LABORATORY AND FIELD TRIAL OF DEVELOPING MEDICINAL LOCAL THAI PLANT PRODUCTS AGAINST FOUR SPECIES OF MOSQUITO VECTORS

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Abstract. Oils of Syzygium aromaticum (clove) and Zanthoxylum limonella (makaen), widely used essential oils for dental caries or flavoring of food in Thailand, were prepared as 10 experimental repellent products in gel or cream form against Aedes aegypti, Culex quinquefasciatus, and Anopheles dirus under laboratory conditions, using the human-arm-in-cage method. Two products that gave the longest-lasting complete protection were selected to examine their repellency against a variety of mosquito species under field conditions. In laboratory tests, 0.1 g of each product was applied to 3x10 cm of exposed area on a volunteer’s forearm, while in field trials, 1.0 g was applied to each volunteer’s leg (from knee to ankle). In the laboratory, the gel dosage form contained 20% clove oil (Gel B) or 10% clove plus 10% makaen oil mixture (Gel E) were promising plant-based repellents against three mosquito species and gave significantly longer complete protection times of 4-5 hours than all other developing products. Therefore, their efficacy in the field was evaluated. Under field conditions, Gel E showed complete protection for 4 hours and gave 95.7% repellency after 5 hours application, whereas Gel B and 20% deet (di-methyl benzamide) provided only 86.8 and 82.7% repellency after treatment, respectively against Ae. aegypti, daytime-biting mosquitoes. For nighttime-biting, the 3 repellents under development yielded equally excellent (average 97.1%) repellency for 5 hours against the predominant Cx. quinquefasciatus and Mansonia uniformis, but they gave 89.0% repellency against Cx. tritaeniorhynchus and Cx. gelidus. This finding demonstrated the effectiveness of Gel B and Gel E products for possible use by low-income rural communities against various mosquito species.

INTRODUCTION

The chemical control of pathogen-transmitting mosquito vectors is likely to be very significant in reducing the incidence of diseases such as malaria, dengue fever (DF), dengue hemorrhagic fever (DHF), filariasis, Japanese encephalitis (JE) and yellow fever, which are still major public health problems for people in the developing world. The main burdens are adverse effects, as insecticide resistance and environmental pollution occur after long-term application (Curtis et al, 1997). Up to the present time, the search for phytochemical strategies from plants, to deplete or incapacitate vector populations, remaining popular research. Personal protection using plant-based repellent is an apparently practical and economical way of preventing the transmission of these diseases to humans. Although deet (di-methyl benzamide)-based repellent is well-known for its excellent repellency against mosquitoes and other biting insects, there has been concern about rare reports of severe reactions to this substance. Moreover, deet does attach to hard plastics and many consumers do not like its odor. For these drawbacks, plant-based products are more favored and citronella from Cymbopogon nardus yields the most popular insect repellents, and has spread world-wide, with various commercial formulations in many concentrations (Curtis et al, 1989; Thorsell et al, 1998; Govere et al, 2000; Fradin and Day, 2002; Moore et al, 2002). Another plant-based product with a lemon-
like odor is derived from Lemon Eucalyptus (*Eucalyptus maculata citriodon*). It was first shown to be an effective repellent in China; *p*-menthane diol (PMD) was the active ingredient (Li *et al*, 1974; Curtis *et al*, 1989). PMD has shown particular promise as a repellent of botanical origin because it gave good mosquito repellency, as long-lasting as deet, and more long lasting than citronella when tests were carried out under laboratory and field conditions. Moreover, its mammalian toxicity is lower than that of deet. In addition, this repellent has been found effective against midges, ticks and the stable fly (Curtis *et al*, 1989; Trigg and Hill, 1996; Govere *et al*, 2000; Moore *et al*, 2002; Trongtokit *et al*, 2004a). The longest-lasting protection of PMD, compared with other plant-based repellents, and its pleasant lemony smell are no doubt important factors in the commercial success of these products. This promising plant-based repellent is an example of finding and developing new phytochemical agents that could be used for controlling mosquito-borne diseases in endemic areas.

Our preliminary study (Trongtokit *et al*, 2004b) clearly demonstrated that essential oils from *Syzygium aromaticum* (clove) and *Zanthoxylum limonella* (makaen) performed as mosquito repellents about equally well as citronella oil. *S. aromaticum* and *Z. limonella* are available in Thailand, as they are local medicinal plants used in folk medicine. Interestingly, a yield of 12.5% w/w of makaen oil is higher than the other plants studied, so it seems to be more cost-effective than the others.

Therefore, the present paper describes the development of appropriate formulations from these oils, including a fixative that would increase efficacy with improved cost-effectiveness. A laboratory study and field trial were carried out to evaluate the efficacy of the developing products.

**MATERIALS AND METHODS**

**Preparation of plant-based repellent formulations**

Clove oil was purchased from Thai-Chai Flavours and Fragrances Industry Co, Ltd (Bangkok, Thailand), but makaen oil was extracted from *Z. limonella* obtained from the north of Thailand, using steam distillation (Trongtokit *et al*, 2004b). Each of 2 formulations was varied to 5 formulas according to active ingredient and concentration. Preparation was carried out at the Department of Pharmacy, Faculty of Pharmacy, Mahidol University. The developing formulas are listed in Table 1.

**Laboratory test mosquitoes**

The tested mosquito species were *Aedes aegypti*, *Anopheles dirus* and *Culex quinquefasciatus*. These mosquitoes were uninfected laboratory strains and were reared for over 10 generations in the insectary of the Insecticide Research Unit at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Thailand. The methods for mass rearing were slightly modified from the procedure mentioned in the manual ‘Rearing techniques for mosquitoes’ (Limsuwan *et al*, 1987). Non-blood-fed 4-5 day-old hungry female mosquitoes were used in laboratory tests.

**Laboratory test procedure**

The study used six human subjects who agreed to take part in testing the repellency of each kind of developing product in the laboratory. This study was approved by ethical committee in 12 January 2004 before processing all experiments.

The repellency of the formulations was evaluated using an arm-in-cage test (Schreck and McGovern, 1989; WHO, 1996). A subject’s arm, wearing a glove, was covered with a rubber sleeve with 3x10 cm window. 0.1 g of each formulation was applied and allowed to dry for 1 minute. The other arm without treatment was used as the control and it was exposed to mosquitos in the cage before each insertion of the treated arm. The mosquitos did not have time to gorge with blood during this control exposure and they remained hungry for exposure of the treated arm. If at least 2 mosquitos landed on the control arm, the repellency test was carried out. The treated arm was exposed for 1 minute to 250 hungry female mosquitos. Every 30 minutes after treatment the treated arm was re-exposed to mosquitos and the time at which the first bite occurred was recorded. Arm exposure at 30-minute intervals continued until two bites occurred and one further exposure was made to check that complete repellency had
indeed failed. Duration (minute) of complete repellency after application of repellent was recorded as the protection time, and was used as a measure of the repellent efficacy. The tests against *Ae. aegypti* were conducted between 0900 hours and 1500 hours, whereas those against *An. dirus* and *Cx. quinquefasciatus* were carried out from 1900 hours to 2400 hours. Of 10 developing products, 2, which gave longest-lasting repellency, were selected for testing under field conditions.

**Field evaluation procedure**

The field evaluations were conducted in various areas near Bangkok, Thailand, during both day and night, to include a wide range of mosquito vectors. Ratchathewi district (Bangkok), Bang Bo (Samut Prakan Province) and Sai Noi (Nonthaburi Province) were selected for testing against nighttime-biting mosquitos. Toong Kru (Bangkok) was chosen for *Ae. aegypti*, the daytime-biting dominant vector species.

The human-bait landing catches were based on a 4x4x4 square design. Four experienced mosquito collectors (2 female, 2 male) sat in 4 different positions and tested 4 treatments. The treatments consisted of 2 developing products, 20% deet in 70% ethanol, and a negative control. The first developing product was a mixture of 10% clove plus 10% makaen oil in a gel form. The second was 20% clove oil in a gel form. Nothing was applied to the legs of the negative control. One gram of the product was applied evenly from knee to ankle of each leg. Shorts and shoes were worn to standardize the exposure area. Other exposed untreated parts of the body were protected against mosquitos attack by a jacket with hood and gloves, covering the thighs with a plastic sheet. Care was taken to minimize contact of the treated legs with clothing or other matter between the hourly tests. At the field site, the testers sat on chairs, at least 10 meters apart and collected all of the mosquitos landing on their legs in the specific area for a 40-minute period. Each exposure period was followed by a 20-minute break before the next mosquito collection was carried out. The tests were run in protected locations with minimal wind disturbance, where mosquito landing or biting activity was high. The test against daytime-biting mosquitos, began at 0830 hours and observation continued to 1130 hours, whereas tests against nighttime-biting mosquitos started from 1630 hours, and the observation time continued for 3 hours (1830-2130 hours). Landing mosquitos were aspirated into paper cups with a piece of cotton wool soaked in 10% glucose solution placed over the net covering. Cups were replaced each hour to record hourly biting rates. The captured mosquitos were brought to the laboratory and identified to species under a stereo microscope. Each individual received a different treatment each night, and sat in a different position every 4th night. A randomized block design was used. The subjects washed their legs with soap after testing, and again the following morning. Washing and use of soap or deodorant after midday were prohibited. Skin irritation was observed in the testing period.

**Data analysis**

The mean protection time was used as a standard measure of the repellency of 10 developing products in gel and cream bases against three mosquito species in the laboratory. Percent repellency in the field trial was calculated (Sharma and Ansari, 1994; Yap et al., 1998). The results were analyzed according to the following formula.

\[
\text{Repellency} = \left(\frac{C-T}{C}\right) \times 100
\]

Where C is the number of mosquitos collected from the control areas and T is the number collected from the treated areas of the subjects.

Data from the field study were normalized using natural log+1, then analyzed with a general linear model (GLM) in the Minitab Statistical Software package (Minitab Inc, State College, PA). The effects of treatment, individual, and position were measured. Possible additive effects from interactions between individual and treatment, position and individual, and treatment and position were also analyzed.

**Ethics approval**

The Faculty of Tropical Medicine, Mahidol University, granted full ethics approval.

**RESULTS**

**Repellency of 10 experimental products against 3 laboratory mosquito spp in the laboratory**

Under laboratory conditions, the duration of
complete repellency of the 10 dosage forms studied, gel and cream in 10% and 20% oil concentrations, was analyzed using the Minitab Statistical program. All observed protection times were transformed \([\log (x+1)]\) and variance analysis by GLM was conducted. The effect of mosquito species \((F=16.47; \text{df}=2, 179; p<0.0001)\), formulation \((F=54.73; \text{df}=1, 179; p<0.0001)\) and active ingredient \((F=24.24; \text{df}=4, 179; p<0.0001)\) on the duration of protection was significant, but the effect of person was vice versa \((F=1.32; \text{df}=5, 179; p=0.258)\). The duration (minute) of complete protection time for each product in the gel form is shown in Fig 1. There was no repellency against the three mosquito species from the gel base (without any active ingredient). Concentration of each active ingredient in the prepared formulation obviously affected the effective duration of action (Figs 1 and 2). Of 5 gel products, Gel B, containing clove oil 20%, and Gel E, containing a mixture of 10% clove oil and 10% makaen oil demonstrated equal repellency \((p>0.05)\), of 4.4 hours and 5.0 hours against \(Ae. aegypti\), 5.10 hours and 5.0 hours against \(Cx. quinquefasciatus\), and 4.5 hours and 4.8 hours against \(An. dirus\), respectively. Moreover, both gel products gave complete repellency significantly longer than other gel products against all three mosquito species \((p<0.05)\).

For the repellent efficacy of products in the cream form (Fig 2), cream b, containing 20% clove oil as the active ingredient, gave the longest protection (4.8 hours) against both \(Cx. quinquefasciatus\) and \(An. dirus\). In addition, cream e, containing the oil mixture of 10% clove and 10% makaen gave a non-significant difference in the complete repellency provided by cream b against \(Cx. quinquefasciatus\) and \(An. dirus\). In contrast, both cream b and cream e showed less protection time than Gel B or Gel E against \(Ae. aegypti\). The gel formulation was 2 times better than the cream formulation for \(Ae. aegypti\), \(Cx. quinquefasciatus\), and \(An. dirus\) \((p<0.05)\).

These initial results clearly demonstrated that Gel B and Gel E performed longest as mosquito repellents, giving at least 4 hours’ repellency against three mosquito species. Therefore, these promising products were evaluated for efficacy under field conditions.

### Table 1

<table>
<thead>
<tr>
<th>Active ingredient, concentration</th>
<th>Formula name</th>
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<tbody>
<tr>
<td>Clove, 10%</td>
<td>Gel A</td>
</tr>
<tr>
<td>Clove, 20%</td>
<td>Gel B</td>
</tr>
<tr>
<td>Makaen, 10%</td>
<td>Gel C</td>
</tr>
<tr>
<td>Makaen, 20%</td>
<td>Gel D</td>
</tr>
<tr>
<td>Clove, 10% plus makaen 10%</td>
<td>Gel E</td>
</tr>
<tr>
<td>No active ingredient</td>
<td>Gel base</td>
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<tr>
<td></td>
<td>Cream a</td>
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<td></td>
<td>Cream b</td>
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Repellency of Gel B and Gel E against mosquito bites under field conditions

In the environment of our experiments, there was no effect from the human factors, including the mosquito capturing ability of each subject and their attractiveness to mosquitoes \((F=0.28; \text{df}=3, 63; p=0.838)\). The sitting position of the subject in each area did not have any significant effect on the results \((F=0.56; \text{df}=3, 63; p=0.647)\). However, there was a significant difference in biting numbers obtained from Ratchathewi, Toong Kru, Bang Bo and Sai Noi \((F=34.91; \text{df}=3, 63; p=0.005)\). The differences in biting numbers after the application of the 4 treatments (Gel B, Gel E, 20% deet, and negative control) were also significant \((F=129.82; \text{df}=3, 63; p<0.0001)\).

Percent repellency of Gel B, Gel E, and 20% deet against daytime-biting mosquitoes in the field at Toong Kru, Bangkok, on September 11th-14th, 2002, is shown in Fig 3. There were no bites by the mosquitoes for at least 3 hours after the application of all products. Among Gel B, Gel E, and deet, Gel E showed greater protection against mosquitoes than the other two products, as it gave the longest-lasting complete repellency (4 hours after application), whereas Gel B or deet gave shorter complete repellency (3 hours). Moreover, 5 hours after application, Gel E still gave better...
repellency (about 95.7%), while Gel B and deet provided only 86.8 and 82.7% repellent activity, respectively. Mosquito collection on the untreated volunteers is presented in Table 2; *Ae. aegypti* was the predominant mosquito species in this area.

Percent repellencies of Gel B, Gel E and 20% deet against night-biting mosquitos at Ratchathewi, Sai Noi, and Bang Bo are shown in Fig 4-6. At Ratchathewi, Bangkok, *Cx. quinquefasciatus* was the predominant species. The 3 repellents yielded equally excellent repellency (5 hours) with almost complete protection (average 97.1%) from mosquito landing and biting. This indicated that Gel B and Gel E provided good mosquito repellency, equivalent to deet, which is the standard repellent. Similarly, at Bang Bo Gel B, Gel E and 20% deet gave non-significant differences in percent repellency (average 97.0%) after 5 hours application; this area had 4 times the mosquito density of Ratchathewi. At Sai Noi, Gel B, Gel E, and deet
presented equal protection against the predominant night-biting mosquitos, *Cx. tritaeniorhynchus* and *Cx. gelidus*. In contrast with the 2 previous sites, all 3 products gave 100% repellency only 2 hours after application, which decreased to an average 89.0% repellency after 5 hours. Regarding with the suspicious effect of repellent products used in this study, it is worth to mention that both laboratory and field tested of all forms of repellent did not show any harmful effect on human skin such as rash, skin irritation, or hot sensation after application.

**DISCUSSION**

The results of the current study, and those of other researchers, showed that the repellency of clove oil against various mosquito vectors is remarkable (USDA, 1954; Barnard, 1999; Trongtokit *et al*, 2004b). In addition to the high cost of clove oil, the potential for using clove or other effective oils as topical mosquito repellents may be limited by user acceptability because they can cause irritant dermatitis and their odor may be unacceptable (Barnard, 1999). The oil combination study was conducted as an alternative to conventional application.

Few other data regarding the comparative repellency of oils and oil mixtures are available to compare with the results of our study. Barnard (1999) reported that, in preventing *Ae. aegypti* or *An. albimanus*, none of the oil combinations repelled longer than their pure constituent oils. For example, pure clove oil gave 3.75-hour mean protection time against *Ae. aegypti*, equal to the mean protection time of 75% clove plus 25% thyme oil (3.75 hours). On the other hand, pure clove oil provided 3.55 hours of mean protection time against *An. albimanus*, which was longer than the 75% clove plus 25% thyme oil mixture (2.25 hours). With regard to our results, the products containing 10% clove plus 10% makaen oil, and 20% clove oil, demonstrated equal repellency against *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. dirus* under laboratory conditions. These findings showed that, although there was no synergistic mechanism of clove plus other oils (makaen, geranium or thyme oil) mixtures, these was a benefit in reducing the cost and improving safety for consumers.

It is important to note the different amounts of active ingredients formulated in gel and cream dosage forms. The laboratory results showed that, of the 10 dosage forms studied, gel products containing 20% clove oil or 10% clove plus 10% makaen oil were promising plant-based mosquito repellents against *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. dirus*, and gave longest-lasting for 4.0-5.0 hours complete protection in the laboratory. The experimental results of this study indicated that the gel dosage form tended to exhibit significantly longer protection time than the cream dosage form. This could be due to the composition of the gel form, consisting of all oil-phase ingredients, which are high molecular weight materials that remain on the skin surface. It demonstrated good durability, with a persistent, soft and emollient feeling. All specific good properties on also sited by Arch Personal Care Products L.P. Cosmetic Ingredients and Ideas Books. On the other hand, the cream form exhibited shorter protection time compared with the gel dosage form, due to rapid evaporation of the water solvent. A controlled-release formulation of mosquito repellent containing deet as the active ingredient has been reported to extend protection against biting mosquitos in the laboratory, prolonging protection time, containing less active ingredient, improving user comfort, reducing odor and plasticizer effects (Gupta and Rutledge, 1991).

With the genus *Cymbopogon*, which yields the most popular repellents in the world, *C. excavatus* gave 100% repellency for 2 hours, when it was evaluated in the laboratory against *An. arabiensis*; its repellency decreased to 59.3% after 4 hours (Govere *et al*, 2000). In Thailand, 25% *C. winterianus* oil in ethanol mixed with 5% vanillin gave 100% protection for 6 hours against *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. dirus*, and compared favorably with 25% deet (Tawatsin *et al*, 2001). Besides, 14% citronella cream gave about 2-hour complete repellency against *Ae. aegypti* (Wasuwat *et al*, 1990). Moreover, a repellent cream containing less than 10% citronella cream provided only 2-hour repellency against *An. minimus*, while a 10% formulation could repel this mosquito species for at least 4 hours under laboratory conditions (Suwonkerd and Tantrarongroj, 1994). Compared with the same
conditions, 20% clove oil or the oil mixture of the gel formulation gave no different repellency from citronella-based repellents. However, the difference in methodology, formulation type and environment of the experiment should be noted.

Other plant-derived substances that have some degree of mosquito repellency include citronella, cedar, verbena, pennyroyal, geranium, lavender, pine, cajuput, cinnamon, rosemary, basil, thyme, allspice, garlic, and peppermint have been reported (Jarratt, 2004; Trongtokit et al, 2004b). The repellency provided by these products is very limited and some studies showed no protection was gained using these plant products. One scientific study using Buzz Away® (containing citronella, cedarwood, eucalyptus, lemongrass, alcohol, and water) and Green Ban® (containing citronella, cajuput, lavender, safrole-free sassafras, peppermint, bergaptene-free bergamot, calendula, soya and tea tree oils) showed essentially no repellency against mosquitoes. However, other studies with Buzz Away® indicated that the product did have repellency for about 2 hours. One plant-based repellent that was released in the US in 1997, Bite Blocker®, (containing soybean oil, geranium oil, and coconut oil) has shown good repellency against Aedes mosquitos for up to 3.5 hours (Jarratt, 2004).

In the field, our results may be compared with the data obtained from a plant-based product contained p-menthane diol (PMD), extracted from Lemon Eucalyptus (Eucalyptus maculata citriodon) as the active ingredient. It has shown particular promise as a repellent of botanical origin in the field, at doses of 0.8-2.0 g/leg, 50% PMD rendered complete protection from biting for 6-7.75 hours (Trigg, 1996) while 20% clove or 10% clove plus 10% makaen mixture in gel form gave shorter complete protection (3-4 hours) against Ae. aegypti, Cx. quinquefasciatus, Cx. tritaeniorhynchus, Cx. gelidus, Ma. uniformis, and other nuisance mosquitos (Table 2). Our results show that our experimental products were not as effective as PMD in repelling mosquitos, but in practice, consumers should note that re-application could provide full protection against evening-biting mosquitos before retiring to a bednet.

The results of this study were clearly better than the efficacy test on 14% citronella cream against Culex mosquitos under field conditions for only 1 hour, and showed that the cream could prevent at least 90% of mosquito attacks in 13/20 volunteers who applied enough cream (1.2 g or more per whole forearm) (Jaruwichitratana et al, 1988). In contrast to our results, the pure citronella oil extracted from C. martini martini (palmarosa) provided 100% repellency for 12 hours against Anopheles mosquitos in the field trial, which was carried out using a pair of volunteers who sat together, one of whom was treated with the oil which the other was not (Ansari and Razdan, 1994). However, the tests utilized pairs of volunteers: one acting as bait and the other as collector (who wore no repellent), therefore, mosquitos would be diverted to the collector giving an inflated measure of repellency.

In summary, gel dosage forms containing 20% clove oil or 10% clove plus 10% makaen oil as active ingredients demonstrated good repellency against day- and night-biting mosquitos under laboratory and field conditions. Therefore, this study clearly indicates the potential of these formulations as effective topical repellents against a wide range of mosquito species. For possible use by low-income rural communities, where the highest incidence of mosquito-borne diseases is reported, our studies have added the cheap and available gel dosage forms containing 20% clove oil or 10% clove, plus 10% makaen oil as the active ingredients to the list of effective plant based repellents.

However, further investigation of traditional plant-based repellents is needed. It is hoped to produce affordable mosquito repellents for use in low-income communities where native plants can be grown and processed with low technology. The use of repellents in combination with insecticide-treated mosquito nets (ITNs) can be expected to be highly complementary, with the repellent affording protection during early-evening feeding and ITNs during late-night feeding. Indeed, it may be that, where the vectors feed in the early evening this is the only means of securing a high level of mosquito-borne disease reduction.

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