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DETOXIFICATION ENZYMES ACTIVITIES IN DELTAMETHRIN AND BENDIOCARB RESISTANT AND SUSCEPTIBLE MALARIAL VECTORS (*Anopheles gambiae)* BREEDING IN BICHI AGRICULTURAL AND RESIDENTIAL SITES, KANO STATE, NIGERIA

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ABSTRACT
Insecticide resistance is an important impediment to malaria control effort. Knowledge of insecticides resistance status is an essential tools to governmental, nongovernmental and insecticides producing industries whose daily challenge is minimization of malaria burden across the globe. Larvae of Anopheles gambiae collected from residential and agricultural areas of Bichi LGA, Kano States, North west Nigeria were reared to adults. Resistance and susceptibility status in the adults mosquitoes were studied by WHO paper bioassay impregnated with diagnostic dose of Deltamethrin and Bendiocarb. The insecticides resistance and susceptible adult mosquitoes of Bichi; residential areas (BR) and agricultural areas (BA) were respectively designated as BRr, BRs, BAr and BAs. Specific activities of insecticides detoxifying enzymes glutathione S transferase (GST), esterase and monooxygenase in resistant and susceptible vectors were measured using standard WHO methods. Although the levels of resistance varied with the insecticides and breeding site, high resistance status of malaria vectors to deltamethrin was recorded in both study sites (> 80% mortality) and incipient resistance (Tolerance) to bendiocarb (>97% mortality) based on WHO results interpretation. Significantly elevated (P<0.05) activities of GST, esterase and monooxygenase were recorded in deltamethrin and bendiocarb resistant strains compared to susceptible species in both BA and BR. The finding of the study established high resistance status to deltamethrin and incipient resistance to bendiocarb in all the study sites which could be linked to indiscriminate use of insecticides in residential sites against malarial vector and other flying insects and agrochemicals for pest control in the agricultural sites. Based on this finding, it may be concluded that insecticides resistance to malaria vector exists in both residential and agricultural areas and measures should be taken to curtail it.
Keywords: insecticides (deltamethrin and bendiocarb) resistance, Bioassay, Malarial vector, detoxification enzymes.

INTRODUCTION

Despite sequential and cumulative efforts to fight the disease, malaria still remains among top three most deadly diseases in the world and the highest most deadly in tropical region (Sachs and Malaney, 2002). An estimated 128 million people are infected with *P. falciparum* in sub-Saharan Africa at any one time. 90% of infections occur in sub-Saharan Africa; 37 million infections (29%) arose from Nigeria (WHO, 2014). *Anopheles gambiae* is the widely anophelene distributed in sub Saharan Africa and is the major malaria vector in Nigeria (Okowa *et al.,* 2009). Efforts at eradicating malaria in Africa have been frustrated by resistance of parasite to antimalarial drugs, lack of alternative and effective antimalarial drugs that are acceptable, affordable and available due to epileptic economic growth and lack of political will bedeviling the region. This necessitates the introduction of vector control measure to serve as the most effective means for limiting the disease transmission (Hemigway *et al*., 2006). Effective vector control largely relies on the use of insecticides to target adults or larvae (Killeen *et al.,* 2002). The World Health Organisation (WHO) approved synthetic pyretheroids as the only insecticides for bed nets while DDT and carbamate are used for indoor residual spray (IRS) in many African countries (Akogbeto *et al*., 2010., Protopopoff *et al.,* 2013). Unfortunately, resistance of mosquitoes against insecticides began to emerge and currently represents the major threat to malaria control program worldwide (Hemigway and Ranson, 2000). The resistance describes the situation in which the vectors are no longer killed by the standard dose of insecticides or manage to avoid coming into contact with the insecticide (WHO, 2012). It is accomplished through any or combination of; increased metabolism of toxic compounds, decreased target site sensitivity, decreased rate of insecticides penetration and increased rate of insecticides excretion but the two most important mechanisms are metabolic or enzymes based detoxification and target site insensitivity (David *et al*., 2013., Nkya *et al*., 2013).

Agricultural fields provide favorable condition for vector breeding and agricultural spray may be accountable for the evolution of insecticide resistant vectors. Several evidence implicated the widespread agricultural use of broad spectrum insecticides to resistance in malaria vectors (Diabete *et al.,* 2002). Genetic mutation at insecticide target site acetylcholinesterase, voltage gated sodium ion channel and ligand gated GABA receptors result in the loss of insecticides sensitivity. Mutation at acetylcholinesterase insecticide binding site causes reduce sensitivity to organophosphate and carbamate in *Drosophila melanogaster* (Mutero *et al.,* 1994) and in *Anopheles gambiae* and *Culex pipiens* (Weill *et al.,* 2002). The pyretheroids and DDT resistance is due to alteration in genetic composition at voltage gated sodium ion channel protein in many insect species (Soderland and Knipple, 2003) including *Anopheles* species (Martinez torres *et al.,* 1998).

Metabolic based resistance is as a result of high expression of genes that code for insecticides detoxifying enzymes mainly GST, esterase and monooxygenase thereby transforming the insecticides into harmless form before reaching their respective target sites. The upregulation of genes responsible for the synthesis of these enzymes in response to xenobiotic exposure have been well documented in many organisms (Misra *et al.,* 2011). Angosin and Dinamar (1963) reported the first case of enzyme induction in which elevated activity of NAD Kinase was observed in *Triatoma infestans* after exposure to DDT. The GSTs metabolize insecticides through reductive dehydrochlorination or by conjugation reaction with reduced glutathione to produce water soluble and readily excretable products. Genomic study of *Anopheles gambiae* and *Drosophila melanogaster* revealed numerous enzyme families of GSTs (Enayati *et al.,* 2005). Cytochome P_{450} monooxygenase metabolises insecticides through O, S and N- alkylhydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation and nitrogen and thioester oxidation. Insect cytochrome P_{450} monooxygenase metabolizes exogenous compounds including insecticides and plant toxins leading to insecticides resistance (Wen *et al.,* 2003) and high tolerance to plant toxins (Li *et al.,* 2002; Wen *et al.,* 2003). Esterase detoxifies organophosphate, carbamates and synthetic pyretheroids through hydrolysis of ester bond and binding of insecticides to the active site of esterase (Crow *et al.,* 2007). This research work was aimed at evaluating the deltamethrin and bendiocarb resistance status and enzyme profile of malarial vectors breeding in residential and agricultural areas of Bichi Local Government in Kano State, Nigeria.

MATERIALS AND METHODS

Materials

All reagents used are of analytical grade obtained from BDH, spectrafuge by Labnet 24d and micro plate reader by Nortek Genesis – MR 6000 were used for the study.

STUDY AREA

Bichi Local Government area is located in Kano State, Nigeria which is ranked second in population with about 9.0 million people and lies between latitude 1.1 30 and 11.5 and longitude 8 30 and 8.5 E.

Larval collection and rearing

The larvae collected from different points in both residential sites (BR) and agricultural sites (BA) in Bichi were reared to adult according to WHO (1998) standard proceedures.

WHO Bioassay

Mosquitoes insecticides diagnostic kit was used to establish susceptibility and resistant status using 0.05% deltamethrin and 0.1% bendiocarb impregnated paper according to WHO procedure (WHO, 1998). For each insecticide, mosquitoes were divided into batches of 20 – 25 per test and exposed to insecticides treated paper for 1hr. the effect of paper treated only with carrier oils were assayed in parallel as control. The knock down rate was recorded at every 10 minutes for 1hour before they were transferred back to the resting tubes for 24 hours when percentage mortality was recorded. Mortality rate between 98 -100% indicate full susceptibility, 80 -97% indicate possible resistance and less than 80% is considered resistant to the tested insecticides.

Enzyme Analyses

Enzymes analyses were carried out using procedure outlined by WHO (1998). Individual mosquitoes were analyzed for protein, esterase, GST and monooxygenase. The mosquitoes were individually homogenized using glass rod in 150µl ice cold distilled water and homogenate was centrifuged at 13000g for two minutes. To obtain specific activities of enzyme, the protein concentration of individual homogenate was determined by the method of Bradford (1976) using Coomasie blue reagent. A quantity of each homogenate (10µl) was mixed with 200µl of Coomasie blue reagent and absorbance was read at 630nm after five minutes incubation.

Esterase assay

Esterase was determined by sprctrophotometric method (Faiz *et al*., 2007). The enzyme hydrolyszes paranitrophenylacetate to acetate and a yellow colour product paranitrophenol which maximally absorbs light at 405nm is formed. A quantity of ten microliter of each homogenate was mixed with 200µl of 1mM paranitrophenyl acetate working solution (100mM paranitrophenyl acetate : 50mM sodium phosphate buffer *pH* 7.4, 1:99) in a microtitre plate well. The absorbance was read at 405nm after ten minutes incubation. An extinction coefficient of $6.53 mM^{-}cm^{-}$ and a path length of 0.6cm was used to convert the absorbance to moles of product. Esterase specific activity was reported as µmolproduct/ min/ mg protein.

GST assay

Glutathione S transferase (GST) was determined following the method of Habig *et al*. (1974).The enzyme catalyses the conjugation of glutathione and chloro 2,4dinitrobenzene to form 2- chloro-4-nitrophenyl glutathione which absorbs light at 340nm.

A quantity of ten microliter of each homogenate was mixed with 200µl reduced glutathione (GSH/I-chloro - 2,4 dinitrobenzene working solution{95 parts of 10mM reduced glutathione in 100mM phosphate buffer *pH* 6.5 + 5 parts of 63 mM chloro-2,4 dinitrobenzene diluted in methanol} in a microtitre plate well. The absorbance was read at 340nm after 10 minutes incubation. An extinction coefficient 5.76mM^{-}cm and a path length of 0.6cm was used to convert absorbance to moles of product. Gst specific activity was reported as CDNB conjugated µmole product min^{-} mg^{-} protein.

Cytochrome P_{450} (Monooxygenase) assay

This was measured by the method of Borgdon *et al.* (1998). The monooxygenase catalyses the reduction of hydrogen peroxide and oxidation of tetramethylbenzedine to form water and oxidized blue color tetramethylbenzidine which absorbs light at 630nm. Twenty microliter of homogenate was mixed with 80µl of potassium phosphate buffer *pH* 7.2 +200µl of 6mM tetramethylbenzidene (TMBZ) working solution{(0.01g TMBZ was dissolved in 5ml methanol and then in 15ml of sodium acetate buffer *pH* 5.0) +25 µl of 3% v/v H_2O_2 solution} in a microtitre plate well. After two hours incubation at room temperature, the absorbance was read at 630nm. By using a standard curve of cytochrome C, a crude estimate of the amount of monooxygenase present was obtained and expressed as equivalent units of cytochrome P_{450}/mg protein

RESULTS

Figure 1and 2 show one hour knock down rate per 10min exposure and % mortality after 24 hrs post exposure period *of Anopheles* mosquitoes to WHO standard paper impregnated with diagnostic dose, deltermethrin (0.05%) and bendiocarb (0.1%) collected from Bichi residential and agricultural sites in Kano state, Nigeria. The percentage susceptibility to all the insecticides ranges from 19% to 96%.

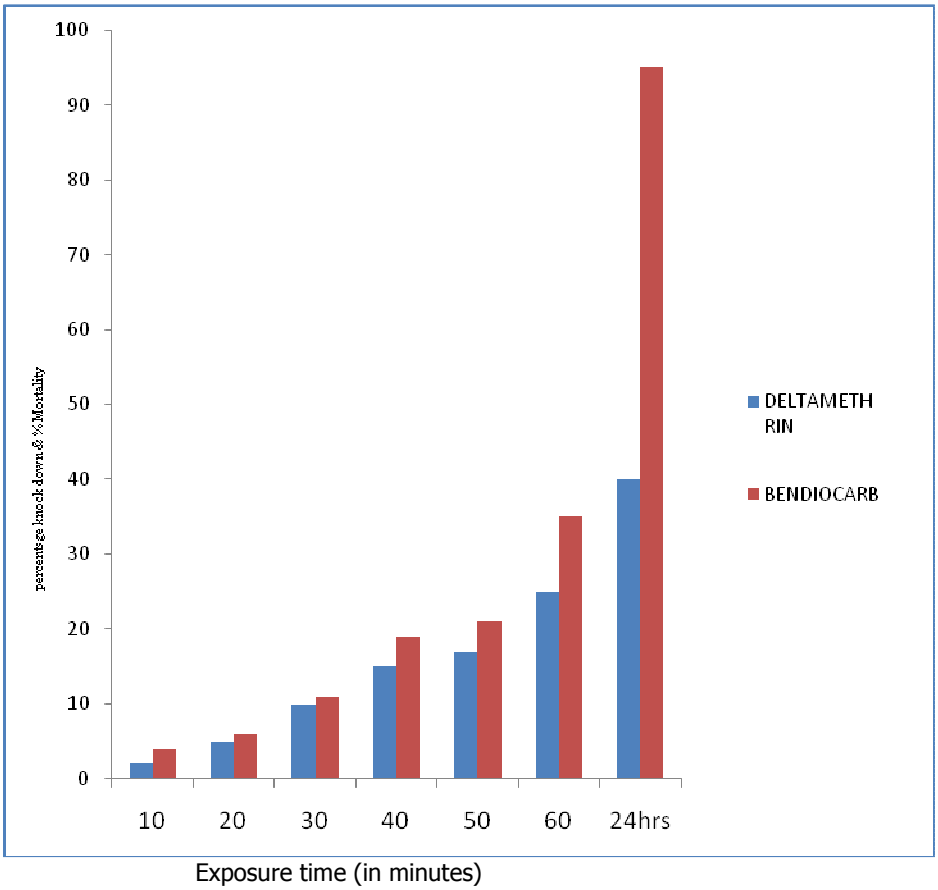

Figure 1: % knock down (10 – 60 mins) and % mortality (24hrs) of *Anopheles* mosquitoes bioassay to **deltamethrin 0.05% and bendiocarb 0.1%** collected from Bichi residential site

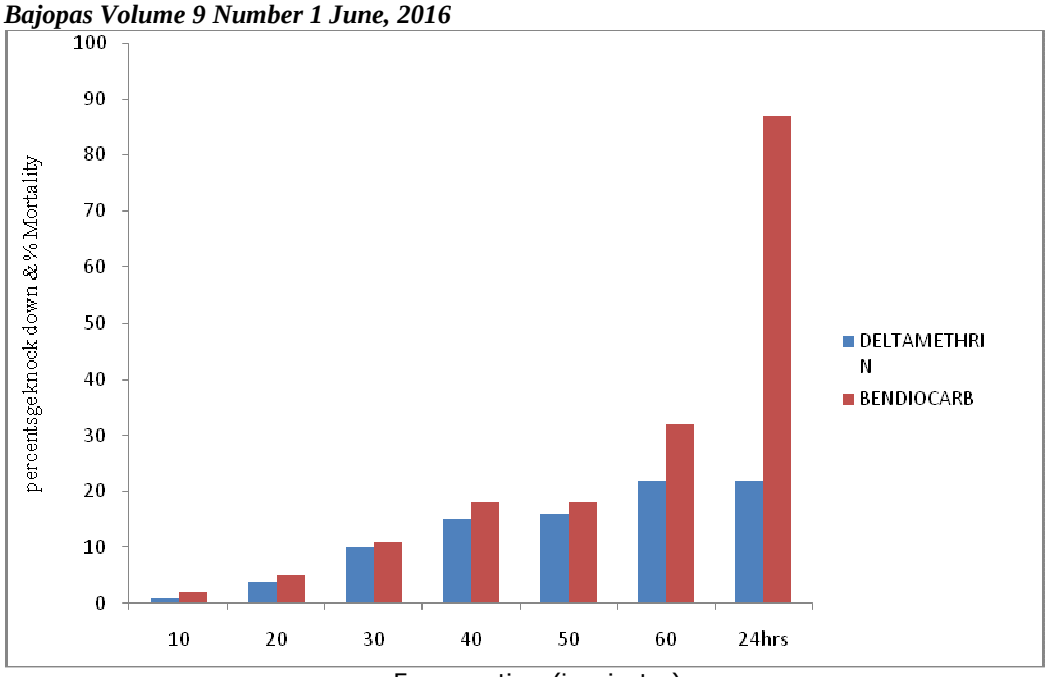

Figure 2: % knock down (10 – 60 mins) and % mortality (24hrs) of *Anopheles* mosquitoes bioassay to deltamethrin 0.05% and bendiocarb 0.1% collected from Bichi agricultural site

Table 1: GST, Esterase and Monooxygenase specific activities (mean ± SD) in *Anopheles* Mosquitoes tested with Deltamethrin collected from Bichi Agricultural and Residential Sites.

Group	No tested	GST (µmole/min/mg protein)	Esterase (umole/min/mgprotein)	Monoooxygenase (nmol/min/mg protein)
BRr	15	0.0127±0.0044^{d}	0.0433±0.0134^{d}	0.4993±0.1686^{d}
BRs	12	0.0091±0.0029^{d}	0.0213±0.0060^{d}	0.3213±0.1260^{d}
BAr	12	0.0305±0.0128	0.0464±0.0079^{l}	0.5597±0.1989^{k}
BAs	12	0.0314± 0.0069	0.0386 ± 0.0046^{l}	0.0434 ±0.0065^{k}

Values with similar superscript indicates significant difference (P<0.05) when the groups were compared

Key:
BRr: Bichi residential site resistant strain
BRs: Bichi residential site susceptible strain
BAr: Bichi agricultural site resistant strain
BAs: Bichi agricultural site susceptible strain

Table 2: GST, Esterase and Monooxygenase specific activities (mean ± SD) In *Anophles* Mosquitoes population tested with Bendiocarb collected from Bichi Residential and Agricuitural site.

Group	**No tested**	**GST (µmole/min/mg protein)**	**Esterase (umole/min/mg protein)**	**Monoooxygenase (nmol/min/mgprotein)**
BRr	12	0.0387 ± 0.0049	0.0485±0.0066	0.5222±0.2695^{f}
BRs	12	0.0345 ± 0.0058	0.0489±0.0089	0.2898±0.0593^{f}
BAr	12	0.0329±0.0059	0.0377±0.0079	0.3922±0.0868^{l}
BAs	12	0.0339 ±0.0064	0.0403±0.0089	0.02830±0.0789^{l}

Values with similar superscript indicates significant difference (P<0.05) when the groups were compared

BRr: Resistant strain from Bichi residential site
BRs: Suceptible strain from Bichi residential site
BAr: Resistant strain from Bichi irrigation site
BAs: Suceptible strain from Bichi Irrigation site

DISCUSSION

This research work revealed high level of resistance to deltamethrin and possible resistance to bendiocarb in *Anopheles gambiae* (Fig 1and2) being the major malaria vector in Bichi, Nigeria, collected from both residential and agricultural sites. This may be associated with increased use of pyretheroids treated bed net and carbamate for indoor residual spray (IRS) in public health control and agricultural application to control pest. Other chemicals sharing the same target site of action with test insecticides may also account or worsen the level of insecticides resistance.

Many studies demonstrated that exposure to environmental xenobiotics result in acquired tolerance to several insecticides with similar mechanisms of action prior to insecticides exposure. *Aedes aegypti* larvae acquired tolerance to organophosphate after been frequently exposed to herbicides atrazine (Boyer et al.,2006). Similarly exposure of *Aedes albopictus* larvae to benzothiozole and pentacholorophenol increased their tolerance to insecticides in many insects and induction of detoxification enzymes as a result of prior exposure to environmental xenobiotics (Feyereisen, 2005., Namotoungou et al, 2012). Cross resistance between DDT and pyretheroids has been established in *Anopheles gambiae* (Hemigway and Ranson, 2000). The result of this study (Figures 1 and 2) show that the deltamethrin resistance status is comparatively higher in agricultural field, this is in consistence with that of several studies from far and near geographical location of the world which implicated agricultural spray as a growing factor in the development of insecticides resistance in various insects species. High resistance status to pyrtetheroids deltamehrin in this study corroborate that of Grant *et al.* (1989). The intermediate resistance to bendiocarb found in this study may be as a result of increased utilization of carbamate in agricultural fields, IRS or as a result of cross resistance to other pesticides applied to target pests. Aikpon *et al*. (2013) repoted bendiocarb resistance in *Anopheles gambiae* population in Nigeria neighbouring country Benin. In Nigeria resistance of *Anopheles gambiae* to common classes of insecticides is well documented (Awolola et al., 2002., Oduola et al., 2012) but little is known until recently regarding carbamate resistance particularly in northern region. This study agrees with that of Oduola *et al.* (2012) who reported carbamate resistance in *Anopheles gambiae* ss resistant to DDT and pyretheroids in Lagos and that of Alhassan *et al*. (2015) who reported 96% and 80% mortality due to bendiocarb exposure in residential and irrigation areas respectively in DDT resistant *Anopheles gambiae* of Auyo town northwest Nigeria. The finding of this work is similar to that of Elissa *et al.*(1993) who reported pyretheroids resistance from Cote d'ivoire and carbamate resistance in 1990. Pyretheriods resistance occurs in many insects other than mosquito such as housefly and cockroach (Awolola *et al.,* 2002, Jirakanjanaki *et al*., 2007). Insecticides resistance is not confined to Africa alone as it was reported from Asia too. DDT resistance in *Anopheles culicifaeces* was reported in India (Dash *et al.,* 2006), resistance to permethrin and cypermethrin was reported in north eastern Thailand (Pimsamurna *et al*., 2009) and resistance to DDT and deltamethrin was reported in China.

Induction of detoxification enzymes in response to xenobiotic exposure have been well documented in many insects (David *et al*., 2013). The elevated activity of GST (Table 1) in deltamethrin resistant strain of Bichi residential site suggests direct or indirect involvement of the enzyme in conferring resistance against deltamethrin. GST role in detoxification of pyretheroids has been basically attributed to its capacity to reduce the peroxidative damage induced by pyretheroids, mainly by detoxifying lipid peroxidation products (Vontas *et al*., 2001). The elevated activity of esterase (Table 1) in both residential and agricultural areas may be the first line of defense against deltamethrin exposure while elevated GST and monooxygenase may be as a result of increased production of esterase metabolic bye product. Esterases have been found over transcribed in *Aedes aegypti* pyretheroids resistant strains (Strode *et al*., 2008) and in response to xenobiotic exposure (Raiz *et al*., 2009). Following the development of transcriptomic tools in mosquitoes (David, *et al.*, 2005., Strode *et al*., 2008) several cytochrome P_{450} over transcribed in pyretheroids resistant mosquitoes were identified (Nkya *et al.*, 2013., David *et al*., 2013). Study showed that cytp6z8 are likely to play a vital role in the clearance of pyretheroids insecticides via further catabolism of pyretheroids derivatives generated by the activity of carboxyl esterase (Alexia *et al*., 2013). The increase in esterase activity in resistant strains corroborates the finding of Desfintianes *et al.* (1989), who reported elevated level of GST and esterase activities in Duala town, Cameroun, where coils and mats containing pyretheroids were extremely used for crop protection and against mosquito bite. A linear relationship between high esterase activity and pyretheroids resistance has been established in insect other than mosquitoes (Jao and Canda, 1974; Jingli and Kun, 1988).

Deltamethrin resistance may not necessarily be metabolic, mutation at sodium ion channel the target site of pyretheroids may also account for it. Leu-phe mutation at sodium ion channel has been extensively documented from West African population of *Anopheles gambiae* (Diabete *et al.,* 2004; Awolola *et al.,* 2007). Induction of GST gene have been reported not only after exposure to organophosphate and organochloride but also against pyretheroids (Yu and Nguyen, 1996; Kostrapoulos *et al., 2001).* The present study also found linear correlation between GST activity and deltamethrin resistance in the irrigation sites, which may be due to excessive agricultural spray. The correlation between high level of GST and high pyretheroids resistance in several insect species including mosquitoes have been reported (Grant *et al., 1989;* Reidy *et al., 1990).*

Studies of insecticides metabolism and resistance triggered the discovery of cytochrome P_{450} monooxygenase in many insects including mosquito. This study implicated the activity of monoxygenase with detoxification of bendiocarb (Table 2). Involvement of thiolate containing enzyme monooxygenase was supported by *in vitro* metabolism studies using various insecticides coupled with P_{450} inhibitors or inducers (Wen and Scott, 1997., Valles, 1998). Alhassan *et al*. (2015) suggests the role of monooxygenase and acetylcholinesterase in conferring resistance against bendiocarb in Anopheles mosquitoes of Auyo town Northwest, Nigeria. The results of the present study agrees with that of several studies which implicated agricultural activities as a selection factor in the development and emergence of insecticides resistance in various insects species.

Nkya *et al*. (2014) reported high transcriptional level in population sampled from agricultural areas suggesting that the intense use of agrochemicals represent a significant selection pressure for mosquito population

CONCLUSION

The study revealed high resistance status to detamethrin and intermediate resistance to bendiocarb in malarial vectors breed in Bichi residential and agricultural areas. This may be as a result of higher activities of detoxifying enzymes; GST, esterase and monooxygenase induced by frequent exposure of the vector to the insectides in the study area.

RECOMMENDATIONS

It is recommended that there is need to carryout molecular specie identification and each species of *Anopheles gambiae* be studied separately as resistant gene expression may likely vary between the specie.

Author's contribution

Safiyanu, M. participated in sample collection, design of the study and WHO bioassay. Alhassan, A.J. participated in biochemical analysis, data analysis and revising the manuscrifts critically. Abubakar, A.B participated in morphological species identification, data analysis and result interpretation.

Conflict of interest

There is no conflict of interest.

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