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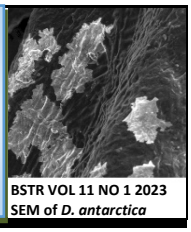

Inhibitive Assay of Insecticides using the Acetylcholinesterase from *Poecilia reticulata* (Peters, 1859)

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ABSTRACT

Poecilia reticulata (P. reticulata) is a species of tropical fish in the family Poeciliidae that is found all over the world and is a favorite in freshwater aquariums. The Malay name for this fish is "Ikan Gapi," although the English term "Guppy" has become more common. The Million Fish, or Rainbow Fish, as it's known in other parts of the world. The goals of this research were to characterize the acetylcholinesterase that will be partially purified from *P. reticulata* brain extract using ammonium sulphate fractionation The screening results show that at 1 mg/L insecticide, acephate, dimethoate, trichlorfon and bendiocarb leads to 100% inhibition, whilst parathion, methomyl and malathion cause >90% inhibition. The AChE from *P. reticulata* is less sensitive to other species as far as the insecticides carbofuran, carbaryl, methomyl, bendiocarb, parathion, diazinon, chlorpyrifos with the exception of malathion, where the sensitivity of malathion to the AChE from was within the range of the AChE from other species such a *E. electricus* , *Pangasius* sp., *Channa micropeltes*, *Clarias batrachus*, *H. nemurus*, *Tor tambroides*, and *Osteochilus hasselti*.

INTRODUCTION

Guppies are a type of tropical fish that formerly inhabited the northeastern region of South America. Male guppies' caudal and dorsal fins are smaller and purely aesthetic compared to those of females. Aquatic bug larvae and benthic algae are just two of the many things that guppy fish enjoy in the wild. This species has been chosen as a model organism in the domains of ecology, evolution, and behavioral studies due to its versatility and capacity to survive in a wide range of environmental and ecological settings. The Guppy is among the most recognizable and beloved species of aquarium fish. They are quiet, cheap, and easy to care for, and they offer a lot of vibrancy to aquariums. It's available in a rainbow of hues. They grow to be between 0.6 and 2.4 inches in length throughout the course of their 2-year lifespan [1]. Similar to humans, guppies are omnivores who consume both plant and animal products. South American guppies are members of the Poeciliidae family of freshwater tropical fish. The common guppy, *Poecilia reticulata*, is its scientific moniker. There are around two hundred subspecies of guppy. Different species have different-sized, shaped, and colored tails. The fish were first spotted in Trinidad in 1866 by a man named Robert John Lechmere Guppy, hence the name. A fish expert called *Girardinus guppii* after he brought it back to the British Museum. The fish's name has evolved from *Lebistes reticulatus* to the current *Poecilia reticulata* at that time. Their high reproductive rate has earned them the nickname "Millions Fish," and their dazzling array of coloration has earned them the name "Rainbow Fish" [2].

For some time now, assays based on the inhibition of ChE have been used for a variety of detection indicators in both living organisms and the natural environment. Land-based pollutants like pesticides, chemical production waste, viruses, and pollutants can accumulate in fish, posing health risks to fishermen, farmers, and consumers [3]. Biomarkers and biosensors for toxicants, particularly insecticides that work by inhibiting cholinesterase activity, have long been researched and developed using fish as a model system. The electric eel and the fruit fly, *Drosophila melanogaster,* for instance, the fish AChE from *Electophorus electricus* and other species are employed in pesticide assays and biosensor technologies. The guppy (*Poecilia reticulata*), tiger grouper (*Epinephelus fuscoguttatus*), Javanese carp (P*untius gonionotus*), and grass carp (*Ctenopharyngodon idella*), all of which are native to Malaysian waters, have the potential to be used as biomarker and biosensor receptors for pesticides especially insecticides in the future. Current studies on the use of acetylcholinesterase for the detection of insecticides centralize on

standard organisms such as *Torpedo californica* or *Torpedo californica*. As these sources are expensive, more local and cheaper sources of the enzymes have been sourced such ass *Oreochromis mossambicus* [4], *Channa micropeltes* or Toman [5], and *Hemibagrus nemurus* or Baung [6]. This research reveals a new potential source of AChE by isolating it from *Poecilia reticulata* brain tissue. The purpose of this research is to assess the sensitivity of the AChE from this species for the detection of insecticides.

MATERIALS AND METHODS

Partially purified AChE preparation

The partial purification of *P. reticulata* AChE was carried out as before [7]. The fish were killed by rapid freezing for around 30 min. Due to its diminutive size, the brain of a fish was obtained by decapitating the animal rather than by dissecting it. The skulls were crushed and weighed in a mortar and pestle. After adding 0.1 M sodium phosphate buffer, pH 7 as a buffer and the antiprotease phenylmethylsulfonyl fluoride (PMSF) at 0.01 mM, the brain sample was homogenized on a homogenizer (Ika's Ultra-Turrax) until it was homogenous, using a buffer-to-sample ratio of 1:4. After that, the homogenate was centrifuged for 20 min at 4 °C and 10,000 ×g in a Sorvall Ultra Pro-TH-641. The whole process was carried out at 4 °C. The supernatant was placed in a 15 mL Falcon tube and frozen at -80 °C. Bovine serum albumin (BSA) was used as the standard when measuring total protein content using the Bradford test [8].

Precipitation of protein by ammonium sulphate

One common method for separating proteins involves the use of ammonium sulfate precipitation, which works by altering the solubility of the protein in a salty medium. Using the nomogram or a standard table, the volume of the protein sample was calculated before the addition of the required amount of ammonium sulphate [9]. Depending on the size of the sample, varying amounts of ammonium sulphate will need to be added. To prevent enzyme degradation caused by the heat generated by this reaction, solid ammonium sulfate was added to the sample while being swirled gently with a magnetic bar in a beaker of ice for 15–20 min.

Then, a dialysis tube was used to remove the salt through the dialysis process. The molecular weight limit of regular dialysis tubing is 10 kDa. After centrifuging the sample at 15,000×g for 10 min at 4 °C, the sample is left in the dialysis tube and the protein is pelleted. In order to prepare the next concentration, the pellet was dissolved in 0.1 M sodium phosphate buffer, the supernatant was collected, and the processes of stirring and centrifugation were repeated. The enzymatic activity of the pellet protein at 30-40% ammonium sulfate saturations was used as a partially purified enzyme preparation [9].

Enzyme assay of ChE

P. reticulata ChE activity was assayed by adapting the method first proposed by Ellman et al. [10]. A 96-well microplate and 405 nm light are suitable for this purpose. The microplate wells were originally placed with 200 µL of sodium phosphate buffer (0.1 M, pH 7.0), 20 µL of DTNB (0.1 mM), and 10 µL of crude ChE, and then incubated for 15 min. Then 20 µL of ATC (2.5 mM) was added to the mixture. Specific activity is reported in terms of mmoles of substrate hydrolyzed per minute per milligram of protein, or units per milligram of protein per hour, with an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$. Below is a formula for determining ChE:

$$\text{Enzyme activity (U)} = \frac{(\text{Absorbance /10 min})}{\varepsilon} \times (\text{TV/TS}) \text{ µl of well}$$

Where,

Δ Absorbance= Change in absorbance reading at 405 nm after 10 min of incubation (Final-Initial)
ε = Specific extinction coefficient = 13.6 mM^{-1} cm^{-1}
TV = Total volume = 250 µL
TS = Total sample = 10 µL

Insecticide inhibition study

ChE inhibition studies were carried out by adding 150 µL of 0.1 M sodium phosphate buffer into a well followed by 25 µL of selected organophosphate and 50 µL of selected carbamates at 1 mg/L. Organophosphate pesticide needs to be activated by adding 5 µL of 1% of bromine water and incubating for 10 min. After 10 min incubation, 20 µL of 0.1 M DTNB and 10 µL of purified ChE were added followed by another 15 min incubation. Then, 20 µL of ATC was added and mixed thoroughly. Reading was taken from a 5 to 10 min incubation period. As a control, 0.1 M sodium phosphate buffer was carried out by replacing the pesticide. All incubation process was carried out at room temperature. The remaining ChE activity was calculated based on the following formula:

$$\% \text{ remaining activity} = \frac{\Delta 405nm \text{ treatment}}{\Delta\ 405nm \text{ control}} \text{ x } 100$$

Half maximal inhibitory concentration (IC_{50})

The concentration of insecticides is divided into two categories. One with a 50% inhibition study was tested with concentration varies from 1-10 mg/L whereas one with more than 50% inhibition study and resulted in negative % of enzyme activity value was tested with a concentration 0.2-1.0 mg/L. Both organophosphate and carbamate insecticide inhibition profile was analysed and IC_{50} value of the insecticide was obtained using GraphPad Prism using the one-phase exponential decay model [11].

Statistical analysis

The following formula was used to determine the percentage of inhibition:

$$\% \text{ Inhibition} = \frac{\text{Test activity of control - test activity of sample}}{\text{Test activity of control}} \text{x } 100\%$$

Means are shown with a standard error of the mean. Graphpad Prism version 3.0 was used for all statistical analysis. Student's t-tests or one-way ANOVAs with Tukey's post hoc test comparisons were used to determine statistical significance across groups. Statistical significance was assumed at the $P<0.05$ level.

Inhibition study of AChE activity

Twelve insecticides from the organophosphate and carbamate classes were evaluated for the inhibition of AChE from *P. reticulata* at 1 mg/L. These insecticides are the type of pesticide that works by damaging an enzyme in the body called acetylcholinesterase. This enzyme is critical for controlling nerve signals in the body. The result shows that all of the 12 insecticides tested show inhibition to AChE of *P. reticulata*. This means AChE from *P. reticulata* is very sensitive to organophosphate and carbamate which gives 50% inhibition to the enzyme activity. The screening results shows that at 1 mg/L insecticide, acephate, dimethoate, trichlorfon and bendiocarb leads to 100% inhibition, whilst parathion, methomyl and malathion cause >90% inhibition (**Fig. 1**).

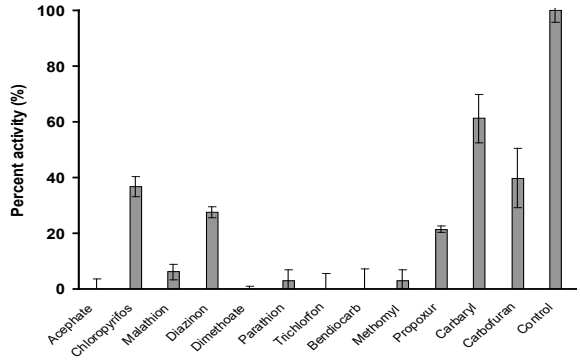

Fig. 1. Inhibition study of AChE from *P. reticulata* exposed to 1 mg/L of various type of insecticide. Error bars represent mean ± standard deviation (n=3).

IC_{50} determination

The IC_{50} was determined to identify the amount of organophosphate and carbamate concentration required to inhibit 50% of partially purified AChE activity. Based on GraphPad Prism, the IC_{50} for the selected insecticides obtained is shown in **Figs. 2** to **7**. It is suggested by this study that the partially purified AChE from the brain extract of *P. reticulata* can act as a good biomarker towards organophosphate and carbamate insecticides. The insecticide molecule binds to an acetylcholinesterase molecule thus making the enzyme inactive and decreasing the enzyme activity.

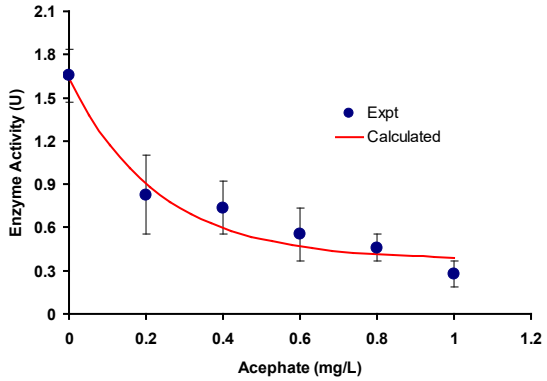

Fig. 2. Acephate inhibition profile to the partially purified AChE from the brain extracts of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

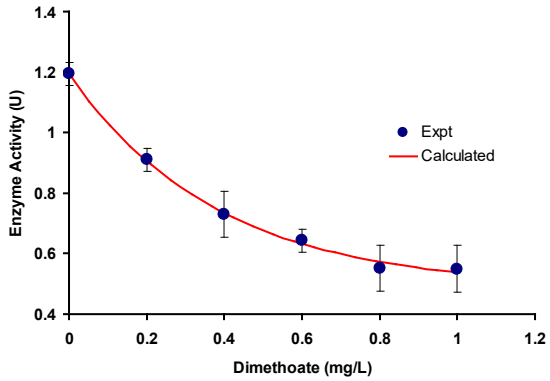

Fig. 3. Dimethoate inhibition profile to the partially purified AChE from the brain extracts of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

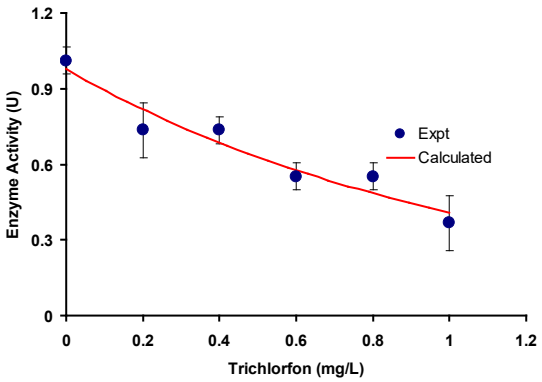

Fig. 4. Trichlorfon inhibition profile to the partially purified AChE from the brain extracts of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

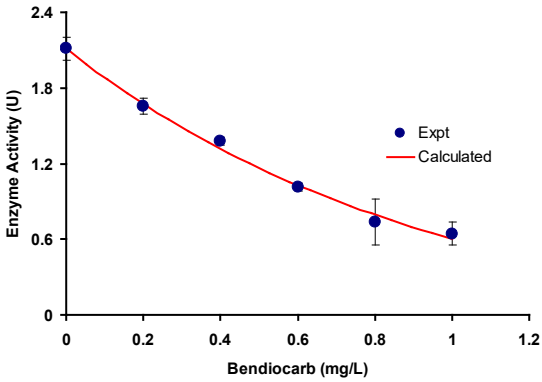

Fig. 5. Bendiocarb inhibition profile to the partially purified AChE from the brain extracts of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

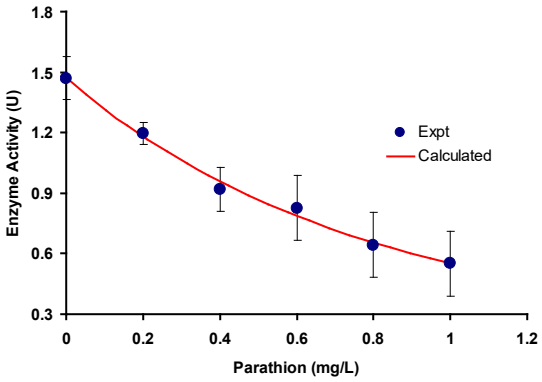

Fig. 6. Parathion inhibition profile to the partially purified AChE from the brain extracts of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

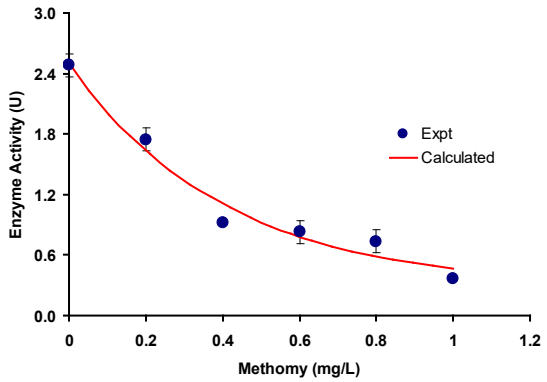

Fig. 7. Methomyl inhibition profile to the partially purified AChE from the brain extracts of *P. reticulata*. Error bars represent mean ± standard deviation (n=3).

The significant difference is usually signified by a non-overlap of 95% confidence interval with $p<0.05$ level. The overlapped confidence interval, on the other hand, does not necessarily show variation or not significant differences at $p<0.05$ level. More data and studies are necessary to assess the non-significance of overlapped confidence intervals [12]. The inhibition study on AChE from *H. nemurus* can be compared in terms of the sensitivity to carbamates and organophosphates from various published results. The AChE from *P. reticulata* is less sensitive to other species as far as the insecticides carbofuran, carbaryl, methomyl, bendiocarb, parathion, diazinon, chlorpyrifos with the exception of malathion, where the sensitivity of malathion to the AChE from was within the range of the AChE from other species such a *E. electricus*, *Pangasius* sp., *Channa micropeltes*, *Clarias batrachus*, *H. nemurus*, *Tor tambroides*, and *Osteochilus hasselti* (**Table 1**).

Table 1. Comparisons of the sensitivity of *P. reticulata* AChE to various insecticides in comparison to other fish AChEs.

Fish species	IC_{50} (mg/L) (95% Confidence Interval)								Ref.
	Carbofuran	Carbaryl	Methomyl	Bendiocarb	Parathion oxon	Malathion oxon	Diazinon oxon	Chlorpyrifos oxon	
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)	[13]
Periophtalmodon 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	[14]
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)	[13]
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)	[15]
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)	[16]
Clarias batrachus	0.006(0.006-0.008)	0.130 (0.12-0.14)	Not done	Not done	Not done	Not done	Not done	Not done	[17]
Tor tambroides	0.0643 (0.0482 - 0.0966)	0.0555 (0.0439 - 0.0754)	0.0817 (0.0571-0.1438)	0.0758 (0.0582-0.109)	Not done	Not done	Not done	Not done	[18]
Puntius schwanenfeldii	1.411	7.045	8.335	0.838	Not done	Not done	Not done	Not done	[19]
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)	[20]
Hemibagrus nemurus	0.0554 (0.0450-0.0721)	0.080 (0.0619-0.01132)	0.0239 (0.0202-0.0293)	0.0991 (0.0873-0.115)	0.0463 (0.0411-0.0531)	0.0455 (0.0416-0.0501)	0.0392 (0.0354-0.0440)	0.0479 (0.0409-0.0578)	[21]
P. reticulata	n.s.	2.474 (2.120-2.971)	0.282 (0.204-0.453)	0.657 (0.401-1.808)	n.s.	0.056 (0.024-0.078)	3.086 (2.411-4.286)	n.s.	This study

Note: All values have a correlation coefficient of at least 0.95. n.s. Not sensitive (IC50>4 mg/L)

CONCLUSION

Crude extract of ChE from the brain of *P. reticulata* was successfully partially purified using ammonium sulphate. The AChE from this fish was tested for its sensitivity to 12 insecticides. Preliminary screening shows that the AChE from *P. reticulata* is very sensitive to the insecticide, acephate, dimethoate, trichlorfon, bendiocarb, parathion, methomyl and malathion. However, the IC_{50} values for the AChE from *P. reticulata* indicated that it is less sensitive to the insecticides carbofuran, carbaryl, methomyl, bendiocarb, parathion, diazinon, chlorpyrifos with the exception of malathion, where the sensitivity of malathion to the AChE from was within the range of the AChE from other published fish species.

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