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Current resistance status of *Aedes aegypti* and *Aedes albopictus* populations in Penang Island, Malaysia

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ABSTRACT

Objective: To determine the current insecticide resistance status of *Aedes* (*Ae.*) *aegypti* and *Ae. albopictus* to four insecticides, namely 0.05% deltamethrin, 0.75% permethrin, 5% malathion and 0.25% pirimiphos–methyl using the World Health Organisation (WHO) susceptibility test kit.

Methods: Adult bioassays were carried out using the standard protocol of the World Health Organisation. All F1 generation urban and suburban field strains of *Ae. aegypti* and *Ae. albopictus* were tested against pyrethroid and organophosphate insecticides, including the presence of piperonyl butoxide (PBO) in four replicates of 25 non–blood-fed female mosquitoes ranging from 3 to 5 days old. The Vector Control Research Unit (VCRU) laboratory strain served as a reference strain.

Results: In this study, 0.05% deltamethrin demonstrated a lower value of knockdown time when 50% of the mosquito population died (KT_{50}) and knockdown time when 95% of the mosquito population died (KT_{95}), which is significantly more effective compared to 0.75% permethrin against adult female *Ae. aegypti* (urban and suburban) and *Ae. albopictus* (urban and suburban) (ANOVA, $P<0.01$). Meanwhile, 5% malathion was a more effective insecticide, amounting to the shorter KT_{50} and KT_{95} compared to 0.25% pirimiphos–methyl against *Ae. aegypti* (urban and suburban) and *Ae. albopictus* (urban and suburban). *Ae. aegypti* urban and *Ae. aegypti* suburban performed a higher resistance ratio (RR) towards both 0.05% deltamethrin and 0.75% permethrin due to the wide use of permethrin in dengue vector control programs in Malaysia. However, *Ae. albopictus* urban and suburban have lower resistance than *Ae. aegypti* urban and suburban towards 0.05% deltamethrin and 0.75% permethrin at 24 hours post-treatment. The addition of PBO with these insecticides successfully reduced knockdown time (KT_{50} and KT_{95}) values of most of the *Ae. aegypti* and *Ae. albopictus* field strains except PBO+0.75% permethrin against *Ae. aegypti* suburban.

Conclusions: The addition of PBO to insecticides has significantly reduced the knockdown time (KT_{50} and KT_{95}) values on most of *Ae. aegypti* and *Ae. albopictus* urban strain except PBO+5% malathion against *Ae. albopictus* urban strain and PBO+0.75% permethrin against *Ae. albopictus* suburban strain in comparison to exposure to insecticides without PBO. *Ae. aegypti* showed a higher resistance ratio of 50 (RR_{50}) when compared with the VCRU laboratory reference strain (susceptible strain) at the exposure to the deltamethrin, including with pre-exposure to PBO. This study found that the addition of PBO with organophosphates (5% malathion and 0.25% pirimiphos–methyl) was significantly more effective than pyrethroids against *Ae. aegypti* and *Ae. albopictus* (urban and suburban) due to their high mortality rate at 24 hours. It can be concluded that the usage of PBO can help reduce resistance alteration in *Aedes* mosquitoes.

KEYWORDS: *Aedes*; Insecticide resistance; Mosquito; Organophosphates; Pyrethroids; Vector control

Significance

The current resistance status of *Aedes* mosquitoes in Malaysia to frequently used pesticides is determined in this study. In Penang Island's urban and suburban districts, it was discovered that piperonyl butoxide combined with pyrethroids and organophosphates effectively decreased the resistance of *Aedes* mosquitoes. The research demonstrated the noteworthy effects of piperonyl butoxide in addition to the two pesticide groups, and it served as a foundational study to assist the government in managing *Aedes* mosquitoes in the field.

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1. Introduction

Dengue fever caused by the dengue virus belonging to the Flaviviridae family, in the genus *Flavivirus*. The dengue virus is transmitted *via* the vector *Aedes* sp. mosquito. The *Aedes* species known to be vectors in Malaysia are *Aedes (Ae.) aegypti* and *Ae. albopictus*. All four dengue virus serotypes circulate in Malaysia[1,2]. Although *Ae. aegypti* is more frequently found in quickly growing areas with less vegetation, *Ae. albopictus* prefers environments with more vegetation and is generally more exophilic than *Ae. aegypti*, these two species share a geographical distribution that overlaps[3]. In 1986, the Vector-Borne Disease Control Division of the Ministry of Health Malaysia (MOH) developed a dengue fever prevention and control guideline, which has since been updated several times. Dengue outbreak is defined as the occurrence of more than one case of dengue fever in a certain locality, whereby the onset date between one case and another is less than 14 days[4].

Despite the overwhelming dengue situation, disease management still relies heavily on symptom treatment and vector control since no effective antiviral has been implemented yet[5]. In general, vector control programs largely deal with reducing the number of *Aedes* immatures and adults *via* insecticide applications. Health authorities in Malaysia usually employ temephos in their routine larval control measures and fogging (thermal or ultra-low-volume sprays) using malathion and permethrin for adult control[6].

Nevertheless, the major drawback of insecticide use in vector control in the long run is the build-up of resistance. Persistent and extensive use of insecticides will induce resistance and cease their effectiveness[6]. In addition, the short lifespan of *Aedes* would further accelerate resistance development after each generation. Insecticide resistance is generally associated with an increased rate of enzymatic detoxification, where enzymes such as cytochrome P450s (P450s), carboxylesterases, and glutathione S-transferases (GST) are upregulated or become more efficient, allowing insects to metabolise and eliminate toxic substances more quickly[7].

There are four major categories of insecticides being used in vector control programmes: organochlorines, organophosphate, carbamates and pyrethroids. Pyrethroid insecticides, known for their fast–acting nature and relatively low toxicity to mammals, are extensively used worldwide in vector control and agriculture sectors[8]. In Malaysia, particularly, the Ministry of Health employs pyrethroids such as permethrin (type Ⅰ pyrethroid) and deltamethrin (type Ⅱ pyrethroid) predominantly for adulticiding in its vector control programmes. Thus, the intense usage of these insecticides has been reported to cause insecticide resistance in *Ae. aegypti* and *Ae. albopictus* populations in Malaysia[9].

Besides that, an inert substance called piperonyl butoxide (PBO) insecticide synergist prevents insects' cytochrome P450s (P450s) from metabolizing pesticides[10]. Studies in Africa and other parts of the world have shown higher mortality in insecticide-resistant *Aedes* pre-exposed to PBO synergist[11]. In Penang Island, there is a paucity of information on the susceptibility status of *Ae. aegypti* and *Ae. albopictus* to the different classes of neurotoxic insecticides used in public health. Previously, resistance status using 0.75% permethrin, 0.05% deltamethrin and 5% malathion were tested in urban areas (Flat Hamna, Kampung Sungai Gelugor and Kampung Tanjung Tokong) in 2021 against *Ae. albopictus*[12]. There is no recent data on the susceptibility status of adult mosquitoes and a lack of information comparing adult *Ae. aegypti* and *Ae. albopictus* in urban and suburban areas using pyrethroids and organophosphates. Thus, our study provided up-to-date information on the status of insecticide resistance of adult *Ae. aegypti* and *Ae. albopictus*. This is crucial for adapting and optimizing vector control strategies based on the current resistance profiles. Therefore, this study is aimed at determining the current insecticide resistance status of *Ae. aegypti* and *Ae. albopictus* to neurotoxic insecticides, which consists of four insecticides from class pyrethroid and organophosphate in urban and suburban areas, Penang Island, namely 0.05% deltamethrin, 0.75% permethrin, 5% malathion and 0.25% pirimiphos-methyl.

2. Materials and methods

2.1. Mosquito colonies collection

Eggs of *Ae. aegypti* and *Ae. albopictus* were obtained by placing ovitraps in Desa Permai Indah (Hamna low-cost flat, urban residential area), Penang (5°21’ N, 100°18’ E) and Taman Indah (Batu Maung low-cost flat, suburban residential area), Penang (5°17' N 100°17' E). Suburban areas are found outside metropolitan centres and have a lower population density. Whereas urban areas are defined by a high population density and a vast amount of human-built infrastructure. Suburban areas are characterized by a mix of residential and commercial development, with more open spaces and larger plots of land compared to urban areas. These sites were selected as the study sites because they are common locations affected by dengue. The mosquito sampling was started from 1st April 2019 until 5th January 2020.

The collected larvae were brought back to the laboratory and were reared in enamel trays containing dechlorinated tap water. The culture was maintained at (28±2) ℃, 70%–85% relative humidity, with a photo–period of 12 h light and 12 h dark. The larvae were fed with a mixture of dog biscuit, beef liver, yeast, and milk powder at a ratio of 2:1:1:1 by weight and prepared as a fine powder at 1 mg/daily[12]. Larvae were allowed to grow into the adult stage (F0 generation).

The emerged adult mosquitoes filial 0 (F0) generation were separated from *Ae. aegypti* and *Ae. albopictus* and placed into two

separate cages. Mosquitoes were morphologically identified using standard morphological identification keys[13]. The adult mosquitoes were provided with a mixture of 10% sucrose solution.

After five to six days of adult emergence, females were provided with blood meal from a restrained rat placed in a resting cage for four hours. This is because not enough female adult samples of filial 0 (F0) were captured from the field, and filial 1 (F1) generation was used in this bioassay study. Two days after blood feeding, a plastic cup lined with moist filter paper was placed inside each cage as an oviposition substrate. The eggs oviposited on the filter paper were collected and air–dried for two days. Eggs were hatched by placing them in enamel trays containing dechlorinated tap water and reared until they developed into the adult stage (F1 generation). The non–bloodfed adult female (F1 generation) of the field–strain mosquitoes were used in this study.

2.2. Adult bioassay on insecticide susceptibility assay

Adult bioassays were tested out according to the standard World Health Organisation (WHO) protocol[14]. All F1 generation urban and suburban field strains of *Ae. aegypti* and *Ae. albopictus* were tested against pyrethroid and organophosphate insecticides including the presence of PBO in four replicates of 25 non–blood fed female mosquitoes ranging from 3 to 5 days old. The insecticides used were: (1) 0.05% deltamethrin, (2) 0.75% permethrin, (3) 5% malathion, (4) 0.25% pirimiphos–methyl, (5) PBO+0.05% deltamethrin, (6) PBO+0.75% permethrin, (7) PBO+5% malathion, (8) PBO+0.25% pirimiphos–methyl. Knockdown effects are part of standardised protocols for assessing insecticide efficacy. Even if a particular insecticide does not cause immediate knockdown, including it in the assessment allows for consistent comparison across different studies and insecticides. Knockdown data could help to differentiate between the modes of action between pyrethroids and organophosphates against mosquitoes. Due to the high resistance usually occurring in *Aedes* mosquitoes in Malaysia, *Anopheles* diagnostic dose was used in this study. Furthermore, some of the *Aedes* diagnostic doses is still at a tentative dose[15]. Controls were done in two replicates for each insecticide using silicone oil for pyrethroid control and olive oil for organophosphate control. While the Vector Control Research Unit (VCRU), Universiti Sains Malaysia susceptible strain was used as a reference for baseline for this susceptible study. The reason why PBO+5% malathion and PBO+0.25% pirimiphos–methyl was conducted was that PBO enhances their efficacy by preventing the mosquitoes from metabolizing and thus detoxifying the insecticide. This makes the insecticide more effective even at lower doses. A 0.5% malathion and 0.25% pirimiphos–methyl was found to have less impact on the knockdown effect in mosquitoes within one hour of exposure due to their slow action, but monitoring for knockdown can still be important for assessing the potential development of resistance. Assessing knockdown and mortality at 24 hours provides a more complete picture of an insecticide's efficacy. An insecticide might not cause knockdown but could still be highly effective in killing mosquitoes over time. If the mosquitoes are exposed to the insecticide over time and show a reduced sensitivity or resistance, this information is valuable for pest control strategies in the field. In addition, the purpose of using PBO+5% malathion and PBO+0.25% pirimiphos–methyl is to assess whether the synergistic effect helps to reduce resistance in mosquitoes and its function in inhibiting the detoxification of enzymes.

Twenty–five mosquitoes in the holding tubes were given one-hour acclimation time. Any damaged, injured, and dead mosquitoes were replaced with healthy ones after the acclimation time. The mosquitoes from the holding tube were then transferred into the test tube containing insecticide–treated impregnated paper. Knockdown data were then recorded every five minutes intervals for one hour once exposed to insecticides. After the exposure period, mosquitoes were immediately transferred back into the holding tubes with 10% sucrose solution–soaked cotton wools as a food source, and the mortality rate was determined after 24 hours at optimum conditions with the temperature of (28±2) ℃ relative humidity of (78±10)% and a photoperiod of 12 hours of light and 12 hours of dark.

Mortality percentage after 24 hours of exposure to insecticides in the adult bioassay test was characterised for the susceptibility status using World Health Organisation (WHO) criteria where mosquitoes are considered: (1) susceptible, if the percentage mortality is between 98% and 100%, (2) incipient resistant if mortality is between 90%–97% and (3) resistant if mortality is < 90%[14]. Resistance ratio (RR_{50} and RR_{95}) was also determined by using knockdown time (KT_{50} and KT_{95}) values of field strain divided by the KT_{50} or KT_{95} values of susceptible laboratory strain[16]. The resistance levels of the adult mosquito were ranked into five categories: susceptible ($RR_{50} < 1$), low resistance ($1 \leq RR_{50} < 10$), moderate resistance ($10 \leq RR_{50} \leq 30$), high resistance ($30 < RR_{50} < 100$) and very high resistance ($RR_{50} \geq 100$)[17].

Mosquito strains that were highly resistant at 24 hours post–exposure to all insecticide treatments were also tested using an insecticide synergist, which is PBO, to assess the involvement of cytochrome P450s in the insecticide resistance mechanisms. Mosquitoes were pre–exposed to 4% PBO for 1 hour before being directly exposed to specific insecticides. In parallel, controls were done by using bioassays with 4% PBO and exposing the mosquitoes to control papers before transferring them to insecticide tubes. All treatments were replicated four times with two sets of control for each insecticide, with and without synergist associated. For the synergistic effect of PBO, the following formula was used:

$$\text{Synergistic ratio } (SR_{50}) = \frac{KT_{50} \text{ of the insecticide}}{KT_{50} \text{ of the (PBO+insecticide)}}$$

The synergistic ratio (SR) classifies the results into three categories: an SR value of 1.00±0.05 is considered to have no obvious effect; an SR value > 1.05 is considered synergistic, and an SR value < 0.95 is considered antagonistic[18].

If the mortality of the control mosquitoes was over 20%, the experiment was discarded. If the control mortality fell between 5% and 20%, the percentage mortality was corrected by using Abbot's formula[19]:

$$\frac{\%\text{ treated mortality}-\%\text{ control mortality}}{1-\%\text{ control mortality}} \times 100\%$$

No correction was required if the control mortality was more than 20%, the experiment needs to be repeated[16]. In this study, there is no Abbot's correction needed for the control mortality.

2.3. Statistical analysis

Insecticide susceptibility was classified based on the criteria that 98%–100% mortality of mosquitoes implies susceptibility, 80%–97% mortality indicates possible resistance that needs to be further confirmed *via* biochemical or molecular assays, and less than 80% mortality implies resistance[20]. IBM SPSS Statistics version 27.0 (IBM Corporation, U.S) was used for the analysis. Regression probit analysis was used to compute the knockdown time (KT_{50} and KT_{95}). *Post hoc* Tukey's HSD test of analysis of variance (ANOVA) was used to compare differences in RR_{50} among different study sites. A *Chi*-square test was used to compare the percentage of mortality between insecticides only and PBO plus insecticides. Synergistic ratio (SR) values were indicated as significant if the statistic tests using *t*–test showed a significant difference between knockdown time (KT_{50} or KT_{95}) values with and without the PBO. A statistical test using ANOVA was conducted to compare the susceptibility levels between the mosquito strains.

2.4. Ethical approval

This study was approved by the Animal Ethics Committee of Universiti Sains Malaysia; approval number: USM/IACUC/2017/(110) (891).

3. Results

3.1. Knockdown time

Table 1 shows the knockdown time of *Ae. aegypti* and *Ae. albopictus* obtained from Penang Island, Malaysia, against various insecticides belonging to two major insecticide groups, namely pyrethroid and organophosphate. From the adult bioassay performed, 0.05% deltamethrin performed the shorter knockdown time (KT_{50} and KT_{95}) value on both *Ae. albopictus* urban strain (KT_{50}=28.36 minutes, KT_{95}=44.07 minutes) and *Ae. albopictus* suburban strain (KT_{50}=20.52 minutes, KT_{95}=44.29 minutes), which demonstrated that 0.05% deltamethrin was obviously more effective against *Ae. albopictus* field strains compared to *Ae. aegypti* field strains (Table 1). However, 0.75% permethrin demonstrated the longest KT_{50} (220.34 minutes) and KT_{95} (342.00 minutes) values on *Ae. aegypti* urban strain and *Ae. aegypti* suburban strain did not record any knockdown for 0.75% permethrin in the study at one–hour intervals. Thus, knockdown time (KT_{50} and KT_{95}) values cannot be calculated.

Meanwhile, 5% malathion performed the shorter knockdown time (KT_{50} and KT_{95}) values on both *Ae. aegypti* urban strain (KT_{50}=64.05 minutes, KT_{95}=93.97 minutes) and *Ae. aegypti* suburban strain (KT_{50}=69.70 minutes, KT_{95}=100.98 minutes) than on *Ae. albopictus* urban strain (KT_{50}=77.42 minutes, KT_{95}=112.59 minutes) and suburban strain (KT_{50}=75.03 minutes, KT_{95}=107.88 minutes), indicating that 5% malathion was significantly effective against *Ae. aegypti* field strains compared to *Ae. albopictus* field strains. Only *Ae. albopictus* suburban field strain showed KT_{50}=84.03 minutes and KT_{95}=109.30 minutes when treated with 0.25% pirimiphos–methyl while other field strains did not show any knockdown (Table 1).

Ae. albopictus suburban strain showed the lowest resistance ratio (RR_{50}) value when treated with 0.05% deltamethrin (RR_{50}=0.86) and 0.75% permethrin (RR_{50}=0.47) compared to other strains, indicating that, *Ae. albopictus* suburban strain was susceptible to 0.05% deltamethrin and 0.75% permethrin as compared to the VCRU laboratory strain (susceptible reference strain). However, *Ae. aegypti* urban strain demonstrated low resistance to 0.05% deltamethrin (RR_{50}=3.17) and moderate resistance to 0.75% permethrin (RR_{50}=12.37). Meanwhile, *Ae. albopictus* suburban strain showed low resistance towards both 5% malathion (RR_{50}=1.09) and 0.25% pirimiphos–methyl (RR_{50}=1.23; Table 1).

The addition of PBO to insecticides has reduced the knockdown time (KT_{50} and KT_{95}) values on most of *Ae. aegypti* and *Ae. albopictus* urban strain except PBO+5% malathion against *Ae. albopictus* urban strain (SR_{50}=0.89) and PBO+0.75% permethrin against *Ae. albopictus* suburban strain (SR_{50}=0.98). This showed that the addition of PBO to insecticides performed a high synergistic effect on most of *Ae. aegypti* and *Ae. albopictus* urban strain. However, it showed an antagonistic effect on *Ae. albopictus* urban strain when treated with PBO+5% malathion and *Ae. albopictus* suburban strain when treated with PBO+0.75% permethrin (Table 1). From Table 2, results from factorial ANOVA indicated that there were significant differences in knockdown time without PBO (F=12.356, df=270, P<0.001) and with PBO (F=27.082, df=270, P=0.001) between mosquito strains, the type of insecticides and the exposure time.

Table 1. Knockdown time of *Aedes aegypti* and *Aedes albopictus* in Penang Island, Malaysia against pyrethroid and organophosphate insecticides.

Insecticide	Mosquito species	KT_{50} (min)(95% *CI*)	KT_{95} (min)(95% *CI*)	RR_{50}/RR_{95}	SR_{50}/SR_{95}	Regression equation	*F* value	*P* value	R^2 value
0.05% Deltamethrin	*Ae. aegypti* laboratory	22.88 (22.16-23.58)	30.55 (29.44-31.93)	1.00/1.00	2.58/1.87	0.55X-1.14	161.06	<0.01	0.78
	Ae. aegypti urban	72.61 (67.07-80.65)	116.30 (103.78-135.04)	3.17/3.81	1.43/1.61	0.13X-1.75	225.89	<0.01	0.83
	Ae. aegypti suburban	83.63 (75.06-98.20)	126.46 (109.01-156.78)	3.66/4.14	1.76/1.84	0.08X-1.16	61.31	<0.01	0.57
	Ae. albopictus laboratory	23.80 (21.32-26.16)	34.89 (31.70-40.18)	1.00/1.00	1.20/0.81	0.54X-1.35	175.66	<0.01	0.79
	Ae. albopictus urban	28.36 (26.99-29.72)	44.07 (41.91-46.72)	1.19/1.26	1.66/1.23	0.55X-3.63	380.13	<0.01	0.89
	Ae. albopictus suburban	20.52 (18.40-22.45)	44.29 (41.22-48.22)	0.86/1.27	1.73/2.44	0.42X+3.72	197.99	<0.01	0.81
0.75% Permethrin	*Ae. aegypti* laboratory	17.81 (17.01-18.59)	23.57 (22.40-25.20)	1.00/1.00	1.73/1.07	0.47X+3.51	81.24	<0.01	0.64
	Ae. aegypti urban	220.34 (ND)	342.00 (ND)	12.37/14.51	2.43/3.06	0.01X-0.02	3.31	>0.01	0.07
	Ae. aegypti suburban	NA	NA	NA	NA	NA	NA	NA	NA
	Ae. albopictus laboratory	42.72 (39.18-46.80)	70.07 (62.94-81.63)	1.00/1.00	1.43/1.14	0.42X-4.59	97.06	<0.01	0.68
	Ae. albopictus urban	35.11 (32.15-38.08)	60.88 (55.75-68.28)	0.82/0.87	2.36/2.60	0.47X-3.57	209.34	<0.01	0.82
	Ae. albopictus suburban	20.07 (16.04-23.40)	51.37 (46.15-58.98)	0.47/2.73	0.98/1.33	0.38X+4.89	129.89	<0.01	0.74
5% malathion	*Ae. aegypti* laboratory	NA	NA	1.00/1.00	NA	NA	NA	NA	NA
	Ae. aegypti urban	64.05 (60.84-68.39)	93.97 (86.58-104.51)	NA	1.44/NA	0.17X-2.77	135.63	<0.01	0.75
	Ae. aegypti suburban	69.70 (65.32-76.19)	100.98 (91.37-115.74)	NA	1.62/1.82	0.12X-2.06	48.64	<0.01	0.51
	Ae. albopictus laboratory	68.80 (61.87-82.92)	99.93 (85.03-132.72)	1.00/1.00	1.24/1.36	0.13X-2.31	24.53	<0.01	0.35
	Ae. albopictus urban	77.42 (68.78-95.26)	112.59 (94.89-150.55)	1.13/1.13	0.89/0.88	0.08X-1.44	24.59	<0.01	0.35
	Ae. albopictus suburban	75.03 (69.11-84.74)	107.88 (95.47-128.83)	1.09/1.08	1.03/1.06	0.09X-1.47	116.80	<0.01	0.72
0.25% Pirimiphos-methyl	*Ae. aegypti* laboratory	NA	NA	1.00/1.00	NA	NA	NA	NA	NA
	Ae. aegypti urban	NA	NA	NA	NA	NA	NA	NA	NA
	Ae. aegypti suburban	NA	NA	NA	NA	NA	NA	NA	NA
	Ae. albopictus laboratory	68.58 (64.71-76.32)	85.37 (77.29-102.24)	1.00/1.00	0.83/0.83	0.06X-1.17	47.27	<0.01	0.51
	Ae. albopictus urban	NA	NA	NA	NA	NA	NA	NA	NA
	Ae. albopictus suburban	84.03 (72.32-132.54)	109.30 (88.11-198.80)	1.23/1.28	1.13/1.14	0.02X-0.36	18.01	<0.01	0.28
PBO+0.05% Deltamethrin	*Ae. aegypti* laboratory	8.86 (6.86-10.51)	16.35 (14.09-20.72)	1.00/1.00	NA	0.23X+14.89	32.91	<0.01	0.42
	Ae. aegypti urban	50.89 (49.51-52.43)	72.03 (68.88-76.01)	5.74/4.41	NA	0.34X-5.57	208.37	<0.01	0.82
	Ae. aegypti suburban	47.60 (46.31-48.98)	68.65 (65.88-72.06)	5.37/4.20	NA	0.39X-6.15	307.98	<0.01	0.87
	Ae. albopictus laboratory	19.89 (16.71-22.63)	42.87 (38.85-48.55)	1.00/1.00	NA	0.42X+4.00	111.77	<0.01	0.71
	Ae. albopictus urban	17.05 (6.69-23.59)	35.76 (28.20-57.46)	0.86/0.83	NA	0.40X+5.83	73.46	<0.01	0.62
	Ae. albopictus suburban	11.83 (ND)	18.16 (ND)	0.59/0.42	NA	0.32X+10.91	39.47	<0.01	0.46
PBO+0.75% Permethrin	*Ae. aegypti* laboratory	10.30 (8.59-11.75)	22.04 (19.89-25.18)	1.00/1.00	NA	0.47X+3.51	81.24	<0.01	0.64
	Ae. aegypti urban	90.79 (ND)	111.83 (ND)	8.81/5.07	NA	0.02X-0.04	2.63	>0.01	0.05
	Ae. aegypti suburban	NA	NA	NA	NA	NA	NA	NA	NA
	Ae. albopictus laboratory	29.83 (26.05-33.34)	61.52 (55.21-71.07)	1.00/1.00	NA	NA	NA	NA	NA
	Ae. albopictus urban	14.89 (ND)	23.41 (ND)	0.50/0.38	NA	0.39X+7.14	55.69	<0.01	0.55
	Ae. albopictus suburban	20.56 (15.42-24.83)	38.67 (33.10-49.15)	0.69/0.63	NA	0.46X+2.67	120.32	<0.01	0.72
PBO+5% Malathion	*Ae. aegypti* laboratory	48.19 (46.91-49.58)	68.75 (65.98-72.16)	1.00/1.00	NA	0.38X-6.22	221.95	<0.01	0.83
	Ae. aegypti urban	44.41 (43.17-45.66)	58.64 (56.54-61.30)	0.92/0.85	NA	0.48X-8.02	180.33	<0.01	0.80
	Ae. aegypti suburban	42.98 (42.07-43.89)	55.58 (54.06-57.40)	0.89/0.81	NA	0.51X-8.49	223.08	<0.01	0.83
	Ae. albopictus laboratory	55.70 (54.10-57.64)	73.30 (69.66-78.33)	1.00/1.00	NA	0.26X-4.79	70.26	<0.01	0.60
	Ae. albopictus urban	86.63 (75.41-110.53)	127.35 (105.36-175.31)	1.56/1.74	NA	0.06X-0.93	23.16	<0.01	0.34
	Ae. albopictus suburban	72.62 (67.45-81.12)	101.47 (90.56-120.00)	1.30/1.38	NA	0.09X-1.56	62.07	<0.01	0.57
PBO+0.25% Pirimiphos-methyl	*Ae. aegypti* laboratory	56.34 (55.07-57.92)	71.59 (68.60-75.66)	1.00/1.00	NA	0.24X-4.56	58.30	<0.01	0.56
	Ae. aegypti urban	57.10 (56.19-58.16)	66.68 (64.68-69.50)	1.01/0.93	NA	0.21X-4.20	41.39	<0.01	0.47
	Ae. aegypti suburban	68.40 (64.46-79.79)	80.57 (72.70-104.21)	1.21/1.13	NA	0.03X-0.65	12.28	<0.01	0.21
	Ae. albopictus laboratory	82.39 (69.68-346.29)	102.70 (80.48-573.41)	1.00/1.00	NA	0.01X-0.19	9.79	<0.01	0.18
	Ae. albopictus urban	140.37 (98.45-467.56)	207.87 (136.76-768.42)	1.70/2.02	NA	0.01X-0.14	8.28	<0.01	0.15
	Ae. albopictus suburban	74.04 (68.07-86.88)	96.27 (84.37-122.62)	0.90/0.94	NA	0.05X-0.92	29.18	<0.01	0.39

KT_{50}=Knockdown time when 50% of the mosquito population died; KT_{95}=Knockdown time when 95% of the mosquito population died; 95% *CI*=95% Confidence interval; NA=Not available because no knockdown; ND=Not determined by probit analysis because knockdown rate <5%; *P* value=Probability value; R^2 value=Regression coefficient value; SE=Standard error; *F* value=Ratio of variances between multiple samples.

3.2. Adult bioassay on insecticide susceptibility assay

Table 3 shows the mortality percentages of *Ae. aegypti* and *Ae. albopictus* obtained from Penang Island, Malaysia, after 24 hours post-treatment, against various insecticides belonging to two major insecticide groups, namely pyrethroid and organophosphate. Various mortality percentages at 24 hours were exhibited when *Ae. aegypti* and *Ae. albopictus* were bioassayed using pyrethroid and organophosphate. Further examination of mortality at 24 hours post–treatment was used as an indication of the susceptibility status of *Ae. aegypti* and *Ae. albopictus*.

Based on WHO guidelines[20], *Ae. aegypti* (urban and suburban) and *Ae. albopictus* (urban) field strains were confirmed to be resistant to all the insecticides (0.05% deltamethrin, 0.75% permethrin, 5% malathion and 0.25% pirimiphos–methyl without PBO) tested as the mortality rates are less than 90%. However, *Ae. albopictus* suburban field strain was confirmed to be resistant to three insecticides (0.05% deltamethrin, 0.75% permethrin and 5% malathion) but susceptible to 0.25% pirimiphos–methyl with the mortality of (100±0.00)% (Table 3). While 0.75% permethrin demonstrated the lowest mortality rate of (4.00±1.63)% for *Ae. aegypti* suburban field strain compared to other strains and insecticides (Table 3).

Table 2. Analysis of variance on *Aedes aegypti* and *Aedes albopictus* knockdown comparing different mosquito strains, insecticides, and time.

Source of variation	Without PBO				With PBO			
	df	MS	*F* value	*P* value	*df*	MS	*F* value	*P* value
Strain	5	26418.14	699.57	<0.01	5	32747.96	1388.86	<0.01
Insecticides	5	127639.36	3379.99	<0.01	5	136792.04	5801.41	<0.01
Time (Minutes)	11	14125.42	374.05	<0.01	11	22366.00	948.55	<0.01
Strain * Insecticide	25	13298.13	352.15	<0.01	25	20337.50	862.52	<0.01
Strain * Time	55	984.66	26.07	<0.01	55	808.61	34.29	<0.01
Insecticide * Time	54	4001.68	105.97	<0.01	54	4829.57	204.82	<0.01
Strain * Insecticide * Time	270	466.61	12.36	<0.01	270	638.58	27.08	<0.01
Error	1318	37.76			1320	23.58		
Total	1744				1728			

df=degree of freedom; MS=mean–squared value.

Table 3. Mortality percentages of *Aedes aegypti* and *Aedes albopictus* in Penang Island, Malaysia at 24 hours post–treatment of various insecticides.

Insecticide		*Aedes aegypti*					*Aedes albopictus*				
		Laboratory	Urban	Suburban	*F* value	*P* value	Laboratory	Urban	Suburban	*F* value	*P* value
No PBO	PY Control	0.00±0.00	0.00±0.00	1.00±1.00	1.00	>0.01	0.00±0.00	0.00±0.00	0.00±0.00	NA	NA
	OP Control	1.00±0.00	0.00±0.00	0.00±0.00	1.00	>0.01	0.00±0.00	0.00±0.00	0.00±0.00	NA	NA
	0.05% Deltamethrin	100.00±0.00 (S)	22.00±2.00 (R)	18.00±1.15 (R)	1202.25	<0.01	100.00±0.00 (S)	67.00±5.74 (R)	76.00±1.63 (R)	24.28	<0.01
	0.75% Permethrin	100.00±0.00 (S)	5.00±3.79 (R)	4.00±1.63 (R)	536.53	<0.01	98.00±1.15 (S)	50.00±7.39 (R)	79.00±6.19 (R)	18.58	<0.01
	5% Malathion	99.00±1.00 (S)	66.00±2.00 (R)	75.00±4.12 (R)	39.68	<0.01	98.00±2.00 (S)	59.00±4.73	85.00±3.00	33.48	<0.01
	0.25% Pirimiphos–methyl	99.00±1.00 (S)	9.00±3.00 (R)	8.00±3.27 (R)	396.34	<0.01	100.00±0.00 (S)	84.00±5.89 (R)	100.00±0.00 (S)	7.39	<0.01
+PBO	PY Control	0.00±0.00	0.00±0.00	0.00±0.00	NA	NA	0.00±0.00	0.00±0.00	0.00±0.00	NA	NA
	OP Control	0.00±0.00	0.00±0.00	0.00±0.00	NA	NA	0.00±0.00	0.00±0.00	0.00±0.00	1.00	>0.01
	0.05% Deltamethrin	100.00±0.00 (S)	48.00±6.32 (R)	70.00±6.00 (R)	26.90	<0.01	100.00±1.00 (S)	99.00±1.00 (S)	100.00±0.00 (S)	1.00	>0.01
	0.75% Permethrin	100.00±0.00 (S)	15.00±6.61 (R)	4.00±2.83 (R)	160.28	<0.01	100.00±0.00 (S)	100.00±0.00 (S)	99.00±1.00 (S)	1.00	>0.01
	5% Malathion	100.00±0.00 (S)	99.00±1.00 (S)	100.00±0.00 (S)	1.00	>0.01	100.00±0.00 (S)	81.00±1.91 (R)	85.00±3.00 (R)	23.76	<0.01
	0.25% Pirimiphos–methyl	100.00±0.00 (S)	100.00±0.00 (S)	100.00±0.00 (S)	NA	NA	100.00±0.00 (S)	100.00±0.00 (S)	100.00±0.00 (S)	NA	NA

Data were expressed as mortality percentage±SE; PY=Pyrethroid; OP=Organophosphate; ND=Not Determined by one–way ANOVA as the values is the same; NA = Not available because no knockdown; SE=Standard error; R=Resistance; IR=Incipient resistance; S=susceptible.

The addition of PBO to all the insecticides showed that *Ae. aegypti* urban and suburban filed strains were confirmed to be resistant to two insecticides (PBO+0.05% deltamethrin, PBO+0.75% permethrin) tested as the mortality rates are less than 90%. However, the recovery happened in *Ae. aegypti* for both urban and suburban strains and confirmed to be susceptible to two insecticides (PBO+5% malathion and PBO+0.25% pirimiphos–methyl) tested as the mortality rates are more than 97%. Meanwhile, *Ae. albopictus* urban and suburban filed strains were confirmed to be resistant to one insecticide (PBO+5% malathion) tested as the mortality rates are less than 90%. However, the exposure to PBO prior to insecticide confirmed the recovery of resistance in *Ae. albopictus* for both urban and suburban strains, and the mosquitoes returned to be susceptible towards three insecticides (PBO+0.05% deltamethrin, PBO+0.75% permethrin, 0.25% pirimiphos–methyl) tested as the mortality rates are more than 97% (Table 3). From Table 4, results from factorial ANOVA indicated that there were significant differences in adult mortality without PBO (F=72.301, df=25, P<0.001) and with PBO (F=102.985, df=25, <0.001) between mosquito strains and the type of insecticides.

Table 4. Analysis of variance on *Aedes aegypti* and *Aedes albopictus* adult mortality at 24 hours comparing different mosquito strains, insecticides, and time.

Source of variation	Without PBO				With PBO			
	df	MS	*F* value	*P* value	*df*	MS	*F* value	*P* value
Strain	5	12116.38	418.88	<0.01	5	2751.58	171.58	<0.01
Insecticides	5	28785.98	995.16	<0.01	5	51405.18	3205.40	<0.01
Strain * Insecticide	25	2091.36	72.30	<0.01	25	1651.58	102.99	<0.01

4. Discussion

In this study, 0.05% deltamethin (type Ⅱ pyrethroid) demonstrated a lower value of knockdown time (KT_{50} and KT_{95}), which is more effective compared to 0.75% permethrin (type Ⅰ pyrethroid) against adult female *Ae. aegypti* (urban and suburban) and *Ae. albopictus* (urban and suburban). This study showed that the effect of 0.05% deltamethin was rapid with a faster knockdown rate recorded against *Ae. albopictus* compared to *Ae. aegypti* urban and suburban strain. However, 100% recovery was observed after 24 hours, resulting in lower mortality rate on *Ae. aegypti* urban and suburban strain, demonstrating that, *Ae. aegypti* in the study area was extremely resistant. This fast recovery rate and lower mortality rate in *Ae. aegypti* indicated a significant level of resistance in this species within the study area. One plausible explanation is the presence of resistance mechanisms in *Ae. aegypti* population that could be attributed to genetic variations or adaptations developed over time, rendering the mosquitoes less susceptible to the insecticidal effects of 0.05% deltamethrin. Mosquitoes can develop resistance to insecticides through metabolic detoxification mechanisms. It can be postulated that, *Ae. aegypti* might have evolved more efficient detoxification pathways, allowing it to recover from the initial exposure to 0.05% deltamethrin and survive at a higher rate compared to *Ae. albopictus*. Moreover, mutations in the target sites of the insecticide within the mosquito's nervous system can lead to insensitivity[21]. It is possible that *Ae. aegypti* has developed mutations that render it less susceptible to the lethal effects of 0.05% deltamethrin. The findings suggest that relying solely on 0.05% deltamethrin for mosquito control in the study area may be less effective against *Ae. aegypti*. Alternative insecticides or integrated vector management strategies may need to be considered to enhance control measures.

Pyrethroids are neurotoxic to insects and act quickly by rapidly paralyzing insects upon contact. This rapid mode of action is known as ‘knockdown’ that paralyzed the insect which is responsible for the reaction of sensitivity in the insect’s nervous system, and mortality can be observed after 24 hours[21]. Pyrethroids can be lethal since they affect both peripheral and central neurons; however, they can also result in a knock-down impact that may involve peripheral intoxication[22].

Pyrethroids listed by the World Health Organisation (WHO) are divided into two groups based on their biological activity that is associated with the absence of alpha–cyano group (type Ⅰ pyrethroid: permethrin) or presence of an alpha–cyano group (type Ⅱ pyrethroid: deltamethrin)[23]. Type Ⅱ pyrethroid (deltamethrin) primarily targets the voltage-gated sodium channel (VGSC) in neuronal membranes and has a higher potency targeting this channel, making it more lethal to insects[24].

In the present study, 5% malathion was a more effective insecticide, amounting to the shorter knockdown time (KT_{50} and KT_{95}) compared to 0.25% pirimiphos–methyl against *Ae. aegypti* (urban and suburban) and *Ae. albopictus* (urban and suburban) strain. However, there was no knockdown on *Ae. aegypti* (urban and suburban) and *Ae. albopictus* urban strain during the one–hour exposure towards 0.25% pirimiphos–methyl. The reason could be the mosquitoes may develop metabolic resistance, where they enhance their ability to detoxify or break down the insecticides. The metabolic pathways involved in processing 5% malathion and 0.25% pirimiphos-methyl may vary between *Ae. aegypti* and *Ae. albopictus*, which can be influenced by environmental factors such as temperature and humidity. A similar study claimed that knockdown time (KT_{50} and KT_{95}) values of 5% malathion were shorter than 0.25% pirimiphos–methyl when tested against adult *Ae. aegypti* field strain from Kampar, Perak[25]. Malathion and pirimiphos–

methyl are organophosphate insecticides that share a similar slower mode of action compared to other insecticide classes. Their slow mode of action does not kill mosquitoes immediately upon contact, but it may take some time, ranging from hours to days, for the full toxic effects to be observed[26]. This is because the disruption of the nervous system and subsequent paralysis occurs gradually as the accumulation of acetylcholine builds up.

In this study, this killing effect has been observed in *Ae. albopictus* suburban strain as the mortality rate was 100% after 24 hours towards 0.25% pirimiphos–methyl. However, the mortality rate for other strains were less than 90% when tested with 5% malathion and 0.25% pirimiphos–methyl. This demonstrates that *Ae. aegypti* and *Ae. albopictus* urban and suburban were resistant to both insecticides. This could be explained by that mosquitoes may develop enhanced metabolic capabilities to detoxify and eliminate insecticides. The enzymes responsible for breaking down 5% malathion and 0.25% pirimiphos–methyl may be upregulated in the resistant strains. A similar finding demonstrated that 0.21% pirimiphos–methyl at 24 hours post–exposure failed to cause mortality greater than 50% in *Ae. aegypti* populations[27]. Full susceptibility of *Aedes* mosquitoes to 0.25% pirimiphos-methyl was reported in Penang[28].

In this study, *Ae. aegypti* urban and *Ae. aegypti* suburban performed a higher resistance ratio (RR) towards both 0.05% deltamethrin and 0.75% permethrin. However, the RR of *Ae. aegypti* urban and suburban cannot be determined when tested with 5% malathion and 0.25% pirimiphos–methyl because there were no knockdown mosquitoes during the one–hour exposure. This is because 5% malathion and 0.25% pirimiphos–methyl may have a slower mode of action, and a slow-acting insecticide compared to 0.05% deltamethrin and 0.75% permethrin. The absence of knockdown may also be due to the mosquitoes having efficient detoxification mechanisms for 5% malathion and 0.25% pirimiphos–methyl. These mechanisms may prevent the rapid incapacitation of mosquitoes observed with pyrethroids. Permethrin and deltamethrin were widely used at Sungai Dua, Penang and this was the main reason for *Aedes* populations resistant to pyrethroids[29].

Extensive use of household pesticides can place the target mosquito population under selection pressure, which could lead to pyrethroid insecticide cross-resistance[30]. Malathion is employed as an adulticide in fogging operations in Malaysia. The resistance that is developing may be a result of droplets that became established in the containers, or it could be a trait that has been passed down to progenies[31]. The other possible reason could be cross–resistance between organophosphate insecticides. Cross–resistance occurs when insects develop resistance to one organophosphate due to mutations or other resistance mechanisms, they may also exhibit reduced susceptibility to other organophosphate insecticides that act on the same target enzyme. This is because the altered enzyme may be less affected by the inhibitory action of other organophosphates, making it more difficult for these chemicals to effectively disrupt the insect nervous system. Resistance could emerge after a few generations if they were treated continually with the same insecticides[32]. Higher resistance ratio levels to neurotoxic pesticides such as organophosphate and pyrethroid were often associated with metabolic modifications, both in terms of the number of enzymes impacted and the degree of activity enhancement. In general, the detoxification enzymes that cause metabolic resistance are involved in the overall metabolism of insects[33].

This study showed that *Ae. albopictus* urban and suburban have lower resistance than *Ae. aegypti* urban and suburban towards 0.05% deltamethrin and 0.75% permethrin at 24 hours post–treatment. Different mosquito species may have inherent differences in their sensitivity to specific insecticides. *Ae. albopictus* may possess a higher susceptibility to the neurotoxic effects induced by 0.05% deltamethrin and 0.75% permethrin compared to *Ae. aegypti*. The target sites for 0.05% deltamethrin and 0.75% permethrin are VGSC, and any mutations or modifications in these channels can lead to resistance. It is possible that *Ae. albopictus* has a lower frequency of knockdown resistance (kdr) mutations, resulting in higher susceptibility to pyrethroids. The two species significantly differ in their resistance profile to pyrethroids, with consistently higher prevalences of resistance observed in *Ae. aegypti* whereas *Ae. albopictus* populations are mostly fully susceptible[34]. Another study pointed out that resistance development in *Ae. albopictus* is more difficult due to genetic differences in the VGSC. However, it is feasible that there are behavioural differences in the two species that result in differing times and amounts of exposure insecticides[35]. A mosquito species' peak activity and the time of adulticide spraying directly affect the amount of pesticide exposure the species receives, which in turn affects resistance. Therefore, the degree to which mosquitoes are exposed to pesticides might vary depending on the management method employed by a mosquito control program[36].

The addition of PBO with the insecticides successfully reduced knockdown time (KT_{50} and KT_{95}) values of for most of the *Ae. aegypti* and *Ae. albopictus* field strains except PBO+0.75% permethrin against *Ae. aegypti* suburban in this study. Thus, it showed the recovery of both mosquito species towards the tested insecticides. *Ae. aegypti* suburban strains may possess metabolic resistance mechanisms that are not effectively inhibited by PBO. If the mosquitoes have highly efficient detoxification enzymes that are not susceptible to PBO, the synergistic effect may be limited. In the event where the metabolic enzyme is the cause of resistance, the addition of PBO to enhance the pyrethroid insecticidal effect *via* inhibiting microsomal mixed function oxidase enzyme is no longer useful in reverting the resistance occurrence in *Ae. aegypti* suburban, indicating no significant role of oxidase in permethrin

resistance[37]. A similar study stated that the addition of PBO) with cypermethrin was able to reduce lethal time 90 (LT_{90}) when tested against three strains of adult *Ae. aegypti*[38]. Another study reported that adding PBO with permethrin and deltamethrin could reduce knockdown time (KT_{50}) value when tested against *Ae. aegypti* from Selangor strain[20]. Piperonyl butoxide is a common pyrethroid synergist, it was utilized prior to exposure to permethrin insecticide. and inhibits the enzyme from the family of esterases and microsomal mixed function oxidase which are mainly associated with metabolic resistance caused by pyrethroid, organophosphate and carbamate exposure[39].

On the other hand, permethrin has been widely used in dengue vector control programmes in Malaysia, and a few reports of resistance were documented nationwide[40,41]. Generally, pyrethroids work by binding to VGSC of neurons, leaving the channels in an activated state, leading to repetitive nerve firing, and the insects will experience convulsions and eventually death. Repeated exposure to pyrethroids could cause amino acid substitutions at the VGSC, conferring resistance to the mosquitoes[42].

Based on this study, we found that the addition of PBO with organophosphates was more effective than pyrethroids alone against *Ae. aegypti* and *Ae. albopictus* (urban and suburban) due to their high mortality rate at 24 hours. *Aedes aegypti* urban and suburban were still showing a high resistance towards 0.05% deltamethrin and 0.75% permethrin even after the addition of PBO due to their low mortality rate at 24 hours. This can be explained by the rapid urbanization and recent regional economic development, insecticides were extensively used in Penang Island for dengue control. Another explanation of the resistance development of *Ae. aegypti* could possibly be due to the proximity they live to human residences. This might have increased the risk of *Ae. aegypti* are exposed to different forms of insecticides, such as aerosol spray from household insecticides or fogging conducted by the Ministry of Health, thus causing them to resist the exposed insecticides[25]. Full susceptibility to pyrethroids was recovered after pre-exposure to PBO synergist, indicating the involvement of metabolic resistance mechanisms[43]. The metabolic mechanism alone can drive mosquito resistance to pyrethroid insecticides[44]. The effect of PBO synergist is more marked with 0.05% deltamethrin than 0.75% permethrin, suggesting the involvement of monooxygenase enzymes in this study.

The dose used in this study is *Anopheles* diagnostic dose, which is higher than the recommended *Aedes* diagnostic dose[15,20]. However, resistance is still prominent in *Ae. aegypti* and *Ae. albopictus* field strains even when the higher dose was used. This suggests that the recommended diagnostic dose for *Aedes* mosquitoes by the World Health Organisation (WHO) is too low to be viable to control *Aedes* mosquitoes here in Malaysia, especially in Penang Island, and needs to be reevaluated.

In conclusion, the synergistic effect of PBO is more effective against organophosphates, which signifies the involvement of cytochrome P450 enzymes (CYPs) in the detoxification of this class. However, CYPs were also known as the activator of organophosphates, and therefore, the increase in mortality after exposure to PBO+ organophosphate was observed in this study. The involvement of cytochrome CYPs in the detoxification of organophosphates with the addition of PBO may enhance the activity of these enzymes to facilitate the breakdown and detoxification of organophosphates. Therefore, inhibiting these enzymes with PBO leads to higher levels of the parent (less toxic) compound, which still has enough intrinsic toxicity to cause increased mortality when not properly detoxified. Therefore, a baseline reference for *Ae. aegypti* and *Ae. albopictus* resistance status against two classes of insecticides, namely pyrethroid and organophosphate in Penang, Malaysia, was established in this study and can be utilized for future monitoring of *Aedes* mosquitoes.

Due to the extensive use of pesticides, *Aedes* mosquitoes have become resistant to pyrethroids in several areas of Penang Island. This study only represented the resistance status of *Aedes* mosquito populations collected from Desa Permai Indah and Taman Indah, Batu Maung Penang. Increased metabolic detoxification and alterations in the sodium channels of mosquitoes, known as kdr mutations, are frequently the causes of this resistance. Rather than being an insecticide, piperonyl butoxide works as a synergist. Although it doesn't directly kill mosquitoes, it increases the effectiveness of pesticides by preventing mosquito detoxification enzymes from working. Consequently, when used in conjunction with pyrethroids or organophosphates, which can overcome resistance by blocking the mosquito's detoxifying enzymes, piperonyl butoxide can play an essential part in managing resistance. By using this method, the usability of current pesticides can be prolonged.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contributions

KHN: Conceptualization, Data curation, formal analysis, investigation, methodology, project administration, writing—original draft; WFZ: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing—review & editing.

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