CHEMICAL PROFILING OF ESSENTIAL OILS FROM *Ruta graveolens* L. AND ASSESSING ITS LARVICIDAL PROPERTIES AGAINST *Culex quinquefasciatus*

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Abstract. Mosquito borne diseases cause a severe menace to the society affecting large populations of people worldwide. Practicing the synthetic insecticides cause toxic effects to non target organism and mosquitoes adapts resistance towards synthetic insecticides. The Integrated Pest Management (IPR) aims to develop insecticides from biological sources as an alternative for chemicals based insecticides. In this context, *Ruta graveolens* is a well know medicinal plant bearing numerous therapeutic properties. The present study investigates the chemical profiling of essential oils and methanolic extract from the leaves of *Ruta graveolens*. The GC-MS analysis identified the presence of cimifugin, psolaren, chalepin, quinoxyfen, methoxsalen, nonanone, decanone, geijerene, octanone and nonanal in the oils and methanolic extract. The larvicidal activity of essential oils and methanolic extract were investigated against the *Culex quinquefasciatus*. The oil and methanolic extract inhibit the growth of IInd instar larvae with 100% mortality with significant LC50 value (oil 9 ppm and methanol extract 17ppm). The oil extract performed superior activity than methanol extract and the oils deteriorate the internal structures and membrane of the larvae observed by histopathological studies. Thus the preliminary studies shed light on the development of *Ruta graveolens* oils as an insecticide to eradicate the larvae of *Culex quinquefasciatus*.

Keywords: culex; diseases; insecticides; larvae; mosquitoes; oils.

INTRODUCTION

Mosquitoes culicidae are the prominent vectors transmit viruses, protozoa, nematodes and causes mosquito borne diseases all over the world [16]. Among the *culicidae* mosquitoes, *Culex quinquefasciatus* having holometabolic metamorphosis (egg-larva-pupae) with nocturnal habits and female are hematophagous [10]. The *Culex quinquefasciatus* cause filariasis in India affecting 25 millions of people with chronic infection [16]. Still now the suppression of *Cx. quinquefasciatus* population is a challenging to the researchers due to its specialty of adaption to various kind of ecosystem.

Synthetic insecticides pyrethroids, pyrroles, organochlorides, organophosphates, carbamates, and phenyl pyrazoles were effective but recently the *Cx. quinquefasciatus* adopted resistance mechanism against the insecticides by changing the metabolism and mutations in the enzymes [14]. In addition, synthetic insecticides cause lethal effects in non-target organism and also pose a threat to the environment [14]. Therefore, it is indeed to control the *Cx. quinquefasciatus* population through innovative strategies avoiding negative effects with prolonged efficacy.

Bio-insecticides derived from plants and microbes are one of the promising approaches to eradicate mosquitoes [28]. The bio-insecticide is eco-friendly, non-toxic and cost inexpensive having broad spectrum of activity against various species of mosquitoes [29]. From time immemorial, plants are used as a repellent against various mosquitoes [26]. Plants having the potential insecticidal attributes and approximately 1500 plant species is insecticide and still under exploration [31]. This is because plants contain bioactive metabolites alkaloids, flavonoids, phenols, triterpenoids and tannins confer defense mechanism against the infection of microbes or insects. Henceforth isolation, identification and structural elucidation of these important molecules can be developed as lead compound in Integrated Pest management.

The most predominant approach of abolishing mosquitoes is larvicides; the approach is eco-friendly, cost inexpensive and target specific. The plant extracts blends with active ingredients and these ingredients and synergistically executes the larvicidal activity with more efficacy and efficiency [6]. For instance extracts of *Syzygium lanceolatum, Argemone mexicana, Echinophora spinosa* and *Euphorbia antiquorum* [1, 2, 12, 22] exhibited strong larvicidal activities against *Cx. quinquefasciatus*.

In this context, herein we have chosen the plant *R. graveolens*, family of *Rutaceae* a perennial plant growing various habitats of terrestrial regions [5]. The plant is effective in treating the diseases of inflammation, rheumatism and dermatitis's [30]. In addition, the plant also reported to have numerous activities like anticancer, antimicrobial, nematicidal, larvicidal, antioxidant [25]. The plant leaves and other parts contain bioactive metabolites having the therapeutic potential needs to investigated exclusively.

Thus, the present study intends to observe the larvicidal potential of crude extract and essential oil from *R. graveolens* in the larvae of *Cx. quinquefasciatus*. The study will be executed by systemic extraction of metabolites using solvents, extraction of oils by hydro-distillation and evaluation in the third instar larvae of *Cx. quinquefasciatus*.

MATERIALS AND METHODS

Collection of Plant material

The plant leaves were collected from the JSS university campus, Ooty, India (Fig. 1). After collection the plant materials were immediately transferred to the lab and washed with distilled water. The plant materials were shade dried in room for 15 days. Further, the dried plant leaves were macerated using a blender for fine powder. The obtained fine powder was used for further experimental studies.

Figure 1. Aerial view of the *R. graveolens* plant

Extraction of oil

The *R. graveolens* was subjected to hydro distillation using Clevenger apparatus. For analysis, 200 g of *R. graveolens* leaf powder mixed with 800 mL of distilled water and operated at temperature of 40 °C for 4 hr. After the time period, the oils were collected and percentage of yield was calculated.

Crude Extract preparation

For crude extract preparation, percolation method was used. For extraction, 100 g of *R. graveolens* leaf powder was mixed with methanol solvent in soxhlet apparatus at 40 °C and continued for 3 cycles of extraction procedure. After the cycle process, the crude residue obtained was concentrated using rotary vacuum evaporator and subjected to further analysis.

Chemical profiling of oil and crude extract of *R. graveolens*

The GC-MS analysis was performed in the instrument GC-MS QP 2010 (Shimadzu). The instrument contains sample and injector and equipped with ion trap mass spectrometer. The column Bpx5 GC (length 30m , thickness and diameter 0.25mm) and helium gas at a flow rate of 1ml/min with an injection volume of 1µL were employed for the analysis. The ion source temperature and injector temperature was maintained at 200 °C and 250 °C. The oven temperature was raised from 60 °C to 300 °C and raised for 30 min at a rate of 15 °C/min. The mass spectrometry was operated in positive ionization mode with a 70 eV ionization energy. The scanning was conducted with mass fragments and the peak and total peak was computed to calculate the percentage of each component. The identified peaks were compared with NIST database for the identification of compounds.

Larvicidal activity of *R. graveolens* extract and oils

Collection of eggs

From the National Centre for Disease Control (NCDC) Mettupalayam (Tamil Nadu, India) the eggs of *Cx. quinquefasciatus* were obtained. After collection of eggs, it is brought immediately to the lab and maintained in 18×13×4 cm plastic containers containing 500 mL of tap water at the condition of 27±2 °C, 75–85 % R.H., 14:10 (L:D) photoperiod. Larvae were fed with a mixture of dog biscuits and yeast (3:1) ratio.

Larvicidal activity

The methodology was adopted from the previously published protocol [12]. Briefly; the methanolic extract (1 g) and essential oil (1 g) were prepared as 1% stock solution. From the stock solution, different concentrations 50, 100, 150, 200 and 250 ppm/L were prepared. 25 larvae of IInd instar *Cx. quinquefasciatus* were introduced into 500 mL glass beaker containing 249 mL of dechlorinated tap water and 1 mL of desired concentrations of methanolic extract and oils were added separately and kept in an environmental chamber at 25°C with a 16:8 light/dark cycle for 24h. Three replicates were set up for each concentration and larvae placed in dechlorinated water considered as control. Mortality was corrected by using Abbott (1925) formula and percentage mortality was calculated.

Histopathological Examination

Based on the results of larvicidal assay, the dead larvae from oil treatment was subjected to histopathological observation. The larvae had been fixed in formalin, dehydrated, and embedded in paraffin. Thin sections were cut and stained with hematoxylin and eosin for the microscopic examination to identify the abnormalities caused in the tissues of by the methanol extract, oil of *R. graveolens*.

RESULTS

R. graveolens leaves were subjected to hydrodistillation process in Clevenger apparatus. The oil extracted from the leaves of *R. graveolens* gave a yield of 10 mL with pale yellow color and aromatic. Likewise, the methanolic extract of *R. graveolens* gave a yield of 2 g. The crude extract was appeared to be dark green in color and viscous.

Chemical profiling of oil and crude extract of *R. graveolens*

The GC - MS analysis identified 10 compounds from the methanolic extract and oil extract from the leaves of *R. graveolens*. From the GC-MS spectrum the identified compounds were compared with NIST database and tabulated (Fig. 2 and 3). Table 1 and 2 infer the compounds identified from the methanolic extract and oil extract from the leaves of *R.graveolens*. The methanolic extract of *R. graveolens* contains cimifugin, psolaren, chalepin, quinoxyfen and methoxsalen and the oils of *R. graveolens* contain nonanone, decanone, geijerene, octanone, nonanal.

Larvicidal activity of oils and crude extract of *R. graveolens*

The oil and methanolic extract of *R. graveolens* were evaluated against the IInd instar larvae of *Cx. quinquefasciatus* at varying concentrations (ppm) for 24hr. From the analysis, it is observed that methanolic extract (ppm) and oil (ppm) delivered the dose response relationship activity against the larvae. At low concentration there was a minimal effect with less mortality and at medium concentration, the mortality level was moderate while at high concentration there was a high mortality rate observed in the IInd instar larvae of *Cx. quinquefasciatus* at 24h. These indicate that methanolic extract and oils have a strong insecticidal property due to the presence of bioactive metabolites in the oil and methanolic extract. The mortality rate in response to the treatment was statistically analyzed and the LC_{50} of oil was 9.68 ppm and methanolic extract LC_{50} 17.28 ppm was determined (Table 3).

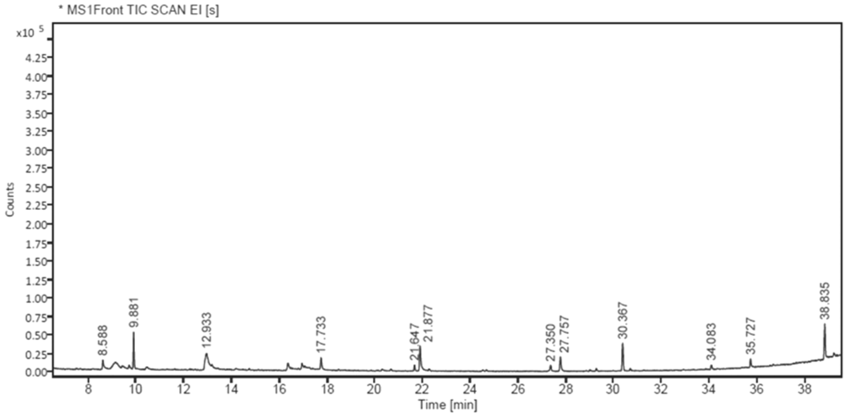

Figure 2. GC-MS spectrum of the methanolic extract from the leaves of *R. graveolens*

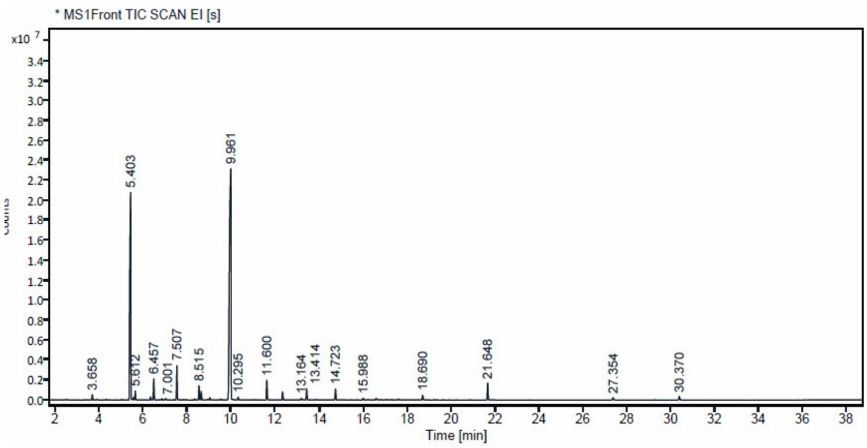

Figure 3. GC-MS spectrum of the oils from the leaves of *R. graveolens*

Table 1. GC-MS spectrum of the identified compound in methanolic extract from the leaves of *R. graveolens*

S. No.	Compound name	Retention time (min.)	Area %	Molecular formula	Molecular weight (g/mol)
1.	Cimifugin	35.72	3.59	$C_{21}H_{24}O_9$	388.40
2.	Psolaren, 3- (α, α dimethyl allel)-	30.36	13.32	$C_{16}H_{16}O_3$	256.28
3.	Chalepin	38.83	16.76	$C_{15}H_{14}O_4$	258.27
4.	2-undecanone	9.87	12.22	$C_{11}H_{22}O$	170.28
5.	Quinoxyfen	34.08	2.46	$C_{15}H_8C_{12}FNO_2$	307.70
6.	Ficusin	21.87	12.02	$C_{11}H_6O_3$	186.16
7.	Ethyl 5-(2H-1,3-benzodioxol-5-ylmethyl)-4,5-dihydro-1,2-oxazole-3-carboxylate	21.64	2.48	$C_{17}H_{19}NO_5$	317.33
8.	2-formyl-9-[βdribofuranasyl] hypoxanthine	12.93	18.39	$C_{11}H_{13}N_4O_5$	346.29
9.	β-D-Glucopyranose	17.73	7.62	$C_6H_{12}O_6$	180.16
10.	Isosorbide dinitrate	8.58	2.76	$C_6H_8N_2O_8$	236.14
11.	Benzamide, 2-methoxy-N-(2-phenylethyl)-N-hexyl-	27.34	2.47		
12.	7H-Furo[3,2-g][1] benzopyran-7-one, 4-methoxy-	27.75	5.91		

Table 2. GC-MS spectrum of the identified compound in oils of *R. graveolens*

S. No.	Compound name	Retention time (min.)	Area%	Molecular formula	Molecular weight (g/mol)
1.	2-Nonanone	5.40	30.73	$C_9H_{18}O$	142.24
2.	2-Nonanol, acetate	8.51	1.10	$C_{11}H_{22}O_2$	186.29
3.	2-Decanone	7.50	2.78	$C_{10}H_{20}O$	156.27
4.	Geijerene	6.45	1.81	$C_{15}H_{24}$	204.35
5.	Psorlen 3-(0,0 dimellylallyl)-	30.37	0.42	$C_{16}H_{14}O_3$	254.28
6.	2-octanone	3.65	0.46	$C_8H_{16}O$	128.21
7.	2-undecanone	9.95	55.27	$C_{11}H_{22}O$	170.29
8.	2-tridecanone	14.72	0.91	$C_{13}H_{26}O$	198.35
9.	Nonanal	5.61	0.66	$C_9H_{18}O$	142.24
10.	Chloroacetic acid, nonyl ester	7.00	0.14	$C_{11}H_{21}ClO_2$	220.73
11.	Acetic acid, nonyl ester	10.29	0.19	$C_{11}H_{22}O_2$	186.29
12.	2-Dodecanone	11.60	1.91	$C_{12}H_{24}O$	184.32
13.	Cyclooctene, 4-methylene-6-(1-propenylidene)-	13.16	0.17	$C_{12}H_{16}$	160.25
14.	1-Octene, 2-methoxy-	13.41	1.10	$C_9H_{18}O$	142.23
15.	Cyclohexanemethanol, 4-ethenyl- α, α, 4-trimethyl-3-(1-methylethenyl)-, acetate	15.99	0.16	$C_{17}H_{28}O_2$	264.40
	3,7-Cyclodecadiene-1-methanol, α, α,4,8-tetramethyl-,[s-(Z,Z)]	15.99	0.16	$C_{15}H_{26}O$	222.36
16.	Benz[f]azulene,1,2,3,3a,4,9,10,10a-octahydro	18.69	0.42		
17.	6-(3-Hydroxy-4-methylphenyl)-2-methylhept-2-2n-4-one	21.647	1.57	$C_{15}H_{20}O_2$	232.32
18.	Benzamide, 2-methoxy-N-(2-phenylethyl)-N-hexyl	27.35	0.19		

Based on the larvicidal activity results, the *R. graveolens* oil performed superior activity than methanol extract and henceforth the oil treated larvae was subjected to histopathological studies.

Histopathology results

The histopathological studies of *R. graveolens* oil essential oils were present in the Fig 4. As illustrated, the midgut larvae of *Cx. quinquefasciatus* are deteriorated by the oils. The control image depict that midgut larvae have normal peritrophic membrane, cuboidal, columnar cells and nucleus remains normal; whereas the *R. graveolens* oil treated larvae have severe abnormalities in the midgut larvae the cells were degenerated, the peritrophic membrane were damaged and internal collapse of the midgut regions were observed.

DISCUSSION

The methanolic extract of *R. graveolens* contains cimifugin, psolaren, chalepin, quinoxyfen and methoxsalen. These compounds are known to possess strong therapeutic properties as reported elsewhere [8, 11, 19, 20, 21, 34]. The oils of *R. graveolens* contain nonanone, decanone, geijerene, octanone, nonanal. The identified compounds belong to aliphatic ketones,

Table 3. GC-MS spectrum of the identified compounds in oils from the leaves of *R. graveolens*

Mosquito species	Plant	Name of the extract	LC_{50} (ppm)	95% confidence limit		LC_{90} (ppm)	95% confidence limit		Slope ± SE	Intercept ± SE	χ^2
				LL	UL		LL	UL			
Cx quinquefasciatus	*R. graveolens*	Methanolic Extract	17.28	13.21	31.41	42.51	25.44	143.92	0.9±0.6	3.2±0.6	2.7

Figure 4. Cross-segment part of the midgut of larvae treated with plant *R. graveolens* oil (**B**) as compared with control (**A**) CU: cuboidal cells, CC: columnar cells, N: nucleus, pM: peritrophic membrane, ES: ectoperitrophic space, MC: midgut content material, M: Muscle tissues, CM: round muscle groups, a.f: adipose fabric

alccohols, aldehyde, esters and unsaturated hydrocarbons [7, 9, 27, 35, 24]. Similar report of *R. graveolens* oils and metabolites extraction reported [32]; but in the present study the chosen plant is located at varying geographical area at high altitude ooty, India - 2,240 meters above sea level. The geographical locations (temperature and other factors) will also vary the chemical constituents present in the plants. Thus, in the present study there was a slight variation in the oil constituents as compared with report of Gina *et al.* [20]. Similarly, larvicidal potency of *R. graveolens* essential oil and methanolic extract were studied in larvae of *Cu. longiareolata*, *Ae. aegypti* and *An. stephensi* and the LC_{50} was determined 199.5 ppm; 31.89 and 66.96 ppm [3, 4, 18, 19]. But herein the present study, the oils and methanolic extract of *R. graveolens* remarkably proved its efficacy in larvae of *Cx. quinquefasciatus* than *Cu. longiareolata*, *Ae. aegypti* and *An. stephensi*. The nature of chemical constituents and its action may vary with mosquito species also. Thus a detailed study is need to be investigated in other species of mosquitoes also.

The active constituents present in the *R. graveolens* essential oil and methanolic extract create lethal toxicity in the larvae by membrane breaching, damaging the intracellular membrane inhibition of DNA/RNA/proteins, failure of respiratory chain and cell death [4]. In addition, other cellular events like inhibition of acetylcholine esterase, ion exchange disruption, nerve cell membrane disruption, hormonal imbalance and inhibition of metamorphosis [23]. Henceforth, we presume that our chemical constituents present in *R. graveolens* essential oil and methanolic extract might put forth above mode of mechanism to cause the toxic effect in the larvae of *Cx. quinquefasciatus.* Based on the larvicidal activity results, the *R. graveolens* oil performed superior activity than methanol extract and henceforth the oil treated larvae was subjected to histopathological studies. This is phenomenon is attributed due to the secondary metabolites of oils [33]. As reported secondary metabolites suppress the growth of larvae by various cascade mechanisms and block the cell cycle. The midgut region of mosquito is an important region where the enzyme secretion, ion transport, absorption of nutrients and defense reaction will occur [13, 36]. In addition the regenerative cells of midgut play an important role in metamorphosis. The plant metabolites disrupt the midgut and regenerative cells of larvae by collapsing the internal architecture and cease the growth process effectively.

The findings of this study can be used to optimize novel insecticides in the Coimbatore vicinity. By better understanding the mosquito diversity, it is possible to develop insecticides indigenously aimed at improving the inhibition of larvae at various ecosystems. The oils of *R. graveolens* must be further processed in various formulations to achieve the desired effects in the larvae breeding sources. Further studies are needed to deepen our understanding of these larvicides with larvae and to develop *R. graveolens* as an effective insecticide in near future.

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