Abstract

This research aims to study the efficacy of Cinnamomum verum (Cv) extracts for ovicidal, larvicidal, and repellent activities against Culex quinquefasciatus mosquito vectors. The active components of C. verum or cinnamon oil by Gas Chromatography (GC) analysis showed the highest cinnamaldehyde at 83.53%. For ovicidal assay, C. verum essential oil at concentrations 12.5, 25 and 50 ppm at 72 h had 100% egg hatch inhibition and had a significant difference when compared to the control group (p<0.05). Larvicidal activity showed that concentrations of 25 and 50 ppm were highly effective in killing 100% mosquito larvae. Morphological changes in egg raft showed a faded color and eggs that seemed to have split from their raft while the larvae changed to a pale white wrinkled body with a destroyed inner tube of the body and were motionless. A Scanning electron microscope study showed that the eggshell and micropyle were wrinkled with the chitin peeled out. After treatment with cinnamon oil, larvae appeared to have a wrinkled body, the thorax and abdominal cuticle were also destroyed with high density of oil particles observed on mouth brushes and obstructing the spiracle. The repellent assay showed that cinnamon oil could repel both male and female mosquitoes for up to 180 min. From the results, it was concluded that cinnamon oil had highly effective repellency against Culex quinquefasciatus adults and insecticidal activity on eggs and larvae stages evidenced by LC50 at 6.59±0.54, 9.07±0.67 and 36.91±7.56 ppm and its morphological changes indicated how the mosquito could not survive after cinnamon oil treatment hence this may be a useful alternative method that is green friendly for controlling mosquitoes in endemic areas.

Introduction

Mosquito-borne diseases is a public health problem in many countries especially in the tropical areas such as India, and some parts of Thailand (WHO, 2014). Since 2017, Sakon Nakhon, located in Northeastern part of Thailand, has had higher cases from mosquito vectors after floods (reported from Sakon Nakhon provincial health office). Culex species, are highly prevalent in both urban and rural areas and are responsible for the transmission of Lymphatic filariasis; a disease which targets lymphatic system (WHO, 1982). The WHO’s recommendation for prevention of vector borne diseases largely depends on vector control, which relies heavily on the use of synthetic chemical insecticides, including Dichlorodiphenyl-trichloroethane, Dieldrin and Malathion (WHO, 2014). The use of chemicals is advantageous because it is

Correspondence: Ratchadawan Aukkanimart, Department of Thai traditional Medicine, Faculty of Natural Resources, Rajamangala University of Technology Isan; Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen; Division of Physiology, School of Medical Sciences, University of Phayao; Udonthani Minicipality Hospital, Udonthani, Thailand.

Tel.: +66.42771460 - Fax: +66.42771460.
E-mail: Ratchadawan.jb@gmail.com

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a fast solution with rapid rate of knockdown and the strong mosquito excitorepellency is convenient but has an impact on the ecosystem such as water sources, housing and air pollution. Currently, there are many kinds of herbs used for preventing mosquitoes or insects such as Cymbopogon citratus, Eucalyptus citriodora oil, Syzygium aromaticum, Curcuma longa and Zingiber officinalis (Phasomkusolsil & Soonwera, 2011; Kalaivani et al., 2012; Madreseh-Ghahfarokhi et al., 2018; Soonwera & Sittichok, 2020). The effects of plant compounds usually exert toxicity by multiple mechanisms of action such as ovicidals, larvicidals, pupericidals, adulticidals, oviposition deterents, repellents, production inhibitors (Amer & Mehllhorn, 2006).

Thai cuisine has many herbs and some of the herbs are widely used to prevent insect infestation. *Cinnamomum verum* or Cinnamon; a medicinal plant belonging to the family Lauraceae, is a small tropical tree that originated in Sri Lanka, East and Middle Asia (Shu et al., 2008). *Cinnamomum verum* is generally used in cooking many dishes. In Thailand, *C. verum* aromatic oils from the roots, bark, leaves, twigs and flowers are used in several aspects including pharmaceutical, food flavoring, cosmetics and repellent manufacturing industries (Ranasinghe et al., 2017; Hamidpour et al., 2015; Wijesekera, 1978; Nabavi et al., 2015). *C. verum* has various active compounds such as Cinnamaldehyde, cinnamate, cinnamic acid transcinnamaldehyde, cinnamyl acetate and eugenol (Singh et al., 2007; Senanayake et al., 1987). Moreover, traditionally cinnamon has been medically used for antitussive, anti-arthritis, anti-oxidant, anti-inflammatory antimicrobial, antifungal activities and reported on as repellent for insects such as Lucilia sericata, Paederus fuscipes, Bemisia tabaci, Megalurothrips sjostedti (Khater & Geden, 2018; Zhang et al., 2016; Abtew et al., 2015; Emilie et al., 2015), and including mosquitoes such as *Anopheles gambiae* (Deletre et al, 2015; Thomas et al, 2017) *Aedes aegypti*, *Anopheles stephensi*, *Culex quinquefasciatus*, (Amer et al, 2006). However, there are a few reports on *C. verum* effects on each life cycle stage of mosquitoes thus the present study aims to investigate the effects of essential oil from *C. verum* on all the stages of a mosquito; on the larvicidal, ovicidal, repellent activities and morphology changes of *C. quinquefasciatus*.

**Figure 1. Cinnamomum verum barks.**

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**Materials and Methods**

**Study area**

This study was carried out at Department of Thai Traditional Medicine, Microbiology and Parasitology Laboratories for mosquito’s cultivation and Plant distillation at Faculty of Industry and Technology, Rajamangala University of Technology Isan, Sakon Nakhon campus from October 2018 – December 2019.

**Plant essential oils preparation**

*Cinnamomum verum* bark (50 Kg) were bought from Traditional shop, Yasothon Province, botanically identified by Botanist of Thai Traditional pharmaceutical unit (Figure 1). *C. verum* bark was extracted by Thermosyphon Distillation (Heat pipe heat exchanger). The machine was set to 100°C for 12 h and then solutions were filtered with 0.45 μm of filter paper, after that cinnamon essential oil was kept and protected from light until use.

**Chemical composition of Cinnamomum verum by GC-MS analysis**

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out to identify the components in cinnamon essential oil extracts using Shimadzu QP2020 NX, Japan with a HP-5MS column (30 m × 0.25 mm, film thickness of 0.25 mm) and GC-MS solution software. He (99.99%, 6 ml/min) was used as the carrier gas, the column temperature was retained at 60°C for 5 min and programmed to 280°C with a rate of 5°C/min, the injector and ion source interface temperatures were 230 and 280°C, respectively, and injection volume 1μl in split mode (1:10). After that, the *C. verum* oil were identified through comparing mass spectrum and retention indices with those given in the library database by Scan mode.

Selective ion monitoring (SIM) was set for quantification and was suitable for identifying the sample components using a mass spectrum for cinnamaldehyde (Sigma®). The sample preparation for evaluating the linearity of the calibration curves in the cinnamaldehyde (Sigma®) solution ranged from 31.25, 62.5, 125, 250 and 500 ppm to confirm the recovery and amounts of compound of samples (*C. verum* oil 50 ppm). The area percentages were calculated by electronic integration of FID peak areas without the use of response factors correction.

**Mosquito test populations**

The *C. quinquefasciatus* strain was obtained from Parasitology and Entomology laboratory in the Faculty of Natural Resources from October, 2018 to January, 2019 and the ethics were approved by Rajamangala University of Technology Isan; ID 52/2561. Next to the water, a straw and a basin were set up at night to catch spawning mosquitoes. This population was brought to the laboratory along with its source water and shifted to a plastic container. The freshly emerged adults were shifted to a plastic cage (30×30×30 cm). After confirmation of the morphological characteristics, the sexes were separated within 24 h of emergence to avoid any chance of mating. All the experiments were conducted in standard laboratory conditions at 25±2°C and 70±5% RH with the 12/12 h (L/D). Adult females were fed on pig blood and adult males were fed on syrup. For mosquito’s population management, other female mosquitoes (3-5 day old) were permitted to mate to obtain the egg and larvae for the insecticidal tests. The adults (both males and females) are then kept in the plastic cage for 4-5 days and maintained with the same condition, females were fed on blood to lay egg. The females will lay eggs two days after they feed.
on blood, egg rafts on the surface of a suitable body of water, methods were adapted from previous study (Richards et al., 2012; Das, Garver and Dimopoulos, 2007; Laurence, 1985). Normally, one day after the female of eggs raft (~100-200 eggs/raft); the egg raft had light white color and was oval shaped; after 2-3 days, the color of eggs raft changed to dark-brown and floated above the water. The 3rd instar larvae was a clear brown long piped body with the stomach having 8-9 segments and it was constantly moving. Larvae were kept in plastic trays (25×35×5 cm) containing 0.5 g of sterilized diet (fish food powder).

**Insecticidal activity of cinnamon oils against egg and larvae stages**

The concentrations of the oils were determined according to a preliminary test and used in the range of 3.125 to 50 ppm and 6.25 to 50 ppm (diluted with 1%acetone in distilled water) for the ovi-cidal and larvicial tests. The experimental conditions at 25±2°C and 70±5% RH with the 12/12 h (L/D). Samples were divided into 6 groups: 1 egg raft/group, after incubation and 10 of the 3rd stage larvae /group exposed with 5 concentrations 3.125 to 50 ppm and 6.25 to 50 ppm of cinnamon oil and diluents control were determined, observed and recorded larvae death at 24 h and 24, 48 and 72h for the and egg hatching testing. The criteria of larval death: loss of tufts of bristles of segments; reduced thickness of the exoskeleton and loss of integrity of the peritrophic membrane, indicated by shrinkage, and loss of definition observed in the internal organs of the larva (Pratti et al., 2015).

All experiments were done in triplicates. The percentage of hatchability and percentage of larva mortality after treatment were calculated, as the formula below (Botas et al., 2017):

**Ovicidal activity**

\[
\% \text{ of mortality} = \frac{\text{Number of hatched larvae}}{\text{Total number of eggs}} \times 100
\]

**Larvicidal activity**

\[
\% \text{ of mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae in treatment}} \times 100
\]

**Repellent test**

The repellent activity of the cinnamon oil was performed according to the methodology described by a previous study (Govindarajan, 2011). One hundred of *C. quinquefasciatus* male and female adults, 4-5 days old, were kept in a net cage (30×80×30 cm³). The repellent assay was divided into 4 groups: i) positive control pyrethrins, KAYARI®, Thaporn marketing co., LTD, Thailand, ii) negative control group, cottons were soaked with syrup for male adult and animal blood for female adult at same concentrations, iii) cinnamon essential oil group at concentration 50 ppm, and iv) olive oil (diluents control) and all groups, cottons were soaked with 1,000 µl of solutions.

Landing counts were recorded at 0, ½, 1, 1½, 2, 2½, 3, 3½, 4, 4½, 5, 5½ and 6 h (s). The petri dishes with soaked cotton were removed and rotated between the exposure intervals to avoid position bias. The mean of percentage repellency for each group were calculated based on the data of the three replicates, following the formula (Govindarajan and Sivakumar, 2012) (2012).

\[
\% \text{ Repellency} = \frac{[T_a - T_b]/T_a] \times 100
\]

Where *Tb* is the number of mosquito landings in the negative control group; *Ta* is the number of mosquito landings in the treated group.

**Statistical analysis**

All data were expressed as means ± standard error of triplicate measurements. Standard Deviation (SD) did not exceed 5% for the majority of the values obtained. The treatment means were subjected to a one-way ANOVA and Bonferroni correction test by SPSS V.16.0 and lethal Concentration 50% (LC50) was subjected to Probit analysis.

**Results**

**Identification active compounds by GC-MS analysis**

Essential oils extracts of *C. verum* were analyzed by GC-MS and their quantitative compositions were determined (Table 1). The main components of essential oils were Cinnamaldehyde, (E)- , trans-.beta.-Ocimene, alpha.-Terpineol, Bicyclo[2.2.1]heptan-2-ol and Benzene, 1-(1-butenyl)-4-methoxy-, trans-, respectively (as shown in Table 1). The standard curve of cinnamaldehyde containing data and the results were represented in linear with a multiple R²=0.996. Quantitative analysis of standard cinnamaldehyde compared to cinnamon oil extracts, 50 ppm, were shown area by area. The % area and retention times (RT) of sample cinnamon oil were cinnamaldehyde, (E)-42585548 (RT 2.759 min), Benzene 6.37 (RT 3.43 min) and Acetic acid 5.30 (RT 3.80 min) while standard Cinnamaldehyde, (E)- at 62.5, 125, 250 and 500 ppm % area were

| Retention time (min) | Compound name                         | Area (%) |
|---------------------|--------------------------------------|----------|
| 10.557              | Cinnamaldehyde, (E)-                 | 83.53    |
| 5.362               | trans-.beta.-Ocimene                 | 2.01     |
| 6.934               | Eucalyptol                           | 0.50     |
| 8.862               | Benzenepropanal                      | 0.77     |
| 9.080               | endo-Borneol                         | 0.66     |
| 9.381               | alpha.-Terpineol                     | 1.49     |
| 10.688              | Bicyclo[2.2.1]heptan-2-ol            | 1.29     |
| 12.054              | Benzene, 1-(1-butenyl)-4-methoxy-, trans- | 4.72    |
| 12.804              | Acetic acid, cinnamyl ester          | 2.67     |
| 29.049              | Unknown                              | 0.68     |
| **Total**           |                                      | **100**  |
568957, 1434372, 5194439 and 9343177, respectively, at the same retention time of 2.75 min as cinnamon oil had area 42585548 (Figure 2).

**The effect of cinnamon essential oil on the hatching of C. quinquefasciatus eggs**

Cinnamon essential oil considerably influenced the viability of *C. quinquefasciatus* eggs in laboratory condition. The cinnamon essential oil at concentrations ranging from 0, 3.125, 6.25, 12.5, 25 and 50 ppm for 0-72 h showed different effects for percentage of egg hatching as 93.13±2.07, 53.84±6.32, 17.05±17.05, 0.00±0, 0.00±0 and 0.00±0%, respectively. The LC₅₀ values of ovicidal assay were 3.31±0.31 ppm (Figure 3) and color changes showed the eggs of *C. quinquefasciatus* after cinnamon essential oil treatment were faded in color and seemed to have split from their raft (Figure 4).

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**Figure 2.** GC-MS SIM chromatogram analysis; standard Cinnamaldehyde, (E)- at concentrations of 62.5, 125, 250, and 500 ppm (a, b, c and d, respectively), in 50 ppm of *Cinnamomum verum* essential oil (e).
The effect of cinnamon essential oil on the survival of *C. quinquefasciatus* larvae

Cinnamon essential oil concentrations at 50% lethal concentration (LC50 values) for larvicidal test were 13.45±0.82 ppm and with 25 and 50 ppm of cinnamon oil extracts having the ability to kill 100% of larvae (Figure 5). Observation of morphological changes after treatment showed significant difference in the appearance from control group. In the control group; third instar larvae had clear brown body with the three regions of head, thorax and abdomen compact. The inner respiratory tube inside the abdominal were long; starting from the thorax to the end of the abdominal segment with the normal 9 segments in the abdomen and siphon is terminal outer tube are a respiratory organ and the surviving larvae had constantly moving. After cinnamon essential oil (treatment group), the larvae were characterized by a pale white wrinkled body with the inner respiratory tube of the body lost as a part of the body and it had decreased movement at 25 and 50 ppm no movements (Figure 6).

Repellent activity of cinnamon essential oil against *C. quinquefasciatus* male and female adults

The repellency test was divided into 4 groups: distilled water (control), olive oil (diluent control), cinnamon oil concentration of 50 ppm and 10% pyrethrin (positive control). Repellency for treatment groups on *C. quinquefasciatus* male and female were observed for up to 180 minutes. Results of repellency bioassay on male *C. quinquefasciatus*, observed at 180 min, of distilled water (control), olive oil (diluent control), 10% pyrethrin (positive control) and cinnamon oil concentration of 50 ppm groups were 0±0.00, 19.57±6.25, 89.66±5.80 and 95.18±4.58, respectively while those of female *C. quinquefasciatus* showed 0±0.00, 13.93±3.74, 86.78±10.36 and 86.07±3.75, respectively with significantly difference to control and olive oil groups. While cinnamon oil had similar effects with pyrethrin, both groups could provide 100% protection up to 0, 30, 60, 90, 120 and 150 min against both *C. quinquefasciatus* male and female (Figure 7).

The Scanning electron microscope study of *C. quinquefasciatus* eggs and larvae

Comparative morphometric and morphological studies of eggs and larvae under scanning electron microscope (SEM) were undertaken, in control and treatment groups (cinnamon oil). The morphology in eggs and larvae of *C. quinquefasciatus* after treatment differed from the egg’s control group which showed a cylindrical shape in raft, rounded head-end and the egg raft was tidily arranged with a slightly wrinkled micropyle (Figure 8a-d). After cinnamon essential oils treatment at a concentration of 50 ppm, the eggs were found to have a wrinkled eggshell, dark skin, chitin peeling out and with withered characteristics, the eggs broke out from the egg terminals of the egg raft and a shrunk micropyle was also observed (Figure 8e-h).

Under scanning electron microscope, *C. quinquefasciatus* larvae control group, the head (Figure 8i,j), thorax (Figure 8s), abdomen (Figure 8t) and spiracle (Figure 8k,l) appeared normal: thick mouth brushes (Figure 8i), the antennae are normally developed (Figure 8j) and siphon and spiracle can pass air in and out (Figure 8k,l). After cinnamon essential oils treatment at a concentration of 50 ppm, the larvae was observed by SEM it was found that the body was wrinkled (Figure 8u-x). In addition, the thorax and abdominal cuticles were also wrinkled (Figure 8w,x), high density of oil particles was observed on mouth brushes (Figure 8m), the antenna were wrinkled (Figure 8n), particles found in cinnamon essential oils obstructed in the respiration on siphon and spiracle (Figure 8o,p).

Discussion

In this study, we investigated the effect of cinnamon essential oil extracts for the elimination of eggs, larvae, pupae and adults of *C. quinquefasciatus*. The essential oil isolated from the bark of *C. verum* was analyzed using GC-MS. The cinnamon essential oil was yellow-light in color and had an odor of cinnamon. Nine compounds accounting for 99.32% of all essential oil were identified. The main active compound in cinnamon essential oil extracts was cinnamaldehyde, (E)- 83.53% and it has the same structure and retention time with cinnamaldehyde standard (Figure. 2). According to a previous study on its chemical compositions, Cinnamomum genus, *C. zeylanicum*, bark essential oil had the...
highest content of cinnamaldehyde at 77.34%, C. verum was found. (E)-cinnamaldehyde at 52.87%, C. cassia was found. (E)-cinnamaldehyde at 62.96% and others (Batiha et al., 2020; Kallel et al., 2019).

The results of the test of C. quinquefasciatus eggs showed that the percentage of hatching from eggs to larvae was 0 ± 0.00 at a concentration 12.5, 25 and 50 ppm at 72 h (Figures 3 and 4). In consistent with the study, Veni et al. (2017) which reported that Terminalia chebula could kill adults of mosquito and inhibit the egg hatching (100% mortality) at 150 μg/ml Ae. aegypti, An. gambiae and C. quinquefasciatus. Similar report of Govindarajan et al. (2011b) showed that the crude extract of Cardiospermum halicacabum extracts of methanol and benzene exerted 100% mortality at 300 ppm against C. quinquefasciatus and Ae. aegypti attained the complete ovicidal activity at 400 ppm. The crude extract of C. pulcherrima exerted 0% hatchability on C. quinquefasciatus, Ae. aegypti and An. Stephensi at 375, 300 and 225 ppm, respectively. In agreement with this, Reegan et al. (2015) also reported that hexane extract of Limonia acidissima had high ovicidal activity at 79.2% and 60% on C. quinquefasciatus and Ae. Aegypti eggs, respectively.

Apart from this, the result of external surface changes by SEM study on C. quinquefasciatus egg hatching after exposure to cinnamon essential oils (Figure 8) showed that chitin wall may have been destroyed by the oils entering via an eggshell pore leading to embryo toxicity thus altering the egg hatching process (Ramkumar et al., 2019).

The results of larvae survival of C. quinquefasciatus after exposure to cinnamon oil at concentration 50 ppm for 24 h showed that 100% of larvae were killed (Figure 5). The death larvae were counted characterized by a pale white wrinkled body with the inner respiratory tube of the body lost as a part of the body and doesn’t movements at all (Figure 6). The results of this study were similar to previous studies where An. gambiae larvae in the control group did not show any damage to their body parts; digestive and respiratory tracts were whole and well (Kringer, 2010). Pratti et al. (2015) reported larvae exposed to essential oil showed a toxic effects on the head, loss of tufts of bristles of segments; reduced thickness of the exoskeleton and loss of integrity of the peritrophic membrane, indicated by shrinkage, and loss of definition observed in the internal organs of the larva, reduced thickness of the

![Figure 5. The percentage of survival larvae of C. quinquefasciatus after treatments. *significantly different p<0.05 of control vs cinnamon oil groups.](image)

![Figure 6. The morphological changes in C. quinquefasciatus larvae. (a, f, k, p) Control, (b, g, l, q) cinnamon essential oil 6.25 ppm, (c, h, m, r) cinnamon essential oil 12.5 ppm, (d, i, n, s) cinnamon essential oil 25 ppm, and (e, j, o, t) cinnamon essential oil 50 ppm, (Ab: Abdomen, C: Caudal hair, T: Thorax, H: Head, AG: Anal Gill).](image)
Figure 7. Repellency test of experimental group on adult males and females. *significantly different p<0.05 of control vs control groups.
Conclusions

This study concluded that cinnamon oil had high cinnamaldehyde ovicidal properties but their efficacies depend on concentrations and mosquito species. Egg hatching inhibition was dose-dependent manner. Overall, cinnamon oil showed a potential on larvicidal and repellent activities on \textit{C. quinquefasciatus}. This study suggests further investigations to study other mosquito species to elucidate species-specific action of these compounds and conduct field trials.

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Figure 8. Scanning electron microscope of eggs surface of \textit{C. quinquefasciatus} of control and after cinnamon oil treatment groups. (AB: abdominal part; An: antenna; Mb: mouth brush; M: Micropyle; S: siphon; Spl: spiracle; T: thorax).
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