

Insecticidal Activities of the Essential Oil of *Aegle marmelos* (Linnaeus, 1800) against *Aedes aegypti* (Linnaeus, 1762) and *Culex quinquefasciatus* (Say, 1823)

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Abstract Essential oil from the leaves of *Aegle marmelos* was investigated for their larvicidal, ovicidal, adulticidal and repellent properties against *Aedes aegypti* and *Culex quinquefasciatus*. Essential oil was extracted from the fresh leaves by hydro distillation method. Different concentrations of the oil were applied against different developmental stages of both the mosquito species. The result of different bioassay showed various responses between the species. The essential oil showed higher efficacy as larvicidal and adulticidal agent against *Cx. quinquefasciatus* (LC50=121.88ppm against larva and 121.50ppm against adult at 72 hour exposure) while as ovicidal and repellent agent, the essential oil showed higher efficacy against *Ae. aegypti* with LC50 value 278.82ppm at 72 hour and 1 hour of protection time respectively. Therefore, it can be inferred that this essential oil is a potential one which could further be used as mosquitocidal agent against both the mosquito species. The GC-MS analysis revealed the presence of β - terpinyl acetate, 5- isopropenyl-2-methyl-7-oxabicyclo (4.1.0) hepten-2-ol and 2,3-pinenediol as major probable constituents of the oil which might play major role against insecticidal activities of the plant oil.

Keywords *Aegle marmelos*, Essential Oil, *Aedes aegypti*, *Culex quinquefasciatus*, Larvicidal, Adulticidal, Ovicidal, Repellent

1. Introduction

Mosquitoes are medically important insects and are considered as major public health pests as they transmit many dreadful diseases to humans and other warm blooded vertebrates [1]. *Aedes aegypti* and *Culex quinquefasciatus* are two important mosquito species under Culicidae family which transmit different diseases like dengue, yellow fever, chikungunya, Japanese encephalitis, and filariasis etc. *Aedes*

aegypti, a mosquito with distinct white bands in legs and abdomen is the common vector of dengue in tropical and subtropical countries [2]. Dengue fever has become an important problem related to public health as the number of its occurrence increases day by day, especially with more drastic forms of this disease such as dengue hemorrhagic fever and dengue shock syndrome along with the involvement of central nervous system [3, 4]. Again, *Culex quinquefasciatus*, a brown-coloured medium-sized mosquito has been established as the vector of *Wuchereria bancrofti* (Cobbold, 1877), avian malaria and arbo viruses including St. Louis encephalitis virus, western equine encephalitis virus, West Nile virus, various protozoan etc. About 1.10 billion people are threatened by this disease in 58 countries worldwide. In India, 19 million people suffer from filarial disease manifestations.

In present time, mosquito control programme faces a serious problem as mosquitoes are developing resistance against different chemical insecticides. Not only the rapid resistance development, these insecticides also possess various side effects on non-target organisms. Therefore, the search for alternative control measures other than synthetic insecticides that pose no or minimal risk to human health and the environment is extensively going on worldwide [5]. From these points, plant based products mainly the extracts and essential oils can be safely included in the integrated mosquito control programme as alternative to chemical insecticide.

Essential oils are volatile substances produced by plant as secondary metabolites which comprise different bioactive compounds like monoterpenoids, sesquiterpenoids, higher terpenoids etc. Essential oils have received more attention as promising insecticide as they show broad spectrum of activity against insect pest along with low mammalian toxicity and biodegradability in the environment [5]. Essential oils also show a broad spectrum of activity against different plant pathogenic fungi ranging from insecticidal, antifedant, repellent, oviposition deterrent, and insect

growth regulatory to antivevector activities [6].

The North-East India holds eccentric position in the world map of plant diversity. Being a part of North- East India, Assam is also rich in both flora and fauna diversity. In Assam, from ancient time various aromatic plants are used for medicinal purpose along with as insects controlling agent.

Aegle marmelos (L), a tree under the Rutaceae family commonly known as “Bael”, is a native plant of India with religious and medicinal importance [7]. The tree has a trunk with spiny branches and green leaves with three leaflets which release a fragrance when bruised [8]. Various parts of this tree already have been widely studied for their medicinal uses like in the treatment of heart diseases, dysentery, inflammation, diabetes, ulcer etc. [8-10]. Besides the medicinal uses, this plant was also studied for their antimicrobial, antifungal and insecticidal properties [8,9]. The effect of leaf extracts of *Aegle marmelos* was also studied against *Anopheles subpictus* (Grassi, 1899) in their oviposition deterrent, ovicidal and repellent activities [7]. Larvicidal activity of the essential oil of *Aegle marmelos* was also reported against *Culex pipiens* (L., 1758) and *Aedes aegypti* [2, 8].

A detailed study was also done regarding the constituents of the essential oil from leaves of *Aegle marmelos*. Some of such previous studies reported presence of various compounds in the essential oil like limonene, ocimene, caryophyllene, α - pinene etc. However no elaborate studies of this plant essential oil was found to be assayed against all the developmental stages of *Aedes aegypti* and *Culex quinquefasciatus*.

Keeping these points in mind, the present investigation aimed at studying the effect of the essential oil from the leaves of *Aegle marmelos* against *Aedes aegypti* (L) and *Culex quinquefasciatus* (Say). Again, GC-MS analysis was also targeted to know about the constitutive compounds of this plant essential oil grown in North Eastern Region of India.

2. Materials and Methodology

2.1. Rearing of *Ae. aegypti* and *Cx. quinquefasciatus*

The eggs of *Ae. aegypti* and *Cx. quinquefasciatus* were collected from ICMR (Indian Council of Medical Research), Dibrugarh, India. The collected eggs were reared in insect culture room, Dept. of Zoology, Gauhati University by following the rearing practices described by Arivoli *et al* [11]. The colonies were maintained at the temperature between 25 – 29° C temperature and 80 – 90% relative humidity. Larvae were fed on larval food (powdered dog biscuit and yeast in the ratio 3:1) and adult mosquitoes on 10 per cent glucose solution. Pupae were transferred to a disposable cup and it is kept inside the cage. In the 4th day after hatching, adult female mosquitoes were blood-fed on restrained albino rat for egg production.

2.2. Collection of Plant Material

Leaves of *Aegle marmelos* were collected from Nalbari district of Assam.

2.3. Extraction of Essential Oil

The essential oil was extracted from the plant with the help of hydro-distillation method using Clevenger apparatus.

2.4. Mosquito Ovicidal Bioassay

The ovicidal bioassay was performed according to the method described by Tennyson *et al* [12] and Puspanathan *et al* [13] with little modifications. For the ovicidal bioassay, 50 eggs of each species were transferred to each of the three replicates of each concentration. Eggs were exposed to the DMSO and water was treated as control. For determination of LC50 values, a wide numbers of concentrations of the oils were tested against the target species. The number of eggs hatched in control and treatments were recorded and the percentage of ovicidal activity was calculated by the following formula-

$$\text{Percent ovicidal activity} = \left(\frac{\% \text{ of eggs hatched in control} - \% \text{ of eggs hatched in treated}}{\% \text{ of eggs hatched in control}} \right) \times 100$$

A total of three replicates of each experiment were carried out.

2.5. Mosquito Larvicidal Bioassay

Screening of the efficacy of essential oils was done by performing bioassay studies against different developmental stages of *Ae. aegypti* and *Cx. quinquefasciatus*. The larvicidal activity of individual essential oil was assayed following the technique described earlier by Tong *et al.* [14] and WHO guidelines [15]. According to the WHO protocol for larvicides testing for laboratory testing, batches of 20 numbers of healthy 4th instar larvae of each species were transferred to the disposable glasses with the depth between 5-10cm having 100ml of water. A series of concentration from the 1000ppm to 10 ppm were used to examine the larvicidal toxicity of the oil. The LC50 values are recorded after 24, 48 and 72 hour exposure. Each concentration was assayed in triplicate along with one negative control group in water and one positive control group with the DMSO. If the pupation occurred in the exposure time or more than 10% larva was died in the control group, the test was repeated. From the data, LC50 values were determined by probit analysis (SPSS 16).

2.6. Mosquito Adulticidal Bioassay

For the adulticidal bioassay, the impregnated filter paper bioassay method described by Ramar *et al* [16] was followed with some modifications. For this assay, 10 specimens of 4-5 days old non blood fed female mosquitoes were selected for

each group. Three replicates were made for each concentration. Based on the preliminary screening, the different concentration of selected essential oil was prepared in 2 ml of acetone and applied on Whatman no.1 filter papers (size 12 x15 cm²), control papers were treated with 2 ml of acetone alone and placed in exposure tubes. 3-5 days old sugar fed mosquitoes are transferred to each tube after the evaporation time of 10 minutes of acetone. The mortality was recorded up to 72 hours. The LC50 value was recorded after the values were pooled for analyses in the log dose probit analysis. If mortality exceeds 20% in the control batch, the whole test should be rejected.

2.7. Mosquito Repellent Bioassay

Repellent bioassay was done according to the method of Barnard *et al* [17]. For the study of repellent activity, the protection time was calculated taking olive oil as control. For this test, three-four days old blood-starved female mosquitoes of each species (50) were kept in a net cage (47 x 35 x 31 cm²). The dorsal side of the arms of the test person was covered with the rubber gloves except an area of 5cm². This area was covered with a muslin cloth. On the cloth 0.5ml of tested solution was applied where olive oil was used as control. After air drying the arms of the test person, the control and treated arms were introduced simultaneously into the cage. The landing of mosquitoes was observed by exposing the treated area inside the cage for 5 minutes after every 30 minutes from 11AM to 4 PM for *Aedes aegypti* and from 8 PM to 11PM for *Culex quinquefasciatus*. The experiment was conducted three times for each concentration. It was observed that there was no skin irritation from the essential oil tested. The protection time was calculated by the following formula

$$\text{Protection time} = \frac{\text{Time of first landing of mosquito in treated} - \text{Time of first landing of mosquito in control}}$$

2.8. GC-MS Analysis of Essential Oil

Sample of essential oil was analyzed using Gas Chromatography (Agilent GC 7890A) and Mass spectrophotometry (Accu TOF GCv from Jeol instrument).

The programme was set as “10:1; 60-1M-8-200-5M-8-275-3M-5-280-HP5-CHC13”. Three major peaks were analyzed with library (NIST) data and the probable constituent compound(s) was identified for the essential oil.

2.9. Statistical Procedures

The data were corrected for the mortalities with the help of Abbott correction factor and were subjected to probit analysis using SPSS software to estimate LC50 values of effective essential oil against the mosquito. Again, if mortality in the controls was found above 5%, results with the treated samples were corrected using Abbott's formula [18].

3. Result

3.1. Ovicidal Activity

During the study of the ovicidal activity of the essential oil against both the target pest, no hatching of larvae were observed till 24 hours. Hatching of larvae were observed from 24 hours to 72 hours. No further hatching was recorded after 72 hour of treatment. Therefore LC50 value of ovicidal activity was recorded at 72 hour of exposure period (Table.4). At 1000ppm concentration, 77.7%, 82.2% and 82.7%, at 500 ppm concentration, 82%, 85.5% and 86.52% hatching was recorded and at 100 ppm concentration hatching of larvae were recorded as 92.3%, 95.6% and 95.6% at 24 hour, 48 hour and 72 hour respectively in case of *Cx. quinquefasciatus*. For *Ae. aegypti*, hatching percentage at 1000ppm concentration was recorded as 21.4%, 27.6% and 37.9%, at 500ppm concentration the hatching percentage were 26.18%, 34.5%, 39.1% and at 100ppm concentration hatching percentage was recorded as 39.29%, 62.03% and 62.05% respectively at 24 hour, 48 hour and 72 hour after treatment (Table1). The LC50 value along with the regression equation is listed in table 2. Ovicidal activity of the plant oil was found higher against *Ae. aegypti* than *Cx. quinquefasciatus*.

Table 1. Hatching percentage of eggs of *Ae. aegypti* and *Cx. quinquefasciatus* after treatment of essential oil of *Aegle marmelos*

No of individuals	Concentration (ppm)	<i>Aedes aegypti</i>			<i>Culex quinquefasciatus</i>		
		24hour (Average ±SE)	48hour (Average ±SE)	72hour (Average ±SE)	24hour (Average ±SE)	48hour (Average ±SE)	72hour (Average ±SE)
150	10	48.79±1.18	79.4±2.11	79.8±0.33	93.08±.32	95.6±.32	96.7±0
	100	39.29±0.59	62.03±0.58	62.05±1.11	92.3±.57	95.6±.32	95.6±0.32
	500	26.18±0.33	34.5±0.87	39.1±1.17	82±1.01	85.5±.67	86.52±0.58
	1000	21.4±1.47	27.6±0.59	37.9±2.11	77.7±.87	82.2±1.45	82.7±0.87

Table 2. Ovicidal activity of essential oil of *Aegle marmelos* against *Aedes aegypti* and *Culex quinquefasciatus*

Time	<i>Aedes aegypti</i>					<i>Culex quinquefasciatus</i>				
	LC50	Regression equation	95%confidence level		Chi-square value	LC50	Regression equation	95% confidence level		Chi-square value
			Lower bound	Upper bound				Lower bound	Upper bound	
72 hour	278.82	Y=3.95+0.43x	.342	.516	72.90	-	-	-	-	-

- : LC50 values could not be determined

Table 3. Survivability of larvae of *Cx. quinquefasciatus* and *Ae. aegypti* after treatment with essential oil

No of individuals	Concentration (ppm)	<i>Aedes aegypti</i>			<i>Culex quinquefasciatus</i>		
		24hour (Average ±SE)	48hour (Average ±SE)	72hour (Average ±SE)	24hour (Average ±SE)	48hour (Average ±SE)	72hour (Average ±SE)
60	10	100±0	100±0	100±0	100±0	100±0	100±0
	100	100±0	100±0	100±0	80±0	70±0.33	50±0.33
	500	100±0	100±0	100±0	20±0.33	10±0.33	10±0.59
	1000	100±0	100±0	100±0	0±0	0±0	0±0

Table 4. Larvicidal activity of essential oil of *Aegle marmelos* against *Culex quinquefasciatus*

Time	<i>Culex quinquefasciatus</i>				
	LC50 (ppm)	Regression equation	95% confidence level		Chi- square value
			Lower bound	Upper bound	
24 hour	185.69	Y=-1.51+2.87x	1.897	3.056	7.86
48 hour	167.42	y = -0.60 + 2.52 x	1.94	3.08	9.04
72 hour	121.88	y = - 0.19 + 2.42 x	1.75	2.81	5.86

Table 5. Survivability of adult *Cx quinquefasciatus* and *Ae.aegypti* after treatment of essential oil

No of individuals	Concentration (ppm)	<i>Aedes aegypti</i>			<i>Culex quinquefasciatus</i>		
		24hour (Average ±SE)	48hour (Average ±SE)	72hour (Average ±SE)	24hour (Average ±SE)	48hour (Average ±SE)	72hour (Average ±SE)
30	10	100±0	100±0	100±0	100±0	90±0	86.6±0.33
	100	100±0	100±0	100±0	83.33±0.33	60±0.58	56.67±0.33
	500	100±0	100±0	100±0	43.33±0.89	36.67±0.58	33.33±0.42
	1000	100±0	100±0	100±0	0±0	0±0	0±0

Table 6. Adulticidal activity of essential oil of *Aegle marmelos* against *Cx. quinquefasciatus*

Time	LC50 (ppm)	Regression equation	95% confidence level		Chi- square value
			Lower bound	Upper bound	
24 hour	288.56	Y=-1.97+2.83x	2.274	3.681	16.22
48 hour	164.67	y = 1.70 + 1.49 x	1.10	1.87	19.89
72 hour	121.50	y = 1.99 + 1.45 x	1.09	1.80	19.17

3.2. Larvicidal Activity

During the study of larvicidal activity of essential oil of *Aegle marmelos*, different concentrations of the oil was prepared and tested against *Ae. aegypti* and *Cx. quinquefasciatus*. In the study it was found that the larval mortality was directly related to the exposure time and concentration of the oil (Table 3). As larvicides, the oil of *Aegle marmelos* showed more effect against *Culex quinquefasciatus* than *Aedes aegypti*. For *Cx. quinquefasciatus* the LC50 values of the oil at 24h, 48h and 72h was recorded as 185.69ppm, 167.42ppm and 121.88ppm respectively while for *Ae. aegypti* no larvicidal effect was observed. The values of sub lethal concentrations are

presented along with the regression equations, 95% confidence level in table-4.

3.3. Adulticidal Activity

No adulticidal effect of the plant oil against *Ae. aegypti* species was recorded till 72 hours after treatment with 100 to 1000ppm concentration. But in case of *Cx. quinquefasciatus*, adults were more susceptible to this oil as it showed LC50 value 288.56 ppm, 164.67ppm and 121.50ppm respectively at 24 hours, 48 hours and 72 hours (Table-6).The survivability of adults after 24 hour, 48hour and 72 hour of treatment is presented in table -5

3.4. Protection Time

Table 7. Protection time of essential oil of *Aegle marmelos* against *Ae. Aegypti* and *Cx. quinquefasciatus*

Species	Control (Olive oil)	Oil (<i>Aegle marmelos</i>)
<i>Aedes aegypti</i>	5 minutes	1 hour
<i>Culex quinquefasciatus</i>	5 minutes	5 minutes

The result of the repellent effect of the essential oil was assessed in terms of determining the protection time. In the repellent study of this essential oil, commercially available olive oil was used as control. The result showed comparatively better protection time of the plant oil against *Ae aegypti* (1 hour) in comparison to *Cx quinquefasciatus* (5 min). In both larvicidal and repellent activities, this essential

oil showed higher efficacy against *Ae.aegypti* than *Cx.quinquefasciatus* (table-7).

3.5. GC-MS Analysis

After extraction of the essential oils from the fresh leaves of *Aegle marmelos*, to identify different constituent compounds, GC-MS analysis was carried out. The identity, retention time, area and percentage composition of the essential oil of *Aegle marmelos* are presented in the table-8. The GC-MS report showed β - terpinyl acetate, 5-isopropenyl-2- methyl-7-oxabicyclo(4.1.0) hepten-2-oland 2,3-pinenediol as probable major constituent of the particular oil (Table 8, fig-1)

Table 8. Different components of essential oil from leaves of *Aegle marmelos* obtained from GC- MS analysis

Component	Area (%)	Molecular weight	Retention index	Chemical formula	Retention time
α -pinene		136	922	C ₁₀ H ₁₆	4.43
β -myrcene		136	979	C ₁₀ H ₁₆	5.36
Bicyclo(3.1.0)hexane, 4- methyl-1-(1-methylethyl		136	873	C ₁₀ H ₁₆	5.64
β - terpinyl acetate	25.82	196	1267.2	C ₁₂ H ₂₀ O ₂	6.1
β -linalool		154	1082	C ₁₀ H ₁₈ O	7
2-cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)		154	1109	C ₁₀ H ₁₈ O	7.87
Cis-verbenol		152	1127	C ₁₀ H ₁₆ O	8.31
crypton	8.14	138	1069	C ₉ H ₁₄ O	9.06
Cis- sabinol	1.64	152	1085	C ₁₀ H ₁₆ O	9.43
Cis- carveol	1.78	152	1206	C ₁₀ H ₁₆ O	9.73
5- isopropenyl-2- methyl-7-oxabicyclo(4.1.0) hepten-2-ol	11.95	168	1169	C ₁₀ H ₁₆ O ₂	10.19
3- tetradecyn-1-ol		210	1673	C ₁₄ H ₂₆ O	10.43
camphenol	7.32	152	1082	C ₁₀ H ₁₆ O	11.06
2,3- pinenediol		170	1276	C ₁₀ H ₁₈ O ₂	11.47
5- isopropenyl-2-methyl-7-oxabicyclo(4.1.0) heptan-2-ol	13.69	168	1169	C ₁₀ H ₁₆ O ₂	11.71
Dipentene oxide		168	1128	C ₁₀ H ₁₆ O ₂	12.34
2,3-pinenediol	9.70	170	1276	C ₁₀ H ₁₈ O ₂	13.05
Piperitone oxide		168	1171	C ₁₀ H ₁₆ O ₂	13.37
Trans-3(10)-caren-2-ol	8.71	152	1131	C ₁₀ H ₁₆ O	13.79
Bicyclo-(3.1.0) hexane-6-methanol,2-hydroxy-1.4.4-trimethyl	3.90	170	1322	C ₁₀ H ₁₈ O ₂	14.59
Caryophyllene oxide	4.63	220	1507	C ₁₅ H ₂₄ O	15.69
12-oxabicyclo(9,1,0)dodeca-3,7-diene 1,5,5,8- tetramethyl		220	1592	C ₁₅ H ₂₄ O	16.09

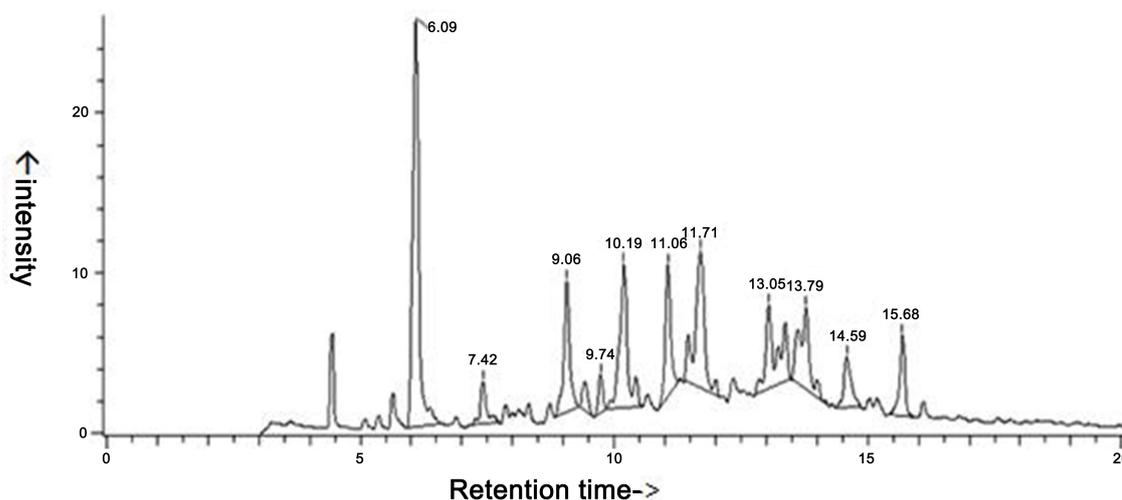


Figure 1. Chromatogram of GC-MS analysis of essential oil of the leaves of *Aegle marmelos*

4. Discussion

In the present study, the essential oil from the leaves of *Aegle marmelos* exhibited insecticidal activities against different developmental stages of the two target species, *Ae. aegypti* and *Cx. quinquefasciatus*. The findings revealed variation in the potentiality of the essential oil in different development stages of the two mosquito species.

In case of *Cx. quinquefasciatus*, the oil showed highest larvicidal activity (LC50 value=121.88ppm at 72 hour) followed by the adulticidal activity (LC50 value=121.50ppm at 72 hour) but no repellent and ovicidal activities was observed. Bioassay against *Ae. aegypti* reported highest ovicidal (LC50 value=278.82ppm at 72 hour) and repellent effect (Protection time=1 hour). As larvicidal and adulticidal agent, the essential oil showed no potent effect against *Ae. aegypti*. Thus the same essential oil showed completely different activity response against the two mosquito species though they belong to the same family "Culicidae".

In the present study the larval stage of *Cx. quinquefasciatus* was recorded as more susceptible among all the developmental stages. This is in conformity with the findings of Fox et al. [19] where they suggested larvae as more responsive to any physical as well as chemical stresses than other developmental stages. The egg stage was found less susceptible among all developmental stages. Like other insects, eggs of mosquitoes are also covered with shell which differs significantly from the integument of the larva in their structure and biochemical constituents which may add difference in the penetration rate of different insecticides to the body [20]. In contrast, bioassay against *Ae. aegypti* revealed that the egg stage is more susceptible among all the developmental stages. Variations in some factors like chitin content of the egg shell, egg volume ratio and egg surface density influence the levels of egg resistance to any stress [21] and these factors vary in different species.

Screening of effective essential oil as repellent is highly valuable for making safe protection from the bite of

blood-sucking mosquitoes. The desired qualities in the design of a repellent mainly include long protection time, low toxicity to humans, and non-irritating to skin [2]. Current study of determining the protection time of the essential oil from the leaves of *Aegle marmelos* against the two target species showed one hour protection time against *Ae. aegypti* while no repellent activity was found against *Cx. quinquefasciatus*. According to previous research, the repellent effect of any essential oil against mosquitoes showed variation due to some factors like temperature, wind, humidity, presence of different phytochemicals etc. [22, 23]. Again, the age of mosquitoes, body size and density in the cage are also responsible for such outcomes [17].

One of the important factors responsible for the insecticidal effect is the major compounds present in the essential oil including its quality and quantity [24]. The compounds including alcohols, aldehydes, fatty acid derivatives, terpenoids, and phenolics may jointly or independently contribute to insecticidal, repellent as well as antifeeding activities [25]. The toxicity of essential oil may be attributed to their major constituents [26]. Report of GC-MS analysis of *Aegle marmelos* oil in the current study showed β -terpinyl acetate, 5-isopropenyl-2-methyl-7-oxabicyclo (4.1.0) hepten-2-ol and 2,3-pinenediol as probable major compounds. This result is highly differing from lots of previous studies conducted in other parts of India which implied limonene as the major compound [8, 9, 27, 28]. Additional study on the same essential oil in other parts of India reported presence of phellandrene, myrcene and eucalyptol as major compound. Again, study on the same essential oil in Egypt by Ibrahim et al. (2015) reported γ -cadinene as major compound. Geographic locations, climatic condition, method of extraction, time of harvesting are some of the factors which influence those variations of the profile of constituents of an essential oil [29-30].

Therefore, essential oil of *A. marmelos* which is inexpensive, easily available at farm level, environmentally safe with low mammalian toxicity can be recommended as a

good alternative to synthetic insecticides against the mosquitoes.

5. Conclusions

The findings of present investigation emphasized the efficacy of *Aegle marmelos* as potent ovicide against *Aedes aegypti* and potent larvicide and adulticide against *Cx quinquefasciatus*. But further studies regarding the mode of action and field application are necessary to provide a futuristic lead product from *Aegle marmelos* for mosquito control.

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