Original Article

Repellency Effects of Essential Oils of Myrtle (*Myrtus communis*), Marigold (*Calendula officinalis*) Compared with DEET against *Anopheles stephensi* on Human Volunteers

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Abstract

Background: Malaria and leishmaniasis are two most significant parasitic diseases which are endemic in Iran. Over the past decades, interest in botanical repellents has increased as a result of safety to human. The comparative efficacy of essential oils of two native plants, myrtle (*Myrtus communis*) and marigold (*Calendula officinalis*) collected from natural habitats at southern Iran was compared with DEET as synthetic repellent against *Anopheles stephensi* on human subjects under laboratory condition.

Methods: Essential oils from two species of native plants were obtained by Clevenger-type water distillation. The protection time of DEET, marigold and myrtle was assessed on human subject using screened cage method against *An. stephensi*. The effective dose of 50% essential oils of two latter species and DEET were determined by modified ASTM method. ED_{50} and ED_{90} values and related statistical parameters were calculated by probit analysis.

Results: The protection time of 50% essential oils of marigold and myrtle were respectively 2.15 and 4.36 hours compared to 6.23 hours for DEET 25%. The median effective dose (ED_{50}) of 50% essential oils was 0.1105 and 0.6034 mg/cm^2 respectively in myrtle and marigold. The figure for DEET was 0.0023 mg/cm^2.

Conclusion: This study exhibited that the repellency of both botanical repellents was generally lower than DEET as a synthetic repellent. However the 50% essential oil of myrtle showed a moderate repellency effects compared to marigold against *An. stephensi*.

Keywords: *Calendula officinalis*, *Myrtus communis*, DEET, *Anopheles stephensi*, Repellent, Essential oils, Iran

Introduction

Malaria is still a major endemic disease in foci located in south and southeast of Iran. The annual malaria cases have been reported from 66075 to 6211 during 1995–2009, indicating the sharp decline of disease. It is unstable with two seasonal peaks mainly in spring and autumn. These areas include the provinces of Sistan and Baluchistan, Hormozgan and Kerman. In this part of the country six anopheline mosquitoes including *Anopheles culicifacies* s.l., *An. stephensi* Liston, *An. dthali* Patton, *An. fluviatilis* s.l., *An. superpictus* Grassi, and *An. pulcherrimus* Theobald are known to be the malaria vectors and *An. sacharovi* (Favre, 1903) and *An. maculipennis* s.l. are considered as malaria vector in northern part of the country (Manouchehri et al. 1992, Zahirnia et al. 1998, 2001, Enayati et al. 2003, Naddaf et al. 2003, Oshaghi et al. 2003abc, Salari Lak et al. 2003, Vatandoost et al. 2004ab, 2005ab, 2006ab, 2008ab, 2009a, 2010, 2011, Sedaghat et al. 2005,

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2003ab, Doosti et al. 2006, 2007, Davari et al. 2007, Abai et al. 2008).

One of the widely used and effective insect repellents is the synthetic compound, N, N-diethyl-m toluamide (DEET) which is generally considered the "gold standard" repellent, providing long-lasting protection of up to 8 hours from time of application. "There are some rare reports of severe reactions in people, additionally DEET melts plastics causing spoilage of equipment, such as glasses and mobile phones, and many consumers find the odor and sensation on the skin unpleasant" (Logan et al. 2010). Therefore researchers are trying to improve the efficacy with reducing the side effects of new generation of repellents. In the past few years, a plant derived repellent, para-methane 3–8, diol (PMD) has been proven to be suitably efficacious and safe to compete with DEET in the field of disease prevention, and repellents have been recognized by WHO as a useful disease prevention tool to complement insecticide-based means of vector control. Many plants have substances that are toxic, antifeedant properties or repellency for insects. Repellent of plant material refers to a plant origin have an inherent and naturally defensive and repellency effects on insects (Maia and Moore 2010). This material includes extracts and essential oils. Their effect is significantly differs from different parts of plants, including flowers, tubers, leaves, fruit, branches and roots.

Myrtle (*Myrtus communis* L.) is a native plant distributed in south, north and central parts of Iran (Rechinger 1996). A study in Iran showed that the myrtle essential oil (*M. communis*) is very active against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* in vitro. This confirms the application of herbal medicines for treating a range of infectious diseases in ancient times (Pourmand et al. 2008). Study of the conditions *in vitro* effect of myrtle essential oil, the extracts form soluble in water, and soluble dichloromethane corn oil was studied on a variety of microbes. It was determined that the oil could prevent the growth of bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Its effect of antiviral ointment containing about 10% of myrtle essential oil on patients with herpes simplex virus was tested (Zolfaghari et al. 1997). The essential oil of myrtle is effective in protection of biting insects. In addition insecticidal action was also observed (Yaghoobi-Ershadi et al. 2006). Marigold also exhibits great effects in treating some skin disorders such as leaving the skin, acne and dermatitis. The *Calendula* ointment are presented and is believed that the spring flowers of this plant had been used for reducing swelling, treat injuries, and a disinfectant material. For domestic use, this plant has been used for soothing effects of mucosal ulcers, swelling of the stomach. The repellency property makes an impact effect on the insects away from humans.

For the first time in the country, the repellency of essentials oils myrtle and marigold which are native in southern Iran, were assessed using *An. stephensi* and compared with DEET on human volunteers under laboratory condition.

Materials and Methods

Mosquitoes

The tested mosquitoes were the established colony of *An. stephensi* obtained from the Insectary of School of Public Health, Tehran University of Medical Sciences, Iran. Mosquitoes were reared and maintained at 27±3 ^{o}C and 80±10% relative humidity (RH) under a 12: 12 (L: D) photoperiod. Larvae were fed on a diet of enriched wheat germ. The adults were maintained in screen cages and fed with 10% aqueous sucrose solution as a source of energy and guinea pigs as blood-feeding female mosquitoes for maturing the eggs. Starved 7 to 10 days old females were used for the repellency tests. The sucrose solution was

picking up from the cage, 12 hour before starting the experiments.

Repellents

The chemical repellent, DEET (N, N-diethyl-meta-toluamid), CAS NUMBER: 134–162 and assay 98.8%, Density: 0.998 g/cm^3 were purchased from Merck Co, Germany. The myrtle (*M. communis*) is evergreen shrubs or small tree which was collected from southern extension of natural habitat in Noorabad district at coordinate 30º 7´E 51º 31´N, 920 meters above sea level, southern Iran. The marigold (*Calendula officinalis* L.) was also collected in suburb of Shiraz City, at coordinates 29°37′N 52°32′E at elevation of 1481 meter above sea level, Fars Province, southern Iran. The plants were identified and the voucher specimens were deposited at the Herbarium of Faculty of Pharmacy, Department of Pharmacognosy. The leaves and flowers of myrtle and the flowers of marigold were dried at room temperature under good ventilation and chopped into small pieces using a knife mill. The essential oil was extracted from the plants using a Clevenger-type water steam distillation apparatus. The distilled essential oils were stored in a refrigerator at 4° C until being used in the experiments which were diluted by absolute ethanol at 50% concentration. The composition of the volatile constituents was established by gas chromatography-mass spectrometry.

Test method

All series of the experiments were carried out in laboratory condition. In the first stage, in order to reveal the probable allergic reaction of chemical and natural repellents to human volunteers, the scratch test was done on skin of the upper arm. The treated skin of arm was observed up to 72 hours for allergic reaction.

The 25% solution of DEET was prepared using absolute ethanol as well as 50% essential oils myrtle and marigold was obtained in same manner and tested against *An. stephensi* on four male volunteers. Observation was based on the variable dose-response of the mosquito to the serial dilutions of the repellents. The procedures for determination of effective dosages of the repellents were adopted by the standard method of American Society for Testing and Material (ASTM E951-94). The testing kit was made of Plexiglas cube at dimension of 4 x 5 x 18 cm having five circles in 29 mm diameters. Before selecting the mosquitoes, the willingness of mosquitoes for introducing in repellency tests were monitored based on determination of biting pressure on untreated, alcohol washed arm which should be at least 10 landings/ probes per 30 seconds. Each of 5 adjacent cells in the ASTM modules was provided with 5 female 7–10 days mosquitoes that randomly selected from a cage containing 200 starved mosquitoes. The effective dose tests were conducted by applying each repellent directly to the human skin. Five circles (29 mm in diameter) were drawn on the volunteer's forearm using a felt tipped pen and a plastic pattern. The drawn circles on the human subjects were treated with 25 µl of the diluents. The serial dilutions were applied on 4 holes as well as the absolute ethanol was applied in control circle. The treated circles were allowed to dry, and then test apparatus containing starved mosquitoes were fixed on the treated skin of the volunteers and opened the slide for exposure with treated skin. The counts of probing and biting were recorded at 1 minute intervals up to 5 minutes. The experiments were done at 2 replicates on right forearm and 2 replicates on left forearm of the volunteers. After each test, the mosquitoes were removed from the test apparatus using aspirator and then transferred into a screened cup. Mortality of mosquitoes was read after 24 hours. The protection and failure times were also determined on human subjects. Before starting the experiments, 1 ml DEET 25%, as well as 1 ml of 50% essential oils both myrtle and marigold were dissolved in

absolute ethanol and spread evenly between the elbow and wrist of a volunteer's arm. The other arm, acting as a control and was treated only with 1 ml of absolute ethanol. After drying the test arm, a rubber glove was put by volunteers and inserted the arms into 50× 50×50 cm cage containing 150–170 starved mosquitoes for 3 minutes. The mosquitoes which landed and attempted to bite were recorded. The procedure was repeated at 30 minutes intervals and was used consistently throughout the experiment. If more than 1 mosquito bite was recorded during an observation, the test of repellency was terminated, and the period of repellent protection was calculated as the time between the repellent application and multiple mosquito bite. If only 1 mosquito attempted to bite during an observation period, any addition mosquito bites during that next observation period (30 min later) confirmed that the initial bite represented the time of repellent failure. The period was repeated up to 10^{th} bites and took into account as failure time. The successive expose of the control arm were made prior to inserting the treated arm in order to provide a standard for comparing mosquito biting activity during the experiments. The similar tests were also repeated on 4 human volunteers.

Statistical analysis

The data were subjected to statistical analyses using SPSS software ver. 11.5. In order to estimate the ED_{50} and ED_{90} values, the cumulative results were subjected to the probit analysis which had been repeated in different days with four volunteers (Finney 1971, 1978). The regression lines were plotted and the ED_{50} and ED_{90} values with confidence limits and regression parameters were calculated. Data were transformed using arcsine $\sqrt{x}$ transformation to meet the normality. Significant differences between three repellents were assessed by ANOVA. The latter test was also used for assessment of the significant differences between protection times of the tested repellents. Means of protection times and ED were compared by the Tukey's honest significance test or games-Howell test depending on significance of Levene's test. The 1% level was employed in tests of significance.

Ethical approval

This study received formal ethical approval from the Medical Ethics and History of Medicine of Research Center, Tehran University of Medical Sciences. Informed consent was taken from each volunteer.

Results

Protection time

The protection time of 50% essential oils myrtle and marigold compared with DEET 25% against *An. stephensi* on human subject is shown in Table 1. The protection time (PT) of botanical repellents was ranged between 4.25–4.40 and 1.00–3.30 hours with mean of PT 4.36 and 2.15 hours respectively with myrtle and marigold essential oils (Table 1). The mean of protection time of DEET was ranged between 6.05–7.00 hours with mean of 6.23 hours. The difference between botanical repellents and DEET was significant compared to both botanical repellents ($P< 0.01$). On the other hand, the difference of protection times of myrtle compared with marigold essential oil were significant ($P< 0.01$) (Fig. 1).

Effective doses

The ED_{50} values (with 95% confidence limits) of the myrtle (*M. communis*) and the marigold (*C. officinalis*) essential oils were 0.1105 (0.0772–0.1399) and 0.6034 mg/cm^2 (0.4464–0.7476), respectively on 4 human subjects. The ED_{90} values with 95% confidence limits were respectively 0.5404 (0.4281–0.7683) and 3.4905 mg/cm^2 (2.6203–5.4534) for myrtle and marigold plants (Table 2). The ED_{50} and ED_{90} values for DEET as a golden repellent were 0.0023 (0.002–0.0027)

and 0.009 mg/cm^2 (0.0071–0.0127). Statistical comparison of the data was revealed that the ED values for essential oils myrtle and marigold is significantly higher than ED values of DEET, showing less repellency of botanical repellents compared with DEET ($P<$ 0.01). The ED_{50} of myrtle was close to ED_{90} of marigold and was significant different (Fig. 2). Comparing the ED_{50} values, it can be concluded that the ED_{50} for DEET is significantly higher than those of ED_{50} for essential oils marigold and myrtle, showing higher repellency effect ($P<$ 0.01). The related dose-response lines, ED values and regression equations for DEET, marigold and myrtle are shown in Fig. 3.

GC-mass analysis

The result of GC-mass analysis showed that the number of chemical contents were 65 and 33 constituents respectively in essential oils marigold and myrtle. The main compounds of marigold were Alpha-cadinol (18.3%), beta-eudesmol (14.5%) and tau-muurolol (13.0%) compared to alpha-pinene (47.8%), 1, 8-cineole (25.9%), linalool (8.4%) and linalyl acetate (4.3%) in myrtle.

Table 1. Protection and failure times values of essential oils both marigold and myrtle compared to DEET against *An. stephensi* on human subjects

Repellents	Protection time (hour)		Failure time (hour)
	Range	Mean ± SE	
DEET 25%	6.05–7.00	6.23 ± 0.16	7.30
Marigold 50%	1.00–3.30	2.15 ± 0.66	3.30
Myrtle 50%	4.25–4.40	4.36 ± 2.18	4.40

Table 2. Parameters of probit analysis on chemical and botanical repellents against *An. stephensi* using standard method (ASTM- E951-94)

Repellents	No. mosquitoes	ED50 (mg/cm^2)	95% C.L. (mg/cm^2)	ED90 (mg/cm^2)	95% C.L. (mg/cm^2)	X^2 (df) ±SE	X^2 (table)	*P*-value	Equation of regression lines
DEET 5%	100	0.0023	0.0020–0.0027	0.009	0.0071–0.0127	16.185(2)±0.221	5.99	<0.05	Y= 5.7588 +2.1903X
Marigold -	100	0.6034	0.4464–0.7476	3.4905	2.6203–5.4530	8.110(2)±0.196	5.99	<0.05	Y= 0.3688+1.6813 X
Myrtle 50%	100	0.1105	0.0772–0.1399	0.5404	0.4281–0.7683	12.043(2)±0.618	5.99	<0.05	Y= 1.7785+1.8589 X

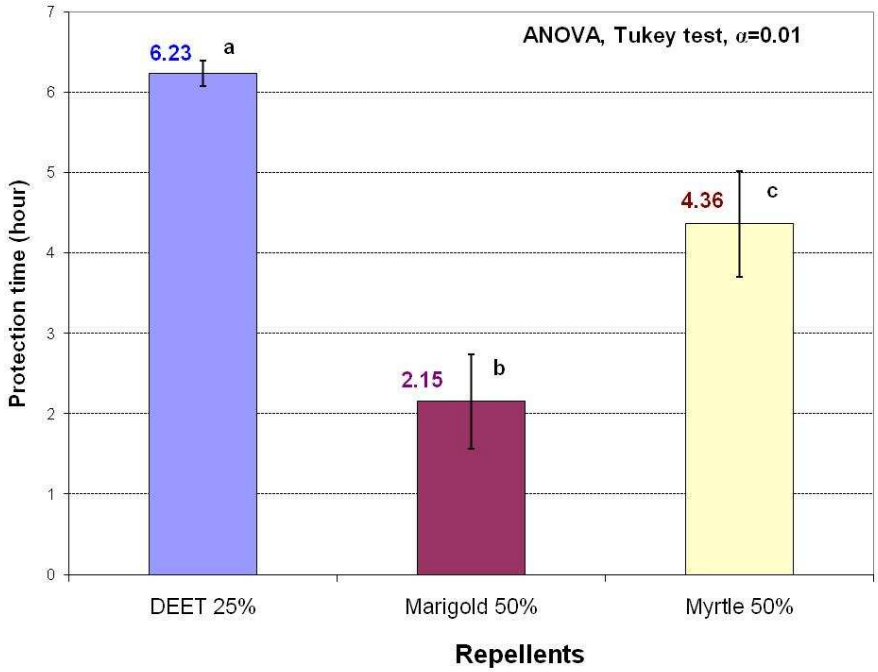

Fig. 1. Statistical comparison of protection time of essential oils of marigold and myrtle as well as DEET on human subjects using *An. stephensi*

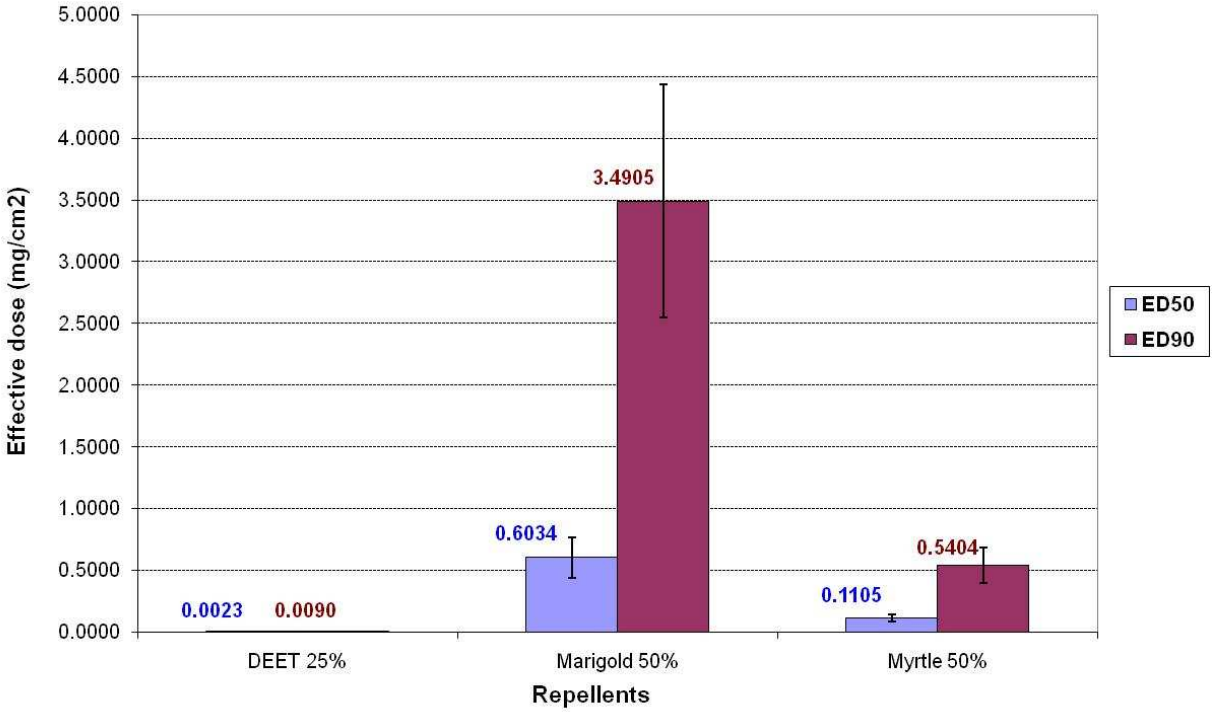

Fig. 2. Statistical comparison of effective doses of essential oils marigold and myrtle as well as DEET on human subjects using *An. stephensi*

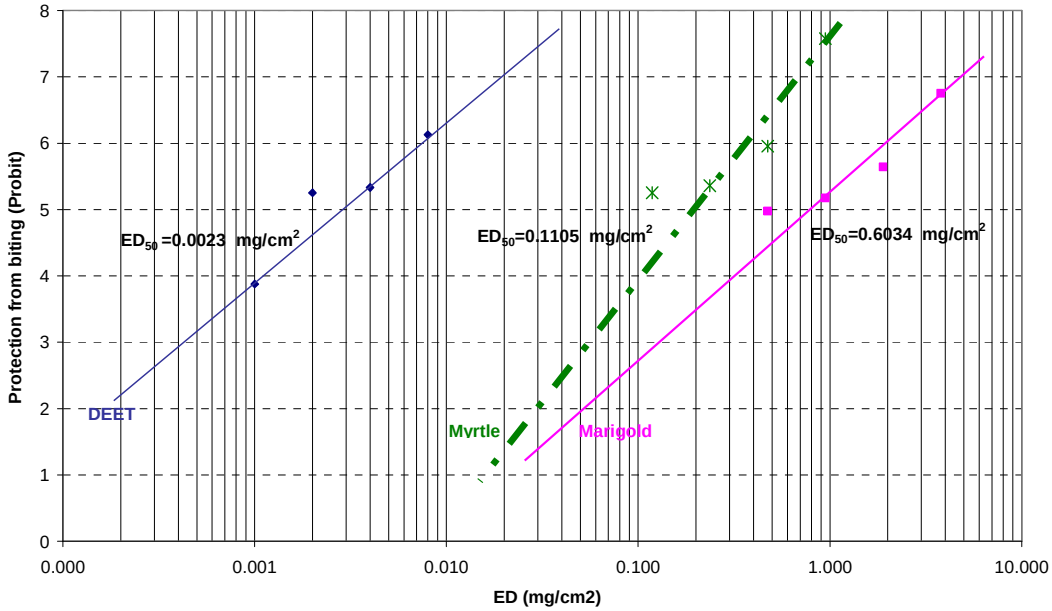

Fig. 3. Dose-response lines and values for three botanical and chemical repellents against *An. stephensi* on human subjects

Discussion

Insect repellents are used to prevent nuisance bites from mosquitoes as well as other blood-feeding arthropods and may aid in lowering disease transmission e.g. malaria, leishmaniasis, filariasis and West Nile virus.

Anopheles stephensi is the main malaria vector in the country and is rearing easily and used for different biological assays such as irritability tests, olfaction studies, bioassay tests for bednets and indoor residual spraying, biological tests for plant extraction and repellents (Hadjiakhoondi et al. 2000ab, 2003, 2005, 2006, Sadat Ebrahimi et al. 2005, Rafinejad et al. 2006, Vatandoost et al. 2006a, 2008b, 2009b, 2011, Davari et al. 2007, Omrani et al. 2010, Shahi et al. 2010, Hanafi- Bojd et al. 2011, Sedaghat et al. 2011). In this study, the effect of essential oils myrtle and marigold as the botanical repellents were compared with DEET. The N,N-diethyl-m-toluamide (DEET) as a broad-spectrum repellent is provide longer-lasting protection against many species of biting arthropods including mosquitoes which has been used worldwide since 1957 (USEPA 1998). It is commonly assumed that plant-based repellents are safer than DEET because they have natural origin (Maia and Moore 2010). The botanical repellents were developed from definite species of plants are environment-friendly, with pleasant natural aroma and less harmful than synthetic repellents which have been reported to cause many undesirable side effects to human.

In our study, 25% DEET provided an average of 6.23 hours of complete protection against *An. stephensi* bites. DEET-based repellents have been shown in other studies to provide complete protection against arthropod bites for as long as 12 hours under laboratory conditions (Fradin and Day 2002) which depend on the concentration, formulation and mosquito species tested (Klun et al. 2006).

Our ED_{50} estimates for DEET is 0.0023 mg/cm^2 on 4 human subjects. On the animal model, the ED_{50} value of DEET was calculated 0.005 mg/cm^2 against *An. stephensi* (Vatandoost 2008b). In the laboratory condition, the mean of relative effectiveness of 50% DEET was showed 97.0% protection against *An. stephensi* on guinea pig and 80.5% protection on human hand (Oshaghi et al. 2003b).

A cream formulation of DEET was evaluated at 10 mg/cm^2 with 96.2% protection against *An. stephensi* and provided protection up to 6.75±0.2 hours up to 4 hours observation (Mittal et al. 2011).

There are no published data describing the repellency of essential oil of marigold. The mean of protection time of 50% myrtle essential oil showed considerable repellency on human subjects and provided 4.36 hours protection against *An. stephensi*, the main malaria vector at laboratory condition. In other study which conducted on 41 natural repellents, the protection time of myrtle essential oil was reported 6.5 hours against *Anopheles* species (Abdelkrim et al. 2006). The past studies revealed that the most natural product-based repellents provided 3 hours protection which is comparable with protection provided by 7 or 15% DEET (Barnard and Xue 2004). The mean of protection time of 50% essential oil marigold provided only 2.15 hours protection against *An. stephensi* bites. In other laboratory study, the values ED_{50} and ED_{90} for myrtle essential oil were respectively calculated as 0.1140 and 0.6711 mg/cm^2 on animal model (rabbit) using K and D apparatus against lab-bred *Phlebotomus papatasi* Scopoli (Yaghoobi- Ershadi et al. 2006). Surprisingly, in our study, the ED_{50} and ED_{90} values for myrtle essential oil against *An. stephensi* was very close to latter studies on animal model with *P. papatasi* (respectively 0.1105 and 0.5404 mg/cm^2) which assessed on 4 male human subjects.

The repellent effects of the essential oils indicated that they contained active constituents which responsible for the repellency activity. The major components of these two essential oils are monoterpenes, primarily 1, 8-cineole and linalyl acetate which detected in moderate percentages (13.0–18.3%) in the essential oil of studied myrtle (*M.communis*) compared to lower percentage (<0.5%) in marigold (*C. officinalis*). The higher repellency and insecticidal effects of the myrtle could be attributed to the major aforementioned constituents. The insecticidal effect of mytle at 1.6 mg/cm^2 was reported 62.2% against lab-bred *P. papatasi* on animal model (Yaghoobi-Ershadi et al. 2006). In marigold (*C. officinalis*), carvacrol and thymol were extracted in low percentage (0.18–0.28%) had been shown to have insecticidal properties. On the other hands, 1, 8-cineole and linalyl acetate could be responsible for the high repellency activity of the essential oil of myrtle (Klocke et al. 1987, Abdurrahman et al. 2006, Günter et al. 2009,).

The protection time provided by 25% DEET (PT=6.23 hours) is contrastable with the results of 50% myrtle essential oil (PT= 4.36 hours) which shows that the myrtle essential oil can be useful and safe in preventing mosquito bites and have potential use as a botanical repellent.

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