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In Vitro Heavy Metals Inhibitive Assay Using the Acetylcholinesterase from *Osteochilus hasselti* (Cyprinid Fish)

Mohd Khalizan Sabullah[1*], Kavilasni Subramaniam[2], Ain Aqilah Basirun[2] and Abubakar M. Umar[2,3]

[1]Faculty of Science and Natural Resources, Universiti Malaysia Sabah,
88400 Kota Kinabalu, Sabah, Malaysia.
[2]Dept of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia.
[3]Department of Biological Sciences, Faculty of Science, Gombe State University,
P.M.B. 027, Gombe, Nigeria.

*Corresponding author: Mohd Khalizan Sabullah
Faculty of Science and Natural Resources,
Universiti Malaysia Sabah,
88400 Kota Kinabalu,
Sabah, Malaysia.
Email: khalizan@ums.edu.my

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ABSTRACT

Rapid industrial development has caused many hazardous toxicants, especially heavy metals, to be released directly or indirectly into the environment which resulted in the polluted environment, mainly the water bodies. There are numerous Malaysian rivers that are largely polluted by heavy metals which can cause negative impact on health of public as well as the environment. Hence, a rapid and simple biomonitoring method will aid in notifying government agencies as well the public to such threat. Accordingly, acetylcholinesterase (AChE) inhibition has been widely used as a biomarker for heavy metals detection. In this study, the potential of AChE from *Osteochilus hasselti* brain as an alternative biosensor for heavy metals detection is measured. We discovered that out of seven heavy metals tested at the final concentration of 5 mg/L, only Hg^{2+}, Ag^{2+}, and Cu^{2+} exerted more than 50% significant inhibition ($p<0.05$) based on ANOVA analysis. The As^{5+} and Cd^{2+} exhibited inhibition by lowering the activity of AChE to less than 50%, displaying no significant difference ($P > 0.05$) compared to each other. Meanwhile, the Cr^{6+} and Pb^{2+} showed no notable inhibitory effect on the activity of AChE. The results exhibited that AChE of *O. hasselti* has the capability to be used as a biosensor for the detection of metal ions.

INTRODUCTION

Within this up-to-date society, the majority of nations are getting a fast-industrial advancement, urbanization, engineering, prospecting activities and deforestation which ends up in ecological matter, primarily land, air and water contamination. In concordance with this, previously scientific studies documented that about 5 to 10% of rivers in Malaysia are actually polluted with pesticides and fertilizer derivatives from substantial implementing agricultural activities in addition to pollutants from household waste materials and from commercial activities [1]. The health issues caused by chronic exposure to heavy metals is costing the country in millions of Ringgits for healthcare as well as to monitor river pollution. Therefore, it is crucial to monitor the pollutant level in the river time to time using efficient as well as cost effective method. As a solution, biomarker was considered to be a reliable method in order to examine the biological response towards environmental hazard so that precautionary measures can be taken. In addition to the affordable and eco-friendly qualities, this technique can reveal the stress degree via bioassay on the organism at numerous phases from a biomolecular, histological to the physiological modification induced by pollutant exposure [1]. Accordingly, the US EPA has recognized microbial- and enzyme-based methods for toxicants biomonitoring [2], however, microbial-based methods such as Microtox and Polytox are inappropriate to be applied in field trial works as they require bulky incubators. In addition, despite the enzyme such as urease [3] and proteases [4–6] could be used, but they are time consuming since each measurement takes more than an hour to complete.

It has been widely known that among the most regularly used biomarkers, acetylcholinesterase (AChE) activity was commonly used to detect and reveal exposure in both vertebrates as well as invertebrates [7]. AChE is the major enzyme responsible for the rapid hydrolysis of acetylcholine at cholinergic synapses and at neuromuscular junctions, resulting the control and modulation of neural transmission. Recently, the analysis of AChE inhibition has been extensively used as a biomarker for the identification of the effect on nervous system

in occupational and environmental medicine. Since, this method has plenty of advantages such as it comprised number of properties significant for the successful application as biomarker of a biological response in human biomonitoring. This method also shows a dose-dependent behaviour to pollutant exposure, sensitive, and the response is easy to measure [8].

Nevertheless, this AChE has only been extensively used as an inhibitive enzyme assay for insecticides, but very limited works have been done on the capability of AChE as an inhibitive assay for heavy metal [9]. Besides, earlier studies use enzyme from the fruit fly *Drosophila melanogaster* and the electric eel *Electophorus electricus* as the source for pesticide bioassay and biosensor technology [10,11]. Nevertheless, these sources are becoming very expensive up to several hundred Malaysian Ringgit per milligram.

Alternatively, the study on fish has been emerging since fish is highly sensitive to toxicants and the *in vitro* use of fish AChE for the bioassay of various toxicants including heavy metals have been reported in multiple studies such as *Electrophorus electricus* [9], *Anabas testudineus* [12], *Periophtalmodon schlosseri* [13], *Lates calcarifer* (cholinesterase from kidney) [14], and *Pangasius hypophthalmus* [15], which exerts better sensitivity compared to other established inhibitive assays mentioned previously. Also, this fish cholinesterase-based assay is a suitable candidate to obtain the data with cost effective, low time consuming, and simple skill procedure [16].

This leads to the need for identifying more sensitive cholinesterase source for the detection of heavy metals which leads to more screening for fish that are more sensitive. Therefore, in this study, AChE was isolated from the brain of freshwater cyprinid fish, *Osteochilus hasselti*, which locally known as 'Nilem' in order to measure the inhibitory effect of metal ions toward the enzyme activity and it is anticipated that AChE from this fish can be part of the new local source of biomarker of those heavy metals.

MATERIALS AND METHODS

Chemicals

Heavy metals such as mercury (Hg^{2+}), silver (Ag^{2+}), copper (Cu^{2+}), chromium (Cr^{6+}), arsenic (As^{5+}), cadmium (Cd^{2+}), and lead (Pb^{2+}), were prepared from Atomic Absorption Spectrometry standard solutions (Merck, Darmstadt, Germany). Acetylthiocholine iodide (ATC), β-mercaptoethanol and 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich.

Extraction and Purification (Affinity Chromatography)

Osteochilus hasselti weighing about 750 g and approximately 20 cm in length were obtained from a local aquaria market in 2010 in Selangor. About 10 g of the brain materials after dissection was homogenized (Ultra-Turrax T25 homogenizer) in 20% (w/v) of 100 mM sodium phosphate buffer at pH 8.0. This is followed by centrifugation at 15 000 ×g to remove unbroken tissues and centrifugation was carried out for 10 minutes at 4 ºC. The next step was to subject the supernatant fraction to one hour of ultracentrifugation at 100,000 ×g (Sorval) at 4 ºC. The obtained supernatant (400 μL) was then further purified via procainamide-sephacryl 6B affinity purification. The column had a dimension of 16 mm diameter and 20 mm height) with a total bed height of about 10 cm. Washing stage was carried out by loading 400 mL of washing buffer (20 mM sodium phosphate buffer; pH 8.0) onto the column with the flow rate calibrated at 1.0 mL/min. This step is crucial in eliminating the unbounded protein to the matrix from the column.

For eluting buffer, 20 mM sodium phosphate buffer at pH 8.0 containing 100 mL of 1 M NaCl was then loaded to elute the AChE of *O. hasselti* which is bounded to the affinity matrix. Fractions of 1 ml were then collected and assayed for activity of enzyme and protein. Fractions exhibiting high AChE activity were then pooled and dialyzed using Sartorius Vivaspin 20 at 2500 rpm at 4 °C overnight. The dialyzed fraction was then concentrated (Viva Spin) and stored at -20 °C for further use.

AChE Activity Determination

The method developed by Ellman *et al*. [17] was utilized to determine AChE activity with reference to an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$ at 405 nm. The reaction mixture consisted of 150 µl of potassium phosphate buffer (100 mM at pH 8.0), heavy metals (50 µl), DTNB (20 µl, 0.067 mM), and enzyme (10 µl). The mixture was incubated at room temperature in the dark for 10 min. Then, 20 µl of acetylthiocholine iodide (0.5 mM stock) was then added. The mixture was left to stand for 10 minutes at room temperature prior to the absorbance reading at 405 nm.

One unit of activity is defined as one µmole ATC hydrolyzed/min. Experiment was conducted in triplicates. IC_{50} of heavy metals was determined using a one phase exponential decay model on Graphpad PRISM 4 using non-linear regression analysis software.

The Effect of Metal Ion

The effects of metal ions on the AChE activity of *O. hasselti* was studied using seven metal ions; Hg^{2+}, Ag^{2+}, Cu^{2+}, Cr^{6+}, As^{5+}, Cd^{2+}, and Pb^{2+}. The reaction mixture contained 50 μL of the metal ion at the final concentration of 1 mg/L preincubated on ice for 10 min with 10 μL of AChE. This is followed by the addition of 150 μL of sodium phosphate buffer (0.1 M, pH 7.5) and 20 μL of DTNB (0.1mM). The mixture was incubated for 15 minutes followed by the addition of 20 μL of the substrate. After 10 minutes of incubation, the absorbance was read at 405 nm.

Data and statistical analysis

The percent inhibition was computed according to following formula:

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

The means ± standard deviations (SE) were analysed using GraphPad Prism version 4.0. Comparison between two or more groups was calculated based on a Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test and $P < 0.05$ was considered statistically significant.

RESULTS

Fig. 1 shows the *in vitro* inhibition of fish AChE activity by Hg^{2+}, Ag^{2+}, Cu^{2+}, Cr^{6+}, As^{5+}, Cd^{2+} and Pb^{2+} by lowering the activity to 93.4, 91.3, 93.3, 4.3, 62.5, 60.7, and 6.2%, respectively. From the result, we can deduce that out of seven heavy metals tested at the final concentration of 5 mg/L, only Hg^{2+}, Ag^{2+}, and Cu^{2+} exerted more than 50% significant inhibition ($p<0.05$) based on ANOVA analysis. The As^{5+} and Cd^{2+} exhibited inhibition by lowering the activity of AChE to

less than 50%, displaying no significant difference ($P > 0.05$) compared to each other. Meanwhile, the Cr^{6+} and Pb^{2+} showed no notable inhibitory effect on the activity of AChE. The heavy metals that have effect on AChE activity were then further tested using various concentrations, and exhibited exponential one phase decay type inhibition curves (**Figs. 2 to 6**).

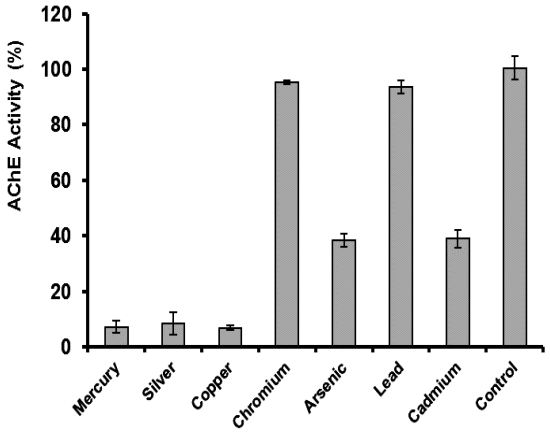

Fig. 1. The effect of metal ions on the activity of the partially purified AChE from *O. hasselti* (data represent means ± sem, n = 3).

Table 1: IC_{50} values for heavy metals that inhibit the fish acetylcholinesterase enzyme activity.

Heavy metals	R^2	IC_{50} (mg /l) (95% CI)
Hg^{2+}	0.989	0.135 (0.121-0.153)
Ag^{+}	0.990	0.250 (0.218-0.293)
Cu^{2+}	0.994	0.150 (0.138-0.165)
As^{5+}	0.988	0.250 (0.216-0.297)
Cd^{2+}	0.986	0.343 (0.284-0.431)

The IC_{50} was calculated using the one phase exponential decay model for the heavy metals that significantly affect AChE activity via GraphPad software 4.0 (GraphPad Software, Inc., San Diego, CA). The calculated values show 0.135, 0.150, 0.250, 0.250 and 0.343 mg/L, in the order of $Hg^{2+} < Cu^{2+}$, $Ag^{2+} < As^{5+} < Cd2^{+}$, respectively, as shown in **Table 1**. From the result, we can deduce that Hg^{2+} displayed high inhibitory effect whereas Cd^{2+} displayed the lowest inhibitory effect upon the AChE activity.

Generally, it is known that non-overlapping confidence interval usually indicates significant difference at the $p<0.05$ level while overlapped interval shows it is non-significant [18]. However, this is not always true where overlapping interval can be the general view that more data and experimentation are needed to evaluate non-significance. Based on the table, the result shows that only Hg^{2+} and Cd^{2+} exhibited significant difference with non-overlapping confidence interval while other heavy metals exhibited overlapping intervals.

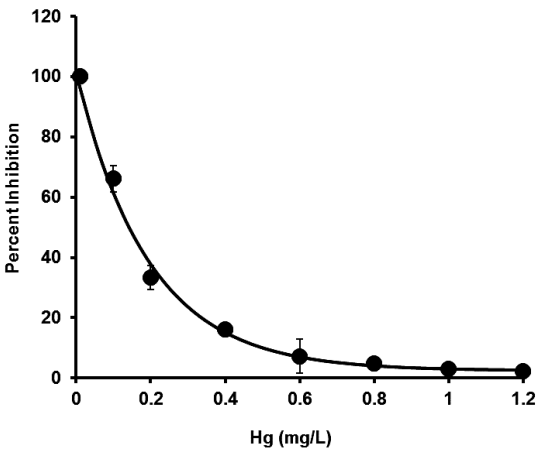

Fig. 2. Effect of mercury on the AChE activity of *O. hasselti*. Values are mean of three replicates ± sem.

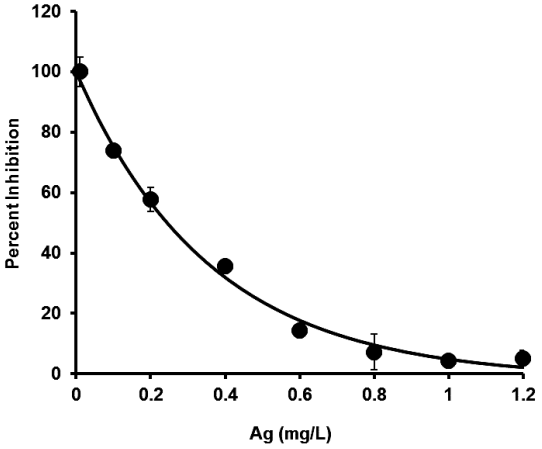

Fig. 3. Effect of silver on the AChE activity of *O. hasselti*. Values are mean of three replicates ± sem.

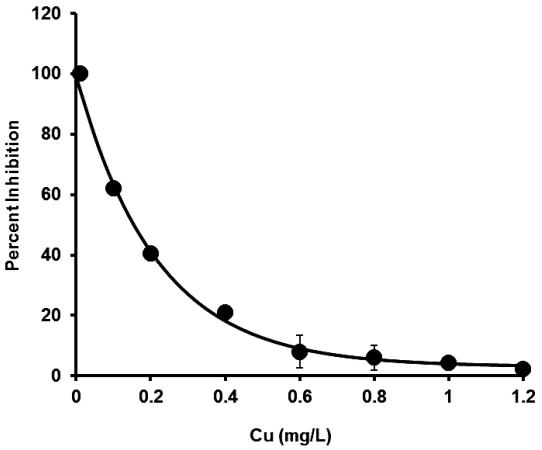

Fig. 4. Effect of copper on the AChE activity of *O. hasselti*. Values are mean of three replicates ± sem.

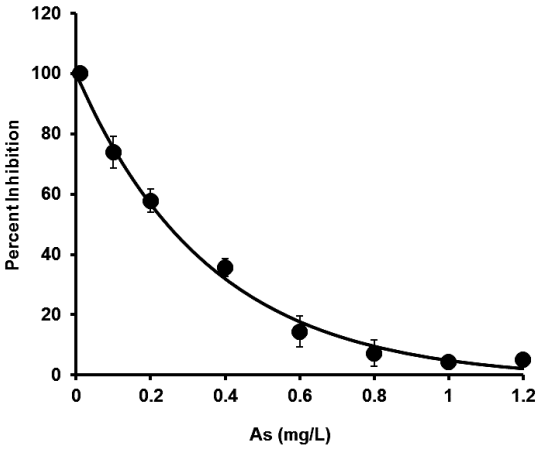

Fig. 5. Effect of arsenic on the AChE activity of *O. hasselti*. Values are mean of three replicates ± sem.

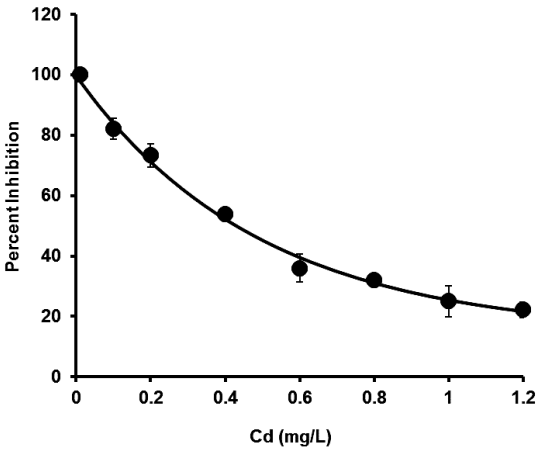

Fig. 6. Effect of cadmium on the AChE activity of *O. hasselti*. Values are mean of three replicates ± sem.

DISCUSSION

It is widely known that fish are highly sensitive to toxicants and this makes the fish a suitable candidate to be used for bioassay or bioindicator along with AChE to indicate the existence of toxicants like heavy metals [9]. Primarily, AChE is considered to be a significant biomarker to detect pesticides, polycyclic aromatic hydrocarbons, detergents, heavy metals, and components of complex mixtures of contaminants as these toxicants have toxic effect on fish and strongly inhibit the activity of AChE. For instance, the heavy metals like Cr, Cu, Cd and Hg has been shown to be highly toxic to *Gambusia affinis* [19], *Mytilus galloprovincialis* [20], and *Pomatoschistus microps* [21], respectively. Therefore, in this study, we intended to study the inhibitory effects of heavy metals like Hg, Ag, Cu, As and Cd with the concentration of 5 mg/L on AChE activity which have been extracted from brain sample of *O. hasselti*. As a result, AChE activity of *O. hasselti* portrayed more than 50% significant inhibition when exposed to Hg^{2+}, Ag^{2+}, and Cu^{2+}.

This finding coincides with the previous study, which showed that Cu, Ag and Hg showed more than 50% significant inhibition towards AChE activity of *Electrophorus electricus* [9]. In addition, Hg and Cu portrayed significant inhibition effect towards AChE activity of *Anabas testudineus* [12]. Although the data showed that Pb and Cr caused no significant inhibition of AChE activity, other studies have proved the toxicity of these metals towards this enzyme. For instance, previous studies showed that Cu, Hg and Pb exhibited 50% inhibition towards AChE activity of *Lates calcarifer* [14] and Cr, Cu, and Hg strongly inhibited *Puntius javanicus* AChE by lowering the activity below 50% [16]. Also, this study exhibited lower inhibition of AChE activity by As and Cd which the toxicity of these metals toward AChE have proven in other studies [22–24].

Previous research has shown that pesticides like carbamate and organophosphate that are also known as nerve agents, inhibit the AChE activity through the catalytic steps involved in carbamylation and phosphorylation at the active site of AChE [25]. In contrast, metal ions bind to amino acid side chains, which include the tryptophan (ring nitrogen), carbonyl groups, cysteine (thiol), serine, methionine (thioether), asparagine threonine, tyrosine (hydroxyl groups) and glutamine, and if these amino acids are involved in the catalytic activity, the enzyme is rendered inactive [13]. This is because the protein groups containing histidine residues are most susceptible to the metal binding as the imidazole group of histidine allows the strongest cation-π attraction which can interact with nitrogenous cations of substrates or free metal ions [16]. Nevertheless, according to Sarkarati *et al.* [26], the inhibition of AChE by free metal ions might be the result of the attraction of the negative charge of amino acid side chains comprised carboxyl groups such as glutamate and aspartate that exist at the catalytic triad of AChE which causes the structural change of the active site.

Previously, it has been reported that Cu and Cd displayed non-competitive inhibitory effect towards AChE activity, whereas Hg act as irreversible inhibitor of AChE activity [26–28]. In addition to it, Hg has high affinity to sulfhydryl groups that can disrupt cysteine bridges presents in AChE which results in highest inhibition effect upon the enzyme [12]. Overall, it can be summarized that the inhibition of enzyme activity by metal ion is associated with some aspects like the blockage of the enzyme active site, alteration of AChE structure as well as the effect of metal ions on the various amino acid sequence, consequently, preventing the formation of enzyme-substrate complex or protein denaturation, either reversible or irreversible [16]. The other mechanisms of heavy metal inhibition is still remains unknown, however, can be hypothesize as to act on the catalytic triad Ser-His-Glu which is usually conserved in AChE [25].

This current study revealed the capability of metal ions to inhibit the AChE activity of *O. hasselti* which makes this fish a suitable candidate to be used for *in vitro* detection of heavy metals. It can be proposed that metals and its exposure conditions portray a significant relationship with the acetylcholine receptor, in so doing affecting its binding efficiency which may also result in the decrease of AChE activity. Therefore, there is a possibility that AChE from brain tissue of *O. hasselti* has the potential to be used as an alternative method for biomonitoring heavy metals.

In conclusion, heavy metal pollution is a great threat to the human kind as well as the environment and the evolving development of an assay for the detection of heavy metals is expected to improve the efficiency of toxicants biomonitoring. The AChE from *O. hasselti* was sensitive and inhibited by

heavy metals resulting in IC_{50} values fall under the level stated by the Maximum Permissible Limit for heavy metals outlined by the Department of Environment, Malaysia. The sensitivity of AChE inhibition by selected heavy metals as per the result of this study suggested a promising biosensor kit to efficiently detect the heavy metals pollution in marine environment. Future work is recommended to study the capability of this AChE to detect other toxicants such detergents, dyes, pesticides, as well as drugs.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Sabullah MK, Ahmad SA, Shukor MY, Gansau AJ, Syed MA, Sulaiman MR, et al. Heavy metal biomarker: Fish behavior, cellular alteration, enzymatic reaction and proteomics approaches. Int Food Res J. 2015;22(2):435–54.
2. Masdor NA, Said NAM. Papain partial purification improves its sensitivity towards heavy metals papain partial purification. Biosci Biotechnol Res Asia. 2012;9(1):236–42.
3. Jung K, Bitton G, Koopman B. Assessment of urease inhibition assays for measuring toxicity of environmental samples. Water Res. 1995;29(8):1929–33.
4. Shukor Y, Baharom NA, Rahman FA, Abdullah MP, Shamaan NA, Syed MA. Development of a heavy metals enzymatic-based assay using papain. Anal Chim Acta. 2006;566(2):283–9.
5. Shukor MY, Masdor N, Baharom NA, Jamal JA, Abdullah MPA, Shamaan NA, et al. An inhibitive determination method for heavy metals using bromelain, a cysteine protease. Appl Biochem Biotechnol. 2008;144(3):283–91.
6. Sahlani MZ, Halmi MIE, Masdor NA, et al. A rapid inhibitive assay for the determination of heavy metals using α-chymotrypsin; a serine protease. Nanobio BioNano *2014*; 1(2): 41-6.
7. Toumi H, Boumaiza M, Millet M, et al. Is acetylcholinesterase a biomarker of susceptibility in *Daphnia magna* (Crustacea, Cladocera) after deltamethrin exposure? Chemosphere 2015; 120: 351–356.
8. Lionetto MG, Caricato R, Calisi A, Giordano ME, Schettino T. Acetylcholinesterase as a biomarker in environmental and occupational medicine: New insights and future perspectives. BioMed Res Int. 2013.
9. Shukor MY, Tham LG, Halmi MIE, Khalid I, Begum G, Syed MA. Development of an inhibitive assay using commercial *Electrophorus electricus* acetylcholinesterase for heavy metal detection. J Environ Biol. 2013;34(5):967-70.
10. Hamers T, Molin KR, Koeman JH, Murk AJ. A small-volume bioassay for quantification of the esterase inhibiting potency of mixtures of organophosphate and carbamate insecticides in rainwater: development and optimization. Toxicol Sci. 2000;58(1):60–67.
11. Gupta SC, Siddique HR, Saxena DK, Chowdhuri DK. Comparative toxic potential of market formulation of two organophosphate pesticides in transgenic *Drosophila melanogaster* (hsp70-lacZ). Cell Biol Toxicol. 2005;21(3–4):149–162.
12. Ahmad SA, Wong YF, Shukor MY, Sabullah MK, Yasid NA, Hayat NM, et al. An alternative bioassay using *Anabas testudineus* (Climbing perch) cholinesterase for metal ions detection. Int Food Res J. 2016;23(4):1446–52.
13. Sabullah MK, Sulaiman MR, Shukor MS, Yusof MT, Johari WLW, Shukor MY, et al. Heavy metals biomonitoring via inhibitive assay of acetylcholinesterase from *Periophthalmodon schlosseri*. Rendiconti Lincei. 2015;26(2):151–58.
14. Hayat NM, Shamaan NA, Sabullah MK, Shukor MY, Syed MA, Khalid A, et al. The use of *Lates calcarifer* as a biomarker for heavy metals detection. Rendiconti Lincei. 2016;27(3):463–472.
15. Aidil MS, Sabullah MK, Halmi MI, Sulaiman R, Shukor MS, Shukor MY, et al. Assay for heavy metals using an inhibitive assay based on the acetylcholinesterase from *Pangasius hypophthalmus* (Sauvage, 1878). Fresenius Env Bull. 2013;22(12):3572–6.
16. Sabullah MK, Sulaiman MR, Shukor MYA, Syed MA, Shamaan NA, Khalid A, et al. The assessment of cholinesterase from the liver of *Puntius javanicus* as detection of metal ions. Sci World J. 2014;2014.
17. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7(2):88–95.
18. Schafer EC, Kleineck MP. Improvements in speech recognition using cochlear implants and three types of FM systems: A meta-analytic approach. J Educ Audiol. 2009;15:4–14.
19. Nasirian H, Mahvi AH, Hosseini M, Vazirianzadeh B, Sadeghi SMT, Nazmara S. Study on the heavy metal bioconcentrations of the Shadegan international wetland mosquitofish, *Gambusia affinis*, by inductively coupled plasma technique. J Environ Health Sci Eng. 2013;11(1):22.
20. Rouane-Hacene O, Boutiba Z, Belhaouari B, Guibbolini-Sabatier ME, Francour P, Risso-de Faverney C. Seasonal assessment of biological indices, bioaccumulation and bioavailability of heavy metals in mussels *Mytilus galloprovincialis* from Algerian west coast, applied to environmental monitoring. Oceanologia. 2015;57(4):362–374.
21. Vieira LR, Gravato C, Soares A, Morgado F, Guilhermino L. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour. Chemosphere. 2009;76(10):1416–1427.
22. Jebali J, Banni M, Guerbej H, Almeida EA, Bannaoui A, Boussetta H. Effects of malathion and cadmium on acetylcholinesterase activity and metallothionein levels in the fish *Seriola dumerilli*. Fish Physiol Biochem. 2006; 32(1):93.
23. Mat-Jais AM, Mohamed MZ. The role of extracellular Ca^ 2^+ on the inhibitory effects of acetylcholinesterase activity in haruan, *Channa striatus* Bloch, brain tissue by heavy metals. Malays Appl Biol. 2000;29(1/2):69–74.
24. Nath S, Roy B, Bose S, Podder R. Impact of arsenic on the cholinesterase activity of grasshopper. Am-Eurasian J Toxicol Sci. 2015;7:173–176.
25. Hayat NM, Ahmad SA, Shamaan NA, Sabullah MK, Shukor MYA, Syed MA, et al. Characterisation of cholinesterase from kidney tissue of Asian seabass (*Lates calcarifer*) and its inhibition in presence of metal ions. J Environ Biol. 2017;38(3):383.
26. Sarkarati B, Çokuğraş AN, Tezcan EF. Inhibition kinetics of human serum butyrylcholinesterase by Cd^{2+}, Zn^{2+} and Al^{3+}: comparison of the effects of metal ions on cholinesterases. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 1999;122(2):181–190.
27. Padrilah SN, Ahmad SA, Yasid NA, Sabullah MK, Daud HM, Khalid A, et al. Toxic effects of copper on liver and cholinesterase of *Clarias gariepinus*. Environ Sci Pollut Res. 2017;24(28):22510–22523.
28. Frasco MF, Colletier J-P, Weik M, Carvalho F, Guilhermino L, Stojan J, et al. Mechanisms of cholinesterase inhibition by inorganic mercury. FEBS J. 2007;274(7):1849–1861.