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Chemical composition and bioefficacy for larvicidal and pupicidal activity of essential oils against two mosquito species

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Abstract
Mosquito species transmit major diseases viz. malaria, dengue, chikungunya, zika, filariasis and various forms of encephalitis; imposing enormous menace to human as well as animals. These diseases cause massive amount of morbidity and mortality across the world. The principal strategy for fighting against these diseases is the vector control including the use of larvicidal against mosquito larvae. Use of essential oils as mosquito larvicide has been suggested by different studies. Four samples of essential oil (EO) were tested for larvicidal as well as pupicidal activity against *Anopheles stephensi* and *Aedes aegypti* and subsequently analysed by gas chromatography- mass spectroscopy (GC-MS). Clove oil shows least LC_{50} and LC_{90} values for larvicidal as well as pupicidal activity against *Anopheles stephensi* species among the four EOs as 15.72ppm, 60.70ppm, for larvae; 46.47ppm, 80.66ppm, for pupae; 45.52ppm, 81.72ppm, against *Aedes aegypti* pupae. Whereas jasmine oil shows best results for larvicidal activity against *Aedes aegypti* larvae as 42.85ppm and 78.18ppm while it shows higher LC_{50} and LC_{90} of 73.52ppm, 399.05ppm against *Anopheles stephensi* larvae. Camphor oil shows high LC_{50} and LC_{90} values against *Aedes aegypti* larvae. It shows high LC_{50} and LC_{90} values against the both species as follows 178.60ppm and 309.7ppm *Anopheles stephensi* pupae and 218.08ppm and 388.29ppm *Aedes aegypti* pupae. All EOs showed moderate to high larval and pupal mortality against the both mosquito species except for camphor, which is least-effective among the others. EOs containing 1,3-ditert-butylbenzene, Linalool, Alpha copane, Artemisia ketone have showed satisfactory larvicidal as well as pupicidal activity . The results signifies that EOs may prove to be an eco-friendly alternative for the vector control in aquatic stages (larvae and pupae) with *Anopheles stephensi* and *Aedes aegypti* as the target species.

Key-words: *Anopheles stephensi; Aedes aegypti*; Essential oils; GC-MS

1. Introduction
Vector-borne diseases caused by pathogens and parasites, still today, is a global public concern since one sixth of the illness and disability suffered worldwide is due to vector-borne diseases, with more than half the world's population currently estimated to be at risk of these diseases [1]. Every year more than one billion people are infected and more than one million people die from vector-borne diseases, including malaria, dengue, schistosomiasis, leishmaniasis, Chagas disease, yellow fever, lymphatic filariasis and onchocerciasis imposing heavy health and economic burden [2]. These infectious diseases are transmitted by vectors, which are living organisms that can transmit the disease between humans or from animals to humans. Generally these vectors are bloodsucking insects, in which, mosquitoes are the most prominent known vector having global ramification. Others include specific species of ticks, flies, sandflies, fleas, bugs and freshwater snails. The poorest segments of least-developed countries are the main victims of these vector-borne diseases causing unprecedented economic and social disruption [2, 3].
Malaria, which is spread by mosquitoes, is one of the most common killers among the vector-borne diseases. In 2012, there were about 207 million cases of malaria and an estimated 627000 deaths worldwide [3]. However, the incidence of malaria is falling gradually in the wake of increased prevention and different control measures. In India, *Anopheles stephensi* is a primary mosquito vector of malaria prefer to thrive in urban settings and is included in the same subgenus as *Anopheles gambiae*, the primary malaria vector in Africa [4]. *Anopheles*

stephensi is a sub-tropical species that predominates in the Indian subcontinent except Nepal and Sri-Lanka [5]. It is estimated that about 12 % of malaria cases in India are due to *Anopheles stephensi* [6]. In rural settings, the larvae of *Anopheles stephensi* generally thrive in varied aquatic habitats *viz.* ponds, streams, swamps, marshes and other sources of standing water [7]. In urban settings, *Anopheles stephensi* breeds in a number of different water-bodies but predominantly in artificial containers, walls, overhead tanks, and ground level water tanks [8].

Apart from malaria, there are other vector-borne diseases, spread by mosquitoes, which have re-emerged in the recent past especially in the tropical countries including India. Dengue, *Aedes aegypti* mosquito being the primary vector, is prominent among them against which no effective antiviral medications exist. WHO estimates pointed out that there may be more than 100 million dengue infections worldwide every year. It is considered to be the most rapidly spreading mosquito-borne viral disease in the world. The bites of infected female mosquitoes transmit the dengue virus to humans, later the infected humans act as a main carrier and multiplier of the dengue virus serving as a source of the virus for uninfected mosquitoes [9-11]. Similarly, Japanese encephalitis virus is transmitted to humans through the bite of infected Culex mosquitoes. Every year an estimated 50,000 cases of Japanese encephalitis recorded worldwide affecting mostly children in the age group of 5 years and below. In the recent past the cases of Japanese encephalitis in India has increased considerably. Moreover, the Indian subcontinent is continuously reeling under the threat of chikungunya. It shares the same vectors, symptoms and geographical distribution as dengue, except for the presence of joint pains. In Asia and the Indian Ocean region, the main vectors of chikungunya are *Aedes albopictus* and *Aedes aegypti* [12-14].

In the 1940s and 1950s insecticides were used in a massive scale at the global level to ouster the main culprit- the mosquitoes. For decades, synthetic pesticides have been utilized for mosquito control by using the tactics of killing, preventing the mosquito from bite or killing the mosquito-larvae at the breeding site. As a result of which many important vector-borne diseases were brought under control. Unfortunately, these diseases re-emerged with retribution due to development of resistance in the vectors against a highly effective class of insecticides that is also the most affordable in several countries. In addition, these insecticides were also posing a severe environmental threat as many of these insecticides are detrimental to the survival of flora and fauna. Some of them are not easily degradable and they have toxic effects [15-18]. Therefore, it is imperative to look for those natural products which are not only effective against vectors but are also eco-friendly. Earlier works of several authors revealed that botanicals can have strong larvicidal activity [19-21]. In this context, EOs is one of the most promising candidate since they are inexpensive and biodegradable in nature. Moreover, there is no reported case of toxicity of these natural products in non-target organisms [17, 18, 22].

There are many reported cases of bioefficacy of EOs which show the larvicidal activity against mosquito species. Govindarajan *et al*., evaluated four EOs of *Cymbopogan citrates*, *Cinnamomum zeylanicum, Rosmarinus officinalis, Zingiber officinale* and all the oils showed promising larvicidal and repellent agent against *Culex tritaeniorhynchus* and *Anopheles subpictus* [8]. But, pupicidal activity of EOs against mosquito species is one aspect which is yet to be studied. Infact, very limited work on the pupicidal activity of EOs has been done till now.

Materials and Methods

Rearing of Mosquitoes

For rearing of *Aedes aegypti* and *Anopheles stephensi*, wooden cages (750 X 600 X 600 mm) were used in the Medical Entomology Division of Defence Research Laboratory, Tezpur. Initially for two days and then at every alternative days the female mosquitoes were fed on rabbits for blood meal. Cotton with 10% sugar solution was provided for nourishment. For egg laying, filter paper strip in a 250 ml beaker containing fresh water were kept in the cages. Collected eggs were transferred to a bowl containing two liters of water for rearing of hatched larvae up to adult stage and were fed on dog biscuits and Brewer's yeast powder in ratio of 1:1 and water was changed on each alternate days. Collected pupae were kept in small cages (750 X 600 X 600 mm) covered with cotton cloth for emerging into the adult.

Bioassay

Larvicidal activity of 4 Essential oil against third instar larvae of *Anopheles stephensi* and *Aedes aegypti* mosquito, were studied according to WHO protocol, [23] with minor modification. Five concentrations of acetone dissolved oils *viz.* 6.25%, 12.5%, 25%, 50%, 100% were prepared in distilled water. Subsequently, 20 larvae were transferred gently to the test medium by topping and with each replicate. The larval mortality was calculated after 24 h of the exposure period.

Alongwith larvicidal activity, we have also bioassayed pupicidal activity against *Anopheles stephensi* and *Aedes aegypti* of the aforesaid EOs in concentrations of 6.25%, 12.5%, 25%, 50% and 100%. The pupal mortality was calculated after 24 h of the exposure period for both the pupae of *Anopheles stephensi* and *Aedes aegypti*.

The experimental data were subjected to statistical analysis in order to estimate the LC_{50} and LC_{90} values.

Essential oils

Four EOs *viz*. jasmine oil derived from flowering parts of *Jasminum officinale*, family Oleaceae; clove oil derived from flower bud, stem, leaf of *Syzygium aromaticum*, family Myrtaceae; camphor oil derived from the wood of the *Cinnamomum camphora*, family Lauraceae; deodar oil derived from Bark, heartwood, leaves of *Cedrus deodara,* family Pinaceae; all EOs were purchased from Surajbala Exports Pvt. Ltd, Gurgaon, New Delhi, and stored at 4°C for further experimentation.

GC-MS study

Gas chromatography-Mass spectroscopy (GC-MS) analysis of the EO were performed on a GC (GC-7890B) and MS (5977A MSD) system of Agilent technologies using Agilent J&W GC column (HP-5 MS UI) having 30 m of length, 0.25 mm of diameter and film thickness of 0.25 µm. Helium (He) was used as carrier gas at flow rate 1ml/min. Oven temperature was programmed from 40-300 ºC at 20 ºC/min. Injector temperature was set at 250 ºC and detector temperature was set at 230 ºC (quard) and 150 ºC (core) respectively.

Results

Larvicidal as well as pupicidal activity of 4 EOs was tested against larvae and pupae of *Anopheles stephensi* and *Aedes aegypti* mosquitoes. Larval and pupal mortality were counted after 24 hrs of treatment with the EOs. Table 5, 6, 7and 8 shows the LC_{50} and LC_{90} values for larvicidal and pupicidal activity against *Anopheles stephensi* and *Aedes aegypti***.** During this study, we have found that, Clove oil showed least LC_{50} and LC_{90} values for larvididal as well as pupicidal activity against *Anopheles stephensi* species among the four EOs as 15.72ppm, 60.70ppm, for larvae; 46.47ppm, 80.66ppm, for pupae; 45.52ppm, 81.72ppm, against *Aedes aegypti* pupae. Whereas jasmine oil showed best results for larvicidal activity against *Aedes aegypti* larvae as 42.85ppm and 78.18ppm while it showed higher LC_{50} and LC_{90} of 73.52ppm, 399.05ppm against *Anopheles stephensi* larvae. Camphor oil showed high LC_{50} and LC_{90} values against *Aedes aegypti* larvae. Camphor oil showed high LC_{50} and LC_{90} values against the both species as follows 178.60 ppm and 309.7 ppm *Anopheles stephensi* pupae and 218.08 ppm and 388.29 ppm *Aedes aegypti* pupae respectively. The GC-MS study of the EOs are given in the table 1,2,3,4 with their chemical name, IUPAC name, structure, formula, mol. wt., retention time and uses. Figure 1,2,3,4 showed the mortality of larvae of *Anopheles stephensi and Aedes aegypti* against different EO samples after 24 hrs. Figure 5,6,7,8 showed the mortality of pupae of *Anopheles stephensi and Aedes aegypti* against different EO samples after 24 hrs respectively.

Among the four EOs, clove oil and deodar oil shows good results for larvicidal as pupicidal activity against the both *Anopheles stephensi* and *Aedes aegypti* mosquito species.

Table 1: Chemical name, IUPAC name, Structure, Formula, Mol. Wt., Retention time of Jasmine oil

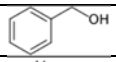
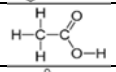
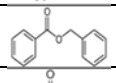
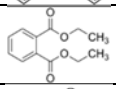
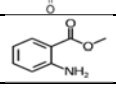
Sl. No.	Chemical Name	IUPAC Name	Structure	Formula	Mol. Wt. (g/mol)	Retention time (mins)
1.	Benzyl alcohol	Phenylmethanol		C_7H_8O	108.14	6.089
2.	Acetic acid	Ethanoic acid		$C_2H_4O_2$	60.05	7.355
3.	Benzyl benzoate	Benzyl benzoate		$C_{14}H_{12}O_2$	212.25	8.206
4.	Diethyl phthalate	Diethyl benzene-1,2-dicarboxylate		$C_{12}H_{14}O_4$	222.24	4.995
5.	Methyl anthranilate	Methyl 2-aminobenzoate		$C_8H_9NO_2$	151.165	4.159

Table 2: Chemical name, IUPAC name, Structure, Formula, Mol. Wt., Retention time of Clove oil

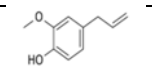
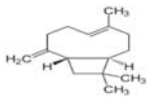
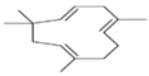
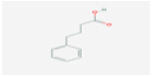
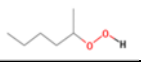
Sl. No.	Chemical Name	IUPAC name	Structure	Formula	Mol wt (g/mol)	Retention time (mins)
1.	Eugenol	2-Methoxy-4-(prop-2-en-1-yl)phenol		$C_{10}H_{12}O_2$	164.20	3.573
2.	Carypohyllene	(1*R*,4*E*,9*S*)-4,11,11-Trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene		$C_{15}H_{24}$	204.36	10.090
3.	Humulene	2,6,6,9-Tetramethyl-1,4-8-cycloundecatriene		$C_{15}H_{24}$	204.36	6.743
4.	Benzene-butanoic acid	2-amino-3,4-dihydroxy-4-(4-hydroxyphenyl)but-anoic acid		$C_{10}H_{12}O_2$	164.20	6.567
5.	2-Hexyl hydroperoxide	Hydroperoxide, 1-methylpentyl		$C_6H_{14}O_2$	118.17	6.141

Table 3: Chemical name, IUPAC name, Structure, Formula, Mol. Wt., Retention time of Camphor oil

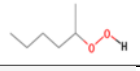
Sl. No.	Chemical Name	IUPAC Name	Structure	Formula	Mol wt (g/mol)	Retention time (mins)
1.	2-Hexyl hydroperoxide	Hydroperoxide, 1-methylpentyl		$C_6H_{14}O_2$	118.17	3.692
2.	Pentane, 3-ethyl-3-methyl	3-ethyl-3-methylpentane		C_8H_{18}	114.23	3.516
3.	3-Hexyl hydroperoxide	Hydroperoxide, 1-ethylbutyl		$C_6H_{14}O_2$	118.17	3.578
4.	Artemisia Ketone	1,5-Heptadien-4-one, 3,3,6-trimethyl		$C_{10}H_{16}O$	152.23	7.345

Table 4: Chemical name, IUPAC name, Structure, Formula, Mol. Wt., Retention time of Deodar oil

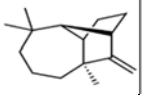
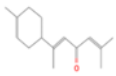
Sl. No.	Chemical Name	IUPAC Name	Structure	Formula	Mol wt (g/mol)	Retention time (mins)
1.	Longifolene	(1*R*,2*S*,7*S*,9*S*)-3,3,7-trimethyl- 8-methylenetricyclo-[5.4.0.0^{2,9}]undecane		$C_{15}H_{24}$	204.36	6.530
2.	(+)-.beta.-Himachalene o xide	(+)-.beta.-Himachalene oxide		$C_{15}H_{24}O$	220.35	7.553
3.	(E)-α-Atlantone	Trans-α-atlantone		$C_{15}H_{22}O$	218.33	7.843
4.	Kuromatsuen	(1*R*,2*S*,7*S*,9*S*)-3,3,7-trimethyl- 8-methylenetricyclo-[5.4.0.0^{2,9}]undecane		$C_{15}H_{24}$	204.35	6.593
5.	Isolongifolene	-(-)- Isolongifolene		$C_{15}H_{22}$	202	6.349

Table 5: LC_{50} and LC_{90} values for larvicidal activity against *Anopheles stephensi* larvae

ESSENTIAL OILS	LARVICIDAL ACTIVITY AGAINST *Anopheles stephensi*	
	LC_{50}	LC_{90}
Clove	15.72	68.70
Deodar	17.15	56.23
Camphor	26.91	70.16
Jasmine	73.52	397.05

Table 6: LC_{50} and LC_{90} values for larvicidal activity against *Aedes aegypti* larvae

ESSENTIAL OILS	LARVICIDAL AGAINST *Aedes aegypti*	
	LC_{50}	LC_{50}
Jasmine	42.86	42.86
Deodar	44.36	44.36
Clove	46.79	46.79
Camphor	114.79	114.79

Table 7: LC_{50} and LC_{90} values for pupicidal activity against *Anopheles stephensi* pupae

ESSENTIAL OILS	PUPICIDAL ACTIVITY AGAINST *Anopheles stephensi*	
	LC_{50}	LC_{90}
Clove	46.47	80.66
Deodar	49.57	90.6
Jasmine	117.29	204.25
Camphor	178.60	309.7

Table 8: LC_{50} and LC_{90} values for pupicidal activity against *Aedes aegypti* pupae

ESSENTIAL OILS	PUPICIDAL AGAINST *Aedes aegypti*	
	LC_{50}	LC_{50}
Deodar	39.88	39.88
Clove	45.52	45.52
Jasmine	125.56	125.56
Camphor	218.08	218.08

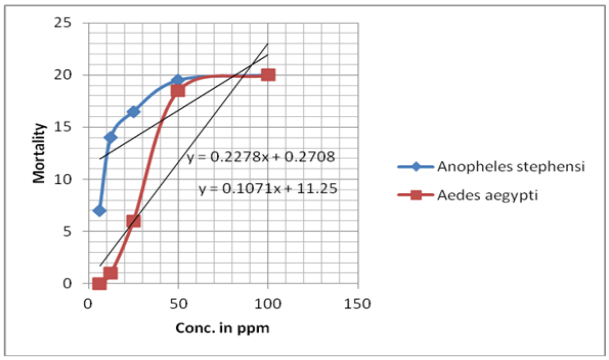


Fig 1: Mortality of larvae of *Anopheles stephensi and Aedes aegypti* against jasmine oil after 24 hrs

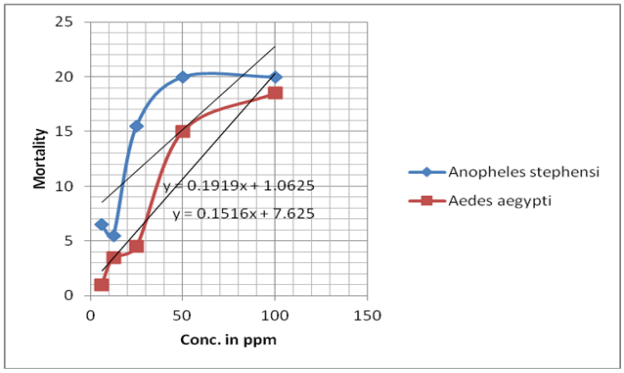


Fig 2: Mortality of larvae of *Anopheles stephensi and Aedes aegypti* against clove oil after 24 hrs

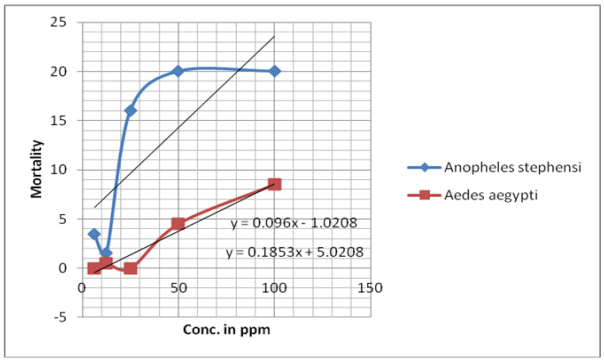


Fig 3: Mortality of larvae of *Anopheles stephensi and Aedes aegypti* against camphor oil after 24 hrs

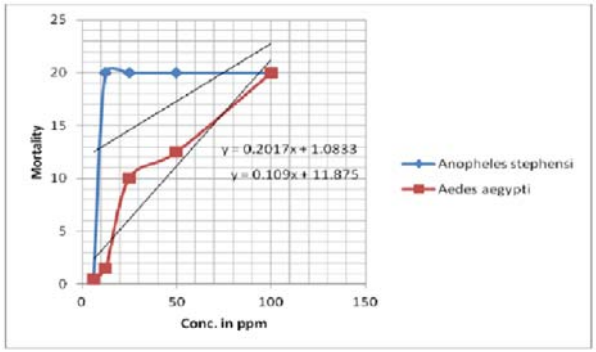


Fig 4: Mortality of larvae of *Anopheles stephensi and Aedes aegypti* against deodar oil after 24 hrs

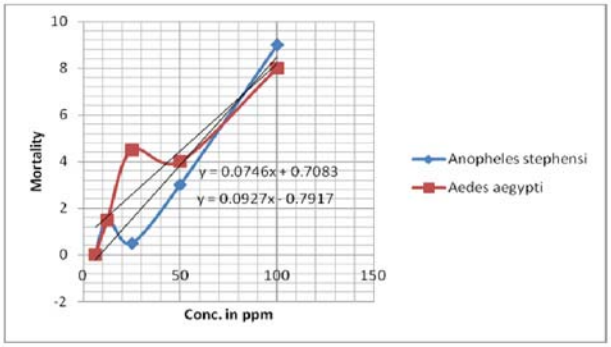


Fig 5: Mortality of pupae of *Anopheles stephensi and Aedes aegypti* against jasmine oil after 24 hrs

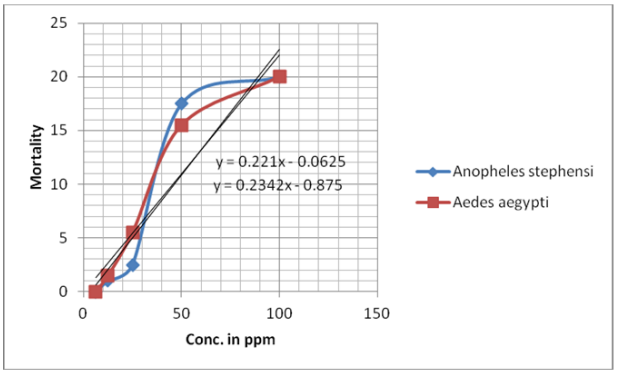


Fig 6: Mortality of pupae of *Anopheles stephensi and Aedes aegypti* against clove oil after 24 hrs

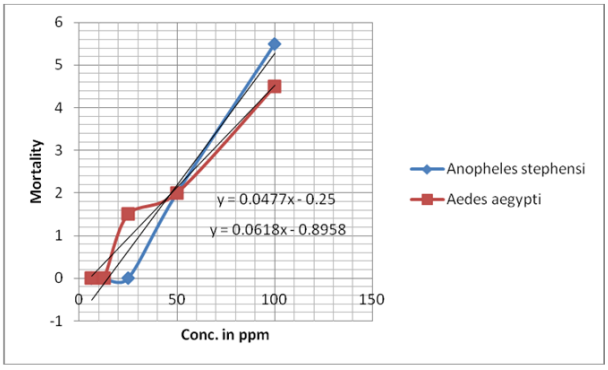


Fig 7: Mortality of pupae of *Anopheles stephensi and Aedes aegypti* against camphor oil after 24 hrs

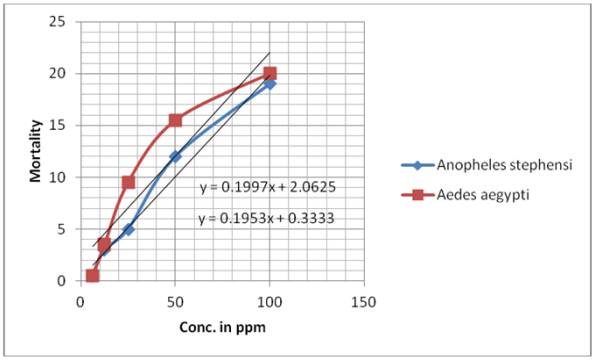


Fig 8: Mortality of pupae of *Anopheles stephensi and Aedes aegypti* against deodar oil after 24 hrs

Discussion

Plant products have proved their utility for various medicinal uses since time immemorial. The bioefficacy of plant EOs against mosquitoes as growth inhibitors, larvicides, adulticides, repellents or oviposition deterrents have been reported by many researchers [16, 24, 25]. Phukerd and Soonwera [10] have found larvicidal and pupicidal activities of EOs from Zingiberaceae plants against *Aedes aegypti* (Linn.) and *Culex quinquefasciatus*. Amer & Mehlhorn [11] have also shown repellency effect of forty-one EOs against Aedes, Anopheles and Culex mosquitoes. Pitasawata *et al*.,[12] found *C. zedoaria* had larvicidal activity against *Aeedes aegypti*. Similarly, we have also found that deodar oil, jasmine oil, clove oil and camphor oil showed moderate to high rate of larval mortality. Some researchers have also reported the pupicidal activity of plant EOs. Dua *et al*. [13] have revealed adulticidal activity of EO of *Lantana camara* leaves against mosquitoes. Ramar *et al*., [14] concluded moderate to good pupicidal activity of EOs from seven plants *viz. Pimpinella anisum*, *Cinnamomum veerum*, *Myrtus caryophyllus*, *Citrus sinensis*, *Thymus vulgaris*, *Ocimum sanctum* and *Vetiveria zizanioides* on *Culex quinquefasciatus* and *Anopheles stephensi*. In conformity with the above Govindarajan mentioned plant extract, this study also revealed that jasmine, deodar and clove oil showed moderate to high larvicidal as well as pupicidal activity against the larvae and pupae of *Aedes aegypti* and *Anopheles stephensi*.

Recently Dharma *et al*., [26] Synthesized and characterized of a novel series of 1, 4-dihydropyridine analogues for larvicidal activity against *Anopheles arabiensis*. They used benzyl alcohol to synthesize of different chemical derivatives for larvicidal activity, in our study, benzyl alcohol was found in jasmine oil as a major chemical component. Barbosa *et al*., [27] studied the larvicidal activity of eugenol and its derivatives against *Aedes aegypti*, Eugenol was identified by GC-MS in our study as a major component of clove oil. Earlier, two chemical humulene and caryophyllene have been reported for mosquito larvicidal activity [28,29]; in our present study, we have also observed these chemical constituent of clove oil by GC-MS. Stappen *et al*., [30]; studied the chemical composition and biological effects of artemisia maritima and artemisia nilagirica essential oils from wild plants of Western Himalaya, the main compounds analysed by GC-MS study in *A. nilagirica* was artemesia ketone where *A. nilagirica* extract showed larvicidal activity against *Aedes aegypti,* in our study, we found artemesia ketone in deodara oil and which showed a good larvicidal potential. In another study, Abbas Ali *et al*., [31] studied the composition, mosquito larvicidal, biting deterrent and antifungal activity of essential oils of different plant parts of *Cupressus arizonica* var. glabra (Carolina Sapphire); where longifolene were a major chemical constituent. Our study also gives positive response of deodara oil against different mosquito larvae and found longifolene as a major chemical constituent in deodara oil by GC-MS technique.

Conclusion

A number of strategies have been employed for control of all these vector-borne diseases, amongst them, vector control is the most potent preventive tool whose full potential is yet to be utilized. To strengthen vector control, WHO is promoting integrated vector management as the best approach and it is working as a complementary measure that can minimize the disease burden aggressively. As a matter of fact, vector control can play a front role in minimizing the severity of vector-borne diseases provided its entire potency is fully harnessed. Study of mosquitoes larvicidal and pupicidal activity of any EO is a serious effort in this direction as they provide safe, eco-friendly, non-irritating and cheap solution to the yet to be conquered problem of vector-borne diseases. The performance of the above studied four EOs against *Anopheles stephenshi* and *Aedes aegypti* is highly encouraging and a new vista has opened to further analyse the role of their individual components.

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Conflict of Interest

All the authors of this research article hereby declare that no conflict of interest exist among each others.

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