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OUTPUT OF THE PROJECT

Output จากโครงการที่ได้รับทุนจาก สกว. มีดังนี้

1. - ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน 1 เรื่อง คือ

Yuwadee Trongtokit, Yupha Rongsriyam, Narumon Komalamisra, Panvipa Krisadaphong, Chamnarn Apiwathnasorn. Laboratory and field trial of developing medicinal local Thai plant products against four species of mosquito vectors. Southeast Asian Journal Tropical Medicine Public Health. 2004, Vol 35, No.2, page 325-333.

- ผลงานกำลังจะถูกตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน 2 เรื่อง คือ

1) Yuwadee Trongtokit, Yupha Rongsriyam, Narumon Komalamisra, Chamnarn Apiwathnasorn. Comparative repellency of thirty-eight essential oils against mosquito bites. ได้รับเอกสารการตอบรับการลงตีพิมพ์ในวารวิชาการนานาชาติเรื่อง Phytotherapy Research (เอกสารตอบรับแสดงใน appendix)

2) Yuwadee Trongtokit, Christopher F. Curtis, Yupha Rongsriyam. Efficacy of repellent products against caged and free flying *Anopheles stephensi* mosquitoes. ได้รับเอกสารการตอบรับการลงตีพิมพ์ในวารวิชาการนานาชาติเรื่อง Journal of the American Mosquito Control Association (เอกสารตอบรับแสดงใน appendix)

2. การนำผลงานวิจัยไปใช้ประโยชน์

จากผลงานวิจัยที่ได้รับการสนับสนุนจากทุนวิจัยองค์ความรู้ใหม่ในการสร้างนักวิจัยระดับปริญญาเอก นับเป็นการสร้างนักวิจัยใหม่จำนวน 1 คนคือ นางสาวยุวดี ตรงต่อกิจ ซึ่ง ได้สำเร็จการศึกษาระดับปริญญาเอกเป็นที่เรียบร้อยแล้ว และจะเริ่มทำงานในวันที่ 1 ตุลาคม 2547 ในตำแหน่งอาจารย์ ที่ภาควิชากีฏวิทยาการแพทย์ คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล กรุงเทพฯ

3. กำลังดำเนินการขอจดสิทธิบัตรการประดิษฐ์ผลิตภัณฑ์สมุนไพรเพื่อใช้ควบคุมยุง จำนวน 2 เรื่อง ดังนี้

1) การขอจดสิทธิบัตรการประดิษฐ์ สารประกอบน้ำมันหอมระเหยมะแข่น (*Zanthoxylum limonella*) และน้ำมันหอมระเหยกานพลู (*Syzygium aromaticum*) มีฤทธิ์ป้องกันยุงกัด

2) การขอจดสิทธิบัตรการประดิษฐ์ สารประกอบที่มีสารสกัดจากต้นทองพันชั่ง (*Rhinacantus nasutus*) มีฤทธิ์ฆ่าลูกน้ำยุง

APPENDIX

Reprint จำนวน 1 เรื่อง และ manuscript จำนวน 2 เรื่อง พร้อมเอกสารตอบรับการตีพิมพ์ดังรายการต่อไปนี้

1. Laboratory and field trial of developing medicinal local Thai plant products against four species of mosquito vectors (reprint)

2. Comparative repellency of thirty-eight essential oils against mosquito bites (เอกสารตอบรับการตีพิมพ์ และ manuscript)

3. Efficacy of repellent products against caged and free flying *Anopheles stephensi* mosquitoes (เอกสารตอบรับการตีพิมพ์ และ manuscript)

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 mosquitos. 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 mosquitos 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-

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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, 1996; 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 mosquitos

The tested mosquito species were *Aedes aegypti*, *Anopheles dirus* and *Culex quinquefasciatus*. These mosquitos 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 mosquitos' (Limsuwan *et al*, 1987). Non-blood-fed 4-5 day-old hungry female mosquitos 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.

$$\% \text{ Repellency} = \frac{(C-T)}{C} \times 100$$

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; df=2, 179; p<0.0001), formulation (F=54.73; df=1, 179; p<0.0001) and active ingredient (F=24.24; df=4, 179; p<0.0001) on the duration of protection was significant, but the effect of person was vice versa (F=1.32; 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.

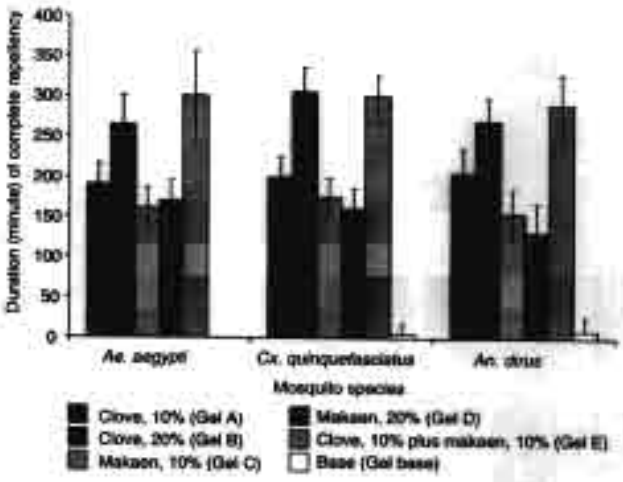

Fig 1–Comparison of the duration (minute) of complete repellency of each product in gel form with each mosquito species.

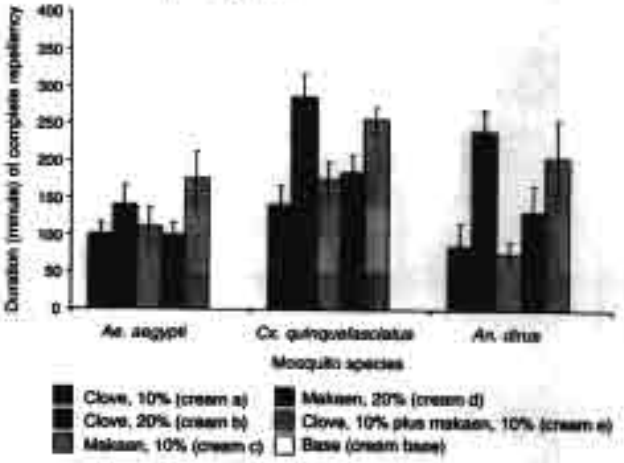

Fig 2–Comparison of the duration (minute) of complete repellency of each product in cream form with each mosquito species.

Table 1
List of gel and cream formulations with different active ingredients and concentrations.

Active ingredient, concentration	Formula name	
	Gel	Cream
Clove, 10%	A	a
Clove, 20%	B	b
Makaen, 10%	C	c
Makaen, 20%	D	d
Clove, 10% plus makaen 10%	E	e
No active ingredient	base	base

Table 2
Mosquitoes captured, biting rate and mosquito species collected hourly on untreated volunteers at various study sites in Thailand, 9-27 September 2002.

Study site	Test date, application time, observation time	Total mosquitos	X± SD of landing rate (landing-man-hour)[a]	Mosquito spp
Ratchathewi, Bangkok	9-12 September 2002, 1730 hours, 1930-2230 hours	819	77 ± 55.6	*Cx. quinquefasciatus*, female (99.63); male (0.12) *Cx. gelidus*, female (0.12) *Ae. aegypti*, female (0.12)
Toong Kru, Bangkok	11-14 September 2002, 630 hours, 0830-1130 hours	216	20 ± 5.8	*Ae. aegypti*, female (60); male (40)
Bang Bo, Samut Prakan	17-20 September 2002, 1630 hours, 1830-2130 hours	3,565	334 ± 101.5	*Ma. uniformis*, female (86.10); male (4.56) *Ma. indiana*, female (2.70) *Ma. annulifera*, female (0.35) *Cx. gelidus*, female (4.63) *Cx. tritaeniorhynchus*, female (1.47) *Cx. sitiens*, female (0.23) *An. vagus*, female (0.27)
Sai Noi, Nonthaburi	23-26 September 2002, 1730 hours 1830-2130 hours	1,735	163 ± 80.0	*Cx. tritaeniorhynchus*, female (23.48) *Cx. gelidus*, female (27.40) *Cx. sitiens*, female (42.49) *Cx. quinquefasciatus*, female (0.79) *Ma. uniformis*, female (1.59) *Ma. indiana*, female (3.27) *Ma. annulifera*, female (0.24) *An. barbirostris*, female (0.32) *An. peditaeniatus*, female (0.24) *An. vagus*, female (0.08) *An. subalbanus*, female (0.08)

[a]n=16, n is the number of catching times/person/hour/night.

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 mosquitos (F=0.28; 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; 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; 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; df=3, 63; p<0.0001).

Percent repellency of Gel B, Gel E, and 20% deet against daytime-biting mosquitos in the field at Toong Kru, Bangkok, on September 11th-14th, 2002, is shown in Fig 3. There were no bites by the mosquitos for at least 3 hours after the application of all products. Among Gel B, Gel E, and deet, Gel E showed greater protection against mosquitos 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

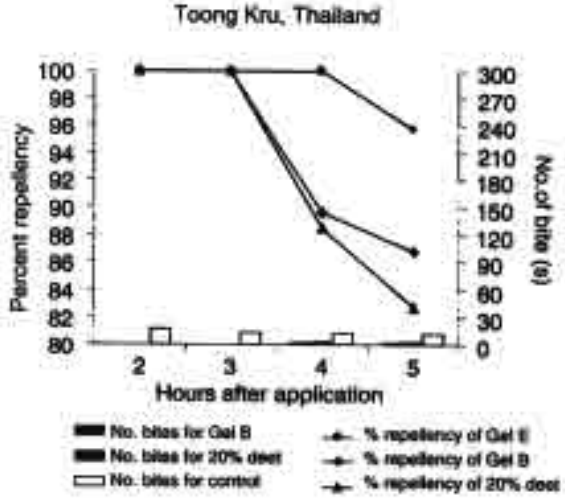

Fig 3–Repellency of Gel B and Gel E compared with 20% deet in ethanol against mosquitos in Toong Kru, Bangkok, Thailand.

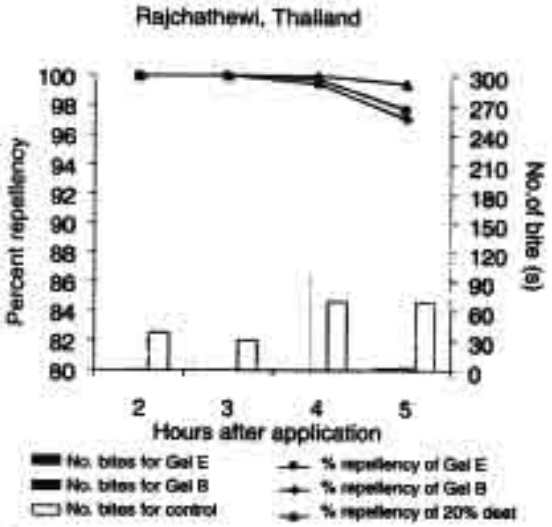

Fig 4–Repellency of Gel B and Gel E compared with 20% deet in ethanol against mosquitos in Ratchathewi, Bangkok, Thailand.

Fig 5–Repellency of Gel B and Gel E compared with 20% deet in ethanol against mosquitos in Sai Noi, Nonthaburi, Thailand.

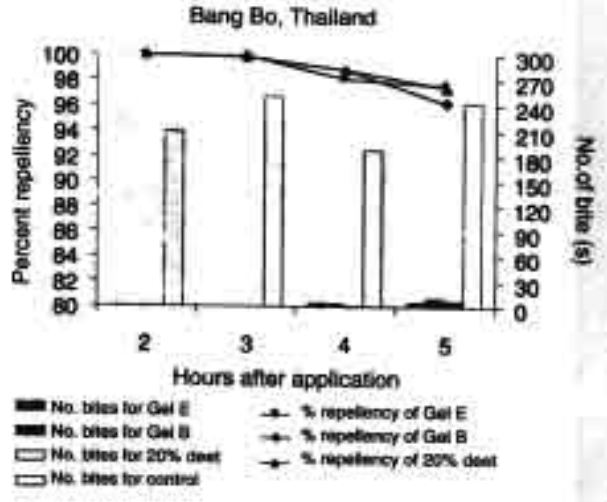

Fig 6–Repellency of Gel B and Gel E compared with 20% deet in ethanol against mosquitos in Bang Bo, Samut Prakan, Thailand.

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 mosquitos. 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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2/02/04

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Comparative repellency of thirty-eight essential oils against mosquito bites

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Introduction

Plants and plant-derived substances have been used to try to repel or kill mosquitoes and other domestic pest insects for a long time before the advent of synthetic chemicals (Curtis *et al.*, 1989). A review on the uses of botanical derivatives against mosquitoes has been presented by Sukumar *et al.* (1991). Essential oils of a large number of plants have been found to have repellent properties against various haematophagous arthropods; some have formed the basis of commercial repellent formulations (Curtis *et al.*, 1989). The repellency of these oils appears to be generally associated with the presence of one or more volatile mono-terpenoid constituents. Although they are effective when freshly applied, their protective effects dissipated relatively rapidly (Buescher *et al.*, 1982a; Rutledge *et al.*, 1983; Curtis *et al.*, 1989). The oils which have been reported as potential sources of insect repellents include citronella, cedar, verbena, pennyroyal, geranium, lavender, pine,

cajeput, cinnamon, rosemary, basil, thyme, allspice, garlic and peppermint. Sharma *et al.* (1993) have reported the effectiveness of neem oil as method of protection from mosquitoes which is safe and does not use synthetic chemicals. In laboratory tests in the USA (Barnard, 1999), thyme and clove oils provided 1.5 to 3.5 hrs of protection against *Aedes aegypti*. Citronella oil, in concentrations ranging from 0.05% to 15%, is used alone or in combination with cedarwood, lavender, peppermint, clove, eucalyptus, and garlic in a number of commercial insect repellent products (Fradin, 1998). Currently, a lemon eucalyptus extract which comes from the plant *Eucalyptus maculata citriodon* with the principal active ingredient *p*-menthane-3,8-diol (PMD) has shown particularly good results in its mosquito repellent properties when tests were carried out under laboratory and field conditions. This repellent has been found to be effective against mosquitoes, midges, ticks, and the stable fly (Curtis *et al.*, 1989; Trigg 1996; Trigg and Hill 1996; Govere *et al.*, 2000). Oils extracted from plants are widely used as fragrances in cosmetics, food additives, household products, and medicines. The U.S. Food and Drug Administration (FDA) generally recognize these as safe.

Recently, there have been many reports concerning the repellent properties of many kinds of essential oils; however, most of the results came

Running title: **Repellency of essential oils against mosquitoes**

Abstract

The mosquito repellent activity of 38 essential oils from plants at three concentrations was screened against the mosquito *Aedes aegypti* under laboratory conditions using human subjects. On a volunteer's forearm, 0.1 ml of oil was applied per 30 cm^2 of exposed skin. When the tested oils were applied at the 10 or 50% concentration, none of them prevented mosquito bites for as long as two hours, but undiluted oils of *Cymbopogon nardus* (Citronella), *Pogostemon cablin* (Patchuli), *Syzygium aromaticum* (Clove) and *Zanthoxylum limonella* (Thai name: Makaen) were the most effective and provided two hours of complete repellency.

From these initial results, three concentrations (10%, 50% and undiluted) of citronella, patchouli, clove and makaen were selected for repellency tests against *Culex quinquefasciatus* and *Anopheles dirus*. As expected, the undiluted oil showed the highest protection in each case. Clove oil gave the longest duration of 100% repellency (2 to 4 hours) against all three species of mosquito.

from artificial (*in vitro*) testing methods using cloth, filter paper, animal membrane or olfactometry but some came from more realistic (*in vivo*) methods utilizing animals or human subjects (Rutledge *et al.*, 1964; Barnard, 2000). Results from different methods cannot be compared directly because these methods yield results strongly related to the laboratory conditions used. The evaluation of repellency should preferably be carried out using human subjects, because laboratory animals may inadequately simulate the condition of human skin to which repellents will eventually be applied (WHO, 1996; Barnard, 2000; Moore, 2003).

In the present study, attempts have been made to characterize the relationship between different concentrations of 38 selected essential oils against *Aedes aegypti* mosquitoes using human subjects with caged mosquitoes. The more promising of the oils were also studied for their repellent activities against *Culex quinquefasciatus* and *Anopheles dirus*.

Material and Methods

Essential oils:

The names and sources of 38 essential oils are presented in Table 1. *Ageratum conyzoides* (leaves and flowers), *Spilanthes acmella* (flowers), *Vitex*

negundo (leaves) and *Zanthoxylum limonella* (seed and fruit) were obtained from northern Thailand. Herbarium specimens were identified by a botanist and deposited at the Forest Herbarium National Park, Wildlife and Plant Conservation Department, Thailand. They were subsequently extracted for essential oils by steam distillation. About one kilogram of fresh plant material at a time was cut into a small pieces and placed in a distillation flask with approximately 3 times as much water and 8-10 glass beads. The distillation chamber was heated to about 120°C and allowed to boil until the distillation was completed. The distillate was collected in a separating funnel with which the aqueous portion could be separated from the oil. The yield of each essential oil is shown in Table 2. These oils were kept at 4°C until they were tested for mosquito repellency.

Apart from the oils extracted from plant material as specified above, another 32 essential oils were purchased from the TCF Co. (Bangkok, Thailand).

Mosquitoes:

The mosquito species tested were *Ae. aegypti*, *An. dirus* and *Cx quinquefasciatus*. These mosquitoes were uninfected laboratory strains and were reared in the insectary of the Insecticide Research Unit at the Department

of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. The methods for mass rearing were slight modifications of the procedure described by Limsuwan *et al.* (1987).

Subjects: This study used three human subjects who agreed to take part in testing the repellency of each kind of oil.

Repellent assay:

The repellency of the essential oils was evaluated by using an arm-in-cage test (Schreck and McGovern 1989; WHO 1996). Each oil was tested undiluted and also was diluted with 70% alcohol to 10% and 50% concentration. An arm was covered with a rubber sleeve with a 3X10 cm window and 0.1 ml of a 10% or 50% concentration or undiluted oil was applied. The treated arm was exposed for 1 minute to 250 hungry female mosquitoes, 4-5 days old. Every 30 minutes after treatment the treated arm was re-exposed to mosquitoes and the time was recorded at which the first bite occurred. Following the method of Schreck and McGovern (1989), the 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 (min.) of complete repellency after application of repellent was used as a measure of the repellency of the essential oils. The arm treated

with the solvent used for the essential oil was used as the control. This control arm was exposed before the start of each assay. The essential oils that provided the longest complete protection time were tested against *An. dirus* and *Cx quinquefasciatus* by the same methods.

Results

Results of the initial screening tests showing the repellent activity of 38 essential oils from plants are given in the Table 3.

Of the essential oils tested, high concentrations of *C. nardus, P. cablin, S. aromaticum* and *Z. limonella* (fruit) were the most effective and provided at least two hours complete repellency against *Ae. aegypti*. The protection times of these oils were less when they were diluted. At 50% concentration, *C. nardus, P. cablin, S. aromaticum* and *Z. limonella* (fruit) showed 50, 60, 70 and 80 minutes protection, respectively and, the repellent activity decreased to 30 minutes or less when diluted to 10%. Based on these results, *C. nardus, P. cablin, S. aromaticum* and *Z. limonella* (fruit) were further studied for effectiveness against two other mosquito species, *Cx quinquefasciatus* and *An. dirus* in comparison with *Ae. aegypti*. The results are presented in Table 4.

The undiluted oil showed the highest protection time in each case. Among the four kinds of oil tested, *S. aromaticum* demonstrated the longest protection time against all three species of mosquito and the order of potency based on the protection time was *Cx quinquefasciatus>An. dirus>Ae. aegypti*. The mean durations of protection from bites for *S. aromaticum* were 240, 210 and 120 minutes against *Cx quinquefasciatus*, *An. dirus* and *Ae. aegypti*, respectively. At 50% concentration *S. aromaticum* provided 120 minutes of complete protection against both *An. dirus* and *Cx quinquefasciatus*. *P. cablin* and *Z. limonella* protected for 120 and 130 minutes, respectively, against *An. dirus*. The protection times of all oils at 10% concentration were less than 120 minutes against all three species of mosquito.

Discussion

Repellency evaluation is preferably carried out using human subjects, as testing repellents on animals or artificial membranes may not give representative data of how the repellent will perform when applied to a human skin (Nicolaides *et al.*, 1968; Cockcroft *et al.*, 1998). Our studies evaluated the repellent activities of 38 oils against *Ae. aegypti* mosquitoes which are anthropophilic, are easy to rear under laboratory conditions and are avid

biters. The tests showed that among 38 undiluted essential oils, the most effective were extracted from *C. nardus*, *P. cablin*, *S. aromaticum* and *Z. limonella* which provided complete repellency for 120 minutes. The results in the reports of United States Department of Agriculture (1952- 1964) also documented the complete repellency of *S. aromaticum* and *C. nardus* for 120 minutes against *Ae. aegypti*.

According to the recommendation of the U.S. Environmental Protection Agency (2003), using *Ae. aegypti* along with a representative human biting species from both the *Anopheles* and *Culex* genera for the laboratory studies of repellent efficacy can provide information on difference in response of the main vector genera of mosquitoes. Rutledge *et al.* (1983) showed that patterns of sensitivity to repellent compounds varied between mosquito genera. Their experiments showed, for 31 repellents, that *Ochlerotatus* (*Ochlerotatus*) *taeniorhynchus* and *Cx pipiens* were significantly more sensitive than were *Ae. aegypti* and *An. albimanus*. Furthermore *Ae. aegypti*, the traditional test species for repellent studies, was an exceptionally poor predictor for the responses of *An. stephensi* to repellents. Curtis *et al.* (1987) showed that *Anopheles* mosquitoes were less sensitive to DEET and other repellent chemicals than *Ae. aegypti*. The present results showed that among the 38 oils

tested, the undiluted oil of *C. nardus*, *P. cablin*, and *Z. limonella* provided better protection against *Ae. aegypti*, *Cx quinquefasciatus* and *An. dirus*. The mean duration of repellency of *S. aromaticum* oil was slightly greater than from the other three oils against *Cx quinquefasciatus* (240 min) or *An. dirus* (210 min).

For oils manifesting mosquito repellency, protection time generally increased with increasing oil concentration. None of the oils prevented mosquito biting for as long as 120 minutes when used at 10% or 50% concentration. It was reported by Li *et al.* (1974) (and summarized in English by Curtis *et al.* (1989)) that, against *Ae. aegypti*, Lemon Eucalyptus oil has a protection time of only one hour but Table 4 showed that *P. cablin*, *S. aromaticum* and *Z. limonella* oils gave 2 hours repellency (Table 4). However, Li *et al.* (1974) showed that the waste distillate of Lemon Eucalyptus contained an active repellent *p*-menthane diol (PMD) and he found that a 15% concentration of PMD obtained from Lemon Eucalyptus oil distillation showed 4.4 hours protection. This was better than 10% of *P. cablin*, *S. aromaticum* and *Z. limonella* oils which gave no more than half an hour protection against *Ae. aegypti* (Table 4). 50% concentration of PMD gave 13 hours protection against this species (Li *et al.*, 1974). At a range of

concentrations applied to humans, protection time of PMD against *Ae. aegypti* was proportional to the amounts applied and was definitely higher than the protection time of oils of *P. cablin*, *S. aromaticum* and *Z. limonella*.

For possible use by low-income rural communities, where the highest incidence of mosquito-borne diseases are reported, our studies have added the cheaply available *P. cablin*, *S. aromaticum* and *Z. limonella* to the list of effective plant based repellents.

Citronella from *C. nardus* belongs to the genus *Cymbopogon* which yield the most popular repellents in the world. In South Africa, *C. excavatus* gave 100% repellency for 2 hours, when it was evaluated in the laboratory against *An. arabiensis* and its repellency decreased to 59.3 % after 4 hours (Govere *et al.* 2000). In Thailand, *C. winterianus* oil, mixed with 5% vanillin, gave 100% protection for 6 hours against *Ae. aegypti*, *Cx quinquefasciatus* and *An. dirus* and compared favourably with 25% DEET (Tawatsin *et al.*, 2001). The pure oil of *C. martinii martinii* (palmarosa) provided 100% repellency for 12 hours against *Anopheles* mosquitoes in a field trial which was carried out by using pairs of volunteers who sat together, one of whom was treated with the oil and other was not (Ansari and Razdan, 1994).

These plants contain varying amounts of several insect repellent chemicals although environmental conditions cause the content of volatile oils in plants to vary greatly. Repellent compounds contained in this group include alpha pinene, camphene, camphor, geraniol and terpenen-4-ol. The most abundant repellent molecules found in the group are citronellal, citronellol and geraniol (Duke, 2000). Buescher *et al.* (1982b) and Rutledge *et al.* (1983) found a synthetic derivative of citronella (a mono-terpene aldehyde), is the main constituent of citronella oil and has been used as the active ingredient of commercial repellents. In addition, their high citronellal content makes the plants of this genus potential candidates for PMD production since citronellal is a precursor of this molecule. The grasses grow readily and rapidly throughout much of the tropics and a simple steam distillation is sufficient to extract the repellent fractions. The plants in this genus are pleasant smelling and are widely used in traditional medicine.

S. aromaticum or "clove oil" was reported the most effective mosquito repellent in the comparison made by Barnard (1999) and in the present study. Barnard showed that this oil gave 90 to 225 minutes of protection against *Ae. aegypti* and 75 to 213 minutes of protection against *An. albimanus*, depending on oil concentration. The major constituents of clove oil are eugenol,

eugenol-acetate, and beta-caryophyllene (Leung and Foster, 1996). Eugenol is repellent to *Ae. aegypti* (USDA 1954) and *An. gambiae* (Chogo and Crank, 1981). Neither eugenol-acetate nor beta-caryophyllene are repellent to *Ae. aegypti* (USDA, 1954), but neither has been tested for repellency to *Anopheles* mosquitoes. Eugenol also acts as an antioxidant in oleogenous foods, as an anticarminative, antispasmodic, and antiseptic in pharmacy, and as an antimicrobial agent (Farag *et. al.*, 1989a, 1989b). Clove oil is used in oriental medicine as a vermifuge, and as an antibacterial and/or antifungal agent (Awuah and Ellis, 2002; Dorman and Deans, 2000). Miyazawa and Hisama (2001) reported that a methanol extract from clove showed an anti-mutagenic effect. Clove oil is very widely used in clinical dentistry in root canal therapy and temporary fillings, and exhibits an anti-microbial activity against oral bacteria that are commonly associated with dental caries and periodontal disease (Cai and Wu, 1996).

For *P. cablin*, there has been no previous report of mosquito repellent activity. This plant has been used against the common cold and as an antifungal agent in traditional medicine. It is cultivated extensively in Indonesia, Malaysia, China and Brazil for its essential oil (patchouli oil), which is important to the perfumery industry. This oil contains many mono-

and sesqui-terpenoids, and several flavonoids and alkaloids (Tsubaki *et. al.*, 1967; Hikino *et. al.*, 1968; Terhune *et. al.*, 1973; Itokawa *et. al.*, 1981). *P. cablin* has as strong an anti-mutagenic effect as *S. aromaticum* (Miyazawa *et. al.*, 2000) and has antibacterial activity (Osawa *et. al.*, 1990) and is used for prevention of emphysema in the convalescent stage (Fu, 1989).

For *Z. limonella*, there are no publications showing its mosquito repellent activity but this plant is mentioned in the website www.indmedplants-kr.org/Zanthoxylum_limonella.htm as some members of this genus have an insecticidal effect. A yield of 12.5% (w/w) of essential oil was obtained with this species which is higher than that of the other plants studied (Table 2) and is likely to make *Z. limonella* more cost effective than the other three plants. Itthipanichpong *et.al.* (2002) reported the chemical compositions of the essential oil distilled from the fruit of *Z. limonella* in Thailand and found the presence of 33 chemical components. Limonene (31.1%), terpin-4-ol (13.9%) and sabinene (9.1%) were found to be the major components. They also reported that the essential oil from the fruit of this plant possessed stimulatory effect on smooth muscle preparations by non-specific mechanisms.

These initial results clearly demonstrated that the essential oils from *P. cablin*, *S. aromaticum* and *Z. limonella* plants performed as mosquito repellents about equally well as citronella oil. As indicated above, these oils are used in medicine, perfumery, and flavouring of food and are considered non-toxic to humans and environmentally friendly.

Further studies are needed to develop appropriate formulations including a fixative, which would increase their efficacy and cost effectiveness. Field trials should be carried out, particularly to evaluate operational feasibility and dermal toxicity over a long period, especially to infants and children. It is important to determine whether widespread use of one of these repellents would produce an overall reduction of vector biting in a community or would simply divert biting from repellent users to non-users.

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Table 1 Name and source of essential oils.

Scientific name	Family	English name	Thai name	Source
Ageratum conyzoides -	Asteraceae	-	Sap rang sap ka	Lab extract
Allium sativum - L.	Alliaceae	Garlic	Kra tearm	Commercial
A.tuberosum - Roxb.	Alliaceae	Oriental garlic	Kui chay	Commercial
Apium graveolens Linne	Umbelliferae	-	Khoun chay	Commercial
Boesenbergia pandurata Roxb Schltr	Zingiberaceae	-	Ka chai	Commercial
Canagium odoratum - Baill. Ex King	Annonaceae	Ylang Ylang	Kar dung nga	Commercial
Cedrus deodara	Pinaceae	Cedar	-	Commercial
Citrus hystrix - Dc.	Rutaceae	Leech lime	Ma krood	Commercial
C.reticulata - Blanco	Rutaceae	Tangerine orange	Soam keuw kwan	Commercial
Cupressus funebris	Cupressaceae	-	Opp chey cheen	Commercial
Curcuma longa - L.	Zingiberaceae	Turmeric	Kha min chan	Commercial
Cymbopogon citratus - (Dc.) Stapf	Poaceae	Lemon grass	Ta kai	Commercial
C.nardus - (L.) Rendle	Poaceae	Citronella grass	Ta kai hom	Commercial
Eucalyptus globulus - Labill.	Myrtaceae	Eucalyptus	Eucalyptus	Commercial
Lavandula angustifolia	Lamiaceae	Lavender	-	Commercial
Litsea cubeba	Lauraceeae	Cubeba	-	Commercial
Mentha arvensis - *L.*	Apiaceae	Japanese mint	-	Commercial

(continued overleaf)

Continuation of Table 1 Name and source of 36 essential oils

Scientific name	Family	English name	Thai name	Source
Mentha piperita - L.	Apiaceae	Pepper mint	-	Commercial
M.spicata - L	Apiaceae	Spear mint	-	Commercial
Myristica fragrans - Houtt.	Myristicaceae	Nutmeg	Chan tade	Commercial
Ocimum basilicum - L.	Apiaceae	Sweet basil	Ho la pa	Commercial
O.sanctum - L.	Apiaceae	Holy basil	Ka prow	Commercial
Pelargonium graveolens	*Geraniaceae*	Geranium	-	Commercial
Pimpinella anisum	Umbelliferae	-	-	Commercial
Pinus sylvestris L.	Pinaceae	-	Soan	Commercial
Piper betle - L.	Piperaceae	Betel pepper	Ploo	Commercial
P.nigrum - L.	Piperaceae	Black pepper	Prik tai	Commercial
Pogostemon cablin - Blanco	Apiaceae	Patchouli	Pim sane bai	Commercial
Sesamum indicum - L.	Pedaliaceae	Sesame	Nga	Commercial
Spilanthes acmella - (L.) Murr.	Asteraceae	Para cress	Pak kard hou wan	Lab extract
Syzygium aromaticum - (L.) Merr.	Myrtaceae	Clove	Khan ploo	Commercial
Vetiveria zizanioides – Nash	Poaceae	Vetiver	Yar fak hom	Commercial
Vitex negundo - L.	Labiatae	Indian privet	Khon tee kha mow	Lab extract

(continued overleaf)

Continuation of Table 1 Name and source of 36 essential oils

Scientific name	Family	English name	Thai name	Source
Zanthoxylum limonella - Alston	Rutaceae	-	Makaen, kam jad torn	Lab extract
Zingiber officinale - Roscoe	Zingiberaceae	Ginger	Khing	Commercial
Z.purpureum – Roscoe	Zingiberaceae	-	Plai	Commercial

Table 2 Description of 5 essential oils that were obtained from steam distillation in the laboratory

Plant	Collection place and date	Part used	Fresh plant (g)	Essential oil (g)	% Yield (w/w)
A. conyzoides	Chaing Rai, 22 January 2002	Leaves	1000	1.25	0.13
A. conyzoides	Chaing Rai, 23 January 2002	Flowers	950	1.14	0.12
S. acmella	Chaing Rai, 26 January 2002	Flowers	1200	1.68	0.14
V. negundo	Bangkok, 10 March 2002	Leaves	1000	1.10	0.11
Z. limonella	ChaingRai, 10 February 2002	Seed	1000	20.80	2.08
Z. limonella	Chaing Rai, 12 February 2002	Fruit	1000	125.00	12.50

Table 3. Repellent activity of 38 essential oils (undiluted or as 10% or 50% dilutions) against *Ae. aegypti* mosquitoes

Oil	Durations (min.) of complete repellency (as defined in the methods section) recorded by 3 volunteers; (with mean of the three)			
	10%	50%	Undiluted	Control*
A. conyzoides (leaf)	0,30,30; (20)	30,30,30; (30)	60,60,60; (60)	0
A. conyzoides (flower)	0,0,30; (10)	30,30,30; (30)	30,30,60; (40)	0
A. sativum	0,0,30; (10)	30,30,60; (40)	60,60,90; (70)	0
A. tuberosum	0	0	0	0
A. graveolens	0	60,60,60; (60)	30,30,60; (40)	0
B. pandurata	0	0	30,30,30; (30)	0
C. ordoratum	0	0	30,30,30; (30)	0
C. deodara	0	0	0	0
C. hystrix	0,0,30; (10)	30,30,30; (30)	60,60,60; (60)	0
C. reticulata	0	0	0	0
C. funebris	0	0	0,0,30; (10)	0
C. longa	0	0,0,30; (10)	0,0,30; (10)	0
C. citratus	0	30,30,30; (30)	30,30,30; (30)	0
C. nardus	0	60,60,60; (60)	120,120,120; (120)	0
E. globulus	0	0	30,30,30; (30)	0
L. angustifolia	0	0	0,0,30; (10)	0
L. cubeba	0	0	0	0
M. arvensis	0	30,30,30; (30)	30,60,60; (50)	0
M. piperita	0	0	30,30,30; (50)	0
M. spicata	0,0,30; (10)	30,30,30; (30)	30,30,30; (30)	0

* in all cases many more than 2 bites were obtained on the untreated control arm during the first exposure just before the time of application of the repellent

(continued overleaf)

Continuation of Table 3. Repellent activity of 38 essential oils in three concentrations on *Ae. aegypti* mosquitoes

Oil	Durations (min.) of complete repellency (as defined in the methods section) recorded by 3 volunteers; (with mean of the three)			
	10%	50%	Undiluted	Control*
M. fragrans	0	0	30,30,30; (30)	0
O. basillicum	0	0	60,60,90; (70)	0
O. sanctum	0	0,0,30; (10)	60,60,60; (60)	0
P. graveolens	0,0,30; (10)	30,30,60; (40)	30,60,60; (50)	0
P. anisum	0	0	0	0
P. sylvestris	0	30,30,60; (40)	60,60,60; (60)	0
P. betle	0	60,60,90; (70)	60,90,90; (80)	0
P. nigrum	0	0	90,90,90; (90)	0
P. cablin	0	60,60,60; (60)	120,120,120; (120)	0
S. indicum	0	0	0	0
S. acmella	30,30,30; (30)	0	30,30,30; (30)	0
S. aromaticum	30,30,30; (30)	60,60,90; (70)	120,120,120; (120)	0
V. zizaniodes	0	0,0,30; (10)	60,60,60; (60)	0
V. negundo	0	0	0,0,30; (10)	0
Z. limonella (Seed)	30,30,30; (30)	60,90,90; (80)	90,90,120; (100)	0
Z. limonella (fruit)	30,30,30; (30)	60,90,90; (80)	120,120,120; (120)	0
Z. purpureum	0	0	60,60,60; (60)	0
Z.officinale	0	0	30,30,60; (40)	0

* in all cases many more than 2 bites were obtained on the untreated control arm during the first exposure just before the time of application of the repellent.

Table 4. Repellent activity of the promising essential oils in three concentrations against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. dirus* mosquitoes

Mosquito spp. and Oil	Durations (min) of complete repellency (as defined in the methods section) recorded by 3 volunteers; (with mean of the three)			
	10%	50%	Undiluted	Control*
Ae. aegypti				
C. nardus	0	30,60,60; (50)	120,120,120; (120)	0
P.cablin	0	60,60,60; (60)	120,120,120; (120)	0
Z. limonella	30,30,30; (30)	60,90,90; (80)	120,120,120; (120)	0
S. aromaticum	30,30,30; (30)	60,60,90; (70)	120,120,120; (120)	0
Cx quinquefasciatus				
C.nardus	30,30,60; (40)	60,90,90; (80)	90,90,120; (100)	0
P.cablin	60,60,60; (60)	60,90,120; (90)	150,150,150; (150)	0
Z. limonella	30,30,90; (50)	90,90,120; (100)	120,180,210; (170)	0
S. aromaticum	30,90,120; (80)	120,120,120; (120)	240,240,240; (240)	0
An. dirus				
C.nardus	30,30,60; (40)	30,30,30; (30)	60,60,90; (70)	0
P.cablin	30,90,120; (80)	90,120,150; (120)	150,180,180; (170)	0
Z. limonella	60,60,60; (60)	60,150,180; (130)	180,180,210; (190)	0
S. aromaticum	60,90,90; (80)	150,150,180; (160)	210,210,210; (210)	0

* in all cases many more than 2 bites were obtained from each mosquito species on the untreated control arm just before the tested arm was treated.

JOURNAL OF THE AMERICAN MOSQUITO CONTROL ASSOCIATION

Kenneth J. Linthicum, Editor
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Mailing address:
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March 7, 2004

Yuwadee Trongtokit
Department of Entomology
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Bangkok, Thailand

Dear Miss Trongtokit:

I am pleased to inform you that the manuscript, "Efficacy of repellent products against caged and free flying *Anopheles stephensi* mosquitoes," No. MS No 5209, is accepted for publication in the *Journal of the American Mosquito Control Association*, subject to the changes recommended by the reviewers. I have enclosed the comments from both reviewers and an annotated manuscript.

Would you please read the comments and consider them carefully. When you return your revision, we would appreciate it if you could submit your revision in electronic format. A paper copy of both the text and the figures should accompany the disk, and should match the disk copy exactly. A 3 ½" high-density floppy disk in either IBM or Macintosh formats should be used. Any word-processing program and most graphics formats are acceptable.

Sincerely,

Kenneth J. Linthicum
Editor

KJL/sl
Enclosures

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Efficacy of repellent products against caged and free flying *Anopheles stephensi* mosquitoes

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Running title: Repellent products against *An. stephensi*

Abstract

The efficacy of 8 commercial repellents and one product which is under development were evaluated on the skin at dosages of 0.65 or 1.7 mg of product/cm², the latter being the industrial standard for deet based repellents. They were applied to the arm or lower legs of a human subject and tested against *Anopheles stephensi* in a cage or flying freely in a mosquito-proof room. In the artificial arm-in-cage tests, a product with 20% p-menthane-3, 8-diol (PMD) provided complete repellency for 7-8 hours, while with another product with 10% this was reduced to only 30 minutes. The natural oils of clove (*Syzygium aromaticum*) (10%) plus makaen (*Zanthoxylum limonella*) (10%) gave protection for 4-5 hours. In a more realistic setting of free flying mosquitoes in a room, products with 20% or 30% PMD gave complete protection for 11 -12 hours, at normal consumer dose of 1.7 mg/cm² or 6 hours at half this rate. With the product with 10% PMD the protection was for less than 2 hours. At the higher dosage rate 40% citronella and hydroxyethyl isobutyl piperidine carboxylate (HIPC) a

relatively new synthetic active provided complete repellency for 7 hours. 50% deet was effective for 30 hours if left undisturbed on the skin.

These results indicate that of the plant-based repellent products, those containing PMD as an active ingredient at greater than 20% give complete protection against *An. stephensi* for 11-12 hours, thus offering an equally effective practical alternative to those containing high concentrations of the synthetic chemical, deet. At normal consumer application rates a single daily application in the later afternoon, will provide full protection during the critical biting times. If required a second application in the morning will cover the much lower risk of daytime biting transmission. The clove and makaen combination could be useful where low cost alternatives are essential.

Keywords: Mosquito repellent, PMD, *Syzygium aromaticum*, *Zanthoxylum limonella*, *An. stephensi*

Introduction

There is a thriving market in mosquito repellents and, on the label of most of the products, claims are made about duration of protection. However, few comparative trials using a standard methodology have been published. Deet (di-methyl benzamide) may be considered as the standard repellent. There has been concerned about rare reports of severe reactions to this substance. Deet does attack hard plastics, and many consumers do not like its odour and favour a plant based product. Citronella from *Cymbopogon nardus* is widely available. Another plant based product with a lemon-like odour is derived from Lemon Eucalyptus (*Eucalyptus maculata citriodon*). It was first shown to be an effective repellent in China and it was shown that *p*-menthane diol (PMD) was the active ingredient (Li et al. 1974 (data summarized in English by Curtis et al. 1989)). PMD containing repellents have now been commercialized in the USA, Europe and Australia. Trials of repellency in laboratory cages and in the field showed that it was approximately as long lasting as deet and more long lasting than citronella (Trigg 1996; Trigg and Hill 1996). The present paper describes comparative tests of several products containing PMD as active ingredients, including one ("Off! Botanicals") in which the active ingredient is derived from

menthol and not from a plant. A new plant product from Thailand was included in the tests.

The products were applied to the arm or legs of a human subject (Yuwadee Trongtokit, the senior author) at the dose of product per unit area which is the industry standard or, in one trial, about half of that dosage. One series of tests was with an arm in a cage, but most were in the more realistic situation of mosquitoes flying freely in a room and with the repellent applied to the lower legs.

The normal way of assessing repellents is by duration of protection (Schreck and McGovern 1989; WHO 1996) with a standard dose applied. This was also used in the present work, though it can be argued that consumers are mainly concerned about achieving reliable protection for relatively short period when at known risk.

Materials and Methods

Repellent products:

A total of 9 repellent products were evaluated for their mosquito repellency (Table 1). Mospel, a product in development by Insecticide Research Unit, Mahidol University, Thailand, were also studied. This product contains 10% clove oil and 10% makaen oil as active ingredient in a

gel form. Clove oil, which was extracted from *Syzygium aromaticum*, and Makaen oil, which was extracted from *Zanthoxylum limonella*, have been previously shown to give a good level of repellency against *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles dirus* (Trongtokit et al. submitted).

Dosage of the repellents

According to the industrial standard for deet based repellents, the application rate for each candidate repellent was 1.0 gm of product per 600 cm^2 which is approximately the area of the skin surface of a forearm from elbow to wrist. This dose is comfortable for the user and covers the skin surface without leaving gaps. For testing on the legs, the skin surface from knee to foot of each leg of the human subject was approximately 1,526 cm^2, so a dosage of 2.6 gm was applied. It was found preferable to weigh the repellents rather than pipetting as some were very viscous and much remained adhering inside a pipette. This dosage from a pump spray was sprayed onto a sheet of aluminum foil and collected on a petrie dish on a balance; pump spraying continued until 2.6 gm had been collected.

Mosquito stock

Laboratory reared *An. stephensi* strain BEECH of Indian origin is colonized in the insectary of the Disease Control and Vector Biology Unit, London School Hygiene and Tropical Medicine. The colony was maintained at a temperature of 27± 2 °C and at a relative humidity of 60-80% under a 12:12 light and dark cycle and provided with horse blood through an artificial membrane.

Hungry female mosquitoes were selected by placing a human hand on the side of a mosquito cage containing 3-8 day old mosquitoes. Then females attracted to the hand were aspirated into a cup to make batches of 30 for each replicate trial.

Bioassay

An arm in a cage

One gram of product was applied onto the forearm (approximate surface is 600 cm^2 from the elbow to the wrist) of a human subject. The treated arm was exposed for 1 minute to 30 hungry female mosquitoes, in a 30x30x30 cm cage, and any mosquitoes landing and biting were counted. Every 30 minutes after treatment the treated arm was re-exposed to mosquitoes. Following the criteria of Schreck and McGovern (1989), the

time was recorded at which the first two bites occurred and one further exposure was made to check that complete repellency had indeed failed. A new cup of 30 mosquitoes was used for each trial at successive time intervals. The other arm without treatment was used as the control and it was exposed to mosquitoes in the cage before each insertion of the treated arm. Numbers of mosquitoes landing and biting in a minute were recorded. The mosquitoes did not have time to gorge with blood during this control exposure and they remained hungry for the exposure for the treated arm. This arm in a cage method was only used for the comparison of Repel Lemon Eucalyptus, Off! Botanicals and Mospel.

Free flying mosquitoes in a mosquito proof room

The room at London School of Hygiene and Tropical Medicine measured 3x2x2.5 m, was maintained at a temperature of 26 ± 2°C and relative humidity of 55-65% and had white tiled walls which could be effectively cleaned. At the beginning of a test, the untreated legs were exposed to mosquitoes for 10 min and the landing, biting mosquitoes were caught and counted to establish the biting rate of a batch of mosquitoes similar to those to be used in the trial.

2.6 gm of repellent product was applied evenly from knee to foot of each leg. Other exposed untreated parts of the body were protected against mosquito attack by wearing a jacket with hood and gloves and shorts covering the thighs. After release of 30 hungry female mosquitoes the subject sat on a bench in the middle of the room for 10 min in each hour and any landing and biting mosquitoes were caught and counted. After finishing a 10 min observation, the mosquitoes were recaptured by exposing two untreated arms on which the mosquitoes readily landed. This showed that tested mosquitoes were still hungry and that the failure to land on the treated legs was because of the repellent treatment. The tested mosquitoes were discarded. A new cup of mosquitoes was used in the next test which was started 50 min after the end of the previous test. The treated legs were exposed at hourly intervals until two bites occurred, and one further exposure was made to check that complete repellency had indeed failed. Care was taken to minimize contact of the treated legs with clothing and furniture between the hourly tests.

Results

An arm in cage

One gram of each of three products was applied onto the forearm and they were evaluated by an arm in a cage. The results are shown in Fig.1. The plant based repellent containing 20% PMD (Repel Lemon Eucalyptus lotion) provided longer lasting complete repellency (5-7 hours before the first bite) than the product containing 10% PMD (Off! Botanicals) which gave complete protection for only 30 min. It was clear that the difference between 10% and 20% of active ingredient made a great difference to the duration of repellency obtained. Mospel, the product which is under development containing 10% clove plus 10% makaen, gave complete repellency for 4.5-5 hours after application (Fig. 1).

Free flying mosquitoes in a mosquito proof room

When 1 gram of product was applied to each of the lower legs, the product containing 20% PMD gave complete repellency for 6-7 hours (Fig. 2) which was much longer than the product containing 10% PMD, which gave complete repellency for 1-2 hours. These durations were somewhat greater than in the arm-in-cage tests, but the marked superiority of the 20% PMD product was confirmed.

The results of repellency tests on six different products against *An. stephensi* flying freely in a room are shown in Fig. 3. Deet, which is the best-known synthetic insect repellent, at 50% concentration of active ingredient, provided the longest-lasting complete protection which continued for 30 hours after application. The plant based repellent products containing 20% - 30% PMD in the form of creams completely repelled *An. stephensi* for up to 11-12 hours; whereas 40% citronella in the oil formulation and 10% clove plus 10% makaen in a gel formulation provided repellency to 7-8 hours. Application of HIPC from a pump spray lasted for about the same time as citronella or clove plus makaen. The repellent containing only 5% citronella was less effective than the other products, with complete repellency for only about 2-3 hours after application. Each of these results was confirmed by carrying out a 2nd replicate.

No skin irritation or dermatitis was observed on the treated skin of the human subject after any of the repellent treatments.

Discussion

Use of insect repellents should have a role in reducing infection with malaria, dengue, filariasis, West Nile virus and other insect-borne diseases (Curtis, 1992 and Fradin, 1998). The mosquito used for these tests was *An. stephensi* which is an important malaria vector in urban India and the Middle East. Many of the people affected by this mosquito can afford to purchase repellents because they are generally less poor than the most malaria affected people of rural Africa and South East Asia. The main motive for most purchases of repellents is avoidance of mosquito nuisance, with malaria as only a secondary consideration. In fact there is still little direct evidence of repellents as an effective means of malaria prevention (Rowland et al. 2004). More such evidence should be sought and investigations should be carried out, with species with different degrees of anthropophily/zoophily, of whether such mosquitoes are diverted from a person using repellent to bite another person or an animal. It many are diverted to animals, repellents could have a significant role in protecting low-income communities from malaria. For them commercial repellents are unaffordable, but there could be an important role for repellents based on plants, which could be grown and processed for use at home.

In most of our studies, the repellent formulations were applied at a rate of 1.7 mg/cm^2. This is the industrial standard for deet based repellents and this dose is comfortable for the user and covers the skin surface without leaving gaps. This dose gave about twice as long protection as did some tests at 38% of the standard dose (Fig. 2 and 3). In addition to conventional arm-in-cage tests the repellents were tested with free flying mosquitoes in a mosquito proof room with repellent applied to each lower leg. This method more closely simulates a field test, but with the advantage that the number of mosquitoes and environmental factors are controlled (WHO, 1996; Moore et al. 2003).

The durations of protection achieved were 50% Deet > 20%-30% PMD > 40% citronella = 10% clove plus 10% makaen = HIPC > 5% citronella.

The efficacy of deet has been evaluated against many mosquito species in many countries under laboratory and field conditions (Bucscher et al. 1982, 1983; Schreck and McGovern, 1989; Barnard et al. 1998; Cockcroft et al. 1998; Debboun et al. 2000; Thavara et al. 2001; Frances et al. 2002). Our study shows that a formulation containing 50% deet gave complete repellency against mosquito bites for as long as 30 hours after a single application, with care taken not to allow contact with clothing or

furniture. Thavara et al. (2001) reported that a lower dose per square cm of skin of a 20% deet product showed repellency for 9.7 hours against *Ae. aegypti*, for 12.7 hours against *Culex quinquefasciatus*, for 14.5 hours against *Cx. tritaeniorhynchus* and for 5.8 hours against *An. dirus*. Higher concentrations of deet have been recommended under circumstances in which the biting pressures are intense, the risk of arthropod transmitted disease is great, or environmental conditions promote the rapid loss of repellent from the skin surface (Maibach et al. 1974; Thavara et al. 2001). However, there are reports of rare severe reactions resulting from the topical use of deet, e.g. contact urticaria syndrome (Maibach and Johnson 1975), three cases of toxic encephalopathy in children (Zadikiff 1979; Edward and Johnson 1987), and skin eruptions in 10 solders after application of 50% deet (Maibach and Johnson 1975; Zandikoff 1979; Reuveni and Yagupsky 1982). After reviewing the pharmacokinetics, formulations, and safety of deet, the result exhibits a good margin of safety (Qiu et al. 1998). Despite the rare adverse effect, considering the vast number of containers sold, the risk of adverse effects were found to be no higher than with commodities such as household bleach (Veltri et al. 1994).

PMD, a mono-terpene of relatively low volatility obtained from lemon eucalyptus, has shown particular promise as a repellent of botanical origin;

its mammalian toxicity is lower than that of deet (Curtis et al. 1989; Trigg 1996; Trigg and Hill 1996, Govere et al. 2000a; Moore et al. 2002). Our results may be compared with the data of Trigg (1996) who showed in the field that at doses of 0.8-2.0 gm/leg of 50% PMD there was complete protection from biting for 6 - 7.75 hours. Fig. 3 shows that, in the present study, by increasing the dose up to 2.6 gm/leg, products with 20%-30% PMD gave protection from free flying laboratory mosquitoes for 11-12 hours. The differences in the methodology, the formulation type and the environment of the experiment should be noted. Our results show that PMD was not as effective as deet in repelling mosquitoes, but in practice, at normal consumer application rates, full protection can be expected for at least 10 hours with either 50% deet or 20% PMD. However, Schreck and Leonhardt (1991) reported that repellent formulations based on lemon eucalyptus were less effective against *Ae. aegypti* (L.), *Ochlerotatus taeniorhynchus* Wiedemann (black salt marsh mosquitoes), and *Ae. albopictus* (Skuse) than deet. In addition, both the lemon eucalyptus product and deet were reported to be ineffective in repelling anopheline species such as *An. quadrimaculatus* Say and *An. albimanus* Wiedemann. These findings emphasise the wide variations in the responses of different mosquitoes to these repellents.

Fig. 3 shows tests with Hydroxyethyl Isobutyl Piperidine Carboxylate (HIPC), which commonly has been known by the name KBR3023 or Bayrepel®, a recently developed piperidine compound which is now the active ingredient in the well know mosquito repellent brand sold under the name Autan. This has been reported as safe and effective for human use (WHO 2001; Yap et al. 2000; Thavara et al. 2001). It has been claimed that this synthetic repellent showed mosquito repellency which equals or exceeds that of deet or PMD (Walker et al. 1996; Thavara et al. 2001; Barnard et al. 2002). However, in our tests, its protection time was shorter than that of 50% deet or 20%-30% PMD against *An. stephensi*.

The use of plants of the *Cymbopogon* genus as insect repellents is widespread throughout the world and the formulations tested represent the range of concentration of citronella which are commercially available. When evaluated in the laboratory with a cage test against *An. arabiensis* by Govere et al. (2000b), the pure oil of citronella gave protection for 2 hours, but this declined to 59.3% after four hours. Fradin and Day (2002) found that the citronella-based repellents containing 0.05%-25% citronella protected for 20 minutes or less against *Ae. aegypti*. Thorsell et al. (1998) reported that a 10% concentration in 70% ethanol 8 hours after application gave 31.9 % repellency against *Ae. aegypti* in the laboratory but gave 99.0 %

repellency against *Oc. communis* and *Ae. cinereus* in a field trial. The above protection times in the laboratory are much shorter than those found in our studies with 5% or 40% citronella using *An. stephensi* flying freely in a room.

It is important to contrast active ingredients diluted in alcohol with commercial formulations. A 5% concentration of citronella in the form of lotion gave the same protection (2-3 hours) as was reported for a 10-50% concentration diluted with 70% ethanol or undiluted (Tawatsin et al. 2001; Trongtokit et al. submitted), whereas 40% concentration in the form of an oil gave 7-8 hours protection. Our study showed that Mospel, containing 10% clove oil plus 10% makaen oil formulated in the form of gel, gave complete repellency for 6-7 hours, but this dose of each oil prepared in 70% ethanol gave repellency for 2 hours or less against *Ae. aegypti*, *Cx. quinquefasciatus* and *An. dirus* (Trongtokit et al. submitted). Similarly, Gupta and Rutledge (1989; 1991) reported that, with sustained-release technology, very marked extension of protection can be achieved. They showed that the two controlled-release repellent formulations containing 33% or 42% deet gave mosquito repellency similar or better than 75% deet in ethanol against *Ae. aegypti*, *Oc. taeniorhynchus*, and *An. stephensi* under field conditions.

40% citronella and 10% clove plus 10% makaen gave protection for 7-8 hours which would be sufficient to protect against evening biting mosquitoes if people use the repellents before retiring to a bednet. However, the strong smell of these products, due to the high concentrations of essential oils, might be unacceptable to consumers. The longer-lasting protection of 20-30% PMD compared with other plant-based repellents and its pleasant lemony smell (as its active ingredient is not an essential oil) are no doubt important factors in the commercial success of these products.

However, further investigation of formulations of plant-based repellents is needed. It is hoped to produce repellents for use in low-income communities where native plants can be grown and processed with low technology to produce affordable repellents for use against biting insects which are a nuisance and vectors of disease.

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Table 1. List of repellents products

Product	Active ingredient and concentration
1. Mosi-guard Natural pump spray (MASTA, UK)	p-menthane diol, 30% from Lemon Eucalyptus
2. Mosi-guard Natural cream (MASTA, UK)	p-menthane diol, 20% from Lemon Eucalyptus
3. Repel Lemon Eucalyptus cream (Jackson, USA)	p-menthane diol, 20% from Lemon Eucalyptus
4. Off! Botanicals lotion (Johnson, USA)	p-menthane diol, 10% synthesized from mtnthol
5. Repel Insect Repellent lotion (Boots, UK)	Citronella oil, 5%
6. Citrepel oil (Chemian Technology Ltd)	Citronella oil, 40%
7. Autan active insect repellent pump spray (Bayer)	Hydroxyethyl Isobutyl Piperidine Carboxylate, *
8. Jungle formula Insect Repellent (Chefaro, UK)	DEET, 50%
9. Mospel (Mahidol University, Thailand)	Clove oil, 10% plus Makaen oil (*Zanthoxylum limonella*), 10%

* Concentration is not specified.

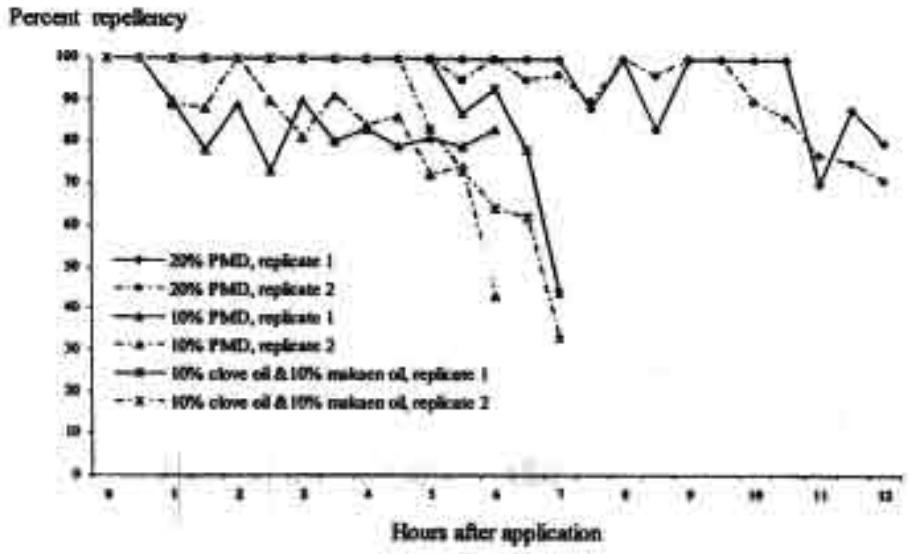

Fig.1. Percent repellency of 20% PMD (Repel Lemon Eucalyptus), 10% PMD (Off! Botanicals) or 10% clove oil plus 10% makaen oil (Mospel) against *An. stephensi* applied to an arm and tested in a cage of mosquitoes. The arm was observed at intervals after application, relative to contemporary control.

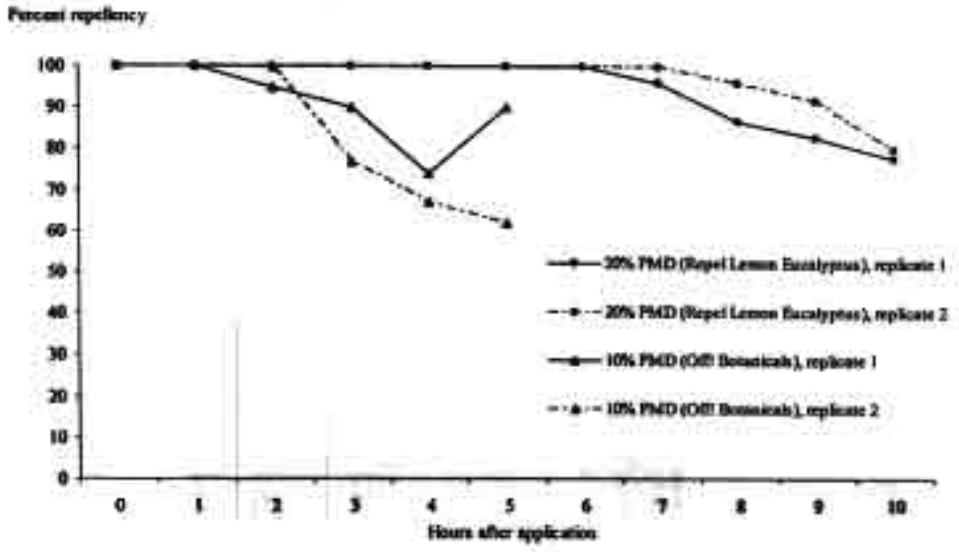

Fig. 2. Percent repellency of 1.0 gm of Repel Lemon Eucalyptus or Off! Botanicals applied to lower legs and with free flying *An. stephensi* in a mosquito proof room, observed at each hour after application, relative to initial biting rate on untreated legs.

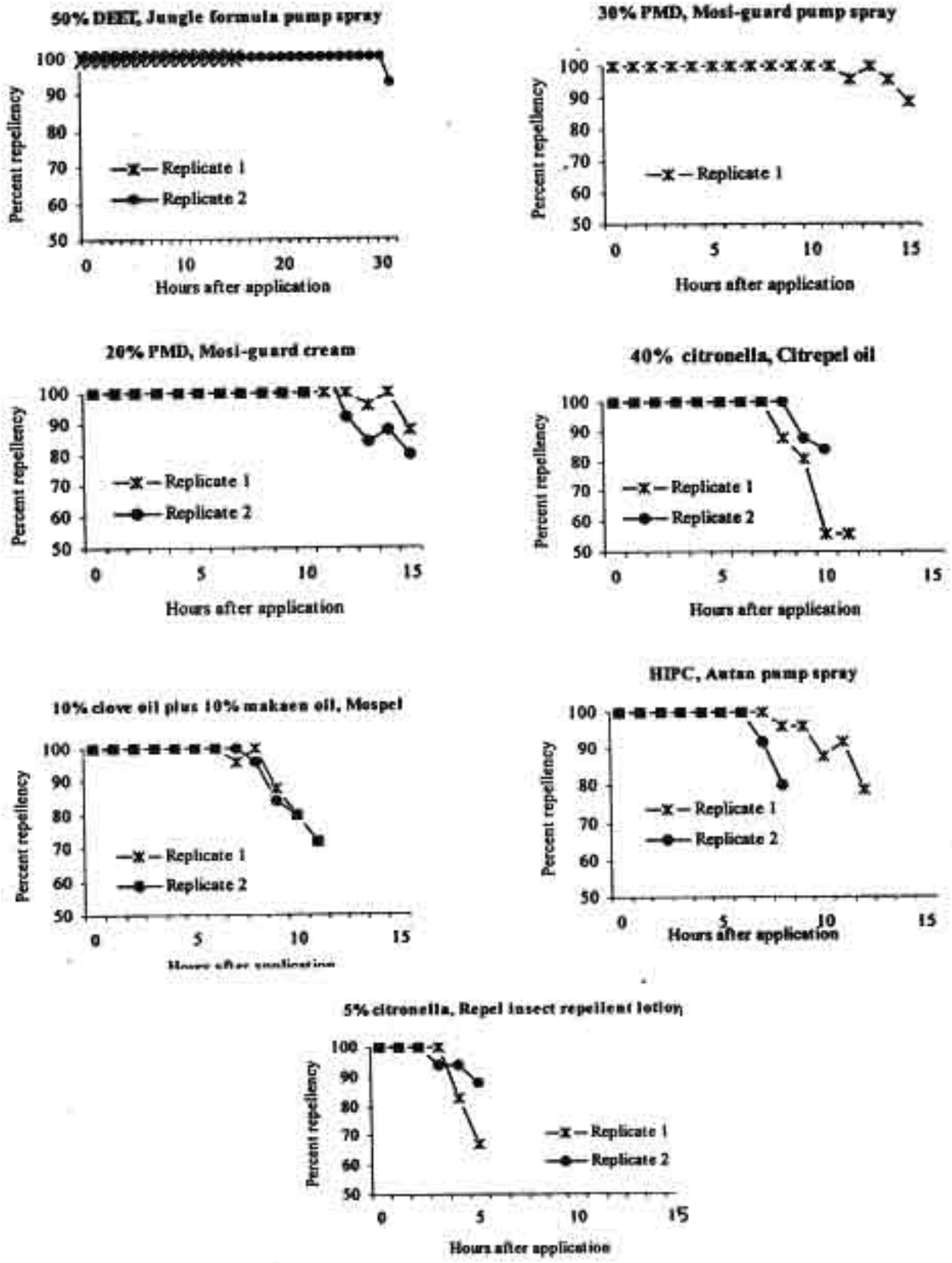

Fig. 3. Percent repellency of 2.6 gm of 7 repellent formulations applied to lower legs and with free flying *An. stephensi* in a mosquito proof room, observed at each hour after application, relative to initial biting rate on untreated legs.