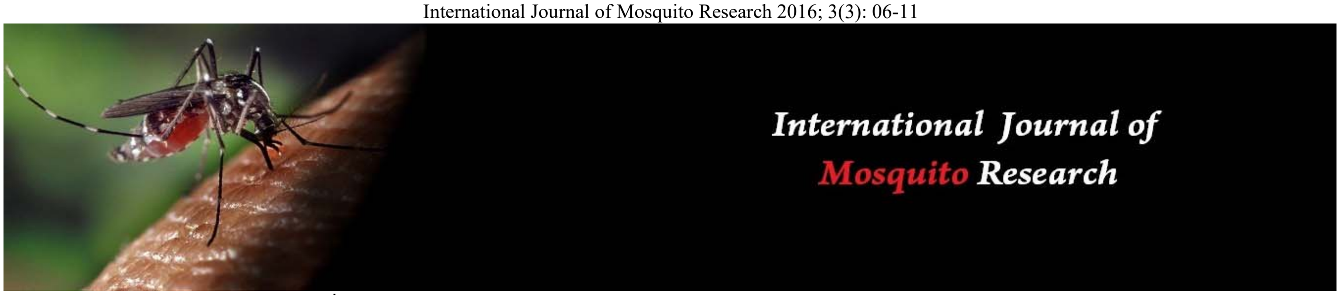

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Evaluation of chemical composition and larvicidal activity of *Coleus aromaticus* essential oil, its major compound carvacrol against *Aedes aegypti, Culex quinquefasciatus* and *Anopheles stephensi* (Diptera: Culicidae)

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Abstract
Vectors are living organisms that can transmit infectious diseases between humans or from animals to humans. Mosquitoes are the best known disease vector that transmits dengue, malaria, lymphatic filariasis and chikungunya. Globally, these major diseases record significant mortality in human and other livestock. Further, due to the high resistance towards chemical insecticides, the mosquito control is now facing a challenge. Phyto-insecticides may help as an alternative to vector (mosquito) control. The major objective of this study was to find out the toxicity of essential oil and carvacrol extracted from *Coleus aromaticus* against *Aedes aegypti, Culex quinquefasciatus* and *Anopheles stephensi*. The phytoconstituents of the essential oil were analyzed by Gas chromatography-mass chromatography (GC-MS) thereby 13 compounds were identified including carvacrol, Dodecane, Tritetracontane and stigamosterol. The larvicidal activity of essential oil and carvacrol were tested with the concentration of 10, 20, 30, 40 and 50µg/ml respectively. The toxicity was observed after 24 hours of treatment. The essential oil had a significant toxic effect against Cx. *quinquefasciatus, Ae. aegypti* and *An. stephensi* with LC_{50} and LC_{90} of 16.84256 (11.45 - 20.71); 48.83991 (43.31 - 57.63), 20.57808 (15.62 - 24.11); 54.75958 (48.24 - 65.42), 23.25586 (18.95 - 26.78); 56.42830 (48.88 - 66.99)µg/ml, whereas carvacrol had 35.67071 (32.565 - 39.228); 64.64680 (57.834 - 75.209), 38.20320 (35.062 - 42.004); 77.93745 (68.746 - 93.804), 40.55376 (36.975 - 45.237); 72.03935 (63.484 - 86.023) µg/ml respectively. There is no observation of mortality in control. The result indicated that essential oil of *C. aromaticus* and its isolates carvacrol have a potential application in controlling of Cx. *quinquefasciatus, Ae. aegypti* and *An. stephensi* instead of synthetic chemicals.

Keywords: *Coleus aromaticus,* carvacrol, GC-MS, essential oil, larvicidal activity, mosquitoes

Introduction
Globally, the vector borne disease of malaria, dengue, schistosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis and onchocerciasis causes over one million deaths in every year [43]. Mosquito bites also cause allergic responses such as urticaria and angioedema [31]. In developing nations like India, malaria is an imperative disease and it causes straight and convoluted mortality among newborn, child and adult. The World Health Organization (WHO) reported that malaria infected ~1.2 billion people in the Southeast Asian region [10]. In malarial epidemiology, out of 2,400 million of the world's population, India contributes 77 percent [20]. *Cx. quinquefasciatus* was found to be a vector of lymphatic filariasis, caused by *Wuchereria bancrofti* and it is endemic in the Indian subcontinent. In 2012, Global Program for eliminating lymphatic filariasis [13] reported that the disease was endemic in 73 countries and anticipated that 1.39 billion peoples were at danger for infection, ~120 million people had infected. *Ae. aegypti* is a vector of dengue viral infection, distributed in the tropical, subtropical zones and closely associated with the human habitat. In 2013, National Vector Borne Disease Control Program (NVBDCP) reported that 74,454 of people affected by dengue with 167 ends. In India, the union health ministry reveals and records the massive increasing of dengue fever in every year [29].
In world wild, the public health authorities and medical entomologists are involving in the mosquito control; a need based important strategy to prevent the mosquito borne disease [4]. The chemical insecticides, including organophosphates and pyrethroids are being utilized for

the control of vectors and mosquito populations [16]. Due to the repeated use of the same chemical insecticides and misusing in inappropriate situations, the developments of insecticide resistance among several insects have been identified [8]. The repeated usage of synthetic chemicals also causes environmental pollution and ecosystem instability, toxic to the human neuron system [35] and affecting nontarget organisms [17]. Considerable skill was achieved in the control of mosquito borne diseases by the utilization of synthetic insecticides and it has also provoked unwanted toxic effect to the environment and human health [21]. Thus, a crucial attention has to be paid for the development of novel, eco-friendly, environmentally safe, biodegradable and low cost natural insecticides [26]. The phytochemicals have been considered as suitable alternative weapons for mosquito control program as they are shown to function as insect repellents, growth regulators, oviposition attractants, smoke toxicity and deterrent activities [1, 3]. Due to these multifunctional properties, the quantity of natural products needed to be controlled the mosquito population is reduced [12]. Plants are a fertile source of novel natural substances that can be applied to develop ecofriendly insecticides [24] which are ecofriendly, easily biodegradable, target specific, lower bioaccumulation and low or sometimes non-toxic to higher animals [42]. Plant constituents, including active compounds and essential oils are studied as alternative potential sources for controlling mosquito and its larvae. In fact, the precise efficiency of larvicidal and repellent activity of essential oil was proven in many plants such as *Zingiber officinalis* against *Cx. quinquefasciatus* [33], Mentha L. and Pulegium essential oil against *Cx. quinquefasciatus* [30], *Toddalia asiatica* against *An. albopictus* [22], *Dianthus caryophyllus, Lepidium sativum, Pimpinella anisum* and *Illicium verum* against *Cx. pipiens* [19], *Saussurea lappa* against *Aedes albopictus* [23], *Allium monanthum* against *Aedes aegypti* [6].

Coleus aromaticus (Benth) is taxonomically described as a dicotyledonous aromatic herbal plant belonging to Lamiaceae family. It is a 20–90 cm height with fleshy leaves distributed throughout India and cultivated in the gardens. It is a folklore medicinal plant and applied by nearly all local people for its therapeutic efficacy to treat malarial fever, hepatopathy, chronic asthma, hiccough, bronchitis, helminthiasis, colic, convulsions and epilepsy [28]. No previous report identified in the larvicidal activity of *C. aromaticus* essential oil and its major compound carvacrol against *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensis*. Hence, this present study determined the larvicidal activities of essential of *C. aromaticus* and its major compound carvacrol against *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensis.*

2. Material and methods

2.1. Plant material

The leaves of *C. aromaticus* (Figure 1) were collected from Kolli Hills of the Eastern Ghats of Tamil Nadu, India and lies between 10° 12′-11° 7′N, 76°-77° 56′E, and altitude 1,300m that is rich in biodiversity. This plant is located in the Namakkal district of Tamil Nadu, India. The collected leaves were shade dried for 20 days (25–37 °C).

Fig 1: *Coleus aromaticus*

2.2. Preliminary processing

The essential oil isolation was carried out by hydrodistillation method using Clevenger type apparatus [7]. The dried leaves were coarsely powdered using a kitchen blender About 25gm of powdered leaves weighed and it was dispensed to 500ml of the distillation flask with 200ml of distilled water. They were immersed for 2 hours, followed by heating at 70-100 °C for 3 hours. The distillate was further transferred to a 250ml separation flask and volatile compounds were taken out from the water phase four times using dichloromethane. The volatile matter was isolated and dried in a Rota-vapor at low temperature to giving brownish-yellow oil. The oil was stored in room temperature until the completion of assays.

2.3. Gas chromatography–mass spectroscopy analysis

Gas chromatography (GC) analysis was carried out using Thermo GC - trace ultra ver: 5.0, thermo MS DSQ II (Thermo scientific, US). The chromatograph was fitted with ZB 5ms capillary standard non-polar column. The injector temperature was set at 260 °C and the oven temperature was initially set at 70 °C. Helium was used as a carrier gas with the flow rate of 1.0 ml/min. One milliliter of the sample was injected in the split mode in the ratio of 1:100. The percentage of the essential oil composition was calculated by the GC peak areas. Gas chromatography–mass spectroscopy (GC-MS) analysis of essential oil was performed using thermo DSQ gas chromatography.

The mass spectrometer was operated in the electron impact mode at 70 eV. The mass spectra were obtained by centroid scan of the mass range from 40 to 1,000 amu. The individual phytocompounds were identified by using the Wiley/ NBS registry of mass spectral database, the NIST (version 3.0) database. Furthermore, the Retention Time (RT) several authentic reference compounds were compared with isolated compounds for identification.

2.4. Maintenance of mosquito larvae

The larval stage mosquitoes of *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* were obtained from the National center for Disease Control (NCDC), Tamil Nadu, India The larvae were kept in plastic trays containing tap water and maintained in the laboratory. All the experiments were carried out at 25 to 29 °C with 80% of relative humidity, 14:10 light and dark photoperiod cycle. Dog biscuit and yeast powder in the proportion of 3:1 were fed to the larvae and further they were maintained and reared in the laboratory.

2.5. Larvicidal bioassay

The bioassay of larvicidal activity of essential oil/carvacrol was evaluated as per the method recommended by World Health Organization [44]. A stock solution (10%) of essential oil and carvacrol in ethanol and a dilution series of 10,20,30,40 and 50µg/ml were prepared. Twenty five of fourth instar larvae were transferred to a disposable paper cups, in 249 ml of water and 1.0ml of essential oil/carvacrol at the concentrations of 10, 20, 30, 40 and 50µg/ml. An equal number of controls were set up simultaneously using tap water. The dead larvae were counted after 24 h of exposure and the percentage of mortality was calculated from the three replicates. The LC_{50} (Lethal concentration that kills 50 % of the exposed larvae) and LC_{90} (Lethal concentration that kills 90% of the exposed larvae) values were calculated after 24 h by probit analysis [11].

2.6. Statistical analysis

The average larvicidal data were determined in order to analysis for calculating LC_{50}, LC_{90} and other statistics at 95 % confidence limits of upper confidence limit (UCL) and lower confidence limit (LCL) values and chi-square test were calculated using the SPSS13.0 (Statistical Package of Social Sciences) software version 13.0.

3. Result and Discussion

3.1. Essential oil chemical composition

In GC-MS analysis, 13 compounds were identified from the essential oil with 99.9%. The major components were identified as carvacrol (27.91 %), Dodecane (24.43), Beta-sitosterol (7.58%), Stigmasterol (6.07%), Phytol (3.87%), Eugenol (3.87%) (Figure 2, Table1). In this study the variations in the chemical profile of *C. aromaticus* essential oil was observed from the earlier reports of Tewari *et al* [39] and Joshi *et al.* [18]. The possible reasons for the differences in essential oil composition are the maturity, seasonal diversity, geographical location, genetic diversity and part of the plants used for essential oil isolation [2].

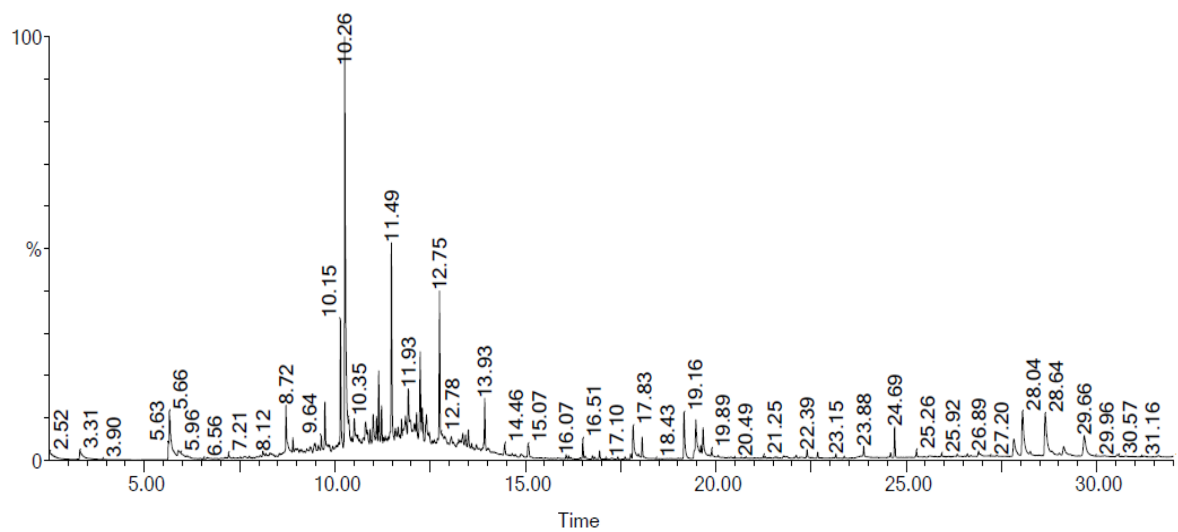

Fig 2: GC-MS analysis of *C. aromaticus* essential oil

Table 1: Composition of essential oil from *C. aromaticus*

S. No.	RT	Area	%	Compound name	Biological properties
1.	5.660	22,090,822.0	6.885	Tert-butyl-[2-[2-(2 methoxyethoxy)ethoxy]ethoxy]dimethylsilane	Anti inflammatory
2.	8.721	14,541,109.0	4.532	Dodecane, 1-fluoro-	Antioxidant
3.	9.741	9,174,847.0	2.859	Sulfurous acid, 2-ethylhexyl tridecyl ester	Disinfectant
4.	10.146	23,697,954.0	7.386	Dodecane, 1-fluoro	Antioxidant
5.	10.257	89,567,592.0	27.914	Carvacrol	antioxidant, anticancer
6.	11.152	12,423,978.0	3.872	Eugenol	Antiseptic
7.	11.487	41,181,784.0	12.835	Dodecane	Antiseptic and anesthetic
8.	12.242	16,843,174.0	5.249	Oxalic acid, 6-ethyloct-3-yl isobutyl ester	Antioxidant, anticancer
9.	12.747	24,663,066.0	7.686	Tritetracontane	Insecticidal
10.	13.933	10,435,310.0	3.252	Tritetracontane	insecticidal
11.	19.160	12,434,523.0	3.875	Phytol	Fragrance
12.	3.875	19,485,996.0	6.073	Stigmasterol	Anticancer and steroid
13.	28.639	24,325,008.0	7.581	Beta-sitosterol	Anti prosthetic, hyperplasia

3.2. Larvicidal activity of essential oil and carvacrol

The larvicidal potential of *C. aromaticus* essential oil and its major compound carvacrol were evaluated against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi*. Among the tested samples, the highest mortality was found in essential oil treated *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* with the LC_{50}; LC_{90} of 16.84256 (11.45-20.71); 48.83991(43.31-57.63), 20.578 (15.62-24.11) ; 54.759 (48.24-

65.42) and 23.255 (18.95-26.78); 56.428 (48.88-66.99)µg/ml, whereas, carvacrol had 35.67071(32.565-39.228); 64.64680 (57.834-75.209), 38.20320(35.062-42.004); 77.93745(68.746-93.804), 40.55376(36.975-45.237); 72.03935(63.484-86.023) µg/ml respectively (Table 2). No mortality was observed in control.

In general, plant essential oil recognized as an important resource for insecticides. Each part of plants contain a mixture of phytochemicals and secondary metabolites with distinctive biological activities (Ghosh, 2012). The diversity in the mosquitocidal activity of plant extracts is probably due to the differences in their active compounds, genetic characteristics of plant species and its harvested conditions [38]. In our result essential oil of *C. aromaticus* showed significant larvicidal activity. This result is also comparable to earlier reports of Sosan *et al* [36] who reported that the 100% mortality was observed in 100, 200 and 300ppm of *Ocimum gratissium, Cymbopogon citrus* and *Ageratum conyzoides* essential oil. Similarly, the dose dependent mortality was observed in the essential oils of *Ipomoea cairica* against *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* with 100% mortality at 100 to 170 ppm [40], *Zingiber officinale* against *Cx. tritaeniorhynchus* and *An. subpictus* with LC_{50}; LC_{90} of 98.83; 57.98, 186.55; 104.23 ppm [14], *Cinamomum camphora*, *Myrtus caryophyllus*, *Eucalyptus globullus* against *Ae. agepti* with higher mortality at 400ppm [32]. The larvicidal activity of mentha, clove and calamus essential oil against *Cx. quinquefasciatus* with LC_{50}:LC_{90} of 39.74; 115.67, 39.80; 149.814, 40.739; 126.035ppm [25], *C. arizonica* against *An. stephensi* with LC_{50} and LC_{90} of 79.30 and 238.89 ppm [27] were documented. In our study, comparatively the lower LC_{50}; LC_{90} were determined in essential oil treated *Cx. quinquefasciatus* (16.84; 48.83) followed *Ae aegypti* (20.57; 54.75) and *An. stephensi* (40.55; 72.03)µg/ml than carvacrol.

From the results it can be concluded that, *C. aromaticus* essential oil and carvacrol are potential for controlling mosquito vectors. These results could encourage the search for active natural compounds offering an alternative to synthetic insecticides. These are environmentally safe and eco-friendly approaches for the vector control. The variations in lethal concentration and lethal time or maybe due to insecticidal ingredients of plants, time of collection and season [37]. The mode of action of essential oils of mosquito larvae is not known but the earlier studies of Rey *et al* [34], David *et al.* [9] stated that the plant chemicals initially affect the midgut epithelium, gastric caeca and Malpighian tubules in mosquito larvae. Also, Usta *et al.* [41] reported that it interfered with the proper functioning of mitochondria at the proton transferring sites. Other possible targets of essential oils are transient receptor type ion channels, acetylcholinesterase and receptors tyramine, octopamine and gamma-aminobutyric acid (GABA). Instead of synthetic insecticides, plant compounds could lead to the development of potent natural mosquitocidal products. Due to volatile nature of plant essential oil, its insecticidal products are easily degraded and it leads lower level of risk to the environment than synthetic insecticides. In summation, the use of natural plant based products by individual and communities would offer local employment and trim down the dependence on expensive imported synthetic products and stimulate local efforts to raise public health [5]. Recent studies have indicated that the essential oil and carvacrol, which is the main constituent of *C. aromaticus* possessed more toxic to the fourth instar larvae of *Ae. Aegypti, Cx. quinquefasciatus* and *An. Stephensis.* Similarly, Govindarajan *et al*. [15] reported that the potent larvicidal activity of thymol from the *C. aromaticus* essential oil itself toward the early third instar larvae of *An. subpictus*, *An. albopictus* and *C. tritaeniorhynchu.*

Table 2: LC_{50}, LC_{90} and chi square analysis of Larvicidal activity of essential oil and carvacrol of *C.aromaticus* against *Ae aegypti. Cx. quinquefasciatus* and *An. Stephensi*

Samples	LC_{50} LCL–UCL (95 % confidence limit), µg/ml	LC_{90} LCL–UCL (95 % confidence limit), µg/ml	χ^2	df
Ae. aegypti				
Essential oil	20.57808(15.627-24.344)	54.75958(48.249-65.426)	3.265	3
Carvacrol	38.20320(35.062-42.004)	77.93745(68.746-93.804)	3.646	3
Cx.quinquefasciatus				
Essential oil	16.84256(11.435-20.716)	48.83991(43.310-57.632)	4.873	3
Carvacrol	35.67071(32.565-39.228)	64.64680(57.834-75.209)	3.331	3
An. Stephensi				
Essential oil	23.25586(18.956-26.783)	56.42830(48.885-66.999)	3.415	3
Carvacrol	40.55376(36.975-45.237)	72.03935(63.484-86.023)	3.071	3

LC_{50} lethal concentration that kills 50% of the exposed larvae, *LC_{90}* lethal concentration that kills 90% of the exposed larvae, *UCL* Upper Confidence Limit (95% fiducial limit), *LCL* lower confidence limit (95% fiducial limit), χ^2 Chi square, *df* degrees of freedom.

4. Conclusion

Based on GC-MS analysis, thirteen compounds from essential oil were analyzed with the major compound of carvacrol. The essential oil and carvacrol from *C. aromaticus* had excellent larvicidal activity against three mosquito vectors of *Ae. aegypti, Cx. quinquefasciatus* and *Ad. stephensi*. Our findings indicated that the essential oil from C. *aromaticus* and its bioactive component may be explored as potential and environmentally benevolent mosquitocides. Further investigations about the mode of action of the constituent effect on nontarget organisms and field evaluation are necessary prior to commercialization. These results will be useful in the search of new selective, biodegradable, and naturally produced mosquito control in the future.

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