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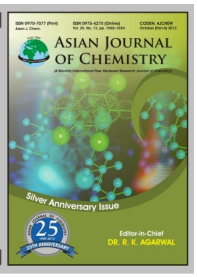

Larvicidal Activities of Five Essential Oils Against *Aedes aegypti* (L.) Larvae (Diptera: Culicidae)

SABIHA FAZAL[1], FARKHANDA MANZOOR[1,*], ASMA ABDUL LATIF[1], NEELMA MUNIR[2], IZZA[1] and MAHNOOR PERVAIZ[1]

[1]Department of Zoology, Lahore College for Women University, Lahore, Pakistan
[2]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

*Corresponding author: E-mail: doc_farkhanda@yahoo.com

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Present study was conducted to evaluate the larvicidal activity of essential oils from *Eucalyptus citriodora* leaves, *Peganum harmala* leaves, *Azadirachta indica* Seeds, *Cocos nucifera* fruit and *Turpentine* tree resin against *Aedes aegypti* mosquitoes larvae. Oils were extracted by steam distillation (Reverse Dean-Stark) method. Different serial dilutions of each oil (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.90, 0.97 ppm) were used to carry out the laboratory bioassays. The percentage mortality of mosquito's species *Aedes aegypti* (L.) when exposed to different concentrations of oils was calculated after 24 h exposure. Similarly relative toxicity (LC_{50}) values of five essential oils against *Aedes aegypti* (L.) were also calculated. All tested essential oils demonstrated significant larvicidal activity against *Aedes aegypti* (L.), with LC_{50} values ranging from 29.013 to 294.980 ppm. Turpentine oil was considered highly toxic as it was capable of killing 100 % larvae of *Ae. aegypti* after 24 h of treatment at concentration of 1000 ppm with lowest LC_{50} values (29.013). It can be concluded from this study that all the essential oils had remarkable larvicidal properties and be used effectively against *Aedes aegypti* (L.) larvae.

Key Words: ***Eucalyptus citrodora, Peganum harmala, Azadirachta indica, Cocos nucifera turpentine, Aedes aegypti* (L.).**

INTRODUCTION

Aedes aegypti (L.) are widely distributed in south East Asia as a potential vector of yellow fever. It is becoming an increasing public health problem with being cause of over 99 % cases of viral hemorrhagic fever[1]. Dengue fever was first noticed in 1950 in Pakistan, while in 1994, first outbreak of dengue fever was reported in the region. Another outbreak was seen in 2005[1]. Approximately 2/5th of world's population is now at risk of dengue transmission according to the World Health Organization[2]. Increasing population movement exacerbated by urbanization and lifestyles has contributed to the proliferation of artificial larval habitat of the mosquito, the worse epidemiological trends seem likely to increase[3].

The eradication of this disease can be controlled by either targeting the mosquito larvae through spraying of stagnant water breeding sites or by killing the adult mosquitoes using insecticides[4]. Larviciding is a successful way of reducing mosquito population in their breeding places before they emerge into adults. The use of pesticide in water, introduces many risks to people and environment.But many of these pesticides such as pyrethroids are very toxic to fish and should not be used in fish or crustaceans ecosystem[5]. The frequent use of synthetic insecticides lead to a subversion of ecosystem and enhanced resistance to insecticide in pests[6,7]. Recently, the environment friendly and biodegradable natural insecticides of plants origin have been receiving attention as an alternative green measure of control of arthropods of public health importance[8].

Natural pesticides are derived from plants and are more effective to mosquitoes. The chemical contents extracted from plant materials can be useful as repellents, larvicides, oviposition attractants, insect growth hormone regulators and deterrent agents[9]. Plant products have been used in many parts of the world for killing or repelling mosquitoes either as extracts or as whole plant. Certain natural products have been investigated for repellent activity against mosquitoes. Extracts of several plants including neem (*Azadirachta indica*), clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) have been studied as possible mosquito repellents[10-14] checked larvicidal activity of essential oil from *Eucalyptus camaldulensis* against *A. aegypti*. Results revealed that essential oil from leaves of *Eucalyptus camaldulensis* is excellent inhibitor against *A. aegypti* larvae. Neem oil is an effective larvicide against *A. aegypti*; it is highly toxic to mosquito larvae and inhibited the development of pupae. The high rates of larval mortality observed at high concentrations were 16 and 32 ppm within 72 h. The oil is also Insect Growth Regulator (IGR). These natural repellents have good efficacy against mosquitoes[15].

Keeping in view the significance of essential oils against *Aedes aegypti* (L.) larvae, present study was conducted with objective to evaluate the larvicidal activity and comparative efficacy of essential oils against the dengue fever mosquito, *Aedes aegypti* (L.).

EXPERIMENTAL

All test were conducted by using larvae of mosquito, *Aedes aegypti* (L.) were collected from NIMRT (National Institute Of Malaria Research and Training) and identified on the basis of the their morphological characters.

To evaluate the larvicidal activity of essential oils following plants were used. *Eucalyptus citriodora* (leaves), *Peganum harmala* (leaves), *Azadirachta indica* (Seeds), *Cocos nucifera* (Fruit) and *Turpentine* (tree resin).

Extraction of essential oils: Oils were extracted by steam distillation (Reverse Dean-Stark method).

Reverse Dean-Stark method: Plant material was put in a 5 dm^3 flasks and placed in an isomantle. Half of the flask was filled with plant material and then water was added to completely immerse the plant material. On the flask mouth, a reverse dean-stark was attached having a coil condenser at the top. Flask was heated which resulted in production of steam that caused the release of oil from the plant material. Steam carrying oil entered into the condenser and condensed liquid dropped into the Reverse Dean-Stark apparatus. The oil floated on the top of water layer which on the addition of more liquid coming from the condenser pushed the water in the bottom through the side arm back into the flask for recirculation. Oil is less dense than water so it floats on the surface of water. Water carrying essential oil from flask was used again and again for the extraction of essential oils. Through separatory funnel, water was separated from the oils. Anhydrous Na_2SO_4 was added to completely remove water from the collected oil which was then weighed and stored for experimentation.

Percentage yield of the essential oils obtained was calculated as:

Percentage yield of essential oil

$$= \frac{\text{Weight of the essential oil obtained}}{\text{Weight of fresh plant material used}} \times 100$$

Evaluation of essential oils with respect to their larvicidal properties: The tests were conducted in Entomology Research laboratory, Department of Zoology, Lahore College for Women University. Three replicates of each oil were prepared by dissolving the suitable amount of oil in distilled water using acetone to produce the stock solution. While 2 mL acetone and 198 mL of distilled water was used in the control replicates[16]. The stock solution of 1000 ppm was prepared by dissolving 1mL of essential oil in 1000 mL of distilled water using 2 mL of 100 % acetone. This solution was used to prepare the serial dilutions of target oil in the concentration of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.90, 0.97 ppm through the dilution of the stock solution with distilled water. While the control replicates contain 0 ppm of oil, 2 mL of 100 % acetone and distilled water.

Bioassay of oil solution: Each replicate containing 200 mL of described oil solution was placed in a 250 mL of glass beaker. Twenty late 3rd instar larvae of target mosquitoes, *Aedes aegypti* (L.) were transferred into the each beaker[17]. After that, the beakers were left or placed on the laboratory table for 24 h. The beakers were observed after 24 h. The number of dead larvae in each beaker was counted after 24 h of contact at room temperature. The larvae were considered dead if they were immobile and unable to reach the water surface.

Calculation of LC_{50} and statistical anaysis: Insect mortality data were corrected by Abbott's formula[18], LC_{50} values (the concentration at which 50 % of larvae were immobilized) were calculated by probit analysis using the PROBIT software Statistical Packages for the Social Sciences while randomized complete blocked design ANOVA were used to detect the significant difference between the treatment in all tests. Means were compared with Duncan's multiple range test.

RESULTS AND DISCUSSION

The percentage mortality of mosquitoes species *Aedes aegypti* (L.) larvae when exposed to different concentrations of oils *i.e., Turpentine, Peganum harmala, Eucalyptus citriodora, Azadirachta indica and Cocos nucifera*, after 24 h exposure are shown in Table-1. Table-2 showed the relative toxicity (LC_{50}) values of five essential oils against *Aedes aegypti* (L.) after 24 h of treatment.

Treatment with turpentine oil: It was revealed from Table-1 that percentage mortality for larvae of *Aedes aegypti* (L.) when exposed to different concentrations of turpentine oil after 24 h of treatment was 0, 0, 20, 30, 40, 85, 85, 100, 100 and 100 % at conc. of 1.95, 3.90, 7.81, 15.6, 31.25, 62.5, 125, 250, 500

TABLE-1
PERCENTAGE MORTALITY ± SE OF *Aedes aegypti* WHEN EXPOSED TO DIFFERENT CONCONTRATIONS OF FIVE ESSENTIAL OILS AND CONTROL UNIT

Concentrations (ppm)	Mortality (%) ± SE					Control
	Turpentine oil.	*P.harmala* oil	*Cocos nucifera* oil	*Azadiracta indica* oil	*Eucalyptus citriodora* oil	
1.95	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.90	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
7.81	20.0 ± 0.0	5.0 ± 0.5	0.0 ± 0.0	0.0 ± 0.5	5.0 ± 0.5	0.0 ± 0.0
15.62	30.0 ± 0.0	10.0 ± 0.0	0.0 ± 0.0	10.0 ± 0.5	15.0 ± 0.0	0.0 ± 0.0
31.25	40.0 ± 0.5	45.0 ± 0.0	10.0 ± 0.5	20.0 ± 0.5	40.0 ± 0.5	0.0 ± 0.0
62.50	85.0 ± 0.5	55.0 ± 0.0	10.0 ± 0.5	25.0 ± 0.0	45.0 ± 0.0	0.0 ± 0.0
125.00	85.0 ± 0.5	65.0 ± 0.0	20.0 ± 0.5	50.0 ± 0.0	55.0 ± 0.0	0.0 ± 0.0
250.00	100.0 ± 0.0	75.0 ± 0.0	40.0 ± 0.5	75.0 ± 0.5	75.0 ± 0.0	0.0 ± 0.0
500.00	100.0 ± 0.0	100.0 ± 0.0	50.0 ± 0.0	80.0 ± 0.5	100.0 ± 0.0	0.0 ± 0.0
1000.00	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0

TABLE-2
RELATIVE TOXICITY OF FIVE ESSENTIAL OILS AGAINST *Aedes aegypti* (L.) AFTER 24 h OF TREATMENT

Essential oils	LC_{50} (ppm)	95 % confidence limits (ppm)	Fit of orobit lines			
		LCL-UCL	Slope ± SE	X^2	df	*p*
Turpentine	29.013	109.76-319.23	2.15 ± 0.26	6.17	5, 4	$p < 0.0001$
P. harmala	60.904	298.90-990.16	1.82 ± 0.21	7.13	7, 2	$p < 0.0001$
C. nucifera	294.980	1201.34-5929.34	1.90 ± 0.28	9.79	5, 4	$p < 0.0001$
A. indica	117.108	575.72-2137.86	1.79 ± 0.22	3.81	7, 2	$p < 0.0001$
E. citriodora	68.900	361.99-1263.39	1.75 ± 0.20	6.42	8, 1	$p < 0.0001$

and 1000 ppm, respectively. It is also shown in table that larval mortality increases with the increase in concentrations. No mortality was observed at control group after 24 h of test period. The LC_{50} value for *Aedes aegypti* (L.) was 29.013 with lower and upper 95 % confidence limits as 109.766 and 319.231, respectively. Analysis of variance revealed that concentrations of turpentine oil tested against *Aedes aegypti* (L.) were significantly different among all treatments (F = 1.745, df = 5, 4; $p < 0.0001$) (Fig. 1).

Treatment with harmal oil: In the same way the percentage mortality of larvae of *Adese aegypti* (L.) when exposed to different concentrations of harmal oil after 24 h of treatment was 0, 0, 5, 10, 45, 55, 65, 75, 100 and 100 % at conc. of 1.95, 3.90, 7.81, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 ppm, respectively. No mortality was observed at control group after 24 h of test period. The LC_{50} value for *Aedes aegypti* (L.) was 60.904 with lower and upper 95 % confidence limits as 298.903 and 990.167, respectively. Analysis of variance revealed that concentration of harmal oil tested against *Aedes aegypti* (L.) were significantly different among all treatments (F = 1.849, df = 7, 2; $p < 0.0001$) (Table-1, Fig. 1).

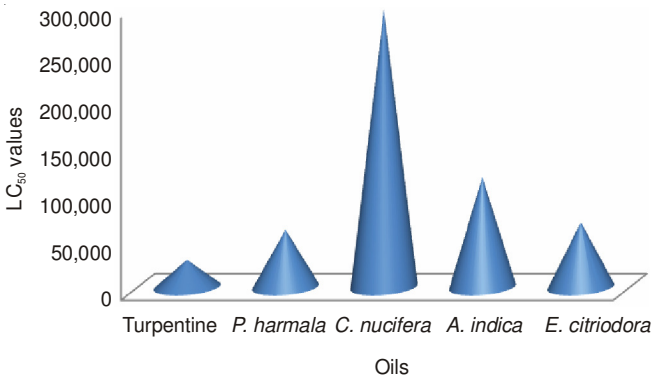

Fig. 1. Graph showing LC_{50} values of five essential oils against *Ae. aegyti* (L.)

Treatment with coconut oil: After 24 h of treatment, the percentage mortality of larvae of *Aedes aegypti* (L.) when exposed to different concentrations of coconut oil was 0, 0, 0, 0, 10, 10, 20, 40, 50 and 100 % at conc. of 1.95, 3.90, 7.81, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 ppm, respectively. No mortality was observed in control group after 24 h of test period. The LC_{50} value for *Aedes aegypti* (L.) was 294.980 with lower and upper 95 % confidence limits as 1201.347 and 5929.348, respectively. Analysis of variance revealed that concentration of coconut oil tested against *Aedes aegypti* (L.) were significantly different among all treatments (F = 1248.974, df = 5, 4; $p < 0.0001$) (Fig. 1).

Treatment with neem oil: Table-1 also reveals the percentage mortality of larvae of *Aedes aegypti* (L.) when exposed to different concentrations of neem oil. The percentage mortality for *Aedes aegypti* (L.) after 24 h of treatment was 0, 0, 0, 10, 20, 25, 50, 75, 80 and 100 % at conc. of 1.95, 3.90, 7.81, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 ppm, respectively. It is also shown in table that larval mortality increases with the increase in concentrations. No mortality was observed at control group after 24 h of test period. The LC_{50} value (Fig. 1) for *Aedes aegypti* (L.) was 117.108 with lower and upper 95 % confidence limit s as 575.721 and 2137.868, respectively. Analysis of variance revealed that concentration of neem oil tested against *Aedes aegypti* (L.) were significantly different among all treatments (F = 14985.131, df = 7, 2; $p < 0.0001$).

Treatment with Eucalyptus oil: In the same way, percentage mortality of larvae of *Aedes aegypti* (L.) when exposed to different concentrations of eucalyptus oil after 24 h of treatment was 0, 0, 5, 15, 40, 45, 55, 75, 100 and 100 % at conc. of 1.95, 3.90, 7.81, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 ppm, respectively. The LC_{50} value for *Aedes aegypti* (L.) was 68.90 with lower and upper 95 % confidence limits as 361.993 and 1263.397, respectively. Analysis of variance revealed that concentration of eucalyptus oil tested against *Aedes aegypti* (L.) were significantly different among all treatments (F = 61414.695, df = 8, 1; $p < 0.0001$) (Table-1).

Public awareness and resulting environmental agencies ruling lead to the removal of some synthetic insecticide from the market. It is because of growing concern for clear environmental and insect population that develop resistance to conventional insecticides[19]. There are a number of alternatives to using chemical pesticides for mosquito larval control. One of alternative method is the use of essential oils. Various studies have revealed that essential oils are not only natural volatile substances, but also mixture of many compounds that are not only used for antimicrobial, antifungal activity but also for insecticidal activity[20]. The essential oil constituents have a long history of application and commercially used in several primary aspects: as aroma in fragrances and perfumes, as pharmaceuticals and as insecticides. There are already numerous studies devoted to biological activities of essential oils from medicinal plants, particularly with respect to their antibacterial, antifungal and insecticidal properties[15,21-23]. Essential oils are presently regarded as a new class of ecological products for controlling insect pests. In the view of recently increased interest in developing plant origin insecticides as an alternative to chemical insecticide, this study was undertaken to access the toxicant potential of five essential oils that are *Turpentine, Peganum harmala, Azadirachta indica, Eucalyptus citriodora, Cocos nucifera* against *Aedes aegypti* (L.). Although essential oils

have been tried as cockroach repellents, antifugal and anti-microbial agents. All the essential oils tested, demonstrated significant larvicidal activity on *Aedes aegypti* (L.), with LC_{50} values ranging from 29.013 to 294.980 ppm. The exposure time is very important in determining the LC_{50} values in tested oils. As the effectiveness of oil is concerned, the results are significantly different for different essential oils. As seen from the Table-1, turpentine was considered highly toxic as it was capable of killing 100 % larvae of *Ae. aegypti* after 24 h of treatment at concentration of 1000 ppm. The percentage mortality for *Ae. aegypti* (L.) after 24 h of treatment was 0, 0, 20, 30, 40, 85, 85, 100, 100 and 100 % at conc. of 1.95, 3.90, 7.81, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 ppm, respectively. The LC_{50} value for *Aedes aegypti* (L.) was 29.013. This oil has the lowest LC_{50} value but has a great effect on *Aedes* larvae within 24 h.

The next effective essential oil was *P. harmala*. The LC_{50} value of *P. harmala* for *Aedes aegypti* (L.) was 60.904. As compared to *P. harmala* oil, *E. citriodora* had a greater LC_{50} value. The LC_{50} value of *E. citriodora* for *Aedes aegypti* (L.) was 68.90. Regarding *A. Indica* and *C. nucifera*, they showed some delayed toxicity but effective with LC_{50} values 117.108 and 294.980, respectively for *Aedes aegypti*. All the essential oils selected showed larvicidal effectiveness. Neverthless upon a comparison of lethal concentration as determining factor in the larvicidal effectiveness of essential oils, it is apparent that their biological effectiveness differs. So according to the larvicidal activity of essential oils against *Aedes aegypti* (L.) , the oils were arranged in the following ascending order of presence (Fig. 1) *i.e*.

Turpentine > P. harmala > E. citriodora > A. Indica > C. nucifera

The findings of our studies are also in co-incidence with Abdelkarim *et al*.[24]. They conducted a study to evaluate the insecticidal properties of *Eucalyptus globulus*, a widely used essential oil , for larvicidal activity against different mosquito species: *Aedes aegypti* (L.) *An. gambiae* and *Cx. quinquefasciatus* (Say) by exposing the IIIrd instar larvae of mosquitoes. Mosquitoes were reared in the laboratory by procedure described by Abdelkarim *et al*.[24]. Oil produces higher mortality in the larvae of *An. gambiae* than in the larvae of *Aedes aegypti* (L.) and no mortality was induced in larvae of *Cx. quinquefasciatus* (Say). They reported the *Eu. globulus* oil have both mosquito larvicidal and repellent properties.

Okumu *et al*.[15] conducted a research to determine the larvicidal and repellent properties of neem and corn essential oils. The research proved that neem oil was effective larvicide against *An. gambiae* larvae. It was highly toxic to mosquito larvae and inhibited the development of pupae. The high rates of larval mortality observed at higher concentrations (16 and 32 ppm of the neem oil formulation) within 72 h after exposure indicates the high toxicity of product. The oil is also a potent insect growth regulator (IGR) which led to 97.5 % increase in larval development time and 97.1 % decrease in pupation at 32 ppm.

Desmond *et al*.[25] worked on coconut oil to check the larvicidal activity of coconut oil. In the research the toxicity of coconut oil was compared with a commercially available oil larvicide. In laboratory bioassays of 4th-stage larvae of *Anopheles farauti* (Laveran) and *Culex annulirostris* (Skuse). Both larvicides were more toxic to *Cx. annulirostris* than to *An. farauti* and the LD_{50} (dose lethal to 50 % of the test organisms) after 24 h exposure indicated that coconut oil was more toxic than commercial oil for both *An. farauti* (LD_{50} = 8.6 µL *versus* 13.0 µL/156 cm^2) and *Cx. annulirostris* (LD_{50} = 1.2 µL *versus* 3.6 µL/156 cm^2). The research showed that coconut oil is the major oil to control the malaria.

Efficacy of turpentine oil assessed as larvicidal agent in this study. The LC_{50} value of turpentine for *Aedes aegypti* (L.) was 29.013. This oil has the lowest LC_{50} value but has a great effect on *Aedes* larvae within 24 h. When the oils were compared for their toxicity, there were significant ($p < 0.05$) difference in the toxicity for all the tested essential oils ($p = 0.01$). The difference in mortality may be due to difference in their chemical constituents and their quality. The quality of volatile oils depends on many factors, *e.g*., plant species, rearing conditions, maturation of harvested plants, plant storage, plant preparation and method of extraction. Thus, these factors should be carefully considered and standardized when extraction of volatile oils is being planned. Since the oil is already used in flavoring, pharmaceuticals, confectionery *etc* and is considered nontoxic to humans, so further studies are needed on formulations against mosquitoes and their efficacy and cost effectiveness.

Nevertheless, further experiment must be undertaken, focused on effects against non-target organisms, the formula of products themselves and practical use in real situations. The present knowledge suggests, however that product based on these essential oils may contribute greatly to a reduction in environmental chemicalisation and to an overall reduction of population density of some significant vectors such as *Aedes aegypti* (L.). Supplementary investigations for the mode of constituent's actions, effects on non-target organisms and old evaluations are necessary. These results obtain are useful in search of more selective, biodegradable and naturally produced larvicidal compounds.

Conclusion

Essential oils from *Turpentine, Peganum harmala, Cocos nucifera, Azadirachta indica* and *Eucalyptus citriodora* were tested against mosquito species *Aedes aegypti* (L.) to develop biopesticide as an alternative to chemical insecticides. In conclusion this study revealed that all the essential oils had remarkable larvicidal properties. The flora of Pakistan has rich aromatic plant diversity with potential for development of natural insecticides for the control of mosquito and other pests. Our findings suggest that essential oils evaluated in this study may be explored as a potential environmental benign disorder.

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