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Laboratory Evaluation of Dimethyl Phthalate against *Anopheles stephensi* and *Culex pipiens*

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Abstract: Two repellents includes Iranian and Merck dimethyl phthalate (DMP) were evaluated against *Anopheles stephensi* and *Culex pipiens*, using American Society for Testing and Material (ASTM) standard ED 951-83 procedure, a free choice method based on the variable dose-fixed time. Also a modified of ASTM method we used for determination of effective dosages of the repellents. In ASTM method there were no significant differences between the two repellents (Iranian and Merck's DMP) as indicated by the ED_{50} and ED_{95} values ($p>0.05$). But, there were significant differences in repellent sensitivity between *An. stephensi* and *Cx. pipiens* at the ED_{95} level. In modified ASTM method there were no significant differences between the two repellents against *An. stephensi*, as indicated by the ED_{50} values ($p>0.05$). But, there are significant differences between the two repellents against *Cx. pipence* based on ED_{50} value. Results of this study showed that the Iranian synthesized DMP has necessary potential and specificity to compare with Merck manufactured product.

Key words: Dimethyl phthalate (DMP), *Anopheles stephensi*, *Culex pipiens*, Effective dose, Repellent

INTRODUCTION

The use of repellents is an important and obvious practical means of preventing the transmission of arthropod born diseases to human (Bernier *et al*., 2005). The most common mosquito repellent formulations available on the market contain deet (N, N-dimethyl-m-toluamide), which has shown good repellency against biting insects (Costantini *et al*., 2004; Roberts and Reigart, 2004). Although effective, deet is not the ideal product, as allergic and toxic effects have been documented (Roberts and Reigart, 2004). To avoid these adverse effects, research on repellents that are derived from plant extracts and some other chemical repellents, to replace deet conducted (Odalo *et al*., 2005; Rajkumar and Jebanesan, 2005). The repellent dimethyl phthalate (DMP) is again attended due to its safety to human (Debboun and Wagman, 2004; Tuetun *et al*., 2005). Since the 1940 DMP, has been used alone or in combination with other repellents, such as Indolan, ethyl hexandiol and dimethyl carbate (Trongtokit *et al*., 2005; Smith *et al*., 1952). During the last few years particular attention has been given to formulation and testing of various combination of some effective repellents (such as DMP) that have been declared safe for application on skin (Kalyanasundram *et al*., 1994; Paul and Sabin, 1994). This study investigates the repellency and effective dosage of DMP, which is synthesized and formulated in Iran, comparing with Germany dimethyl phthalate against *Anopheles stephensi* and *Culex pipiens* using human bait methods. Also we used two methods including: American Society for Testing and material Standard ED951-83 (EPA, 1999) and modified model of ASTM method.

MATERIALS AND METHODS

Chemicals: Dimethyl phthalate (DMP) was synthesized at military medicine Institute in Iran and compared with standard insect repellent purchased from Merck, Germany: dimethyl Phthalate, which is widely used in commercially available preparation (USEPA, 1980).

Mosquitoes: The laboratory colonies of *An. stephensi* and *Cx. pipiens* (Diptera: Culicidae) established at Tehran

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Medical Sciences University (TMSU) was used for repellent tests. The laboratory colonies was maintained at 20-30°C, 80-90% relative humidity with a photoperiod of 12 h light, 12 h dark. Larvae were fed with Bemax powder and adults colony were provided with 10% sucrose. They were periodically blood fed on restrained Guinea pig. The 5-10 days nulliparous females, which starved 12-24 h before test, used for testing.

Test method: In laboratory repellent test, dimethyl phthalate was evaluated against *An. stephensi* and *Cx. pipeins* when applied to human skin. Observation was made on the response of the mosquitoes to a graded series of dosages. The protocol specified an estimated amount of repellent to be applied to the skin to produce a given level of effectiveness against a mosquito test population. The procedures for determining effective dosages of the repellents were the American Society for testing and material standard ED 951-83,94 (ASTM) (EPA, 1999, 2000) and a modification of the ASTM method.

Tests were based on the variable dose-fixed time, free choice method described by Buescher *et al.* (1982) and similar to the method described by Coleman *et al.* (1994). Five circles (29 mm in diameter) were outlined on the volunteer's forearm using a plastic template and a felt-tipped pen. These areas were treated Randolf with 25 μL of the diluents, one control and four serial dilutions of the dimethyl phthalate in absolute ethanol. Two plastic cages which divided into 5 compartments (4×5×18 cm) and one without division into compartments, were provided for testing (Fig. 1 and 2).

After the treatment had dried for 5 min, plastic cage with matching cutouts on its floor while containing 10 nulliparous 5-10-days old female mosquitoes was secured over the area with rubber bands.

Fig. 1: The modified cage (single dose) (original)

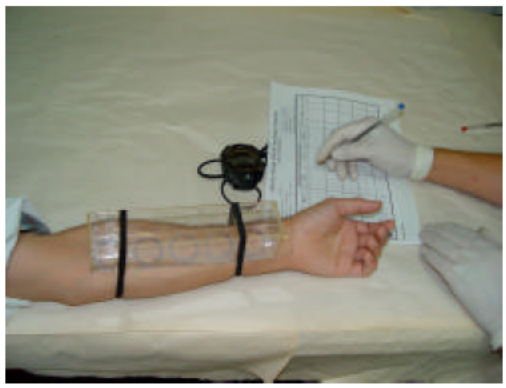

Fig. 2: Effective dose evaluation with standard (ASTM) method (original)

The number of mosquitoes biting on each test site was recorded per minute for 5 min. Tests were conducted three ties on each repellent-treated area and completed within 25 min of repellent application. The experiments with two models of cages conducted twice on each of the four human volunteers (4 males). All tests were conducted at least two ties on different days to obtain an estimate of the ED_{50} and ED_{95} values.

Statistical analysis: The median (ED_{50}) and 95% effective dose (ED_{95}) expression in micrograms of repellent per square centimeter of skin area with 95% Confidence Interval (CI) and comparing repellent effects of Iranian and Merck's DMP against *An. stephensi* and *Cx. pipiens* were estimated by the probity plan procedure, using Statistical Analysis System (SAS). Significant differences were determined by comparing the 95% of CI_S among ED_S. Comparing of *An. stephensi* and *Cx. pipiens* sensitivity was conducted with t-test.

RESULTS

ASTM method: In ASTM method there were no significant differences between the two repellents (Iranian and Merck's DMP) as indicated by the ED_{50} and ED_{95} values ($p>0.05$). The results demonstrated that, there were significant differences in repellent sensitivity between the *An. stephensi* and *Cx. pipiens* as revealed with the ED_{95} level ($p<0.05$) (Table 1).

Table 1: Comparative results on effective dosages of two repellents (μg cm^{-2}) against *An. stephensi* and *Cx. pipiens* with ASTM method

	ED_{50} (95% CI)		ED_{95} (95% CI)	
Species	DMP_{Iran}	DMP_{Merck}	DMP_{Iran}	DMP_{Merck}
An. stephensi	1.91 (1.44-2.36)	2.13 (0.58-5.89)	23.33 (14.66-50.89)	25.12 (7.88-84.40)
Cx. pipiens	1.45 (1.01-6.28)	1.59 (0.51-4.56)	6.89 (3.25-27.8)	7.24 (3.19-24.55)

Modified ASTM method: In modified ASTM method, also there were no significant differences between the two repellents against *An. stephensi*, as demonstrated by the ED_{50} values ($p>0.05$). The results indicated that, there are significant differences between the two repellents against *Cx. pipence* based on ED_{50} value (but not at the ED_{95} level) (Table 2).

Comparing of two method of tests based on results of effective dose of Iranian DMP against *An. stephensi*, indicated, there is no significant differences between these methods ($p>0.05$). But in modified ASTM method the range of effective dose (interval between ED_{50} and ED_{95} values) is wider than ASTM method (Table 3).

The slope of log dose-probit lines used to obtain the ED_{50} and ED_{95} values were consistently the same for two repellents, but the slope of log dose-probit lines in ASTM, is more than modified methods. In chart the large slope value indicated, there is no tolerance to the repellents (Fig. 3-6).

Biting pressure of *An. stephensi* and *Cx. pipiens* on human volunteers were measured 26.5 (SE = ±0.9) and 14.8 (SE = ±1.2), respectively.

Table 2: Comparative results on effective dosages of two repellent (μg cm^{-2}) against *An.stephensi* and *Cx. pipiens* with modified ASTM method

	ED_{50} (95% CI)		ED_{95} (95% CI)	
Species	DMP_{Iran}	DMP_{Merck}	DMP_{Iran}	DMP_{Merck}
An. stephensi	2.78 (1.23-4.55)	3.15 (1.52-5.00)	29.74 (16.8-86.61)	34.54 (19.26-105.75)
Cx. pipiens	0.42 (0.24-0.59)	3.44 (1.50 -*)	10.08 (6.26-23.06)	21.60 (14 -*)

Table 3: Comparison of ASTM and modified methods base on effective dosages of Iranian DMP (μg cm^{-2}) against *An. stephensi*

Methods of test	ED_{50} (CI95%)	ED_{95} (CI95%)	Lines
Standard ASTM	2.13 (0.06-5.06)	25.12 (7.88-36.44)	Y = -0.5041+1.53X
modified ASTM	3.15 (1.52-5.00)	34.54 (19.26-105.75)	Y = 0.7881+1.58X

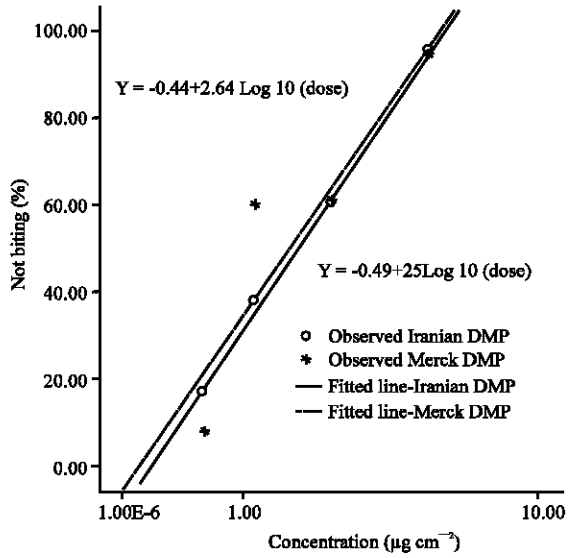

Fig. 3: Log dose-probit lines for Iranian and Merck's DMP against *Cx. pipiens* by standard method (serial dilutions)

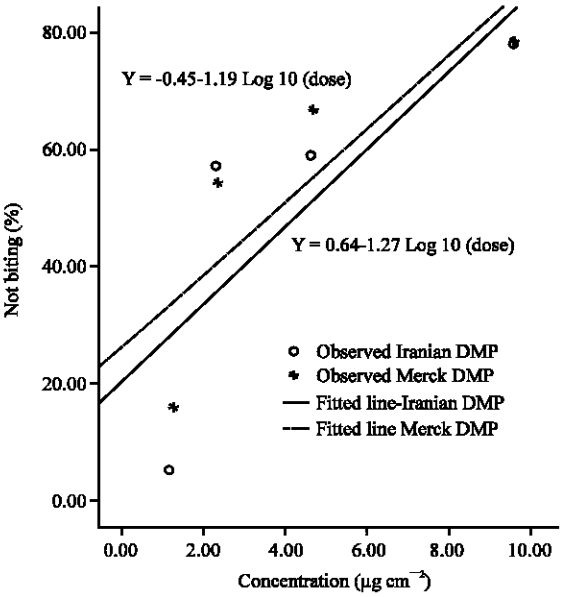

Fig. 4: Log dose-probit lines for Iranian and Merck's DMP against *Cx. pipiens* by modified method (single dose)

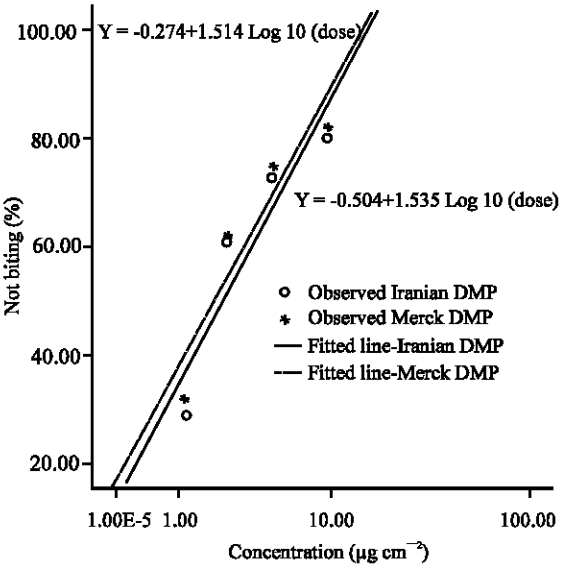

Fig. 5: Log dose-probit lines for Iranian and Merck's DMP against *An. stephensi* by standards method (serial dilutions)

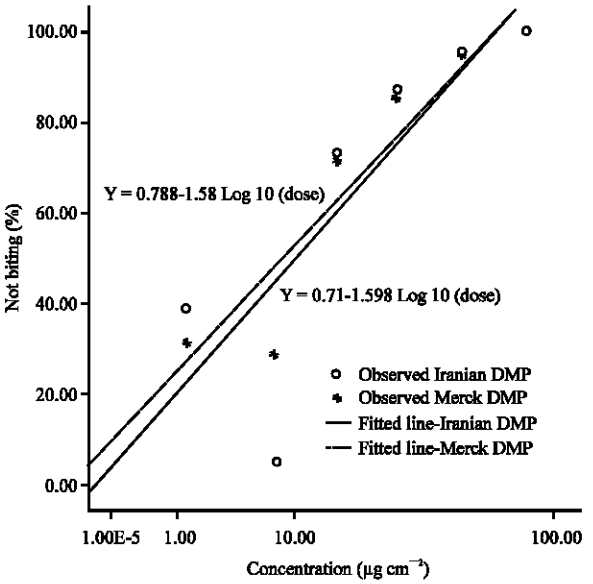

Fig. 6: Log dose-probit lines for Iranian and Merck's DMP against *An. stephensi* by modified method (single dose)

DISCUSSION

The Comparison of ED_{50} and ED_{95} of Iranian and Merck's DMP against *An. stephensi* and *Cx. pipiens* indicated that there is no significant difference between two repellents. So Iranian synthesized DMP has equal efficacy and could be used instead of active ingredient of DMP purchased from Merck, Germany. According to Buescher theory, ED_{50} is a good indicator to compare repellents and is used for practical goals and determining the percentage of active ingredient of repellents. So in this study ED_{50} is used for comparing repellents types. We measured ED_{50} of Iranian and Merck's DMP against *An. stephensi*, 2.13 and 1.91 respectively. Another similar study, which used standard method, measured 1.90 for ED_{50} of DMP against *An. stephensi* (Robert *et al*., 1991). Also comparing the result of ED_{50} and ED_{95} of repellent, demonstrated that *Cx. pipiens* is more sensitive to repellants in Comparison with *An. stephensi*. Similar studies have shown that some of the *Culex* sp. like *Cx. pipiens* and *Aedes* sp. like *Aedes taeniothynchus* are more sensitive to repellents than *Anopheles* sp. (Rutledge *et al*., 1983; Barnard, 1999). So generalizing the measured data from one species to others is unreliable (Robert *et al*., 1991). Because of different mosquitoes' behavior, especially human biting propensity, ideal species must be selected.

Other studies have shown that the mosquito's species, which have suitable biting pressure on the subjects, must be used. So antropophilic species like *Aedes aegypty* is the best selection for human subjects (Barnard, 1999). Undoubtedly, growth facility and availability of species are indications of selection. In this study evaluation of repellents was made on human subjects, *Cx. pipiens* and *An. stephensi* was used. Based on the result of our study, *An. stephensi* was distinguished as ideal species for repellents test in the laboratory, approximately after 12 h starvation; biting pressure was measured 26.5 per 30 sec. Which is standard biting pressure are more than 10 biting (landing or probe) during 30 sec in the laboratory (EPA, 2000).

Table 4: Comparison of standard (ASTM) and modified method base on effective dose (ED_{50}-ED_{95}) (µg cm^{-2}) against *An. stephensi* in other surveys

	(ED_{50}-ED_{95}) (µg cm^{-2})	
Repellents	Standard ASTM (Coleman *et al.*, 1994)	Modified ASTM (Klun and Debbone, 2000)
DEET	(0.56-3.99)	(0.13-8.44)
AI3-37220	(0.27-3.90)	(0.12-8.16)
AI3-35765	(2.63-5.53)	(0.39-30.10)

Culex mosquitoes, especially *Cx. pipiens* are one of the ornithophiles. That give erratic or negative responses, so they are not ideal for repellents test in the laboratory (Barnard, 1999). In our study, usage of this species was very difficult for lab test, because after 12-24 h of starvation, in some ties, they have a little propensity to human biting and their biting pressures was lower than standard value for repellent test. Therefore in most cases, they were starved for 26-36 h until receiving biting pressure to standard value.

Based on our survey and other trails, *Cx. pipiens* is not a good species for repellents test in human subjects, because it has some systemic errors. So *An. stephensi* is suggested for screening and repellents study in the laboratory.

As mentioned in methods and materials section, ASTM is based on using of 4 serial dilutions and a solvent in one test, simultaneously and randomly which is according to free choice method (Buescher method). Cage model test in modified method was similar to standard model, although the cage was divided in to 5 parts distinctly (Pitasawat *et al*., 2003).

The results demonstrated that there is no significant difference between ED_{50} and ED_{95} values of standard ASTM and modified ASTM, based on *An. stephensi*, but effective dose ranges are wider in modified models. This situation is considerable in other studies. In the Klun and Debbone model (K and D), which is a modified model, widening of range of effective dose is shown and ED_{50}-ED_{95} are more distinct (Table 4).

Current study had similar results comparing to previous studies, which is indicated that ED_{50}-ED_{95} values of 2 repellents are wider in modified model than in standard model.

It is noticeable that one of the major existing problem of standard ASTM model is using serial dilutions of repellents in one test simultaneously and concomitantly.

In modified models for omitting this effect, test cages had been divided, so interaction of repellents evaporation is omitted completely. It must be noticed that EPA-ASTM did not approved modified methods like K and D as a standard method (EPA, 2000). Based on scientific advisory panel meeting April, 2000 held in Virginia, USA, modified model like K and D has a test cage with enclosed area which does not provide for free flow of repellents vapors from the surface and eventual dissipation of repellents vapor into immediate environment. It is probably that some repellents may have indicated higher confounding repellency (EPA, 2000). So, ranges of the effective dose (ED_{50}-ED_{95}) would be wider.

In the present study, this problem was lower than other studies because the cage was modified unaerodynamically and it had some necessary fenestrate for repellents vapors.

In spite of the problem, cage studies, which determine ED_{50} and ED_{95} of repellents, are reliable for comparing and screening of different repellents. Therefore this screening can be done with modified or standard method, because effective dose in both methods are proportional. With both methods relative difference of effective dose of different repellents can be indicated.

Finally the present study indicated that Iranian synthesized DMP has adequate potency and specificity to compare with Merck's DMP in mosquitoes repellency.

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