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The formulation of the essential oil of *Piper aduncum* Linnaeus (Piperales: Piperaceae) increases its efficacy as an insect repellent

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Abstract

The essential oil (EO) of *Piper aduncum* Linnaeus, known as 'sireh lada' to locals Malaysian, has the potential to be used as an alternative to synthetic insect repellents such as N,N-diethyl-meta-toluamide. However, the EO's efficacy as a repellent decreases after application due to the high volatility of its active ingredients. A number of studies have showed that optimizing the formulation of plant-based EOs can improve their efficacy as repellents. The present study sought to evaluate the effectiveness of 10% *P. aduncum* EO in ethanol and in three different semisolid formulations: ointment, cream and gel. These formulations were tested on *Aedes aegypti* under laboratory conditions. Each formulation was applied to the subject's hands, which were then inserted into a cage containing 25 nulliparous *A. aegypti*. The number of mosquitoes landing on or biting each subject's hand was recorded, and the repellency percentage, landing/biting percentage and protection time for each of the formulations were compared. There were no statistically significant differences between the semisolid EO formulations with regards to the repellency percentage and the landing/biting percentage at 4 h post-application. All three semisolid EO formulations were able to repel >65% of the *A. aegypti* at 4 h post-application. The EO ointment formulation provided a protection time (182.5 ± 16.01 min) that was statistically significantly longer than that associated with the EO gel formulation (97.5 ± 14.93 min). Meanwhile, the EO cream formulation provided a protection time of 162.5 ± 6.29 min. As the EO cream and ointment formulations displayed better repellent properties than the EO gel formulation, they appear to be the most promising *P. aduncum* EO formulations to be developed and commercialized as alternatives to synthetic repellents.

Keywords: *Aedes aegypti*, *Piper aduncum*, N,N-diethyl-meta-toluamide, ointment, cream, gel

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Introduction

Insect repellents are substances that can prevent insects such as mosquitoes from flying near, landing on or biting human or animal skin. They can act either locally or at a distance (Blackwell *et al.*, 2003; Choochote *et al.*, 2007). As female mosquitoes can spread pathogens to humans via their saliva

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during the blood-feeding process, reducing human–vector contact using repellents can prevent or reduce the transmission of vector-borne diseases (Vatandoost & Hanafi-Bojd, 2008; Meepagala *et al.*, 2013).

The use of repellents is an economical method for preventing vector-borne disease and it provides an alternative to methods of vector control that use synthetic insecticides (Gupta & Rutledge, 1994). Repellents are widely available and they are considered to be important aids for personal protection, especially in tropical regions where mosquito-borne diseases are a major health problem (Golenda *et al.*, 1999; Frances &Wirtz, 2005). Many travelers use repellents for personal protection against mosquito bites. Travelers from non-endemic countries are advised to use repellents for personal protection against dengue fever (DF), which is a mosquito-borne disease and one of the most frequently reported diseases in post-travel illness registries (Meltzer & Schwartz, 2009). Data from the GeoSentinel database (one of the surveillance systems that monitor infectious diseases among travelers) indicate that about 2.1% of illnesses among travelers are due to DF (Schwartz *et al.*, 2008) and DF is a major reason for hospitalization among travelers (Stienlauf *et al.*, 2005). UK guidelines recommend that travelers who visit endemic areas such as Malaysia should apply a repellent to exposed skin (Health Protection Agency, 2007). For travelers to areas where malaria is endemic, it is advised that personal protection methods against mosquito bites are used in addition to malaria chemoprophylaxis (Goodyer & Song, 2014).

The gold-standard insect repellent is N,N-diethyl-meta-toluamide (DEET), which is the main active ingredient in most repellent products that are currently on the market. It is a synthetic repellent and it is mainly available in concentrations of 10 to 35% (Gillij *et al.*, 2008; Katz *et al.*, 2008). DEET has excellent repellent properties and it can be found in a variety of formulations such as solutions, gels, creams, rub-on sticks, lotions and impregnated wipes (Golenda *et al.*, 1999; Govere *et al.*, 2000). However, its impacts on human health and on the environment have led to a gradual increase in research on repellents derived from natural sources for use as alternatives to synthetic repellents (Choochote *et al.*, 2007; Katz *et al.*, 2008; Nerio *et al.*, 2010).

Piper aduncum Linnaeus, also known as *P. angustifolium*, *P. celtidifolium*, *P. elongatum*, *P. multinervium* and *P. stevensonii* (Burger, 1971; Ciccio & Ballestero, 1997; Olander *et al.*, 1998), is a shrub with alternate leaves and spiky flowers. It can reach a height of up to 7 to 8 m. *Piper aduncum* has traditionally been used in medicinal and culinary applications, and its essential oil (EO) is a well-known insecticide and molluscicide, in addition to having antibacterial activity (Pohlit *et al.*, 2006). A previous study demonstrated that its extract is effective against adult *Aedes aegypti* Linn. (Hidayatulfathi *et al.*, 2004). According to Misni *et al.* (2008), *P. aduncum* EO has the ability to prevent mosquito bites and so it may be useful as a mosquito repellent.

Repellents act against insects in the vapor phase. Therefore, their effectiveness depends on their volatility and their persistence on the skin during activities such as washing. Plant EOs tends to offer short protection times as repellents due to the volatility of their phytochemical compounds. Therefore, regular reapplication of these repellents is often required (Logan *et al.*, 2010). Formulation optimization is important in order to prolong the repellent effect of an EO (Amer & Mehlhorn, 2006; Nerio *et al.*, 2010).

Piper aduncum EO has the potential to be used as an alternative to the synthetic repellents that are currently on the market. As the active ingredients in *P. aduncum* EO are volatile, preparation of the EO into semisolid formulations may prolong the repellency effect of the EO, thus providing improved protection against mosquito bites. In this study, we investigated the efficacy of *P. aduncum* EO in three different semisolid formulations against *A. aegypti* under laboratory conditions in order to determine the optimum vehicle for the EO. The effectiveness of the EO as a repellent was also compared with the gold-standard repellent, DEET.

Materials and methods

Chemicals

Prepared ointment formulations were purchased from Hovid Bhd (Malaysia). Cetostearyl alcohol, cetomacrogol 1000 and triethanolamine were obtained from R&M Chemicals (UK). Paraffin oil was obtained from Sigma-Aldrich (Germany) and Polysorbate 80 was obtained from Sigma-Aldrich (USA). 1,2-Propanediol (99%), carbopol 940 and DEET (98%) were supplied by Acros Organics (USA) and ethanol (95%) was obtained from Merck (Germany). Anhydrous sodium sulfate was obtained from Fisher Scientific (UK).

Piper aduncum *EO extraction*

Piper aduncum leaves were obtained from a forest area along a street named Batu 13 Gombak in Selangor, Malaysia, and the identification of the species was confirmed by the Malaysian Forest Research Institute (FRIM). The EO was extracted using a hydro-distillation method (FRIM, unpublished data) and it was dried over anhydrous sodium sulfate. The voucher number for this plant, obtained from the National University of Malaysia (Universiti Kebangsaan Malaysia, UKM), was UKM b 29778.

Gas chromatography analysis

The chemical constituents of the *P. aduncum* EO were analyzed using a gas chromatograph equipped with a mass spectrophotometer (GC/MS, Hewlett-Packard GC-MSD 5890 series 2). The column used was a BPX5 column (with a size of 30 m × 0.25 mm and a film thickness of 0.25 µm) and helium was used as the carrier gas with a flow rate of 0.5 cm s^{-1} injector temperature of 250°C. The chemical components were further analyzed using a second GC/MS (Agilent PEG 44DI number 7) equipped with a time-of-flight (ToF) machine (LECO). The column used was an Rtx-5MS column (with size of 30 m × 0.25 mm and a film thickness of 0.10 µm). The chemical constituents of the EO were identified by comparing the retention index of each of the constituents with data from the National Institute of Standards Technology library. The quantity of each of the constituents in the EO, which was presented as a percentage, was determined from the associated GC peak areas.

Formulation preparation

Three semisolid formulations of 10% *P. aduncum* EO were prepared: an ointment, a cream and a gel. The ointment formulation was made from cetomacrogol emulsifying wax

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(cetostearyl alcohol and cetomacrogol 1000) (30% w/w), liquid paraffin (20% w/w) and white soft paraffin (50% w/w). The cream formulation was made from emulsifying wax (30% w/w), paraffin oil (20% w/w), 1,2-propanediol (30% w/w) and distilled water (quantity sufficient (q.s.)). The gel formulation consisted of carbopol 940 (1.5% w/w), distilled water (q.s.), polysorbate 80 (1% w/w) and triethanolamine (q.s.). The ointment, cream and gel formulations were all compared with 10% EO in ethanol (which was used as a vehicle). Three positive controls consisting of 10% DEET were also prepared using the same semisolid formulation bases as for the EO. Each formulation was prepared without a repellent to be used as negative controls.

Experimental mosquitoes

Aedes aegypti (a strain classified as susceptible by the World Health Organization) were provided by the Institute for Medical Research in Malaysia. They were reared at the insectarium in the Department of Biomedical Science at UKM, under laboratory conditions (at a temperature of 25–30°C and a relative humidity of 60–70%). The larvae were reared in trays of dechlorinated water; the first and second instar larvae were fed liver powder and the third and fourth instar were fed half-baked cow liver. The adult mosquitoes were maintained in screened cages; they were fed a 10% sucrose solution and they were given access to a blood meal by placing a live guinea pig inside the cage for 2 h in order to obtain the eggs.

Repellent testing

Testing was conducted based on Standard and Industrial Research Institute of Malaysia (SIRIM) protocols (SIRIM, 2007). Four human subjects were used to test each EO formulation and each DEET positive control. The test was carried out in triplicate for each subject for all formulations tested. Each subject served as both the test subject and the subject for the negative control, with the EO formulation or DEET positive control tested on one hand and the negative control tested on the other. For example, for a trial of EO in cream, the subject had EO in cream applied to one hand and cream only to the other. The subjects were all female UKM students aged 20–25 years old who agreed to take part in the study. The study was approved by the UKM Human Ethics Committee (UKM 1.5.3.5/244/NN-143-2011).

Testing was carried out during the peak biting time for *A. aegypti* (8 am to 1 pm). An area of 3×8 cm^2 was drawn on each of the subject's hands, and 0.5 g of the repellent formulation (either the EO or DEET) was applied within this area on one hand. As a negative control, the formulation without repellent was applied to each subject's other hand in the delineated area. Each subject's hands were covered with rubber gloves such that only the 3×8 cm^2 areas were exposed to the mosquito bites. Each subject's hands were then inserted into a $60 \times 60 \times 60$ cm^3 test cage containing 25 nulliparous female *A. aegypti*, which were 5–7 days old and had fasted overnight. The mosquito landing/biting incidence within a 5-min exposure period was calculated and recorded. This procedure was repeated every 30 min until 4 h post-application (to give nine 5-min exposure periods). The protection time, repellency percentage and mean landing/biting percentage were calculated as follows:

Repellency percentage (%) = $((C–T)/C) \times 100$; where C, the number of mosquitoes that landed on the negative control hand; T, the number of mosquitoes that landed on the treated hand; landing/biting percentage = $(LB/N) \times 100$; LB, the number of mosquitoes that landed on or bit the treated hand; N, the total number of mosquitoes used in the test = 25; protection time, the time from the application of the repellent until the first two mosquito landing/biting incidences (within one of the 5-min exposure periods) or a landing/biting incident in one 5-min exposure period followed by a confirmatory landing/biting incident in the next exposure period.

Statistical analysis

Statistical analysis was conducted using SPSS software version 22. A split-plot analysis of variance (SPANOVA) with Bonferroni pairwise comparisons was carried out in order to analyze the statistical significance of the differences in both the repellency percentage and the landing/biting percentage between each of the three semisolid formulations of EO and EO in ethanol and between each of the three semisolid formulations of EO and their respective positive controls (DEET in ointment, cream and gel). A one-way analysis of variance (ANOVA) was used to compare the differences in the protection time between each of the three semisolid formulations of EO and EO in ethanol. Tukey's *post hoc* test was used in the one-way ANOVA analysis. Student's *t*-test was used to compare the differences in the protection time between each of the three semisolid formulations of EO and their respective positive controls. The level for statistical significance was set at $P < 0.05$ for all the statistical analyses. The results are displayed in terms of the means and standard errors of the mean (SEMs).

Results

Gas chromatography analysis

The chemical constituents of the *P. aduncum* EO are shown in table 1. The major constituent of the EO was apiol, which represented about 38.01% of the constituents detected in the EO. The other main constituents were methyl isobutyl ketone (8.26%), piperitone (3.34%) and caryophyllene (2.45%).

Repellency percentage of P. aduncum *EO formulations*

The repellency percentages of the various *P. aduncum* EO formulations are shown in table 2. The repellency percentage of EO in ethanol was statistically significantly different compared with EO in ointment, cream and gel ($F_{3,12} = 5.20$, $P = 0.016$). EO in ethanol provided >90% repellency at 30 min post-application. After that, its effectiveness started to decrease; it provided 80.31 ± 2.81% repellency at 2 h post-application, which was statistically significantly lower than for EO in ointment (95.68 ± 2.95%) and EO in cream (98.25 ± 1.75%), but not statistically significantly different when compared with EO in gel (90.86 ± 2.27%). The EO in cream formulation was able to repel 84.74 ± 6.81% of the mosquitoes at 3.5 h post-application but the difference was not statistically significant when compared with EO in ointment (72.34 ± 11.77%) and EO in gel (73.09 ± 12.91%). At 4 h post-application, EO in ointment (78.41 ± 7.96%), EO in cream (71.92 ± 5.79%) and EO in gel (67.71 ± 12.64%) still repelled >65% of the mosquitoes, while EO in ethanol was only able to provide 44.91 ± 5.54% repellency, though the differences were not statistically significant. However, at 4 h post-application, DEET still provided excellent repellency against

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Table 1. Chemical constituents of *Piper aduncum* essential oil (EO).

Chemical constituents	Percentage (%)
Apiole	38.01
Methyl isobutyl ketone	8.26
Piperitone	3.34
Caryophyllene	2.45
Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-	1.45
α-Caryophyllene	1.42
Terpinen-4-ol	1.40
γ-Terpinene	1.20
Caryophyllene epoxide	1.04
Globulol	0.90
1,5-Cyclooctadiene, 3,4-dimethyl-	0.80
α-Phellandrene	0.80
α-Pinene	0.74
Myristicine	0.66
α-Muurolene	0.58
cis-Calamenene	0.58
β-Cymene	0.45
Pentadecane	0.45
Copaene	0.42
α-Humulene epoxide II	0.39
γ-Muurolene	0.39
α-Terpinene	0.34
α-Cubebene	0.31
α-Farnesene	0.28
(+)-Cyclosativene	0.25
Germacrene D	0.16
Cyclohexene, 4-methyl-3-(1-methylethylidene)-	0.15
(-)-Spathulenol	0.14
1,2,3,4,4α,5,6,8α-octahydro-4α,8-dimethyl-2-(1-methylethenyl)-,[2R-(2α,4αα,8αα)]-naphthalene	0.14
α-Myrcene	0.12
c-Elemene	0.10
Elemicine	0.10
Furan, 2-ethyl	0.04
2-Decanone	0.02
Borneol	0.01

Table 2. Repellency percentage of *Piper aduncum* essential oil (EO) in ethanol, ointment, cream and gel formulations from 0 to 4 h post-application.

Time (min) after repellent application	Repellency percentage (% ± SEM)			
	EO in ethanol	EO in ointment	EO in cream	EO in gel
0	96.97 ± 2.20	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
30	93.98 ± 2.48	100.00 ± 0.00[1]	100.00 ± 0.00[1]	100.00 ± 0.00[1]
60	89.56 ± 4.09	100.00 ± 0.00[1]	100.00 ± 0.00[1]	97.09 ± 1.55
90	84.52 ± 2.66	98.43 ± 0.92[1]	99.67 ± 0.33[1]	96.49 ± 1.71[1]
120	80.31 ± 2.81	95.68 ± 2.95[1]	98.25 ± 1.75[1]	90.86 ± 2.27
150	70.08 ± 8.93	93.25 ± 2.57[1]	90.40 ± 1.91	84.60 ± 3.97
180	64.50 ± 11.49	80.11 ± 8.03	88.29 ± 2.74	82.55 ± 5.02
210	53.84 ± 2.76	72.34 ± 11.77	84.74 ± 6.81	73.09 ± 12.91
240	44.91 ± 5.54	78.41 ± 7.96	71.92 ± 5.79	67.71 ± 12.64

[1]Statistically significantly different from EO in ethanol (at the same time after application).

A. aegypti. At 4 h post-application, the DEET ointment, cream and gel formulations were, respectively, statistically significantly better than EO in ointment ($F_{1,6} = 8.75$, $P = 0.025$) (fig. 1a), EO in cream ($F_{1,6} = 25.71$, $P = 0.002$) (fig. 1b) and EO in gel ($F_{1,6} = 7.27$, $P = 0.036$) (fig. 1c).

Landing/biting percentage of P. aduncum *EO formulations*

The landing/biting percentage associated with EO in ethanol was statistically significantly different compared with that for the EO in semisolid formulations ($F_{3,12} = 6.02$, $P = 0.010$), as shown in table 3. At 60 min post-application, the landing/biting percentage for EO in ethanol (10.33 ± 4.09%) was statistically significantly higher than for EO in ointment (0.00 ± 0.00%) and EO in cream (0.00 ± 0.00%). Two hours post-application, the landing/biting percentages for EO in ointment (1.33 ± 0.94%), EO in cream (1.33 ± 1.33%) and EO in gel (8.33 ± 1.67%) were statistically significantly lower than for EO in ethanol (17.00 ± 2.06%). At 4 h post-application, the landing/biting percentage for EO in ethanol was the highest (41.67 ±

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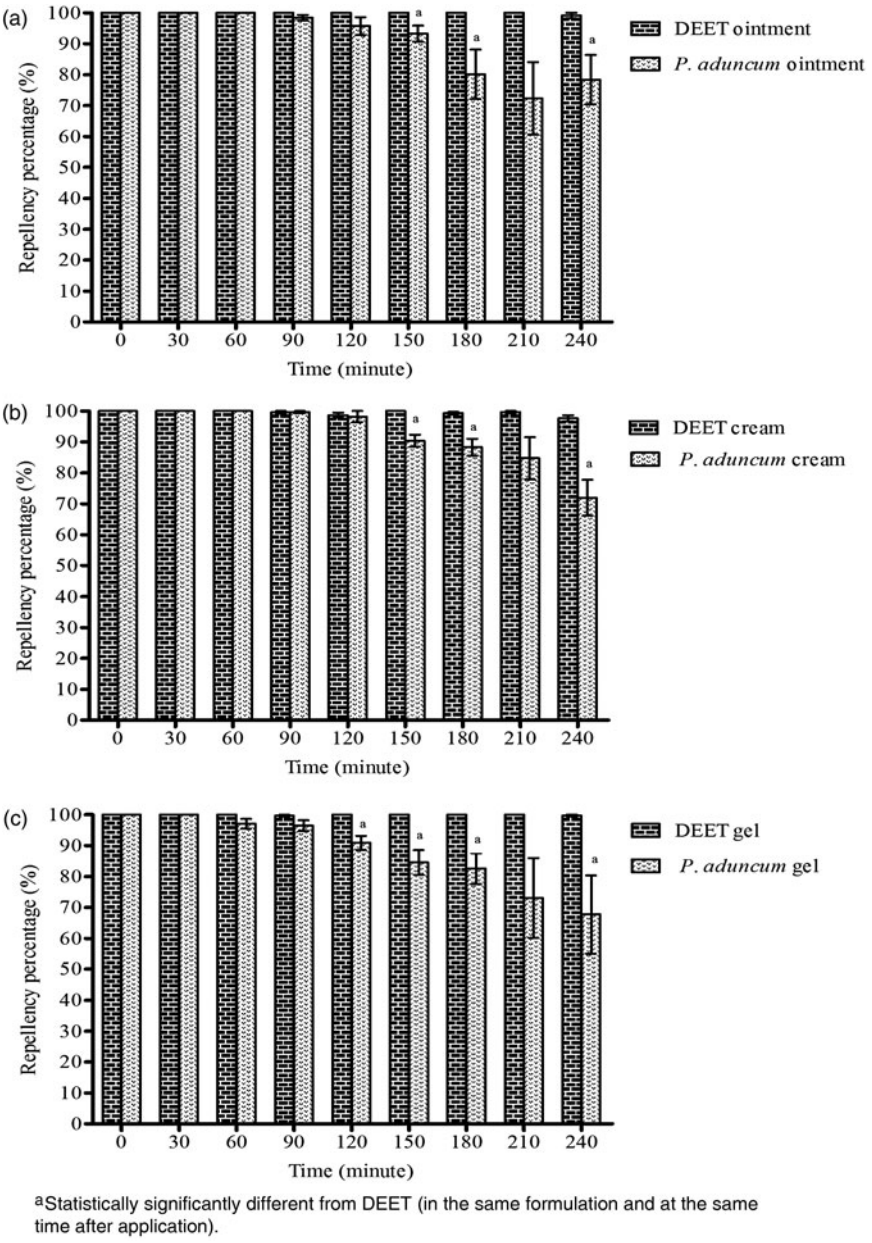

Fig. 1. (a) Repellency percentages of *P. aduncum* EO and DEET in ointment formulations; (b) Repellency percentages of *P. aduncum* EO and DEET in cream formulations; (c) Repellency percentages of *P. aduncum* EO and DEET in gel formulations

5.61%), but it was not statistically significantly different compared with those for EO in ointment (14.00 ± 5.47%), EO in cream (20.33 ± 5.74%) and EO in gel (24.67 ± 8.25%). As for the positive controls containing DEET, the landing/biting percentages were statistically significantly different compared with the respective EO formulations: EO in ointment ($F_{1,6} = 7.94$, $P = 0.030$) (fig. 2a), EO in cream ($F_{1,6} = 10.14$, $P = 0.019$) (fig. 2b) and EO in gel ($F_{1,6} = 9.32$, $P = 0.022$) (fig. 2c).

Protection times of P. aduncum *EO formulations*

The differences in protection times (as shown in table 4) between EO in ethanol and EO in ointment, cream and gel were statistically significant ($F_{3,12} = 11.21$, $P = 0.001$). The protection time for EO in ointment (182.5 ± 16.01 min) was statistically significantly greater than those for EO in ethanol (52.5 ± 27.5 min) and EO in gel (97.5 ± 14.93 min). EO in cream also provided a statistically significantly greater protection time (162.5 ± 6.29 min) compared with EO in ethanol but it was not statistically significant different compared with EO in ointment and EO in gel. The difference in protection time between EO in gel and EO in ethanol was not statistically significant.

The protection times (as shown in table 4) for EO in ointment, cream and gel were statistically significantly lower than DEET ointment (240 ± 0 min, $t(3) = 3.59$, $P = 0.037$),

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Table 3. Landing/biting percentage for *Piper aduncum* essential oil (EO) in ethanol, ointment, cream and gel formulations from 0 to 4 h post-application.

Time (min) after repellent application	Landing/biting percentage (% ± SEM)			
	EO in ethanol	EO in ointment	EO in cream	EO in gel
0	3.00 ± 2.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
30	5.67 ± 2.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
60	10.33 ± 4.09	0.00 ± 0.00[1]	0.00 ± 0.00[1]	2.67 ± 1.44
90	14.33 ± 3.19	0.67 ± 0.38[1]	0.33 ± 0.33[1]	3.33 ± 1.59[1]
120	17.00 ± 2.06	1.33 ± 0.94[1,2]	1.33 ± 1.33[1,2]	8.33 ± 1.67[1]
150	23.67 ± 7.15	3.33 ± 1.28[1]	7.00 ± 1.13	14.00 ± 3.38
180	28.00 ± 9.27	12.00 ± 5.16	10.33 ± 3.29	15.33 ± 4.27
210	33.33 ± 1.72	13.67 ± 4.43	14.00 ± 7.11	20.67 ± 8.94
240	41.67 ± 5.61	14.00 ± 5.47	20.33 ± 5.74	24.67 ± 8.25

[1]Statistically significantly different from EO in ethanol (at the same time after application).
[2]Statistically significantly different from EO in gel (at the same time after application).

DEET cream (240 ± 0 min, $t(6) = 12.32$, $P = 0.001$) and DEET gel (240 ± 0 min, $t(3) = 9.54$, $P = 0.002$), respectively.

Discussion

Insect repellents are used for protection from insect bites, including mosquito bites, and they are considered to be a major tool for reducing the transmission of vector-borne diseases and the irritation due to mosquito bites (Osimitz & Grothaus, 1995; Fradin & Day, 2002). A previous study by Misni *et al.* (2008) showed that *P. aduncum* EO has the ability to repel *A. aegypti*. However, the volatility of its active ingredients shortens its effectiveness after application. In order to address this issue, we formulated the EO using different bases and tested the relative efficacy of each formulation to determine which was the optimal vehicle for the prolonged efficacy of the EO as a topical repellent.

This study has shown that 10% *P. aduncum* EO in ethanol was less effective as a repellent than EO in semisolid formulations. Directly after application, EO in ethanol was able to repel >95% of the mosquitoes. However, as expected, 30 min after application the effectiveness of the EO in ethanol started to decrease due to the volatility of the active ingredients. Plant-based repellents often have low protection times compared to synthetic repellents. A recent study showed that seven out of 13 plant-based repellents exhibited a repellency percentage of <90% against mosquitoes at 30 min after application (Bissinger *et al.*, 2014). Sritabutra *et al.* (2011) found that the protection time of plant-based EO formulations against *A. aegypti* was only 60–98 min. Other studies conducted on the potential of plant-based EOs as repellents concluded that their effectiveness against mosquitoes could only last for 2 h or less (Aisien *et al.*, 2004; Phasomkusolsil & Soonwera, 2010) due to the rapid evaporation of their active ingredients.

However, when the EO was prepared using three semisolid formulations, its effectiveness as a repellent increased because the volatile compounds evaporated more slowly. As mentioned previously by Oyedele *et al.* (2000), EO is released more quickly from liquid formulations (e.g., ethanol) compared to semisolid formulations. Semisolid formulations appear to have a controlled-release effect on EOs. Various oil-based formulations, creams mixed with polymers and microencapsulation formulations can allow the controlled release of the volatile repellent compounds, thereby increasing the duration of their effectiveness (Gupta & Rutledge, 1991). Plant extracts and EOs usually react against *A. aegypti* in the vapor phase (Browne, 1997; Zhu *et al.*, 2001) and their effectiveness only lasts for relatively short duration (Rozendaal, 1997; Barnard 2000). However, their efficacy as repellents has been shown to increase when the EO is formulated with base or fixative substances such as vanillin, paraffin oil, mustard and coconut oil (Das & Ansari, 2003; Oyedele *et al.*, 2002). The formulation may be able to retain the EO active ingredients on the skin surface for an acceptable protection time (Mukesh *et al.*, 2014).

When the EO was in the ointment and cream formulations, it repelled 100% of the *A. aegypti* mosquitoes for 1 h, compared with only 30 min when it was in the gel formulation. All three semisolid formulations provided good efficacy against *A. aegypti*, repelling >80% of the mosquitoes for up to 210 min post-application (in the case of the cream formulation) and 180 min post-application (in the case of the gel and ointment formulations). These durations were not statistically significantly different from each other. Bissinger *et al.* (2014) studied a synthetic repellent p-menthane-3,8-diol (PMD) and they found that 10% PMD in lotion and spray formulations were able to repel >90% mosquitoes for 60 and 90 min, respectively. This indicates that the *P. aduncum* EO in semisolid formulations is as effective as the synthetic repellent PMD at repelling mosquitoes since the EO could repel >80% of the mosquitoes for more than 2 h. All the semisolid formulations were able to repel >65% of the mosquitoes 4 h post-application. In contrast, a recent study showed that a cream formulation containing *Lantana camara* Linnaeus methanol crude extracts and hexane and ethyl acetate fractions could only repel >60% of the mosquitoes up to 2.5 h post-application (Keziah *et al.*, 2015).

The landing/biting percentage for each of the EO formulations increased with post-application time, with EO in ethanol being associated with a higher landing/biting percentage at 4 h compared with EO in ointment, cream and gel, though the differences were not statistically significant. As expected, the DEET formulations had a very low landing/biting percentage at 4 h, confirming its well-known persistent repellent activity.

The ointment formulation of the EO offered 3 h of protection time, approximately the same as the cream formulation, while the gel yielded a lower protection time. A previous study by Chou *et al.* (1997) evaluated the efficacy of DEET in several different formulations and found that DEET in a rub-on stick formulation exhibited the highest repellency compared with DEET in cream and DEET in aerosol spray. The

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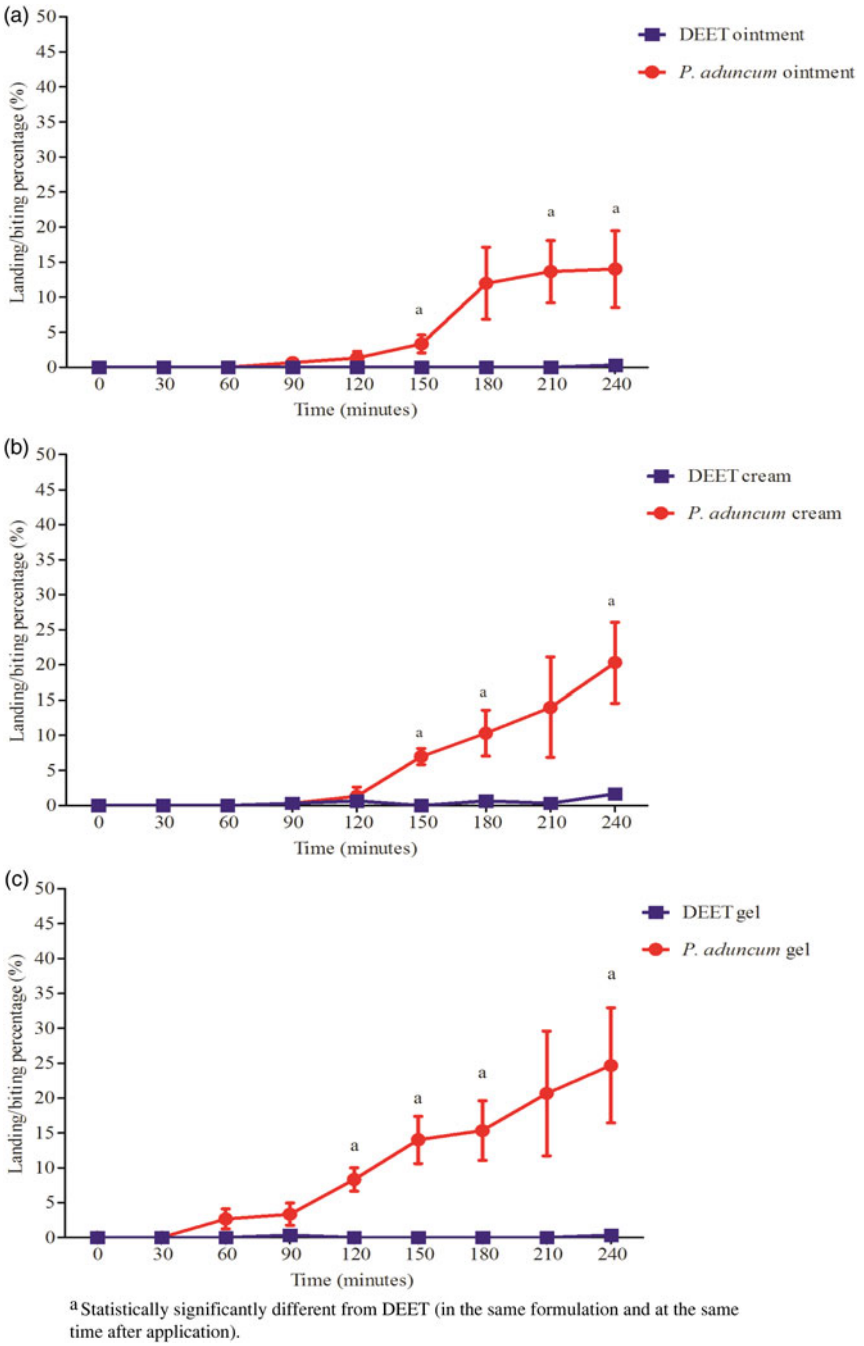

Fig. 2. (a) Landing/biting percentages of *P. aduncum* EO and DEET in ointment formulations; (b) Landing/biting percentages of *P. aduncum* EO and DEET in cream formulations; (c) Landing/biting percentages of *P. aduncum* EO and DEET in gel formulations

oleaginous base of the stick formulation may facilitate the retention of DEET in the vehicle. As the base is not absorbed by the skin, the DEET remained on the skin surface for a long period of time, thereby prolonging the protection time. In addition, 15% lemongrass oil, a natural repellent, in an ointment base was able to prolong the efficacy of the lemongrass oil more than other bases (Oyedele *et al.*, 2002). However, this ointment only deterred mosquitoes for 1 h, which is a much lower protection time than that associated with *P. aduncum* EO. The ointment formulation of *P. aduncum* EO might provide better protection against *A. aegypti* than some commercial plant-based repellents. A study on repellent effectiveness by Fradin & Day (2002) showed that two commercial repellents containing 10% citronella oil (Natrapel® which contains 10% citronella oil and Green Ban for People® which contains 10% citronella oil and 2% peppermint oil) provided protection times of <30 min. Moreover, Bissinger *et al.* (2014) found that six out of ten commercial repellents, which are exempt from

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Table 4. Protection time of *Piper aduncum* essential oil (EO) (in ethanol, ointment, cream and gel formulations) and DEET (in ointment, cream and gel formulations).

Formulation	Protection time (min ± SEM)
EO in ethanol	52.5 ± 27.50
EO in ointment	182.5 ± 16.01[1–3]
DEET in ointment	240.00 ± 00.00
EO in cream	162.5 ± 6.29[1–3]
DEET in cream	240.00 ± 00.00
EO in gel	97.5 ± 14.93[3]
DEET in gel	240.00 ± 00.00

[1]Statistically significantly different from EO in ethanol.
[2]Statistically significantly different from EO in gel.
[3]Statistically significantly different from DEET in the same formulations.

the requirement for registration in the USA under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), failed to provide >90% efficacy even at 30 min post-application.

The efficacy of the active ingredients in repellents is influenced by the formulation of the active ingredients, which regulates their evaporation rates and protection times and affects the persistence of the repellent's (Omotosho *et al.*, 1986; Florence & Attwood, 1990; Novak & Gerberg, 2005). The affinity of the active ingredients to the base and the viscosity of the formulation are other factors that can affect release of the active ingredients from the formulation (Florence & Attwood, 1990; Oyedele *et al.*, 2000, 2002).

No adverse skin reactions occurred after application of the *P. aduncum* EO formulations to the skin of the all subjects who participated in the study. As the sample was limited, further testing is required to fully ascertain the dermatological safety for the EO formulations, but we feel that it is a positive sign that none of our subjects suffered any skin reactions.

Conclusion

From the present study, we can conclude that the ointment and cream formulations were able to retain the *P. aduncum* EO on the skin for longer periods of time compared to the gel formulation. Repellent formulations containing DEET still provide superior repellency against *A. aegypti* compared with *P. aduncum* EO. Nevertheless, *P. aduncum* EO ointment and cream formulations were able to provide adequate protection against mosquito bites (over 2 h of protection time). According to the Thai Industrial Standards Institute (TISI), repellent formulations must provide a minimum of 2 h of protection against mosquitoes in order to be sold in Thailand (TISI, unpublished data). A minimum 2 h of protection also is required by the Food and Drug Administration for labeling in the USA (Thavara *et al.*, 2002). The cream and ointment formulations displayed better repellent properties than the other formulations so they are the most promising vehicles for *P. aduncum* EO for further development and commercialization as alternatives to synthetic repellents.

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Disclosure

No potential conflicts of interest exist.

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