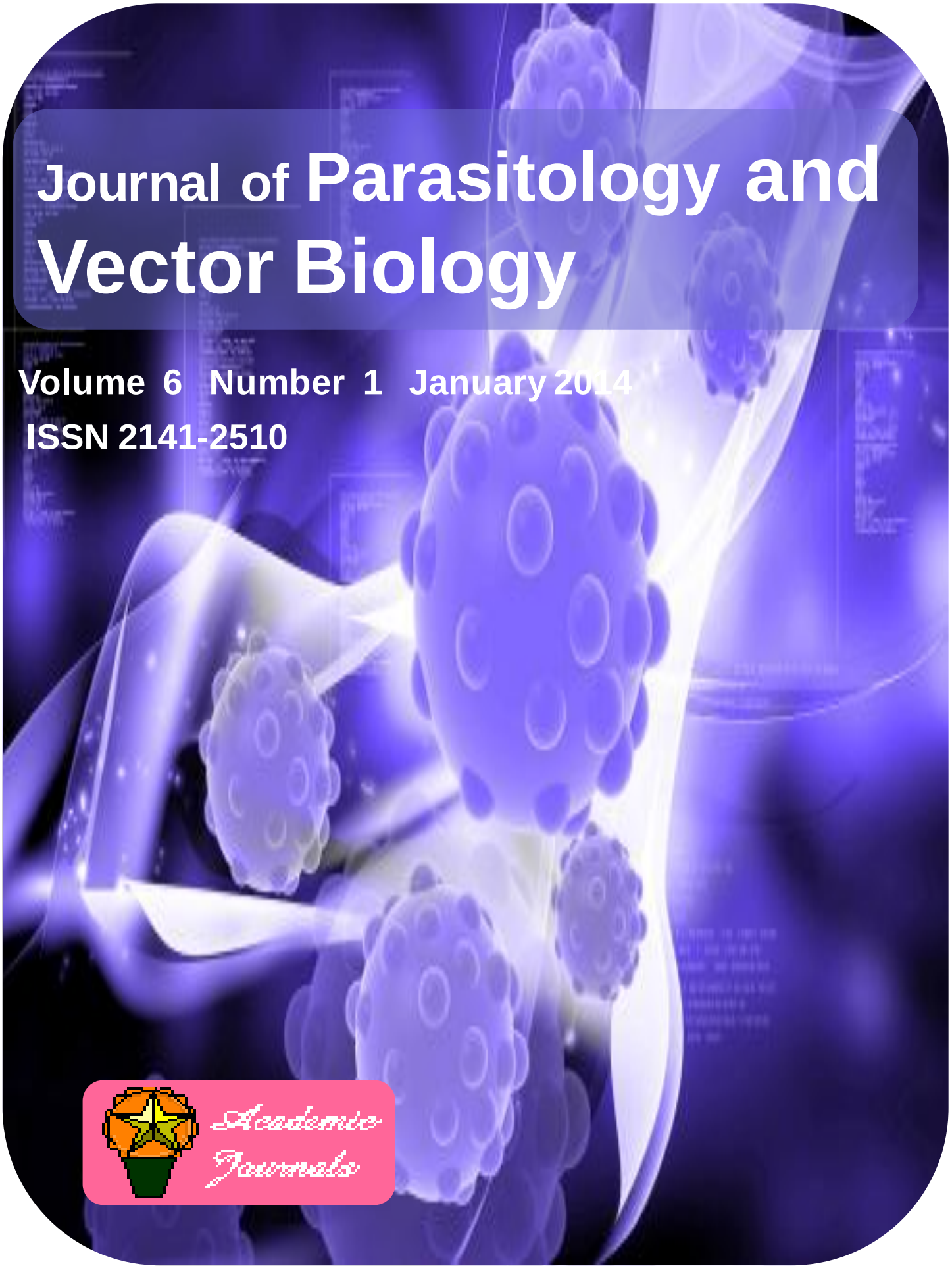

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Dynamics of insecticide resistance and effect of synergists piperonyl butoxide (PBO), S.S.S-tributylphosphorotrithioate (DEF) and ethacrynic acid (ETAA or EA) on permethrin, deltamethrin and dichlorodiphenyltrichloroethane (DDT) resistance in two *Anopheles gambiae s. l.* populations from Southern Benin, West Africa

Nazaire Aïzoun[1,2]*, Rock Aïkpon[1,2], Virgile Gnanguenon[1,2], Roseric Azondekon[1], Frédéric Oké-Agbo[1], Gil Germain Padonou[1,2] and Martin Akogbéto[1,2]

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Permanet 2.0 distribution was made free in October, 2008 and May, 2009 in Oueme Department. OlysetNet distribution was also made free in July, 2011 throughout the entire country by Beninese National Malaria Control Programme to increase coverage of long-lasting insecticidal nets (LLINs). We investigated the dynamics of insecticide resistance in *Anopheles gambiae* from southern Benin and the metabolic resistance mechanisms involved. Larvae and pupae of *A. gambiae s. l.* were collected from the breeding sites in Littoral and Oueme Departments. Centers for Disease Control and Prevention (CDC) susceptibility tests were conducted on unfed female mosquitoes aged 2 to 5 days old with stock solutions of permethrin, deltamethrin and dichlorodiphenyltrichloroethane (DDT). CDC biochemical assays using synergists were also carried out. *A. gambiae* Ladji populations were resistant to permethrin and DDT in 2008 and in 2013. *A. gambiae* Sekandji populations were susceptible to deltamethrin in 2008 and in 2013 whereas these populations were resistant to this product in 2010. *A. gambiae* Sekandji populations were resistant to DDT in 2008 and in 2013. The DDT resistance level in *A. gambiae* Ladji and Sekandji populations recorded in 2013 was higher than the one observed in 2008. The metabolic resistance conferred by detoxifying enzymes is an indication of phenotypic resistance to both DDT and pyrethroids.

Key words: Dynamics, piperonyl butoxide, S.S.S-tributylphosphorotrithioate, ethacrynic acid, insecticide, vectors, resistance.

INTRODUCTION

Malaria vector control in Africa relies heavily on the organochlorine, dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides such as permethrin, deltamethrin (Zaim et al., 2000). The first cases of pyrethroid resistance

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were recorded in West Africa more precisely in Côte d'Ivoire (Elissa et al., 1993). Many other cases have been described in West Africa (Akogbeto and Yakoubou, 1999; Chandre et al., 1999), in Benin (Chandre et al., 1999; Diabaté, 1999), in Burkina Faso (Fanello et al., 2003) and in Mali. In West Africa, the first cases of dieldrin resistance in *Anopheles gambiae* were recorded in Burkina Faso in 1960 (Coz et al., 1968). Ten years later, the identification of cases of DDT resistance was reported in Togo, Senegal, Nigeria (OMS, 1976). In Benin, Akogbeto and Yakoubou (1999) suspected the emergence of DDT resistance recorded in *A. gambiae* from meridian regions, to be related to two phenomena: (i) the massive use of DDT and dieldrin for house-spraying applications in southern villages from 1953 to 1960 during World Health Organization (WHO) pro-grammes of malaria eradication (Joncour, 1959) and (ii) the massive use of organo-chlorine in agricultural settings during the 1950s (OMS, 1976). However, some cross-resistance exists between different groups of insecticides and emergence of resistance in vector populations is a major threat for the sustainability of malaria prevention through vector control in Africa (N'Guessan, 2009).

In West Africa, *A. gambiae* resistance to the four major classes of insecticides available for public health has been reported (Chandre et al., 1999; Elissa et al., 1993; Akogbeto and Yakoubou, 1999; Awolola et al., 2002; Fanello et al., 2003; Diabate et al., 2002). Pyrethroids are the only option for net treatment due to their relative safety for humans at low dosage, excito-repellent properties, rapid rate of knock-down and killing effects (Zaim et al., 2000). However, the success of the control methods is threatened by resistance to the main insecticides such as pyrethroids in malaria vectors.

Malaria vector resistance to insecticides in Benin is conferred by two main mechanisms: (1) alterations at site of action in the sodium channel via the *kdr* mutations and (2) an increase of detoxification and/or metabolism through high levels of multi-function oxidases (MFOs) and non-specific esterases (NSEs) (Corbel et al., 2007; Djogbénou et al., 2009; Djègbé et al., 2011; Aïzoun et al., 2013a).

A total of 48,819 Long-Lasting Insecticidal Nets, Permanet 2.0 (LLINs) were distributed to 47,524 households, with particular attention to children under-five and pregnant women, in October, 2008 and May, 2009 in the framework of President's Malaria Initiative of the U.S. Government in Oueme Department (Padonou et al., 2012). In addition, Beninese National Malaria Control Programme has implemented large-scale and free distribution of LLINs (OlysetNets) in July, 2011 through-out the entire country to increase coverage of LLINs. It is crucial that information on current status of *A. gambiae s.l.* permethrin, deltamethrin and DDT resistant populations should be investigated. This will properly inform control programs of the most suitable insecticides to use and facilitate the design of appropriate resistance anagement strategies.

Padonou et al. (2012) have shown that the main mechanism of resistance to pyrethroids is the mutation Leu1014F *kdr* allele in Seme district including Sekandji location. However, it would be useful to check if metabolic resistance conferred by detoxifying enzymes is also present in such *A. gambiae* populations. In fact, that will help to investigate multiple insecticide resistance mechanisms in *A. gambiae* Sekandji populations. In addition, Corbel et al. (2007) reported on multiple insecticide resistance mechanisms in *A. gambiae* Ladji populations. These authors also mentioned that an experimental hut study carried out at Ladji location in 2004 showed a rather low efficacy of permethrin treated nets at WHO recommended dosages against *A. gambiae* (Corbel et al., 2004) and this result underlines the need to investigate the role of enzymes in *A. gambiae* insecticide resistant populations through the use of classical synergists.

The main goal of this study was to explore the involve-ment of cytochrome P450 mono-oxygenases, esterases and glutathione S-transferases (GSTs) in permethrin, deltamethrin and DDT resistant *A. gambiae s.l.* populations from southern Benin by using classical synergists from 2008 to 2013.

METHODOLOGY

Study area

The study was carried out in the South of Benin, more precisely in Ladji location, in the Cotonou district of Littoral Department and in Sekandji location, in the Seme district of Oueme Department (Figure 1). The choice of the study sites took into account the economic activities of populations, their usual protection practices against mosquito bites, the LLINs distribution recently in these localities and peasant practices to control farming pests. So, deltamethrin and permethrin were the two pyrethroid insecticides used in malaria vector control throughout LLINs distribution recently by Beninese National Malaria Control Programme. In addition, Permanets 2.0 were only distributed in Oueme Department whereas OlysetNets were distributed throughout the entire country. These factors have a direct impact on the development of insecticide resistance in the local mosquito vectors. Cotonou is characterized by a tropical coastal guinean climate with two rainy seasons (April to July and September to November). The mean annual rainfall is over 1,500 mm. Oueme has a climate with two rainy seasons (March to July and September to November). The temperature ranges from 25 to 30°C with the annual mean rainfall between 900 and 1,500 mm.

Mosquito sampling

A. gambiae s.l. mosquitoes were collected during the rainy seasons (March to July and September to November, 2008, 2010 and March to July, 2013) across Sekandji in the Seme district selected in south Benin. *A. gambiae s.l.* mosquitoes were also collected during the rainy seasons (April to July and September to November, 2008 and April to July, 2013) across Ladji in the Cotonou district selected in south Benin. Larvae and pupae were collected on breeding sites using the dipping method. They were then kept in separated labeled bottles related to each locality. The samples were reared up

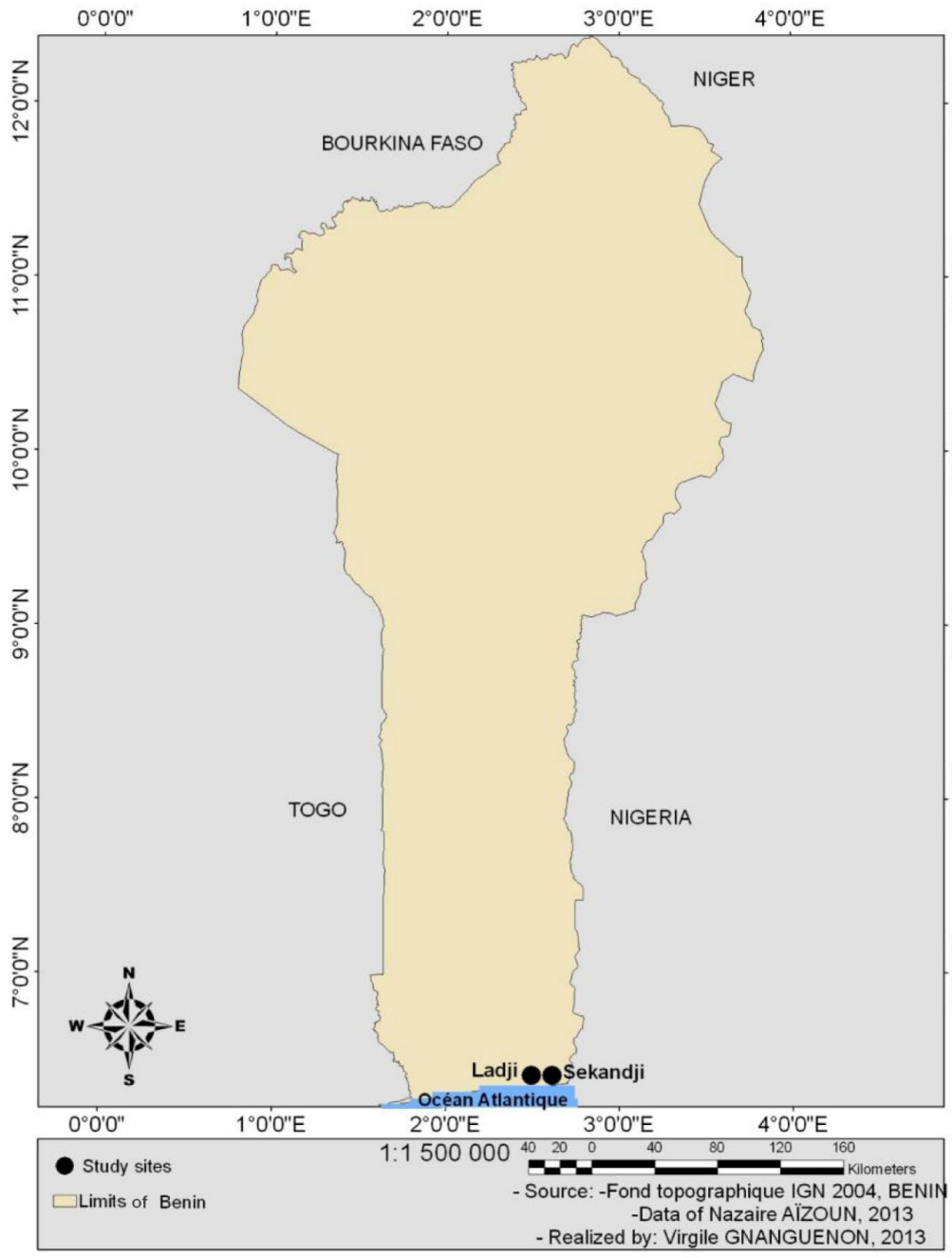

Figure 1. Map of the study area.

to adult emergence at the Centre de Recherche Entomologique de Cotonou, Benin (CREC) insectary. *A. gambiae* Kisumu, a reference susceptible strain was used as a control for the bioassay tests. Susceptibility tests were carried out following CDC protocols on unfed female mosquitoes aged 2 to 5 days old reared from larval and pupal collections. All susceptibility tests were conducted in the CREC laboratory at 25±2°C and 70 to 80% relative humidity.

CDC protocol

The principle of the CDC bottle bioassay is to determine the time it takes an insecticide to penetrate an arthropod, traverse its intervening tissues, get to the target site, and act on that site relative to a susceptible control. Anything that prevents or delays the compound from achieving its objective of killing the arthropods contributes to resistance. Diagnostic doses that were applied in the current study were the doses recommended by CDC (Brogdon and Chan, 2010). These doses were checked on the *A. gambiae* Kisumu susceptible reference strain before being applied to field populations. For *A. gambiae s.l.*, the diagnostic dose of 12.5 µg per bottle for deltamethrin and of 21.5 µg per bottle for permethrin were used for the same diagnostic exposure time of 30 min whereas the diagnostic dose of 100 µg per bottle for DDT was used for a

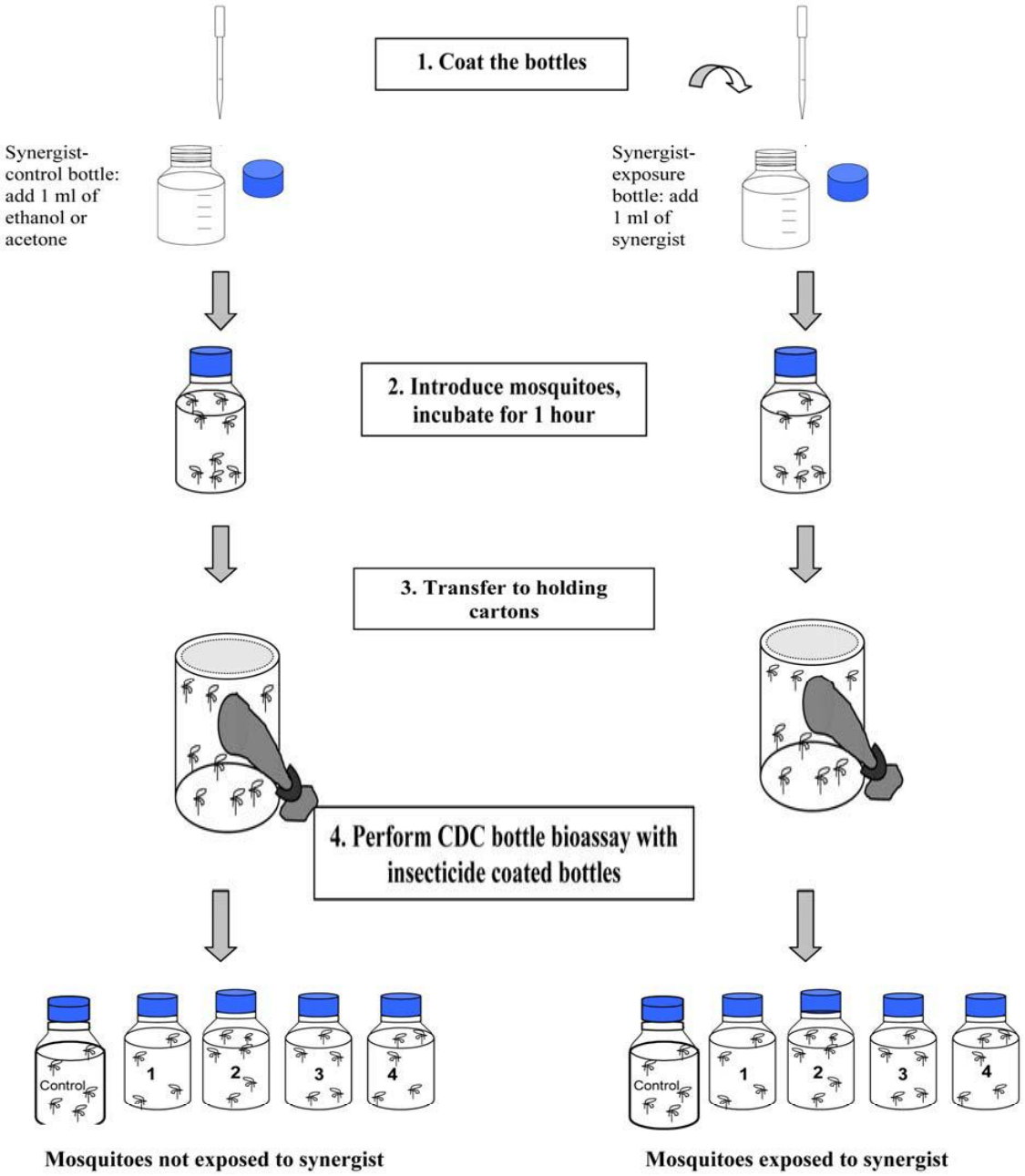

Figure 2. Diagram for performing the CDC bottle bioassay with synergists [CDC: Methods in *Anopheles* Research, 2010].

diagnostic exposure time of 45 min. The choice of deltamethrin was justified by its use on Permanet 2.0 that was distributed free by the NMCP in October, 2008 and May, 2009 in Oueme Department, whereas permethrin is the insecticide used on OlysetNets that were distributed free by the NMCP in July, 2011 across the entire country. DDT was tested because of its intensive use in the past as well as to assess cross-resistance with permethrin and deltamethrin in localities surveyed.

The solutions were prepared and the bottles coated according to the CDC protocol (Brogdon and Chan, 2010). Fifteen to 20 unfed female mosquitoes aged 2 to 5 days old were introduced into four 250 ml Wheaton bottles coated with insecticide and one control bottle coated with acetone only. The number of dead or alive mosquitoes was monitored at different time intervals (10, 20, 30, 40, 50, 60 min) in 2008 and (15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min) in 2010 and 2013. This allowed us to determine the total percent mortality (Y axis) against time (X axis) for all replicates using a linear scale.

Biochemical assays using synergists

Synergists were used according to the protocol described by CDC (Brogdon and McAllister, 1998; Brogdon and Chan, 2010) following the procedure outlined in Figure 2. Samples that showed high resistance to permethrin in 2008 in Ladji location from the Cotonou

district were exposed to the effects of two synergists: S.S.S-tributylphosphorotrithioate (DEF) (125 µg per bottle), which inhibits esterase activity and piperonyl butoxide (PBO) (400 µg per bottle), which inhibits oxidase activity. Piperonyl butoxide (PBO) is a pyrethroid synergist whereas S.S.S-tributylphosphorotrithioate is used in combination with PBO in order to explore multiple insecticide resistance mechanisms in *A. gambiae* Ladji populations. These two synergists were used separately and in combination. In a similar way, samples that showed high resistance to deltamethrin in 2010 in Sekandji location from the Seme district were exposed to the effects of these two same synergists (PBO and DEF). In addition, the samples that showed high resistance to DDT in 2013 in Sekandji locality were also exposed to the effects of the synergist: Ethacrynic acid (ETAA or EA) (80 µg per bottle), which inhibits glutathione S-transferases activity. This synergist was used in combination with DDT alone. Ethacrynic acid (ETAA or EA) is an organochlorine synergist such as dichlorodiphenyltrichloroethane (DDT). Approximately 125 mosquitoes were used for each synergist assay. The number of dead or alive mosquitoes was monitored at different time intervals (0, 10, 20, 30, 40, 50, 60 min) in 2008 and (0, 15, 30, 35, 40, 45, 60, 75, 90, 105, 120 min) in 2010 and in 2013. This test allowed us to compare the obtained percentages of dead mosquitoes (Y axis) against time (X axis) before the addition of the synergist (s) to those obtained after the addition of the synergist (s) (Figure 2).

Data analysis

The resistance status of mosquito samples was determined according to the CDC criteria (Brogdon and McAllister, 1998; Brogdon and Chan, 2010). The susceptibility thresholds at the diagnostic time of 30 min for pyrethroids and 45 min for organochlorines are: Mortality rate = 100%: the population is fully susceptible, Mortality rate < 100%: the population is considered resistant to the tested insecticides. Abbott's formula was not used in this study for the correction of mortality rates in the test-bottles because the mortality rates in all controls was always less than 5% (Abbott, 1987). To appreciate the effects of synergists PBO and DEF on *A. gambiae* Ladji and Sekandji permethrin and deltamethrin resistant populations in 2008 and in 2010, respectively and the effect of synergist ETAA on *A. gambiae* Sekandji DDT resistant populations in 2013, we used a Kruskal-Wallis test. The significance level was set at 5%. The software R-2.15.2. (R Development Core Team, 2011) was used for the statistical analysis.

RESULTS

Evolution of *A. gambiae* resistance to insecticides in Ladji and Sekandji locations from 2008 to 2013

The Kisumu strain (control) confirmed its susceptibility status as a reference strain. All female mosquitoes of *A. gambiae* Kisumu that were exposed to CDC bottles treated with permethrin 21.5 µg per bottle and DDT 100 µg per bottle in 2008 and in 2013, were dead and none of them could fly after 30 and 45 min, which represent the susceptibility threshold times or diagnostic times clearly defined by the CDC protocol. This confirmed that this strain was fully susceptible to these products in 2008 and 2013. In similar way, all female mosquitoes of *A. gambiae* Kisumu that were exposed to CDC bottles treated with deltamethrin 12.5 µg per bottle in 2008, 2010 and in 2013 and with DDT 100 µg per bottle in 2008 and in 2013 were dead and none of them could fly after 30 and 45 min. This confirmed that this strain was fully susceptible to these products in 2008, 2010 and 2013.

A proportion of the *A. gambiae* Ladji population 28.56 and 11.67% in 2008 and 2013, respectively continued to fly again in the bottles following 30 min exposure to CDC bottles treated with permethrin. In addition, a large proportion of this population 91 and 97.68% in 2008 and 2013, respectively continued to fly again in the bottles following 45 min exposure to CDC bottles treated with DDT. This confirmed that *A. gambiae* Ladji population was highly resistant to these products (Table 1). No *A. gambiae* Ladji population was exposed to CDC bottles treated with permethrin 21.5 µg per bottle in 2010 (during our survey period) because it was difficult to collect a sufficient number of larvae and pupae of *A. gambiae* mosquitoes during this year in Ladji locality (Table 1).

A large proportion of the *A. gambiae* Sekandji populations (26%) continued to fly again in the bottles following 30 min exposure to CDC bottles treated with deltamethrin in 2010. This confirmed that these populations were resistant to this product (Table 1). Conversely to this resistance recorded in 2010, these populations were susceptible to the same product in 2008 and in 2013 when they had the same behavior facing deltamethrin as Kisumu susceptible reference strain. A large proportion of the *A. gambiae* Sekandji populations 85.92 and 97.15% in 2008 and 2013, respectively continued to fly again in the bottles following 45 min exposure to CDC bottles treated with DDT. This confirmed that these populations were highly resistant to this product (Table 1).

Effects of synergists PBO and DEF on *A. gambiae* Ladji populations resistant to permethrin in 2008

The data presented in Figure 3 show that after the addition of synergist PBO and DEF to permethrin 21.5 µg per bottle, the percentage of dead mosquitoes from Ladji is higher than the one obtained with permethrin alone. The use of either PBO or DEF synergist in bottles treated with permethrin 21.5 µg per bottle did not eliminate permethrin resistance, but significantly reduced the level with the mortality rate increasing from 71.25 to 84% ($p = 0.0053$) or from 71.25 to 80.80% ($p = 0.0425$), respectively. The use of the synergist combination DEF + PBO did not give the same result as the one obtained with PBO alone ($p = 0.0132$) or DEF alone ($p = 0.0017$). In addition, the use of synergist combination DEF + PBO did not restore the susceptibility in *A. gambiae* Ladji populaions by rendering them susceptible to permethrin 21.5 µg per bottle as the reference strain Kisumu. These results suggest an implication of both mono-oxygenases and esterases in resistance of *A. gambiae* to pyrethroids.

Table 1. Mortality of *Anopheles gambiae* from the localities of Ladji and Sekandji after one and two hours exposure to CDC bottles treated with permethrin (21.5µg/bottle), deltamethrin (12.5µg/bottle), and DDT(100µg/bottle) from 2008 to 2013.

Locality	Year	Insecticide	Number tested	Mortality (%)	Resistance status
Kisumu (Ctrl)	2008	Permethrin	200	100	S
	2008	DDT	200	100	S
	2013	Permethrin	37	100	S
	2013	DDT	35	100	S
Ladji	2008	Permethrin	334	71.25	R
	2008	DDT	100	9	R
	2013	Permethrin	60	88.33	R
	2013	DDT	86	2.32	R
Kisumu(Ctrl)	2008	Deltamethrin	200	100	S
	2008	DDT	200	100	S
	2010	Deltamethrin	110	100	S
	2013	Deltamethrin	25	100	S
	2013	DDT	35	100	S
Sekandji	2008	Deltamethrin	86	100	S
	2008	DDT	72	14.08	R
	2010	Deltamethrin	47	74.07	R
	2013	Deltamethrin	41	100	S
	2013	DDT	70	2.85	R

Ctrl : Control

Effects of synergist ETAA on *A. gambiae* Ladji populations resistant to DDT

The analysis of Figure 4 shows that after the addition of synergist EA to DDT 100 µg/bottle, the percentage of dead mosquitoes from Ladji is slightly higher than the one obtained with DDT alone. The use of synergist EA in bottles treated with DDT 100 µg/bottle did not eliminate DDT resistance, and the mortality rate increased from 02.32 to 8.62% (P = 0.1179). These results show that GSTs may play a little role in *A. gambiae* Ladji resistance to DDT.

Effects of synergists PBO and DEF on *A. gambiae* Sekandji populations resistant to deltamethrin in 2010

The analysis of Figure 5 shows that after exposure to the synergist PBO prior to exposure to deltamethrin 1.25%, the percentage of dead mosquitoes from Sekandji on PBO was higher than that obtained with deltamethrin alone. The PBO synergist did not eliminate deltamethrin resistance but significantly reduced the level with the mortality rate increasing from 74.07 to 90.90% (p = 0.0122). The mortality rate recorded with deltamethrin + DEF was similar to the one obtained with deltamethrin alone (p = 0.5388). In addition, the use of the synergist combination DEF + PBO did not give the same result as the one obtained with PBO alone (p = 02.99) or DEF alone (p = 0.0004). In addition, the use of synergist combination DEF + PBO did not restore the susceptibility in *A. gambiae* Sekandji populaions by rendering them susceptible to deltamethrin 1.25% as the reference strain Kisumu. These results suggest an implication of mono-oxygenases in resistance of *A. gambiae* to pyrethroids.

Effect of synergist ETAA or EA on *Anopheles gambiae* Sekandji populations resistant to DDT in 2013

The analysis of Figure 6 shows that after the addition of synergist ETAA to DDT 100 µg per bottle, the percentage of dead mosquitoes from Sekandji is lower than the one obtained with DDT alone. The use of synergist ETAA in bottles treated with DDT 100 µg per bottle did not eliminate DDT resistance, and the mortality rate decreased from 2.85 to 0% (P = 0.5114). These results show that GSTs may play no role in *A. gambiae* Sekandji resistance to DDT.

DISCUSSION

A. gambiae Ladji populations were resistant to permethrin

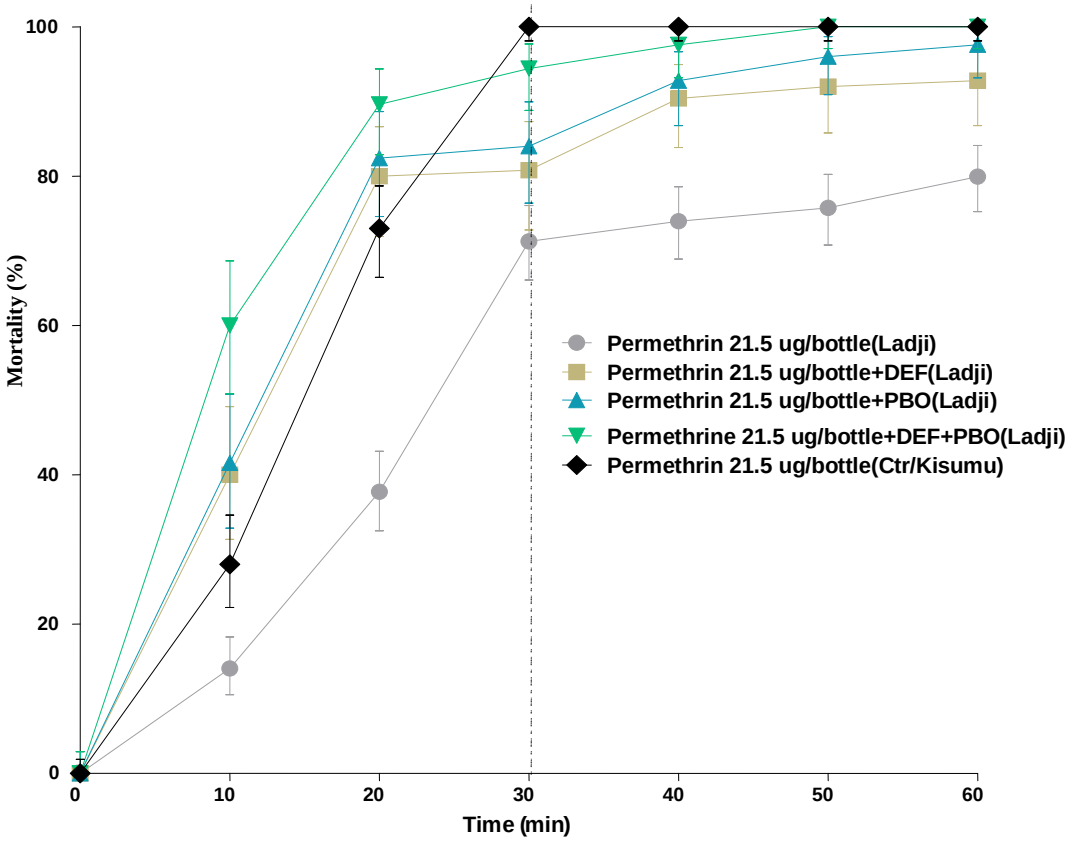

Figure 3. Effects of synergists PBO and DEF on *Anopheles gambiae* Ladji populations resistant to permethrin in 2008.

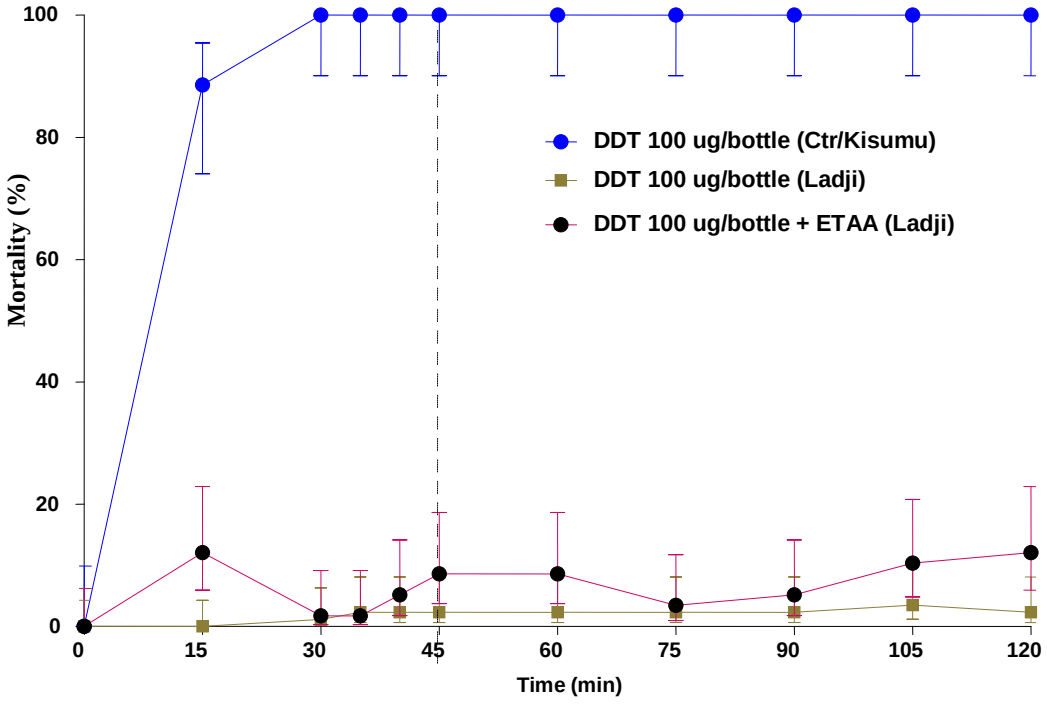

Figure 4. Effects of synergist ETAA on *Anopheles gambiae* Ladji populations resistant to DDT.

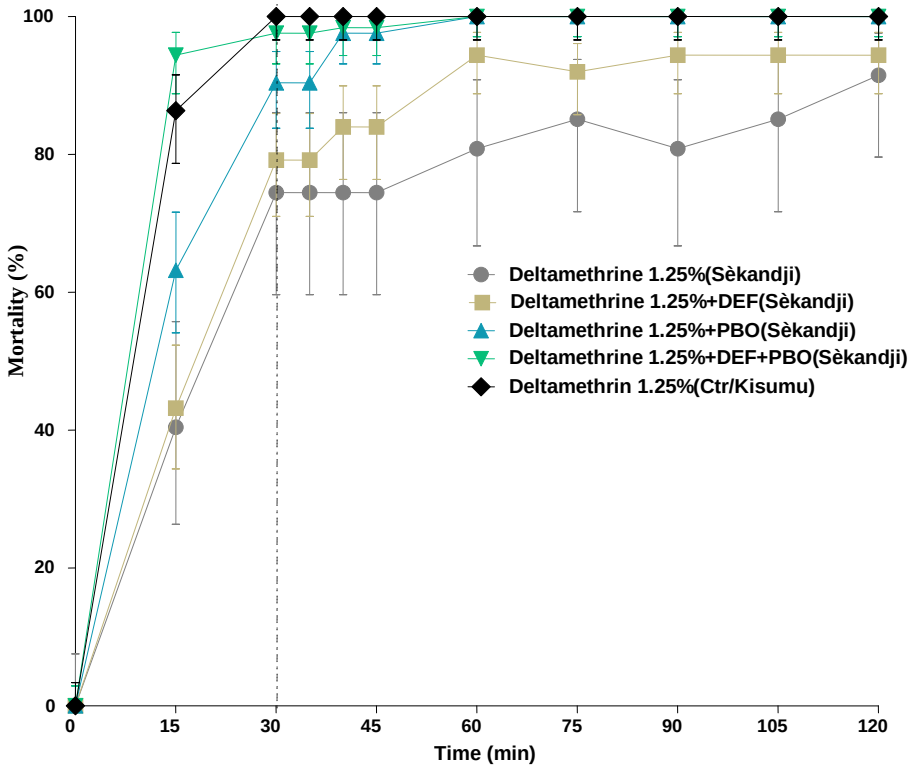

Figure 5. Effects of synergists PBO and DEF on *Anopheles gambiae* Sekandji populations resistant to deltamethrin in 2010.

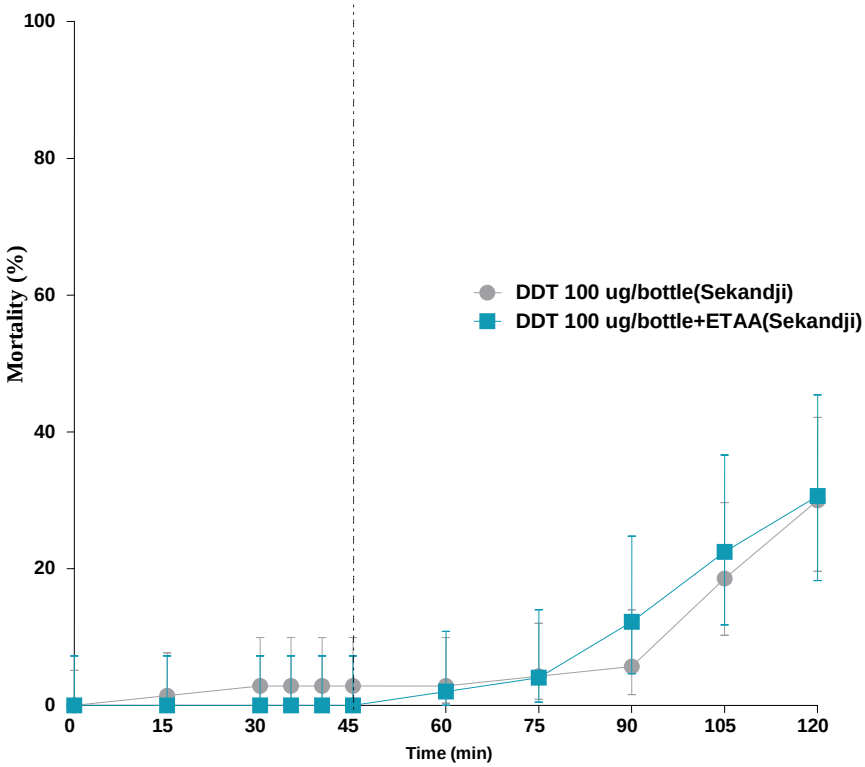

Figure 6. Effect of synergist ETAA or EA on *Anopheles gambiae* Sekandji populations resistant to DDT in 2013.

in 2008 and still remained resistant to the same product in 2013. The pyrethroid resistance observed in *A. gambiae* Ladji populations may be due to the presence of several environmental pollutants and pesticide residues from the neighbouring peri-urban cities and farms to the coastal location of Ladji, a peri-urban locality located in the town of cotonou. This locality is crossed by the Nokoue Lake's streams. These streams sweep and converge these environmental pollutants and pesticide residues in Ladji locality. These xenobiotics available in larval breeding sites in Ladji may be one of the possible factors selecting for pyrethroid resistance in *A. gambiae* populations in this locality. A similar pattern was already observed with *A. gambiae* Agbalilamè permethrin resistant populations (Aïzoun et al., 2013a). In the current study, in 2008, metabolic resistance conferred by detoxifying enzymes such as cytochrome P450 mono-oxygenases and esterases were found to play a role in *A. gambiae* Ladji permethrin resistant populations.

In addition, after the addition of both synergists PBO and DEF on *A. gambiae* Ladji permethrin resistant populations, we have not still obtained fully susceptibility. These results showed that there were other resistance mechanisms which were not synergizable by PBO and DEF. In southern Benin, Corbel et al. (2007) have already reported on multiple insecticide resistance mechanisms in *A. gambiae* Ladji populations. Among these mechanisms, there were mixed function oxidase (MFO) and α-esterase with the presence of *Kdr* at high frequency (80%). However, even if the Leu-Phe *kdr* mutation is the most important resistance mechanism in these *A. gambiae* Ladji populations, metabolic resistance conferred by detoxifying enzymes is also an indication of phenotypic resistance to permethrin.

A. gambiae Ladji populations were resistant to DDT in 2008 and still remained resistant to the same product in 2013 with an increase in the resistance level. According to Akogbeto and Yakoubou (1999), the emergence of DDT resistance recorded in *A. gambiae* from meridian regions was related to two phenomena: the massive use of DDT and dieldrin for house-spraying applications in southern villages from 1953 to 1960 during WHO programmes of malaria eradication and the massive use of organochlorine in agricultural settings during the 1950s (OMS, 1976). In addition, previous studies conducted by Akogbeto et al. (2006) and Corbel et al. (2007) have already showed a cross-resistance to pyrethroid and DDT in *A. gambiae* Ladji populations. A recent study conducted in 2013 showed that GSTs may play a little role in *A. gambiae* Ladji DDT resistant populations using CDC biochemical assays (Aïzoun et al., 2013b). This result could explain in part the other resistance mechanisms involved in *A. gambiae* Ladji permethrin resistant populations because this permethrin resistance was not synergizable by PBO and DEF in 2008. This result also shows that the three categories of enzymes namely esterases, cytochrome P450 mono-oxygenases and glutathione S-transferases which were typically involved in insecticide resistance in malaria vectors were all present in *A. gambiae* Ladji populations.

A. gambiae Sekandji populations were susceptible to deltamethrin in 2008 and 2013. But in 2010, these populations were resistant to this product. According to Padonou et al. (2012), the deltamethrin resistance recorded in *A. gambiae* Seme populations in 2010 was not due to PermaNet2.0 distribution by National Malaria Control Programme (NMCP), and the high frequency of the mutation L1014F *kdr* allele could explain this resistance. These authors also mentioned that the selective pressure exerted by the promotion of mosquito nets by the Health Ministry and the free distribution of LLINs in Oueme region, causing the *kdr* increase within *A. gambiae* populations is doubtful. The *kdr* frequency recorded in Seme district including Sekandji locality by Padonou et al. (2012) in 2010 was 0.87. During the same year, metabolic resistance conferred by cytochrome P450s was detected in *A. gambiae* Sekandji deltamethrin resistant populations in the current study. A recent study carried out by Aïzoun et al. (2013a) in Seme district, precisely in Agbalilame locality also suggested an implication of mono-oxygenases in resistance of *A. gambiae s.l.* to pyrethroids. Indeed, Padonou et al. (2012) showed that the main mechanism of pyrethroid resistance in *A. gambiae* Seme was L1014F *kdr* mutation. As it was observed with *A. gambiae* Ladji populations, even if the Leu-Phe *kdr* mutation is the most important resistance mechanism in *A. gambiae* Sekandji populations collected in Seme district, metabolic resistance conferred by detoxifying enzymes is also an indication of phenotypic resistance to deltamethrin.

A. gambiae Sekandji populations were resistant to DDT in 2008 and still remained resistant to the same product in 2013 with an increase in the resistance level. The cause of this resistance level increasing was the same as the one observed with *A. gambiae* Ladji DDT resistant populations. In fact, Sekandji is also located in the meridian region like Ladji. In the current study, after the addition of synergist ETAA on *A. gambiae* Sekandji DDT resistant populations, the mortality rate decreased and Glutathione *S*-transferases therefore may play no role in these *A. gambiae* Sekandji DDT resistant populations. A similar pattern was already observed with *A. gambiae* Bohicon and Parakou populations (Aïzoun et al., 2013b). In some cases, the use of synergists at the same time as the application of insecticide could inhibit the penetration of the insecticide through the cuticle, therefore reducing the amount of insecticide entering the insect's body (Martin et al., 1997), the result of which was that the toxicity effect would also be reduced.

Conclusion

This study shows that *A. gambiae s.l.* populations from southern Benin were resistant to both DDT and pyrethroids.

The resistance level of these populations to these products has varied or changed in an interval of five years. Even if the Leu-Phe *kdr* mutation is the most important resistance mechanism in these *A. gambiae s.l.* populations, metabolic resistance conferred by detoxifying enzymes is also an indication of phenotypic resistance to both DDT and pyrethroids in southern Benin.

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Short Communication

Evaluation of larvicidal activity of the different extracts against important species of mosquito: *Anopheles stephensi*

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The aim of this work was to compare larvicidal activity of insecticidal plants against malaria vector mosquito *Anopheles stephensi.* The larvicidal activity of *Lantana camara* Linn. and *Bauhinia racemosa* Lam., extracted in petroleum ether, chloroform and ethyl acetate were tested against mosquito larvae of *A. stephensi*. Late third or early fourth instar larvae were used for the screening. These extracts were used for determining the larvicidal activity using World Health Organization (WHO) method for evaluation of new larvicidal agents. The petroleum ether extract of *L. camara* showed highest larvicidal activity in comparison to petroleum ether extract of *B. racemosa*, and ethyl acetate extract of *B. racemosa* showed highest larvicidal activity in comparison to chloroform extract of *L. camara* against the mosquito vector *A. stephensi*. No mortality was observed in control. The result suggests the use of the plants in insect control as an alternative method for minimizing the noxious effect of some pesticide compounds on the environment. Thus, the extracts of whole plant of *L. camara* Linn. and leaf extracts of *B. racemosa* Lam. may deliver promising, more selective and biodegradable agents.

Key words: *Lantana camara*, *Bauhinia racemosa, Anopheles stephensi*.

INTRODUCTION

Mosquitoes are one of the most medicinally significant vectors and they transmit parasites and pathogens which continue to have devastating impact on human beings (Maheswaran et al., 2008). Several numbers of species belong to genera *Anopheles, Culex, Aedes* and vectors for the pathogens of various diseases like malaria, filaria, Japanese encephalitis, dengue, and yellow fever. Thus, one of the approaches for control of these vector-borne diseases is the interruption of disease transmission by killing mosquitoes or preventing mosquito bites (Das, 1989). Herbal products which have proven potential as insecticides or replicants can play an important role in the interruption of the transmission of mosquito-borne diseases both at the individual and community level. However the discovery, development and use of synthetic organic insecticidal chemicals with persistent residual action not only over shadowed the use of herbal products as insecticides of choice against mosquitoes but also become the major weapon for mosquito control (Sakthivadivel and Daniel, 2008). The extensive use of synthetic organic insecticides during the last decade has resulted in environmental hazards and also in the development of physiological resistance in most vector species. This has necessitated the need for research and development of environmentally

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safe, biodegradable, low cost indigenous methods for vector control, which can be used with minimum care by individual and communities in specific situations (Singh et al., 2006). The plant *Lantana camara* Linn. (Verbenaceae) and *Bauhinia racemosa* Lam. (Caesalpiniaceae) are described in Ayurveda and Siddha, as a potent drug against a variety of ailments. These plants are widely distributed and cultivated in various parts of India (Yoganarasimhan, 2000; Varier, 2006; Prajapati et al., 2007; Ivan, 1999; Singh and Himadri, 2005; Raveendra and Martin, 2006).

MATERIALS AND METHODS

Collection of plant material

The plants were collected during flowering stage in the month of July to August from Nilgiris. Then, their identification was established with the aid of an expertise botanist and by making comparison with herbarium sheets of the authentic sample. Many of the defensive components are biodegradable, with non-residual effect on the biological environment hence; an attempt has been made in the present investigation to identify plants with the potential to control vector mosquitoes.

Extraction

The plant *L. camara* Linn. and *B. racemosa* Lam. were powdered and extracted in soxhlet, with petroleum ether, chloroform and ethyl acetate. The extracts were concentrated under reduced pressure to a semisolid mass. These extracts were used for determining the larvicidal activity against mosquito larvae.

Biological assay

Larvicidal activity was evaluated in accordance with World Health Organization (WHO) for the evaluation of new larvicidal agents (WHO, 1985). The larvae of *Anopheles stephensi* was obtained and reared from the neonates in National Institute of Communicable Diseases, Southern India branch field station located at Mettupalayam (District Coimbatore of Tamil Nadu State), at 28 ± 2°C with a photoperiod of 12 h light and dark, and 80 ± 10% relative humidity. A brewer's yeast powder mixed with an equal quantity (w/w) of ground dog biscuit was used in the laboratory as food for the larvae. The late third or early fourth instar larvae were collected according to larval size and the degree of chitinization of respiratory siphon (Cheng et al., 2003). Different concentrations of the extracts were prepared in 1 ml of acetone for each experiment. All experimental exposure was done in 500 ml glass beaker in triplicate. Twenty-five (25) larvae were collected with a pasture pipette, placed on a filter paper for removal of excess of water and placed in 250 ml dechlorinated tap water containing various concentration of crude extracts. Three controls in triplicate were setup, one with acetone (1 ml), the other with distilled water (250 ml).

The beakers were covered with muslin cloth to avoid entry of any foreign material. Sufficient control was also kept for each extracts. The observed mortality (crude mortality) was recorded at 24 h of exposure to test solution. From this crude mortality, percentage crude mortality was obtained. Subsequently, controlled mortality, if any, was recorded and percentage crude mortality was obtained. The percentage crude mortality was corrected by using Abbot's formula. The corrected probit mortality and expected mortality was also obtained but no control mortality was recorded during the experiment, so Abbot's formula was not used.

Statistical analysis

Lethal concentration (LC_{50} and LC_{90}) values and their 95% confidence limits were estimated by fitting a probit regression model to the observed relationship between percentage mortality of larvae and logarithmic concentration of the substance. Separate probit models were fitted for each extract (Finnely, 1971). All analysis was carried out using the statistical package for social sciences (SPSS) software, version 13.0.

RESULTS AND DISCUSSION

Seven different concentrations of test solution ranging from 50 to 350 ppm for petroleum ether extract and six concentrations of test solution ranging from 50 to 300 ppm for chloroform extract for *L. camara* Linn. and six different concentrations of test solution ranging from 50 to 300 ppm for petroleum ether extract and five different concentrations of test solution ranging from 40 to 200 ppm for ethyl acetate extract were subjected to 24 h bioassay, using late 3rd or early 4th instar larvae of *A. stephensi.* Based on observations made in the 24 h bioassay studies among the different plant extracts, the petroleum ether extract of *L. camara* Linn. was more potent than petroleum ether extract of *B. racemosa* Lam. and ethyl acetate extract of *B. racemosa* was more potent than chloroform extract of *L. camara* Linn. for vector mosquito and were identified as efficient against them. The results from the *A. stephensi* larvicidal bioassay using different extracts of two different plants, the most active extract against late third or early fourth instar larvae of *A. stephensi*, were the petroleum ether extract of *L. camara* and Ethyl acetate extract of *B. racemosa*. The results of susceptibility of larvae for the extracts were given in Tables 1 and 2; and Figures 1 and 2.

Conclusion

The use of the plants in insect control offers a safer alternative to synthetic chemicals and can be obtained by individuals and communities easily at a very low cost. Moreover, these results could be useful in the search for newer, more selective and biodegradable larvicidal natural compounds.

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The authors are thankful to the J.S.S. College of Pharmacy, Ooty, Tamil Nadu and National Institute of

Table 1. Observed and expected mortality of *Anopheles stephensi* larvae exposed to *Lantana camara* with petroleum ether and chloroform extracts. Expected mortality is based on probit regression analysis.

Concentration (µg/ml)	No. of Larvae		Mortality (%)		Expected mortality			Probit (mortality) = a + b × concentration	χ^2, DF, P-value	LC_{50} (95% CI)	LC_{90} (95% CI)
	Exposed	Dead	Crude	Corrected	Probit	Dead	%				
Petroleum extract											
50	75	17	22.7	22.7	-0.80	15.8	21.1	-1.3270+0.0105×conc.	χ^2=1.56, DF=5, P=0.9	126.7 (112.0-139.7)	248.9 (231.2-271.8)
100	75	30	40.0	40.0	-0.28	29.3	39.1				
150	75	44	58.7	58.7	0.25	44.8	59.8				
200	75	56	74.7	74.7	0.77	58.5	78.0				
250	75	67	89.3	89.3	1.30	67.7	90.3				
300	75	73	97.3	97.3	1.82	72.4	96.6				
350	75	75	100.0	100.0	2.35	74.3	99.1				
Chloroform extract											
50	75	8	10.7	10.7	-1.95	1.9	2.6	-2.7408+0.0158×conc.	χ^2=39.9, DF=4, P<0.005	173.8 (120.2-235.3)	255.0 (205.5-435.0)
100	75	9	12.0	12.0	-1.16	9.2	12.3				
150	75	13	17.3	17.3	-0.37	26.7	35.5				
200	75	44	58.7	58.7	0.42	49.7	66.2				
250	75	73	97.3	97.3	1.21	66.5	88.7				
300	75	75	100.0	100.0	2.00	73.3	97.7				

DF = degrees of freedom, conc. = concentration, CI = confidence interval.

Table 2. Observed and expected mortality of *Anopheles stephensi* larvae exposed to *Bauhinia racemosa* with petroleum ether and ethyl acetate extracts. Expected mortality is based on probit regression analysis.

Concentration (µg/ml)	No. of Larvae		Mortality (%)		Expected Mortality			Probit (mortality) = a + b x concentration	χ^2, DF, P-value	LC_{50} (95% CI)	LC_{90} (95% CI)
	Exposed	Dead	Crude	Corrected	Probit	Dead	%				
Petroleum ether extract											
50	75	11	14.7	14.7	-1.21	8.4	11.2	-1.8238+0.0122×conc.	χ^2 = 9.7, DF=4, P=0.046	149.3 (121.2-175.3)	254.3 (220.5-316.0)
100	75	24	32.0	32.0	-0.60	20.5	27.3				
150	75	31	41.3	41.3	0.01	37.7	50.2				
200	75	49	65.3	65.3	0.62	54.8	73.1				
250	75	69	92.0	92.0	1.23	66.7	89.0				

Table 2. Conted

300	75	75	100.0	100.0	1.84	72.5	96.7				
						Ethyl acetate extract					
40	75	12	16.0	16.0	-1.14	9.5	12.7				
80	75	25	33.3	33.3	-0.43	25.1	33.5				
120	75	42	56.0	56.0	0.28	45.9	61.2	-1.8513+0.0178×conc.	χ^2 =5.9, DF=3, P=0.11	104.0 (95.2-112.5)	176.1 (163.3-193.1)
160	75	60	80.0	80.0	1.00	63.0	84.1				
200	75	75	100.0	100.0	1.71	71.7	95.6				

DF = degrees of freedom, conc. = concentration, CI = confidence interval.

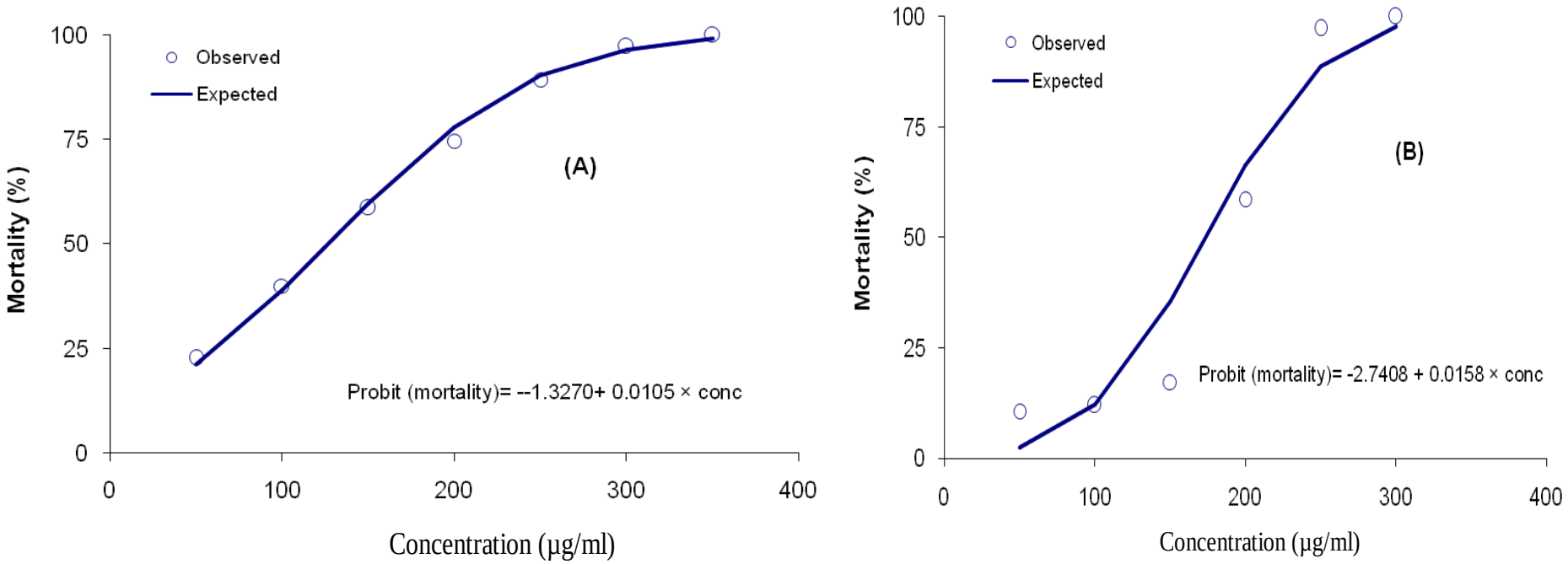

Figure 1. Relationship between *Anopheles stephensi* larval mortality and concentration of *Lantana camara* with (A) petroleum ether and (B) chloroform extracts. Expected values are based on probit regression analysis. Conc = concentration.

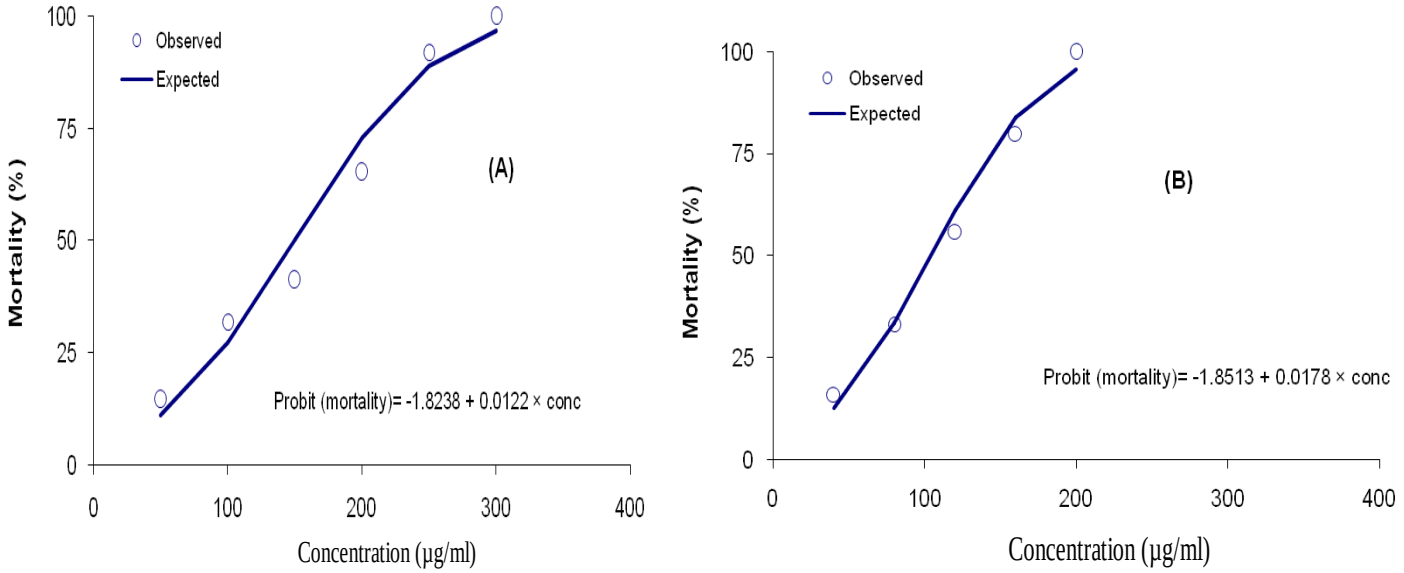

Figure 2. Relationship between *Anopheles stephensi* larval mortality and concentration of *Bauhinia racemosa* with (A) petroleum ether and (B) ethyl acetate extracts. Expected values are based on probit regression analysis. Conc = concentration.

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