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Study on larvicidal activity of crude extracts of *Ruta graveolens* against *Aedes aegypti* and *Anopheles stephensi*

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Abstract
The present study was conducted to identify the larvicidal activity of *Ruta graveolens* against larval cultures of *Anopheles stephensi* and *Aedes aegypti*. The larvicidal bioassay of each plant extract was conducted. The % of mortality was calculated. The petroleum ether extract showed maximum larvicidal activity against both the larvae when compared to the hexane and methanol extract. The petroleum ether extract was then subjected to TLC using the solvent system petroleum ether: chloroform: acetone at 0.5: 1.5: 0.5 ratios. The larvicidal activity of the pure compound was checked and the % mortality of different fractions was calculated. It was observed that the fraction 1 showed 100% mortality against *An. stephensi* and 80% mortality against *Ae. aegypti* and toxicity was not observed for the cream prepared for fraction 1. The functional groups were analyzed using FTIR analysis.

Keywords: *Ruta graveolens*, *Anopheles stephensi, Aedes aegypti*, larvicidal bioassay, mortality, FTIR analysis.

1. Introduction
A variety of organisms like insects and arthropods affects the human health by causing various fatal diseases. The insects related to epidemiology include mosquitoes, houseflies, sandflies, rat flea, tse-tse fly etc., and among these insects, mosquitoes play an important role. They are the causative agent of various fatal diseases like malaria, filariasis, encephalitis, yellow fever and dengue [6]. The mosquito species belonging to genera *Anopheles*, *Culex*, and *Aedes* are the major causative agents of these diseases [1]. Mosquitoes also cause allergic responses that include local skin and systemic reactions such as angioedema in humans [13].
Mosquito-borne diseases have an economic impact, which include loss in commercial and labor outputs, particularly in countries with tropical and subtropical climates; however, no part of the world is free from vector-borne diseases [4].
Aedes aegypti is the vector for an arbo-virus which causes dengue fever, which is endemic to Southeast Asia, the Pacific island area, Africa, and the Americas. This mosquito also spreads yellow fever in Central and South America and West Africa. It has been recently studied that more than 50 million people are at risk of dengue virus exposure worldwide. Annually, there are about 2 million infections, 500,000 cases of dengue hemorrhagic fever, and 12,000 deaths [7]. Reproduction of *Ae. aegypt*i occurs in water storage jars, drums, tanks, flower pots etc [11].
Anopheles stephensi is a vector of urban malaria in several countries of the Middle East and Indian sub-continent [5]. It is estimated that around 3.4 billion people are at risk of malaria, of which 1.2 billion are at high risk.
Plants, which are considered as a rich source of bioactive compounds [17] may be used as an alternative source of mosquito control agents. Natural products of plant origin with insecticidal properties have been tried in the recent past for the control of varieties of insect pests and vectors [3].
Ruta graveolens, (Ruta derived from Greek "reuo" means to set free) commonly called rue, is a dicot herb that belongs to the *Rutaceae* family. It is native to Europe, specially the Mediterranean region. *R. graveolens* contains many secondary metabolites such as furocoumarins, furoquinolines and acridone alkaloids, mainly present in the leaves, especially before blooming. The volatile rue oil has a pungent smell and bitter taste, and possesses antibacterial activity against *Micrococcus pyogenes var aureus* and *Escherichia coli*.
R. graveolens is used for medicinal preparations in homeopathic, ayurvedic, and unani [14] because this herb is so efficient against various diseases. It has been extensively used in

treatment of leucoderma, vitiligo, psoriasis, multiple sclerosis, cutaneous lymphomas and recently reported to possess anti-inflammatory and anticancer activity [12].

In the present study the larvicidal activity of various extracts of *R. graveolens* were studied and TLC was employed to separate the various components of the highly active extract. The larvicidal activity of the pure components was carried out and FTIR analysis was carried for the most effective component.

2. Materials and Methods

2.1 Collection of plant materials

The leaves of *R. graveolens* were collected from Munnar and Wayanad, Kerala and Gudalur, Tamilnadu.

2.2 Preparation of plant extract

Leaves of *R. graveolens* was washed with dechlorinated water, shade dried and powdered with the help of an electric blender. The test material (30g) was macerated with different organic solvents viz., hexane, petroleum ether and methanol for 48h. The mixture was then filtered through a Whatman No.1 filter paper and the filtrate was evaporated to collect crude extract.

2.3 Collection of mosquito larvae

Ae. aegypti and *An. stephensi* larvae were collected from National Centre for Disease Control field station of Mettupalayam, Tamil Nadu. The larvae were kept in plastic bottles containing tap water. They were maintained at 27 ± 2°C and 75–85 % relative humidity under 14:10 h light and dark cycles. The mosquito larvae were observed under the microscope.

2.4 Larvicidal activity of crude extract

Larvicidal bioassay was carried out by the method of WHO (1996). Bottles containing 100 ml of water and 10 numbers of early IV instar mosquito larvae of *Ae. aegypti* and *An. stephensi* were taken separately to treat in various concentrations of plant extracts. Four different step fold concentrations (250, 125, 62.5 and 31.25µg) of each plant extract were taken to carry out the experiments, with three replicates. Control was maintained by 1 ml of solvent in 99 ml of water and mortality was recorded after 24h separately. Larvicidal experiments were carried out separately under controlled laboratory conditions. The percentage of mortality was calculated using this formula,

$$\% \text{ of mortality} = \frac{\% \text{ mortality in test concentration} - \text{mortality in control}}{100 - \text{mortality in control}} \times 100$$

2.5 Purification of the active principles

Purification of secondary metabolites using TLC with the standardized solvent system was performed and the active principles were separated as fluorescent bands. R_f value of the different bands were calculated by:

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

2.6 Larvicidal activity of purified compounds

The 11 separated purified components were analyzed for its larvicidal activity. Bottles containing 25 ml of water and 5 numbers of early IV instar mosquito larvae of *Ae. aegypti* and *An. stephensi* were taken separately. The purified components of *R. graveolens* were added to the bottles. One milli litre of the solvent was added to the control as well. The percentage mortality was calculated.

2.7 Cream preparation

A mosquito repellent cream was formulated based on the standard methods using the most effective extracts. A measured quantity of petroleum jelly was melted in a vessel and heated to 65°C in a water bath. At this stage the extract was added to the molten petroleum jelly and prepared jelly was applied to the selected people for checking toxicity by skin irritation test.

The skin irritancy of formulation was evaluated in 9 healthy adult volunteers (8 females and 1 males; age range from 21-37 years). The area of application was first cleaned using ethanol. The formulation to be tested was applied to the skin of the volunteers. The cream was applied for 4h of exposure, after which the treatment sites were gently wiped with wet gauze to remove excess test material and then washed with distilled water. The sites of application were examined for irritation.

2.8 FT- IR analysis

IR- spectral analysis was performed using Shimadzu FTIR 8300 instrument, Japan. Potassium bromide pellet was prepared by mixing 1 mg of the fraction 1 of the petroleum ether extract of *R. graveolens* with 100 mg of anhydrous potassium bromide. The spectra were recorded from 400 to 4000 cm^{-1}.

3. Results

3.1 Preparation of plant extract

The weight of crude extract obtained after the process of maceration using the solvents hexane, petroleum ether and methanol is shown in Table 1.

Table 1: The yield of crude extract of *R. graveolens*

Solvent Used	Crude Extract (in g)
Hexane	0.06
Petroleum Ether	0.46
Methanol	2.62

3.2 Collection of mosquito larvae

The mosquito larvae collected from National Centre for Disease Control field station of Mettupalayam was observed under the microscope and photographs were taken (Fig 1 and 2).

Fig 1: Microscopic image of larvae of *Ae. aegypti*

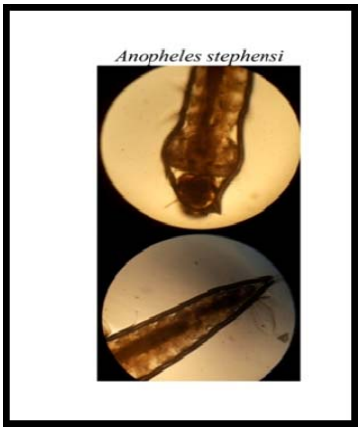

Fig 2: Microscopic image of larvae of *An. stephensi*

3.3 Larvicidal activity of crude extract

The larvicidal activity of the different crude extracts of *R. graveolens* was observed. The larvicidal activity was given in terms of percentage of mortality (Table 2).

The petroleum ether extract of *R. graveolens* showed maximum inhibition against both the larval species. The maximum, 100% inhibition was shown at 250 µg of the extract and minimum inhibition of 48.98% was shown at 31.25 µg against *An. stephensi*. Simultaneously, the maximum inhibition against *Ae. aegypti* was 79.59% at 250 µg of the petroleum ether extract.

The LC_{50} value of petroleum ether extract against *An. stephensi* was found to be 31.89 and against *Ae. aegypti* was 66.96 (Table 2). This results clearly shows that the petroleum ether extract have potent larvicidal activity against mosquitoes.

Table 2: % mortality of crude extracts on mosquito larvae

Larval Species	% Mortality (%)														
	Hexane (µg)					Petroleum Ether (µg)					Methane (µg)				
	250	125	62.5	31.25	LC_{50}	250	125	62.5	31.25	LC_{50}	250	125	62.5	31.25	LC_{50}
Anopheles stephensi	-	-	-	-	-	100	69.38	59.18	48.98	31.89	0.2	0.1	-	-	-
Aedes aegypti	-	-	-	-	-	79.59	69.38	48.98	38.77	66.96	-	-	-	-	-

3.4 Purification of active principles

The different components of the petroleum ether extract of *R. graveolens* were separated by the process of thin layer chromatography (Fig 6) and the R_f value of the different components was calculated (Table 3).

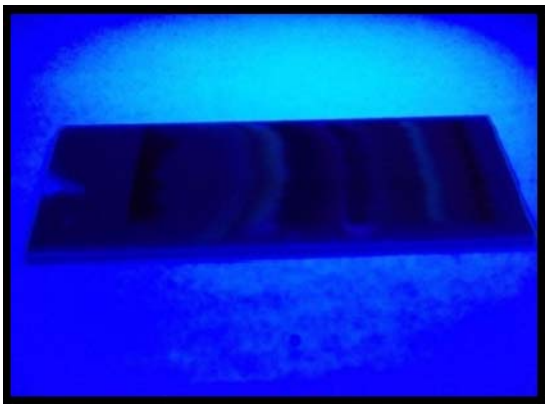

Fig 6: Thin layer chromatography

Table 3: R_f value of different fractions of *R. graveolens*

Fractions	Distance Travelled By The Solvent Front(cm)	Distance Travelled By The Compound (cm)	R_f Value
F1	9.5	1.5	0.157
F2	9.5	2.8	0.294
F3	9.5	3.5	0.368
F4	9.5	3.8	0.4
F5	9.5	4.4	0.463
F6	9.5	5	0.526
F7	9.5	5.5	0.578
F8	9.5	6	0.631
F9	9.5	6.5	0.684
F10	9.5	8.5	0.894
F11	9.5	9.4	0.989

3.5 Larvicidal activity of pure compounds

The different fractions of *R. graveolens* after thin layer chromatography showed larvicidal activity towards the mosquito larvae. The first fraction showed 100% mortality towards *An. stephensi* and 80% mortality towards *Ae. aegypti* and other fractions showed larvicidal activity but at a minimal percentage. The fraction F10 and F11 did not show any activity towards both the larvae. To the control, petroleum ether was added and the larvicidal activity was not observed (Table 4).

Table 4: % mortality of pure compounds on mosquito larvae

Fraction	% Mortality (%)	
	An. stephensi	*Ae. aegypti*
F1	100	80
F2	80	80
F3	80	60
F4	80	60
F5	60	60
F6	60	40
F7	40	40
F8	40	20
F9	40	0
F10	0	0
F11	0	0

3.6 Toxicity Study

The petroleum ether extract was mixed with petroleum jelly and cream was prepared. The toxicity of the formulation was tested on 9 healthy volunteers (8 female and 1 male) within an age group of 21-37. After 4h of exposure, the volunteers did not develop any sign of irritation (Fig 7).

Fig 7: Cream preparation

3.7 FT-IR analysis

FT-IR analysis of the fraction 1 of petroleum ether extract of *R. graveolens* is shown in Fig.8. Intense signals and their respective functional groups of fraction 3 1 of petroleum ether extract of *R. graveolens* are shown in Table 5.

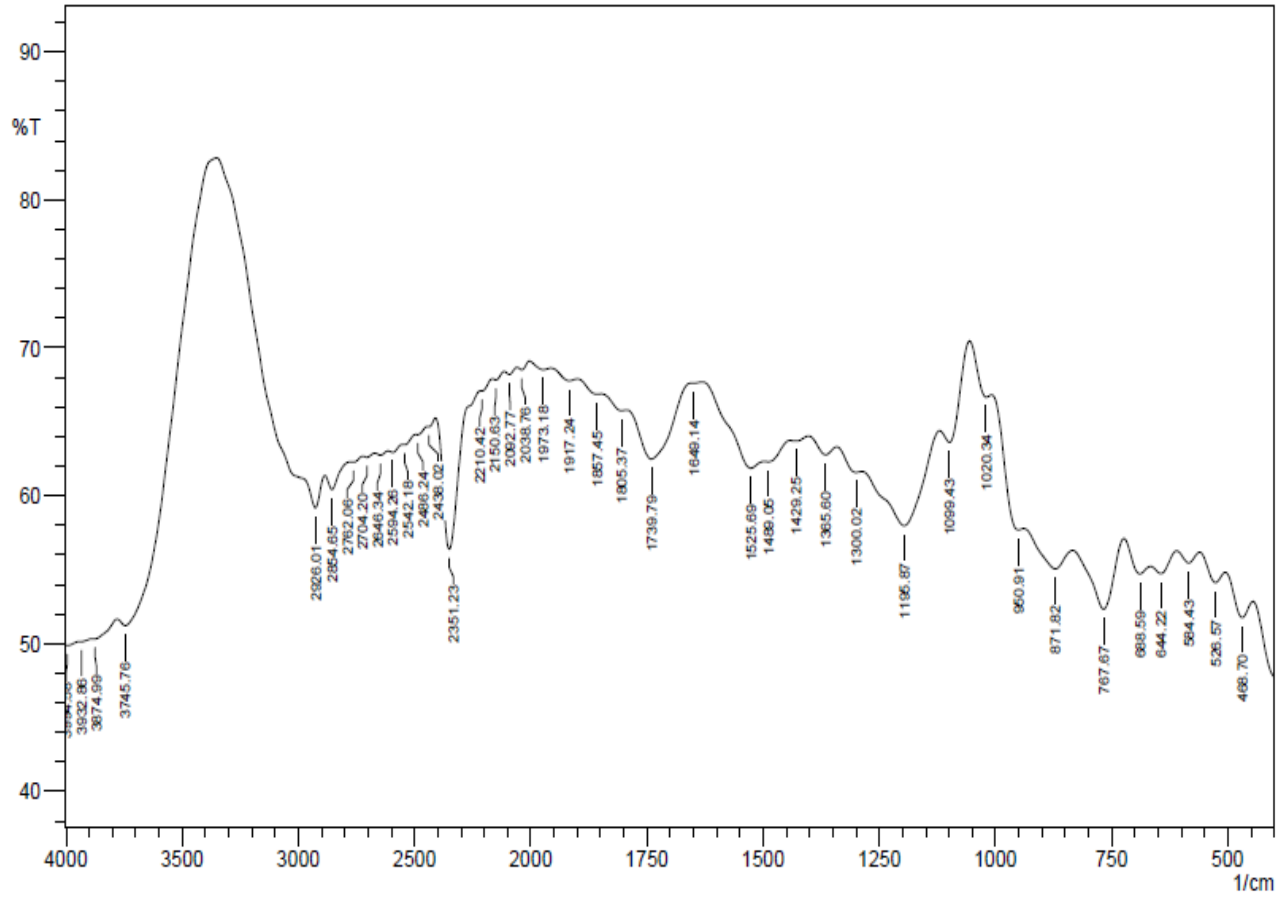

Fig 8: FTIR analysis of fraction 1 of petroleum ether extract of *R. graveolens.*

Table 5: FTIR analysis of Fraction1

Sl.no	Peak	Ref. peak	Functional group
1 2 3	468.7 526.57 584.43	800–400	C–X stretching (X = F, Cl, Br or I)
4 5 6 7 8 9	644.22 688.59 767.67 871.82 950.91 1020.34	600-1000	Alkenes-C–H out-of-plane bending
10 11	1099.43 1195.87	1000-1275	In-plane C–H bending
12 13	1300.02 1365.6	1300–1000	C–O stretching
14 15 16	1429.25 1489.05 1525.69	1600-1400	C=C stretching
17	1649.14	1660–1620	Nitrate NO asymmetric stretching
18	1739.79	1650–1800	First overtone C–H stretching
19 20 21 22	1805.37 1857.45 1917.24 1973.18	2000–1700	Overtone and combination bands
23	2038.76		
24	2090.77		
25	2150.63	2130–2100	Aromatic isonitrile –N=C stretching
26 27 28	2210.42 2351.23 2438.02	2200–2450	Combination C–H stretching
29	2486.24		
30	2542.18		
31	2594.26		
32	2646.34		
33 34	2704.2 2762.06		Aldehyde C–H stretching
35 36	2854.65 2926.01		alkanes - Methylene asymmetric C–H stretching
37 38 39 40	3745.76 3874.99 3932.86 3994.58	above and 3700 –3200	Si–OH stretching

4. Discussion

Plant secondary compounds have been the major source of thorough investigation in an effort to discover new sources of botanical insecticides. The secondary metabolites of plants (such as steroids, alkaloids, terpenoids, saponins, phenolics, essential oil, etc.) are associated with a wide range of biological activities [2]. Naturally occurring botanical compounds contain a broad range of chemical active ingredients that can interfere with the biological processes of the mosquito and thus interrupt its life cycle and dispersal and reduce harm to humans and animals.

In the present study, three solvents, hexane, petroleum ether and methanol were used. Among the three solvents used here, the petroleum ether solvent is the most suitable for *R. graveolens,* while the remaining solvent such as hexane and methanol showed low susceptibility towards the mosquito larvae.

The petroleum ether extract exhibited a high mortality against the larvae of both *An. stephensi* and *Ae. aegypti*. The high mortality might be due to the chemical constituents present in extracts that arrest the metabolic activities of the larvae. The variations in extraction solvents, mosquito species or exposure period affects the variation in the susceptibility of the extracts to mosquito larvae [16].

According to the present study, the petroleum ether extract of *R. graveolens* possess activity against both the larvae but, higher activity was shown against *An. stephensi.* The petroleum ether crude extract showed 100% mortality against *An. stephensi.* Sharma *et al.,* reported that the petroleum ether extract of *Artemisia annua* leaf possess larvicidal effects with LC_{50} values of 16.85 ppm against *A. stephensi* [16]. Leaves of *Argemone mexicana*, *Jatropha curcus* and *Pergularia extensa* causes 100% mortality at 250 ppm of each extracts against *C. quinquefasciatus* [9].

The studies done by Jayapal *et al.,* shows that the petroleum ether extract of *Aloe vera* showed larvicidal activity against *Ae. aegypti* [8]. 34% mortality was noted at I instar larvae by the treatment of *A. vera* at 80 ppm, whereas it has been increased to 89% at 400 ppm of *A. vera* leaf extract treatment. The LC_{50} value of I instar was 162.74 ppm, II instar was 201.43 ppm, III instar was 253.30 ppm, and IV instar was 300.05 ppm, respectively. The LC_{90} value of I instar was 442.98 ppm, II instar was 518.86 ppm, III instar was 563.18 ppm and IV instar was 612.96 ppm, respectively.

In this study, it was observed that, higher concentrations of methanol crude extract showed minute larvicidal activity against *An. stephensi.* Recent studies on the larval and pupal mortality of *An. stephensi* after the treatment of methanol

extract of *Clerodendron inerme* leaf extract showed 22% mortality at I instar larvae as a result of treatment at 20 ppm; in contrast, it was increased to 81% at 100 ppm of *C. inerme* leaf extract of larval and pupal mortality of *A. stephensi* (I–IV instars) after the treatment of methanol extract of *Acanthus ilicifolius* at different concentrations (20–100 ppm) [10].

5. Conclusion

The outcome of present study concluded the larvicidal property of three solvents (hexane, petroleum ether and methanol) extracts of *R. graveolens*. Among them, petroleum ether extract exhibited highest mortality rate compared with other solvents. The active principles of the petroleum ether extract were separated by thin layer chromatography and the activity of pure compounds was tested. The toxicity of the petroleum ether extract was done by preparation of cream. Further, the functional groups of bioactive petroleum ether extract were identified by performing FT-IR analysis. The overall results suggest that chloroform extracts of *R.graveolens* contain potential larvicidal agent and may be used for controlling *An. stephensi* and *Ae. aegypti* mosquito vectors.

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