PRELIMINARY STUDY OF HERBAL TOPICAL LOTION REPELLENT MADE OF BETEL LEAVES (*Piper betle*) AND PATCHOULI OIL (*Pogostemon cablin*) MIXTURE AGAINST YELLOW FEVER MOSQUITO (*Aedes aegypti*)

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Received 25 February 2014/Accepted 7 December 2014

ABSTRACT

Exploration of plants as natural repellent against yellow fever mosquito (*Aedes aegypti*) that transmits dengue virus to human is still under way. Betel leaves (*Piper betle*) could be used as repellent material. The study was aimed to test the repellency, potency, and safety of topical lotion repellent of betel and patchouli oil mixture against yellow fever mosquito. This study used nulliparous of 3-5 days old female yellow fever mosquito. Before the study, irritation test was conducted as safety test. Five treatments with five replications was implemented to seek the most effective repellent substance i.e. DEET lotion for positive control, betel leaves and patchouli oil mixture lotion, betel leaves only lotion, patchouli oil only lotion and base lotion only. The substance effectiveness was determined based on the rejection of yellow fever mosquito to bite human's arms and was analyzed using protective percentage. The irritation test showed no safety concerns. Protective percentage analysis showed that modified betel leaves lotion had mean protective power of over 90% for 6 hours. The data showed that the modified lotions had the same protective percentage as the DEET (ratio: PP (betel+patchouli)/PP (DEET) =0.98; 95% confidence interval 0.93, 1.04; *p*-value=0.50). This indicated that betel leaves mixed with patchouli oil is potential to be used as safe repellent against yellow fever mosquito.

Keywords: *Aedes aegypti*, betel leaves, DHF, formulations, lotion, repellent, yellow fever mosquito

INTRODUCTION

Utilization of synthetic insecticide can cause a resistance of the exposed mosquitoes towards the insecticide compounds when used continuously. Insecticides commonly used are available in spray, burnt, electric and lotion forms. Spray, burnt, and electric insecticides have dangerous effects to health through inhalation which vapor may easily enter the respiratory system and may even enter the blood stream. Various effects including nerve, liver, and respiratory disorders may happen. Long-term use may cause cancer (Zaim & Guillet 2002).

Lotion repellent is one of alternative insecticides that do not harm the respiratory system because it is directly applied to the skin. The lotion is used to discourage mosquitoes from biting human's skin. Generally, the marketed lotion contains active chemical substance called diethyl toluamide (DEET). This substance is only allowed in Indonesia at the concentration of 15%. DEET has negative effects i.e. skin irritation, hives (urticaria), and even brain malfunction (encephalopathy). The use of essential oils as direct repellents is less effective because of its volatile nature. Therefore, alternative repellent form may be developed to lengthen the repellent's durability.

In this study, essential oil used as fixative agent in developing mosquito repellent lotion was patchouli oil extracted from patchouli (*Pogostemon cablin*) . Lotion is a clear preparation that does not leave stain on its user. In addition, lotion is a liquid form, thus facilitating the active ingredient inside the lotion to be evenly absorbed through the skin providing protection to its user. Adding fixative

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agent to repellent substance increases the potency of repellent lotion to function as long as DEET lotion. Patchouli oil has 100% protection against yellow fever mosquito (*A. aegypti*) for 2 hours under laboratory condition. Betel leaves (*Piper betle*) are among therapeutic plants growing in Indonesia. The leaves contain saponin, phenolic, alkaloid, and substance that can be used as mosquito repellent.

Eugenol is essential oil extracted from clove leaves proven to control mosquito larvae and adult mosquitoes or repel mosquitoes. Betel leaves contain lower concentration of eugenol (30%) compared to eugenol concentration in clove leaves (71%). The objective of this study was to test the repellency, potency and safety of topical lotion repellent of betel and patchouli oil mixture against yellow fever mosquito.

MATERIALS AND METHODS

Rearing Nulliparous Mosquitoes

Colonies of yellow fever mosquito (*A. aegypti*) were reared in the insectarium at Laboratory of Health Research, Ciamis Unit of Vector Borne Diseases Research, National Institute of Health Research and Development, Indonesian Ministry of Health at Pangandaran, Ciamis, Indonesia. The hatched larvae were held in plastic trays and larval diet was added to each tray. Newly emerged pupae were transferred to screen cage (size 30x30x30 cm^3) and emerged as adult mosquitoes. These adult mosquitoes were kept in the insectarium with ambient temperature (25-30 $^{\circ}C$) and were provided with soaked cotton balls containing 5% multivitamin solution.

Production of Betel+Patchouli Mixture Lotion

The first lotion base solution was produced by weighing 3 g of Carbopol Ultrez 10, adding it to 10 mL water until it dispersed. Stir the solution completely until there were no trapped air bubbles. Finally, add 0.50 g trietanolamine into the solution. This was called the first lotion base solution. The next step was adding 2 g of glycerine to the first lotion base solution while being stirred to achieve homogenous substance. Then, the remaining 0.25 g of trietanolamine was added. This was called the second lotion base solution. The next step was diluting 0.10 g methyl paraben in 10 mL of water which was then added to the second lotion base solution and stirred to achieve homogenous mixture. This was called the final lotion base solution. Afterwards, 2 mL of betel oil in 95% ethanol was mixed with 0.2 mL patchouli oil in 95% ethanol and then was added to the final lotion base solution while being stirred until the solution became homogenous to obtain the formula of anti-mosquito lotion with optimal concentration. After all of ingredients were homogenously mixed, 80 mL distilled water was added to the mixture. Subsequently, pH was measured and verified at the range of 4-7.

Test on Human Subject

For lotion safety tests, 20 adult male and female volunteers with age range of 25-45 years old and body weight range of 50-70 kg were recruited. For the efficacy tests, other five adult male volunteers with age range of 25-45 years old and body weight range of 50-70 kg who had no history of allergy to arthropod bites were recruited. Before signing an informed consent form, the volunteers were interviewed and explained about the methodology, probable discomforts and remedial arrangements.

Testing the Biological Stability and Safety

Lotions were stored in closed vials for up to six months and stability of the fraction was determined in room conditions at varied temperatures. Testing lotion safety was conducted on 20 volunteers. Their skin was applied with 4 treatments for 4 days, one treatment each day. The treatments were DEET, modified (betel + patchouli mixture) lotion, betel lotion (2% betel mixed with lotion), patchouli lotion (0.2% patchouli mixed with lotion). Lotion was applied and the reaction was observed for 6 hours after application.

Laboratory Repellent Bioassay

The lotions were tested for repellency against yellow fever mosquito (*A. aegypti*) by following Indonesian Pesticide Commission guidelines under laboratory conditions. Six hundred nulliparrous 3 to 5 days old female mosquitoes were taken from a stock cage using an

aspirator and placed inside six cages. Each cage had a length of 50 cm, width 35 cm and height 40 cm and was made of nylon netting, iron wire framed, with 2 holes on the front to insert hands. Each cage consisted of 100 mosquitoes. The night before being exposed to the blood meal they were deprived of the multivitamin solution and supplied with water pads only. Six hours prior to the blood feeding, the water soaked cotton pads were removed. The assays were done in a well-ventilated room that is equipped with 6 tables and 6 chairs, with temperature of 26-30 ^{o}C and relative humidity around 60-80%.

Thirty minutes before starting the test, the left forearm of a human subject was treated with repellent. There were 5 test repellents i.e. lotion base, DEET, betel+patchouli mixture, betel 2%, and patchouli 0.2%. Each repellent was tested on one subject. One gram of repellent was equally applied on 650 cm^2 of skin surface area between the left elbow and wrist. The area between the elbow and wrist served as negative control by applying water on the skin surface. A latex glove was worn over the hand to protect from mosquito bites. Tests were conducted by placing the repellent-treated (left) forearm into a test cage for 5 minutes, and any mosquitoes that had taken a blood meal were replaced. The control (right) forearm was placed into the test cage for 5 minutes, immediately after placing the repellent-treated (left) forearm into the test cage. These tests were conducted at 60 minutes intervals for 6 hours starting from the moment when the treatment was applied (hour 0: 08.00 am), and the number of landing mosquitoes was recorded. Landing mosquitoes were blown from the arm, in an effort to stop any blood from being taken. Repellency data were presented as protective percentage (PP). The whole procedure was repeated on the same volunteers in 5 separate days. During testing, body temperature of the human subject, as well as ambient temperature, humidity and lighting were checked.

Each test repellent was tested on 35 separate occasions (each hour for 0-6 hours, repeated in 5 days). For each test repellent, the protection percentage at each of the 35 occasions was calculated using the following formula:

$$PP = 100 \times \frac{C - T}{C}$$

where:

C = the number of mosquito landings on the control (right) forearm

T = the number of mosquito landing on the repellent-treated (left) forearm

Statistical Analysis

To calculate confidence intervals for the PP for each hour, the percentile of confidence interval were set on a parametric bootstrap with 10,000 replications. It was assumed that the number of landings on each arm is in accordance with Poisson distribution with mean determined by the sample mean from the 5 days. There was no over dispersed data in the data distribution.

The PP for different test repellents was calculated using a ratio. The 95% confidence interval was determined using the percentile parametric bootstrap with 10,000 replications using similar assumptions as with the PP confidence intervals.

RESULTS AND DISCUSSION

Safety and Preference

Based on human safety test, 10 subjects thought that the betel+patchouli lotions did not give any effects, 6 subjects thought that the betel+patchouli lotion was good, and 4 subjects thought that the betel+patchouli lotion was very good. DEET and betel+patchouli lotion did not show any irritations. In the patchouli lotion test, 1 out of 20 subjects experienced skin rash. In the betel lotion test, 2 out of 20 subjects showed skin rash (Table 2).

Efficacy Experiment

Subjects had body temperature ranged between 36-36.5 ^{0}C, environmental temperature was 27-28 ^{0}C, humidity was 89-95%, and lighting was 12-87 lux. The environment conditions were considered as confounding variables because they might tamper with the condition of the mosquitoes and human subjects, and it was hard to control. Efficacy experiment showed that human subjects applied with DEET and lotion had stable body temperature during experiment.

Table 1. Overall impression of betel+patchouli mixture lotion from 20 subjects

Subject	Overall impression			
	Not good	Regular	Good	Very good
1		+		
2		+		
3			+	
4				+
5			+	
6			+	
7		+		
8		+		
9				+
10			+	
11			+	
12				+
13		+		
14				+
15		+		
16		+		
17		+		
18			+	
19		+		
20		+		

Table 2. Number of adverse events by treatment out of 20 subjects tested (with percent).

Treatment	Skin rash	Itch	Swollen	No adverse events
DEET	0	0	0	20 (100%)
Modified (betel+patchouli mixture) lotion	0	0	0	20 (100%)
Betel lotion	2 (10%)	0	0	18 (90%)
Patchouli lotion	1 (5%)	0	0	19 (95%)

According to the results, DEET and modified betel lotion had an estimated protection percentage of more than 90% from yellow fever mosquito (*A. aegypti*) for the 6-hour duration. Although the number of mosquito landings decreased quite drastically during the fourth hour, since a similar finding occurred in the control group, it did not clearly influence the magnitude of protection. During the fourth hour, when the test performed after 12:00, the activity of the mosquitoes started to decrease. This might be explained by the cycle of mosquito's life, where their resting period lied after 12:00. Thus, the mosquito perching started to decrease (Chadee 2013). Betel leave contains various chemical compounds, including saphonine, phenolic, alkaloids, and substance which can be used as repellents (Paluch *et al*. 2010). Betel leave commonly contains 30% eugenol (Bhalerao *et al*. 2013) which has been proven for controlling

larvae and adult mosquitoes (Eliningaya 2008). Other study also reported that eugenol derived from clove leaves (70-93%) has a potential to repel mosquitoes. Although the composition of betel leaves were not as eugenol-rich as clove leaves, it also has the potential to repel yellow fever mosquito (*A. aegypti*) after some modifications. Betel lotion mosquitoes repellent produced in this study is targeting *palpi* and antennae of mosquitoes because these two structures on mosquito body are most sensitive to the aroma of modified lotions. The aroma of plant extracts could cover the scent of the human body and thus impairing the mosquito's ability to detect the presence of humans (Stella et al. 2010). Based on the safety test, this preliminary test showed that our modified repellent is potentially safe to be used in repellency test.

The control arms landing were comparable between the 4 subjects who got the test repellents, while the control arm of subject who got the base only had substantially fewer mosquito landings.

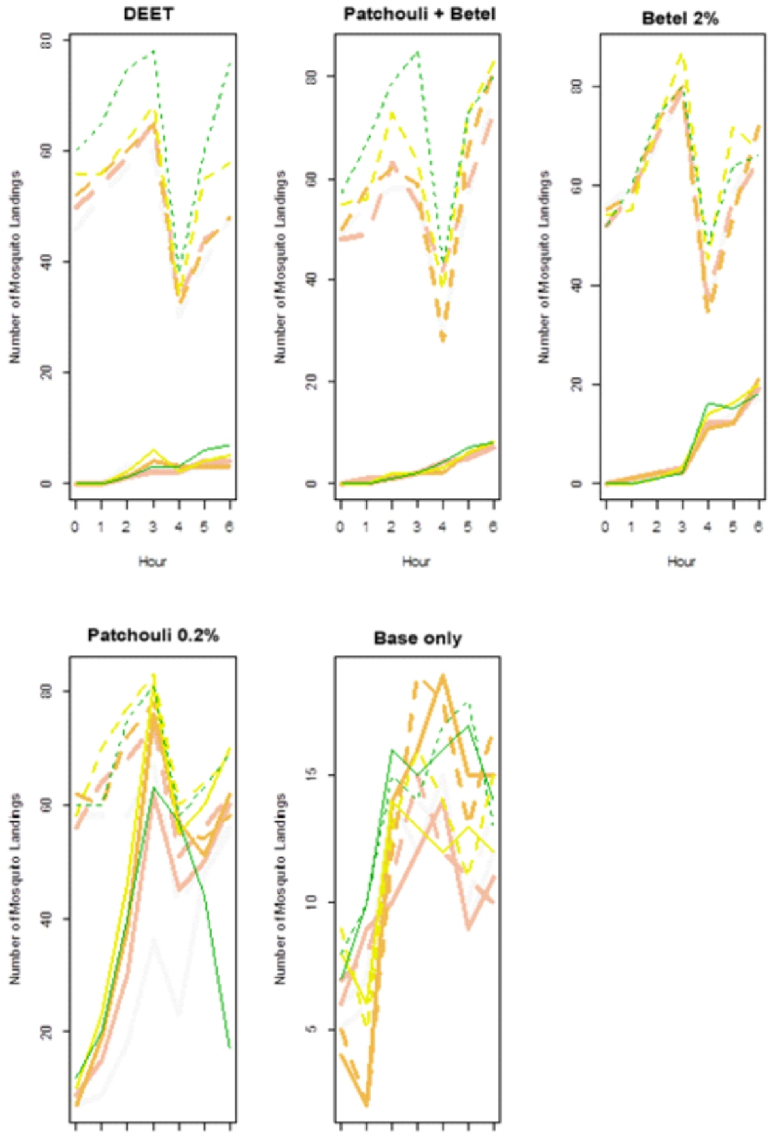

Figure 1. Number of mosquito landings for the control arm (dotted lines) and the repellent-treated arm (solid lines) for each of the 5 days (different colors)

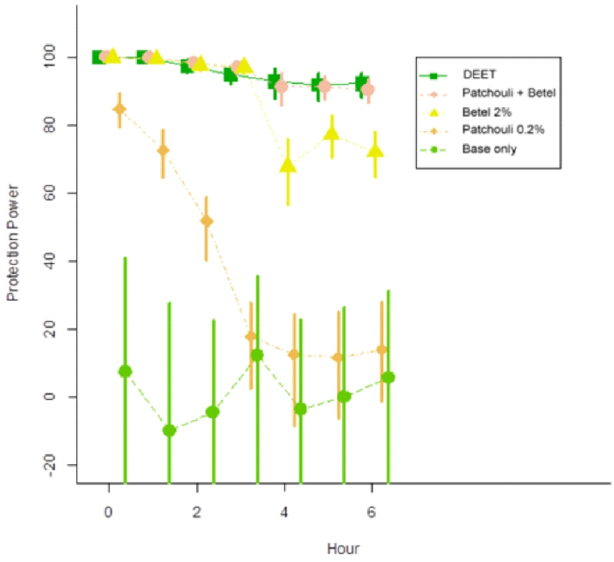

Figure 2. Mean protection power with 95% confidence intervals (some CIs were hidden, since they were not as large as the mean symbol). The points were slightly shifted at each hour so that the different test repellent points do not overlap

The mean protection percentage (PP) for each test repellent was presented with 95% confidence intervals. Mixture of betel+patchouli lotion performed very similarly to DEET at every hour, while the betel 2% only matched the DEET until hour 3, by hour 4 its PP dropped substantially. At 6 hours, DEET and betel+patchouli had PP around 90%. DEET had PP = 92.2% (95% CI 88.1; 95.2), while betel+patchouli had PP = 90.4% (95% CI 86.7; 93.4). Both DEET (p = 0.29) and betel+patchouli (p = 0.85) were not significantly different from 90%. The "base only lotion" provided no protection, and the patchouli 0.2% provided poorer protection compared to other active repellents.

The betel+patchouli had about the same protection power as the DEET (ratio: PP (betel+patchouli)/PP (DEET) = 0.98; 95% confidence interval 0.93; 1.04; p-value = 0.50), while betel 2% alone had significantly less protection (ratio: PP (betel)/PP (DEET) = 0.78; 95% CI 0.71; 0.86; $p < 0.001$).

The data analysis was expected to be uninterrupted by environmental aspect. For the comparison of PP between different test repellents, each test repellent was tested on only one subject. This is a limitation of the design. However, each subject was acting as his or her own control, so systematic biases on the landing counts (e.g. caused by personal odor of a subject) might affect both the control and the repellent-treated arm. These biases might cancel out when PP was used. Moreover, the systematic bias caused by always starting the test with the control arm at each hour. For comparing PP for different test repellents, both test repellents will have that same bias, and it may be canceled out in the comparison.

DEET performed well. The modified lotion could be considered as a potential repellent due to its 90% protective power for 6 hours and lack of safety concerns. Although DEET protective power was high, betel lotion might work as an alternative for DEET. The main problem of natural repellent is durability. Several studies have shown that natural ingredients rarely fulfill the requirements of repellent effectiveness. However, the modification of formulation by adding patchouli oil as a fixative increased the potential of natural ingredients to be used as repellent.

CONCLUSIONS

Modified betel (betel+patchouli mixture) lotion had shown repellent potential for yellow fever mosquito (*Aedes aegypti*) mosquitoes with estimated mean protection power of 90.4% at 6 hours. This study has opened up the possibility for further research to assess the effects of this repellent formulation, and modifications of other natural repellent plants.

ACKNOWLEDGEMENTS

Authors would like to thank to Michael Fay for statistical assistance. The author would also like to thank to INA RESPONDS Infectious disease group mentors, Ibu Emil, Ibu Inge, and Mr. Joshi for their suggestions, member of Loka litbang Ciamis for their contribution in this research assays and INA RESPONDS for their assistance in making manuscript writing workshop. This research was funded by Balitbangkes as one of RISBINKES project.

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