Entomology Division of Defence Research Laboratory, Tezpur, India, for the rearing of the yellow fever mosquitoes *Aedes aegypti* (Diptera: culicide). The female mosquitoes were fed New Zealand albino rabbits for blood meal initially for two days and then every alternative day. For nourishment, cotton with 10% sugar solution was provided. Filter paper strip in a 250 ml beaker containing fresh water was kept in the cages for egg laying. Collected eggs were transferred to a bowl containing two litres of water for the rearing of hatched larvae up to the adult stage. The larvae were fed on dog biscuits and Brewer's yeast powder in a ratio of 1:1 and water were changed on each alternate days. Collected pupae were kept in small cages covered with a cotton cloth for emerging into the adult [23, 24].

Maintenance of rabbits

Rabbit used for the blood meal to the mosquitoes was individually housed. The temperature and humidity of the animal room were maintained at 24±2°C and 60±10% respectively. Lighting was artificial, the sequence being 12 h light, 12 h dark. For feeding, conventional laboratory diets were used with an unrestricted supply of drinking water.

Larval Bioassay

The third instar larvae of *Aedes aegypti* mosquito was used for the larvicidal property of the EOs, according to the WHO protocol [25]. In every 100 ml of the glass beaker, twenty-five 3rd instar larvae were transferred. The essential oils were tested at 4 different concentrations as follows 10, 50, 100 and 500 mg/l respectively. Oils were dissolved in acetone as a stock solution and added to the beakers to produce the desired concentration in 100 ml of dechlorinated water. For deltamethrin (positive control), the test concentrations [26] were 0.00001, 0.00005, 0.00010, 0.00050 mg/l respectively [27]. The test was replicated three times for each test concentrations. A total number of dead larvae in each beaker were counted after 24 h, and percentage of larval mortality was calculated.

Biochemistry

Groups of the 50 fourth instar larvae of *Aedes aegypti* were exposed to 100 ml of LC_{50} of citronella, eucalyptus and patchouli oil for 1 h. The control group was exposed to 100 ml of double distilled water with 0.5% acetone. A solution of 10 ml 20 mM Tris buffer with pH 7.3 was used to homogenate the larvae and were centrifuged for 3 min at 1500 rpm. The supernatant was used for the determination of total protein, glucose and urea contents by using the Biochemical analyzer (Corralyzer 100, Tulip group, India).

Histopathology

Exposed (citronella, 500 mg/l after 24 h) and control (untreated) were collected and fixed in 10% formalin solution. After 48 h of formalin fixation, the larvae were preserved in 70% ethanol. Larvae were embedded in paraffin wax at 58-60 °C to prepare sections of 5µm thick by a microtome (Spencers, Fully automated microtome, Model no.: 1010-SMT-118) and stained with hematoxylin and eosin. Images (40X) were digitally acquired using a light microscope (ZEISS Scope A1, Axio Cam ERc 5s, Germany).

Mass spectrometric identification

Sample preparation

A stock solution of 1000 mg/l concentration was prepared in n-hexane for essential oil as well as standard deltamethrin. The samples were further diluted to 100 mg/l for evaluation under gas chromatography-mass spectroscopy (GC-MS). GC (GC-7890B) and MS (5977A MSD) system of Agilent technologies and Agilent J&W GC column (HP-5 MS UI) having 30 meters of length, 0.25 mm of diameter and the film thickness of 0.25 µm were used. Helium was used as carrier gas at flow rate 1ml/min. Oven temperature was programmed from 40-300 ºC at 20 ºC/min. The injector temperature was set at 250 ºC and detector temperature was set at 230 ºC (quad) and 150 ºC (core) respectively [28]. Identification of the components in the test samples was done with the aid of NIST-14 mass spectra search software.

Statistical analysis

The average larval mortality data were subjected to regression analysis using probit table. The biochemical data were submitted to one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison tests.

Results

Larval bioassay

Among the three EOs, the highest larvicidal activity was observed in patchouli oil, with LC_{50} (lethal concentration

required to kill 50% of the population) values 25.14 mg/l respectively. The order of the EOs with their LC_{50} values (in mg/l) after 24 h of exposure were, eucalyptus (51.93)> citronella (38.37)> patchouli (25.14). Table 1 represents the relative toxicity of EOs against the 3rd instar larvae of *Aedes aegypti* mosquitoes.

Table 1: Relative toxicity of essential oils with standard pesticide against 3rd instar larvae of Aedes aegypti mosquitoes after 24 h of treatment

Essential oils	LC_{50} (mg/l)	95% confidence limits (mg/l)	Fit of probit line		
		Lower-Upper	R square	P-value	t-stat
Citronella	38.37	0.006-5.436	0.902	0.049	0.431
Eucalyptus	51.93	1.018-4.6022	0.957	0.021	6.749
Patchauli	25.14	2.501-2.975	0.999	0.0004	49.67
Deltamethrin (Positive control)	0.00001	0.753-3.273	0.959	0.020	6.877

Biochemistry

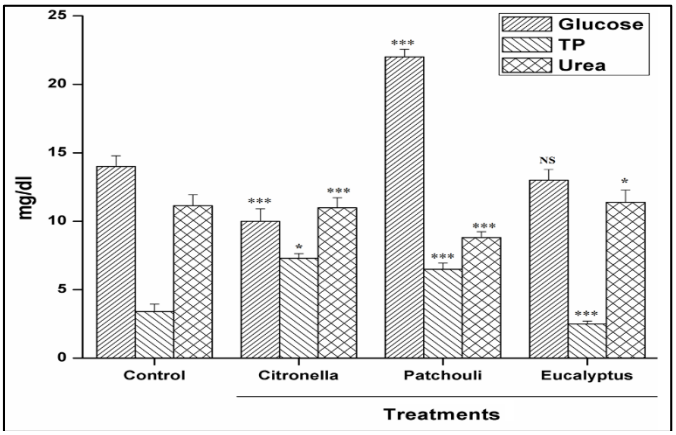
Citronella and eucalyptus oil decreased the glucose level by 28% and 7.1% as compared to control respectively. Patchouli oil increased the glucose level by 57.1% respectively.

Total protein was increased by 114.7% and 91.1% for citronella and patchouli oil, whereas eucalyptus oil decreased the protein level by 26.4% in the 4th instar larva, as compared to the control.

There were no significant changes in urea level, except the patchouli oil (decreased 20.9%) as compared to the control respectively (Table 2, Fig 1).

Table 2: Biochemical parameters for the mosquito larvae treated with essential oils

Treatments	Glucose (mg/dl)		Total protein (TP) (mg/dl)		Urea (mg/dl)	
	Mean	±SD	Mean	±SD	Mean	±SD
Citronella	10	0.91	7.3	0.33	10.98	0.74
Eucalyptus	13	0.78	2.5	0.21	11.39	0.89
Patchouli	22	0.56	6.5	0.45	8.81	0.43
Control	14	0.78	3.4	0.55	11.14	0.80



*** = $P<0.001$
** = $P<0.01$
* = $P<0.05$
NS = not significant

Fig 1: Biochemical parameters. Effect of essential oils on the contents of glucose, total protein and urea in *Aedes aegypti* larvae (Means ± SD, n=6)

Histopathology

The larvae of the control group showed a well developed brush border, basal membrane, digestive cells, intestinal epithelial cells, nucleus and peritrophic membrane. On the contrary of the control group, the mid gut portion of the citronella oil (500 mg/l) treated larvae showed a serious damage in brush border, digestive cells, basal membrane, epithelium, and peritrophic membrane (Fig.2).

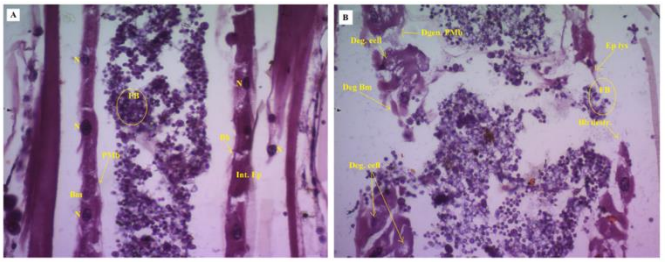


Fig 2: Histopathology of mosquito larvae. Longitudinal section of the midgut of 3rd instar larvae of *Aedes aegypti* mosquito (a) Larval midgut of the control group showing well developed brush border (Bb), basal membrane (Bm), digestive cells (DC), food bolus (FB), intestinal epithelial (Int. ep), nucleus (N) and peritrophic membrane (PMb). (b) Midgut of larvae exposed to 500mg/L citronella oil, showing destructive brush border (Bb destr.), degenerative digestive cells (Deg. DC), degenerative basal membrane (Deg. Bm) and cells (Deg. cells), epithelial lyses (Ep lys), degeneration in peritrophic membrane (Degn. PMb), distribution of food bolus (FB) etc.

Mass spectrometric analysis

From the GC-MS data, β-Citral, citronellal, citronellol and δ-pinene were found as the major aromatic compounds in citronella oil; eucalyptol, linalool, and o-cymene in eucalyptus oil and patchouli alcohol, seychellene in patchouli oil respectively. Standard larvicide deltamethrin was also identified by mass spectroscopy (Fig 3). Table 3 represents the identified components of the three EOs by GC-MS along with their chemical formula, KI, and RT with respect to NIST-14.

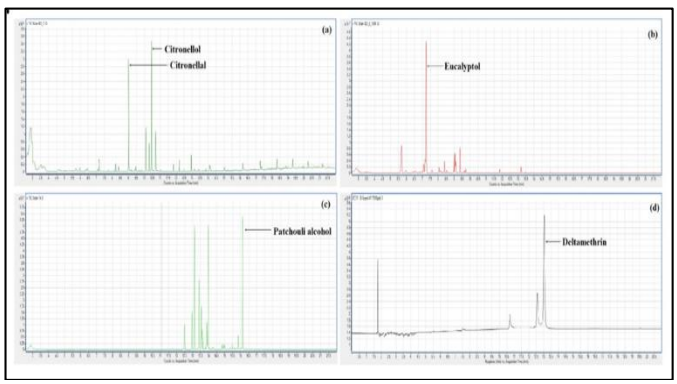


Fig 3: Identification of major chemical compositions by GC-MS. (a) citronella oil, (b) eucalyptus oil, (c) patchouli oil, (d) deltamethrin (positive control)

Table 3: Identified components of essential oils, with their formula, KI, RT and probability (%) with respect to NIST

Sl. No.	Essential oils	Identified components	Chemical Formula	KI*	RT**	Probability (%) with respect to NIST-14
1	Citronella	Linalool	$C_{10}H_{18}O$	1099	8.217	57.5
		Citronellal	$C_{10}H_{18}O$	1153	9.024	50.3
		Citronellol	$C_{10}H_{20}O$	1228	10.106	47.7
		β-Citral	$C_{10}H_{16}O$	1240	10.313	51.9
2	Eucalyptus	Eucalyptol	$C_{10}H_{18}O$	1032	7.235	94
		δ-Pinene	$C_{10}H_{16}$	929	8.684	24
		o-Cymene	$C_{10}H_{14}$	1022	7.09	26
5	Patchauli	Patchouli alcohol	$C_{15}H_{26}O$	1660	16.099	62
		Seychellene	$C_{15}H_{24}$	1459	13.403	51

KI*= Kovats Index
RT**=Retention Time

Discussion

Herbs with insecticidal properties have several advantages over the use of synthetic insecticides. Natural products are easily obtainable and the production cost is very less as compared to the other synthetic marketed products. The development of insect resistance is slow and they do not leave residues in environment, as they are obtained from renewable resource and quickly degrade [29]. Therefore, using EOs to control mosquitoes is an environmentally safe option as compared to synthetic pesticides. EOs from citronella, calamus, thymus and eucalyptus have been promising enough in killing mosquito larva [30, 31, 32, 33, 34, 35]. In our study, the LC_{50} values for citronella oil against the larvae of *Aedes aegypti* was recorded as 38.37 mg/l and for eucalyptus oil, the LC_{50} value was 51.93 mg/l respectively. Manimaran *et al*. (2012) suggested the LC_{50} values of citronella oil as 47.21and 80.93 mg/l, against the larvae of *Aedes aegypti and Anopheles stephensi* mosquitoes [36] whereas in our study the LC_{50} values of eucalyptus oil was found to be 47.61 mg/l for *Aedes aegypti* respectively. They also reported that, after 24 h of exposure time, 1000 mg/l concentration of patchouli oil kills 68% larvae of *Culex quinquefasciatus* mosquitoes. In the current study, patchouli oil showed the best result among the three EOs. It recorded the LC_{50} values of 25.14 against the *Aedes aegypti* larvae after 24 h of exposure. A study [37] reported the LC_{50} value of 161 mg/l against the larvae of *Aedes aegypti*, for eucalyptus oil. Dorta *et al*. (1993) reported the LC_{50} of deltamethrin against *Aedes aegypti* and *Anopheles stephensi* as 0.000008 mg/l and 0.005 mg/l [27]. Deltamethrin, a synthesised product of pyrethrum is an established and potent insecticide. In our study, deltamethrin was used as a standard larvicide (positive control) and the LC_{50} values against the third instar larva of *Aedes aegypti* were found to be 0.00001 mg/l respectively. As compared to deltamethrin, though the three EOs are less potent in case of larvicidal activity, they could be an alternative to reduce the pyrethroid resistance in mosquitoes and which do not cause any harmful effects to the environment. The variation in LC_{50} values in a different research study for the same test and standard substances might be due to some developmental, environmental, experimental or other factors. In an another study, [38] among the 10 plant oils (lemongrass, palmarosa, cedarwood, citronella, clove, nutmeg, eucalyptus, orange, pine and tulsi) orange oil was found to exhibit the highest larvicidal activity with LC_{50} values of 85.93 mg/l, followed by tulsi (92.48), palmarosa (88.78), and nutmeg (93.62) against the larvae of *Aedes aegypti*. A research study [37] reported the LC_{50} values (mg/l) of oils of peppermint (346), eucalyptus (161), basil (659), neem (117), and ginger (152) against the larvae of *Aedes aegypti* respectively. In our study, the EOs showed satisfactory results against the third instar larvae of *Aedes aegypti*.

Till date, the mode of action of EOs for the insecticidal activity is not clearly defined. One hypothesis [40] suggested that inhalation of EOs can kill insects. Another hypothesis [41] is that the monoterpenes act on cytochrome P450. It has been reported that some terpenoids have the property to inhibit the acetylcholinesterase activity. But further investigation will require for understanding the real mechanism of action of these EOs [42].

The decrease in the glucose level in the citronella and eucalyptus oil treated larvae observed in our study might be due to stress caused by the exposure to the components present in the essential oil. de Melo *et al*. (2018) reported the decrease in glucose level in *Culex quinquefasciatus* larvae exposed to linoleic acid [43]. Increase in glucose level in the patchouli oil treated group has been observed.

Reduction of the total protein levels indicates a negative effect of eucalyptus oil on the lipid metabolism of *Aedes aegypti* larvae. After exposure to insecticides, *Anopheles stephensi*, *P. turionellae* and *Tenebrio molitor, and* reduce protein levels [44]. In our study, decline in protein content by eucalyptus oil might probably be due to the plant essential oil interfering with the hormones that regulate protein synthesis.

Histopathological report of the citronella oil treated larvae showed a remarkable changes in the mid gut epithelium. Destructive brush border, degenerative digestive cells, degenerative basal membrane and cells, epithelial lyses, degeneration in peritrophic membrane, bursting of the food bolus to different mid gut sections in the exposed larvae were observed. Ndione *et al*. (2007) studied the toxicity of neem oil against the *Aedes aegypti* Linnaeus 1762 larvae and reported on various damage in the mid gut epithelium of the exposed larvae [45].

Wei & Wee (2013) reported identification of 20 chemical components in citronella oil [46] by GC-MS where 6-octenal, 3, 7-dimethyl- or citronellal represented the major chemical compounds (29.6%). In our study carried out by GC-MS, linalool, citronellal, citronellol, β-Citral were the major chemical compounds identified in citronella oil. A study reported by Jimenez-Carmona & de Castro (1999), the major ingredient identified in eucalyptus oil were α-pinene, β-pinene, β-myrcene, eucalyptol [47]. Here, we also identified the major chemical composition in eucalyptus oil as δ-pinene,

eucalyptol, and o-cymene. Patchoulol or patchouli alcohol ($C_{15}H_{26}O$) is a sesquiterpene alcohol found in patchouli oil. In our investigation, the compounds present in patchouli oil are patchoulol, δ-guaiene, α-guaiene, and seychellene. These compounds are the same as those found by Betts (1994) in the analysis of a commercial sample of patchouli essential oil [48]. The GC-MS data from the present study reveals that there are many oxygenated monoterpenes and related identical compounds present in the EOs. Thus the activity of the oils against the mosquito larvae may be due to the synergistic effect of certain constituents.

Conclusions

The findings of the present investigation revealed that citronella, eucalyptus and patchouli oil have larvicidal effect which is concentration-dependent for the *Aedes aegypti* larvae. This could be used as a green pesticide. EOs can kill mosquito larvae by causing biochemical and histopathological alterations. Further research should be conducted with isolated active compounds from the essential oils.

List of abbreviations

EOs: essential oils, GC-MS: gas chromatography-mass spectrometry, LC_{50}: lethal concentration required to kill 50% of the population, mg/l: milligram per litre, WHO: World Health Organization, mM: milli mol, ml: millilitre, h: hour, min: minute, rpm: rotation per minute, µm: micrometre, °C: degree Celsius, NIST: National Institute of Standards and Technology, KI: Kovats Index, RT: Retention Time, mg/dl: milligram per decilitre, SD: standard deviation, TP: total protein

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Availability of data and materials

The datasets generated and analyzed during the current study is available from the first author on a reasonable request.

Authors' contributions

HH designed, analyzed the toxicological data regarding larva toxicity and chemical composition of the EOs. HK was a major contributor in writing the manuscript. SK and SK operated and interpreted the GC-MS data. KZ, VT, DRB, and PC extended their help by guiding us throughout the whole research study. All authors read and approved the final manuscript.

Ethics approval and consent to participate:

The Institutional Animal Ethics Committee (IAEC) approval number that has approved the use of rabbits for mosquito rearing is IAEC/DRL/16/2014.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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