OPEN ACCESS Journal of Innovative Biology and Environmental Sciences

Research Article

Repellency of Essential Oils and Effect of Sensory Organ Ablation in Three Mosquito Species in Lahore, Pakistan

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Abstract | In the present study, varied concentrations of essential oils (*Oscimum bascilus, Juniper berries, Citrus reticulate* and *Menthe piperata*), mix oils and creams as repellents was investigated against three mosquito species: *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* by direct testing on humans. The protection time, percentage of repellency, landing and biting of mosquitoes was calculated. *Aedes aegypti* and *Anopheles stephensi* were studied for effect of ablation of mosquito organs on repellency of 3 essential oils. About 30% formulation of mix oils induced a higher repellency effect towards *Aedes aegypti* (75%) and *Culex quinquefasciatus* (69%). Highest efficacy of *Citrus reticulate* was observed against *Anopheles stephensi* (75%) while the highest landing and biting percentage at 30% concentration was 49% and 47% of *C. reticulate* for *Aedes aegypti* and 45% and 41% for *Culex quinquefasciatus* against cream. Efficacy of all concentration of essential oils was significant for three mosquito species. Repellency, landing and biting of mosquitoes of three mosquitoes was significant at all concentration of essential oils and cream. It was concluded that *Citrus reticulate* and *Oscimum bascillus* are environment friendly and suitable to be used as green repellents for mosquito control. Ablation of organs showed that the maxillary bulb was the important organ observed for repellency in *Aedes aegypti* mosquitoes whereas, no responsible organ was observed in *Anopheles stephensi* mosquitoes.

Received | April 9, 2024; **Accepted** | June 27, 2024; **Published** | June, 2024
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Citation | Manzoor, F. 2024. Repellency of Essential Oils and Effect of Sensory Organ Ablation in Three Mosquito Species in Lahore, Pakistan. *Journal of Innovative Biology and Environmental Sciences*, 4(1): 47-55
Keywords | Vector, Mosquitoes, Essential oils, Green repellents, Protection time

Introduction

Infections transmitted by the bite of infected arthropod are called vector-borne diseases. Many arthropod species such as ticks, mosquitoes, bugs and blackflies are involved in transmission of vector borne diseases of public health importance. Prevalence of vector-borne disease is profoundly restricted to socioeconomic status of those countries. Highest rates of infection were observed in countries located in the tropics and subtropics regions (Kalita *et al*., 2013). In Pakistan

differences in population of mosquitoes was due to inadequate sanitary conditions, regional climatic changes and rapid urbanization. Population density of *Culex quinquefasciatus* (*Cx. Quinquefasciatus*) was observable from February to May but due to the low temperature it was decreased in December and January (Manzoor *et al*., 2013). *Aedes aegypti* (*Ae. aegypti*) carrier of dengue virus causes dengue hemorrhagic fever however, life time immunity from that particular serotype achieved when someone infected with a certain serotype but other serotypes may cause infection later (Chew *et al*., 2012). Four distinct serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4) as the cause of dengue infection have been reported. However, worldwide major cause of infection in humans was serotypes 2 and 3 (Manzoor *et al*., 2017).

Mosquito control and personal protection from mosquito bites are currently the most important measures to prevent these diseases. The common approach for the control of mosquito vectors and reducing the transmission of human pathogens is based on the chemical insecticide-based intervention measures (Das *et al*., 2003). Naturally occurring botanical compounds contain a broad range of chemical active ingredients which can intervene in all biological processes of the mosquito, thus interrupt its life cycle and dispersal and reduce harms to humans and animals (Geetha and Roy, 2014).Use of synthetic insecticides for controlling mosquitoes is among one of the many approaches developed to control the mosquitoes. Synthetic insecticides are preferred due to their quick action but also discouraged for the development of resistance and adverse effect on environment. In contrast use of plant extracts for controlling mosquitoes are readily biodegradable and less hazardous. Use of essential oils as mosquito larvicides and as repellent of insect pests is widely used in vector control programs (Pavela *et al*., 2019; Su, 2022).Though essential oils are best insect repellent but due to rapid volatility protection time of these oils decreased and requires repeated application. Topical application of repellents for personal protection is a common practice however a number of environmental and user related physiological factors effects on control of mosquito bite (Chattopadhyay *et al*., 2015; Haris *et al*., 2023).

Soonwera and Phasomkusolsil (2024) determined repellency activity of Thai essential oils derived from ylang ylang (*Cananga odorata* (Lamk.) and lemongrass (*Cymbopogon citratus* (DC.) against two mosquito vectors, *Ae. aegypti* (L.) and *Cx. quinquefasciatus* (Say) and compared with two chemical repellents. All herbal repellent exhibited higher repellent activity. Oil of *Mentha piperata* (*M. piperita* L.) (Peppermint oil) was evaluated for larvicidal activity against different mosquito species *Ae. aegypti*, *Anopheles stephensi* (*An. Stephensi*) and *Cx. quinquefasciatus* by exposing 3rd instar larvae of mosquitoes. *Cx. quinquefasciatus* was most susceptible followed by *Ae. aegypti* and *An. Stephensi* resulting in 100% mortality within 24h for *Cx. quinquefasciatus*, 90% for *Ae. aegypti* and 85% for *An. Stephensi* (Ansari *et al*., 2000). Kumar *et al*. (2011) determined larvicidal efficiency of *M. piperita* against dengue vector. The bioassays displayed remarkable repellent properties of *M. piperita* against adults *Ae. aegypti*.

Following study evaluated protection period of three mosquito species *Ae. aegypti*, *Cx. quinquefasciatus* and *An. stephensi* at three concentrations of four different oils extracted from plants one mixture and one cream of all four oils and recorded protection period, the percentage of repellency, landing percentage and biting percentage of three mosquito species at different concentrations. This

study also observed the number of landing and biting mosquito with the effect of ablation of different sensory organs.

2. Materials and Methods

2.1. Mosquito strains

Three mosquito strains were used in present study: *Ae. aegypti*, (Linnaeus) (Diptera: culicidae), called Yellow Fever Mosquito. *An. stephensi*, (Liston) (Diptera: Culicidae), one of the most important malarial vector and *Cx. quinquefasciatus*, (Say) (Diptera: Culicidae), vector of filariasis and encephalitis. Mosquito strains were obtained from the Jinnah Bagh (Lawrence garden) and Lahore College for Women University (LCWU) by trapping techniques.

2.2. Selection of plant species

Four plant species belonging to different families were selected for this study because most of them are known or used traditionally as mosquito repellents. Those were *Oscimum bascillus* (Basil) *(O. bascillus)*, *Juniper berries* (*J. berries*), *Citrus reticulate* (Mandarin orange) (*C. reticulate*), *Mentha piperata* (Peppermint) (*M. piperata*).

2.3. Extraction of essential oils

The extraction procedure for essential oils was performed as described by Tawatsin *et al.*, (2001). Essential oils were extracted from each plant by steam distillation. About 2kg fresh plant leaves were cut into small pieces and placed in a distillation flask with water and 10 glass beads. The distillation chamber was heated in a liquid paraffin bath at about 120ºC until the distillation was completed. The distillate was collected in a separate funnel in which the aqueous portion was separated from the essential oil (oily phase). The aqueous phase (lower layer) was slowly drawn off until only the oil layer remained. This procedure was repeated until at least 5ml of essential oil was collected. Each essential oil was kept in a screwed-cap glass vial at 4ºC for further experimentation.

2.4. Preparation of the oils for the test

Four oils *O. bascillus*, *J. berries*, *C. reticulate*, *M. piperata* were used in this trial. In the first stage the oils were tested to detect their repellent properties against the *Ae. aegypti*; *An. stephensi*; *Cx quinquefasciatus* by preparing three concentrations (10%, 20% and 30%). The fifth mixture contain all four oil while the sixth mixture contains cream having base cream. The seventh was the control group on which no treatment was done.

2.5. Repellency assay

The tests were conducted in the entomology laboratory LCWU, within insectary maintained at 28±2ºC and relative humidity 70±10%. The repellent effect of essential oils was evaluated using the human-bait technique. Depending on the efficacy, the testing period lasted up to eight hours. Since the target mosquitoes were day or night biters, *Ae. aegypti* was tested from 8 to 16 hours, while *An. stephensi* and *Cx. quinquefasciatus* were tested between 16 to 24 hours. For testing, 0.1 ml of test material (oil) was applied onto a 30cm^2 marked area of a human volunteer forearm. Each arm was covered by a paper sleeve with a 30cm^2 exposed area corresponding to the marked and treated site. After treatment, a human volunteer arm was introduced every 30 minutes into a mosquito cage containing 50 nulliparous female mosquitoes being between 5 and 15 days old and left the arm there for 2 minutes. If at least 2 mosquitoes bite the hand of the control person the repellency test was carried out, otherwise the test was not conducted. On each day only one repellent preparation was tested, in order to leave time for residues to be lost from the skin before the next test.

The duration period between the application of a repellent and the first 2 bites or 2 bites in successive observations was recorded as the protection time. The percentage of repellency was calculated at the end of every test by using the formula that was mentioned by Curtis and Hill (1988).

$$\% \text{ Repellency} = \frac{C - T}{C} \times 100$$

Where C is the total number of mosquitoes landing and/or biting at the control area ($30cm^2$ on a human volunteer forearm without repellent material) and T is the total number of mosquitoes landing and/or biting at a treated area.

The percentage of landing mosquitoes was calculated at the end of every test by using standard formula (Thavara *et al*., 2001).

$$\% \text{ Landing} = \frac{L}{100} \times 100$$

L represents the total number of landing mosquitoes at the end of test.

The percentage of biting mosquitoes was calculated at the end of every test by using this formula:

$$\% \text{ Biting} = \frac{B}{100} \times 100$$

B is the total number of biting mosquitoes at the test end.

2.6. Anesthetization of mosquitoes

Mosquitoes were anesthetized by exposing five individuals for 5 seconds in a 50ml glass bottle to current of CO_2 gas released from other controlled glass bottle containing CO_2 dry ice. This treatment was done for 3 minutes of anesthetization period (Elawami and Dent, 1995).

2.7. Ablation of mosquito organs

Two anthropophilic mosquitoes (*Ae. aegypti* and *An. stephensi*) were tested against 50 female (15 days old). Test group was divided into five cages sized i.e. 10 females in each cage. The five groups of mosquitoes were treated as (group **1**: without antenna; group **2**: without maxillary bulbs; group **3**: without proboscis; group **4**: without frontal tarsus; group **5**: normal females as control). The organs of mosquitoes were ablated using micro shears under binocular after mosquito anesthetization.

2.8. Statistical Analysis

Data collected in laboratory was analyzed on SPSS 20 and one way ANOVA test was applied to compare repellency at different concentrations and result was considered significant at $p<0.05$. The variables examined were protection period, repellency %, landing % and biting % of mosquitoes.

3. Results

3.1. Repellency properties of oils

3.1.1. Protection period and percent repellency

The protection periods of the oils, cream at 10%, 20%, 30% concentration against the three mosquito species were evaluated and mix oil formulation was found as the best one in all trials. *Ae. aegypti* with maximum protection period at 10% (250), 20% (290) and 30% (320) was recorded. The *Cx. reticulate* formulation was the best in all trials, for *An. stephensi* with the maximum protection period at 10% (280), 20% (320) and 30% (350 minutes) was recorded. The mix oil formulation was the best one in all trials, for *Cx. quinquefasciatus* with the maximum protection period at 10% concentration (300) 20% concentration (340) and for 30% (380 minutes) was recorded in Table 1. The protection period of *Ae. agypti* at 10% concentration of oils ranged between 250 for mix oils and only 100 for *Citrus reticulate*.

As far as percentage of repellency of the three mosquito species at three

concentrations 10%, 20%, 30% was also recorded in Table 1. The percentage of repellency of the oils, cream at 10%, 20%, 30% concentration against the three mosquito species were examined and the highest repellency was shown by *An. stephensi* at three concentrations of oils i.e. 75% at 30%concentration of *C. reticulate*. The second highest repellency was recorded by *Ae. agypti* 71% for mix oils. The highest repellency for *Ae. aegypti* was observed at 10% concentration for mix oils (68%) while for *An. stephensi* it was 68% for *C. reticulate* while for *Cx. quinquefasciatus* (62%) for mix oils. Similar pattern of repellency was observed at 20% concentration for *Ae. aegypti* with mix oils (71%) while for *An. stephensi* it was 73% for *C. reticulate* however, in case of *Cx. quinquefasciatus* mix oils (66%). Analogous repellency pattern was found at 30 % concentration. In *Ae. aegypti*, 75% was observed for mix oils while *An. stephensi* (75%) for *C. reticulate* however in case of *Cx. quinquefasciatus* 90% for mix oils was observed (Table 1).

Table 1: The protection period and percentage of repellency of tested oils at 10%, 20%, 30% concentration against the three mosquito species.

	10% concentration						20% concentration						30% concentration					
	Aedes		*Anopheles*		*Culex*		*Aedes*		*Anopheles*		*Culex*		*Aedes*		*Anopheles*		*Culex*	
Material*	PP	%R	PP	%R	PP	%R	PP	%R	PP	%R	PP	%R	PP	%R	PP	%R	PP	%R
O.B	120	46	180	50	280	58	160	49	200	54	310	62	180	53	230	59	350	66
J.B	230	62	250	61	280	58	280	65	290	66	310	62	310	69	330	70	350	66
C.R	100	44	280	68	280	58	130	50	320	73	310	62	160	54	350	75	350	66
M.P	140	51	230	57	280	58	140	56	260	61	310	62	180	58	290	65	350	66
M.O	250	68	270	64	300	62	290	71	300	69	340	66	320	75	350	73	380	69
Cr	180	55	200	53	260	50	240	58	220	57	300	57	270	62	240	61	340	60
Ct	90	20	120	30	200	40	90	20	120	30	200	40	90	20	120	30	200	40

*O.B = *Oscimum bascillus*, J.B = *Juniper berries*, C.R = *Citrus reticulate*, M.P = *Mentha piperita*, M.O = Mix oils, Cr. = Cream, Ct = Control

The statistical analysis reveals that repellency of three mosquitoes were significant at 10% concentration of five oils and cream where (F=8.553, df= 20, *p*= 0.000). The data were also significant for oils at 20% concentration for 5 oils and cream (F=11.221, df=20, *p*= 0.000). Data were significant for oils at 30% concentration for three mosquito species (F=14.180, df= 20, *p*= 0.000).

3.1.2. Landing percentage

In *Ae. aegypti* the highest landing percentage (56%) at 10% concentration for *C. reticulate* while the percentage of biting mosquitoes is highest for the *C. reticulate* (51%). In *An. stephensi* the percentage of landing mosquitoes ranged between 26% (*C. reticulate*) to 50% (*O. bascillus*) while the biting rate was highest for *O. bascillus* (46%). In *Cx. quinquefasciatus* the percentage of landing rate was 40% for *C. reticulate* but the percentage of biting was 39% for *O. bascillus* (Table 2). The statistical analysis reveals that landing of three mosquitoes were significant at 10% concentration (F=8.17, df=20, *p*=0.001), at 20% concentration (F=10.65, df=20, *p*=0.00) and at 30% concentration for three mosquito species (F=12.67, df=20, *p*=0.00) of five oils and cream. The statistical analysis reveals that biting of three mosquitoes were significant at 10% concentration (F=8.29, df= 20, *p*=0.001), at 20% concentration (F=10.37, df = 20, *p*=0.000) and at 30% concentration (F=10.27, df= 20, *p*=0.000) of five oils and cream.

Table 2: The percentage of landing and the percentage of biting mosquitoes after application of the oils at10, 20% and 30% concentration tested against three mosquito species.

	10% concentration						20% concentration						30% concentration					
	Aedes		*Anopheles*		*Culex*		*Aedes*		*Anopheles*		*Culex*		*Aedes*		*Anopheles*		*Culex*	
Material*	L%	B%	L%	B%	L%	B%	L%	B%	L%	B%	L%	B%	L%	B%	L%	B%	L%	B%
O.B	54	49	50	46	42	39	51	44	46	42	38	35	47	44	42	41	35	32
J.B	38	34	39	35	42	36	35	30	35	31	37	32	32	30	31	29	32	30
C.R	56	51	32	28	42	35	52	47	29	24	40	31	49	47	26	23	37	32
M.P	49	44	43	38	42	37	44	41	39	34	38	33	41	40	35	34	35	31
M.O	32	28	36	32	38	33	27	24	32	29	34	29	22	20	28	25	31	28
Cr	45	42	47	42	50	44	41	38	43	38	47	41	37	38	40	37	45	41
Ct	80	72	70	63	60	54	80	72	70	63	60	54	80	72	70	63	60	54

*O.B = *Oscimum bascillus*, J.B = *Juniper berries*, C.R = *Citrus reticulate*, M.P = *Mentha piperita*, M.O = Mix oils, Cr. = Cream, Ct = Control

3.2. Effects of organ ablation on repellent sensation

Effect of organ ablation on repellent sensation was observed quite differently in *Ae. aegypti* and *An. Stephensi*. The highest biting on the oil treated area in the case of without antenna was observed for *C. reticulate* (7) in *Ae. aegypti*, however, 12 *An. stephensi* without maxillary bulbs fed in the *M. piperata* area and in the case of without tips of the proboscis only 1 mosquito biting for both species was recorded in all types of treatment. In the group without the tip of the frontal tarsus 7 *An. stephensi* bites in *M. piperata* area whereas in control group 13 biting of *An. stephensi* was recorded for *M. piperata* area (Table 3).

The highest landing was recorded as 13 *An. stephensi* without antenna on *M. piperata* area and 15 *Ae. aegypti* without maxillary bulbs were landing on *C. reticulate* area. In the case of the mosquito group without the tips of the probiscis 5 *Ae. aegypti* were landing on the *C. reticulate* area and 4 on *M. piperata*. Moreover, 9 *Ae. aegypti* without the tip of the frontal tarsus were observed on *C. reticulate* and 9 *An. Stephensi* were observed on *M. piperata*. But in control group only 15 *An. stephensi* were landing on the *M. piperata* and 14 *Ae. aegypti* were landing on *C. reticulate* (Table 3).

Table 3: Shows the number of biting and landing of *Ae. aegypti* and *An. stephensi* with an effect of organ ablation when human skin was treated with different essential oils.

Material**	Without antennae				Without Maxillary bulbs				Without probocsis				Without Frontal tarsus				Normal females			
	Aedes		*Anopheles*		*Aedes*		*Anopheles*		*Aedes*		*Anopheles*		*Aedes*		*Anopheles*		*Aedes*		*Anopheles*	
	B*	L*	B	L	B	L	B	L	B	L	B	L	B	L	B	L	B	L	B	L
J.B	4	7	6	12	8	10	10	10	1	3	1	3	2	4	4	6	5	9	11	12
C.R	7	10	5	10	11	15	9	9	1	5	1	4	6	9	3	5	8	14	9	10
M.P	5	8	8	13	9	13	12	12	1	4	1	3	3	6	7	9	6	12	13	15

*B= No. of Biting, L= No. of Landing, **J.B= *J. berries*, C.R = *C. reticulate*, M.P = *M. piperata*

4. Discussion

The quality of essential oils, such as yield, chemical constituents and physical properties depends on many factors including plant species (variety), cultivating conditions and maturation of harvested plants, plant storage, plant preparation and methods of extraction. Inconsistency in the different exposure

periods in the mosquito cage was observed when determining the repellency of mosquito under laboratory conditions. Previous studies used short exposure times of one minute only. A clear substantial difference in repellency was observed during different exposure periods in this study. The minimum protection time (2 hrs) needed against *Ae. aegypti* and *Cx. quinquefasciatus* specifically as mosquito repellents.

The protection period of the three mosquito species depend directly on concentration of essential oils. In *Ae. Aegypti* the protection period at 10% concentration for the oil *J. berries* was 230 and it was increased to 280 at 20% concentration and at 30% concentration the protection period of the same oil was 320. The highest protection period observed in *Cx. Quinquefasciatus* (370) for mix oils then the second highest protection period recorded in *An. stephensi* (350) for mix oils and *Ae. Aegypti* (320) for mix oils at 30% concentration. The highest repellency shown by *Cx. Quinquefasciatus* (80%) for mix oils at 30% concentration then *An. stephensi* (73%) preceeded by *Ae. Aegypti* (71%) for mix oils. The highest landing percentage was observed in *Ae. Aegypti* (55%) for *C. reticulate* at 30% concentration then *Cx. Quinquefasciatus* (42%) and *Anopheles stephensi* (32%). The highest biting percentage was observed in *Ae. Aegypti* (51%) for *C. reticulate* at 30% concentration followed by *Cx. Quinquefasciatus* (35%) and *An. stephensi* (29%).

This study evaluated the difference in biting and landing mosquitoes separately in each trial. Therefore the protection time was determined as the time from material application until the first two bites. The percentage of repellency depended on the total number of landing and biting mosquitoes in treated and control areas. If the protection time was long and the material had feed-deterrent properties, the mosquitoes should more often land than try to bite. If the protection period of the material is long, the percentage of repellency should increases because of the small number of landing mosquitoes.

The response of the three mosquito species in this study was tremendously different. *Cx. quinquefasciatus* was more sensitive for all oils and mixtures, while *Ae. aegypti* was tolerant to many oils and oils mixtures compared to *An. stephensi*. Barnard (1999) explained the differences in responses of mosquito species for their preference of food sources. *Ae. aegypti* is an anthropophilic species with high biting pressures in laboratory bioassays while *Cx. quinquefasciatus* is an ornithophilic biter thus it has only small appetite in laboratory trials, while *An. stephensi* is a general mammalophilic and it ranged in the middle between other two species.

In the *Ae. aegypti* tests the increase of percentages of landing and biting mosquitoes in the mosquitoes groups that lacked some organs as compared to normal mosquitoes became significantly clear. However, the responsible organs for repellent sensation in the *An. stephensi* are surely different from those demonstrated in *Ae. aegypti* mosquitoes.

5. Conclusion

It was concluded that *Citrus reticulate* and *Oscimum bascillus* are environment friendly and suitable to be used as green repellents for mosquito control. Ablation of organs showed that the maxillary bulb was the important organ observed for repellency in *Aedes aegypti* mosquitoes whereas, no responsible organ was observed in *Anopheles stephensi* mosquitoes.

6. Acknowledgments

The author is grateful to technical staff of Entomology Research Laboratory, Department of Zoology, LCWU, Lahore for their help in maintenance of the mosquito colonies and their support while carrying out study on essential oils.

7. Conflict of Interest

The authors declare that there is no conflict of interests.

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