International Journal of Agricultural Technology 2015 Vol. 11(3):669-681
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Efficacy of Essential Oils from Citrus Plants against Mosquito Vectors *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say)

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Soonwera, M. (2015). Efficacy of essential oils from Citrus plants against mosquito vectors *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say). International Journal of Agricultural Technology 11(3):669-681.

Abstract The eight essential oil derived from citrus plants (*Citrus aurantifolia* (Christm.&Panz.) Swingle, *Citrus aurantium* L., *Citrus hystrix* DC., *Citrus maxima* (Burm.f.) Merr., *Citrus medica* L. var *sarcodaclylis* Swingle, *Citrus reticulate* Blanco, *Citrus sinensis* Osbeck and *Citrofortunella microcarpa* (Bunge) Wijnands were evaluated for repellent activity against female adult of *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say), and compared them with chemical repellent (IR3535 12.5 w/w; Johnon's Baby Clear Lotion ®). Each herbal essential oil in ethyl alcohol was applied at 0.33 μl/cm^2 on the forearms of volunteers. On the protection time (in minutes), biting rate (%) and protection (%) revealed that essential oil of *C. aurantifolia* was effective as repellent and feeding deterrent against *Ae. aegypti* (65.0±22.91 minutes protection times, 1.5% biting rate and 98.5% protection) and *Cx. quinquefasciatus* (71.7±5.8 minutes protection times, 1.7% biting rate and 98.3% protection). Thus, repellent activity indicated the order of protection time and biting rate against two mosquito species in eight essential oils as *C. aurantifolia* > *C. microcarpa* > *C. maxima* > *C. reticulate* > *C. sinensis* > *C. hystrix* > *C. aurantium* >*C. medica* var *sarcodaclylis*. Meanwhile, the period of protection time against two mosquito species of all herbal essential oil was higher than IR3535 (3.0±0 minutes for *Ae. aegypti* and *Cx. quinquefasciatus*).

Keywords: Essential oils, Repellency, *Aedes aegypti*, *Culex quinquefasciatus*

Introduction

Mosqutioes are responsible for spreading serious human diseases, especially *Aedes aegypti* (Linn.) and *Culex quinquefasciatus* (Say). *Ae. aegypti*, is the primary carrier for viruses that cause dengue fever, dengue hemorrhagic

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fever, yellow fever and chikungunya fever (Sakulku *et al*., 2009; Yang *et al*., 2009; WHO, 2011). Meanwhile, outbreaks of dengue fever and dengue hemorrhagic fever have been reported by WHO- South-East Asia Region (SEARO) from Bhutan, Bangladesh, Indonesia and Sri Lanka (in 2004 - 2005) (Tjahjani, 2008). In Thailand, it is estimated that more than 200,000 people suffer annually from dengue attacks (Juckkapan, 2009; Ministry of Public health, Thailand, 2013).

In addition, *Cx. quinquefasciatus* is the principal rector of Japanese encephalitis (JE), Lymphatic filariasis caused by *Wuchereria bancrofti*, heartworm in dogs and its also causes annoyance and dermatitis (Ramaiah *et al*., 2006; Nitatpattana *et al*., 2008; Du Ponte *et al*., 2009). Conseguently, *Ae. aegypti* and *Cx. quinquefasciatus* constitute one of the most serious pests for humans and animals.

The control of mosquito vectors and reducing the transmission of human pathogens are based on the chemical insecticides, especially chemical repellents. Thus, chemical repellents are considered to be a useful of reducing and preventing the mosquito vectors, deterring an insect from flying and landing, and biting human and animal skin. Unfortunately, chemical repellents are not safe for human, especially children because they may cause skin irritation, hot sensation rashes or allergy (Das *et al*., 2003), such as DEET (N,N-diethyl-M-methyl benzamide) and IR3535 (ethyl butylacetyl aminopropionate) may be unsafe for children possibly causing encephalopathy (Abdle-Rahman *et al*., 2001). Besides, DEET is also know to damage plastic and synthetic materials (Kang *et al*., 2009).

Therefore, there is an urgent need to develop new repellents for controlling mosquito vectors which are more environmentally friendly, biodegradable, non-toxic effects on human and domestic animals (Kumar *et al*., 2011; Rabha *et al*., 2012). The mosquito repellents base on plant extracts, or plant essential oils may be a possible alternative as one of the methods in preventing mosquito vectors also compatible with human life and environment. However, plant essential oils are reported to have repellency against mosquito adults, such as essential oils from *Citrus aurantifolia*, *Citrus sinensis*, *Cinnamomum xeylanicum*, *Cymbopogon nardus*, *Cymbopogon citratus*, *Curcuma aromatic*, *Eucalyptus citriodora*, *Eucalyptus globules*, *Mentha piperita*, *Ocimum basilicum*, *Piper aduncum*, *Syzygium aromaticum*, *Zanthoxylum limonella*, *Zingiber officinalis* and *Vitex negundo* (Das *et al*., 2003; Choochote *et al*., 2005; Gleiser *et al*., 2011; Karunamooethi *et al*., 2008, 2010; Prajapati *et al*., 2005; Yang and Ma, 2005; Phasomkusolsil and Soonwera, 2010a; Nour *et al*., 2009; Pushpanathan *et al*., 2008; Tjahjani, 2008).

Moreover, mosquito repellents based on Thai indigenous plant oils have demonstrated good efficacy against *Ae. aegypti*, *Anophele minimus* and *Culex quinquefasciatus* in the laboratory condition (Phasomkusolsil and Soonwera, 2010b, 2011).

This study investigates the repellency of essential oils derived from eight species of Rutaceae plants against *Ae. aegypti* and *Cx. quinquefasciatus* and to compare them with chemical repellent (DEET) under laboratory conditions.

Materials and methods

Plant materials and essential oils

The eight species of Rutaceae plants were used in this study, are shown in Table 1. All of plants were identified, authenticated and submitted at Plant Production Technology Section, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL). Each plant material was extracted for essential oils by water distillation. All essential oil was dissoled in ethyl alcohol and were kept at room temperature defore testing.

Table 1. List of Rutaceae plants were extracted for essential oils and used in this study.

Scientific Name	Part Used	Location
Citrus aurantifolia (Christm. X Panz.) Swingle	fruit	Phetchaburi, Thailand
Citrus aurantium L.	fruit	Phetchaburi, Thailand
Citrus hystrix DC.	fruit	Nakhonratchasima, Thailand
Citrus maxima (Burm.f.) Merr.	fruit	Nakhonratchasima, Thailand
Citrus medica L. Swingle	fruit	Chumphon, Thailand
Citrus reticulata Blanco	fruit	Chiangmai, Thailand
Citrus sinensis Osbeck	fruit	Chiangmai, Thailand
Citrofortunella microcarpa (Bunge) Wijnands	fruit	Chumphon, Thailand

Chemical repellent

IR3535, 12.5% w/w Ethyl butylacetylaminopropionate; Johnon's Baby Clear Lotion Anti Mosquito® is a common chemical repellent for children in Thailand. It was purchased from IDS Manufacturing Co. Ltd, Thailand.

Test mosquitoes

Ae. aegypti and *Cx. quinquefasciatus* eggs were obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS). They were maintained in Entomology and Environmental Laboratory, Plant Production Technology Section, Faculty of Agricultural Technology, KMITL a glass up (1,000 ml). The eggs were hatched in with 500 ml of water and then transferred batches of 200 larvae to white plastic trays (24x30x6 cm). Fish food (OPTIMUM®, crude protein min 28%) was added for 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar larvae at 8.00 am and 4.00 pm each day to each tray for the successive two weeks until pupation of all larvae. The pupae were collected weekly and keep in a insect cage (30x30x30 cm) for adults emergence. Adults of two mosquito species were fed on 5% glucose solution and maintained at 32.50 ± 1.20 ℃, 64.80 ± 3.50% RH with a photoperiod of 12h light followed by 12h dark (12L : 12D) Nulliparous female of 4-5 days-old were used for repellency tests. Before testing, the glucose solutions were removed from insect cage for 12h.

Bioassay

The repellency of eight essential oils were evaluated using the human-bait technique following Thai Industrial Standards Institute Guidelines (TISI, 2009). Human volunteers were recruited from the Healthy students and staff of Entomology and Environment Laboratory, Plant Production Technology Section, Faculty of Agricultural Technology, KMITL. The volunteers signed an informed consent from after having received a full explanation of the lest objectives. The research proposal approved by the research committee of Faculty of Agricultural Technology, KMITL. The timing of the tests depended on the mosquitoes, for *Ae. aegypti* was tested during the daytime from 8.00 am to 4.00 pm, while *Cx. quinquefasciatus* was tested during night time from 4.00pm to 12.00 pm (Phasomkusolsil and Soonwera, 2010, 2011).

Before testing the volunteers arms were washed and cleaned thoroughly with distilled water and used the left arm for treatment and the night arm for control. Both arms of volunteers were covered with rubber sleeve with a window area of 30 cm2 (3x10 cm) on the ventral part of forearm 0.1 ml of each test repellent was applied to the treatment area of left forearm of each volunteer and allowed to dry on the skin for 1.0 minute. After applying the test repellent, the volunteer was instructed not to rub, touch or wet the treated area. The right arm acting as a control, was exposed for up to 30 seconds to mosquito cage (30x30x30 cm) containing 250 nulliparous female mosquitoes (4-5 days old). The test was conducted for 3 minutes. If no mosquito bite occurred within 3 minutes, the forearm was then taken out and the test was repeated every 15 minute intervals. This was continued until at least two mosquito bites occurred during the 3-minute study period. The experiment was completed after two mosquitoes had bitten. The protection time was the time from repellent application until the study was stopped.

Data analysis

The median protection time was used as a standard measure of repellency of the essential oils and IR3535 against *Ae. aegypti* and *Cx. quinquefasciatus*. Differences in significance were analyzed by one-way analysis of variance (ANOVA) and Duncan’s New Multiple Range Test (DMRT). Percentage of mosquito biting or landing was calculated for each test using the following formula (Phalsomkusolsil and Soonwera, 2010).

$$\% \text{ Biting} = [\text{ B}/250] \times 100$$

Where B is the total number of biting or landing by the end of the test. The test was carried out 3 times per test solution.

Percentage of protection was calculated for each repellent using the following formula

$$\text{Percentage of protection} = 100 - \frac{\text{No. of mosquitoes biting or landing}}{\text{No. of mosquitoes released}} \times 100$$

Results

The repellency results (as show in minute of protection time) for eight essential oils repellents, chemical repellent (DEET 20% w/w, positive control) and ethyl alcohol 70% (negative control) assessed by mean protection time against *Ae. aegypti* under laboratory conditions is shown in Table 2. The essential oils from *C. aurantifolia* and *C. microcarpa* had the best efficiency against *Ae. aegypti*; in which the mean protection times were 65.0±22.9 minutes, 1.5% biting rate and 98.5% protection and 61.7±2.9 minutes, 1.6% biting rate and 98.4% protection, respectively. The mean protection times of essential oil from *C. maxima*, *C. reticulate*, *C. sinensis*, *C. hystrix*, *C. medica* and *C. aurantium* against *Ae. aegypti* were 45.0 ±6.93, 21.0±6.93, 20.95±8.67, 20.50±8.66, 11.67±5.75 and 10.0±8.66 minutes, biting rate of 2.3, 1.3, 1.2, 1.5, 1.5 and 1.7% and protection of 97.7, 98.7, 98.5, 98.5 and 98.3%, respectively. The period of protection time against *Ae. aegypti* of all essential oil was higher than ethyl alcohol (negative control) and IR3535 (positive control). While, ethyl alcohol (negative control) showed no repellency activity against *Ae. aegypti*, in contrast ethyl alcohol showed the highest biting percentage at 28.7% and protection of 71.3%.

Table 2. Repellency of eight essential oils from Citrus plants, positive control (IR3535) and negative control (ethyl alcohol) against female adult of *Aedes aegypti* at 0.33 µl/cm^2.

Test repellents	Protection time (mean±SD) (min)	Biting rate	% Protection
C. aurantifolia	65.0±22.9b[1/]	1.5	98.5
C. aurantium	10.0±8.7e	1.7	98.3
C. hystrix	20.5±8.7d	1.5	98.3
C. maxima	45.0±6.9c	2.3	97.7
C. medica	11.7±5.8e	1.5	98.5
C. reticulata	21.0±6.9d	1.3	98.7
C. sinensis	20.9±8.7d	1.2	98.8
C. microcarpa	61.7±2.9b	1.6	98.4
Negative control (ethyl alcohol 70%)	0f	28.7	71.3
Positive control (IR3535 12.5% w/w)	3.0±0f	25.2	74.8

[1/]means of protection time / biting rate in each column, followed by the same letter are not significantly different (one-way ANOVA and Duncan's multiple Range Test, P<0.05)

The mean repellency in minutes for eight essential oils, negative control (ethyl alcohol) and positive control (IR3535) against *Cx. quinquefasciatus* is shown in Table 3. There were significant differences in repellency among essential oils and chemical repellent (IR3535) (P<0.05). The essential oils from *C. aurantifolia* and *C. microcarpa* also showed the best effiency against *Cx. quinquefasciatus*, with the protection times of 71.7±5.8 and 70.0±8.7 minutes, 1.7 and 1.4% biting rate and 98.3 and 98.6% protection, respectively. The protection times of essential oils from *C. medica*, *C. hystrix*, *C. sinensis*, *C. reticulate*, *C. aurantium* and *C. maxima* against *Cx. quinquefasciatus* were 46.0±1.3, 45.0±8.7, 42.8±7.8, 40.9±8.6, 21.6±8.1 and 16.0±1.3 minutes, and 1.3, 1.7, 1.3, 1.4, 1.4 and 1.3% biting rate and 98.7, 98.3, 98.7, 98.6, 98.6 and 98.7% protection, respectively. However, the mean protection times against *Cx. quinquefasciatus* of all essential oils was higher than chemical repellent (positive control) and ethyl alcohol (negative control). Meanwhile, negative control (ethyl alcohol) showed no repellency activity against *Cx. quinquefasciatus*, but exhibited the highest biting rate of 20.8% and 79.2%

protection. In contrast, all essential oil showed a low biting percentage (1.33–1.43 %).

Table 3. Repellency of eight essential oils from Rutaceae plants, positive control (DEET) and negative control (ethyl alcohol) against adult female of *Culex quinquefasciatus* at 0.33 µl/cm^2.

Test repellents	**Protection time (mean ±SD) (min)**	**Biting rate**	**% Protection**
C. aurantifolia	71.7 ±5.8b[1/]	1.7	98.3
C. aurantium	21.8 ±8.1d	1.4	98.6
C. hystrix	45.0 ±8.7c	1.7	98.3
C. maxima	16.0 ±1.3e	2.3	98.7
C. medica	46.0 ±1.3c	1.3	98.7
C. reticulata	40.9 ±8.6c	1.4	98.6
C. sinensis	42.8 ±7.8c	1.3	98.7
C. microcarpa	70.0 ±8.7b	1.4	98.6
Negative control (ethyl alcohol 70%)	0f	20.8	79.2
Positive control (IR3535 12.5% w/w)	3.0 ±0f	20.5	79.5

[1/]means of protection time / biting rate in each column, followed by the same letter are not significantly different (one-way ANOVA and Duncan's multiple Range Test, P<0.05).

Discussion

In our study showed that all essential oil from Citrus plants gave mean protection time, mean biting rate and protection percentage against *Ae. aegypti* ranged from 10.0 ±8.7 to 65.0 ±22.9 minutes, biting rate ranged from 1.2 to 2.3% and protection ranged from 98.3 to 98.8%. For *Cx. Quinquefasciatus*, all essential oil also showed protection times ranged from 16.0 ±1.3 to 71.7 ±5.8 minutes, biting rate ranged from 1.3 to 1.7% and protection ranged from 98.3 to 98.7%. However, the responses of two mosquito species to the eight essential oils were differcnt, *Cx. quinquefasciatus* was sensitive to all essential oil than *Ae. aegypti*. Moreover, Tawatsin *et al*. (2006) have reported that the essential oils extracted from 18 plant species, belonging to 11 families were more effective against the night-biting mosquitoes (*Anopheles dirus*, *Cx. quinquefasciatus*) exhibiting repellency for 4.5–8.0 hrs. than against *Ae. aegypti* (0.3–2.8 hrs). The essential oil from *C. aurantifolia* was highly effective as repellent and feeding deterrents, this essential oil exhibited the protection time against two mosquito species more than 1 hour (65.0–71.7 minutes), but biting percentage was less than 1.80%. Unfortunately, the mean protection times against *Ae. aegypti* and *Cx. quinquefasciatus* for all essential oil was lower than the Thai Industrial Standards Institute determined time of greater than 2 hrs (120 minutes) (TISI, 2010). Besides, Amer and Mehlhorn (2006) defined that if the protection time of repellent is long and the biting percentage is low, the repellent had good efficiency in repelling mosquitoes and deters biting. In contrast, the protection time is short but the biting percentage is low, than the repellent is more a feeding deterrent than a repellent. If the protection time is long but the biting percentage is high, then the repellent is more a repellent than a feeding deterrent. In the result of this study, essential oil from *C. aurantifolia* showed a low protection time (< 2 hours.) against two mosquito species, while the biting percentage exhibited less than 1.80%, this indicate that *C. aurantifolia* oil is rather feed deterrent than repellent. However, essential oils from other Rutaceae plants in this study also rather feed deterrent (< 2.30 % biting rate) than repellent (< 1 hour protection time). Moreover, plant essential oils have been reported to have repellent activity against mosquito vectors include citronella, cedar, verbena, pennyroyal, geranium, lavender, pine, cinnamon, rosemary, basil, thyme and peppermint. Most of these essential oils provided short-lasting protection usually lasting less than 2 hours (Koul *et al*., 2008). The essential oils from clove (*Syzygium aromaticum*) and *Zanthoxylum limonella* were the most effective and provided 2 hours of complete repellency against mosquito vectors (Das *et al*., 2003; Shapiro, 2012).

Besides, many researchers suggested the synergistic effects among constituents of plant essential oils and mixtures of oils as well as the search of new additives that could make longer the protection times, represents an important tool to replace the chemical repellents used today (Nerio *et al*., 2010).

Moreover, all essential oil in this study provided more protection time and protection percentage against two mosquitoes than IR3535. Our data indicated that all essential oil can be considered to be used as green repellent against mosquito vectors, they are safe for human, children, and domestic animals and environmental friendly. Meanwhile, chemical repellents such as DEET, IR3535 are not safe for publicuse. Moreover, DEET, with symptoms varying from mild to severe and may cause dermal toxicity in infants and children (Kang *et al*., 2009). On the other hand, the mosquito repellent base on plant essential oils exhibited low toxicity to human, animal other non-target organisms, environmental friendly and mosquito resistance will develop more slowly to mosquito repellent base on plant essential oils (Koul *et al*., 2008; Regnault-Roger *et al*., 2012; Isman, 2006).

In conclusion, this study demonstrated the potential of essential oils derived from *C. aurantifolia*, *C. aurantium*, *C. hystrix*, *C. maxima*, *C. medica*, *C. reticulate*, *C. sinensis* and *C. microcarpa* for use as mosquito repellents against *Ae. aegypti* and *Cx. quinquefasciatus*. Moreover, they are also good and safe for humans and children.

Acknowledgements

The authors are highly grateful to Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand and The National Research council of Thailand (NRCT) for providing financial assistance to carry out this study. Thanks are extended to the Department of Entomology, Armed Forces Research Institute of Medical Sciences (AFRIMS) for providing the eggs of *Ae. aegypti* and *Cx. quinquefasciatus*. Grateful thanks are due to the volunteers from Plant Production Technology Section, Faculty of Agricultural Technology, KMITL for their assistance in repellent test.

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(Received: 7 January 2014, accepted: 21 February 2015)