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Assessment of Acetylcholinesterase (AChE) from *Oreochromis mossambicus* (Cuvier, 1831) as a Source of Enzyme for Insecticides Detection

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ABSTRACT

In this work we assess the potential of acetylcholinesterase (AChE) from *Oreochromis mossambicus* (Toman) as a sensitive test for the presence of insecticides. The partial purification and characterization of a soluble AChE from *Oreochromis mossambicus* brain tissues using affinity chromatography gel (procainamide–Sephacryl S-1000) showed that the partially purified AChE was most active on acetylthiocholine (ATC) but had low activities on propionylthiocholine (PTC) and butyrylthiocholine (BTC), indicating that the partially purified fraction was predominantly AChE. Soluble AChE was partially purified 9.27-fold with a 91.12% yield. The partially purified AChE displayed the highest activity on ATC at pH 7 and at 30°C using 0.1 M Tris buffer. The enzyme exhibited Michaelis-Menten kinetic constants, K_m, for ATC, BTC and PTC at 36, 77 and 250 µM, respectively, and the maximum velocities, V_{max}, were 18.75, 0.12 and 0.05 µmol/min/mg protein, respectively. Moreover, the AChE from *Oreochromis mossambicus* presented comparable sensitivity to carbamates and organophosphates insecticides than that from *Electrophorus electricus* and many other fish AChE by comparing half maximal inhibitory concentration values. Therefore, the enzyme is a valuable source for insecticides detection in Malaysian waters at lower cost.

INTRODUCTION

The extensive use of organophosphate (OP) and carbamate insecticides is a concern due to the neurotoxicity properties of the compounds [1]. These compounds inhibit the activities of important enzymes, such as cholinesterases, which are needed for functional nervous systems [2,3]. The rapid increase in production and use of organophosphorus (OP) and carbamate pesticides has raised concerns about their potential to cause harm to human and non-target wildlife populations. Pesticides enter waterways from agricultural and urban run-off, movement through soil into water courses and after direct application [4]. Aquatic organisms have been widely used as biomarkers to detect various pesticides and toxicants, which inhibit activities of cholinesterases [5–7]. An example of the effect of pesticides on fish has been tested on a snakehead fish, *Channa striata* [8]. Similar types of fish that are natives to Malaysian waters, such as tiger grouper (*Epinephelus fuscoguttatus*), Javanese carp (*Puntius gonionotu*) and grass carp (*Ctenopharyngodon idella*) [9–11] are therefore potentially useful as biomarker agents for pesticides or insecticides. Mussels [12] have been also employed to detect the presence of pollutants by linking it to the inhibition of cholinesterase activities.

Acetylcholinesterase (AChE) enzyme is regarded as a biomarker in evaluating the effects of pollutants and environmental monitoring [13]. Two classes of cholinesterase, i.e. acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), can be distinguished functionally, primarily on the basis of substrate specificity [3]. AChE hydrolyzed acetylcholine much faster than other choline esters and is

inactive on butyrylcholine. BChE, on the other hand, can hydrolyze both butyrylcholine and acetylcholine although at a much lower rate [14,15]. The role of acetylcholinesterase is the hydrolysis of acetylcholine into choline and acetic acid, acetylcholine as a neurotransmitter and therefore responsible for the normal neural functioning of sensory, therapeutic, and muscular systems. In fish, acetylcholinesterase inhibition resulted in difficulty in respiration, feeding and swimming [16]. Inhibition of the esterase activities resulted in the accumulation of acetylcholine in the synapses and too much stimulation of muscarinic and nicotinic receptors [17].

Additional levels of acetylcholine over stimulate the muscarinic and nicotinic receptors in the central and peripheral nervous systems and also the neurotransmitter junctions, resulting in various signs of poisoning which include body temperature fluctuations, changes in the heart rate, blood pressure, muscle twitching, and tremors. Sometimes dead may occur due to the cessation of respiration as a result of the effects of anticholinesterase activity in both the central and peripheral nervous systems [17]. Many pollutants cause changes in the activity of acetylcholinesterase in fish [18]. Nevertheless, this enzyme is easily inhibited by these insecticides, providing a convenient and rapid means of monitoring the presence of pollutants in the environment.

A previous *in vivo* work on a snakehead fish has shown that the brain cholinesterase activity from the organism is very sensitive to insecticides [19] and hence can be a replacement for the expensive *Electrophorus electricus*, which is commonly used as a biosensor for the detection of insecticide [6]. The main aim of this work is to assess the sensitivity of AChE from *Oreochromis niloticus* on carbamates and organophosphates assay *in vitro*. The sensitivity of AChE from *Oreochromis niloticus* in comparison of AChE from *Electrophorus electricus* will be then evaluated. This work proves that AChE from *Oreochromis niloticus* has the potential to be a cheaper and local source of AChE for detection of insecticides in the tropics.

Materials and Methods

Chemicals

Carbofuran, methomyl, carbaryl, parathion, malathion, diazinon, bendiocarb, chlorpyrifos, acephate, dimethoate and trichlorfon were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Bromine, acetylthiocholine iodide (ATC), propionylthiocholine chloride (PTC), β-mercaptoethanol, procainamide hydrochloride, 1,4-butanediol diglycidyl ether and sodium borohydride were purchased from Sigma-Aldrich. 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and butyrylthiocholine iodide (BTC) were purchased from Fluka Chemie GmbH.

Commercial AChE preparation from eel (*E. electricus*, 349 units/mg solid) was purchased from Sigma (St. Louis, USA). Biorad Protein Assay was purchased from Bio-Rad Laboratories Inc. Vivaspin4 was from Vivascience. All other chemicals used in this study were of the analytical or special grade.

Specimen

Oreochromis niloticus was used as the fresh water test organisms in this study. The fish, weighing 900-1200 g and measuring approximately 36 cm in length, were obtained from Snoc International Sdn Bhd, Selangor, Malaysia. The fish was killed by decapitation, and the whole brain was dissected out immediately. Approximately one gram of brain was homogenized in 20% (w/v) of 0.1 M sodium phosphate buffer pH 8.0 using an Ultra-Turrax T25 homogenizer fitted with a Teflon pestle. Phenylmethylsulfonyl fluoride was used to inactivate and remove unwanted serine proteases. The brain suspension was homogenized, and the crude extract was centrifuged at 15,000×g for 10 minutes at 4ºC to remove debris. The homogenate was subsequently centrifuged at 100,000×g in a Sorval® Ultra Pro 80-TH-641 for an hour at 4ºC to separate the cytosol and membrane components. The supernatant `was used in the next purification procedures [3,6].

Preparation of Affinity Chromatography Columns

Epoxy (Bisoxirane) Activation

Affinity procainamide chromatography was prepared according to the modified method of Tham *et al.* [6]. Briefly, 100 mL of Sephacryl S-1000 (settled gel, Sigma, St. Louis, USA) was washed with 1 L of deionized water in a sintered glass tunnel, dried, and then transferred to a 500-ml beaker. The gel was suspended in 75 ml of 0.6 M NaOH containing 150 mg sodium borohydride and stirred. Approximately 75 ml of 1,4-butanediol diglycidyl ether was slowly added with constant stirring.

The mixture was left stirred at room temperature overnight. The activated gel was then thoroughly washed with water to remove excess reagent until there was no longer evidence of an oily film on the surface of the gel, representing the remaining epoxy compound. Acetone was used to aid in the complete removal of bisoxirane groups. The gel was resuspended in water for ligand coupling.

Ligand Coupling of Procainamide–Sephacryl S-1000 gel

The epoxy-activated Sephacryl S-1000 was washed with deionized water on a sintered glass filter. The gel slurry was transferred onto a coupling solution of 12 mM of borate buffer (pH 11.0) containing 0.2 M of procainamide. The pH was then adjusted to 12 by the addition of 1.0 M NaOH. The mixture was incubated at 25°C for 96 hours on a shaking incubator.

The gel was washed in sequence with 10 volumes each of 0.1 M sodium acetate (pH 4.5), 12 mM sodium borate (pH 10) and deionized water. The excess active groups on the gel were blocked by suspending the gel in 100 ml of 1.0 M ethanolamine (pH 9.0). The mixture was stirred at room temperature for 6 hours. Finally, the gel was washed thoroughly with 1 L of 1.0 M NaCl followed by 5 L of deionized water.

Screening of carbamates and organophosphates as AChE inhibitor

Carbamates and organophosphates were sourced from PESTANAL (Sigma-Aldrich International GmbH). OPs were activated prior to assaying according to the modified method of Villatte *et al.* [20]. The pesticide (25 µl) was incubated in 5 µl of 0.01 M pure bromine solution at room temperature for 20 minutes. 20 µl of 5% ethanol was added to stop the activation process. Preliminary experiments showed that bromine and ethanol at the given concentration did not inhibit AChE activities [21].

The half maximal inhibitory concentration (IC_{50}) was determined using at least five different concentrations of carbamate and OPs. The assay mixture contained 150 µl of potassium phosphate buffer (0.1 M, pH 8.0), DTNB (20 µl, 0.067 mM), carbamate (50 µl) and enzymes (10 µl). The mixture was incubated in the dark for 10 minutes at room temperature. ATC (20 µl, 0.5 mM) was subsequently added. The mixture was left to react at room temperature for 10 minutes before the absorbance was read at 405 nm [6].

RESULTS

The effect of insecticides on AChE activity

Screening of insecticides showed that all the carbamates and the OPs gave strong inhibition with no significant difference among them but carbofuran and carbaryl show closer to 100% inhibition to AChE activity. IC_{50}s for various insecticides of previous studies are shown in **Table 1** in comparison with another source of AChE. Non-overlap of confidence interval usually signifies significant difference at the $p<0.05$ level while overlapped interval does not make necessary means difference or no significant differences at the $p<0.05$ level. An overlapped confidence interval provides a general view that more data and experimentation are needed to assess non-significance (Schenker and Gentleman, 2001).

Based on this premise, the AChE from *Oreochromis mossambicus* showed comparable sensitivity to carbamates and organophosphates than that from *E. electricus*. The AChE from *Oreochromis mossambicus* was more sensitive to the carbamates carbaryl, and carbofuran than that from *E. electricus.* The latter was more sensitive to the carbamate bendiocarb while methomyl showed similar sensitivity towards both sources with an overlapped confidence interval. As for organophosphate insecticides, *Oreochromis mossambicus* was more sensitive to parathion and chlorpyrifos than *E. electricus* while the latter was more sensitive to malathion and diazinon while parathion from *Channa micopeltes* showed similar sensitivity towards both sources with an overlapped confidence interval (**Table 1**).

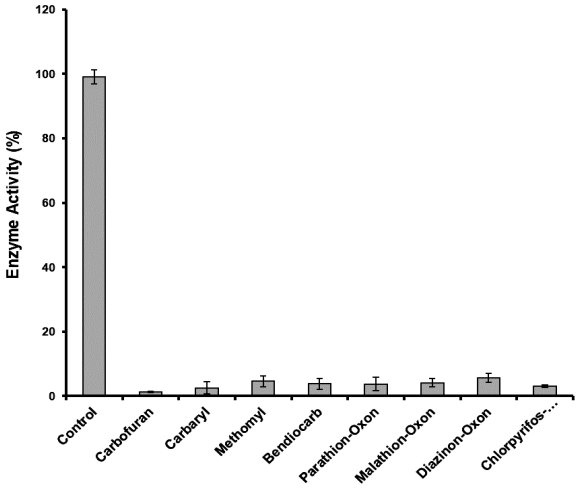

Fig. 1. Effect of various pesticides (1 mg/L) on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

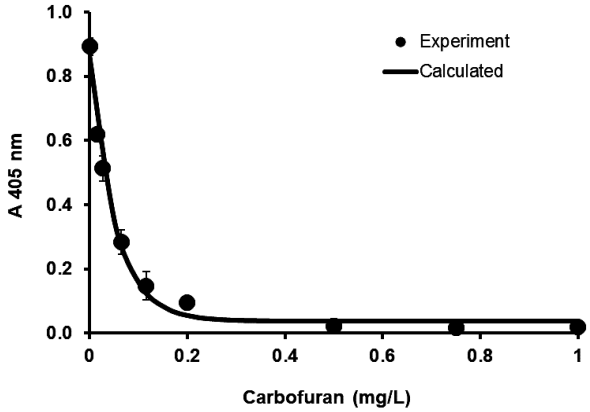

Fig. 2. Effect of carbofuran on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

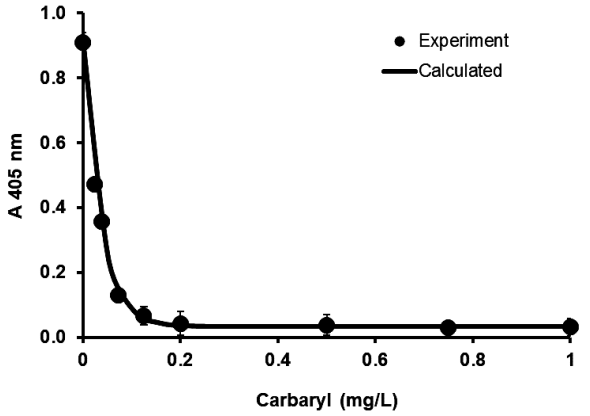

Fig. 3. Effect of carbaryl on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

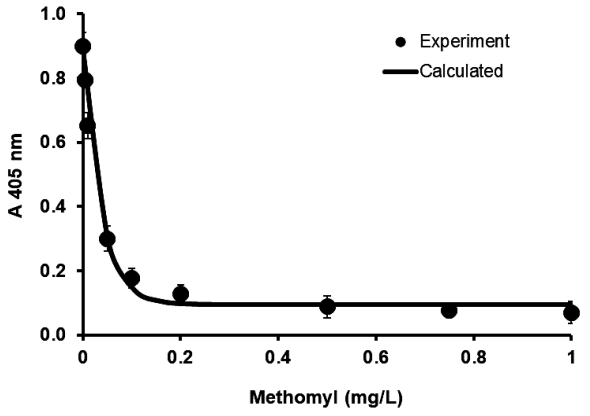

Fig. 4. Effect of methomyl on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

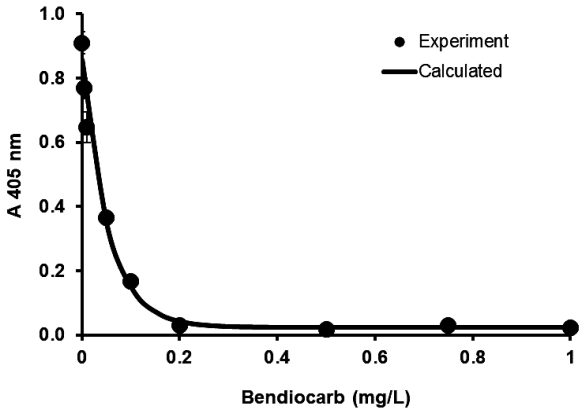

Fig. 5. Effect of bendiocarb on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

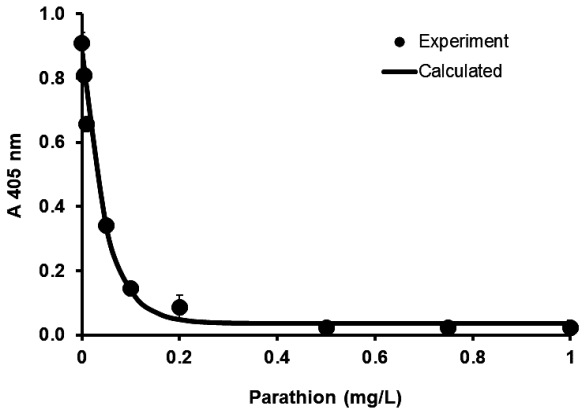

Fig. 6. Effect of parathion on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

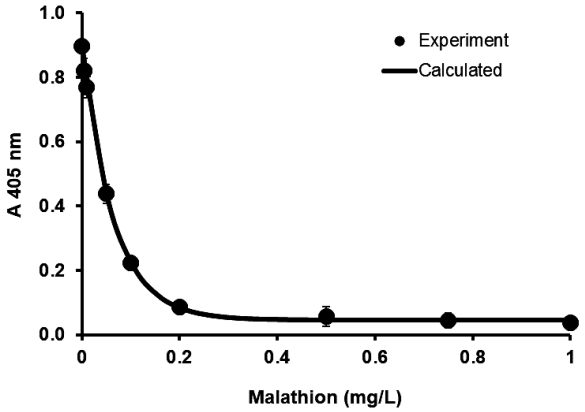

Fig. 7. Effect of malathion on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

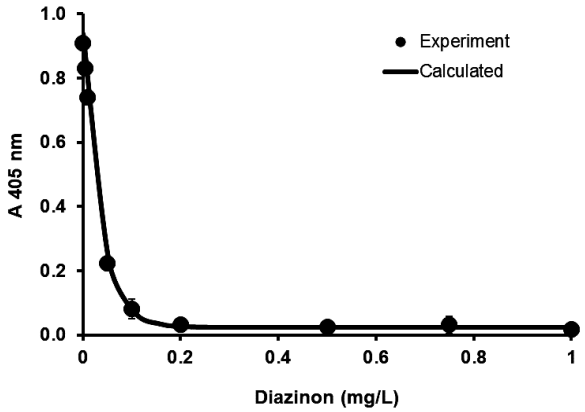

Fig. 8. Effect of diazinon on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

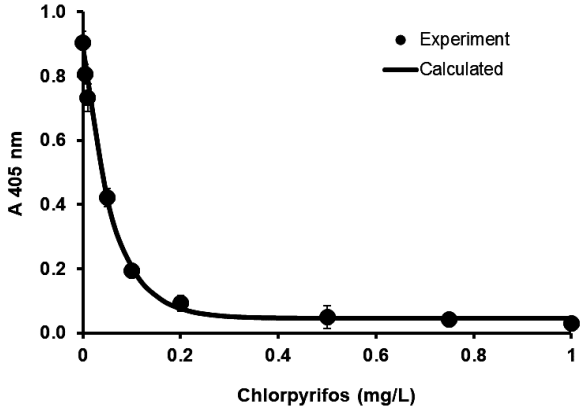

Fig. 9. Effect of chlorpyrifos on partially purified AChE from *Oreochromis mossambicus.* Data is mean± standard error (n=3).

Table 1. Comparisons of the sensitivity of *Oreochromis mossambicus* AChE to various insecticides in comparison to other fish AchEs.

	IC_{50} (mg/L) (95% Confidence Interval)								
Fish species	Carbof--uran	Carb-aryl	Meth-omyl	Bend-iocarb	Parathion-oxon	Malathion-oxon	Diazino n-oxon	Chlorp-yrifos-oxon	Author
Electrophorus electricus	0.0060 (0.0063-0.0065)	0.1330 (0.1220-0.1450)	0.0260 (0.0240-0.0280)	0.0150 (0.0150-0.0160)	0.0680 (0.0660-0.0690)	0.0140 (0.0130-0.0140)	0.1770 (0.1690-0.1860)	0.0600 (0.0550-0.0650)	[22]
Periophtal-modon schlosseri	0.0450 (0.0399-0.0517)	0.1124 (0.1025-0.1245)	0.0567 (0.0504-0.0648)	0.0633 (0.0537-0.0773)	Not done	Not done	Not done	Not done	[23]
Lates calcarifer	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	nil
Osteochilus hasselti	0.0550 (0.0515-0.0670)	0.0497 (0.0414-0.0620)	0.0845 (0.0747-0.0973)	0.0470 (0.0409-0.0553)	0.0660 (0.0580-0.0766)	0.0681 (0.0592-0.0802)	0.0991 (0.0906-0.1094)	0.0632 (0.0570-0.0709)	[22]
Pangasius sp.	0.006 (0.0058-0.0065)	0.061 (0.043-0.105)	0.016 (0.015-0.017)	0.012 (0.011-0.013)	0.047 (0.041-0.055)	0.011 (0.008-0.015)	0.081 (0.074-0.089)	0.029 (0.023-0.039)	[24]
Channa micropeltes (Toman)	0.0081 (0.0074-0.0089)	0.07922 (0.0697-0.0917)	0.0192 (0.0178-0.0208)	0.0379 (0.0341-0.0427)	0.0316 (0.0279-0.0363)	0.0242 (0.0192-0.0327)	0.0599 (0.0554-0.0652)	0.0522 (0.0418-0.0693)	[25]
Clarias batrachus	0.006 (0.006-0.008)	0.130 (0.0012-0.0014)	Not done	Not done	Not done	Not done	Not done	Not done	[6]
Tor tambroides	0.0643 (0.0482 - 0.0966)	0.0555 (0.0439 - 0.0754)	0.0817 (0.0571-0.1438)	0.0758 (0.05815-0.1090)	Not done	Not done	Not done	Not done	[26]
Puntius schwanenfeldii	1.411	7.045	8.335	0.838	Not done	Not done	Not done	Not done	[27]
Puntius javanicus	0.035 (0.030-0.045)	0.031 (0.026 - 0.040)	0.090 (0.077 - 0.108)	0.045 (0.039 - 0.054)	0.151 (0.122 - 0.198)	0.063 (0.053 - 0.078)	0.103 (0.084 - 0.132)	0.202 (0.178 - 0.232)	[28]
Present study	0.03605 (0.03201-0.04127)	0.02482 (0.02305-0.02687)	0.02482 (0.02189-0.03052)	0.03658 (0.03194-0.04280)	0.03272 (0.02894-0.03765)	0.04486 (0.04145-0.04888)	0.02455 (0.02238-0.02720)	0.04168 (0.03774-0.04654)	

Note: All values have a correlation coefficient value of at least 0.95.

DISCUSSIONS

Oreochromis mossambicus is highly prized for its medicinal properties in healing wounds of internal organs and is widely found in Malaysian fresh water aquatic bodies [29]. The results from this study will be useful for comparison purposes on other local fish species and as a precursor for the development of an assay for insecticide pollutants. The purification method that uses a custom made procainamide based affinity gel is an efficient partial purification technique that has been employed elsewhere [6,30,31].

The insecticides screening results showed that the AChE from this organism could be further developed into a sensitive inhibitive assay for insecticides. The bromine oxidation technique in this work was adequate to fully oxidize the organophosphates. However, oxonation using bromine is limited to OP compounds that require oxidative desulfuration for activation. OP compounds that are oxygen analogue in the active form are activated by other procedures [20].

Between the CBs, carbofuran is a comprehensive range systemic insecticide which is commonly used throughout the world. Carbofuran has been detected in ground, surface, and rain water due to its widespread use [32].The use of carbofuran is limited to oil palm plantation but is broadly applied in a paddy field, vegetables and fruits in Malaysia making their detection important [33]. Begum *et al*. [4] reported the toxic effect of carbofuran on freshwater teleost (*Clarias batrachus*), the findings explain that, toxicity increases due to the increase in carbofuran concentration and time of exposure. When exposed *in vivo,* each of these insecticides is toxic to fish, and the mechanism of inhibition is probably through inhibiting the functions of cholinesterases [34]. For example, diazinon is toxic to *Oncorhynchus mykiss* (rainbow trout), *Poecilia reticulata* (guppy), *Brachydanio rerio* (zebra fish) and *Cyprinus carpio* (carp), and guppy exhibited the strongest toxicity due to it having the highest rate of bioactivation of diazinon [35–37].

Carbaryl is toxic to fish as it causes changes in both physiology as well as the behaviour of the fish and mostly sprayed towards the period of crop harvesting. Its exposure causes significant inhibition of cholinesterase in the muscle of rainbow trout (*Oncorhynchus mykiss*) [37]. Exposure to methomyl pesticide at different concentrations resulted to mortalities and the maximum concentration of 10 ppm for 96 hours causes 100% mortality in *Oreochromis niloticus,* it is also indicated that mortality increases as the methomyl concentration is increases as well as the time of exposure [38]. Diazinon and chlorpyrifos persist longer in the aquatic environment as compared to marathon and carbaryl, the degree of pesticides usage and method of application especially in agriculture have been of great concern to scientists [39].

Parathion is reported to inhibit acetylcholinesterase activity of zebra fish (*Danio rerio*) after exposure to sub-lethal concentrations; there was a correlation between the AChE inhibition and the parathion concentration by which higher concentrations causes more inhibition [40]. Malathion exposure causes biochemical alterations in the liver *Labeo rohita*; there was a reduction in total, soluble and structural protein. Also the activity of acetylcholinesterase was hindered [41].

The organophosphates are not toxic on their own and need activation by oxonation. This is accomplished using bromine water in vitro and enzymes in vivo. Parathion is converted to O, O-diethyl O-(4-nitrophenyl) phosphorothioate in microsome [42]. Trichlorfon, acephate and dimethoate were not inhibitory to AChE. This is because these pesticides are not from the class of phosphorothionates and they cannot be oxonated by bromine [43].

Pollutions such as azo dyes [44–46], detergents [47–53], hydrocarbons [54–59], heavy metals [60] and insecticides [61–65] are serious health threats, and the development of an assay for insecticides is hoped to increase the biomonitoring efficiency of toxic xenobiotics. Previous studies have shown that species of the genus *Oreochromis* spp. could be used as a biomarker for monitoring the presence of insecticide *in vivo* [66–72] and there is a possibility that AChE from this organism could be used as a new source of AChE for the detection of insecticides *in vitro*.

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