EFFICACY OF REPELLENT PRODUCTS AGAINST CAGED AND FREE FLYING *ANOPHELES STEPHENSI* MOSQUITOES

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Abstract. The efficacy of 9 repellents (8 commercial repellents and one product under development) was evaluated on the skin at dosages of 0.65 and 1.7 mg of product/cm^2, the latter dosage being the industrial standard for deet based repellents. The repellents were applied to the arm or lower leg of a human subject and tested against *Anopheles stephensi* in a cage or flying freely in a mosquito-proof room. In the cage tests, a product with 20% p-menthane-3, 8-diol (PMD) active ingredient provided complete repellency for 7-8 hours, while with 10% PMD had complete repellency for only 30 minutes. The natural oils of clove (*Syzygium aromaticum*) (10% active ingredient) plus makaen (*Zanthoxylum limonella*) (10% active ingredient) gave protection for 4-5 hours. In the case of free flying mosquitoes, products with 20% and 30% PMD gave complete protection for 11-12 hours at a dosage of 1.7 mg/cm^2 or 6 hours at half the dosage, while the product with 10% PMD afforded protection for less than 2 hours. At the higher dosage rate 40% citronella and hydroxyethyl isobutyl piperidine carboxylate, a new synthetic compound, provided complete repellency for 7 hours. Fifty percent deet (*N,N*-diethyl-3-methylbenzamide) was effective for 30 hours if left undisturbed on the skin.

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 (*N,N*-diethyl-3-methylbenzamide) may be considered as the standard repellent. There has been concern about rare reports of severe reactions to this substance. Deet melts hard plastics, and many consumers do not like its odor and the sensation it creates on the skin.

Plant extract based products are favored by many consumers. Citronella from *Cymbopogon nardus* is an example of a widely available product with repellency properties. 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 and that *p*-menthane-3,8- 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 PMD in laboratory cages and in the field showed that it was 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. Mospel, a new plant product from Thailand, was included in the tests. The products were applied to the arm or leg of a human subject at the dosage, product per unit area, which is the industry standard and, in one trial, at 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

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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 was a product in development by the Insecticide Research Unit, Mahidol University, Thailand. This product contains 10% clove oil and 10% makaen oil as the active ingredients in a gel form. Clove oil, extracted from *Syzygium aromaticum*, and makaen oil, 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*, 2005).

Dosage of the repellents

According to the industrial standard for deet based repellents, the application dosage for each candidate repellent was 1.0 g 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 g was applied. It was found preferable to weigh the repellents rather than pipetting them. The very viscous nature of the compounds made them hard to apply at a uniform rate when pipetting. This dosage from a pump spray was sprayed onto a sheet of aluminum foil and collected on a Petri dish on a balance; pump spraying continued until 2.6 g 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 of Hygiene and Tropical Medicine. The colony was maintained at a temperature of 27± 2ºC at a relative humidity of 60-80% under a 12:12 light and dark cycle and provided with horse blood through an artificial membrane.

Feeding 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.

Arm exposure cage test

One gram of product was applied onto the forearm of a human subject. The treated arm was exposed for 1 minute to 30 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 test procedure was only used for the comparison of Repel Lemon Eucalyptus, Off! Botanicals and Mospel.

Walk-in exposure room test

The room at London School of Hygiene and Tropical Medicine measuring 3x2x2.5 m was maintained at a temperature of 26±2ºC 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 minutes 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.

An aliquot of 2.6 g 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, gloves and shorts covering the thighs. After release of 30 female mosquitoes the subject sat on a bench in the middle of the

room for 10 minutes in each hour and any landing and biting mosquitoes were caught and counted. After finishing a 10-minute 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 minutes 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.

The results were analyzed according to the following equation:

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

where C is the number of mosquitoes collected from the control area and T is the number collected from the treated area of a subject.

RESULTS

Arm exposure cage test

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 minutes. 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).

Walk-in exposure room test

When 1 g 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 only 1-2 hours. These durations were somewhat greater than in the arm exposure cage tests, but the marked superiority of the 20%

Table 1
List of repellent 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 menthol
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[a]
8. Jungle formula Insect Repellent (Chefaro, UK)	DEET, 50%
9. Mospel (Mahidol University, Thailand)	Clove oil, 10% plus makaen oil (*Zanthoxylum limonella*), 10%

[a]Concentration not specified.

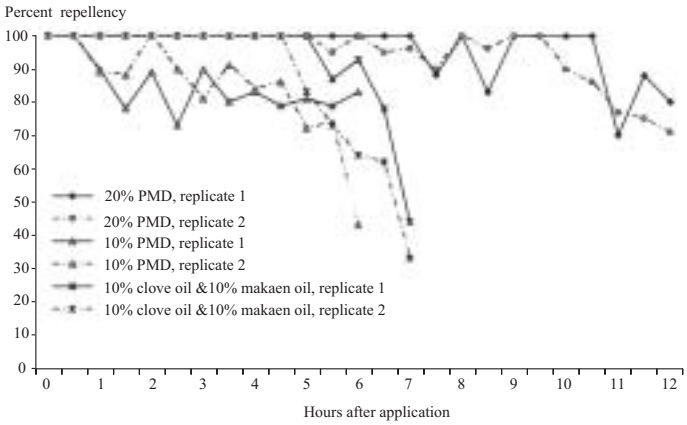

Fig 1–Percent repellency of 20% PMD (Repel Lemon Eucalyptus), 10% PMD (Off! Botanicals) or 10% clove oil plus 10% makaen oil (Mospel) against *An. stephensi* applied to an arm and tested in a cage of mosquitoes. The arm was observed at intervals after application, relative to contemporary control.

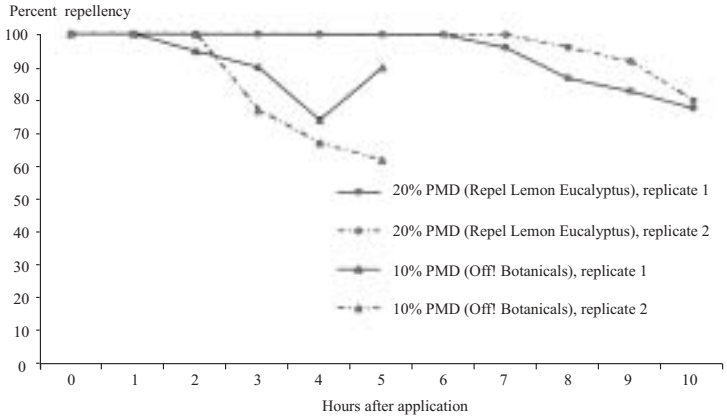

Fig 2–Percent repellency of 20% PMD (Repel Lemon Eucalyptus) and 10% PMD (Off! Botanicals) applied to lower legs and with free flying *An. stephensi* in a mosquito proof room, observed at each hour after application, relative to initial biting rate on untreated legs.

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 hydroxyethyl isobutyl piperidine carboxylate (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, providing 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 application 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; Fradin, 1998). *Anopheles stephensi*, the mosquito used for these tests, 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 Southeast Asia. The main motive for most purchases of repellents is avoidance of mosquito nuisance, with the contracting of 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).

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

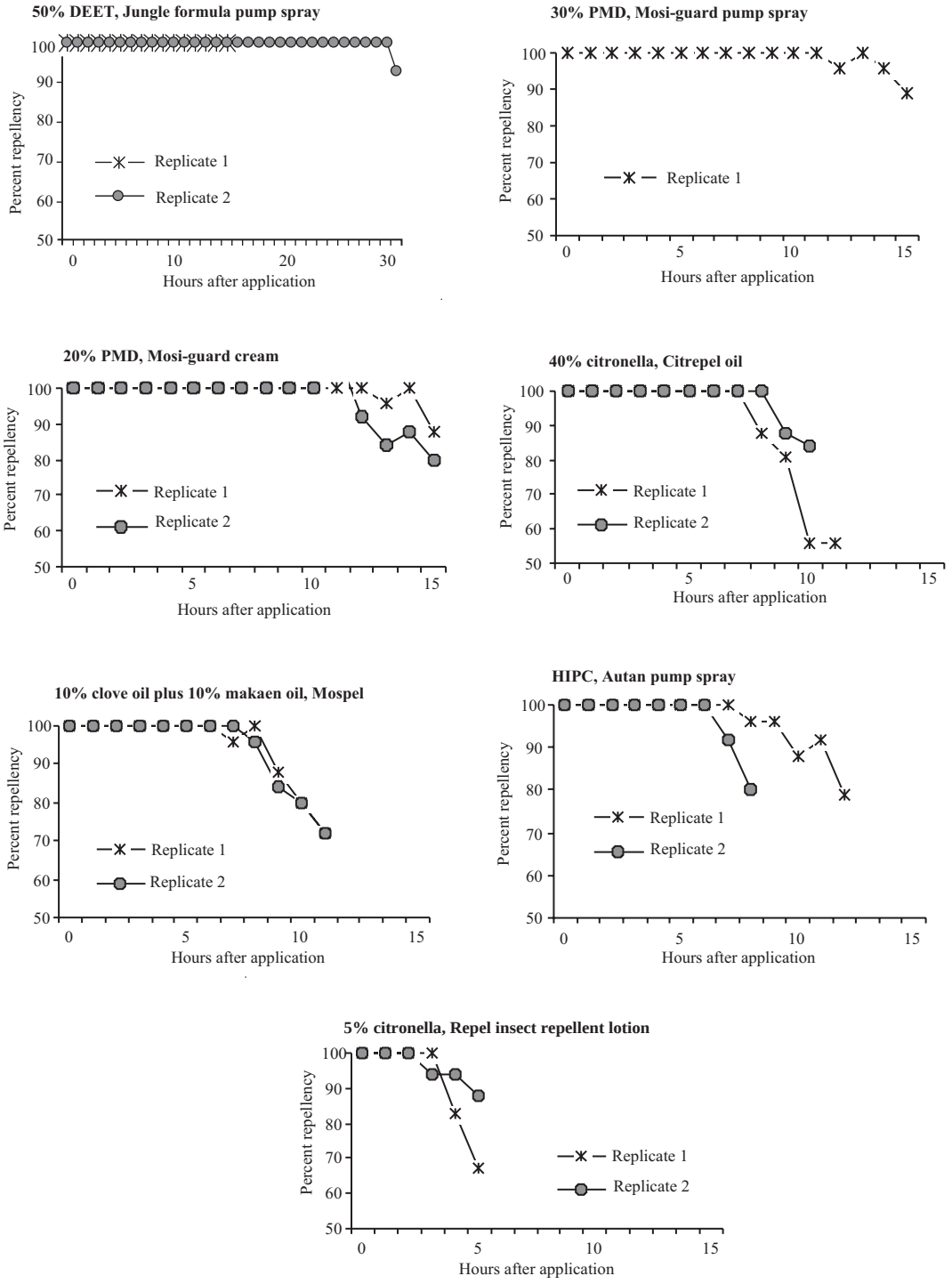

Fig 3–Percentage repellency of 2.6 g of 7 repellent formulations applied to lower legs and with free flying *An. stephensi* in a mosquito proof room, observed at each hour after application, relative to initial biting rate on untreated legs.

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 (Figs 2 and 3). In addition to conventional arm exposure 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).

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 (Buescher *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. Thavara *et al*, (2001) reported that a lower dose per cm^2 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, *eg* 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, Qiu *et al* (1998) found deet to exhibit a good margin of safety. 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 g/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 g/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 is commonly 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 (Yap *et al*, 2000; Thavara *et al*, 2001; WHO, 2001). It has been claimed that this synthetic repellent showed mosquito repellency equal to or exceeding 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*, the pure oil of citronella gave protection for 2 hours, but this declined to 59.3% after four hours (Govere *et al*, 2000b). 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% citronella 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 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*, 2005), 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*, 2005). The gel dosage form of Mospel showed significant repellency under field conditions in Thailand. This gel provided complete protection for 4 hours and gave 95.7% repellency after 5 hours application against *Ae. aegypti*, daytime-biting mosquitoes. For nighttime biting, the gel yield average 97.1% repellency for 5 hours against the predominant *Cx. quinquefasciatus* and *Mansonia uniformis*, but it gave 89.0% repellency against *Cx. tritaeniorhynchus* and *Cx. gelidus* (Trongtokit *et al*, 2004). 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.

Forty percentage 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 in 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 investigations of formulations of plant-based repellents are needed. There is a need 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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