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Insecticidal Activities of *Piper retrofractum* Extracts Against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae)

Acting. Lt.Tatchai Subsuebwong* Siriluck Attrapadung** Rutcharin Potiwat**
Raweewan Srisawat** Narumon Komalamisra***

ABSTRACT

Mosquito borne diseases such as dengue fever, filarial are endemic disease of more than hundred countries worldwide. It causes millions of morbidity and mortality of people. Vector control by using chemical insecticides is one important approach to reduce the infected vectors. Plant derived insecticide is an alternative to control by chemical insecticide. In this study was to investigate adulticide and larvicide properties of *Piper retrofractum.* Essential oil was extracted from a mixture of fresh ground fruit and water by hydrodistillation. Adulticide activities were determined by applying drops of essential oil in acetone to the thoraces of mosquitoes. The results showed an LD_{50} of 8.86% ± 0.69 and an LD_{95} of 19.16% ± 2.3 for adult *Aedes aegypti* and an LD_{50} of 6.95% ± 0.51 and an LD_{99} of 14.44% ± 2.49 for adult *Culex quinquefasciatus*. Crude extracts were prepared by macerating dried fruit with 3 organic solvent, methanol, ethyl acetate, hexane and aqueous. Larvicide activities of the diluted crude extracts were determined using third instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus*. The crude organic solvent extracts were diluted to the test concentrations with ~~in~~ acetone, and the crude aqueous extract with distilled water. The methanol extract showed the highest larvicidal activity for *Ae. aegypti*, the LC_{50} being 2.2 ppm. The LC_{50}s of the hexane, ethyl acetate, and aqueous extracts for *Ae. aegypti* were 2.7, 2.9, and 297.4 ppm, respectively. The LC_{50}s for *Cx. quinquefasciatus* were similar, being 2.1, 2.4, 2.9, and 431.3 ppm for the methanol, ethyl acetate, hexane, and aqueous extracts, respectively.

Keywords: Piperaceae, *Piper retrofractum,* Larvicide, Adulticide, Mosquito control

* *Student, Master of Science Program in Tropical Medicine, Faculty of Tropical Medicine, Mahidol University*

** *Lecturer, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University*

****Associate Professor, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University*

Introduction

Mosquito control strategies are generally focused on controlling mosquitoes in either the adult stage or the larval stages of the life cycle. Various physical, biological, and chemical methods have been used to control mosquito vectors, and the method used in a particular area will depend on the specific situation in that area and the budget available. Chemical control methods are popular and widely used in many countries because they are easy to perform and effective. Paul Hermann Muller discovered DDT in 1874 (WHO, 1979). DDT has been widely used to control a range of insect pests, including insect vectors, but DDT use was restricted in 2001 because of the serious negative impacts it has had on the environment. The regular and widespread use of insecticides has resulted in insect vectors increasingly developing resistance to insecticides (Gakhar *et al*., 2013). Temephos, an organophosphate pesticide, is commonly coated onto the surfaces of sand particles that are then spread in campaigns to control dengue (CDC, 2014). Temephos is very effective at killing mosquito larvae, but its unpleasant smell, tendency to cause films to form on water surfaces, and concern about the health risks it poses to humans through its presence in drinking water mean that the general public may not easily accept the use of temephos to control insect vectors. There is currently great interest in studying the insecticidal properties of plants. Insecticides extracted from plants can be used as alternatives to synthetic insecticides for controlling insect vectors. Tropical plants have great biodiversity, meaning that there is the strong possibility that some plants can be used to develop new insecticides.

The Piperaceae family contains plants with spicy flavors. The family contains roughly 3,920 species in 13 genera. The two main genera in the Piperaceae family are the *Piper* genus, which contains 2,000 species, and the *Peperomia* genus, which contains 1,600 species. Piperaceae plants are commonly found in South East Asia, and many have long been used in cooking and traditional medicines (Chaveerach *et al*., 2006). The major chemical constituents of the essential oils of Piperaceae plants are beta-sitosterol, cineole, terpinan-4-ol, 1-betacaryophyllene, piperodione, sitisterol, and venerol (Banerji, 2002; Kubo, 2013).

Many of the plants in the Piperaceae family have adulticidal and/or larvicidal activities toward insects. For example, ethanol extracts of *Piper betle* can kill the larvae of *Aedes aegypti*, with an LC_{50} of 177.6 ppm (Komalamisra *et al*., 2005). Ethanol extracts of *Piper longum*, *Piper ribesoides, Piper sarmentosum,* and *Piper nigrum* have been found to have LC_{50} concentrations of 2.2, 4.0, 8.1, and 0.9 ppm, respectively, toward *Ae. aegypti* larvae (Chaithong *et al*., 2006; Simas *et al*., 2007). Hexane extracts of *P. longum*, *P. nigrum* (white pepper), and *P. nigrum* (black pepper) have been found to have LC_{50} concentrations of 0.017, 0.024, and 0.007 ppm, respectively, toward *Ae. aegypti* larvae (Kumar *et al*., 2010). The essential oil of the fruit of *P. longum* has been found to have LC_{50} concentrations, in solution, of 6.21 and 6.35 $\mu g.mg^{-1}$ toward females of a field strain and a laboratory strain, respectively, of *Ae. aegypti* (Chaiyasit *et al*., 2006). In the study presented here we investigated the adulticidal activity of the essential oil of *Piper retrofractum* fruit and the larvicidal activities of crude extracts of the fruits toward culicine mosquito vectors.

Objective of the study

To evaluate adulticide and larvicide activity of *P. retrofractum* extracts against *Ae. aegypti* and *Cx. quinquefasciatus*.

Material and Methods

Plant material

Mature *P. retrofractum* fruits were collected from three houses in Huay-sathon village, Tha-mi District, Chanthaburi Province, Thailand, in April and May 2014. The plants and fruits had not been treated with synthetic insecticides because the owners grew the plants to use the fruits in their own kitchens. Specimens of the plants were identified by Dr. Chalermphol Suwanphakdee from Kasetsart University, Thailand and the specimens were archived at the Queen Sirikit Botanical Garden, Mae-rim District, Chiangmai Province, Thailand as voucher specimen QBG No.79271.

Essential oil preparation

The essential oils were extracted by hydro distillation method. Each fruit was approximately 5 cm long and 1 cm in diameter. A 1 kg sample of the fresh *P. retrofractum* fruits was washed with tap water, air dried, then ground in a blender. The ground fruit and 2 L of distilled water were added to a distillation apparatus, and the essential oil was extracted by distilling the mixture for 3 hours. The product was kept at 4 °C until use.

Preparation of crude extracts

The crude extracts were extracted by maceration methods with 4 different solvents as methanol, ethyl acetate, hexane or aqueous. Four 10 g samples of the dried fruits were crushed in a blender and then each sample was macerated with 500 mL of a solvent for 1 day. Each mixture was then filtered and the solution was concentrated (using a rotary evaporator for the solutions in organic solvents and by lyophilization for the aqueous solution). The crude extracts were kept at 4 °C until use.

Mosquito strains

Susceptible strain of *Ae. aegypti* and *Cx. quinquefasciatus* were provided by the Insecticide Research Unit, Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. The rearing procedure involved putting the eggs in a 25 cm × 50 cm × 10 cm tray containing 700 mL of dechlorinated tap water. Twenty-four hours after the eggs had hatched, 200 late first instar larvae were transferred to a new tray to be reared, to avoid the larvae becoming overcrowded. The larvae were provided with Hikari® Lionhead fish-food pellets (Kyorin®;Himeji, Japan). Third instar larvae were selected for the larvicidal activity tests. The remaining larvae continued to be reared to the adult phase. The adults were maintained at 28±2 °C until they were used in an experiment, and they were provided with 10% sugar solution soaked cotton

Adulticidal activity

The adulticidal activity against *Ae. aegypti* and *Cx. quinquefasciatus* was determined by topically applying a solution of the essential oil in acetone on pronotum of adult *Ae. aegypti* and *Cx. quinquefasciatus* followed a protocol described by the World Health Organization (2006) with slight modifications. The working solutions of essential oil in acetone were prepared in 5%, 7%, 9%, 11%, 13%, and 15%. Fifty female mosquitoes in each species, non-blood fed, 2-5 days old were used in each concentration. It was anesthetized with ether and then placed on a cold plate before tested. A 0.5 µL drop of an essential oil in acetone was applied to the pronotum of each mosquito using a Burkard® hand microapplicator (Burkard Scientific®; Uxbridge, United Kingdom). After exposure, the tested mosquitoes were transferred in to plastic cups provide with 10% sugar solution soaked cotton placed on covered net and held in appropriate condition for 24 hr. Damage or unrecovered mosquitoes were removed . Two control groups were used, the mosquitoes in one were exposed to acetone and the mosquitoes in the other were untreated. Three replicates of each test, using mosquitoes from separately reared batches, were performed.

Larvicidal activity

Larvicide activities were determined by diluted crude extracts in distilled water with third instar larvae using a method following World Health Organization guidelines (WHO, 2005) with slight modifications. Each extract was diluted to give the desired test concentration, and each test was performed on 100 third instar *Ae. aegypti* larvae and 100 third instar *Cx. quinquefasciatus* larvae. The concentration of organic solvent extract in acetone were 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ppm and the water crude extract dissolved in distilled water were 150, 200, 400, 600, 800, and 1000 ppm. The mortality was determined after the larvae had been exposed for 24 hours. Three replicates of each test, using larvae from separately reared batches, were performed. The Median Lethal Dose (LD_{50}) and Median Lethal Concentration (LC_{50}) were calculated using Probit analysis (Finney, 1987).

Results

The essential oil extracted from the fresh *P. retrofractum* fruit was clear, had a sweet, spicy odor, and was soluble in acetone. A total of 1.25 mL of essential oil was extracted from 1 kg of the fresh fruit by distillation (0.125% yield.). Exposure to 15% essential oil in acetone for 24 hrs was found to cause more than 90% mortality in both *Ae. aegypti* (92.7% mortality) and *Cx. quinquefasciatus* (98.7% mortality). No mortality occurred in either species in the acetone control and untreated control groups

The LD_{50} were 8.86% ± 0.69 for *Ae. aegypti* and 6.95% ± 0.51 for *Cx. quinquefasciatus*, as shown in Table 1.

Table 1 LD_{50} and LD_{95} values of *P. retrofractum* essential oil against *Ae. aegypti* and *Cx. quinquefasciatus* by topical application after 24 h.

Species	Lethal Dose (% of essential oil in acetone)	
	LD_{50}±SE	LD_{95}±SE
Ae. aegypti	8.86 ± 0.69	19.16 ± 2.30
Cx. quinquefasciatus	6.95 ± 0.51	14.44 ± 2.49

The crude aqueous, methanol, and ethyl acetate extracts were yellow and contained particles, but the crude hexane extract was red and oily. The 10 g samples of dried *P. retrofractum* fruit gave crude extracts of 14% yield when methanol was used, 9% yield when ethyl acetate was used, 6% yield when hexane was used, and 20% when aqueous was used. The LC_{50} concentrations for *Ae. aegypti* larvae after 24 hours of exposure were 2.2, 2.9, and 2.7 ppm for the crude methanol, ethyl acetate, and hexane extracts, respectively, diluted in acetone, and 297.4 ppm for the aqueous extract diluted in distilled water as shown in Table 2. The LC_{50} concentrations for *Cx. quinquefasciatus* larvae after 24 hours of exposure were 2.1, 2.4, and 2.9 ppm for the crude methanol, ethyl acetate, and hexane extracts, respectively, diluted in acetone, and 431.3 ppm for the aqueous extract diluted in distilled water as shown in Table 2.

Table 2 LC_{50} values for *Ae.aegypti* and *Cx. quinquefasciatus* larvae exposed to methanol, ethyl acetate, hexane and aqueous extracts of *P. retrofractum* for 24 h.

Solvent	Lethal Concentration (ppm) ± SE			
	Ae. aegypti		*Cx. quinquefasiatus*	
	LC_{50}	LC_{95}	LC_{50}	LC_{95}
Methanol	2.21 ± 0.28	4.45 ± 1.13	2.11 ± 0.4	5.70 ± 0.45
Ethyl acetate	2.96 ± 0.57	5.61 ± 1.06	2.47 ± 0.11	6.30 ± 0.47
Hexane	2.77 ± 0.26	5.79 ± 0.60	2.90 ± 0.25	4.18 ± 0.75
Aqueous	297.39 ± 0.78	629.65 ± 1.43	431.28 ± 1.12	1,621 ± 1.73

Discussion

The Piperaceae family contains 3,920 species, and at least 17 species have been found to have insecticidal properties (Marques and Kaplan, 2015). Piperidine alkaloids are active ingredients in many Piperaceae species (Mishra, 2010; Hieu *et al*., 2014) but the concentrations of these alkaloids vary depending on the species, geographic area, stage of growth, and season (Bao *et al*., 2014). Piperidine alkaloids can enter an insect through ingestion (Paula *et al*., 2000) and the alkaloids can inhibit egg hatching (Tavares *et al*., 2011) or act as adulticides (Rassami, 2011) or larvicides (Chansang *et al*., 2005). The insecticidal activities of essential oil and crude extracts of *P. retrofractum* fruits against *Ae. aegypti* and *Cx. quinquefasciatus* were determined in this study.

The estimated yield of the essential oil used in the adulticide tests was 0.125%, which is five times lower than the yield achieved using the same method from the closely related species *P. longum* (Choochote *et al*., 2006) The *Cx. quinquefasciatus* adults were affected more strongly by exposure to the essential oil than were the *Ae. aegypti* adults, the LD_{50} being 6.9% for *Cx. quinquefasciatus* and 8.8% for *Ae. aegypti* as is shown in Table 1. Seizures occurred in the mosquitoes that had been exposed to the essential oil, once they had recovered from the anesthetic, some of the mosquitoes lost control of and then actually lost their legs and wings. These effects continued for 1 hour, then some of the mosquitoes died. Essential oil extracted from *P. longum* had LD_{50} of 6.21 and 6.35 $\mu g.mg^{-1}$ against laboratory strain and field strain of adult *Ae. aegypti* females in a previous study by Choochote *et al.* (2006). *P. longum* appears to have a stronger adulticidal activity than did *P. retrofractum* (which is closely related) in our study the LD_{50} of *P. retrofractum* essential oil was 8.8%, which is equivalent to 16.56 $\mu g.\ mg^{-1}$. Essential oil extracted from *P. retrofractum* will therefore be suitable for controlling the polluted breeding species *Cx. quinquefasciatus.* Essential oil extracted from *P. retrofractum* has been used in shampoo for treating *Pedicular capitis* in humans (Rassami, 2011). Further study is required for using *P. retrofractum* essential oil to control *Rhipicephalus sanguineus* and *Ctenocephalides canis* in pets.

The LC_{50} concentrations for the organic solvent extracts were not statistically different from each other, but the LC_{50} for the aqueous extract was much higher. The *Ae. aegypti* larvae were 1.5 times more sensitive than the *Cx. quinquefasciatus* larvae to the aqueous extract. The methanol extract had the strongest larvicidal activity for both *Ae. aegypti* and *Cx. quinquefasciatus*. The methanol extracts LC_{50} for *Ae. aegypti* larvae was 142 times lower than the LC_{50} of aqueous extract. The methanol extract LC_{50} for *Cx. quinquefasciatus* was 188 times lower than the LC_{50} of aqueous extract. The larvae responded to the crude extracts within four hours. After that time they moved less than before, became unable to swim to the water surface, and some eventually died. The modes of action the crude extracts was interesting, and need further studies. The larvicidal activities of species in the Piperaceae family have been reported many times. For example, an ethanol extract of *P. betle* had an LC_{50} of 177.62 ppm against *Ae. aegypti* larvae (Komalamisra *et al*., 2005), an ethanol extract of *P. longum* was found to have an LC_{50} of 2.23 ppm against *Ae. aegypti* larvae (Chaithong *et al*., 2006) and also ethanol extracts of white and black *P. nigrum* were found to have LC_{50} concentrations of 0.356 and 405 ppm, respectively (Kumar *et al*., 2010). The results of our study agreed with the results of a study by Chansang *et al.* (2005) who found that crude extracts of dried ripe *P. retrofractum* fruit were larvicides for *Ae. aegypti* and *Cx. quinquefasciatus* with LC_{50} of 79 and 135 ppm, respectively. The synthesis of the compounds responsible for the insecticidal activities of the *P. retrofractum* extracts could possibly be developed to new insecticides for mosquito control. However, the crude extracts should be tested against other mosquito species, such as *Ae. albopictus*, *Cx. tritaeniorhynchus*, and *Anopheles* spp., including other agricultural insects. Since it is the editable plant and the ease preparation of the crude extract by maceration made it can apply in the field to control the pest insects easily. It is eco-friendly and can be the alternative to the chemical synthetic insecticide.

Conclusion

Essential oil extracted from *P. retrofractum* fruit was an adulticide for *Ae. aegypti* and *Cx. quinquefasciatus*, the LD_{50} values being 8.8% for *Ae. aegypti* and 6.9% for *Cx. quinquefasciatus*. The crude methanol extract of dried *P. retrofractum* fruits was a stronger larvicide for *Ae. aegypti* and *Cx. quinquefasciatus* than that were the other crude extracts. A methanol extract of *P. retrofractum* could potentially, therefore, be developed for use in mosquito control programs in urban and rural areas.

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