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Thanaka (*Limonia acidissima*) and Deet (di-methyl benzamide) mixture As a mosquito repellent for use by Karen women

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Abstract. The prevention and treatment of drug-resistant malaria is becoming increasingly difficult. On the Thai-Myanmar border multi-drug resistant strains of falciparum malaria are increasing and, because the malaria vector *Anopheles* bite outdoors during early evening, insecticide house-spraying or impregnated bednets provide only limited protection. Therefore, the protective efficacy of repellent formulations containing di-methyl benzamide (deet) and permethrin against local vectors was estimated, when applied to the skin, and their acceptability amongst pregnant Karen women who are at relatively high risk from malaria was assessed. Human landing catches of mosquitoes showed that almost complete protection was achieved using different formulations of 20% deet and 0.5% permethrin for up to 6 h. All-night collections from human subjects indicated that this repellent combination reduced exposure to malaria parasites by at least 65 and 85% for those transmitted by *Anopheles minimus* and *An. maculatus*, respectively, the two principal vectors in this area. Pregnant women in the camps preferred repellents which were mixed with 'thanaka', a root paste made from pulp of the wood apple tree, *Limonia acidissima*, used locally as a cosmetic. Apart from a temporary warming sensation where repellent thanaka was applied to the skin, the repellents were well tolerated. An intervention trial is currently in progress to determine whether deet mixed with thanaka can protect pregnant women against malaria in this part of the world. Bioassays using a laboratory strain of *Aedes aegypti* demonstrated that thanaka is itself slightly repellent at high dosages and the mixture with deet provides protection for over 10 h. This treatment would therefore also provide some personal protection against dengue, which is increasing locally, transmitted by *Ae. Aegypti* and *Ae. Albopictus* biting during the daytime.

Key words. *Aedes aegypti*, *Anopheles maculatus*, *Anopheles minimus*, *Limonia acidissima*, deet, di-methyl benzamide, permethrin, dengue, malaria, mosquito repellents, thanaka, Thailand.

Introduction

Malaria is a major cause of illness and death for pregnant women and is an important cause of low birth weight (Gilles *et al.*, 1969; McGregor, 1984; Brabin, 1991; Dolan *et al.*, 1993; Menendez, 1994). Along the Thai-Myanmar border, about 37% of Karen women experience at least one attack of malaria during pregnancy and, despite early detection and treatment, this infection results in material anaemia and low birthweight children (Nosten *et al.*, 1991). The treatment of pregnant women in this area is becoming progressively more difficult since falciparum malaria has developed resistance to a wide range of antimalarial drugs (White, 1992). Most infections nowadays fail to respond to treatment with chloroquine or mefloquine; there is a 20% failure rate when pregnant women are treated with quinine (F. Nosten, unpublished data) and doxycycline is contra-

indicated during pregnancy. As antimalarials fail, other methods of protection need to be developed. Several other mosquito-borne diseases are also increasingly important in Thailand, notably dengue transmitted by *Aedes aegypti* (L.) and Japanese encephalitis transmitted by various mosquitoes.

Vector control and personal protection against mosquitoes are important options. Control programs are often directed at killing endophilic mosquitoes by spraying insecticides indoors or by using insecticide-impregnated bednets (IBNs). However, the initial success of house-spraying in Thailand has declined as local malaria vector *Anopheles spp.* have apparently adapted to avoid the insecticide by biting earlier and more outdoors (Somboon *et al.*, 1995) and home-owners are increasingly reluctant for their houses to be sprayed (Nutsathapana *et al.*, 1986). IBNs have been only partially successful in the Karen community, reducing anaemia in pregnant women, but having no demonstrable effect on incidence of parasitaemia (Dolan *et al.*, 1993). One reason for the limited efficacy of IBNs may be due to infective bites received outside the nets.

If mosquito biting during the early evening accounts for this, then insect repellents could be effective for personal protection against malaria vectors and other mosquitoes. The present investigation was undertaken to determine the efficacy and acceptability of various formulations and dosages of deet (di-methyl benzamide, formerly di-ethyl toluamide) and permethrin, applied to the skin as insect repellents, for protecting pregnant women against mosquitoes on the Thai-Myanmar border. Deet was selected as it can be protective against a wide range of biting insects (Curtis, 1989) and is the most widely used repellent in the world today (Veltri *et al.*, 1994). It is also long-lasting, remaining active on the skin for 4- h and is safe for use by adults (WHO, 1991). The addition of permethrin, a pyrethroid insecticide with repellent qualities (Lindsay *et al.*, 1991), to the mixture was thought likely to enhance protection by killing mosquitoes landing on treated skin. We also developed a popular formulation of repellents based on the traditional local cosmetic thanaka, *Limonia acidissima* L. (Rutaccae).

Materials and Methods

Study area and population

Field studies took place at Shoklo (17°38' N, 97°50' E). 120 km north of Mae Sod town, in an area of hill forest on the Thai-Myanmar border. During our study in 1994, Shoklo harboured a community of *c.* 4500 displaced people of the Karen ethnic group. Shoklo and the study population were described by Nosten *et al.* (1987). Houses were constructed from bamboo with a roof made of leaves. Pregnant women were recruited from adjacent camps at Bono, Mae Salit and Klee Ma Klu.

Fig. 1. Thanaka (*Limonia acidissima*) applied to the face.

Mosquito biting activity

Three catching stations of a style similar to Karen houses were constructed in different parts of the camp. Landing mosquitoes were collected from the exposed limbs of adult male collectors using small tubes and torchlight. All collectors were offered prompt diagnosis and treatment of fever – although none were ill during the study. At each station two collectors sat outdoors and two indoors from 18:00 hours until midnight. At midnight the teams were replaced by teams of two men sitting indoors at each station until 06.00 hours the following morning. In addition, two men collected mosquitoes outdoors at each station from 05.00 to 07.00 hours. This routine was thought to give relevant information on the numbers of mosquitoes biting during periods when people were indoors and outdoors during the night. Collections were made for 2 consecutive nights every fortnight from May to October 1994, i.e. throughout the rainy season.

Treatment preferences and adverse reactions

Focus group discussions (WHO, 1992) were held with Karen women and used as a pilot study to assess the acceptability of a range of different repellent formulations. Then groups of twenty-six to thirty-one pregnant women were recruited from antenatal clinics at three camps: Bono, Klee Ma Klu and Me Salit. Each woman received one of five treatments containing 20% deet and 0.5% permethrin as repellents: (i) Repel plus® (The

Boots Company Plc, Nottingham, U.K.) spray formulation (ii) Repel plus® roll-on formulation, (iii) thanaka, 1.128 g/ml in water. (iv) thanaka 1.128 g/ml in 70% ethanol, (v) repellents diluted in coconut oil. Thanaka suspended in water (1.128 g/ml) was used as a control. Thanaka is a paste made from pulp of the wood apple tree, *Limonia acidissima*, habitually used by the Karen as a cosmetic applied to the face (Fig. 1) and arms. Fifty ml of each formulation was prepared in a glass bottle, weighed and randomly distributed to the women. They were asked to mix the contents of the bottle by shaking and to apply each solution to their legs and arms at dusk each night. Bottles were returned one week later and weighed to measure how much of the formulation had been used. A questionnaire survey was used to record the responses of the women to each treatment.

Repellency field trial

The trial was carried out on the edge of the camp, next to the forest, in September and October 1994. Three repellent mixtures of 20% deet and 0.5% permethrin were tested: (i) commercial spray formulation (Repel Plus®), (ii) thanaka mixed with water (1.128 g/ml), and (iii) thanaka mixed with 70% ethanol (1.128 g/ml). Solutions of 70% ethanol alone and thanaka mixed with water (1.128 g/ml) served as two controls. Exposure to mosquitoes was measured using adult male human subjects. For each trial, the collectors applied 4 ml of the test or control solutions to their exposed limbs (0.75 ml to each forearm, 1 ml to each leg and 0.25 ml to each foot), nightly. Pair of men with the same treatment sat next to each other and collected mosquitoes using a sucking tube and torchlight from 18.00 hours to midnight. Each pair sat 10 m from the next, with chairs arranged along a straight line. To identify the effects related to each treatment and allow for other sources of variation, the trial was based on a series of Latin squares (Fisher, 1942). At the end of the 25 night trial, each pair of men had used each treatment in each of the seating positions.

Bioassays

Laboratory experiments were carried out using a strain of *Aedes aegypti* (L.) from the London School of Hygiene and Tropical Medicine. The first assay was designed to test whether thanaka repelled mosquitoes. Batches of thirty-five female mosquitoes 1-2 weeks old, previously fed only on sultanas and water, were placed in a cage measuring 38 x 52 x 67 cm and allowed access to water, but not food. The following morning at 08.30 hours, a human volunteer inserted their right forearm, with their hand protected by a surgical glove, into the test cage. The number of mosquitoes settling on the forearm was counted after 30 s. Immediately thereafter, 50 mg of thanaka was made into a paste with 2 ml of water, applied to the same arm, and dried in a warm air current. The arm was reintroduced into the cage and the number of mosquitoes settling on the treated forearm after 30 s was recorded. This procedure was repeated for thanaka dosages of 100, 250, 500 and 1000 mg on the same forearm. Approximately 1 hour after the primary dose, the untreated left forearm was exposed for 30 s. To assess whether there had been a deterioration in the attempted feeding activity of *Ae. Aegypti* over time. The treated right arm was re-exposed hourly for the following 6 hours. At the end of this period, the untreated arm was reintroduced to assess feeding avidity. Thanaka tests were replicated at all five dosages using the same adult human volunteer on six occasions.

The second assay was performed to determine whether thanaka prolonged the effective life of deet compared with a water-based deet formulation. The procedure was similar to that described above, but used two cages of mosquitoes to compare the repellency of each treatment. One forearm was treated with a 20% deet solution (containing 155 µl of 97% deet in 0.75 ml of water), the other with the same volume of deet diluted in 1.845ml of water and mixed with 1 g of thanaka. The number of mosquitoes landing on both arms after 30 s was recorded pre-application and 0, 2, 4, 6, 8 and 10 h after applying the repellents. This experiment was also replicated on six separate occasions.

Statistical analysis

Hourly counts of mosquitoes collected at each station were halved to produce an estimate of biting rates per person. In all analyses, counts were log transformed [$\ln(n + 1)$] to normalize the data. The average number of mosquitoes collected by one man each hour was calculated separately for indoors and outdoor collections during the study. Each hourly mean was then back transformed to produce a geometric mean. The proportion of mosquitoes biting before midnight was adjusted to take into account the fact that most adult people went indoors at 21.00 hours and came outside again at 05.00 hours.

In the repellency trial the protective efficacy for each treatment was calculated as:

$$\Sigma[(ma^c - ma^i) / ma^c]100/ n,$$

where ma^c is the number of mosquitoes collected on the control subject, ma^i is the number with the repellent and n is 25, the number of nights in the trial.

For analysis of the bioassay results, proportions were arcsin transformed to normalize the distributions. Comparisons between treatments were made using the Mantel-Haenszel summary test for the six 2 x 2 tables.

Results

Biting activity

Among 288 female anopheline mosquitoes collected landing on unprotected human subjects, the three major malaria vectors comprised 174 *Anopheles maculatus* Theobald, 77 *An. minimus* Theobald and 12 *An. dirus* Peyton & Harrison. The nocturnal biting activity of the two most abundant vectors, *An. maculatus* and *An. minimus*, is shown in Fig. 2. Overall, 65% of *An. minimus* came before midnight and 22% outdoors before 22.00 hours, when people normally go to bed. The respective figures were 85 and 66% for *An. maculatus*.

Treatment preferences

Responses of pregnant women to different treatments are summarized in Table 1. Women used greater quantities of some treatments than others (ANOVA, $F = 12.86$, d.f. = 51.59 $P < 0.001$). Significantly less roll-on Repel plus ® formulation was used when compared individually with the other treatments (least significance test, $P < 0.005$).

Adverse effects

The use of repellents mixed with thanaka was just significantly associated with a transient warming sensation of the skin (Table 2), when compared with the solution of thanaka alone, i.e. $X^2 = 4.02$, $P < 0.045$ for repellents mixed with thanaka and water. Fisher's exact test. $P = 0.05$ for repellents mixed with thanaka and ethanol.

Repellency field trial

Significant differences were found between the protective efficacy of all treatments, including the controls (ANOVA, $F = 136.5$, d.f. = 4, $P < 0.001$). However, all repellents were similarly effective (ANOVA with only repellents, $F = 1.068$, d.f. = 2, $P = 0.356$). The protective efficacy of 20% deet plus 0.5% permethrin against all mosquitoes (anophelines plus culicines) was: (i) 98.8% with the Repel plus ® spray formulation (95% ci = 96.7-99.9%), (ii) 99.6% with thanaka and water (95% CI = 98.3-100%), and (iii) 98.2% with thanaka and ethanol (95% CI = 95.9-99.6%). Similar numbers of mosquitoes were obtained from collectors using either of the two control solutions, the geometric mean (GM) total mosquitoes/pair/night being: for thanaka with ethanol, GM = 2.11 (95% CI = 1.78-2.44) or ethanol alone, GM = 2.44 (95% CI = 2.12-2.76). Similar numbers of anophelines were also caught using either of the two controls: thanaka with ethanol, GM = 0.34 (95% CI = 0.13-0.55) or ethanol alone, GM = 0.25 (95% CI = 0.07-0.44).

Bioassays

Increasing doses of thanaka applied to the forearm resulted in significantly fewer *Ae. Aegypti* females landing on the skin (Fig. 3; X^2 for trend = 70.5 d.f. = 1, $P < 0.001$) in the laboratory experiments. Part of this reduced feeding avidity may be explained by a general decline of mosquito landing activity during the course of this experiment, as significantly fewer mosquitoes landed on the untreated arms at the end of the experimental period, compared to the beginning (46% reduction; $X^2_{M-H} = 10.6$, d.f. = 1, $P = 0.001$). Nonetheless, repellency was demonstrated as significantly fewer mosquitoes landed on the forearm treated with the higher dose of 500 mg (61% reduction; $X^2_{M-H} = 8.14$, d.f. = 1, $P = 0.004$) and 1000 mg of thanaka (61% reduction; $X^2_{M-H} = 8.10$, d.f. = 1, $P = 0.004$).

compared with the untreated arm at the end of the period. The repellent properties of the 1000 mg dose of thanaka deteriorated over the six hour follow-up period (Fig. 4; X^2 for trend = 18.6, d.f. = 1, $P = 0.001$). Breakdown of protection became apparent 4 h post-application, because landing rates at this time were significantly greater than immediately after application ($X^2_{M-H} = 15.4$, d.f. = 1, $P = 0.001$).

Both deet formulations provided complete protection against mosquitoes immediately after application (Fig. 5). However, the repellency of deet in thanaka lasted at least 10 h following application and was significantly more repellent at this time than deet in water ($X^2_{M-H} = 8.3$, d.f. = 1, $P = 0.004$).

Fig. 2. Biting cycles of the two prevalent anophelines in Shoklo.

Time of biting

Fig. 2. Biting cycles of the two prevalent anophelines in Shoklo.

Table 1. Treatment preferences expressed by pregnant Karen women.

Response		Treatment					
		Thanaka And water (control)	Repellents ^a thanaka ^b and water	Repellents ^a thanaka and ethanol	Repellents ^a Spray formulation	Repellents ^a roll-on formulation	Repellents ^a in coconut oil
Liked (%)	smell	84.6 (22/26)	83.3 (25/30)	74.1 (20/27)	66.7 (20/30)	51.9* (14/27)	41.9* (13/31)
Liked (%)	feel	88.5 (23/36)	75.9 (22/29)	78.6 (22/28)	86.7 (26/30)	63.0 (17/27)	58.1* (18/31)
Amount used/day (%)		4.26 (3.24-5.60)	5.27 (4.33-6.68)	7.57 (6.25-9.17)	2.59 (1.89-3.55)	1.65 (0.00-2.81)	5.28 (4.05-6.89)
Preferred ^d (%)		-	51.3 (78/152)	-	17.1* (26/152)	20.4* (31/152)	3.3* (5/152)

² 20% deet and 0.5% permethrin, ^b thanaka 1.128 mg/ml. ^c 95% CIs are shown in parentheses. Significant difference between the control and test groups are indicated thus * = $P < 0.05$. ** = $P < 0.01$, ^d eight women did not have a preference and stated that they would use any of the repellents, and four did not want to use any of the repellents.

Table 2. Adverse effects reported by pregnant women using repellents and control formulations.

Response %		Treatment*					
		Thanaka and water (control)	Repellents, thanaka and water	Repellents, thanaka and ethanol	Repellent spray formulation	Repellent roll-on formulation	Repellents in coconut oil
No reaction	appreciable	80.8 (21/26)	80.0 (24/30)	92.9 (26/28)	70.0 (21/30)	63.0 (17/27)	66.7 (20/30)
Dizziness/head- ache/nausea and other symptoms		19.2 (5/26)	20.0 (6/30)	7.1 (2/28)	30.0 (9/30)	37.0 (10/27)	33.3 (10/30)
No skin reaction		80.8 (21/26)	60.0 (18/30)	57.1 (16/28)	80.0 (24/30)	70.0 (19/27)	80.0 (24/30)
Warm sensation of		7.7	33.3*	28.6*	16.7	22.2	13.3

skin						
	(2/26)	(10/30)	(8/28)	(5/30)	(6/27)	(4/30)
Skin redness	3.8	0	0	0	0	0
	(1/26)	(0/30)	(0/28)	(0/30)	(0/27)	(0/30)
Skin itching	0	6.7	7.1	0	3.7	3.3
	(0/26)	(2/30)	(2/28)	(0/30)	(1/27)	(1/30)
Other skin reactions	7.7	0	7.1	3.3	3.7	3.3
	(2/26)	(0/30)	(2/28)	(1/30)	(1/27)	(1/30)

* Significant differences between the control and treatment groups are indicated thus * $P < 0.05$.

Thanaka dose (mg)/arm

Fig. 3. Proportion of *Ae. Aegypti* landing on the right arm (solid bars) before and after treatment with different dosages of thanaka, and on the left untreated arm (open bar) at the end of the study period.

Fig. 4. Proportion of *Ae. Aegypti* landing on the right arm (solid bars) after treatment with a 1 g dose of thanaka, and on the left untreated arm (pen bars) at the beginning and end of the study period.

Fig. 5. Proportion of *Ae. Aegypti* landing on the right arm (solid bars) before and after treatment with a 1 g dose of thanaka and deet, and on the left arm (open bars) before and after treatment with deet.

Discussion

Repellents are best applied in the early evening for protection against malaria vectors since, at least in this part of Thailand, a substantial proportion of vector biting occurs before midnight - as reported by workers in other parts of the country (Harbach *et al.*, 1987; Ratanatham *et al.*, 1988; Upatham *et al.*, 1988; Somboon *et al.*, 1995). In field tests, deet plus permethrin gave almost total protection against mosquitoes from dusk until midnight and we estimated that this would reduce exposure to the risk of malaria transmission by up to 65% for *An. minimus* and 85% for *An. maculatus*. Our bioassay result show that when deet is mixed with cosmetic thanaka the period of repellency is prolonged, indicating that protection may be substantially greater than these figures suggest. A combination of repellents and IBNs could result in an appreciable decline in malaria transmission, with continuing risks occurring mostly early in the morning after people leave the protection of their nets. On the other hand, repellents divert mosquitoes from users, and may increase the risk of exposure to non-users.

Fewer mosquitoes landed on people using thanaka than those without thanaka. However, the difference was small and only reached statistical significance under controlled laboratory conditions. Our bioassay results showed that, at high dosages, thanaka itself was weakly repellent for about 3 h after application. As the cosmetic appearance of thanaka skin treatment is a very visible pale 'make-up' layer, its protective value may have been due to physical deference (and masking of attractant odours) rather than true repellency. The addition of deet to thanaka provided long-lasting protection against mosquitoes under both field and laboratory conditions. Bioassays demonstrated that thanaka plus deet provided protection for about 10 h. Substantially longer than the 3 h protection when deet was used alone. Presumably the thanaka powder layer on the skin was acting as a slow release formulation for deet.

Repellent preparations mixed with thanaka were preferred by pregnant women, since this is a popular cosmetic used by Karen people. Traditionally, thanaka paste is mixed with water and applied mainly to the face. Although thanaka is used most commonly by women, children and men also use it sometimes. As well as a cosmetic, it may also be used to remove small spots or lesions which appears on the skin. This may be an effective form of antiseptic, as the leaves and fruit of *Limonia acidissima* contain fungicides and bactericides (Bandara *et al.*,

1988, 1990; adikaram *et al.*, 1989). Interestingly, thanaka may also have antiplasmodial activity (M. Simmonds, personal communication) and, at high concentrations, it is insecticidal (Bandara *et al.*, 1990).

Although deet and permethrin mixed with thanaka was protective and well accepted by most women, we identified a number of potential problems related to its use. Firstly, women reported a temporary warm feeling when large amounts of the repellent mixture was applied to the skin. This is of some concern, since contact dermatitis due to deet has been reported in a few sensitive individuals (Lamberg & Mulrennan, 1969; Maibach & Johnson, 1975; Reuveni & Yaagupsky, 1982; Von Mayenburg & Rakowski, 1983; Amichai *et al.*, 1994). Although three women stopped using the repellents prematurely, most women used the mixture daily for one week. Secondly, large amounts of thanaka repellent mixture were used by women in our study, well over twice the amount compared with the roll-on commercial formulation. It was unclear, however, whether this was the actual amount of thanaka applied or whether some was hoarded or used by other members of the family. Thirdly, during this study we asked individuals not to apply the mixture to the face, only the exposed parts of the legs and arms. Thus, although we offered a substance which was popular amongst the Karen, we asked people to use it in an unusual fashion, which may cause problems with compliance or correct application during extended use of the repellent. Lastly, little is known about the effect of these compounds on the human foetus. To our knowledge, one birth abnormality associated with deet (and chloroquine prophylaxis) has been reported (Schaeffer & Peters, 1992). Yet, on the other hand, this repellent has apparently been used safely by millions since 1956 and some 30% of the population of the United States use it each year (Veltri *et al.*, 1994), many of whom are pregnant. Moreover, there is no evidence that deet has any reproductive nor developmental toxicity in rats (Wright *et al.*, 1992) and it is rapidly excreted from the foetus (Robbins & Chermiack, 1986). On the evidence to date it appears that deet is safe to use by pregnant women.

Repellents may prove to be a particularly suitable method for protecting pregnant women against malaria in the Karen camps, as compliance is likely to be high in this group of well-motivated women. Moreover, because women usually begin attending these clinics at the beginning of the second trimester, when the foetus is less vulnerable to teratogenicity, any risk to the foetus should be reduced. The cost of chemicals for the thanaka repellent was 7 US cents/day or US\$12.60 for protecting one woman for the last 6 months of her pregnancy. This compares favourably with the high cost of mefloquine prophylaxis of about US \$32 for the same period. We have therefore commenced an intervention trial to assess whether a mixture of deet and thanaka can protect pregnant women from malaria, transmitted by nocturnal *Anopheles* mosquitoes in this part of the world. The efficacy of deet and thanaka mixture as a repellent against *Ae. Aegypti* also merits daytime evaluation for personal protection against potential transmission of dengue and other arboviruses.

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