



Insecticidal activities of *Citrus aurantifolia* essential oil against *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT

Background: In the recent time, global attention for the control of vectors has shifted from chemical insecticides to botanicals. In the present investigation, authors attempted to evaluate the efficacy of peel and leaf essential oil (EO) of *Citrus aurantifolia* against *Aedes aegypti*.

Results: The results revealed that both the oils possess more ovicidal activity (LC50 value of 5.26 ppm and 17.71 ppm for leaf and peel oil respectively at 72 h) than larvicidal activity. As larvicide, the essential oil from the peel of *Citrus aurantifolia* showed rapid effect with LC50 value of 128.81 ppm at 24 h which reduced to 106.77 ppm at 72 h while the leaf oil showed slow effect with LC50 value of 188.59 ppm, 107.37 ppm and 104.59 ppm at 24 h, 48 h and 72 h respectively. Again, the two essential oils did not show significant adulticidal activity. GC–MS analysis of both the oils recorded presence of different compounds. As a major constituent compound of the leaf EO of *Citrus aurantifolia*, citral was tested for their ovicidal, larvicidal and adulticidal activities against *Aedes aegypti*. The result showed highest ovicidal activities (LC50 value of 4.84 ppm at 72 h) of citral followed by larvicidal (LC50 value of 87.02 ppm at 24 h) and adulticidal (LC50 value of 103.88 ppm at 24 h) activities.

Conclusion: From this study, it can be concluded that the essential oil extracted from the leaf and peel of *Citrus aurantifolia* and one of its major constituent compound citral can be included in the mosquito control programme of *Aedes aegypti*.

1. Introduction

Plants possess enormous number of defensive chemical compounds which are known by the general name “secondary metabolites”. These metabolites play a pivotal role in plant defense system as well as in its relationship with other plants, herbivores, pathogens and pollinators [1]. Essential oil (EO)s are aromatic, liquefied, and volatile substances which are produced by different plant parts viz. flowers, buds, seeds, leaves, twigs, barks, herbs, woods, fruits and roots. They are the complex natural mixtures of lipophilic substances [2] containing about 20–60 compounds were two or three major compounds accounting 20–70% of the total oil compared to others. At present approximately 300 EOs are commercially exploited for pharmaceutical, food, sanitary, cosmetic including perfume industries [1]. As environmentally safe and target specific, in recent times attraction has been grown for usage of natural products like EOs, and therefore development of better understanding on their mode of action is necessary for new applications in human health, agriculture and environment.

EO has been getting amazingly wide application in the industries

like flavoring and fragrance along with pharmaceuticals due to their high aroma. Not only in the perfume industry, EOs are also widely studied for their antibacterial, antiparasitic, antifungal as well as insecticidal activities [3–5]. Again, being a complex mixture of different compounds, EO achieves another advantage as insecticide from the point of resistance development by the target insects ([6], Sutthanont et al. 2010, [7,8]). These properties of EOs attract the scientific community towards the development of eco- friendly and target specific biopesticides. Again, due to the lipophilic natures of EOs, they can interfere with various biochemical, physiological and behavioral functions of an organism [9] by passing easily through the cell membrane. Some of the earlier studies have elaborately discussed about such different targets of EO in different insect body systems [10,11]. Among those, nervous systems, digestive system, developmental pathway, oviposition activity, various ion channels and some specific enzymes are important. So, EOs and their derivatives can be used as an alternative to synthetic insecticides in controlling different vectors and pest.

Citrus species (Rutaceae), native of the tropical regions of South-east Asia and China, is a great source of EOs which possesses numerous oil

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glands in different body parts. Lots of Citrus species have already been exploited commercially mainly for fresh consumption as fruit and fruit juice. Their byproducts which are treated as waste, is an important source of different bioactive compounds with potential use for animal feed and health care [12]. Among the citrus byproducts, EOs has been produced and exploited in a large scale for a long period. Insecticidal uses of these byproducts are also well established throughout the literature [13–17].

With the growing concern for the mosquito borne diseases, urge for different botanicals as mosquitocidal agent has increased. In this context, Citrus derived EO is also studied to some extent [18–20]. Among such target vectors, members of *Culex*, *Anopheles* and *Aedes* genera are important as they are the key transmitter of different pathogens. Although, with the advancement of medical sciences the epidemics of *Culex* and *Anopheles* borne diseases has somewhat reduced in recent years, but the epidemics of *Aedes* borne disease is still emerging. Control of such diseases totally depends on vector control programme. Therefore, the present investigation was designed to investigate the toxicity of *Citrus aurantifolia* leaf and peel EOs against the dengue vector- *Aedes aegypti* under laboratory conditions.

2. Methodology

2.1. Collection of plant materials

The plant was selected based on traditional knowledge of indigenous people and review study. Selected plant parts i.e. leaf and peel of *Citrus aurantifolia* was collected from Kamrup district of Assam for evaluating their mosquitocidal potentiality against *A. aegypti*. Collected plant species were identified with the help of the curator, Department of Botany, Gauhati University, and the voucher specimen (accession number 18496A) was submitted.

2.2. Essential oil extraction

Leaves and peels of *Citrus aurantifolia* (500 g) were cut into small pieces and washed with tap water. The EO was extracted through hydro distillation method using Clevenger's apparatus. The washed plant materials were placed in round bottom flask with 2–3 l of water and allowed to heat over heating mantle for about 5–6 h setting the thermostat at 50 °C. The EO vapors released by the heating process condense with the cold water stream and deposited in the recuperating channel. EOs were collected in clean glass vials and traces of anhydrous sodium sulphate [21] was added to absorb moisture content and then it was stored at 4 °C for further study.

2.3. Rearing of *Aedes aegypti*

Egg strips of *A. aegypti* were collected from Regional Medical Research Center (RMRC- ICMR), Dibrugarh for establishment of *Aedes aegypti* colony in the department of Zoology, Gauhati University. The colony was maintained between 25–29 °C temperature and 80–90% relative humidity following the rearing practices described by Arivoli et al. [22]. The collected egg strips were released in plastic trays containing tap water for hatching. The larvae were fed on finely powdered dog biscuits and yeast powders (3:1) while adults were fed on 10% glucose solution. After 3–4 days of adult emergence, adult female mosquitoes were blood-fed on albino rat for egg production between 10 am- 4 pm. A beaker wrapped with filter paper from inside surface having 200 ml of tap water was kept in the mosquito cage for egg laying

2.4. Screening of essential oils

2.4.1. Ovicidal assay

The ovicidal bioassay was performed following the method of Samidurai et al. [23] with little modifications. For the bioassay,

initially, 1000 and 100 ppm concentration of each EO was tested. Later based on the results of these two concentrations, a series of different concentrations ranging between 1–1000 ppm was prepared using equal amount of DMSO as emulsifying agent and applied to the respective disposable plastic cup (depth 2.5 cm). For each replicate of all tested concentration, fifty numbers of 7–14 days old eggs of *A. aegypti* were released. Two controls one positive with DMSO treated water and one negative control with water only were set. The number of eggs hatched in control and treatments were recorded after 24 h, 48 h, 72 h of treatment and hatching percentage was calculated. As the hatching of the eggs continued to 72 h, hence the percentage of ovicidal activity after 72 h was calculated by the following formula

Percent ovicidal activity

$$= \frac{\% \text{ of eggs hatched in control} - \% \text{ of eggs hatched in treated}}{\% \text{ of eggs hatched in control}} \times 100$$

2.4.2. Larvicidal activity

The standard World Health Organization procedure (WHO guidelines, 2005) was used with slight modification for studying the larvicidal activity of the selected EOs. For laboratory testing, 20 numbers of healthy 4th instar larvae were transferred to each replica. Initially two concentrations of each oil i.e. 100 and 1000 ppm were applied and based on the results, a wide range of concentrations (1 ppm to 1000 ppm) were prepared and tested in triplicate against *Aedes aegypti* larvae to calculate their respective sub-lethal concentration (LC50). For preparing each concentration of selected oils, DMSO was used as emulsifying agent. Mortality of the larva was recorded from 1 h to 6 h at interval of one hour and at 24 h, 48 h and 72 h after treatment. The LC50 value was calculated after 24, 48 and 72 h exposure period. Each concentration was assayed along with one negative control (water only) and one positive control (DMSO treated water). If the pupation occurred during exposure period and if more than 10% larvae died in the control group then the test was repeated. If mortality occurred in the control groups between 5–10% then, Abbot's correction formula (1925) was used.

2.4.3. Adulticidal assay

The impregnated filter paper bioassay method described by Ramar and Ignacimuthu- Paulraj [24] was followed with some modifications for evaluating the adulticidal potentiality of selected EOs against *Aedes aegypti*. Initially, two concentrations of each EOs (100 ppm and 1000 ppm) were prepared using acetone as solvent. 2 ml of each prepared solution was applied on Whatman no.1 filter papers (size 12 × 15 cm [25]) and allowed to evaporate acetone for 10 min. Control filter paper was treated with 2 ml of acetone alone. After evaporation of the solvent both the EO treated and control filter paper was placed in cylindrical tubes (depth 10 cm). After that ten numbers of 3–4 days old non blood fed mosquitoes were transferred in each replica of each treatment. Mortality was recorded at 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 24 h, 48 h and 72 h respectively. Sub-lethal concentration (LC50) value was recorded for 24 h, 48 h and 72 h of exposure period using probit analysis. If mortality exceeds 20% in the control batch, the whole test was repeated. Again, if mortality in the controls was above 5%, results with the treated samples were corrected using Abbott [25].

2.5. Analysis of effective essential oil components

Gas chromatography (Agilent 7890A) and mass spectrometry (Accu TOF GCv, Jeol) analysis of the selected essential oils were performed to identify the constituent compounds of each EO. GC was equipped with a FID detector and a capillary column (HP5- MS). The carrier gas was helium at a flow rate of 1 ml/ min. The GC programme was set for both EO as Split 10:1; 60-2M-6-200-5M-10-270-1M-10-280-HP5-CHCl3.

2.6. Identification of major terpene compounds of different essential oils

The major compounds of each EO were chosen based on their area percentage of the GC- chromatogram and mass spectrometry results and comparing the results with NIST library.

2.7. Bioassays of terpene compounds against *A. aegypti*

Efficacy of the selected terpene compounds against *A. aegypti* were screened through different bioassay viz. ovicidal, larvicidal and adulticidal following the methods described by Samidurai et al. [23] WHO guidelines [26], and Ramar and Ignacimuthu- Paulraj [24] respectively as mentioned above.

2.8. Statistical analysis

The LC50 values of selected EOs and the compounds were calculated using SPSS (Version 16) and Minitab software (Finney 1971). Standard error was calculated using MS- EXCEL.

3. Results

3.1. Bioassay of crude essential oil

3.1.1. Ovicidal activity

In the present study, crude EOs of both the leaf and peel of *Citrus aurantifolia* was found to have promising ovicidal effect against the same target pest with LC50 value of 17.71 ppm and 5.26 ppm respectively (Supplementary Table 1, Fig. 1) at 72 h exposure time. The result showed that the leaf EO of *Citrus aurantifolia* possessed higher ovicidal potentiality than the peel EOs against the same development stage of *Aedes aegypti*.

3.1.2. Larvicidal activity

Good larvicidal properties of both the selected oils of *Citrus aurantifolia* were recorded against 4th instar larvae of *Aedes aegypti*. The peel EO was found to have rapid toxic action in comparison to leaf EO as the LC50 values for these oils were found as 128.82 ppm and 188.59 ppm respectively (Supplementary Table 1). But with prolongation of exposure time, larvicidal activity was found to increase in case of the leaf oil in comparison to the peel oil as the LC50 dose at 72 h was recorded as 104.59 ppm and 106.77 ppm respectively (Supplementary Table 1, Fig. 1).

3.1.3. Adulticidal activity

Both the selected EOs of *Citrus aurantifolia* were found with no significant adulticidal activities against *Aedes aegypti* though time dependent enhancement of toxicity was noticed (Fig. 2) from 24 h to 72 h. The LC50 value could not determine for both the tested oil due to the low observed mortality of adult *Aedes aegypti* even at the highest

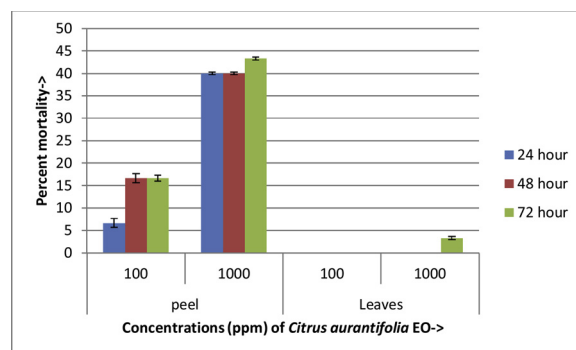


Fig. 2. Mortality percentage of adult *Aedes aegypti* at two different concentrations (100 & 1000 ppm) of leaf and peel essential oil of *Citrus aurantifolia*.

concentration applied.

3.2. GC–MS analysis of the essential oil

The result of the GC- MS analysis showed presence of 31 different compounds in the crude leaf oil and 26 compounds in the peel oil of *Citrus aurantifolia* (Tables 1 and 2; Figs. 3 and 4). Based on the area percentage, citral and limonene were determined as the major compounds present in the leaf EO while limonene and palatinol-1C were found as major compounds of peel oil of *Citrus aurantifolia*. Again, limonene and farnesol were found as common constituent in both the oils (Figs. 3 and 4).

3.3. Bioassay of terpene compounds

The study regarding the insecticidal activities of citral, the major compound of the leaf EO of *Citrus aurantifolia* against *Aedes aegypti* revealed that this compound is more toxic as ovicidal with LC50 value of 4.84 ppm at 72 h exposure period (supplementary Table 2, Fig. 5). Again, as larvicidal and adulticidal agent also, citral showed potential result with LC50 value of 87.02 ppm and 103.88 ppm respectively after 24 h of treatment (supplementary Table 2, Fig. 5).

4. Discussion

Present investigation showed remarkable potentiality of the leaf and peel EO of *Citrus aurantifolia* against eggs and larvae of *Aedes aegypti* though they were not found much active as adulticides. These findings showed similarity with our previous study with the same mosquito by applying the *Citrus grandis* oil (Sarma et al. 2017a). This observed toxicity may be due to the encapsulation of the mosquito eggs by shell which increases the exposure of the eggs to stresses while larvae or other stages could easily escape this through dispersal or migration [27,28]. Again, variation in the body structure of each development

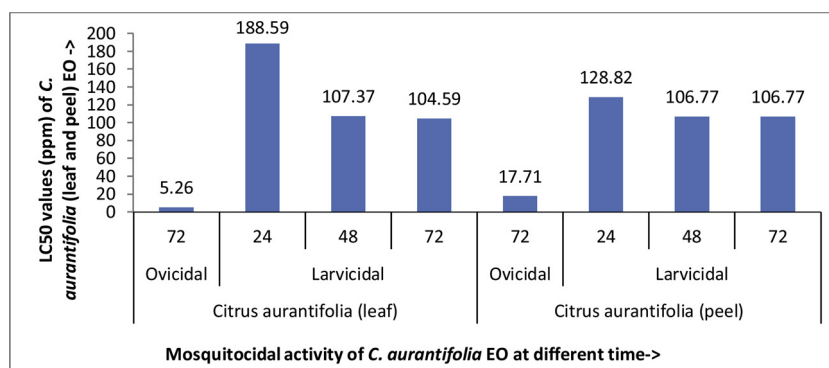


Fig. 1. Ovicidal and larvicidal activity of leaf and peel essential oil of *Citrus aurantifolia* against *Aedes aegypti*.

Table 1
Different constituent compounds of the essential oils from the leaves of *Citrus aurantifolia*.

Component	Molecular weight	Retention index	Chemical formula	Area (%)	Retention time
6- methyl- 5 hepten-2-one	126	938	C ₈ H ₁₄ O	–	6.39
Furan, tetrahydro-2,2- dimethyl-5(1- methylethyl)	142	919	C ₉ H ₁₈ O	–	6.61
Limonene	136	1019.8	C ₁₀ H ₁₆	11.59	7.38
Cis-linalool oxide	170	1064	C ₁₀ H ₁₈ O ₂	–	8.48
β- linalool	154	1081	C ₁₀ H ₁₈ O	3.5	9.22
Trans-p- Mentha-2,8 dienol	152	1113	C ₁₀ H ₁₆ O	0.64	9.78
Limonene epoxide	152	1139	C ₁₀ H ₁₆ O	2.17	10.09
β- citronellal	154	1132	C ₁₀ H ₁₈ O	2.83	10.39
Decanol	156	1183	C ₁₀ H ₂₀ O	6.19	11.71
Cis- geraniol	154	1215	C ₁₀ H ₁₈ O	5.47	12.4
Citral	152	1208	C ₁₀ H ₁₆ O	13.46	12.63
Geraniol	152	1249	C ₁₀ H ₁₆ O	10.59	13.34
Epoxy- linalooloxide	186	1224	C ₁₀ H ₁₈ O ₃	–	13.64
1,2-15,16- diepoxyhexadecan	254	1792	C ₁₆ H ₃₀ O ₂	1.60	14.08
1,2- Cyclohexanediol, 1-methyl-4-(1-methylethenyl)	170	1321	C ₁₀ H ₁₈ O ₂	11.33	15.4
Geraniol acetate	196	1352	C ₁₂ H ₂₀ O ₂	5.82	15.79
Neric acid	168	1316	C ₁₀ H ₁₆ O ₂	0.92	16.1
2- Isopropenyl-5-methylhex-4-enal	152	1092	C ₁₀ H ₁₆ O	–	17.59
2-Octen-1-ol 3,7- dimethyl- isobutyrate	226	1437	C ₁₄ H ₂₆ O ₂	–	18.1
Epoxy- linalooloxide	186	1224	C ₁₀ H ₁₈ O ₃	–	18.27
1,2-15,16- Diepoxyhexadecane	254	1792	C ₁₆ H ₃₀ O ₂	–	19.47
3,7- Nonadien-2-ol,4,8- dimethyl	168	1329	C ₁₁ H ₂₀ O	2.37	21.22
Cis- linalool oxide	170	1064	C ₁₀ H ₁₈ O ₂	–	21.83
9-(3,3- Dimethyloxiran-2-yl)-2,7- dimethylnona-2,6- dien-1-ol	238	1751	C ₁₅ H ₂₆ O ₂	–	24.05
2- Butyloxycarbonyloxy-1,1,10-trimethyl-6,9-epidioxydecalin	326	2021	C ₁₈ H ₃₀ O ₅	–	25.67
1b,5,5,6a- tetramethyl- octahydro-1-oxa-cyclopropa(a)inden-6-one	208	1445	C ₁₃ H ₂₀ O ₂	–	26.08
Cyclopropanemethanol, 2-methyl-2-(4- methyl-3-pentenyl)	168	1280	C ₁₁ H ₂₀ O	1.65	31.58
4,8-Decadienal,5,9- dimethyl	180	1373	C ₁₂ H ₂₀ O	0.89	31.78
3,7- nonadien-2-ol,4,8- dimethyl	168	1329	C ₁₁ H ₂₀ O	3.44	33.03
Farnesol	222	1658	C ₁₅ H ₂₆ O	–	33.53
Cholestan-3-ol, 2- methylene-(3β, 5α)	400	2652	C ₂₈ H ₄₈ O	–	35.07
1,1- methylcyclopentyl acetate	142	1001	C ₈ H ₁₄ O ₂	–	4.47

stages of the same insect species may be another possible reason for such differences in toxicity with each life stages [29]. Supporting the present findings previously, Soonwera [30] and Sarma et al. [28]; Sarma et al. [31] also stated that the variation of biochemical constituents between integument of the mosquito larva and the egg shell may also add difference in the penetration rate of different insecticides

to the body of target insect. The adult stage of *Aedes aegypti* was found less prone to both EOs compared to other developmental stages of the target mosquito in the present investigation. Behavioral and habitat difference along with the differences in body structure might make the adult stage more resistant to the insecticide than any other developmental stages [29,31]. Thus from the current investigation, it can be

Table 2
Different constituent compounds of the essential oils from the peel of *Citrus aurantifolia*.

Component	Molecular weight	Retention index	Chemical formula	Area (%)	Retention time
1,3-Methyl-1-hexanol	116	895	C ₆ H ₁₆ O	–	6.1
Octanal	128	982	C ₈ H ₁₆ O	1.16	6.74
Limonene	136	1014	C ₁₀ H ₁₆	12.85	7.39
1,2-Diethylcyclobutane	112	801	C ₈ H ₁₆	2.23	8.51
Linalol	154	1081	C ₁₀ H ₁₈ O	2.43	9.22
Limonene oxide	152	1031	C ₁₀ H ₁₆ O	3.44	10.11
Citronellol	154	1132	C ₁₀ H ₁₈ O	–	10.41
Nonyl alcohol	144	1149	C ₉ H ₂₀ O	2.09	11.02
Terpineol	154	1172	C ₁₀ H ₁₈ O	3.58	11.56
Cis-4-decanol	154	1170	C ₁₀ H ₁₈ O	4.09	11.72
Cis- Cavacrol	152	1207	C ₁₀ H ₁₆ O	5.28	12.31
Carvone	150	1220	C ₁₀ H ₁₄ O	9.13	12.76
2- Isopropenyl-5-methyl-4- hexanal	152	1092	C ₁₀ H ₁₆ O	5.48	13.34
Limonene dioxide	168	1294	C ₁₀ H ₁₆ O ₂	–	13.99
4- Isopropenyl-1- methyl-1,2-cyclohexanediol	170	1321	C ₁₀ H ₁₈ O ₂	1.28	15
Oleic acid	282	–	C ₁₈ H ₃₄ O ₂	0.76	16.17
α-Farnesene	204	1486	C ₁₅ H ₂₄	–	16.93
α-Bisabolol	222	1683	C ₁₅ H ₂₆ O	2.41	18.5
Caryophyllene oxide	220	1576	C ₁₅ H ₂₄ O	2.18	20.13
Acetate (1,3,7-trimethyl-2,6- Octadienyl) ester	210	1387	C ₁₃ H ₂₂ O ₂	1.22	21.23
Palatinol-1C	278	1819	C ₁₆ H ₂₂ O ₄	13.26	25.3
4,8-Dimethyl-3,7-nonadien-2-ol	168	1329	C ₁₁ H ₂₀ O	–	33.03
2-Methyl-2- (4-methyl-3-pentenyl) cyclopropyl methanol	168	1280	C ₁₁ H ₂₀ O	3.64	33.07
Farnesol	222	1658	C ₁₅ H ₂₆ O	–	34.12
2- Methylenecholestan	400	2652	C ₂₆ H ₄₈ O	–	34.88
1-Hepta triacotanol	536	3942	C ₃₇ H ₇₆ O	1.76	38.27

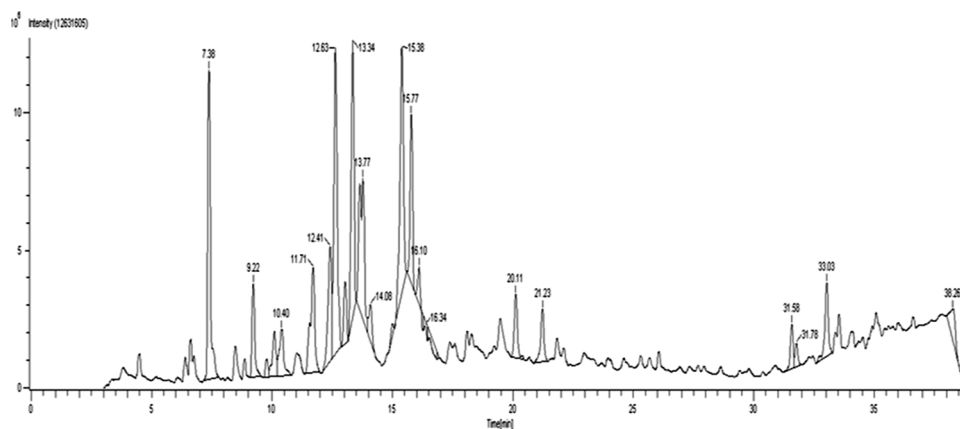


Fig. 3. GC chromatogram of leaf essential oil of *Citrus aurantifolia*.

concluded that the insecticidal activity of the plant EOs against *A. aegypti* may depend upon two factors viz., dose of the plant oils and period of exposure.

Variation in the activity of the two selected oils from the same plant against the same target stage of *Aedes aegypti* was another finding of the current study which may arise due to the difference in composition in the two oils. It was previously mentioned that bioactivity of an EO is totally influenced by their chemical composition [32]. Chemically, the EO is a mixture of different major and minor aromatic compounds [33] which can be broadly categorized in to four groups-terpenes, terpene derivatives, hydrocarbons and other miscellaneous compounds [34]. But the proper documentation of the occurrence and distribution of these volatile compounds was possible with the adoption of simple and sensitive Gas- chromatography and mass spectrometry technique.

For linking the observed insecticidal activity with the constituents of EOs, present study was also aimed to analyze the composition of leaf and peel EO of *Citrus aurantifolia* by GC-MS. The results showed organ wise variation in the EO composition of the same selected plant species. This finding showed similarity with the study of Prasad et al., [35] where they mentioned such type of variation in case of leaf and rind oil of *Citrus maxima*. Dominance of limonene was observed in both the selected oils in this study which was in line with the previous study of Razzaghi-Abyaneh et al. [36] and Mansour et al. [37]. Again, some contradiction was found regarding the probable major constituents of the same essential oil which may be due to influence of some factors like geographic locations, method of extraction, time of harvesting [38].

The major constituent compounds of the essential oil generally influence the overall activity of EOs [39]. Hence, in the present study the bioactivity of citral, a major compound of leaf EO *Citrus aurantifolia* investigated against *Aedes aegypti*. Likewise, the crude oil, citral also showed the highest affect as ovicide followed by larvicidal and adulticidal activities though the overall efficacy was found higher in the pure

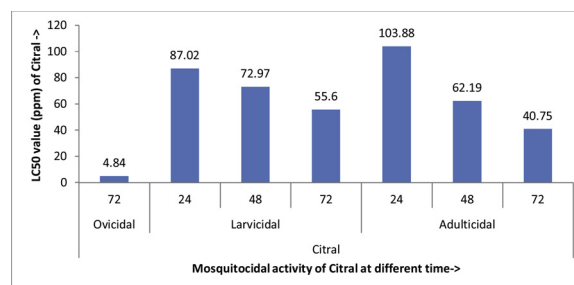


Fig. 5. Bioactivity of citral against *Aedes aegypti*.

compound. Earlier studies reported highest ovicidal activity of Citral, but the exact cause of death is still unknown [40–42]. Molecular structure including position and type of functional group as well as bond position etc. of a terpene compound also influences their activity [33]. Again, lethal action of this compound against larva may be mediated by interfering different metabolic activities as proved for other citrus derived limonoids [43]. The observed high adulticidal activity of citral showed similarity with other study of Yang et al. [44]. This observed toxicity may be due to inhalation by the insects [44,45].

Throughout the literature it was mentioned that the crude EO is more effective than the individual major compound as crude oils are mixture of different compounds ([6] Sutthanont et al., 2010; [7]; Pavela 2015). But contradicting this fact, the present investigation showed higher insecticidal potentiality of citral than the source crude oil. From this result it can be concluded that, in some cases, minor compound may reduce the efficacy of major compound in an EO.

Thus the present investigation established remarkable potentiality of *Citrus aurantifolia* leaf and peel essential oils along with citral, a major compound against different development stages of *Aedes aegypti*.

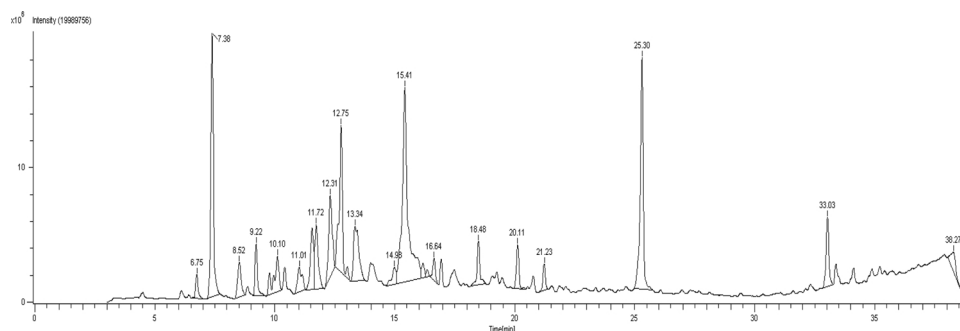


Fig. 4. GC chromatogram of peel essential oil of *Citrus aurantifolia*.

5. Conclusion

From the current investigation on the insecticidal activity of *Citrus aurantifolia* and one of their major compounds against the common dengue vector- *Aedes aegypti*, it can be concluded that these oils or compounds can be incorporated in vector control programme. But for more convenient outcomes, field trials should be attempted along with their detailed mode of action.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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