

Kinetics of Acetylcholinesterase Inhibition of *Quisqualis indica* Linn. Flower Extract

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Abstract

Rangoon creeper (*Quisqualis indica* Linn.) is a plant of the family Combretaceae. In Thai traditional medicine, its seeds contain oil and quisqualic acid that act as anthelmintic. Its flowers are used against diarrhea and eaten as vegetable. The flower extract gave high total polyphenol contents and showed strong antioxidant activity. In the search for new acetylcholinesterase inhibitors from plant origin, it was demonstrated that methanolic extract of *Q. indica* flower exhibited this activity. The extract inhibited electric eel acetylcholinesterase in dose dependent manner with an IC_{50} value of 0.77 $\mu\text{g/ml}$. The Michaelis-Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide was 0.034 mM. The K_m value in the presence of the extracts (K_{mapp}) at first decreased, and then increased by 60-88.9%. The V_{max} was 0.017 $\mu\text{M/min}/\mu\text{g}$ protein. The V_{max} value in the presence of the extracts (V_{maxapp}) decreased by 2.8-52.3%. The estimated value of K_i was 1.41 mM, respectively. The Lineweaver-Burk plot, Dixon plot and their replots showed combination of the mixed and partially noncompetitive inhibition.

Key Words: *Quisqualis indica* Linn; Michaelis-Menten constant; Lineweaver-Burk plot; Dixon plot

Introduction

The turnover regulation and level of acetylcholine in neurons and synaptic junction play an important role in a number of neural diseases, particularly Alzheimer's disease (AD), myasthenia gravis (MG) and anticholinesterase poisoning (Soreq and Seidman, 2000). Acetylcholinesterase (AChE) is a substrate specific enzyme degrading neurotransmitter acetylcholine in nerve synapses. According to cholinergic hypothesis, cholinesterase inhibitors enhance the signal transmission in nerve synapses by

prolonging the effect of symptomatic acetylcholine and beneficial for curing CNS degenerative diseases (Coulthard et al., 2006).

Currently, the effective chemicals for AD therapy are AChE inhibitors, which elevate the attenuated acetylcholine concentrations in the AD-affected brain by enhancing cholinergic function (Alexopoulos, 2005). Although the use of AChE inhibitors (e.g. donepezil, rivastigmine and galantamine), a symptomatic treatment of AD, has been shown as beneficial to cognitive, functional and behavioral

symptoms of the disease, it also causes the adverse effects due to cholinergic stimulation in the brain and peripheral tissues (Zarotsky, 2003). Therefore, the searching for new AChE inhibitors, particularly edible flowers which may cause lower side effects is very interesting for extensively investigated.

Rangoon creeper (*Quisqualis indica* Linn.) is a plant in family Combretaceae. It is a tropical flowering vine up to 12 feet long. Its blossom blooms in warm months. Its flowers are white at first then gradually change to pink and finally turn to red. The blossom bunches start to fragrance in the evening through the night. Its shuttle shape seeds contain oil (Brill and Well, 1971) and quisqualic acid that acted as anthelmintic (Ishizaki et al., 1973), for driving roundworms and threadworms especially *Ascaris*. The toxicity studies in mice showed no acute toxicity with the LD₅₀ value of water seed extract that equivalent to more than 20 g/kg/day of seeds. Toxicity studies in rat showed the LD₈₀ value of water extract of seed equivalent to 20 g/kg/day of seeds (Chivapat et al., 1998). The abnormal symptoms of rat after treatment with water seed extract were seizures following by respiratory arrest and death. The quisqualic acid which was an excitatory amino acid (Zaczek and Coyle, 1982) could cause neurotoxic and seizures as it was injected into limbic of dog (Rondouin et al., 1987), applied topically to cerebral cortex of rat (Addae and Stone, 1988), injected into amygdala of cat (Kaijima et al., 1987) and induced hippocampal seizures of cat (Funda et al., 1985). Quisqualic acid induced neuronal necrosis in stratum and hippocampus of 7 days old rat (Silverstein et al., 1986). In Thai traditional medicine, *Q. indica* seeds were broken and boiled in water, after that the seeds were ground to powder and mixed with honey to make pill or were sliced into thin pieces and were fried with eggs for anthelmintic. Its leaves could cure abscess and its flowers were used as food and antidiarrhea. Our previous studies showed that methanol extract of flowers gave high total polyphenol content (Limmatvapirat et al., 2006) and exhibited strong antioxidant activity (Wetwitayaklung

et al., 2007). There were research works suggested that the phytochemical antioxidants might act as AChE inhibitors (e.g. flavonoids and other phenols) (Ji and Zhang, 2006; Kim et al., 2004). Therefore, this study was hypothesized that *Q. indica* flower extract could inhibit the electric eel AChE. To the best of our knowledge, this is the first investigation of the kinetics of AChE inhibition of *Q. indica* flower extract.

Materials and Methods

Plant

The *Q. indica* flowers were collected from the herbal garden of Faculty of Pharmacy, Silpakorn University, Nakhon-Pathom, in April 2006. The voucher specimens were deposited at the Department of Pharmacognosy, Silpakorn University, Nakhon-Pathom, Thailand.

The dried *Q. indica* flowers were ground through size No.20 mesh and then the 72-g powder was macerated in 1,000 ml of 95% methanol, stirred with magnetic bar at room temperature for 72 hrs and then filtered. The filtrate was evaporated with a rotary evaporator until dried. The crude extract was kept at 4°C.

Chemicals

Electric eel acetylcholinesterase type III (AChE), acetylthiocholine iodide (ASCh), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 99.9% dimethylsulfoxide were purchased from Sigma-Aldrich, (St. Louis, MO, USA). 85% *O*-Phosphoric acid was purchased from Ajax Finechem (NSW, Australia). Coomassie Blue G250 and lyophilized bovine serum albumin (>96% BSA) were obtained from Fluka Chemie GmbH (Buchs, Switzerland).

Assay of protein content of AChE

The protein content of AChE preparation was estimated by Bradford method using BSA (0-40 µg/ml) as a standard (Bradford, 1976). All experiments were done in triplicate (n = 3).

***In vitro* analysis of AChE activity**

In order to select the proper concentration of enzyme, the AChE activity was measured *in vitro* by Ellman method (Ellman et al., 1961), and each assay was done in triplicate ($n = 3$). The assay contained 1 ml of mixture of 0.25 mM ASCh and 0.25 mM DTNB in 50 mM sodium phosphate buffer pH 8 and 200 μ L of AChE in different concentrations (0.01-0.243 μ g/ml). The final volume was adjusted to 3 ml with 50mM sodium phosphate buffer pH 8. The enzymatic reaction of AChE was the hydrolysis of acetyl group of ASCh and gave thiocholine (SCh) as the product. The SCh could react with DTNB to form 5-thionitrobenzoate, a colored anion, which absorbed UV at 412 nm. The absorbances were measured at 0, 0.5 min and every 1 min interval starting from 0.5 min (0, 0.5, 1.5, ..., 20.5). The rate of product formation (ΔA) was measured by the difference of absorbance (A) in every 1 min time intervals within 20.5 min. Then the product formation was calculated for each AChE concentration.

The effect of plant extract on AChE substrate hydrolyzation.

For studying the effect of plant extract on AChE activity, the enzyme was preincubated with each plant extract for 10 min before the addition of ASCh.

Estimation of the IC_{50} value

The concentration of the extract that inhibited 50% of AChE activity (IC_{50}) was estimated by method described by Kamal et al., 2000 and Alhomida et al., 2000. The method was performed by plotting % activity and %inhibition of AChE versus extract (inhibitor) concentrations on the same graph. The concentration at the intersection of these two curves was the IC_{50} value.

The assay contained 200 μ l of 0.0948 μ g/ml AChE, chosen from *in vitro* analysis of AChE activity, 1 ml of mixture of 0.25 mM DTNB and 0.25 mM ASCh in 50mM sodium phosphate buffer pH 8 and 200 μ l of plant extract in reaction concentration range of 0-2.22 μ g/ml (final

concentration). The final volume was adjusted to 3 ml with the 50mM sodium phosphate buffer pH 8.

Estimation of kinetic parameters

Michaelis constants (K_m) were determined by means of substrate concentration at $1/2V_{max}$ of v and substrate concentration plot and Lineweaver-Burk plot over ASCh concentration range of 0.025-0.25 mM ($1/ASCh = 4-40 \text{ mM}^{-1}$), while v and V_{max} were velocity and maximum velocity, respectively. The assay contained 200 μ l of 0.0948 μ g/ml AChE, 1ml of mixture of 0.25 mM DTNB and different concentrations of ASCh (0.025-0.25 mM) in 50mM sodium phosphate buffer pH 8. The final volume was adjusted to 3 ml with the 50 mM sodium phosphate buffer pH 8.

The assay conditions for measuring the inhibition activity of plant extract were the same as IC_{50} assay plus with 200 μ l of plant extracts. The concentrations of plant extracts were in range 0 - 4.25 μ g/3 ml of assay for each fixed concentration of ASCh.

The kinetic values were applied by transforming data of Lineweaver-Burk plot, Dixon plot, $1/V_{maxapp}$ versus extract concentration plot and $1/V_{maxiapp}$ versus $1/ASCh$ concentration plot. The V_{maxapp} was the maximum apparent velocity of the AChE at the given concentration of extract (inhibitor). The V_{maxapp} was obtained from the intersection at ordinate of Lineweaver-Burk plot. The $V_{maxiapp}$ was the maximum apparent velocity of the AChE in the presence of extract at the given concentration of ASCh and $V_{maxiapp}$ was obtained from the intersection at ordinate of Dixon plot.

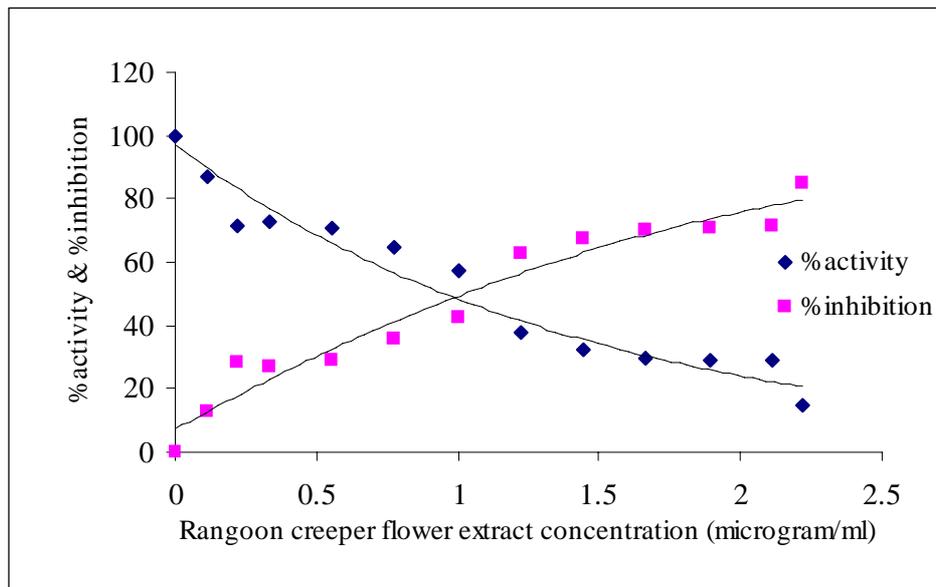
All graphs were plotted by Microsoft Excel. The correlation coefficient, slope, and intercept were obtained by linear regression and non linear regression analysis.

Results

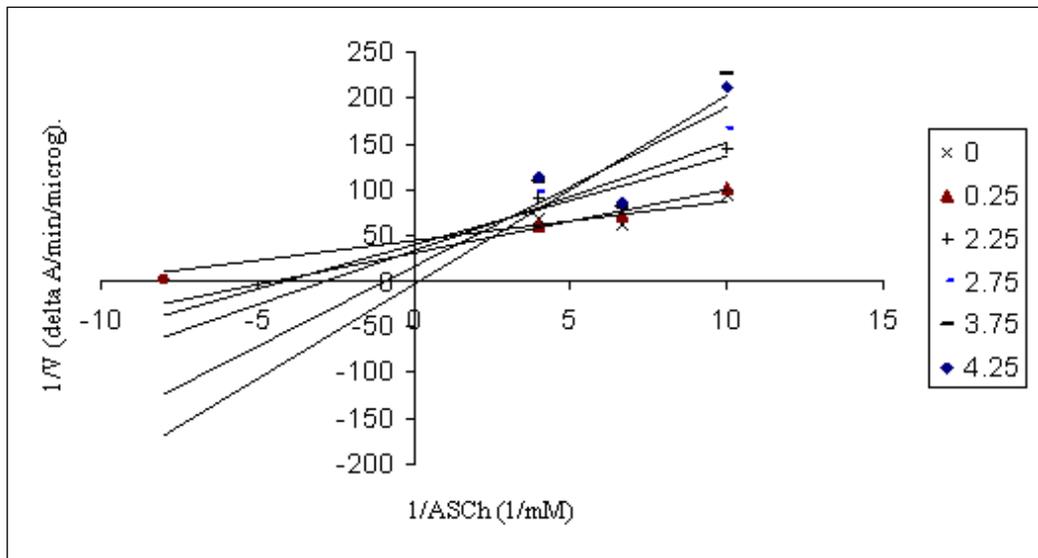
The percentage yield of the methanolic extract obtained from *Q. indica* flower was 24.5%. The plot between ΔA and incubation time (20.5 min) of different AChE concentrations showed linearity

relationship ($r^2 > 0.95$). The relationships between ΔA and AChE concentrations within 7 min indicated good linearity ($r^2 > 0.99$). From this plot, the optimum condition as AChE concentration ($0.0948 \mu\text{g/ml}$) and incubation time (7 min) were chosen to provide linearity of AChE activity in further kinetic studies.

The data of AChE inhibition by extract at different concentrations were presented in Figure 1a. The IC_{50} value of extract which was obtained from intersection point of %activity and %inhibition versus extract concentration curves equalled to $0.77 \mu\text{g/ml}$.



a)



b)

Figure 1 a) The %inhibition (■) and %activity (◆) of AChE versus *Q. indica* flower extract concentration. b) The Lineweaver-Burk plots in the absence and presence of different concentrations of *Q. indica* flower extract (0 (x), 0.25 (▲), 2.25 (+), 2.75 (-), 3.75(-) and 4.25 (◆) $\mu\text{g/3ml}$).

The inhibitory activities by plant extract were shown in Figure 1b (Lineweaver-Burk plot). This plot represented the inhibition type of the extract as noncompetitive inhibition (Bisswanger, 2002). From the plot, K_m and K_{mapp} values were obtained from intersection of abscissa and $1/V_{maxapp}$ values were obtained from intersection of ordinate. The values of K_{mapp} at first decreased then increased by 60-88.9%

(Table 1). The K_m value from Lineweaver-Burk plot was 0.032 mM.

The secondary replot of Lineweaver-Burk plot was the $1/V_{maxapp}$ versus extract concentration plot (Figure 2). The intersection of abscissa values of this plot was K_i (= the dissociation constant of AChE-ASCh-inhibitor complex into AChE-ASCh complex and inhibitor) and the plot showed K_i value of 1.41 mM.

Table 1 Kinetic constants of *Q. indica* flower extract on AChE activity obtained from Lineweaver-Burk plot.

Extract concentration ($\mu\text{g}/3\text{ ml}$)	K_{mapp} (mM)	% increase of K_{mapp}	V_{maxapp} $\mu\text{M}/\text{min}/\mu\text{g}$	% decrease of V_{maxapp}
0	0.032	0	0.017	0
0.25	0.036	-11.76	0.016	2.79
2.25	0.013	60.49	0.011	35.88
2.75	0.008	74.63	0.0095	42.42
3.75	0.0035	88.89	0.0079	52.28
4.25	0.0036	88.94	0.0080	51.90

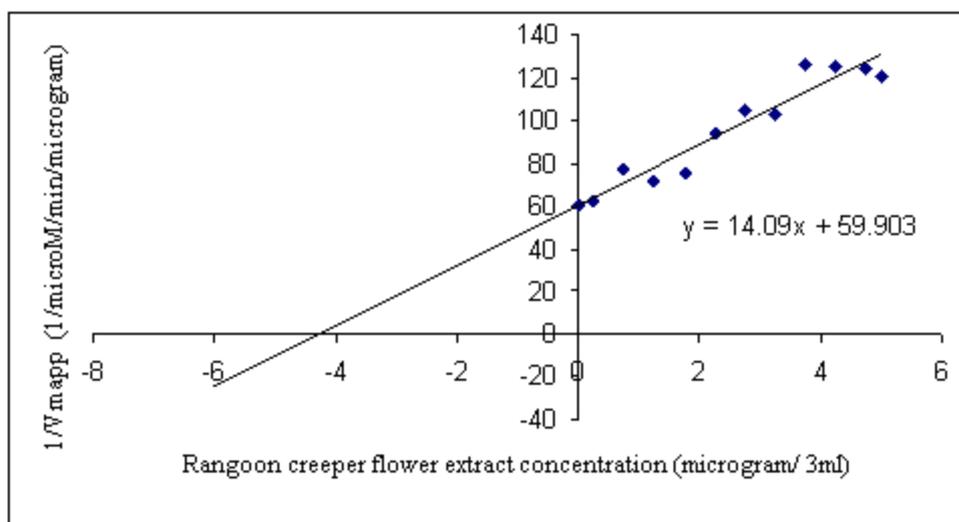


Figure 2 The secondary replot of Lineweaver-Burk plot (plot between the $1/V_{maxapp}$ versus various concentrations of *Q. indica* flower extract).

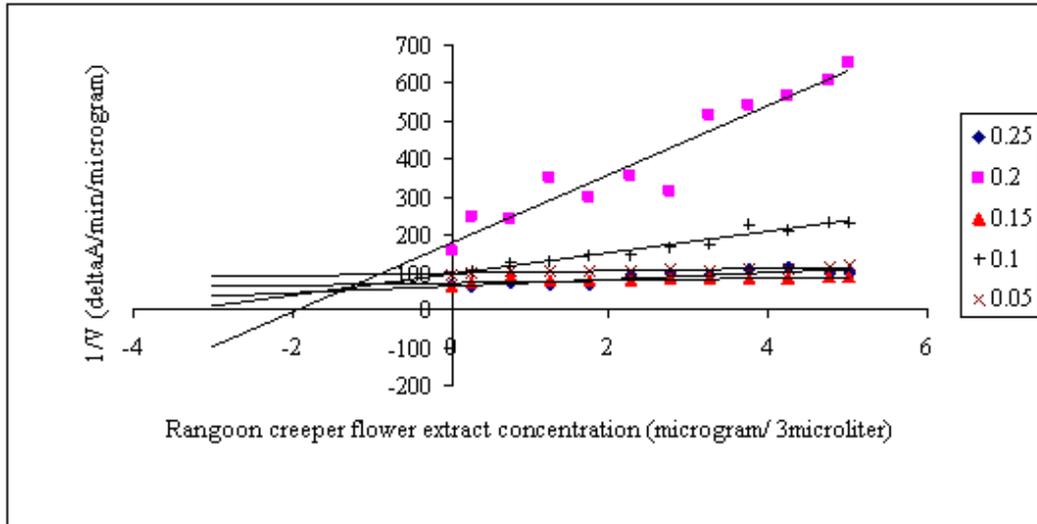


Figure 3 The Dixon plot *Q. indica* flower extract, in the presence of different ASCh concentrations (0.05 (×), 0.1 (+), 0.15 (▲), 0.2 (■) and 0.25 (◆) mM).

From the Dixon plot (Figure 3), $V_{maxiapp}$ and K_{lapp} were obtained. The K_{lapps} was calculated by linear regression analysis from Dixon plot. The K_{lapp} values increased from 42 to 94% (Table 2). The $1/V_{maxiapp}$ values were obtained from intersection of ordinate of Dixon plot. From the secondary replot of Dixon plot

(Figure 4), the intersection of abscissa values was $(-)/K_m$. The value of K_m calculated from $(-)/K_m$ was 0.036 mM. Then the mean value of K_m of extract was 0.034 mM. The estimated kinetic constants, K_m , K_i and V_{max} , were shown in Table 3.

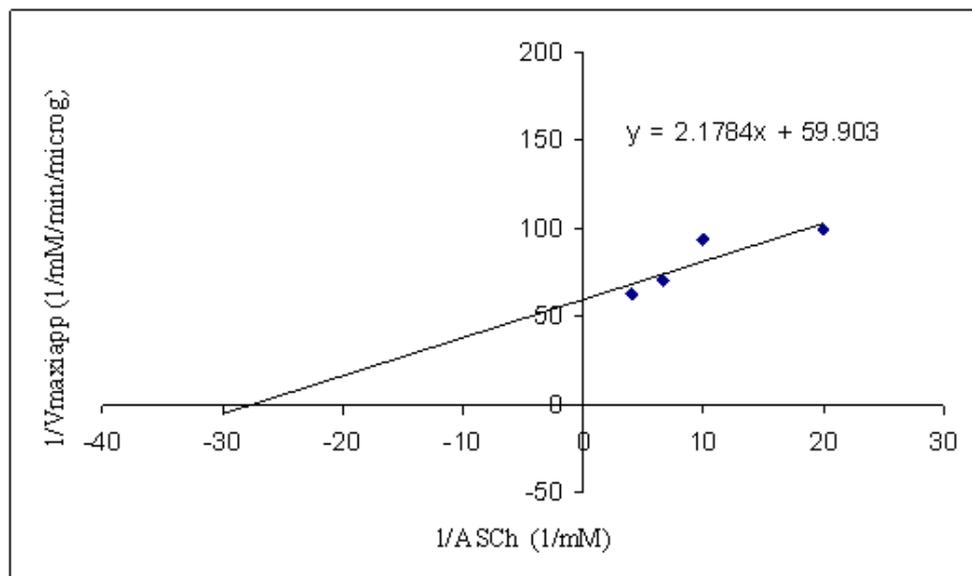


Figure 4 The secondary replot of Dixon plot (plot between the $1/V_{maxiapp}$ versus $1/ASCh$ concentration)

Table 2 Effect of extract at different concentration of ASCh on K_{iapp} , slope and $V_{maxiapp}$ of AChE obtained from Dixon plot.

ASCh concentration (mM)	K_{iapp} (mM)	% increase of K_{iapp}	$V_{maxiapp}$ $\mu\text{M}/\text{min}/\mu\text{g}$	% increase of $V_{maxiapp}$
0.05	11.43	0	0.010	0
0.10	1.12	90.21	0.011	5.18
0.15	6.61	42.17	0.014	39.07
0.25	2.30	94.33	0.016	56.88

Table 3 The estimated kinetic constants from various plots and replots.

Type of plots	K_m (mM)	V_{max}	K_I (mM)
Lineweaver-Burk	0.032	0.017	-
$1/V_{maxapp}$ VS [extract]	-	-	1.41
$1/V_{maxiapp}$ VS $1/[\text{ASCh}]$	0.036	-	-

Discussion

The data and plots (Figure 1b – 4) of *Q. indica* flower extract indicated the combination of the mixed and partially noncompetitive inhibition (Bisswanger, 2002). The mechanism of inhibition revealed that the extract might compete with ASCh for binding at substrate binding site of AChE or combined with AChE or with AChE-ASCh. In case of high concentration of ASCh, the extract may bind to the secondary binding site of AChE. This was confirmed by the increasing of K_{mapp} and decreasing of V_{max} as the extract concentrations were increased. From K_I value, they suggested that the extract had low affinity for AChE (Alhomida et al., 2000).

The percentage inhibition of AChE of *Q. indica* flower extract increased upon the concentrations of extract. In previous report (Brossi, 1986), (-)-physostigmine salicylate showed inhibitory effect on electric eel AChE with IC_{50} value of 1.65×10^{-3} $\mu\text{g}/\text{ml}$. In this study, the flower extract of *Q. indica* exhibited inhibitory effect on that enzyme with an IC_{50} value of $0.77 \mu\text{g}/\text{ml}$. So, the flower

extract of *Q. indica* showed low AChE inhibitory activity when compared to the remark high potency physostigmine salicylate.

Methanolic flower extract of *Q. indica* contained some active compounds that exhibited the AChE activity inhibition. However, this result might be due to the synergistic effect of many compounds in this extract. Further purification and isolation should be performed for profoundly understanding the mechanism of AChE inhibitory activity. For this study, it is the first time screening the inhibition of acetylcholinesterase in *Q. indica* extract. So, the crude extract was used and further purification such as the elimination of tannin was not conducted. Somehow, some type of tannins might show the activity that should be further studied.

Conclusion

Acetylcholine is one of the most important neurotransmitter in either central or peripheral nervous system and the inhibition of AChE has been proposed as biomarker for the neurotoxicity (Rickwood and

Galloway, 2004). In this study, we have shown for the first time that *Q. indica* flower extract was dose-dependently inhibited the AChE activity in a noncompetitive manner. However, this extract showed slight inhibition of AChE activity (IC_{50} value of 0.77 μ g/ml).

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