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Biological stability of repellent activity of lemon grass (*Cymbopogon citratus* (DC.) Staph), and citronella grass (*Cymbopogon nardus* (Linn.) Rendle) oils against *Aedes aegypti* (Linn.) and *Anopheles dirus* (Peyton and Harrison)

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Abstract The biological stability of repellent activity to female mosquitoes of the stored samples (kept at 4^{o}C and ambient temperature (25-30^{o}C) for 3 and 6 months) of 10% *Cymbopogon citratus* and *C. nardus* in soybean oil seemed to be comparable to that of the fresh sample (≤1 month). Topical application was made on the forearms of volunteers and was assessed by the protection time. Testing *Aedes aegypti* females against stored samples of *C. citratus* and *C. nardus* demonstrated that the product stored at 4^{o}C for 3 months showed the longest repellent activity (66 and 48 min), followed by those kept at ambient temperature for 3 months (48 and 36 min) and for 6 months at 4^{o}C (42 and 30 min) and ambient temperature (36 and 30 min), respectively. Repellent testing of *C. citratus* against *Anopheles dirus* stored at 4^{o}C for 3 and 6 months showed higher efficacy than those kept at ambient temperature. Meanwhile, there were not significant differences in the efficacy of fresh and stored samples of *C. nardus* oil exerting protection against *An. dirus.* The biological stability of *C. citratus* and *C. nardus* oils may prove this product as an alternative to essential oils in the development of green repellents.

Keywords: essential oil, biological stability, repellent, protection time, *Aedes aegypti, Anopheles dirus*.

Introduction

Aedes aegypti is a primary vector of dengue viruses in urban areas of Thailand. Dengue fever (DF) and dengue haemorrhagic fever (DHF) are vector-borne diseases of public health importance in tropical, subtropical, and temperate regions of the world (Gubler, 1998; Pancharoen *et al*., 2002). In

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Southeast Asia, dengue haemorrhagic fever (DHF) is a major cause of mortality among children (Halstead, 1980) while dengue fever (DF) occurs epidemically and affecting older children and adults (World Health Organization, 1997). *Anopheles dirus* is the main malaria vector in Thailand (Rosenberg *et al*,. 1990), and other countries in mainland Southeast Asia, including Burma, Cambodia, and Bangladesh (Rosenberg and Maheswary, 1982). *An. dirus* has proven extremely difficult to control due to a diverse array of host seeking behaviors and preferences, biting patterns and larval breeding habitats (Pates and Curtis, 2005).

Personal protection is particularly important because most mosquito control efforts have been directed towards indoor spraying, bed-netting, and eradication of mosquito breeding sites. Therefore, insect repellent using is one of the most efficient ways to prevent disease transmission by biting insects, particularly by mosquitoes (Gupta and Rutledge, 1994). Currently, the use of synthetic chemicals to control insects and arthropods raises several concerns related to environment and human health. Synthetic chemicals have been developed in order to protect human from mosquito bites, being DEET (N,N-diethyl-m-toluamide) not only a broad spectrum repellent, but also the most effective and persistent on skin (Isman, 2006). However, human toxicity has been reported with DEET, with symptoms varying from mild to severe (Briassoulis *et al*., 2001). It is irritating to mucous membranes, and concentrated formulations dissolve plastic. DEET may be unsafe for children possibly causing encephalopathy (Abdel-Rahman *et al*., 2001). Among those chemicals, essential oils from plants belonging to several species have been extensively tested to assess their repellent properties as a valuable natural resource.

Repellent properties of essential oils and extracts from genus *Cymbopogon* are also well documented. This genus produces the most used natural repellents in the world (Trongtokit *et al*., 2005). *Cymbopogon citratus* is a tall perennial grass that grows in warm temperate and tropical regions of the world. Its common names include lemon grass, lemongrass, barbed wire grass, silky heads, and many others (Kazembe and Chauruka, 2012). The insecticidal (Arias *et al*., 1992), antimicrobial (Syed *et al*., 1995), and the therapeutic properties (Akendengue, 1992) of its oil and extracts have been reported. *Cymbopogon nardus* is known for their rich essential oil content and widely used in food or drinks, mosquito repellent, perfumery and health and care products (Jantan *et al*., 1999). Citronella (*C. nardus*) essential oil has been used for over fifty years both as an insect repellent and an animal repellent. Combining few drops each of citronella, lemon (*Citrus limon*), rose (*Rosa damascena*), lavender and basil essential oils with one litre of distilled water is

effective to ward off indoor insect pests (Koul *et al*., 2008). However, the repellent effects of natural oils do not usually last as long as DEET which can protect mosquito bites for up to 6 hours (Frances, 1987; Debboun *et al*., 2000; Barnard and Xue, 2004). The level of protection provided will be directly proportional to the period of time that has passes from the time of application. Since the level of protection is known to fall off with time, the proportionality constant will in the case be negative.

As a part of our studies on aduticidal, larvicidal and ovicidal activity against *Ae. aegypti* and *An. dirus* from *C. citratus* and *C. nardus* oils were previously reported (Phasomkusolsil and Soonwera, 2011, 2012, 2013). The objective of this research was to evaluate the stability of formulations developed by laboratory of Entomology and Environment Programme, Plant Production Technology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL). The formulations were tested after storage at varied temperature. The information resulting from these investigations may highlight the importance of natural products as a potential source of biologically active agents as personal protection against mosquito protection.

Materials and methods

Mosquito cultures and rearing conditions

Ae. aegypti and *An. dirus* eggs were obtained from the Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand. These colonies were reared in the laboratory of Entomology and Environment Programme, Plant Production Technology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok. Eggs were submerged in 1.5 L of distilled water in plastic trays (size 30x35x5 cm). 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) and emerged as adults. Mosquitoes were kept in the room with ambient temperature (25-30^{o}C). Adults were provided with soaked cotton balls containing a 5% multivitamin solution.

Five (5) to seven (7) day-old female mosquitoes were used in these experiments. The night before being exposed to the blood meal they were deprived of the multivitamin solution and supplied with water pads only. Six (6) hours prior to the blood feeding, the water soaked cotton pads were removed.

Plant materials

The plant materials were collected from Lemon grass (*C. citratus*), and Citronella grass (*C. nardus*). They were provided by the medicinal plant laboratory of KMITL. Each plant material had the essential oils extracted by the following water distillation method (Charles and Simo, 1990). One kilogram of dried and finely ground material from each plant was placed in an extraction column connected to a round-bottomed distillation flask containing distilled water. The flask was heated to approximately 100^{o}C and allowed to boil until distillation was completed. The liquid formed, together with the distillate oils, were collected in a separating funnel. The mixture was then allowed to settle for 1 day, after which the water (lower) layer was slowly drawn off until only the oil remained. These essential oils were prepared as 10% solutions in soybean oil.

Human volunteers

Five adult volunteers of both sexes, 25-45 years old, weight 50-70 kg, who had no history of allergic reaction to arthropod bites were recruited. Before signing an informed consent form, the volunteers were interviewed and instructed on the methodology, probable discomforts to subjects and remedial arrangements.

Testing the biological stability

Both essential oils were stored in closed vial up to six months and stability of the fraction was determined in conditions that varied in temperature (4^{o}C and 25-30^{o}C) and time storage (3 and 6 months time intervals). In these testing, the fresh sample of essential oils which kept at ambient temperature for $\leq$1 month were carried out in parallel for comparison.

Laboratory repellent bioassay

The oils were tested for repellency against *Ae. aegypti* and *An. dir*us following Thai Industrial Standards Institute (TISI) guidelines (1986) under laboratory conditions. The timing of the tests depended on whether the target mosquitoes were day- or night-biters; *Ae. aegypti* was tested from 8 am to 4 pm, while *An. dirus* was tested between 4 pm and 12 pm. Two hundred and fifty non-blood fed starved female mosquitoes were randomly selected and placed in an experimental cage (30×30×30 cm) and left to acclimatize for 1 hour. Human volunteers wore gloves and plastic sleeve with a 3×10 cm

window on the ventral part of the forearm after cleaning their arms with distilled water. Before the start of each exposure period, the mosquitoes were tested for their readiness to bite by placing an untreated bare hand of a volunteer into a test mosquito cage for up to 15 sec for *Ae. aegypti* and for up to 30 sec for *An. dirus* (Thavara *et al*., 2001). The mosquitoes were blown from the hand before any blood was taken. If at least two mosquitoes had bitten the hand of the control person, the repellency test would be carried out; otherwise, the test would not be conducted.

Therefore, only a restricted zone of the skin was exposed to the mosquitoes. An aliquot of 100 μl of the test samples was pipetted onto a 30 cm^2 test area of one forearm of each 5 human volunteers and was allowed to dry for 5 minute at room temperature. The test was conducted for 3 minute. The total number of mosquitoes biting on the treatment was recorded. If no mosquito bite occurred within 3 minutes, the forearm was then taken out and the test was repeated every 30-minute interval. The experiment was completed after two mosquitoes had bitten. The study period was carried out every 30 minutes until fewer than 2 mosquitoes bit during the 3 minute study period, at which time the study was stopped. The protection time was the time from repellent application until the study was stopped. On each day, only one repellent preparation was tested to assure that residual material has disappeared from the skin before the next test (Curtis and Hill, 1988).

Statistical analysis

In laboratory repellent bioassay, the mean protection time was used as a standard repellency measure of the test samples against *Ae. aegypti* and *An. dirus* in the laboratory. Differences in significance were determined by comparing the range of protection time of each sample and Duncan's multiple comparisons by SPSS for Windows (version 16.0).

Results

The repellent activities of *C. citratus*, and *C. nardus* oils kept at 4^{o}C and ambient temperature (25-30^{o}C) and for different durations ($\leq$1, 3 and 6 months) against *Ae. aegypti* mosquito were given in Table 1. The result of the protection time of *C. citratus* and *C. nardus* oils kept at 1 month gave the highest repellency for the longest lasting period against *Ae. aegypti* for 72 and 60 min, respectively. Meanwhile, there were moderate repellent activities of *C. citratus* and *C. nardus* oils kept at 4^{o}C for 3 months, the protection time were 66 and 48 min, respectively. Moreover, the protection time of *C. citratus* and *C. nardus* oils kept at ambient temperature (25-30^{o}C) for a period of 3 months, there were

48 and 36 min, respectively. In addition, storage for 6 months led to a decrease in repellency of both *C. citratus* and *C. nardus* oils, the protection time were 42 and 30 min (at 4°C) and 36 and 30 min (at ambient temperature), respectively. Table 2 summarizes the repellencies against *An. dirus* of the fresh (≤1 month) and stored samples at 4°C and ambient temperature (25-30°C) for 3 and 6 months of *C. citratus*, and *C. nardus* oils. The result indicated that the fresh essential oil (≤1 month) of *C. citratus* provided up to 2 hours (132 min) protection against *An. dirus.* In addition, *C. citratus* oil stored at 4°C for 3 and 6 months provided equal protection times of 102 min. Correspondingly, *C. nardus* oil stored at 4°C and ambient temperature for 3 and 6 months yielded protection times of 1.5 hour (90-78 min), which were no significant difference from the fresh product (1.5 hour).

Table 1. Repellency against *Ae. aegypti* of the fresh and stored samples (kept at 4°C and ambient temperature; 25-30°C for 3 and 6 months) of *C. citratus*, and *C. nardus* oils

Months in storage	**Temperature in storage (°C)**	**Protection time[1/] (min)**	
		C. citratus oil	*C. nardus* oil
≤1 Month	25-30°C	72.0±16.4a	60.0±21.2a
3 Months	4°C	66.0±13.4ab	48.0±16.4ab
	25-30°C	48.0±11.0bc	36.0±13.4b
6 Months	4°C	42.0±16.4c	30.0±0.0b
	25-30°C	36.0±13.4c	30.0±0.0b
CV (%)		27.5	32.9

[1/] Means protection time in each column followed by the same letter are not significantly different (P>0.05, by one-way ANOVA and Duncan's Multiple Range Test).

Table 2. Repellency against *An.dirus* of the fresh and stored samples (kept at 4°C and ambient temperature; 25-30°C for 3 and 6 months) of *C. citratus*, and *C. nardus* oils

Months in storage	**Temperature in storage (°C)**	**Protection time[1/] (min)**	
		C. citratus oil	*C. nardus* oil
≤1 Month	25-30°C	132.0±16.4a	90.0±30.0a
3 Months	4°C	102.0±16.4b	90.0±0.0a
	25-30°C	90.0±0.0bc	84.0±13.4a
6 Months	4°C	102.0±16.4b	84.0±13.4a
	25-30°C	78.0±16.4c	78.0±16.4a
CV (%)		14.6	20.5

[1/] Means protection time in each column followed by the same letter are not significantly different (P>0.05, by one-way ANOVA and Duncan's Multiple Range Test).

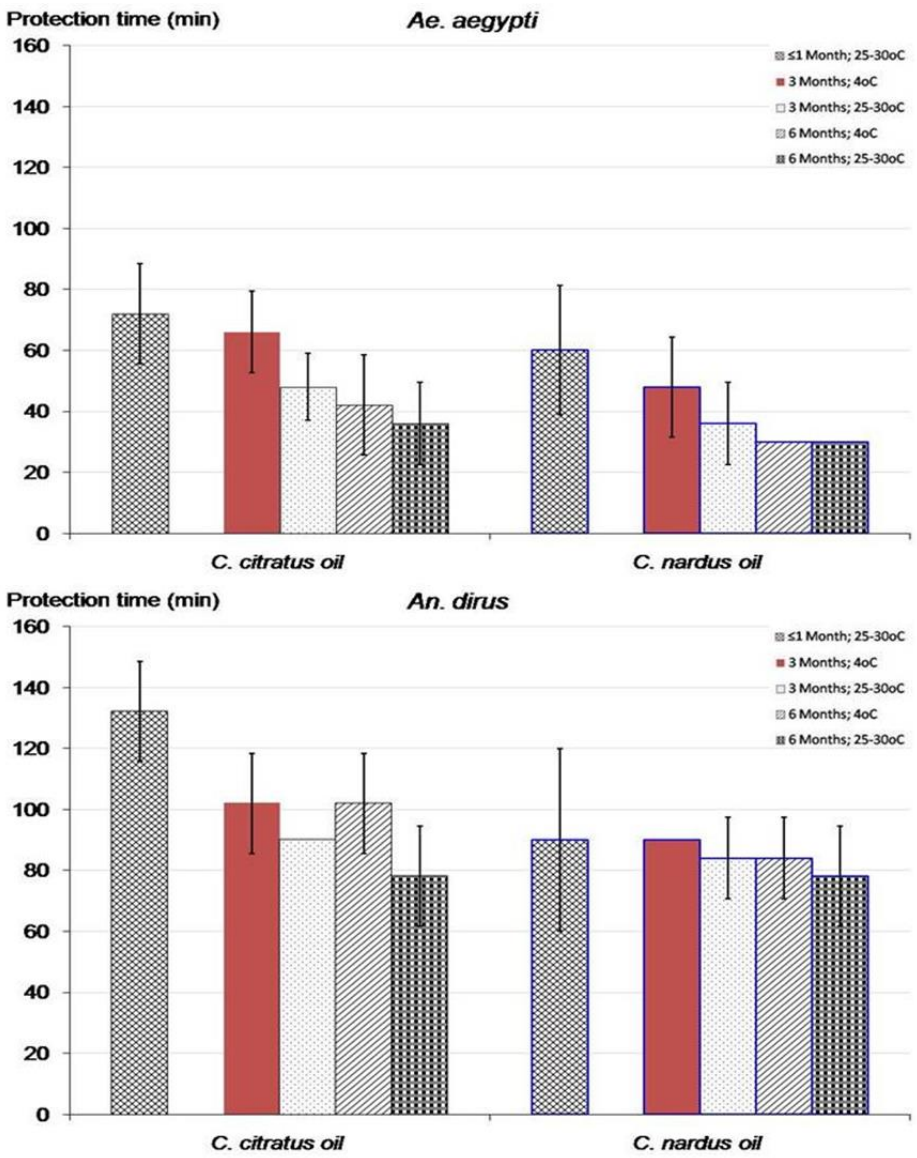

Fig. 1. Repellency against *Ae. aegypti* and *An.dirus* of the fresh and stored samples (kept at 4^{o}C and ambient temperature; 25-30^{o}C for 3 and 6 months) of *C. citratus*, and *C. nardus* oils.

Discussion

One limitation in practical applications of botanical-based repellents is the unstable physical and biological properties of the active principles that possibly influence the bioactivity against mosquitoes. Apart from inherent stability, the lack of persistent repellent action of botanical-based products

commonly found after production and/or use for a period of time was probably associated with the variation in transport and storage conditions. Practical repellent products should maintain their bioactivity for an appropriate period of time, despite being under variable conditions (Tuetun *et al*., 2009). Smith (1966) proved that the length of time a repellent remained effective depended on the rate at which it was lost by rubbing, evaporation or absorption. For investigating biological stability, *C. citratus*, and *C. nardus* oils kept at conditions that varied in temperature and time storage exhibited varying degrees of repellency. After keeping at 4^{o}C and ambient temperature (25-30^{o}C) for 3 and 6 months, the physical properties such as color and odor of all stored samples were unchanged and similar to those of the fresh sample, showing no color and a pleasant odor.

Conclusion

Stability test of *C. citratus* and *C. nardus* oils against *Ae. aegypti* stored at 4^{o}C for 3 months showed the longest repellent activity and these oils were decreased during six months at 4^{o}C and ambient temperature (25-30^{o}C). In addition, stability test of *C. citratus* oil against *An. dirus* stored at 4^{o}C for 3 and 6 months showed higher efficacy than those kept at ambient temperature and *C. nardus* oil was stable for six months at 4^{o}C and ambient temperature.

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