MATERIALS AND METHODS

1. Rearing of *M. domestica* and *C. megacephala* in laboratory

The colonies of *M. domestica* and *C. megacephala* were originated from the numerous adult flies collected from two fresh markets in Muang district, Chiang Mai province, northern Thailand, by using sweeping net. The caught flies were transferred into small black net cage (16×16×16 cm) and then transported to fly rearing room of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, for identification and colonization. Before identification, flies were anesthetized by placing in refrigerator set at 4°C for 15 min. The anesthetized flies were identified under dissecting microscope followed the taxonomic keys of Tumrasvin and Shinonaga (1977), Kurahashi and Chowanadisai (2001). *M. domestica* and *C. megacephala* were separated into each standard cage (30×30×30 cm) screened with black net cloth (25 meshes/mm^2); sixty adult flies in equal sex ratio in each cage. Both of *M. domestica* and *C. megacephala* adults were reared with two kinds of food including (I) the mixture of 10% (w/v) sugar solution 985 ml and multivitamin syrup 15 ml and (II) fresh pork liver as both food source and oviposition site. Besides, *M. domestica* were provided with the combination of rice polish, chaff and water at weight ratio of 2:1:1, and 40 g of this mixture was placed in a 9-cm glass plate, as the supplementary food and oviposition site. Small pieces of fresh pork liver were changed daily while the mixture of 10% sugar solution and mutivitamin syrup, and the supplement food were changed every two days. Subsequently, the oviposition sites were observed daily for the presence of fly eggs. If so, the eggs were transferred into a 12×15×6 cm transparent plastic box, and 40-g fresh pork liver was provided as larval food. For over crowed prevention, each box consisted of thirty larvae. A lid of box was cut as rectangle approximately 3/4 of the total area and replaced with finest silk screen cloth (100 meshes/mm^2) for ventilation as well as to prevent other small insects entering to oviposite in the rearing box. The box was covered by its lid and both parts were sealed tightly with adhesive paper tape to prevent the larvae crawled out. These rearing boxes were kept

under ambient temperature and atmospheric condition in the rearing cabinet at the fly rearing room, Department of Parasitology, Faculty of Medicine, Chiang Mai University. Liver was replaced daily until some third-instar larvae developed to be prepupa, the non-feeding period. The box having pupae was still covered and tightly sealed until some emerged to be adults. Then, the box containing some adult flies was placed in the rearing cage and the lid was taken out for releasing the adults within the cage. The next generation of flies was reared in the same manner as previously described. Flies after the eighth generations were graded as the laboratory colony and used to assess the toxicity of eucalyptol.

2. Assessment of eucalyptol toxicity

The commercial eucalyptol was purchased from Sigma-Aldrich® (Switzerland). This eucalyptol was produced for R&D (Research and Development) use only, not for drug, household or other use. Its properties are 98% purity and 0.92 g/ml of density.

The assessment of eucalyptol toxicity against *M. domestica* and *C. megacephala* was performed using the same methods; topical application method for adults (WHO, 1980; Saito, et al. 1992) and dipping method for third-instar larvae (Matsumura, 1985).

2.1 Topical application method for assessment eucalyptol toxicity against adults *M. domestica* and *C. megacephala*

Four-days old adult flies were individually transferred from the rearing cage to small cage (16×16×16 cm) using a transparent test-tube. This cage was then covered with transparent plastic bag in order to anesthetize them by fumigant with 5 L of CO_2 for ~3 min. Flies were separated into male or female group. Flies of each sex were randomized divided into 7 groups, 20 flies per group and transferred into each small cage (16×16×16 cm) for recovery before being treated with eucalyptol. Eucalyptol solutions were immediately prepared in small glass bottle by serially 2-fold dilution method using absolute ethanol as the solvent. Concentration series started at the concentrated (100%), 50%, 25%, 12.5% and 6.25% (v/v)

of eucalyptol, respectively. These denoted 0.902, 0.451, 0.226, 0.113 and 0.056 g/ml, respectively. All dilutions were mixed well by using an auto-pipette and then the bottles were immediately and tightly closed with their caps. Flies in each group were re-anesthetized by the previously described CO_2 fumigant method prior to test with topical application. Anesthetized flies were gently placed onto plastic ice cube, then 1.0 µl of each diluted eucalyptol concentration was individually topical applied at the dorsal thorax of each flies using a Hamilton Digital Microsyringe™ (Hamilton; USA) as shown in Table 2.

Control flies were divided into 2 groups; the first group was treated with absolute ethanol while the another group was untreated. Both control groups were anesthetized twice similar to the manner of treated groups. After being tested, all flies, including the control groups, were transferred into each rearing cage provided with adult fly foods. Mortality in each fly group was assessed at 24-hr period after exposure by softly stimulating each fly with tip of the pen, with those had not showed any respond or move being considered deadly. The topical application of each sex of each fly species was carried out in three replications. The LD_{50}, LD_{95} and LD_{99} values of toxicity were determined based on mortality data at 24-hr assessment, and Probit analysis (Harvard Programming; Hg1, 2) was used for analyzing the dosage-mortality response. The alive flies after being tested with each concentration of eucalyptol were further determined of their life span in 2.1.1.

Table 2. Assessment of eucalyptol toxicity on adult flies by topical application method

Group	Sex of flies	Chemical and concentration (%)
1	male	Not Done (control group)
2	male	Absolute ethanol (control group)
3	male	eucalyptol (100%)
4	male	eucalyptol (50%)
5	male	eucalyptol (25%)
6	male	eucalyptol (12.5%)
7	male	eucalyptol (6.25%)
8	female	Not Done (control group)
9	female	Absolute ethanol (control group)
10	female	eucalyptol (100%)
11	female	eucalyptol (50%)
12	female	eucalyptol (25%)
13	female	eucalyptol (12.5%)
14	female	eucalyptol (6.25%)

2.1.1 Life span of alive flies after being tested with eucalyptol using topical application

Males and females of all alive flies after being tested with the same concentration of eucalyptol were pooled together and maintained within the same cage. Adult foods were provided the same as previously described and mortality of flies were investigated daily compared with those in those two control groups. All flies in the rearing cages were maintained under the same ambient condition including quality and quantity of food. Replacement of food was performed in the same manner as previously described until all flies were dead. The life span of flies in each group was summarized and analyzed comparing with control groups using Mann Whitney *U* test.

2.2 Dipping method for third-stage larvae of *M. domestica* and *C. megacephala*

The third-stage larvae of *M. domestica* and *C. megacephala* used in this experiment were 3 days-old after hatching from the same batch of egg. Each species was randomized into 7 groups (20 larvae/group) and reared in each rearing box. Eucalyptol solutions were immediately prepared in a ceramic bowl by serially 2-fold dilution method using absolute ethanol as the solvent. Concentration series started at the concentrated (100%), 50%, 25%, 12.5% and 6.25% (v/v) of eucalyptol, respectively. These denoted 0.902, 0.451, 0.226, 0.113 and 0.056 g/ml, respectively. Bowl containing each concentration of eucalyptol was tightly covered with the lid until they were used for dipping method. For the experiment, larvae of each group were wrapped with voile cloth (49 meshes/mm^2) and gently dipped into eucalyptol solution, whereas those of control group were dipped in the absolute ethanol (Table 3).

Table 3 Assessment of eucalyptol toxicity on third-stage larvae by dipping method

Group	Chemicals and concentration (%)
1	Absolute ethanol (control group)
2	eucalyptol (100%)
3	eucalyptol (50%)
4	eucalyptol (25%)
5	eucalyptol (12.5%)
6	eucalyptol (6.25%)

After being dipped in eucalyptol solution for exactly 30 second, the larvae were transferred into the rearing box containing larval food. Mortality of each larva was assessed at 24-hr period by touching each fly with paint brush (no. 0), with those had not moved being considered deadly. The dipping experiments of each fly species were carried out in three replications. The LD_{50}, LD_{95} and LD_{99} values of toxicity were determined based on mortality data at 24-hr assessment, and Probit analysis (Harvard Programming; Hg1, 2) was used in analyzing the dosage-mortality response. The alive larvae after being tested with each concentration of eucalyptol were further determined of their emergence and adult sex ratio.

To determine the effect of eucalyptol on fly larvae, those dying only due to 100% eucalyptol were observed any surface changing by means of scanning electron microscope.

2.2.1 Emergence of adult flies after third-instar larvae being dipped with each concentration of eucalyptol

The living third-stage larvae after being dipped in each concentration of eucalyptol and absolute ethanol were reared in each rearing box. The maintenance of larvae until adult was the same manner previously described in method 1. Once emergence occurs, adult flies were counted and sexed. Chi Square test was used to compare the emergence rate of tested groups with control group.

2.2.2 Surface ultrastructure of third-stage larvae of *M. domestica* and *C. megacephala* after being treated with 100% eucalyptol using scanning electron microscope (SEM)

The mortal larvae, after tested with concentrated eucalyptol by using dipping method, were ultrastructurally investigated using SEM comparing with the control larvae that were dipped in absolute ethanol.

Larvae from treated and control groups were primary fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) at 4°C for 24 hr in an individual small glass bottle covered with foil. After that, they were rinsed with phosphate buffer (2 times of 10-min interval). The rinsed larvae were fixed (post fixation) in 1% osmium tetroxide (OsO_4) at room temperature for 2-3 hr. After 2 times rinsing with phosphate buffer, they were dehydrated with gradually different concentration of 10%, 30%, 50%, 70%, 80%, 90%, 95% and absolute ethanol. They were maintained in each step of dehydration for 2 hr except absolute alcohol for 1 hr and replaced it twice. In last step of dehydration, acetone was applied instead of absolute ethanol 2 times, each at 30-min interval. The critical point drying was performed thereafter. These specimens were attached to brass holders, using carbon two-component glue. They were coated with gold in a high-vacuum sputtering apparatus and stored in a desiccator until use. The specimens were observed with a JEOL-JSM840A scanning electron microscope (Japan) at an

accelerating voltage 20 kV. Photographs were made using Kodak® Verichrome Panchromatic film VP 200 (New York, USA).

The surface changes of tested larvae was observed and compared with those of the control. Photographs were taken according to the obvious differences of both larva groups as the scientific evidences.