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The effect of *Piper aduncum* Linn. (Family: Piperaceae) essential oil as aerosol spray against *Aedes aegypti* (L.) and *Aedes albopictus* Skuse

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Abstract. The bioefficacy of *Piper aduncum* L. essential oil formulated in aerosol cans was evaluated against *Aedes aegypti* and *Aedes albopictus* in a simulated room. The aerosol spray test was based on the Malaysian test standard for aerosol (MS 1221:1991UDC 632.982.2 modified from WHO 2009 methodology) and examined the knockdown effect within 20 minutes of exposure. Mortality rate after 24 hour of holding period was also determined. A commercial aerosol spray (0.09% prallethrin 0.05% d-phenothrin) was also tested as a comparison. Our results showed that the knockdown effect of the commercial aerosol spray and *P. aduncum* essential oil spray (8% and 10% concentrations) was significantly higher in *Ae. albopictus* adult females, when compared with that of *Ae. aegypti* adult females ($P<0.05$). There was a significant difference in knockdown between commercial aerosol spray and essential oil spray for both *Aedes* spp. ($P<0.05$). The essential oil induced significantly higher mortality in *Ae. aegypti* (80%) than in *Ae. albopictus* (71.6%) ($P<0.05$). The commercial aerosol spray caused 97.7% and 86.5% mortality against *Ae. aegypti* and *Ae. albopictus* respectively ($P<0.05$). Based on these data, *P. aduncum* essential oil has the potential to be used as an aerosol spray against *Aedes* spp.

INTRODUCTION

The incidence of dengue fever (DF) and dengue hemorrhagic fever (DHF) has increased dramatically over the last decades. It has become endemic in more than 100 countries and more than 2.5 billion people are at risk (WHO, 2008). There is yet no effective drug or vaccine for treatment of DF and DHF. The method to control dengue largely depends on preventing transmission by *Aedes aegypti* and *Aedes albopictus*. The authorities and public still depend on the conventional measures of dengue vector control such as public involvement, environmental management, biological and chemical control of both the adults and the immature *Aedes* mosquitoes and personnel protection against mosquito bites (Chua *et al.*, 2005). According to Ansari (2007), the control strategy which is simple, non-toxic and can be handled by people themselves is ideal. One such way is to use commercial aerosol as a personnel protection tool against mosquitoes.

At present, pyrethroids in aerosol canister are widely used for controlling adult mosquitoes in household and also by thermal fogging and ULV (Ultra-Low-Volume) spraying at community level. Therefore, some concern has been expressed as to the possibility that mosquitoes may develop resistance to pyrethroids due to selection pressure (Samboon *et al.*, 2003). Study in Thailand by Somboon *et al.* (2003) indicated that permethrin and deltamethrin had some degrees of ineffectiveness against *Ae. aegypti*. Moreover, the large–scale use of synthetic insecticides over the last seven decades has led to health problems in non-target organisms, including human, as well as observable negative effects on the environment (Xu *et al*., 2005; Tao *et al*., 2006). These problems have prompted renewed interest in safe plant-derived insecticides that can be used to control mosquito populations without residual effects on non-target organisms or the environment.

Recently, plant essential oils have been examined for their insecticidal activities such as their adulticidal fumigant toxicity (Yang *et al*., 2005), repellent activity (Norashiqin *et al*., 2008; 2009; Trongtokit *et al*., 2004) and also their mosquito growth-regulating activity (Jeyabalan *et al*., 2003). *Litsea elliptica (*Sulaiman *et al*., 2006), *Piper aduncum* (Sulaiman *et al*., 2004) and *Melaleuca cajuputi* (Sulaiman *et al*., 2002) extract have been found to have adulticidal activity when formulated into ULV spraying in the field. In this study, we examined the insecticidal activity of *P. aduncum* essential oil in aerosol cans for spraying against the adult *Ae. aegypti* and *Ae. albopictus* in a simulated room of an ordinary Malaysian house.

MATERIALS AND METHODS

Extraction of *Piper aduncum*

Leaves of *P. aduncum* were collected from Gombak (latitude: 3º 17' 60 N; longitude: 101º 42' E), a secondary forest 25 km from Kuala Lumpur city. The essential oil was obtained by hydro distillation using Turbo Extractor Distillator at Chemical Engineering Pilot Plant (CEPP), Universiti Teknologi Malaysia, Malaysia.

Formulation of *P. aduncum* essential oil aerosol spray

The essential oil was formulated in aerosol cans in different concentrations viz: 1%, 2%, 3%, 4%, 5%, 8% and 10% by combining 40% of odourless kerosene and 60% of Liquefied Petroleum Gas (LPG) as solvent and propellant respectively by Sara Lee Malaysia Sdn. Bhd. A commercial aerosol spray containing prallethrin (0.09% w/w) and d-phenothrin (0.05% w/w) was used as a positive control, while for the negative control, 40% odourless kerosene and 60% of LPG were used.

Test mosquitoes

An established colony of *Ae. aegypti* (susceptible strain) which originated from Institute for Medical and Research (IMR) of Malaysia and *Ae. albopictus* (laboratory strain) which originated from Kota Tinggi, Johor (latitude: 1º 43' 47" N; longitude: 103º 54' 0" E) were reared at the insectarium of Department of Biomedical Science, Universiti Kebangsaan Malaysia. Nulliparous 3-7 days old sugar-fed mosquitoes *Ae. aegypti* and *Ae. albopictus* were used for testing the effects of *P. aduncum* essential oil.

Aerosol spray testing

The aerosol spray test was based on Malaysian Standard (MS 1221:1991UDC 632.982.2) for aerosol spray testing methodology. The experiment was conducted inside a room which was modified from a Peet Grady chamber, simulating a living room of human residence (Figure 1). The room (3.48 m x 4.23 m x 2.19 m) is made of concrete and the floor is covered with white ceramic tiles. The roof is mounted with five iron hooks to suspend mosquito-holding cages. A door (2.05 m x 1.50 m) is located in front of the chamber

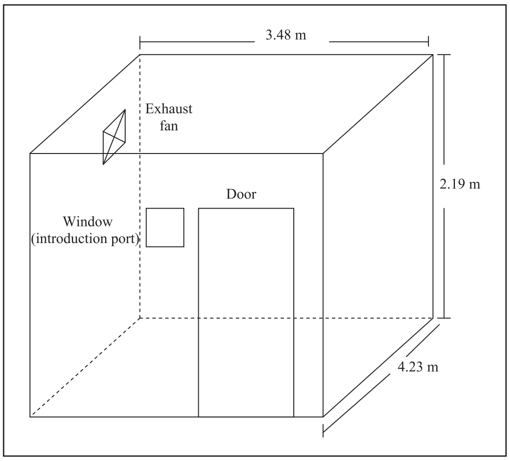

Figure 1. Test room modified from a Peet Grady chamber and simulating a living room of human residence

and a small window (0.24 m x 0.28 m) for spraying is fitted to one side of the chamber. The other side is set up with an exhaust fan for ventilation purposes. All tests were conducted at temperature ranging from 26ºC to 28ºC and 65% to 85% relative humidity. Before a test was conducted, the chamber was cleaned from residue using 10% acetone or detergent. The chamber was considered to be contaminated and unsatisfactory for test purpose when test mosquitoes held for a one hour period with food and water but without insecticide treatment, knockdown/mortality was more than 10% within 10 to 20 minutes after being released into the test chamber.

Aedes spp. were confined in five cylindrical cages covered with mosquito netting (25 cm long x 18 cm diameter), each with 25 female mosquitoes and suspended from hook fixed onto the ceiling of the chamber (2.2 m from the floor). Mosquitoes were provided with 10% sugar solution soaked cotton wool placed on the top of each cage. The aerosol can was weighed before and after spraying to record the actual weight of the insecticide released. The content was shaken vigorously to achieve homogeneity of its content. The aerosol spray was discharged through the introduction port (window) for 5 seconds. All window and door of the chamber were closed for 20 minutes after the mist was introduced. The numbers of test mosquitoes paralyzed or knocked down were counted following spray application at indicated intervals of 1, 5, 10, 15 and 20 minutes.

At the end of 20 minutes, the exhaust fan was switched on for two minutes to remove the remaining aerosol vapour. Thereafter, cages were removed from the chamber; mosquitoes were transferred to plastic containers covered with mosquito netting and 10% sucrose dip cotton wool and kept in a clean room. The mortality and/or recovery of the mosquitoes were recorded after 24 hours. The mortality was based on a combination of dead and moribund mosquitoes over the total number of the mosquitoes in the containers.

Droplet analysis

The droplet size of the aerosol spray was measured using Teflon-coated slides. A slide each was placed at four corners and centre of the chamber before spraying. Immediately after spraying, droplets of the aerosol content that adhered to the Teflon surface were measured using the light microscope. Teflon slides were examined as soon as possible or within 48 hours post-spraying. A total of 200-300 droplets were counted for each experiment for determining the droplet size of insecticidal sprays (Carlisle & Rathburn, 1970).

Data analysis

Data were analysed by using log-probit programme (Raymond, 1985) to determine the value of concentrations that can cause 50% and 90% knockdown and mortality from the tested mosquito populations. ANOVA (Statistical Package for the Social Sciences, SPSS version 12.1) was used to compare the differences between essential oil and commercial aerosol spray and between both species.

RESULTS

Aedes spp. showed the knockdown effect immediately after 60 seconds post-spraying of 2% to 10% *P. aduncum* essential oil (Tables 1 and 2). At a concentration of 10%, *P. aduncum* essential oil gave the highest knockdown effect of 33.2% and 36.3% compared to the commercial aerosol spray which gave knockdown effect of 22.9% and 24.4% against *Ae. aegypti* and *Ae. albopictus* after 1 minute post-spraying respectively. At the end of the observation time (20 minutes post-spraying), *Ae. albopictus* showed the highest percentage of knockdown (2.8 to 72.4%), while for *Ae. aegypti* the percentage ranged between 4.1 to 74.8%. However, *P. aduncum* essential oil still induced lower knockdown effect compared to the commercial aerosol spray which gave 78.7% and 90.7% knockdown 20 minutes post-spraying against *Ae. albopictus* and *Ae. aegypti* respectively.

Tables 2 and 3 showed that at concentration of 1% to 5%, *P. aduncum* essential oil showed high KT_{50}, ranging from 67.0 to 2633.3 minutes and KT_{90} of 279.4 and

Table 1. Knockdown effect of *Piper aduncum* essential oil against *Aedes* spp. at regular interval of exposure

Mosquito	Concentration (%)	% Knockdown				
		1 minute	5 minutes	10 minutes	15 minutes	20 minutes
Ae. aegypti	1	0	2.1	6	2.8	4.1
	2	0.3	0.8	1.9	3.3	4
	3	0.8	5.5	8	10.9	12.1
	4	5.1	15.3	20.9	24.8	34.5
	5	6.1	15.8	21.2	27.2	35.9
	8	19.5	35.1	58.5	62.8	69.2
	10	33.2	45.1	58.5	68	74.8
	Positive control	22.90	55.20	73.10	82.10	90.70
	Negative control	0	0	0	0	0
Ae. albopictus	1	0	0.4	0.9	2	2.8
	2	0.5	1.9	4.3	5.1	6.5
	3	0.6	4.9	10	12.3	13.1
	4	0	1.1	4.8	9.3	13.3
	5	5.4	19.6	24.7	29.6	33.4
	8	24.4	44.8	55.6	66.1	75.9
	10	36.3	50.3	62.1	66.3	72.4
	Positive control	24.40	36.80	55.6 0	69.60	78.7
	Negative control	0	0	0	0	0

Positive control (0.09% prallethrin and 0.05% d-phenothrin)
Negative control (Kerosene and LPG)

Table 2. The KT $_{50}$ and KT_{90} of *P. aduncum* essential oil and commercial aerosol spray (0.09% prallethrin, 0.05% d-phenothrin) against *Ae. aegypti*

Mosquito	Concentrations (%)	Knockdown Time (minutes)		Slope ± standard (%)
		KT_{50} (95% CI)	KT_{90} (95% CI)	
Ae. aegypti	1	2633.3 (IR)[a,b]	98517.0 (IR)[a,b]	0.8 ± 0.4
	2	1635.6 (IR)[a,b]	10262.8 (IR)[a,b]	0.9 ± 0.5
	3	374.5 (105.9-19394.6) [a,b]	10262.8 (949.2-19801820)[a,b]	0.9 ± 0.2
	4	101.5 (101.5-473.1)[a,b]	4304.2 (770.1-190409.1)[a,b]	0.8 ± 0.2
	5	67.0 (39.4-297.6)[a,b]	279.4 (104.2-4769.9)[a,b]	2.1 ± 0.5
	8	7.5 (5.8-9.7)[a,b]	114.2 (63.9-289.9)[a,b]	1.1 ± 0.1
	10	4.5 (3.0-6.1)[a,b]	162.3 (73.9-676.9)[a,b]	0.8 ± 0.1
	0.09% prallethrin and 0.05% d-penothrin	3.5 (2.8-4.2)[b]	25.4 (19.7-335.3)[b]	1.5 ± 0.1

IR = Impossible range. [a] significant compared to commercial aerosol spray (p<0.05). [b] significant compared to both *Aedes* spp.
IR = Immposible range. * significant compared to commercialized aerosol spray.

Table 3. The KT 50 and KT90 of *P. aduncum* essential oil and commercial (0.09% prallethrin, 0.05% d-phenothrin) aerosol spray against *Ae. albopictus*

Mosquito	Concentrations (%)	Knockdown Time (minutes)		Slope ± standard error
		KT50 (95% CI)	KT90 (95% CI)	
Ae. albopictus	1	972.4 (IR)[a,b]	12061.1 (IR)[a,b]	1.2 ± 0.2
	2	1357.5 (146.5-451390100000)[a,b]	47758.6 (1039.7-9.7E+22)a,b	0.8 ± 0.4
	3	116.9 (51.8-728.2)[a,b]	7966.5 (1090.0-83330.1)[a,b]	0.7 ± 0.1
	4	61.6 (36.4-16508.6)[a,b]	1389.4 (393.7-16508.6)[a,b]	1.0 ± 0.2
	5	45.7 (31.7-104.6)[a,b]	149.6 (74.9-782.5)[a,b]	2.5 ± 5.3
	8	5.7 (4.3-7.4)[b]	105.3 (58.1-277.4)[a,b]	1.0 ± 0.1
	10	3.6 (2.1-5.3)[a,b]	235.5 (89.5-1629.1)[a,b]	0.7 ± 0.1
	0.09% prallethrin and 0.05% d-penothrin	5.9 (3.0-11.6)[b]	83.5 (15.4-534.0)[b]	1.1 ± 0.2

IR = Impossible range. a significant compared to commercial aerosol spray ($p<0.05$), b significant compared to both *Aedes* spp.

98517.0 minutes for *Ae. aegypti* and 45.7 to 1357.5 minutes and KT_{90} of 149.6 to 47758.6 for *Ae. albopictus*, respectively. However, at concentrations of 8 and 10%, *P. aduncum* essential oil showed lower KT_{50} and KT_{90} for both *Aedes* spp. At concentration of 10%, *P. aduncum* essential oil showed the lowest KT_{50} value of 3.6 minutes which was significantly different when compared to the commercial aerosol spray (5.9 minutes) against *Ae. albopictus* ($p<0.05$). The commercial aerosol spray indicated the lowest KT_{90} of 83.5 minutes compared to 10% *P. aduncum* essential oil (235.5 minutes) against *Ae. albopictus* and KT_{90} of 25.4 minutes for the commercial aerosol spray compared to 162.3 minutes for 10% *P. aduncum* essential oil against *Ae. aegypti*. There was significant difference for each concentration of essential oil and commercial aerosol spray when compared between both *Aedes* spp. ($P<0.05$).

Figure 2 showed that commercial aerosol spray gave significantly higher mortality (86.5% and 97.9%) compared to the essential oil spray (8% and 10%) which induced 71.6% and 80% mortality against *Ae. albopictus* and *Ae. aegypti* respectively ($P<0.05$). The

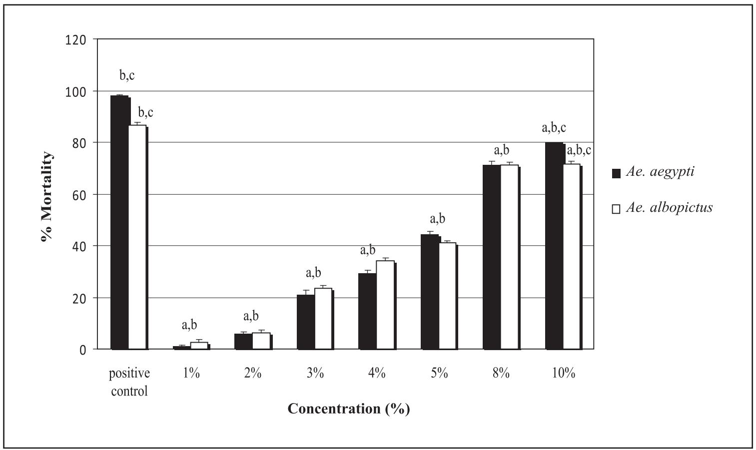

Figure 2. Mortality of *Aedes* spp. 24 h post application with *Piper aduncum* essential oil and commercial aerosol spray (0.09%-pralethrin, 0.05% d-phenothrin). Vertical lines denote standard errors of the means
a = significant difference compared to each concentration of *P. aduncum* essential oil ($p<0.05$)
b = significant difference between the *P. aduncum* essential oil and commercial aerosol spray ($p<0.05$)
c = significant difference between *Ae. aegypti* and *Ae. albopictus* ($p<0.05$)

mortality was significantly higher in *Ae. aegypti* (80%), compared to *Ae. albopictus* (71.6%). There was no significant difference between LD_{50} and LD_{90} of *P. aduncum* essential oil against both *Ae. aegypti* and *Ae. albopictus* species respectively ($P>0.05$) (Figure 3). *Ae. albopictus* exhibited the lowest LD_{50} (5.5%) compared to the LD_{50} (5.6%) of *Ae. aegypti*. However, the LD_{90} value for *Ae. aegypti* was the lowest (12.3%) compared to the LD_{90} value for *Ae. albopictus* (12.7%). Analysis of volume median diameter (VMD) showed that *P. aduncum* essential oil aerosol spray ranged between 7.6 - 8.6 microns (Table 4). There was no significant difference in droplet size from each test concentration of essential oil and the commercial aerosol spray (0.09% prallethrin and 0.05% d-phenothrin) ($P>0.05$). The rate of discharge was also consistent for each concentration of *P. aduncum* essential oil (range: 2.7-3.2 mg/s).

DISCUSSION

Aerosol is the most widely used spray technology for household insecticide in a pressurized can, which is a simple system and well developed technology. It was first

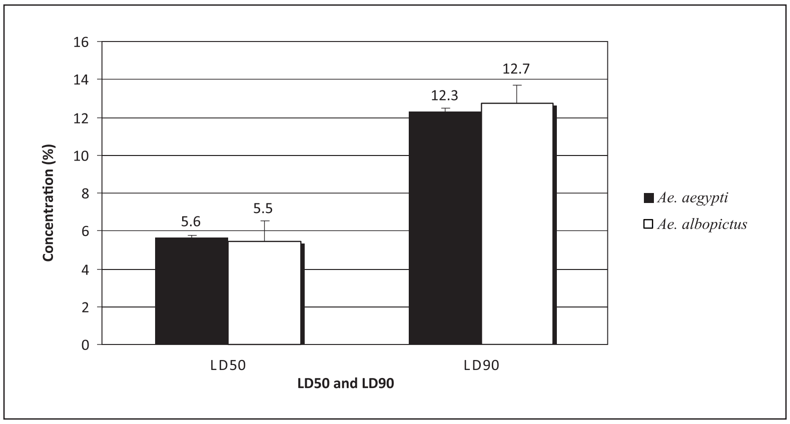

Figure 3. The LD_{50} and LD_{90} of *P. aduncum* essential oil aerosol spray against *Aedes* spp. 24 h post application. Vertical lines denote standard errors of the means

Table 4. Volume median diameter (VMD) and mean rate of discharge (mg ± sem) of *P. aduncum* essential oil and commercial aerosol spray

Concentration (%)	Amount of aerosol (mg)	Rate of discharge (mg/s)	Volume median diameter (VMD) (mean ± sem) μ
1	15.4 ± 0.5	3.1	7.6 ± 0.2
2	14.9 ± 0.9	3.0	8.5 ± 0.1
3	15.7 ± 0.3	3.1	8.0 ± 0.7
4	14.8 ± 1.0	3.0	8.5 ± 0.3
5	15.8 ± 1.0	3.2	7.6 ± 0.1
8	15.9 ± 0.6	2.7	8.0 ± 0.7
10	15.7 ± 0.7	3.1	8.0 ± 0.5
Positive control	15.4 ± 0.9	3.1	8.6 ± 0.5
Negative control	15.9 ± 1.1	3.2	8.2 ± 0.4

Positive control (0.09% prallethrin and 0.05% d-phenothrin)
Negative control (kerosene and LPG)

developed by Goodhue & Sullivan (1943) for practical use during the 2nd World War and has been improved and used widely in many fields ever since. Studies using aerosol spray were conducted since the early 40s and these studies were focused on pyrethroid-based aerosol spray. Up till today, there have been no studies on new formulations incorporating botanical sources as active ingredients in aerosol insecticide sprays against household pests. Results from this study showed that essential oil of *P. aduncum* has the potential as an aerosol spray against *Aedes* spp.

At higher concentrations (8% and 10%), *P. aduncum* essential oil had shown better knockdown effect which is as good as commercial aerosol spray. Thus, *P. aduncum* essential oil in the formulation of aerosol spray exhibited rapid knockdown properties. In a previous study by Hidayatulfathi *et al.* (2004), the standard WHO bioassay adulticidal test showed that at 5% *P. aduncum* essential oil, the knockdown effect (59.09%) occurred after 60 minutes exposure against adult *Ae. aegypti*. The present study indicated that at higher concentrations (8% and 10%), KT_{50} of *P. aduncum* essential oil were lower than 10 minutes (range between 3.64 minutes and 7.52 minutes). However, *P. aduncum* essential oil exhibited higher value of KT_{90} compared to commercial aerosol spray (0.09% prallethrin and 0.05% d-phenothrin). This is because the active ingredients of the commercial aerosol spray have both knockdown and killing effect. Prallethrin has an outstanding knockdown effect while d-phenothrin is the killing agent associated with knockdown properties (WHO, 2004). The combination of these components even at lower concentration, gave a better knockdown effect compared to the *P. aduncum* essential oil alone in the aerosol formulation. In addition, the inherent toxicity of pyrethroid to insects is much higher compared to insecticides of botanical origin.

Many studies were conducted on adulticidal activity of plant extract against mosquitoes. Since no studies on effects of plant extracts in aerosol can were conducted, the present study indicated that *P. aduncum* essential oil aerosol has the potential in causing knockdown and mortality of *Aedes* spp. Insecticide droplets released from the aerosol can impinge on the flying mosquito to deliver a lethal dose (WHO, 2001). Flying mosquitoes will be killed because of the direct contact with the insecticide droplets. WHO (2001) also reported that treatment using space spray is more effective at times when the insects are active because droplets are more likely to impinge on flying mosquitoes than those that are at rest. Several studies were conducted on using botanical extracts against adult mosquitoes through direct contact. According to Choochote *et al.* (2006), *Piper longum* Linn., *Piper ribesoides* Wall and *Piper sarmentosum* Roxb. extracts showed adulticidal effect against *Ae. aegypti* by topical application at concentrations of 0.26 µg/mg, 0.15 µg/mg and 0.14 µg/mg respectively. Hexane extract of *Curcuma aromatica* Salisb. A, also showed mortality effect at concentration of 10.05 µg/mg by topical application (Choochote *et al.*, 2005). The present results showed that LC_{50} and LC_{90} of *P. aduncum* essential oil is 5.6% and 12.3% against *Ae. aegypti* respectively. Previous study showed that adulticidal effect of the hexane fraction of *P. aduncum* gave LC_{50} of 0.2 mg/cm^2 against *Ae. aegypti* and 5.32 mg/cm^2 for LC_{90} (Hidayatulfathi *et al.*, 2004). Jeyabalan *et al.* (2003) had reported the adulticidal effect of *Pelargonium citrosa* Van Leenii on *Anopheles. stephensi* Liston with LC_{50} and LC_{90} value of 1.56% and 5.22% respectively.

Field evaluation of *P. aduncum* extract applied as ULV treatment against dengue vectors showed high mortality (80%) 24 h post-spraying (Sulaiman *et al.*, 2004). *Litsea elliptica* Blume (Family Lauraceae) extract (0.01 gm/m^2) in application of ULV spraying also showed high mortality (96.7%) against *Ae. aegypti* 24 h post-spraying (Sulaiman *et al.*, 2006). These findings showed that plant extract as aerosol application (aerosol can and ULV) exhibited killing effect against dengue vectors in the laboratory or in the field. The KT_{90} of *P. aduncum* essential oil at concentrations 5% to 10% ranged between 1.8 hr to 4.7 h. However, after 24 hours the mortality was lower than 90%. Observation of commercial aerosol spray also showed similar trend mainly for the *Ae. albopictus*. This finding indicated that *P. aduncum* essential oil and commercial aerosol caused some of the knockown mosquitoes recovered after 24 hours of treatment.

Pyrethroids act mainly by contact, passing through the cuticle of the insect and reaching their nervous ending. Then the insect will no longer be able to control their movement and the initial excitability is seen followed by convulsion and paralysis (knockdown) which eventually leads to death. In the meantime, the enzymatic mechanism in the insect's cell strives to metabolise the xenobiotic compound (detoxification system) then leads the insect to recovery (Yamamoto, 1973). The mechanism of toxicity of *P. aduncum* essential oil against insect is yet to be studied. However, in general, essential oil acts on nervous system similar to pyrethroid-based insecticides (Enan, 2001). The presence of a synergist such as piperonyl butoxide (PBO) could increase the insecticidal efficacy of pyrethroids which inhibited the enzyme responsible for detoxification (Martin *et al.*, 1997). Hence, further study incorporating PBO in formulation of essential oil in aerosol spray should be done in order to increase the effectiveness of the formulation. When compared between both *Aedes* spp., there was not much difference in their knockdown and mortality response towards each concentration of *P. aduncum* essential oil, showing that the degree of toxicity of *P. aduncum* essential oil to both *Aedes* spp. is similar.

Volume median diameter (VMD) is the droplet diameter where 50% of the aerosol volume is in larger droplets and 50% is in smaller droplet (Mount, 1998). The efficacy of aerosol depends entirely on the interaction of insecticide droplet in the air with the flying mosquitoes (Newton & Reiter, 1992). Our results showed that the VMD was in the range of 7.6 – 8.5 microns in each of the concentration tested. Thus, the concentration of *P. aduncum* essential oil did not affect the VMD. The optimum VMD ranges between 8 – 15 microns for aerosol (Mount, 1998). Larger droplets tend to settle under gravity whereas the smaller droplets do not impact readily on solid surface and remain suspended in the air for extended period of time (WHO, 2001). The efficacy of the aerosol insecticide depends on various factors including the formulation, particle behavior, droplet size, method of application and environmental behavior. Generally, an insecticidal aerosol formulation must have both rapid knockdown efficacy and strong killing efficacy against insect pests (Tsuda & Okuno, 1985). Temperature and relative humidity can be significant factors determining the success or failure of a vector control operation because they relate to the particle evaporation (Akesson & Yates, 1973).

This study found that *P. aduncum* essential could be useful as an active ingredient in aerosol spray because of high knockdown and killing effect against *Aedes* spp. The aerosol spray of *P. aduncum* essential oil under semi-simulated laboratory conditions should be studied further under field conditions to evaluate the field effectiveness of *P. aduncum* essential oil.

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