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Repellent Effect of Extracts and Essential Oils of *Citrus limon* (Rutaceae) and *Melissa officinalis* (Labiatae) Against Main Malaria Vector, *Anopheles stephensi* (Diptera: Culicidae)

***MA Oshaghi[1], R Ghalandari [2], H Vatandoost [1], M Shayeghi[1], M Kamali-nejad [2], H Tourabi-Khaledi [2], M Abolhassani[1], M Hashemzadeh[1]**

[1]*Dept. of Medical Entomology, School of Public Health & Institute of Health Research , Tehran University of Medical Sciences, Iran*
[2] *School of Pharmacy, Shaheed Beheshti University of Medical & Health Sciences, Tehran, Iran*

Abstract
Repellet effect of extracts and essential oils of *Citrus limon* (L.) Burm.F., (lemon) and *Melissa officinalis,* (balm) were evaluated against *Anopheles stephensi* in laboratory on animal and human and compared with synthetic repellent, N,N-diethyl-3-methylbenzamide (Deet) as a standard. Results of statistical analysis revealed significant differences between oils and extracts ($P< 0.05$) against the tested species, thus oils were more effective than extracts. There was no significant difference between Deet and lemon oil, whereas the difference between lemon and melissa oils was significant. Relative efficacy of lemon oil to Deet was 0.88 whereas it was 0.71 for melissa oil. The results were found marginally superior in repellency for animals than human. Due to advantages of botanic compounds to synthetic compounds we recommend lemon essential oil as an effective alternative to Deet with potential as a means of personal protection against mosquito vectors of disease.

Keywords: *Repellent, Anopheles stephensi, Citrus limon, Melissa officinalis*

Introduction

The repellent properties of plants to mosquitoes and other pest insects were well known before the advent of synthetic chemicals (1). However, the most commonly used insect repellents are synthetic chemicals that mostly have contained Deet (N, N-diethyl-3-methylbenzamide) in their formulations. Although Deet is an effective repellent against a broad spectrum of insects, however there are disadvantages associated with the use of Deet, which stem principally from its activity as a solvent of paints, varnishes, and some plastic and synthetic fabrics. There have also been concerns over toxicity of Deet (2-4). A variety of botanical substances have been evaluated for their repellency against mosquitoes (5). In ancient medicine Lemon citrus (*Citrus limon* Burm) and melissa (*Melissa officinalis* L.) have long been used as natural insect repellents in Iran (personal observation) and the world (5-6). These plants are cultivated widely in tropical, subtropical, and temperate parts of the world especially in parts of Iran and neighbouring countries where malaria is endemic. In this paper we report the repellent effects of extracts and essential oils of lemon citrus and melissa in comparison with a solution formulation of Deet against *An. stephensi* Liston (Diptera: Culicidae). This species is the main vector of malaria in the Middle East, including Iran and Indian sub continent.

Materials and Methods

Mosquitoes The mosquitoes of *An. stephensi* obtained from a well-established laboratory

***Corresponding author:** Tel: +98 21 6112606, E-mail: oshaghi@ lycos.com

colony from School of Public Health & Institute of Health Research, Tehran University of Medical Sciences. Mosquitoes were reared and maintained at 27±3° C and 80±10% Relative Humidity (RH) under a 12:12 (L:D) photoperiod. Larvae were reared on a diet of floating catfish food. The adults were maintained in screen cages on 10% sucrose solution but 24 hours before experiments the sucrose solution was removed from the cages. Repellency was tested against 3-5-day-old, blood-starved mosquitoes, and for each test 35 mosquitoes were used.

Repellent In this paper we compared the efficacy of alcoholic extracts and oils of *M. officinalis* and *C. limon*. They were identified based on plant taxonomy keys by botanist of Agriculture Department, Tehran University. Mellisa cultivated in the farm of Karaj, Dept. of Agriculture, affiliated to Tehran University and its leaves were used to prepare extracts and oils. Lemon fruits were purchased from the market. Lemon oil was obtained from fresh skin and extracts from whole dried fruit. The method of maceration was used to prepare extracts from the plants. They converted to powder and then 100 g of powder was macerated with 1 litre ethanol. After deletion of extra alcohol, 14 g and 12 g lemon and mellisa extracts respectively obtained. Essential oils were obtained from the plants using the method explained earlier (7). Distillations were performed by adding adequate double distilled water to 100 g of the plants in Clevenger apparatus. Using this protocol we were able to produce 2 ml lemon oil and 0.3 ml mellisa oil. The extract or oil products were obtained as required and subsequently diluted to 20% concentration. To prepare different concentrations, the products were further diluted using alcohol as diluent. Extract and essential oil solutions were formulated on a volume- volume basis at a concentration of 3% and 1%, respectively. The compounds were applied as 4-ml aliquots of ethanol solution and were spread evenly over the animal skin or hands of volunteer as explained previously (8, 9). After a period of 30 min, one treated subject at a time was exposed to 35 adult female mosquitoes for 30 minutes. Fifteen percent Deet in ethanol solution was used as a standard to evaluate the candidate compounds against the mosquito species.

Experiments Two series of experiments were carried out in laboratory condition. In the first series, the extracts and essential oils were tested on animal guinea pigs. The animals were laboratory reared albino males with average 400-450 g weight. A 4×6 cm of animal abdomen hairs was removed then washed and cleaned by ethanol. Treatments were 4 ml of either the extracts containing 0.12 and 0.04 g of active ingredients of extracts and oils respectively, Deet (standard), or ethanol (control). After treatment, the animal was bound on top of the cage in which the treated position was exposed to mosquitoes for thirty minutes. Each test was repeated three times replacing new mosquitoes and new animal, and number of bites through the fabrics was recorded. The compounds, which revealed significant efficacy, were selected for the second series of tests. The second series of tests were performed on the skin of volunteers. Based on low repellent efficacy of extracts, only oils were tested in second experiment. For these evaluations, hands of volunteers from wrist to tips of fingers were again treated with 4 ml of either of the selected compounds (i.e. oils of lemon and melissa), Deet, or ethanol and exposed to mosquitoes in the cage. Twelve subjects participated for three replicates, thus for each replicate four subjects were treated by either of mentioned compounds. To count number of bites, all mosquitoes were aspirated from the cage, anaesthetised with carbon dioxide and crushed on paper to determine the number of individuals that obtained blood meal. The number of bites in treated and control tests in each trial was recorded and the mean percentage protection from bites was calculated. The percentage protection, defined as the average number of bites received by subjects in each trial relative to that

of control, was calculated as (control-treatment)/control × 100. To compare the repellent efficacy of the compounds we used a paired *t*-test to determine if there were significant differences at 5% level between the candidates and between the candidates and the standard.

Results

Results of laboratory study on animals comparing lemon extract, lemon oil, melissa extract, melissa oil, and Deet against caged population of *A. stephensi* are presented in table 1. Results showed that oils were significantly more effective than extracts. Repellent efficacy of both oils in animal tests was as similar as Deet. Although Deet with 97% effectiveness was more effective than lemon (92.70%) and melissa (92.67%) oil, however, these differences were not significant. The lemon extract with the rate of 56% and melissa extract with the rate of 66% were significantly less protective than oils. Results of tests on human skin comparing oils products and Deet are summarised in table 2. Mean repellent efficacy of Deet (80.5%) against *A. stephensi* was higher than Melissa and Lemon oils (60.00 and 71.16% respectively), but the difference was not significant in case of lemon oils (71.16%). Melissa oil with 60% protective rate was significantly less protective than lemon oil and Deet ($P<0.05$).

Table 1: Relative repellent effectiveness of 3% extracts and 1% oils of lemon citrus and melissa against laboratory mosquitoes of *Anopheles stephensi* on guinea-pigs in the laboratory

Repellent	I Replicate	II Replicate	III Replicate	Mean (%) ± SE	Ratio to Deet
Lemon extract	29.41	58.8	62.15	56.12 ± 18.01	0.58**
Lemon oil	95.90	90.00	92.00	92.70 ± 3.0	0.96
Melissa extract	71.42	83.3	74.2	76.31 ± 6.21	0.79**
Melissa oil	93.50	84.50	100	92.67 ± 7.78	0.95
Deet	97.00	100	94.00	97.00 ± 3.0	1
Control	0	0	0	0	0

* t- test of paired test data at 5% level of confidence, same letters indicate no significant difference

Table 2: Relative repellent effectiveness of 1% melissa and lemon citrus essential oils against laboratory mosquitoes of *Anopheles stephensi* on hands of human skin

Repellent	I Replicate	II Replicate	III Replicate	Mean (%) ± SE	Ratio to Deet*
Melissa oil	63.50	59.50	57.00	60.00 ± 3.27	0.74**
Lemon oil	71.00	69.50	73.00	71.16 ± 1.76	0.88
DEET	82.00	80.00	79.50	80.50 ± 1.32	1
Control	0	0	0	0	0

* t- test of paired test data at 5% level of confidence, same letters indicate no significant difference

Discussion

The present study clearly showed that the application of lemon citrus repellent compounds gave acceptable percentage biting protection against *A. stephensi* which is less than but not significant with the percentage protection seen in Deet under similar conditions. No adverse effects on the skin or other parts of the body of the human volunteers were observed during the study period and through one month after application. This study showed that oils provide better protection than

extracts; however it is known that extracts are more stable than oils and could be kept for longer time than oils. Secondly oils are more prone to irritate human skin, and finally extracts are less expensive to produce than oils. Therefore it is recommended to improve the existing methods to provide better quality extracts. There are many reports indicated that low concentrations of repellent materials are attractant to mosquitoes (10-11). On the other hand, many materials that are normally thought as attractants have been reported to be repellent at high concentrations (12-13). In the present study, because of time limitation, a 3% concentration of extracts were used but and it would be worth to evaluate higher concentrations of extracts. Also it is recommended to test repellent efficacy of individual components of the extracts to identify the most effective ingredient. Moreover these compounds have to be tested in field condition to evaluate their efficacy and effective durability. Between-species differences in sensitivity to repellents have been widely documented (14-15). In this work, we have tested the repellents against only one species and do not know if these compounds are protective against other mosquito species or medically important insects. However studies on the efficacy of lemon citrus oils against sandflies of *Luzomyia youngi* showed that this compound gave higher protection than Deet and Citronella (6). Also another study showed that the essential oils of six plants, mainly *Citrus* species, were highly toxic against insects of *Liposcelis bostrychopia* (16). The repellents of lemon citrus and melissa have a lemon smell and unlike Deet, do not have undesirable dissolving properties. Citronelol, the most prevalent component alcohol, and linalool which are parts of main active ingredients of lemon and melissa in the distillate have been among the main active ingredients of other botanical repellents such as citrosa and eucalyptus (17-21). It is known that different cultivars may contain different components with different characters at different places. There are some reports on the local Iranian cultivar components (22-27), however, it is highly recommended to do further experiments to identify which part or parts of these components have got the most repellent or toxic effect against insects. There are several studies on the larvicidal activities of Iranian herbs against *A. stephensi.* In these experiments authors tested different essential oils of plants against *A.stephensi* larvae and calculated LC_{50} values. They concluded that in some extent these herbal extracts can be used for malaria vectors control (28).

It is concluded that because of the simplicity of production, cheapness, and protective activity, we recommend lemon essential oil as an effective alternative to Deet with potential as a means of personal protection against mosquito vectors of disease.

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