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Sublethal Effects of Ethanol Extract of *Ocimum sanctum* on Laboratory Bred Population of Dengue Mosquito *Aedes aegypti* L. (Diptera: Culicidae)

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Abstract

Objectives: *Aedes aegypti* is a vector of several diseases which cause serious discomfort and fatality to human being. Current investigations were carried out to assess sublethal effects of ethanol extract of the leaves of *Ocimum sanctum* against *A. aegypti* in order to find ecofriendly alternatives for integrated vector management".

Methods: Lethal and sublethal effects of ethanol extract of *O. sanctum* against fourth instar larvae were assessed by using WHO protocol. The studies were extended to evaluate the effects of the extract on growth and development of the treated larvae. The chemical components present in the extracts were analyzed by Gas chromatography and mass spectroscopy (GC-MS).

Results: Exposure of fourth instar larvae of *A. aegypti* revealed moderate toxicity. However, the treated larvae exhibited mortality during subsequent days of exposure. The treatment with the extract also has adverse impact on growth and development of the larvae. Consequently there was increase in the larval duration in the treated larvae. Larva-pupa intermediate and pupa-adult intermediate were the developmental anomalies observed in the treated fourth instar larvae. This suggested presence of juvenile hormone mimic or juvenile analogue activity in the ethanol extract of *O. sanctum* leaves. Presence of farnasene, a derivates of the intermediates in the juvenile hormone biosynthesis was reported in the GCMS analysis of the extract.

Conclusion: Our studied indicated moderate lethal and efficient sublethal effects of ethanol extract of *O. sanctum* against *A.aegypti.* The bioactive compounds present in the extract, individually or synergistically may be used in formulating a strategy for the mosquito management.

Keywords

Aedes aegypti; Ocimum sanctum; Sublethal; Growth and development

Introduction

Aedes aegypti L. (Diptera: Culicidae) is the primary vector of diseases like dengue fever [1,2], yellow fever [3], chikungunya [4,5] and Zika virus [6]. The incidence of dengue fever has increased dramatically around the world, specifically in last decade. One recent estimate indicated 390 million dengue infections per year worldwide of which 96 million manifest clinically with severity of disease [7]. In fact the actual number of dengue cases is much more; many cases are misclassified and underreported [8]. In 2016, dengue outbreak occurred worldwide; the region of Americas reported more than 2.38 million cases [9]. Official records of Union health ministry of India reported a significant increase in the number of dengue cases in last few years [10]. It was estimated that that 3.9 billion people, in 128 countries, are at risk of infection with dengue viruses [11].

In the absence of effective vaccine against many viral disease transmitted by A. aegypti, control of the mosquito population is the only mean to prevent the spread of the diseases. Synthetic insecticides are widely used as larvicide in mosquito control programs worldwide. However, they are toxic to non-target organisms, have high mammalian toxicity and affect the environment by contaminating soil, water, and air [12]. In past few decades mosquito populations have developed resistance against a variety of synthetic organic chemicals. A. aegypti has developed resistant against deltamethrin [13], an extensively used insecticide in mosquito control programs [14].

Plant possesses a variety of phytochemicals. Many of these are secondary metabolites and are used for protection against herbivore insect predators. Therefore, they are natural candidates for the discovery of new compounds to combat insect menace [15]. Plant based chemicals have an advantage over the synthetic chemicals as they are target specific, biodegradable, environmental friendly and relatively nontoxic to human; also chances of development of resistance in insects against plant products are less [16,17].

Ocimum sanctum commonly called tulsi in India is "*queen of the herbs*" [18]. Essential oils present in the leaves of *O. sanctum* have been reported to possess wide range of activities in insects. Many species of *Ocimum* have larvicidal activity against *A. aegypti* and *Culex quinquefasciatus* [15,19,20]. Besides antifeedent activity [21] and repellent activity [22] of *Ocimum* has also been reported against *Aedes* and other insects. Some of the bioactive compounds present in *Ocimum* function as insect growth regulators and disrupt growth and development in insects [23].

Most of the studies on *O. sanctum* were focused on larvicidal activity and lethal effects on *A. aegypti.* Very few reports were available on delayed toxicity and sublethal effects of plant extracts on *A. aegypti.* Growth, development and reproduction are important components of insect life; aberration of these can hamper the insect population. Therefore, a schematic research on implication of ethanol extract of *O. sanctum* against fourth instar larvae of *A. aegypti* was initiated in search for effective affordable natural products which can be used in integrated vector management programme of *A. aegypti.*

Materials and Methods

Collection of plant material

The leaves of *O. sanctum* were collected from the botanical garden of Deshbandhu College, Kalkaji, New Delhi, India (28.5403° N,

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77.2544° E), during April-May, 2017 and brought to the laboratory in polythene bags. The leaves were washed thoroughly with tap water in order to remove dust or any other particles stuck to them. These were kept in shade at temperature (30 ± 2°C) for about 30 days to dry completely.

Preparation of ethanol extract

Extract of *O. sanctum* was prepared by cold extraction method [24]. The dried leaves were mechanically grinded with the help of electric grinder to make a fine powder. The powder of the leaves was mixed with five times ethanol (weight to volume) and kept for 24h at a temperature 27°C. The extract was filtered by using Whatman filter paper No 1. The residue after first extraction was extracted again twice in the same manner. Subsequently, the extracts were pooled, and concentrated using a vacuum rotary evaporator (Buchi) at 45°C. After complete evaporation of the solvent, the concentrated extract was collected and stored as 10% stock soulution in a refrigerator at 4°C for further use.

Rearing of mosquitoes

The present investigations were employed on the dengue fever mosquito, *A. aegypti.* The stock culture was obtained from International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. The colony was maintained in an insectary at temperature 28 ± 1°C, 80 ± 5% relative humidity and 14 L: 10 D photoperiod [25]. Adults were provided with freshly soaked deseeded raisins. Periodic blood meals were provided to female mosquitoes for egg maturation. The eggs were collected in a bowl lined with Whatman filter paper and were allowed to hatch in enamel trays filled with dechlorinated water. Larvae were provided with a mixture of yeast powder and grinded dog biscuits in a ratio on 1:3. The pupae were collected in enamel bowl filled with dechlorinated water and transferred to the cloth bag cages for adult emergence.

Larval toxicity bioassay

The protocol prescribed by WHO [8] was adopted to conduct bioassay of *O. sanctum* leaf ethanol extract against fourth instar larvae of *A. aegypti.* Laboratory reared *A. aegypti* larvae were used for the larvicidal bioassay. Twenty-five fourth instar larvae were kept in an enamel bowl (capacity 500mL) containing 249 mL of dechlorinated water and 1mL of ethanol leaf extract of *O. sanctum.* Concentrations of 400 ppm, 200 ppm, 100 ppm and 50 ppm of the extract were used for the bioassay. In control, 1 mL of ethanol was mixed to 249 mL of dechlorinated water. Normal food was provided to the experimental larvae. Mortality in the control and experimental set ups was recorded after 24h of exposure. The experiments were continued to observe day wise mortality, larval duration, and developmental abnormalities in the larvae and the pupae. The various developmental abnormalities were photographed using Nikon SMZ745T stereo zoom microscope The control mortality, if any, was corrected by using Abbott‟s formula [26]. All the experiments, unless specified, were replicated five times.

Statistical analysis

SPSS version 19 software was used for analysis of data. Anova: one factor was used to test the significant difference between groups. Tukey post hoc test was used to identify the significant difference within the group. Results with $p<0.05$ were considered to be statistically significant [27].

GC-MS Analysis of the Ethanol Leaf Extract of *Ocimum sanctum*

The phytochemicals present in the ethanol extract of *O. sanctum* were analyzed through gas chromatography and mass spectroscopy (GC-MS) [28]. The concentrated extract of *O. sanctum* was dissolved in ethanol and injected into the Gas chromatography unit (Shimadzu GC-MS QP2010). The injector temperature was maintained at 250 °C. The detector used was flame ionization detector which was maintained at 280 °C. The pressure of the carrier gas, nitrogen was kept at 10 psi. The oven temperature was set at 60 °C to 280 °C with a gradual increment of 10 °C per min. The injected extracts were eluted in the DB-5 MS column of 30m long and 0.25 mm inner diameter and the eluted constituents were detected by flame ionization detector. The GC chromatogram was recorded. From the graph, the compounds were identified by comparing the data with the existing software libraries like WILEY08, NIST08 and NIST08s [29].

Result

The present study was carried out with an aim to develop a safe and environment-friendly strategy for mosquito management. Efficacy of ethanol extract of *O. sanctum* was assessed for survival and longevity of early fourth instar larvae of *A. aegypti.* The results of larvicidal bioassays showed moderate toxicity of the ethanol extract of *O. sanctum* against early fourth instars larvae of *A. aegypti*. In a population of the fourth larval instar, 80.00%, 24.00%, 4.80%, and 4.00% mortality was observed after 24h of the exposure to 400 ppm, 200 ppm, 100 ppm and 50ppm respectively (Table 1). Exposure to 400ppm of the ethanol extract of *O. sanctum* also caused mortality during subsequent days. It was recorded that at this concentration the mortality on second, third, fourth and fifth day was 5.6%, 0.8%, 1.6% and 0.8% respectively (Table 2).

Ethanol extract of *O. sanctum* has an effect on the larval duration (Figure 1). Normally the fourth instars larvae developed into pupa after 2.61 days. However, the larval duration of the surviving fourth instar increased to 3.68, 3.62, 3.68 and 4.45 days after treatment with 50ppm, 100 ppm, 200 ppm and 400 ppm respectively. The results were statistically significant ($P< 0.001$).

Treatment of the fourth instar larvae with the extract also resulted in formation of larva-pupa intermediate. The pupae formed from the treated larvae sometime moulted into pupa-adult intermediate. The body of the fourth instars larva of *A. aegypti* is differentiated into head,

Table 1: Larvicidal activity of ethanol extracts of *Ocimum sanctum* against fourth instar larvae of *Aedes aegypti.*

Concentration (ppm)	Percent mortality after 24 hr of exposure* (Mean ± S. E.)	F value	*P* -value
50	4^{a} ± 2.53	82.368	≤0.001
100	4.8^{a} ± 2.33		
200	24^{b}± 6.32		
400	80^{c} ± 4.0		
Means followed by the same letter in a column are not significantly different at P<0.05 (ANOVA followed by Tukey test). *Average of five replicate, 25 fourth instar larvae per replicate			

Table 2: Day wise larvicidal activity of ethanol extract of *Ocimum sanctum* on fourth instar larvae of *Aedes aegypti*.

Concentration	Day wise mortality* (Mean ± S. E.)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
50 ppm	4±0.06	0	0	0	0.8 ± 0.02	0.8 ± 0.02
100 ppm	4.8 ± 0.05	0	0	0	0	0.8 ± 0.02
200 ppm	24 ± 0.14	0.8 ± 0.02	0	0	0	-
400 ppm	80 ± 0.09	5.6 ± 0.09	0.8 ± 0.02	1.6 ± 0.04	0.8 ± 0.02	-
*Average of five replicate, 25 fourth instar larvae per replicate						

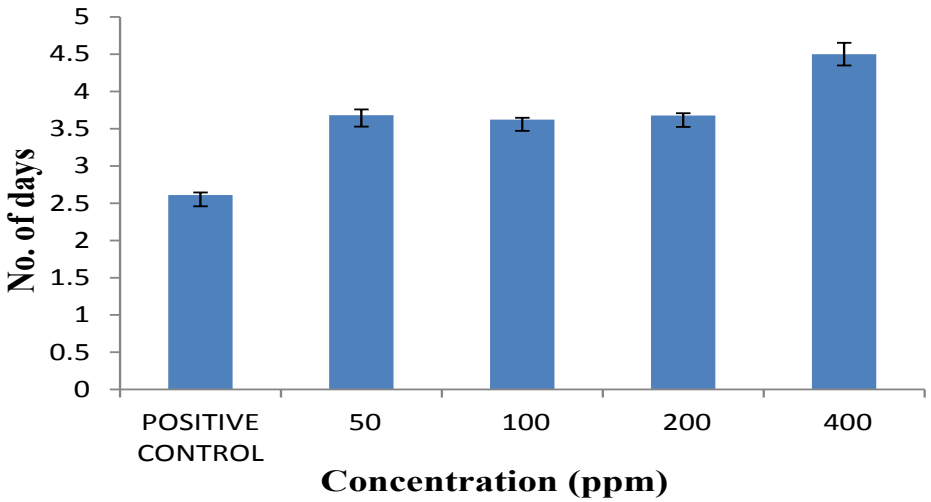

Figure 1: Influence of ethanol extract of *Ocimum sanctum* on larval duration of fourth instars larvae of *Aedes aegypti*.

thorax and abdomen. A pair of eye spot is present in head region and respiratory siphon and tracheal gills are present on last abdominal segment. The body is covered with chitinous setae (Figure 2a). Body of pupa is divided into cephalothorax and abdomen. Cephalothorax region possesses single median eye, a pair of respiratory siphon and rudimentary appendages. The abdomen is attached to the cephalothorax at an angle which gives a characteristic comma shape to the pupa. Tracheal gills were present on last abdominal segment (Figure 2c). In case of the larva-pupa intermediates body divided into cephalothorax and abdomen. Like pupa a single median eye, a pair of respiratory siphon and rudimentary appendages were present in cephalothoracic region. The chitinous setae were absent. However, the abdomen was attached to the cephalothorax in a straight line. Consequently, characteristic comma shape of the pupa was absent in the larva-pupa intermediate. Like fourth instar larva, the respiratory siphon was also observed on the last abdominal segment of larva-pupa intermediate (Figure 2b). Pupa-adult intermediates retained most of the pupal characters but showed presence of one pair of wings and three pairs of jointed legs in the cephalothoracic region (Figure 3). Formation of larva-pupa intermediate was dose related. The results were statistically significant ($P< 0.007$) (Table 3). Pupa-adult intermediate was observed in the experimental set ups of treatment with 100ppm. GC-MS analysis of ethanol extract of *O. sanctum* revealed the presence of 51 compounds (Figure 4). Some of the compounds like eugenol, caryophyllene, alpha.-farnesene, farnesyl acetone and geranylgerniol have been described for their insecticidal, larvicidal, mosquitocidal, insect deterrent, and insect repellant activity (Table 4) [30-37].

Discussion

Mosquitoes affect the external and internal environment of human being by creating a biting nuisance that deter outdoor activities and by transmitting pathogens of deadly disease. Although, many mosquito species are not important in pathogen transmission, they take blood meal from a vertebrate host in order to complete the gonotropic cycle of ovaries and development of eggs [38]. Mosquito borne diseases can be controlled by the preventing mosquito bite to human beings; this can be achieved mainly by controlling the mosquito population. Since five decades synthetic chemicals have been used as a mean to control the mosquito population. Synthetic chemicals had larvicidal, adulticidal, ovicidal, oviposition-deterrent and repellent activities. Extensive use of synthetic organic chemicals resulted in environmental hazards and the development of resistance in mosquito species. This has necessitated the need for search and development of environmentally safe and low cost methods for mosquito control. Phytochemicals alter the different life stages of mosquito hence may be used as a potential weapon against the mosquito population.

Present study evaluated the potential role of ethanol extract of *O. sanctum* in the management of mosquito population by altering the growth and development of the fourth instar larvae. The study revealed that *O. sanctum* ethanol leaf extract had moderate larvicidal activity. These results are in agreement with that of [39] who reported the larvicidal activity in acetone, chloroform, ethyl acetate, hexane and methanol leaf and flower extract of *Ocimum* against *A. aegypti* and *Culex quinquefasciatus*. He reported moderate larvicidal activity in leaf extract of *Ocimum* [40] studied larvicidal effects of methanol extract of *O. canum* and acetone extract of *O. sanctum* against the

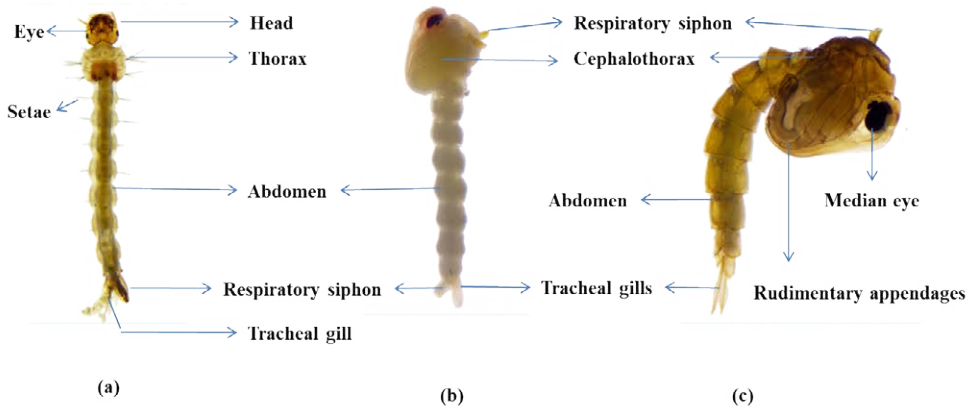

Figure 2: Developmental anomalies in the *Aedes aegypti* after treatment with ethanol extract of *Ocimum sanctum*(a.) Normal fourth instar larva (b.) larva-pupa intermediate (c.) Normal pupa.

[Images were taken on Nikon SMZ 745T Stereozoom microscope].

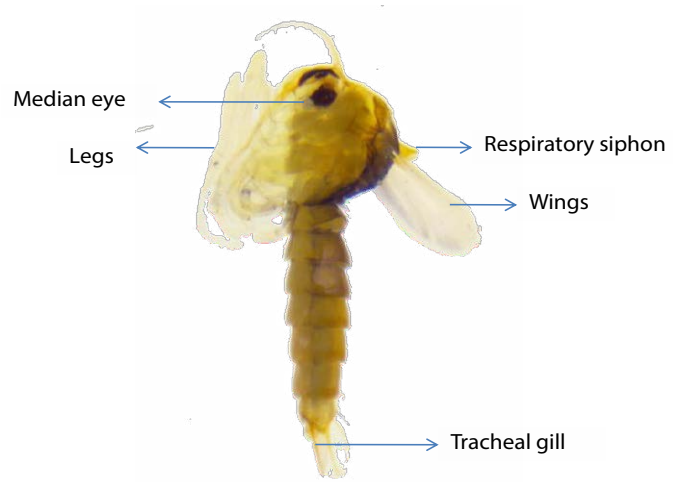

Figure 3: Pupa-adult intermediate of *Aedes aegypti* formed after treatment of fourth instar larva with the ethanol extract of *Ocimum sanctum.*

Table 3: Influence of ethanol extract of *Ocimum sanctum* on Larvae-pupa intermediate formation in the fourth instar larvae of *Aedes aegypti.*

Concentration (ppm)	Percent larva pupa intermediate* (Mean ± S. E.)	F value	*P*-value
50	$0.0^{a} \pm 0.0$	6.625	0.007
100	$4.0^{bc} \pm 2.31$		
200	$6.67^{c} \pm 1.33$		
400	$0.0^{a} \pm 0.0$		

Means followed by the same letter in a column are not significantly different at P<0.05 (ANOVA followed by Tukey test). *Average of three replicate, 25 fourth instar larvae per replicate

larvae of *A. aegypti* (LC50=99.42 and 81.56 ppm) and against *C. quinquefasciatus* (LC50=44.54 and 38.30 ppm). Kelm and Nair [41] investigated that hexane extract of *O. sanctum* contained compounds like eugenol which was responsible for mortality in the fourth instar larvae of *A. aegypti.*

Sub-lethal effects of ethanol extract of *O. sanctum* were assessed by considering day wise mortality, larval duration and growth and development impairment. Although the extract could not inhibit the development completely, it mislead or the deranged the physiological cues which were required for the development of a normal adult. The ethanol extract of *O. sanctum* significantly prolonged the larval duration of *A. aegypti.*fourth instar larva. Nathan, et al. [42] reported similar activity in the leaf and seed extract of *Melia azedarach* against *Anopheles stephensi.* The inability of the fourth instar larva to molt in the pupa and formation of larva-pupa intermediate and moulting of pupa into pupa-adult intermediate indicated the presence of some JH mimic or JH analogue in the ethanol extract of *O. sanctum.* Parallel results of ethanol extract of *O. sanctum* leaf ethanol extracts were reported by Summarwar and Pandey [21] against the larvae of *Spodoptera litura.* Several developmental anomalies were reported in many insects as a sequel to plant extract treatment by many researchers. Kodandaram [43] reported the dose dependent effects

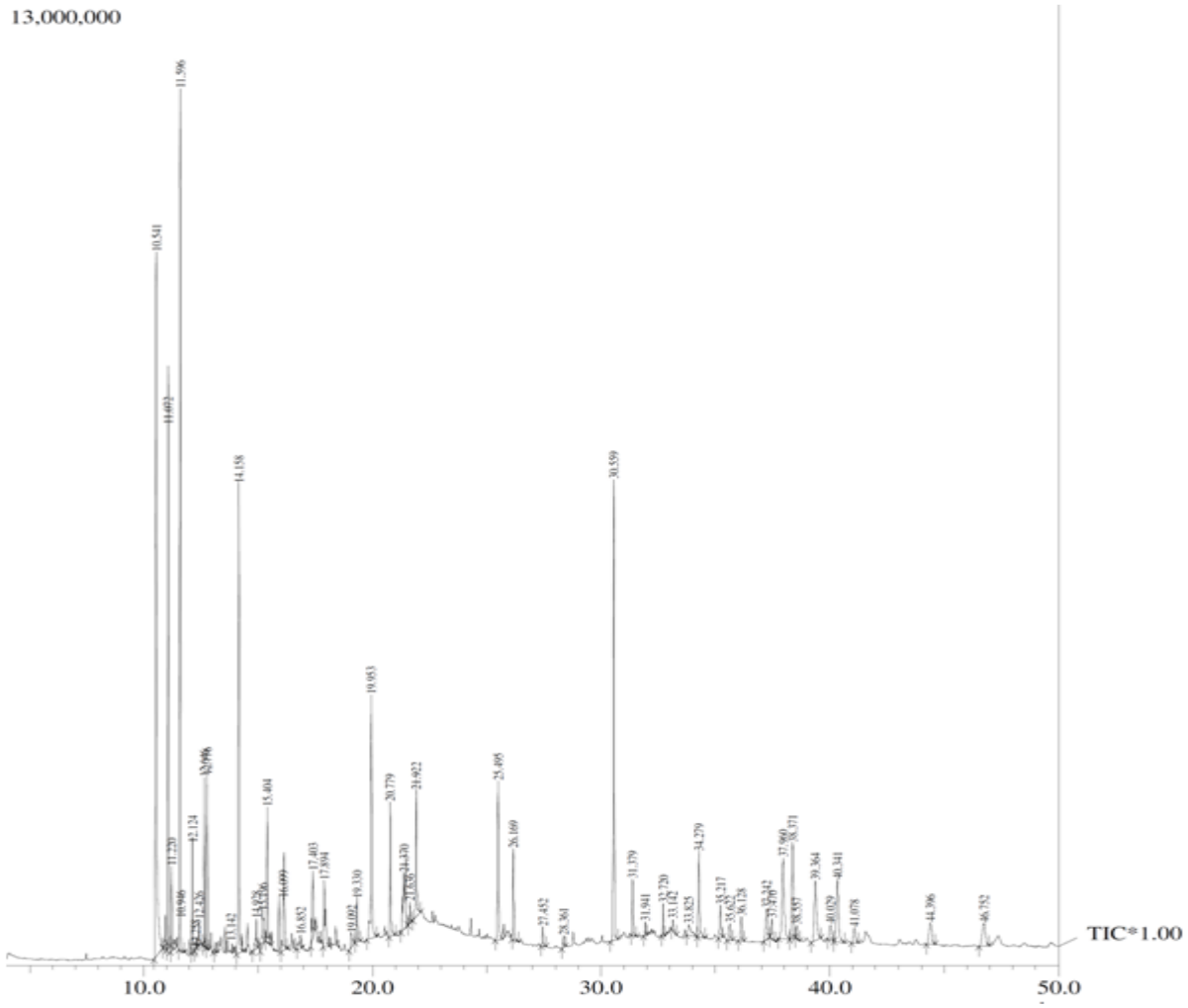

Figure 4: GC-MS Histogram of ethanol extract of *Ocimum sanctum.*

Table 4: GC-MS analysis of ethanol extract of *Ocimum sanctum.*

S. No	Name of the Compound	Molecular Formula	Compound Nature	Activity	References
1	Eugenol	$C_{10}H_{12}O_2$	-	Insecticide, larvicide, juvabional	[30].
2	Caryophyllene	$C_{15}H_{24}$	Sesquiterprnes	Antimicrobial, antioxidant, anti-tumor, antibacterial, anti-inflammatory, fungicide, insecticidal, antifeedant, insectifuge, larvicide, mosquitocide	[30-32]
3	Alpha.-farnesene	$C_{15}H_{24}$	Terpenoid	Insect deterrent, high mortality	[33]
4	Farnesyl acetone	$C_{18}H_{30}O$	-	Insect repellant	[34]
5	Geranylgeraniol	$C_{20}H_{34}O$	-	Bactericidal and precursors of Mevalonic acid pathway.	[31,35- 37]

of anonin, imidacloprid, karanjin, achook, and ecomeem against *D. koenigii.* Umamageswari [44] observed ecdysis inhibitor activity in the extracts of *Acorus calamus*, *Ocimum sanctum*, Neem, *Parthenium* and *Eucalyptus* against *Dysdercus cingulatus*.

GC-MS analysis of the *Ocimum* leaf ethanol extract revealed 51 peaks. Some of the important chemical compounds observed in the extract were eugenol, caryophyllene, geranylgerniol, hexadecanoic acid, etstigmasterol, alpha-farnesene, farnesyl acetone. Devendran and Balasubramanian [45] reported the presence of eugenol (43.88%), caryophyllene (26.53%), cyclopentane, cyclopropylidene-(1.02%), cyclohexane, 1,2,4-triethenyl (15.31%), octadecane, 1,1-dimethoxy-(2.04%) and benzene methanamine, N,N,a,4-tetramethyl-(2.04%) in hydroalcoholic extracts of O. sanctum. Methanol extract of *O. sanctum* showed the presence of three phytocomponents viz methyl-isoeugenol, caryophyllene and eugenol [46] reported the presence of terpenes, phenols, caryophyllene, eugenol, farnesene, geraniol, pthalic acid, terpieol and germacrene D in GC-MS analysis of essential oil of *O.sanctum*. Our study indicated that phytoconstituents present in the ethanol extract have chemical compounds responsible for insecticidal, growth and developmental disruptors against *A. aegypti.* eugenol has been reported to possess juvabional activity in a variety of insects [44]. Geranylgeraniol present in the extract acted as intermediate of mevalonic acid pathway of

juvenile hormone biosynthesis [31]. These compounds individually or synergistically acted as a JH mimic or analogue and effect the growth and development.

Present investigation explored the prospective role of ethanol extract of *O. sanctum* as larvicide and growth disruptor against A. aegypti. The investigations suggested potential sublethal effects of the extract. The growth and development of *A. aegypti* population in natural environment can be impaired by using the extract. In this way the mosquito population can be hampered specially during early stages. The results however may vary depending on the weather conditions and environmental intricacies.

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