BIOACTIVITY OF CITRUS SEED FOR MOSQUITO-BORNE DISEASES LARVAL CONTROL

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Abstract. This study aimed to determine the activity of citrus-seed extract against *Ae. aegypti* and *Cx. quinquefasciatus* larvae. The results indicated that ethanol citrus-seed extract showed the best killing effect on *Ae. aegypti* larvae, followed by local liquor, and water, with LC_{50} of 2,267.71, 6,389.22, and 135,319.40 ppm, respectively, whereas against *Cx. quinquefasciatus* larvae, the LC_{50} were 2,639.27, 5,611.66, and 127,411.88 ppm, respectively. Temephos was tested against *Ae. aegypti* larvae; the LC_{50} was 0.00057 ppm, which was nearly 4,000,000 times less than ethanol citrus-seed extract. When ethanol citrus-seed extract and temephos were tested with Nile tilapia (*Oreochromis niloticus*), a standard environmental organism, using LC_{50} of *Ae. aegypti* larvae at 2,267 and 0.00057 ppm, respectively, fish mortality was 0%. The results suggested that ethanol citrus-seed extract had no harmful effect on the fish, and that temephos, which is recommended by WHO, was safe for use in drinking water. However, when the LC_{50} dose that killed *Ae. aegypti* larvae for local liquor (6,389 ppm) and water extract (135,319 ppm) were tested with fish, the mortality rates were 35% and 100%, respectively. On the whole, the results suggested that ethanol citrus-seed extract is environmentally friendly and can be used in the control of *Ae. aegypti* and *Cx. quinquefasciatus* larvae.

INTRODUCTION

Aedes aegypti is a very important disease vector, transmitting the arbovirus causing dengue hemorrhagic fever (DHF) in humans (Rigua-Perez *et al,* 1997). *Culex quinquefasciatus* is an important urban mosquito that plays a major role in the transmission of *Bancroftian filariasis* in Myanmar (Subra, 1980). At present, many Burmese laborers come to work in Thailand, and some of them have microfilariae in their blood; *Cx. quinquefasciatus* have been infected with *Wuchereria bancrofti* in the laboratory (Rongsriyamy, personal communication). The natural population of the two vectors has increased with the growth of towns and poor sanitation (WHO, 1982). The current recrudescence of these diseases is due to the greater number of breeding places in today's throwaway society, and to the increasing resistance of mosquitoes to current commercial insecticides.

Temephos has been used for controlling DHF vectors in Thailand since 1970. The sand-granule formulation of temephos is an effective larvicide (Phanthumachinda, 1974). The WHO has recommended a dosage of Abate 1% SG for eliminating larval populations at a ratio of 1 gram: 10 liters of water (Bang *et al,* 1972). However, the control of mosquito-borne diseases is becoming increasingly difficult because the effectiveness of vector control has declined, due to the development of resistance in mosquitoes against currently used insecticides (Chandre *et al,* 1998). *Ae. aegypti* has also developed resistance to a variety of insecticides in subtropical and tropical regions of the World (Curtis, 1991). The abundant use of insecticides might also impact fish, amphibians, aquatic arthropods, and other aquatic organisms. Therefore, they should be used carefully; alternative replacement botanical insecticides are needed for emergency vector control. Due to widespread insect resistance to insecticides, harmful residues in the environment and high cost, the use of synthetic insecticides has led to a search for safe alternative mosquito-control methods. Botanical insecticides may provide a safe and effective short-term control technology for mosquito larvae (Sukuma *et al,* 1991).

Citrus reticulata is a favorite orange in Thailand and is classified in the Mandarin group (Setpakdee, 1998). Klocke and Kubo (1982) reported that citrus seeds contain limonoid glucosides (limonin, nomilin, and obacunone). Limonoids have attracted attention due to their insect antifeedant, and growth-regulating activity (Champagne *et al,* 1992), and are present in large quantities in the byproducts of the citrus industry (Klocke and Kubo, 1982). The limonoids in citrus seed

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have been reported in insect control. This aroused interest for larval control in two species of mosquitoes, *Ae. aegypti* and *Cx. quinquefasciatus*. Therefore, the use of plant extracts in insect control is an alternative pest-control method, minimizing the noxious effects of pesticide compounds on wildlife, livestock, non-target insect species, and the environment (Fatope *et al,* 1993).

MATERIALS AND METHODS

Mosquitoes and extractions

In this study, *Ae. aegypti* and *Cx. quinquefasciatus* were used. Both species were colonized in the insectarium of the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University.

Citrus seeds were collected from the orange juice industry in Sam Phran District, Nakhon Pathom Province. They were washed, dried in an oven at 60 ºC for 2 days and finely powdered. Each sample (100 g) was macerated in 400 ml of 95% ethanol, 40% local liquor and water at room temperature for 24 hours and then filtered (Kongkathip, 1994).

Bioassay test of citrus-seed extracts and temephos

The bioassay test was done according to the World Health Organization (1970) methodology. Serial dilution concentrations of citrus-seed extract with various solvents were performed. One ml of the extract was added to a test cup and water added to a total volume of 250 ml. Twenty-five early 4th instar larvae of both species were used per cup and control solution, with 4-cup replications for each concentration. Temephos was tested only with *Ae. aegypti* larvae. The mortality rates were recorded after 24 hours. Three subsequent sets of the experiment were carried out for each test.

Toxicity test of citrus-seed extracts and temephos with fish

The toxicity of citrus-seed extracts and temephos were tested with Nile tilapia fish. Citrus-seed extracts and temephos at LC_{50} were added to a fish-box, with a total 5 liters of water and 20 fish per fish-box. Fish mortality was recorded every hour for 24 hours. Further observations of effects and mortality were recorded every 6 hours for 24 hour. There were two replications for each concentration.

Data analysis

LC_{50} was analyzed by Probit analysis (Finney, 1989) with SPSS for Windows and log-dosage probit line. If the control mortality rates were between 5-20%, the percentage mortality should be corrected by Abbott's formula, as follows:

$$\text{\% corrected mortality} = \frac{\text{\% observed mortality - \% control mortality}}{\text{100- \% control mortality}} \times 100$$

RESULTS

Bioactivity of citrus-seed extracts and temephos

Based on the 24-hour exposure results obtained from citrus-seed extracts on 4th instar larvae, the LC_{50} values of ethanol citrus-seed extract for *Ae. aegypti* and *Cx. quinquefasciatus* larvae were 2,267.71 and 2,639.27 ppm, respectively (LC_{95} = 3,925.70 and 4,587.43 ppm). The LC_{50} regardingcitrus-seed extract in local liquor for *Ae. aegypti* and *Cx. quinquefasciatus* larvae were 6,389.22 and 5,611.66 ppm, respectively (LC_{95} = 12,157.89 and 11,141.77 ppm). The LC_{50} of citrus-seed extract in water for *Ae. aegypti* and *Cx. quinquefasciatus* larvae were 135,319.40 and 127,411.88 ppm, respectively (LC_{95} = 274,122.06 and 273,195.62 ppm) (Table 1). The LC_{50} value of temephos with *Ae. aegypti* larvae was 0.00057 ppm and LC_{95} was 0.116 ppm.

Toxicity of temephos and citrus seed extracts with fish

To determine the effect of citrus-seed extracts on Nile tilapia, the fish were exposed for 48 hours at the LC_{50} of citrus-seed extract against *Ae. aegypti* and *Cx. quinquefasciatus*. At 0.00057 ppm of temephos solution, no fish died. LC_{50} of ethanol citrus seed solution for *Ae. aegypti* and *Cx. quinquefasciatus* larvae were 2,267 ppm and 2,639 ppm, respectively. Fish mortality was 0%. When the LC_{50} of local liquor citrus seed solution for *Ae. aegypti* (6,389 ppm) and *Cx. quinquefasciatus* (5,611 ppm) larvae were tested, fish mortality was 35% and 30%, respectively. LC_{50} of water citrus seed solution were 135,319 and 127,411 ppm, respectively. All fish died in the first hour (Table 2). In the control container, no fish died and the surviving fish had normal characteristics.

DISCUSSION

Citrus-seed extracts in different solvents were tested against *Ae. aegypti* and *Cx. quinquefasciatus* larvae. The results showed that different solvent extracts exhibited different effects on *Ae. aegypti* and *Cx. quinquefasciatus* larvae. Ethanol citrus-seed extract yielded LC_{50} of 2,267.71 ppm (LC_{95} = 3,925.70 ppm) and 2,639.27 ppm (LC_{95} = 4,587.43 ppm) for *Ae. aegypti* and *Cx. quinquefasciatus* larvae. With local liquor, LC_{50} was 6,389.22 ppm (LC_{95} = 12,157.89

Table 1
The effects of citrus seed extracts on *Ae. aegypti* and *Cx. quinquefasciatus* larvae.

Citrus seed extract in various solvents	*Ae. aegypti*		*Cx. quinquefasciatus*	
	LC_{50} (ppm)	LC_{95} (ppm)	LC_{50} (ppm)	LC_{95} (ppm)
Ethanol	2,267.71 (2,084.41-2,481.42)	3,925.70 (3,579.68-4,386.22)	2,639.27 (2,456.78-2,847.70)	4,587.43 (4,242.33-5,021.25)
Local liquor	6,389.22 (5,559.14-7,461.43)	12,157.89 (10,507.95-14,726.39)	5,611.66 (5,240.06-6,032.53)	11,141.77 (10,318.67-12,163.06)
Water	135,319.40 (117,540.83-160,824.95)	274,122.06 (233,041.93-339,843.12)	127,411.88 (111,126.32-150,078.48)	273,195.62 (233,694.60-334,441.09)

Table 2
Toxicity of temephos and citrus seed extracts against Nile tilapia fish.

Larvicides	Concentrations at LC_{50} of *Ae. aegypti* (ppm)	% mortality of fish	Concentrations at LC_{50} of *Cx. quinquefasciatus* (ppm)	% mortality of fish
Temephos	0.00057	0	-	-
Ethanol extract	2,267	0	2,639	0
Local liquor extract	6,389	35	5,611	30
Water extract	135,319	100	127,411	100

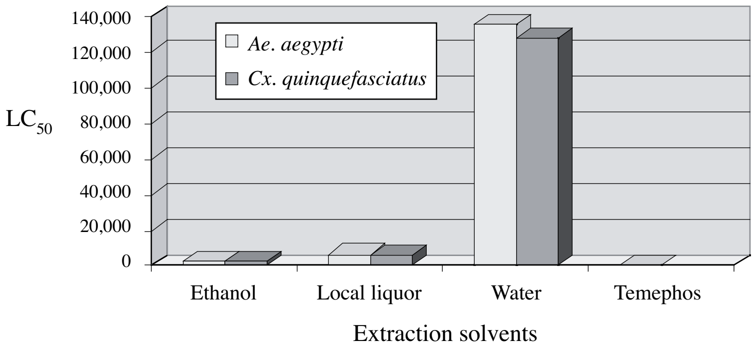

Fig 1- Histogram showing comparison of LC_{50} of citrus seed extracts and temephos on early 4th instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* at 24-hour exposure.

ppm) and 5,611.66 ppm (LC_{95} = 11,141.77 ppm), whereas 135,319.40 ppm (LC_{95} = 274,122.06 ppm) and 127,411.88 ppm (LC_{95} = 273,195.62 ppm) were obtained for water extracts (Fig 1). Based on LC_{50}, ethanol extract was the most potent, followed by local liquor and water extract. The active ingredients in citrus seed were more completely extracted in ethanol and local liquor than water. Limonoids are polar compounds that can dissolve well in organic solvents but dissolve less readily in water (Jayapraksha *et al,* 1977). Water extract was the least effective, as very high concentrations were needed for *Ae. aegypti* and *Cx. quinquefasciatus* larvae control. LC_{50} at 24 hours showed that ethanol citrus-seed extracts were about 60 and 48 times more efficient than water extract against *Ae. aegypti* and *Cx. quinquefasciatus*, respectively. Ethanol citrus-seed extracts were about 3-2 times more potent than local liquor against *Ae. aegypti* and *Cx. quinquefasciatus*. Local liquor citrus-seed extracts were about 21 and 23 times more efficient than water extract against *Ae. aegypti* and *Cx. quinquefasciatus*. These results were supported by Junjanwit (1999), who studied *Pinus merkusii* in ethanol and water extract, and found that ethanol extract was more effective against mosquito larvae than water extract. The results revealed that ethanol leaf extracts were 8, 34, and 2 times more efficient than water for *Ae. aegypti, Cx. quinquefasciatus* and *An. maculatus* larval control. Nicharat (2001) reported that bracken fern in ethanol extract was about 700, 450, and 300 times more potent than water extract. Tuntaprasart (1996) studied yam-bean leaf and seed extracts in different solvents, and ethanol showed the best activity. However, it was noted that water extract did not possess any larvicidal activity. It was clear that ethanol was the best solvent to extract the active ingredient from each plant. Citrus-seed extraction seemed to be possible for *Ae. aegypti* larval control. It was easy to get free of charge, and safe, except for drinking water. The control of *Cx. quinquefasciatus* larvae was more difficult because they prefer polluted water outside the house. There was a need for more citrus-seed extract than in clear water due to decay.

From this study, the LC_{50} of temephos against *Ae. aegypti* larvae was 0.00057 ppm, which was nearly 4,000,000 times that of ethanol citrus-seed extract (Fig 1). However, Paeporn (2003) reported the LC_{50} for *Ae. aegypti* against temephos was 0.00091 ppm, which was higher than this study, as a different strain of *Ae. aegypti* was used.

To determine whether citrus-seed extract can be used in combination with fish or not, the question is whether the concentration that kills larvae can be safe for fish, or not. Therefore, the fish were treated with ethanol citrus-seed extract at 2,267 ppm (LC_{50} of *Ae. aegypti*) and 2,639 ppm (LC_{50} of *Cx. quinquefasciatus*). None of the fish died, at all. However, when the Nile tilapia fish were treated with local liquor extract at 6,389 ppm (LC_{50} of *Ae. aegypti*) and 5,611 ppm (LC_{50} of *Cx. quinquefasciatus*), fish mortality was 35% and 40%, respectively. Moreover, when water citrus-seed extract at 135,319 ppm (LC_{50} of *Ae. aegypti*) and 127,411 ppm (LC_{50} of *Cx. quinquefasciatus*) were tested with the fish, all of the fish died in the first hour. Citrus-seed extract could pass into the blood circulation system by diffusion through the gills and small intestine, and then be accumulated in many organs, such as the liver, kidney and adipose tissues (Spear and Pierce, 1979). Sakulku (1996) also reported the acute toxicity of *Derris trifoliata* leaf extract with Nile tilapia. The static non-renewal test was used to determine LC_{50} during 95 hours. All dead fish showed similar behaviors to those observed in this study.

The results suggest that citrus-seed ethanol extract had no harmful effect on the fish as well as temephos, which is recommended by WHO to be safe for use in drinking water. The study provided evidence that ethanol citrus-seed extract is an environmentally friendly medium for controlling *Ae. aegypti* and *Cx. quinquefasciatus* larvae.

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