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THE EFFECT OF PIPERONYL BUTOXIDE ON THE SUSCEPTIBILITY STATUS OF *Anopheles gambiae s.l.* TO PUBLIC HEALTH INSECTICIDES IN A NIGER DELTA ZONE

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ABSTRACT – Present level of resistance of *Anopheles gambiae sensu lato* and the effectiveness of PBO-bednet insecticides were examined in the lowland forest vegetation of Rivers State, Nigeria. Sample collection was achieved in the rainy season. Female *Anopheles gambiae s.l.* were tested. Technical grade insecticides included bendiocarb, propoxur, permethrin, deltamethrin, lambda-cyhalothrin, alpha-cypermethrin, primiphos-methyl and dichlorodiphenyltrichloroethane. Dead and active mosquitoes after 15, 30, 35, 40, 45, 60, 75, 90, 105, 120 minutes were recorded. Some resistant insecticides were further subjected to piperonyl butoxide (PBO) synergist bioassay. Analysis of variance checked for significant variations in dead mosquitoes to the insecticides tested. Bendiocarb proved active for indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) impregnation. Addition of PBO-synergists to pyrethroids was effective and should be used for IRS and LLINs. The study presented reference point data when observing the level of insecticide resistance in Rivers State.

Keywords: Anopheles gambiae s.l., insecticides, lowland forest, mortality, resistance, Rivers State

INTRODUCTION

Malaria is a life-endangering sickness which is endemic in sub-Saharan Africa, causing severe suffering and death, particularly in Nigeria which holds the highest record of sickness and death from the disease (WHO 2020).

Presently, the curbing of malaria is dependent on the usage of some insecticides to keep the disease vector, female *An. gambiae s.l.* under check. This has led the World Health Organization (WHO) to recommend some public health insecticides for use and they fall into various classes. Public health insecticides are insecticides used for the health concern of a large number of persons, as in a community. They include classes such as pyrethrum, carbamate, organochlorine and organophosphate. These classes have different kinds of insecticides under them, such as permethrin, propoxur, DDT, primiphos-methyl, etc. (WHO 2020).

Some of these insecticides have been used in larviciding as a means of larval source management (LSM) and control of the malaria vector, long-lasting-insecticide-nets (LLINs) and indoor residual

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spraying (IRS). Some classes of insecticides used include insect growth regulators and microbial larvicides (Ekerette and Ebere 2017; 2018), used in larval source management. Chemical insecticides include the organochlorines, organophosphates and carbamates which function in IRS and pyrethroids which have also functioned in LLINs.

The use of these insecticides in large-scale campaigns and implementations of *An. gambiae s.l.* control, have experienced simultaneous steady rise in resistance of the vectors of malaria to these public health chemicals and also its use in agriculture as pesticides, has exposed the insecticides to the vectors of malaria, even when it was not intended for them in those agricultural settings. This has resulted in decline in the effectiveness of these insecticides in the control of the disease vectors.

The continuous steady rise in resistance by malaria vectors to the limited WHO approved public health insecticides has raised alarm in the control of these malaria vectors. Consequently, there have been the unavoidable need for the routine surveillance of the malaria vectors resistance status to the limited WHO approved public health insecticides.

Up-to-date information on the spread of the resistance in most parts of Africa is not available, which is necessary for the proper preparation of malaria vector control campaigns in the continent. In Nigeria, few studies have been carried out in some parts of the country: south, south-east, south-west and north-east (Ebere et al 2019; Opara et al 2017; Adeogun et al 2017; Umar et al 2014).

There has been a very intensive search for replacement, with effective insecticides in the malaria vector control, in the face of declining efficacy in the current public health insecticides. Experimental works have shown that using synergist and insecticides together, is a good alternative to control resistant mosquitoes (Corbel et al 2010; Pennetier et al 2013). Piperonyl butoxide (PBO) is a frequently used synergist, as it is responsible for hindering the action of cytochrome P450 monooxygenase enzyme directed at pyrethroids and DDT (Bingham et al 2007; Moores et al 2009). Pyrethroids have been used in LLINs because they are safe, acceptable, and suitable for such purpose, though they disintegrate speedily when exposed to sunlight (WHO 2005). Two pyrethroids: deltamethrin and permethrin have been commonly used in LLINs impregnation.

An alternative method in the vector management of malaria has been in the impregnation of LLINs with a combination of piperonyl butoxide (PBO) and pyrethroids synergist. PBO has functioned in impeding the role of P450 monooxygenase and esterase enzymes, their action in insecticide oxidative detoxification, in vectors of malaria (Demkovich et al 2015). LLINs impregnated using PBO synergist have been more effective in killing pyrethroid resistant vectors in comparison to LLINs with no PBO synergist (N'Guessan et al 2010). The synergist action of these two chemicals has the capability to hinder the activity of oxidase enzymes in the vectors. These enzymes function by detoxifying the insecticide within the vectors, resulting in resistance to the insecticides. However, with the addition of PBO, this oxidase activity is hindered thereby increasing the susceptibility of the vectors to the insecticides.

Proper usage of the inadequate obtainable insecticides, for malaria vector control will depend on full knowledge of the resistance level of the active vectors of malaria. Full understanding on extent of insecticide resistance in an area endemic to malaria will generate invaluable information, that will be useful in IRS planning and LLINs impregnation. Periodical monitoring of the resistance status of these insecticides is vital in malaria vectors resistance management and planning of intervention in a particular endemic area.

Currently, there is paucity of data on the resistance level of vectors of malaria to WHO approved insecticides within the zone covered by the lowland forest vegetation in Rivers State, Nigeria, except for a few localized works carried out by (Ebere et al 2019; Ebere 2010; 2013; 2015), in few parts of the vegetation zone, all of which needs to be updated and their current resistance status determined. This will remove the limitation in ability to properly manage and curb vectors of malaria in the lowland forest vegetation zone of the State.

Therefore, the goal of this study was to make available data that reflect present level of resistance and to find out the efficacy of PBO-deltamethrin and PBO-permethrin synergists, on the population of *An. gambiae s.l.* in the lowland forest region of Rivers State, Nigeria, since they have been used in the impregnation of LLINs.

MATERIALS AND METHODS

Study Area

Lowland forest vegetation located in Niger delta of Nigeria, made up of Rivers State and some other states. It is coordinated by longitude 6’50”E and latitude 4’45”N (Figure 1) and enclosed by Atlantic Ocean. Mean temperatures range from 25 °C to 28 °C (77 °F to 82 °F). It has two well-known seasons: November to April (dry season) and May to October (rainy season), often with a break from rainfall in August. Subsistence farming and fishing are the major sources of livelihood for the occupants.

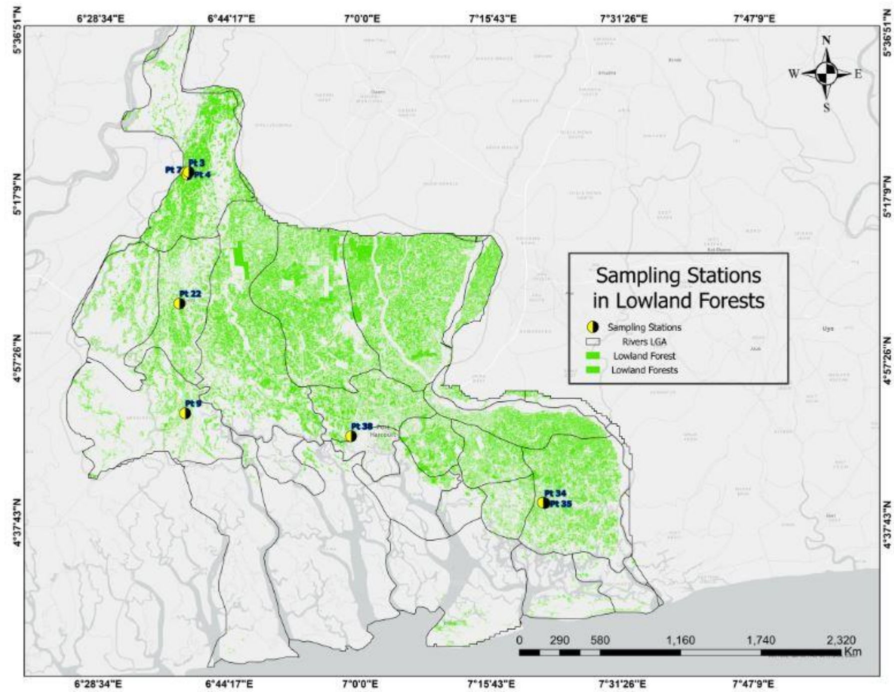

Figure 1. Map of Rivers State showing the lowland forest vegetation zone and sampled breeding sites of *An. gambiae s.l.*

Mosquito Sampling

Prospective breeding locations of *Anopheles* mosquitoes witnessed sampling from the months of May 2019 to December 2019 and also from the months of May 2020 to October 2020. This made certain that samples were collected during the period of heavy rainfall, which had larvae in large quantity. A typical dipper (400ml) which had one (1m) handle served for collecting larvae from different Anopheles breeding spots (WHO 2005) using the normal dipping method. The several breeding locations from which samples collection was carried out is presented in Figure 1.

Rearing of Mosquito Larvae

Larvae of mosquito from diverse locations where they bred within the vegetation zone were brought together and preserved alive in plastic cans (in which the top were perforated for ventilation), capable of holding breeding water from the sites. The larvae were taken to the insectary of Malaria Vector Surveillance and Insecticide Resistance Monitoring Laboratory of the Department of Animal and Environmental Biology, Rivers State University, Port Harcourt, Nigeria. The larvae were nurtured to maturity (imago) using the techniques of (Gerberg et al 1994). The mosquito larvae were sorted and placed in de-chlorinated water held in plastic containers. The containing bowls were enclosed using nets secured with elastic bands, kept on a stage holding water beneath it, to stop creeping organisms from reaching and eating up the larvae. Larvae were nourished using ground biscuits at interval of two days, observed for emergence of adult. Recently developed imago were sorted, females separated from males. The imago were kept in transparent cages in which the top was covered with net and nourished continuously using 10% glucose solution as long as they were kept, before the bioassay. The temperature of the laboratory was maintained at 26°C to 29°C and relative humidity at 74% - 82%. Female *Anopheles* mosquitoes were used for the bioassay. The parental larvae from the lowland forest ecological zone were sampled and used for experiment.

Morphological Identification of *An. gambiae s.l.*

Morphologically, *An. gambiae s.l.* was sorted from other anopheline mosquitoes aided by morphological identification keys of (Gillies and De-Mellion 1968; Gillies and Coetzee 1987).

Insecticide Susceptibility Bioassays

Insecticide exposure test was done using the normal Centers for Diseases Control (CDC) practice (CDC n.d.). The technical grade insecticides were provided by the National Malaria Elimination Programme (NMEP), of which the following were used: Pyrethroids (Permethrin 21.5µg/ml, Lambda-cyhalothrin 12.5µg/ml, Alpha-cypermethrin 12.5µg/ml, Deltamethrin 12.5µg/ml), Organophosphate (Primiphos-methyl 20µg/ml), Carbamates (Bendiocarb 12.5µg/ml, Propoxur 12.5µg/ml), Organochlorine (Dichlorodiphenyltrichloroethane, DDT 100µg/ml). The test was done using the Wheaton bottles (250ml). The mosquitoes used in the experiment were female imago, aged between 3 to 5 days and had not fed on blood. By means of an aspirator (diameter-1cm, length-60cm: half rubber and half glass materials). Control and four replicate bottles each had 25 mosquitoes put into them.

The quantity of the dead and living mosquitoes at 15, 30, 35, 40, 45, 60, 75, 90, 105 and 120 minutes was noted. There was no need to extend the time of the experiment after 2 hours. The mean percentage mortality (Y axis) against time (X axis) graph, was plotted by means of the linear scale. No need for test result correction with Abbott's formula, since for the 2 hours duration, no mortality was found in the control. Mosquitoes were regarded as dead when they could not stand anymore. Flying mosquitoes at the end of the 2 hours showed that they were living and resistant to the tested insecticide. Mosquitoes were considered 'dead' if they could not stand. The Wheaton bottles were gradually and softly rotated when count of the dead mosquitoes was done [21]. Motionless mosquitoes that slide through the curved bottle, were without difficulty considered, dead. The number of dead mosquitoes were calculated in the early evaluation periods of the bioassay, while the amount of living ones were calculated as few stayed alive later. Conclusively, the percentage of dead mosquitoes at the diagnostic time (dead mosquitoes/total of mosquitoes in the assay) was of utmost significant in the evaluation of the graph (CDC n.d.).

Piperonyl Butoxide (PBO) Synergist Test

Piperonyl butoxide synergist test was done using the normal Centers for Diseases Control (CDC) practice (CDC n.d.). The same numbers of about 125 mosquitoes a piece, was put into the synergist-control bottle and the synergist-exposure bottle; the mosquitoes were set aside for 1 hour to let the synergist to act. At the expiration of the 1-hour-exposure period, the mosquitoes were moved to two holding cages: the synergist-control and the synergist-exposed. From there they were easily moved into the insecticide-treated Wheaton bottles. The CDC bottle experiment was accomplished using one set of insecticide-coated bottles (one control and four replicate bottles) for the synergist-control mosquitoes and a different set (one control and four replicate bottles) for the synergist-exposed mosquitoes; the figures for the two groups of experimented mosquitoes were matched for similarity and differences.

Statistical Analysis

Two-way analysis of variance was applied to probe for significant variations in mortality to the insecticides. Significance was considered at a probability level of <0.0001. Mean separation test was carried out using Tukey's multiple comparison.

RESULTS

Susceptibility of An. gambiae s.l. to the Insecticides in Lowland Forest Vegetation

The results (Figure 2) show that after 1 hour, percentage mortality of *An. gambiae s.l.* to: bendiocarb-100, propoxur-96, alpha-cypermethrin-89, lambda-cyhalothrin-79, dichlorodiphenyltrichloroethane-65, deltamethrin-0, primiphos-methyl-1 and permethrin-2. After 2 hours, percentage mortality of *An. gambiae s.l.* to: bendiocarb-100, propoxur-96, alpha-cypermethrin-90, lambda-cyhalothrin-81, dichlorodiphenyltrichloroethane-69, deltamethrin-14, primiphos-methyl-1 and permethrin-2. The result tested with the eight insecticides (p = 0.0001) revealed that within 15 minutes of the test period of 120 minutes, bendiocarb recorded 100% mortality (Figure 2). The highest mean mortality was 22.7$\pm$7.5 for bendiocarb, narrowly trailed by 21.2$\pm$7.1 for propoxur and among the lowest mean mortality was 0.2$\pm$0.1 for primiphos-methyl, closely followed by 0.3$\pm$0.2 for permethrin and 0.3$\pm$1.1 for deltamethrin. Tukey's multiple comparison showed no significant variation between propoxur and alpha-cypermethrin (p = 0.9994) and both fell within possible resistance that needs to be confirmed (when mortality is between 80%-97%), according to WHO recommendations. Tukey's multiple comparison showed no statistical variation between lambda-cyhalothrin and DDT (p = 0.9983) and resistance status (when mortality is less than 80%), was suggested for both based on WHO recommendations, though at 120 minutes, lambda-cyhalothrin recorded a mortality of 81%. There was significant difference among the pyrethroids (p = <0.0001) (Table 1).

Table 1. Analysis of variance mortality based on type of insecticides in the lowland forest vegetation.

Insecticide	**Lowland Forest Vegetation**
	Mean ± Standard Deviation
Alpha-cypermethrin (ACM)	19.9 ± 6.6
Deltamethrin (DM)	0.3 ± 1.1
Lambda-cyhalothrin (LCT)	14.4 ± 7.7
Permethrin (PM)	0.3 ± 0.2
Bendiocarb (BDC)	22.7 ± 7.5
Propoxur (PPX)	21.1 ± 7.1
Primiphos-methyl (PPM)	0.2 ± 0.1
Dichlorodiphenyltrichloroethane (DDT)	12.9 ± 5.8
P – value	<0.0001
Tukey's Multiple Comparison (P - value)	
ACM vs DM	<0.0001
ACM vs LCT	0.2831
ACM vs PM	<0.0001
ACM vs BDC	0.9321
ACM vs PPX	0.9994
ACM vs PPM	<0.0001
ACM vs DDT	0.0708
DM vs LCT	<0.0001
DM vs PM	>0.9999
DM vs BDC	<0.0001
DM vs PPX	<0.0001
DM vs PPM	>0.9999
DM vs DDT	<0.0001
LCT vs PM	<0.0001
LCT vs BDC	0.0149
LCT vs PPX	0.0898
LCT vs PPM	<0.0001
LCT vs DDT	0.9983
PM vs BDC	<0.0001
PM vs PPX	<0.0001
PM vs PPM	>0.9999
PM vs DDT	<0.0001
BDC vs PPX	0.9979
BDC vs PPM	<0.0001
BDC vs DDT	0.0019
PPX vs PPM	<0.0001
PPX vs DDT	0.0158
PPM vs DDT	<0.0001

PBO-Deltamethrin comparison with Deltamethrin alone in Lowland Forest Vegetation

After 1 hour, percentage mortality of *An. gambiae s.l.* to: PBO-deltamethrin synergist-81, deltamethrin alone-0, and after 2 hours, percentage mortality of *An. gambiae s.l.* to: PBO-deltamethrin synergist-89, deltamethrin alone-14 (Figure 3).

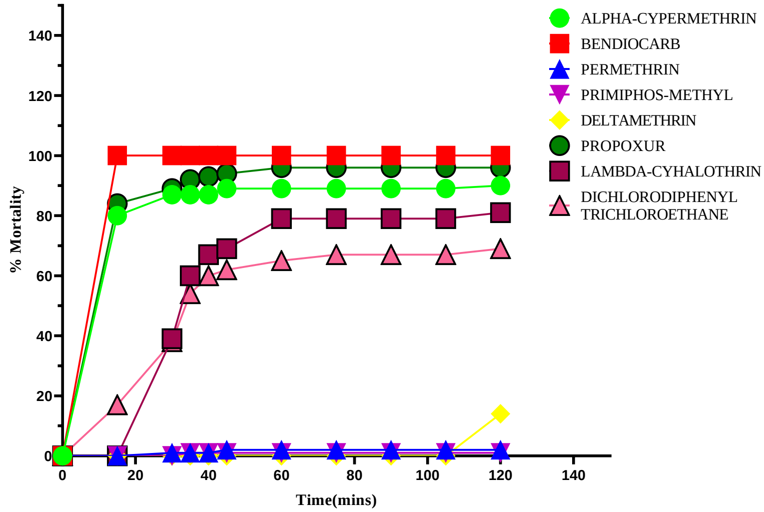

Figure 2. Susceptibility of the insecticides in lowland forest vegetation.

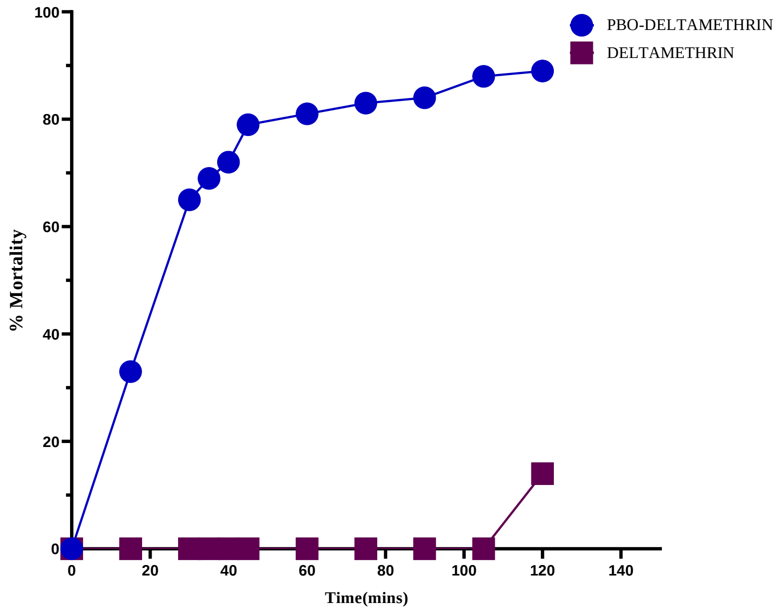

Figure 3. PBO-deltamethrin comparison with deltamethrin alone in lowland forest vegetation.

PBO-Permethrin comparison with Permethrin alone in Lowland Forest Vegetation

Results (Figure 4) showed that after 1 hour, percentage mortality of *An. gambiae s.l.* to: PBO-permethrin synergist-83, permethrin alone-2, and after 2 hours, percentage mortality of *An. gambiae s.l.* to: PBO-permethrin synergist-90, permethrin alone-2 (Table 2).

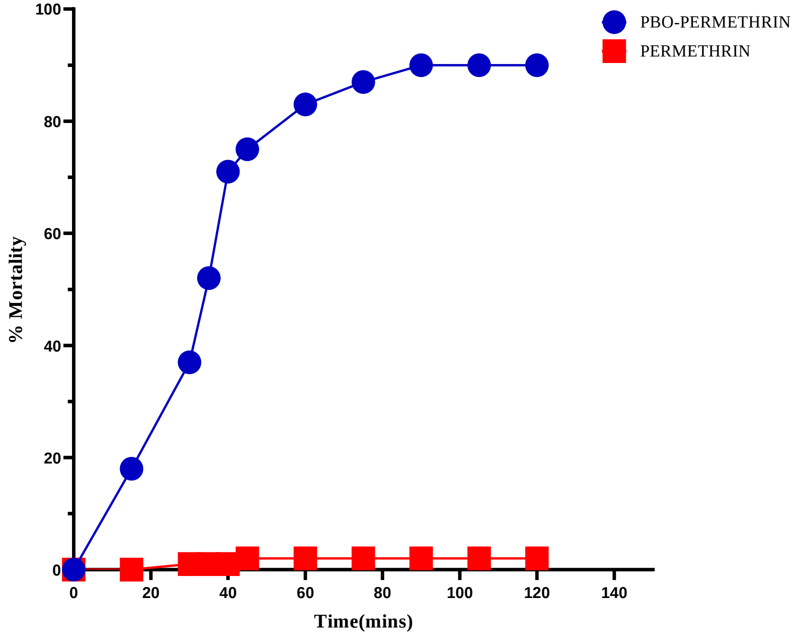

Figure 4. PBO-permethrin comparison with permethrin alone in lowland forest vegetation.

Table 2. Analysis of variance of piperonyl butoxide (PBO) synergist.

Piperonyl Butoxide (PBO) – Insecticide in comparison with Insecticide alone in the Lowland Forest Vegetation		
Insecticide	Deltamethrin	Permethrin
	Mean ± Standard Deviation (M±SD)	
PBO-Insecticide	16.9±6.8	15.8±8.0
Only Insecticide	0.3±1.1	0.3±0.2
P-value	<0.0001	<0.0001
t-value	7.940	6.411
PBO – Deltamethrin with PBO - Permethrin		
P-value	0.7235	
t-value	0.3589	

PBO-Bednet-Insecticides Synergist Performance in Lowland Forest Vegetation

After 1 hour, percentage mortality of *An. gambiae s.l.* to: PBO-permethrin synergist-83, PBO-deltamethrin synergist-81, and after 2 hours, percentage mortality of *An. gambiae s.l.* to: PBO-permethrin synergist-90, PBO-deltamethrin synergist-89 (Figure 5).

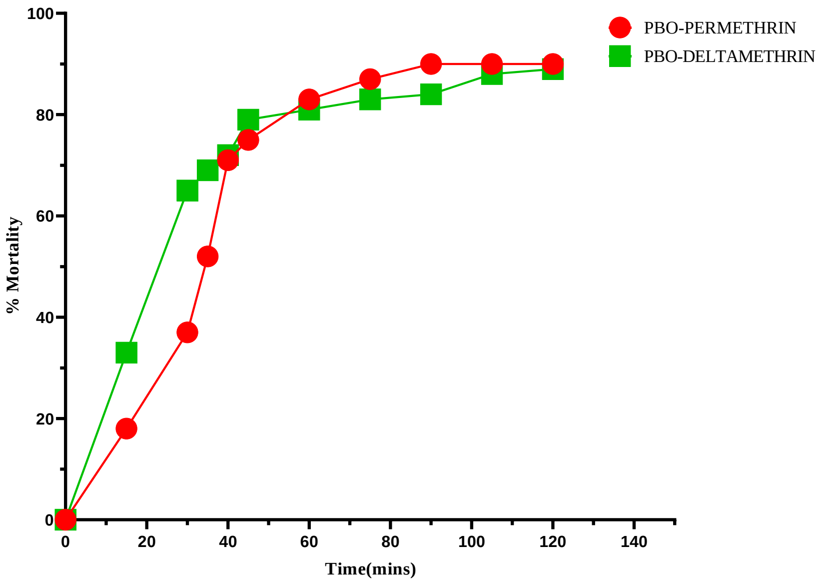

Figure 5. PBO-bednet-insecticides synergist performance in lowland forest vegetation.

DISCUSSION

The lowland forest zone of Rivers state includes these local government areas: Emuoha, Ikwerre, Etche, Omumma, Obio/Akpor, Okirika, Ogu Bolo, Eleme, Oyigbo, Tai, Gokana and Khana. From the result in this study, *An. gambiae s.l.* was completely vulnerable to the carbamate, bendiocarb while susceptibility to propoxur was marginal because both of them are newly formulated chemicals that have not had much exposure to the environment, though propuxur is older than bendiocarb in existence and usage. Percentage mortality of 83 - 95 was recorded in Jigawa state, Nigeria between 2013 and 2014 (Abdu et al 2017). There might have been more exposure of bendiocarb over time in Jigawa state for resistance to be built compared to this current study. *An. gambiae s.l.* was resistant to DDT, primiphos-methyl and the pyrethroids (deltamethrin, permethrin, alpha-cypermethrin and lambda-cyhalothrin). DDT and primiphos-methyl have been exposed and used in the environment for a long time and *An. gambiae s.l.* has steadily developed resistance to them hence they were not effective against the vector. Carbamates were highly effective in the lowland forest vegetation, though there was complete susceptibility with bendiocarb when likened to propoxur.

In Nigeria, there have been reports of complete susceptibility to bendiocarb in Emouha, Ikwerre and Etche local government areas (Ebere et al 2019; Ebere 2013), other areas that have recorded complete susceptibility to bendiocarb include Port Harcourt, Ibadan and Bauchi state (Umar et al 2014; Ebere and Nwakama 2016; Okorie et al 2015), while the mangrove vegetation of Rivers state consisting of Port Harcourt, Akuku Toru, Opobo/Nkoro, Andoni, Degema, Asari-Toru and Bonny local government areas recorded 99% susceptibility to bendiocarb (Ekerette and Ebere 2022). There was also report of complete susceptibility in West African Mali (Cisse et al 2015). Contrastingly, resistance to bendiocarb was recorded in Burkina Faso, Benin, Cote d'Ivoire and Tanzania (Kisinza et al 2017; Manguin 2013).

The degree of resistance to DDT in this study was reported elsewhere in Nigeria (Ebere et al 2019; Adeogun et al 2017; Umar et al 2014; Ebere 2015) and in other parts of Africa like, Cote d'Ivoire, Tanzania and Mali (Cisse et al 2015; Kisinza et al 2017; Camara et al 2008). Resistance to multiple classes of insecticides witnessed in the present study was reported in some parts of Nigeria: Oduoha-Emuoha community, Rivers state, Bauchi state (Ebere et al 2019; Umar et al 2014; Oduola et al 2010; 2012; Riveron et al 2015) and in Africa: Cote d'Ivoire, Benin, Burkina Faso (N'Guessan et al 2003; Corbel et al 2007; Djogbenou et al 2008; Dabire et al 2009).

Tukey's multiple comparison, assisted to differentiate between old and new chemical compounds of pyrethroids as reflected from the p-values (Table 1). The old compounds are permethrin and deltamethrin (within the context of this study) while the new compounds are alpha-cypermethrin and lambda-cyhalothrin (within the context of this study). *An. gambiae s.l.* have over time been able to develop or build-up resistance to permethrin and deltamethrin (older pyrethroids) owing to usage of home-made insecticides of non-standard chemical configuration and common domestic insecticides, could have added to raise the insecticide resistance capacity in the indigenous mosquito populace. This incessant vulnerability of *An. gambiae s.l.* to pyrethroids, in addition to other normally used insecticides put together, makes the mosquito to become intensely resistant to the insecticides (Ebere et al 2019), on the contrary, *An. gambiae s.l.* have just started to build-up or develop resistance to lambda-cyhalothrin and alpha-cypermethrin (newer pyrethroids). The resistance build-up in these newer pyrethroids is revealed in their mortalities: alpha-cypermethrin, 90% and lambda-cyhalothrin, 81%. These failed to meet WHO recommendations for insecticides that indicate susceptibility (when mortality is between 98%-100%) but were found in the category of those that possible resistance is suggested and which needs further confirmation (when mortality is between 80%-97%). Similar trend was observed from the carbamates as bendiocarb was more effective than propoxur (Figure 2) but no statistical difference in the action of bendiocarb over propoxur as reflected in their p-value (Table 1).

Synergists are enzyme inhibitors of insecticide detoxification enzymes. Synergists are available for the metabolic detoxification enzymes: esterases, oxidases and glutathione S-transferases (CDC n.d.). There was significant difference between PBO-deltamethrin synergist and deltamethrin alone (Table 2). PBO has been engaged in the role of preventing the action of esterases and P450s enzymes and has a part in insecticide oxidative detoxification in insects (Demkovich et al 2015). The result from this present study agrees with (N'Guessan et al 2010), that LLINs impregnated with piperonyl butoxide have displayed greater level efficiency against pyrethroid resistant vectors of malaria than nets with pyrethroids alone. This result agrees with the report of [36] in Nigeria, of the high efficiency of LLINs treated with deltamethrin and PBO together, on resistant *An. gambiae s.l.* when likened to ordinary treated nets with no PBO. From a study in Mozambique, deltamethrin and PBO combined, demonstrated the ability to be additionally active against resistant *An. funestus* and *An. gambiae s.l.* (Abilio et al 2015; Riveron et al 2018). In Kolokope, Togo in West Africa, resistance to *An. gambiae s.l.* showed 14.8% mortality record when deltamethrin was singly used but after it was boosted with PBO, the mortality due to deltamethrin increased from 14.8% to 100% (Ketoh et al 2018).

There was significant difference between PBO-permethrin synergist and permethrin alone (Table 2). PBO has functioned in impeding P450s enzymes and their known established duty in insecticide oxidative detoxification in insects (Demkovich et al 2915) was observed earlier in PBO-deltamethrin. This result agrees with the report of (Ketoh et al 2018) from Kolokope, Togo in West Africa, of the resistance to permethrin by *An. gambiae s.l.*, mortality noted was 7.5% but after being boosted with piperonyl butoxide, the mortality shown by permethrin increased from 7.5% to 92%. In Kpome, south of Benin Republic, permethrin combined with piperonyl butoxide was treated on *An. colluzzi*, mortality went up from 19.27% to 69.67% (Akoton et al 2018).

There was a very small change in mortality between PBO-permethrin synergist and PBO-deltamethrin synergist (Figure 5) but no significant difference between them (Table 2). The average mortality for PBO-permethrin synergist was 15.8 ± 8.0 and for PBO-deltamethrin synergist was 16.9 ± 6.8. The small difference in the susceptibility range in this result (Table 2) was not a surprise because they originated from the pyrethriod class, also the extent of resistance of *An. gambiae s.l.* to the pyrethroids synergist witnessed in the lowland forest vegetation could be linked to the selection pressure put forth by the widespread usage of mosquito coils and aerosols (pyrethroids) which was seen applied by residents in the community for vector control on the indigenous mosquito populace (Okorie et al 2015).

CONCLUSION

In the region of the lowland forest vegetation of Rivers state, *An. gambiae s.l.* populace was 100% susceptible to the carbamate, bendiocarb while resistant to another carbamate, propoxur. They were also resistant to primiphos-methyl, DDT and the following pyrethroids: alpha-cypermethrin, deltamethrin, lambda-cyhalothrin and permethrin. The increase in the efficacy of the pyrethroids with adding of the synergist shows that the PBO additive could possibly increase the susceptibility level of the vectors and hence should be encouraged. Results from this study suggests that bendiocarb could be used in addition to the PBO-pyrethroid synergists for IRS and LLINs impregnation respectively, in the vegetation zone. This study make available reference point information for checking the level of insecticide resistance in Rivers State.

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STATEMENT OF AUTHORSHIP

Ekerette, I.B. designed and conducted the research. Ebere, N. supervised the research and proofread the initial manuscript.

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