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Protective Effects of *Thunbergia laurifolia* (Linn.) on Organophosphorous (chlorpyrifos)-Induced Cholinesterase Dysfunction

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Abstract

Organophosphorous (chlorpyrifos) has been widely used in pest control. It is considered to be a functionally irreversible inhibitor of cholinesterase (ChE), since the time necessary to liberate the enzyme from inhibition may be in excess of time required for the synthesis of new ChE. This study was designed to investigate the effects of total phenolic content, total antioxidant capacity, and free radical scavenging activities in a cell free system present in *Thunbergia laurifolia* (Linn.) (TL) and further examine their ability against chlorpyrifos-induced ChE dysfunction *in vitro* (blood and brain of mice) studies. The results showed that TL has the highest phenolic content, which is a significant positive correlation with its antioxidant activity (R^2 = 0.99). Additionally, the effects of 50 % inhibitory concentration of TL on scavenging free radical activities (IC_{50}) for hydroxyl, superoxide, hydrogen peroxide radical and nitric oxide in the cell free system were 1.47±0.02, 2.23±0.08, 1.66±0.09 and 1.00±0.07 mg/mL, respectively. We found TL showed a potential inhibition of chlorpyrifos-induced ChE dysfunction in a concentration dependent manner. These data suggest that TL exhibits anti-oxidant and, at least in part, neuroprotective properties against chlorpyrifos-induced ChE alteration. We strongly believe that these antioxidant constituents are prospective novel nutriceuticals.

Keywords: Organophosphorous (chlorpyrifos), cholinesterase (ChE), *Thumbergia laurifolia* (Linn) (TL), free radical scavenging

Introduction

Organophosphate (OP) insecticides, lubricants, and plasticizers present potential health and safety hazards to individuals, associated with their manufacture, formulation, application, and field use [1,2]. Organophosphate, when used as an insecticide, inhibits mainly cholinesterase (ChE), the enzyme responsible for degradation of neurotransmitter acetylcholine (ACh). Accumulation of acetylcholine (ACh) at the synapses results in excessive stimulation of cholinergic receptors in postsynaptic cells, leading to cholinergic toxicity [3]. In addition to neurotoxicity, the adverse effects of OP exposure may alter other body functions. The ability of OP pesticides to interfere with the immune system and exert immunotoxic effects, through both anti-cholinergic and non-cholinergic methods, has been recognized in recent years [4]. Organophosphates have become the most widely used pesticides since the removal of organochlorine pesticide from use. Consequently, non-target species, including humans working as

occupational workers in roles such as insecticide formulators and farm workers, are frequently affected by organophosphates. The inhibition of ChE is generally regarded as a standard biochemical marker and a mainstay for the fast initial screening of organophosphate and carbamate toxicity [5]. Many studies have shown that oxidative stress may be induced in humans and animals during acute OP exposure alongside the characteristic inhibition of ChE [6,7]. Further, it is evident that antioxidant-rich plant extracts offer protection against OP-induced neurotoxicity, as evident from reduced ChE inhibition [8].

Plants, rich in antioxidants including polyphenolic compounds, tocophenols, and carotenoids, are able to retard the progress of many oxidative stress related chronic diseases in humans. Therefore, antioxidant compounds are potential novel nutriceuticals, due to their role in protecting the body from oxidative stress and nitrosative stress, and are derived either from normal metabolic processes or from external sources [9]. *Thunbergia laurifolia* (Linn) (TL) is a Thai medicinal plant known for its anti-mutagenic, anti-inflammatory, and anti-pyretic properties [10,11]. Tangpong and Satarug stated that TL leaf extract reduced Pb uptake-induced neuronal cell death and memory loss in mice; the anti-oxidant activities of the TL leaf extract might account for these effects [12].

This study aimed to determine the total phenolic content and scavenging properties of the Thai traditional medicinal plant TL against organophosphate (chlorpyrifos)-induced ChE dysfunction *in vitro* (blood and brain of ICR mice) studies. From this perspective, and considering the powerful effects present, the assessment of dietary antioxidants from traditional plants can reduce/prevent chlorpyrifos exposure-induced ChE inhibition.

Materials and methods

Chemicals and preparation of *Thunbergia laurifolia* (Linn.) aqueous extract

Chlorpyrifos (0-0 diethyl 0-3, 5, 6- trichloro-2-pyridyl), and all chemicals, were purchased from Sigma-Aldrich, St. Louis, MO, USA. TL leaves were collected during April-May from Nakhon Si Thammarat, Thailand. Leaves were air dried and ground in a blender to a fine powder. TL leaf powder, 100 g, was extracted with 1,000 mL of boiling water for 15 min. The TL leaf aqueous extracts were filtered by Whatman No.1 and then lyophilized using a freeze dryer at –20 °C for 20 h (Eyela, Tokyo, Japan). The powder was stored at 20 °C until it was used. Chlorpyrifos aqueous stock solution was freshly prepared in distilled water prior to use.

Total phenolic content and total antioxidant capacity of *Thunbergia laurifolia* (Linn.) aqueous extract

The total phenolic content of the extracts was measured according to the Folin-Ciocalteu method [13]. The concentrations of phenolic content in the TL extracts were expressed as gallic acid equivalents (GAEs). Briefly, 12.5 µL of extract of different concentrations (0.1, 0.25, 0.5, and 1 mg/mL) and control (distilled water was used instead of extract) were added to a 96-well microplate. Then, 12.5 µL of Folin-Ciocalteu's phenol reagent was added to each well. After 5 min, 125 µL of saturated sodium carbonate (Na_2CO_3) solution (~7.5 %) was added to the mixture. The reaction mixtures were incubated at room temperature for 30 min. Distilled water was used as a blank. All assays were conducted in triplicate. The absorbance was determined at 765 nm with a microplate reader. Gallic acid solutions with concentrations ranging from 0 to 100 mg/L were used for calibration. A dose response linear regression was generated by using the gallic acid standard absorbance, and the levels in the samples were expressed as gallic acid equivalents (mg of GAEs/mg dry weight).

2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), or ABTS, also forms a relatively stable free radical, which decolorizes in its non-radical form. The analysis of $ABTS^{\cdot+}$ scavenging activity was determined according to the method of Rice-Evan *et al*. [14]. In this method, an antioxidant is added to a pre-formed ABTS radical solution and, after a fixed time period, the remaining $ABTS^{0+}$ is quantified with a microplate reader at 734 nm. $ABTS^{0+}$ was produced by reacting 7 mM ABTS in H_2O with 4.9 mM potassium persulfate ($K_2S_2O_8$) and stored in the dark at room temperature for 12 - 18 h. The $ABTS^{0+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm. Then, 180 µL of $ABTS^{0+}$ solution was added to 20 µL of TL solution in distilled water at different concentrations (0.1 - 1 mg/mL). The

absorbance was recorded 3 min after mixing, and the percentage of radical scavenging was calculated for each concentration relative to a blank containing no scavenger. The extent of decolorization was calculated as percentage reduction of absorbance. For preparation of a standard curve, different concentrations of trolox were used and expressed in millimole of trolox equivalent per gram of dry weight (mM TEAC/g dw).

The correlation coefficients (R^2) of the total antioxidant capacity and the total phenolic content of TL aqueous leaf extract were considered.

Hydroxyl radical assay

Hydroxyl radical assay was performed according to the Fenton-type reaction [15]. The reaction mixture contained 1 mL of 0.1 mM methyl violet, 0.5 mL of 5 mM $FeSO_4$, 0.5 mL of 1 %H_2O_2, and 2 mL of Tris buffer (pH 4.0). 10 mL and 0.5 mL of extract (TL) were then added. The absorbance of the reaction mixture was measured at 565 nm using a spectrophotometer. The scavenging activity (D) of the sample on hydroxyl radicals was calculated according to the following formula;

$$D\text{-}[As\text{-}A0/A\text{-}A0] \times 100\ \% \quad (1)$$

DC_{50} is defined as the concentration of sample required to scavenge 50 % of ROS.

Superoxide anion assay

One hundred µL of 3 mM pyrogallol and 3 mL of Tris buffer (pH 8.2) was mixed with 0.5 mL of the sample at 10 mg/Ml, and the auto-oxidation rate of pyrogallol was measured by determining the changes in the absorbance at 325 nm in 4 min using a UV-Vis spectrophotometer. The scavenging activities (D) of the sample on superoxide anion was calculated by comparing DA1/min (without sample) and DA2/min (with sample) of the pyrogallol system, according to the following formula [15];

$$D = [\Delta A1 - \Delta A2/\ \Delta A1] \times 100\ \% \quad (2)$$

Nitric oxide scavenging capacity assay

The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2), producing $NO^{\bullet}$. Under aerobic conditions, $NO^{\bullet}$ reacts with oxygen to produce stable products (nitrate and nitrite), which can be determined using Griess reagent. The absorbance of the chromophore that forms during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthyl ethylenediamine dihydrochloride can be immediately read at 550 nm. 4 mL of extract or standard solutions of different concentrations were taken in different test tubes, and 1.0 mL of sodium nitroprusside (5 mM) solution was added. They were then incubated for 2 h at 30 °C to complete the reaction. 2 mL solution was withdrawn from the mixture and mixed with 1.2 mL of Griess reagent (1 % Sulfanilamide, 0.1 % naphthylethylene diamine dihydrochloride in 2 % H_3PO_4), and the absorbance of the solutions measured at 550 nm using a spectrophotometer against a blank [16]. Ascorbic acid was used as standard. The percentage (%) inhibition activity was calculated from the following equation;

$$[(A0 - A1)/A0] \times 100. \quad (3)$$

where A0 is the absorbance of the control, and A1 is the absorbance of the extract or standard. IC_{50} was calculated by the linear regression method.

Hydrogen peroxide scavenging effects

The ability of the extract to scavenge hydrogen peroxide was assessed by the method of Dan *et al.* [15]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer. Extracts (TL) at a concentration of 20 µL were added to H_2O_2 solution (80 µL), and the total volume was made up to 100 µL. The absorbance of the reaction mixture was recorded at 230 nm in a spectrophotometer. A blank solution,

containing phosphate buffer without H_2O_2, was prepared. The extent of the H_2O_2 scavenging of the extracts (TL) was calculated;

$$\% \text{ of scavenging of hydrogen peroxide} = [(A0 - A1)/A0] \times 100. \quad (4)$$

where A0 is the absorbance of the control, and A1 is the absorbance of the extract or standard.

Determination of cholinesterase (ChE) activity

The AChE and BChE assays were performed according to the colorimetric method [17,18], using acetylthiocholine iodide and *s*-butyrylthiocholine iodide as substrates. For the enzyme source, mouse brains, red blood cells, and plasma samples were used to determine the activities. The rates of hydrolysis by AChE and BCHE were monitored spectrophotometrically. Each sample or standard solution (500 µL) was mixed with an enzyme solution (500 µL). After incubation at 37 °C for 15 min, the absorbance was measured at 405 nm immediately after adding Ellman's reaction mixture (3.5 ml; 0.5 mM acetylthiocholine iodide, 1 mM DTNB) or *s*-butyrylthiocholine iodide in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. A blank reaction was measured by substituting saline for the enzyme.

Statistical analysis

Data were expressed as mean ± SEM. The data obtained was analyzed using the Student-*t*- test and one way *ANOVA*, which need to compare treatment groups. The p values of ≤ 0.05 were considered to identify statistically significant levels.

Results and discussion

Total phenolic content and antioxidant activity

Table 1 showed that TL aqueous leaf extract (0, 0.05, 0.1, 0.2 mg/mL) contained total phenolic content and total antioxidant capacity. The correlation between the antioxidant activity and the total phenolic content was determined. The antioxidant capacity of the extract appears to be largely influenced by the content of the total phenolic compound ($R^2 = 0.996$) (**Figure 1**).

Table 1 Total phenolic content and total antioxidant capacity of *Thumbergia laurifolia* (Linn.) aqueous leaf extract.

Concentration of *Thunbergia laurifolia* (Linn.) (mg/mL)	**Total Phenolic content (mg GAE/mg dw)**	**Total antioxidant capacity (mM TEAC/g dw)**
0.05	6.11±0.59	206±11.84
0.1	10.12±0.34	390±12.46
0.2	19.3±1.24	694±11.84

Data are presented as mean ± SEM, mgGAE/mg dw; milligram gallic acid equivalent per milligram dry weight, mM TEAC/g dw; millimolar trolox equivalent antioxidant capacity per gram dry weight.

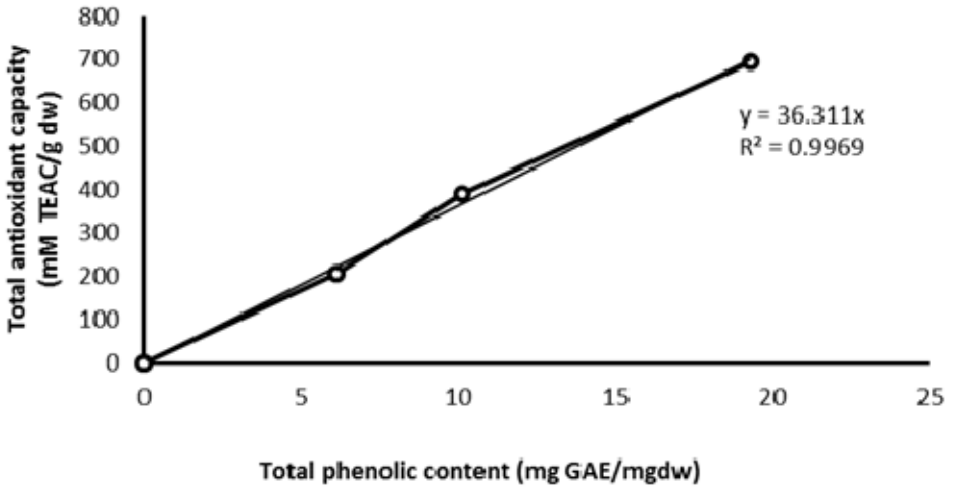

Figure 1 Linear correlation of trolox equivalent antioxidant capacity (mM TEAC/g dw,Y) versus the total phenolic content (mg GAE/mg dw, X) of *Thunbergia laurifolia* (Linn.) aqueous leaf extract.

Radical scavenging activities in cell free system

Table 2 provides a breakdown of selected extracts possessing antioxidant properties which could react with free radicals (hydroxyl, hydrogen peroxide, superoxide, and nitric oxide). In this study, the TL amounts obtained (1.47, 2.23, 1.66 and 1.0 mg/mL) were the amounts of antioxidant material required to scavenge 50 % of free radicals in the hydroxyl radical, superoxide, hydrogen peroxide, and nitric oxide scavenging assay. According to these results, TL aqueous extract showed all radical scavenging activities.

Table 2 Hydroxyl radical, superoxide anion, hydrogen peroxide, and nitric oxide scavenging assay in cell free system.

Free radical	***Thunbergia laurifolia* (Linn.) IC_{50}±SEM (mg/mL)**
Hydroxyl radical	1.47±0.02
Superoxide anion	2.23±0.08
Hydrogen peroxide	1.66±0.09
Nitric oxide	1.0±0.07

Data are presented as mean ± SEM.

Cholinesterase (ChE) *in vitro* test

Organophosphates (chlorpyrifos) are widely used for pest control, and act primarily by inhibition of insect and mammalian ChE. Our findings are consistent with chlorpyrifos significantly inhibiting ChE activity ($p < 0.05$). An inhibition of more than 50 % was observed at concentrations equal to, or higher than, 5 µg/mL (**Figure 2**). Chlorpyrifos at higher concentrations tested *in vitro* (5 and 20 µg/mL) promoted significant decrease in ChE activity (1636 ± 10.39 U/L) and (620 ± 12.45 U/L), respectively.

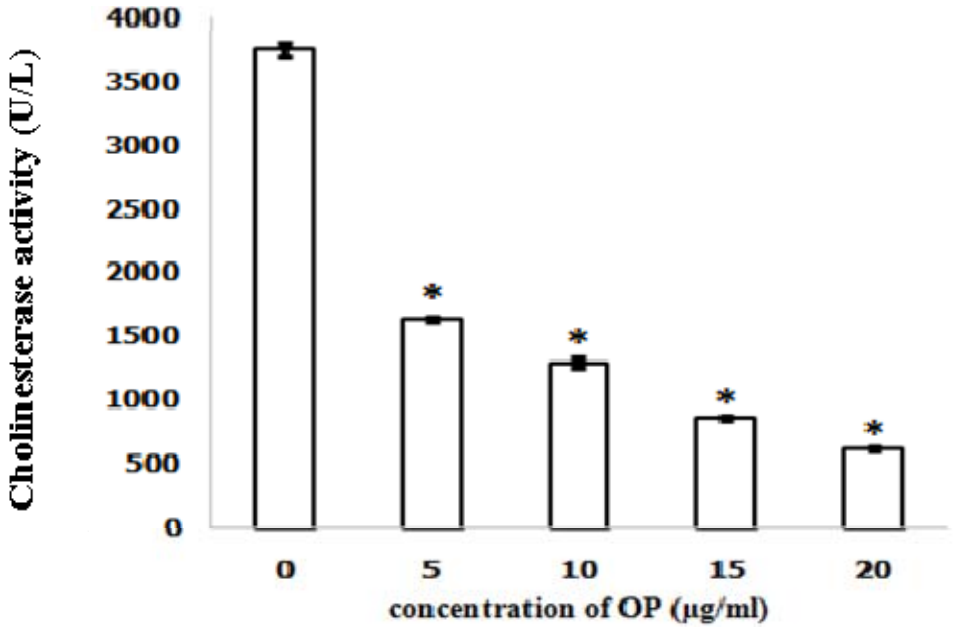

Figure 2 Organophosphate (OP)-inhibited cholinesterase (ChE) activity *in vitro* study. Data are presented as mean±SEM. *$p < 0.05$, significant difference compared between untreated group and OP group.

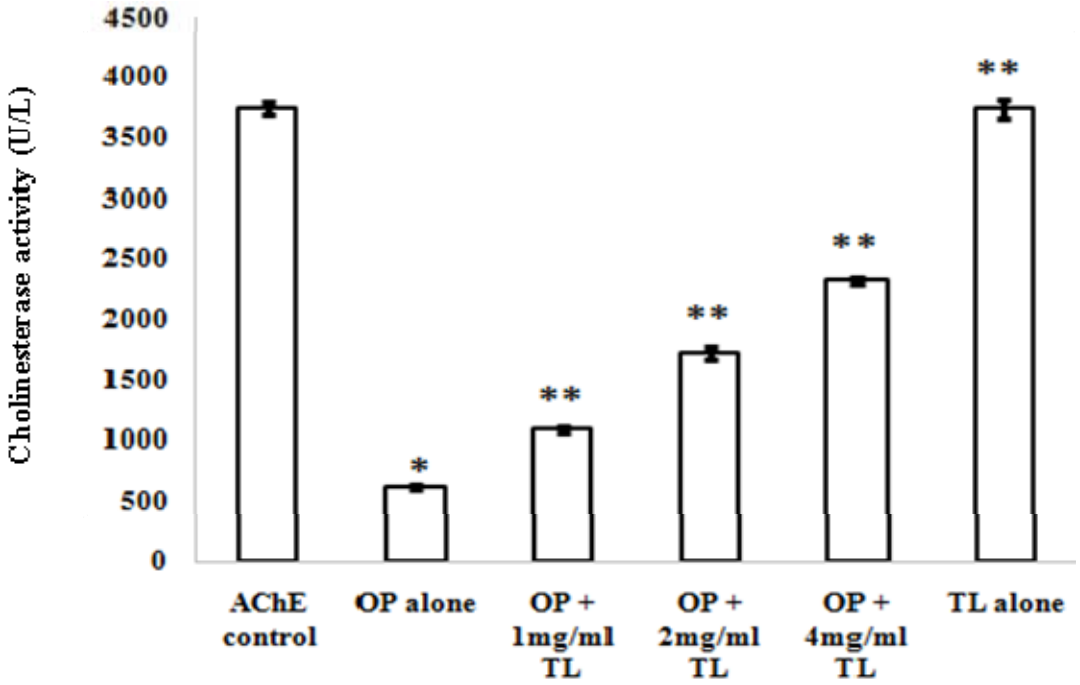

Figure 3 Protective effect of *Thunbergia laurifolia* (Linn.) against Organophosphate (OP)-induced cholinesterase (ChE) activity *in vitro* study. Each value is expressed as mean± SEM. *$p < 0.05$, compared with the control group. **$p < 0.05$, versus OP treated group.

***In vitro* effects of *Thunbergia laurifolia* (Linn.) aqueous extracts against organophosphorous (chlorpyrifos)-induced cholinesterase (ChE) activity**

In order to determine whether the extract can attenuate neurotransmitter system damage, we measured the activities of ChE. According to **Figure 2**, chlorpyrifos at higher concentrations tested *in vitro* studies promoted significant decreases in ChE activity. Interestingly, on the other hand, treatments with extracts restored the activities of ChE ($p < 0.05$) (**Figure 3**). There was no significant difference in the activities of ChE between the control group and the extract-treated group.

***Thunbergia laurifolia* (Linn.) aqueous leaf extract protects cholinesterase activity in chlorpyrifos-treated mice brains and red blood cells**

As shown in **Figures 4** and **5**, the activity of ChE was obviously decreased in mouse brains and red blood cells treated with chlorpyrifos, as compared with the controls ($p < 0.05$). However, there was significantly increase in the activities of ChE in the co-treatment of chlorpyrifos with TL aqueous leaf extract at 2 mg/mL or 4 mg/mL ($p < 0.05$). There is no significant difference between the TL control group and the normal group.

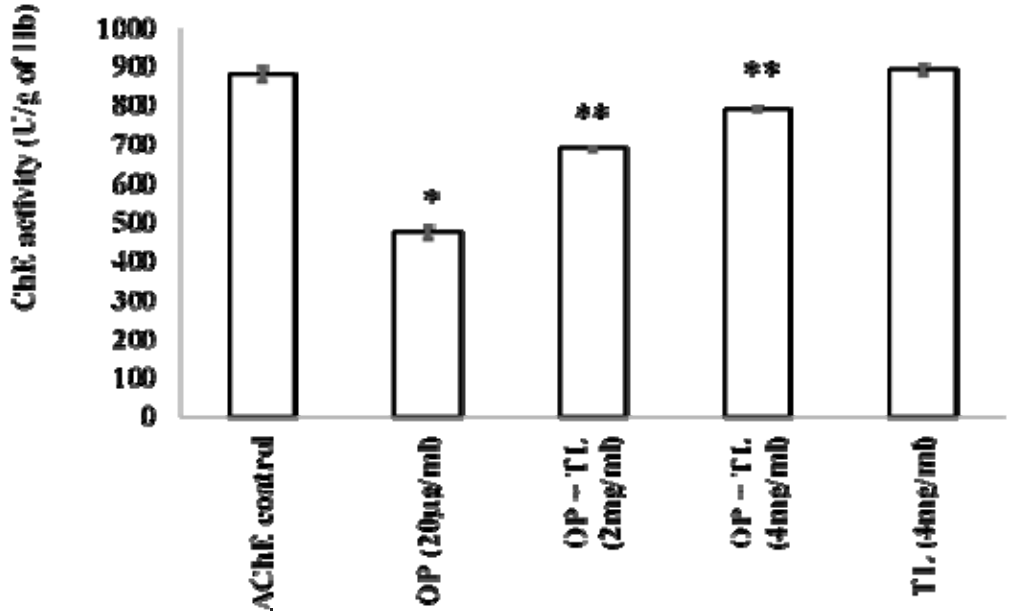

Figure 4 Protective effect of *Thunbergia laurifolia* (Linn.) against organophosphate (OP)-induced cholinesterase (ChE) activity in mouse red blood cells. Each value is expressed as mean± SEM. *$p < 0.05$, compared with the control group. **$p < 0.05$, versus OP treated group.

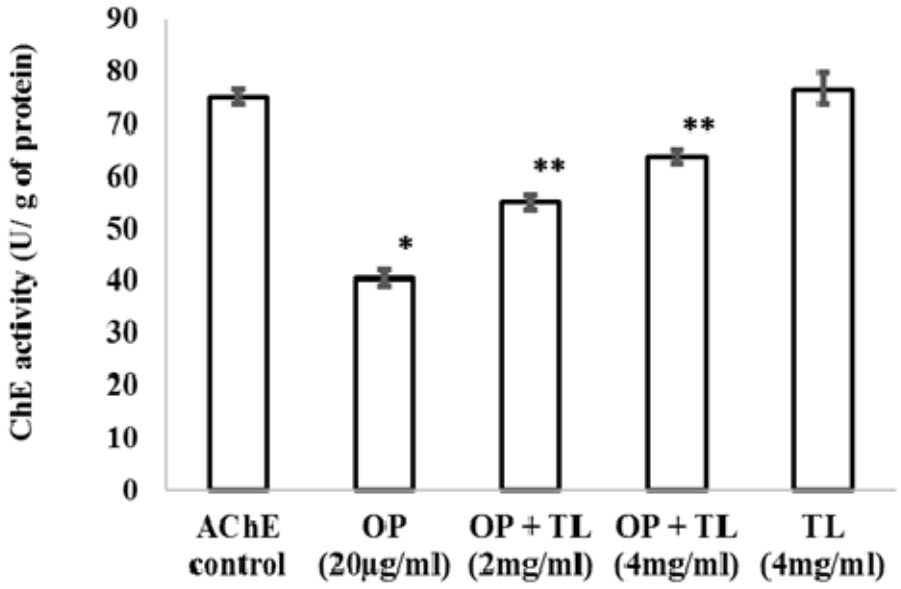

Figure 5 Protective effect of *Thunbergia laurifolia* (Linn.) against OP-induced cholinesterase (ChE) activity in mouse brains. Each value is expressed as mean ± SEM. *$p < 0.05$, compared with the control group. **$p < 0.05$, versus OP treated group.

Discussions

It is well known that phenolic compounds contribute to quality and nutritional value, in terms of modifying color, taste, aroma, and flavor, and also in providing health benefit effects. They also serve in plant defense mechanisms to counteract reactive oxygen species in order to survive, prevent molecular damage, and prevent disruption by microorganisms, insects, and herbivores [19]. However, the correlation between total phenolic content and antioxidant activity is not clear. Our results show that the correlation between the total phenolic content and the antioxidant activity of the extracts had correlation coefficients of $R^2 = 0.996$, 0.97, 0.92, and 0.84. This suggests that the antioxidant capacity of the extracts results from the contribution of the phenolic compounds. They may also have metal chelating potential [20].

Superoxide radicals are generated by direct auto-oxidation of O_2 during mitochondria electron transport reaction. O_2• so formed is catabolized to produce H_2O_2 by superoxide dismutase (SOD), a metalloprotein enzyme. It is considered to be the least reactive type of ROS, and the most commonly produced free radical in humans [21]. However, it is normally formed first, and its effects can be magnified because it produces other kinds of free radicals and oxidizing agents. Hydrogen peroxide has strong oxidizing properties. It can be formed by many oxidizing enzyme, such as SOD, which can cross cellular membranes and may slowly oxidize a number of intracellular compounds. H_2O_2 is reduced to water enzymatically by catalase (in the peroxisomes) and by glutathione peroxidase (both in the cytosol and mitochondria). Hydrogen peroxide itself is not very reactive; however, it can sometimes be toxic to cells, because it may give rise to hydroxyl radical in them [22]. Hydroxyl radicals are the most reactive radical molecules, and can damage cell membranes and lipoproteins by lipid peroxidation. Reactive nitrogen species (RNS, e.g., nitric oxide, $NO^{\bullet}$) play a vital role in the generation of free radicals. $NO^{\bullet}$ is an abundant reactive radical which has a role in diverse physiological processes, such as neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation, and immune regulation. Overproduction of reactive nitrogen species is called nitrosative stress [23]. **Table 2** depicts the scavenging activities of the TL aqueous extract IC_{50}, which shows antioxidant activities,

proving its capacity to scavenge superoxide, hydrogen peroxide, and hydroxyl radical. TL also provided a breakdown, as selected extracts possess antioxidant properties which could react with free radicals (nitric oxide). In this study, TL (1.0±0.07 mg/mL) amounts were obtained, which were the amounts of antioxidant material required to scavenge 50 % of free radicals in the nitric oxide scavenging assay system.

Organophosphorous (chlorpyrifos) (OP) is widely used for pest control, and acts primarily by inhibition of insect and mammalian ChE. OP pesticide has to be designed for enzyme ChE inhibition in a dose dependent manner at the neuromuscular junction in the central nervous system and peripheral nervous system [24]. Our findings are consistent with chlorpyrifos significantly inhibiting AChE activity in a concentration dependent manner. Many reports have indicated that OP compounds cause inhibition of ChE, as well as induce oxidative stress marked by increased lipid peroxidation, altered antioxidant enzyme activities, and reduced glutathione [25,26]. According to the statistical analysis, significant *in vitro* inhibition was obtained from the lowest concentration tested. *In vitro* effects of TL extracts against chlorpyrifos-induced ChE dysfunction showed amelioration of chlorpyrifos-induced ChE inhibition by TL aqueous extracts, which could be due to the high antioxidant activity of its constituents. Green tea extract, which is rich in antioxidants, has been shown to protect against OP compound toxicity [27]; also, antioxidant rich *Decalepis hamiltonii* roots offer protection against OP (dichlorvos)-induced neurotoxicity, as evident from reduced ChE inhibition. The role of antioxidants is to neutralize the excess of free radicals, to protect the cells against their toxic effects, and to contribute to disease prevention [28].

Conclusions

Antioxidant rich extracts offer protection against OP (chlorpyrifos)-induced ChE dysfunction. The exact mechanism of prevention is not clear, but could involve the mitigation of oxidative stress. The antioxidant compounds are potential novel nutriceuticals, and need further experimental investigation for their use as therapeutic agents for treating neurotoxicity involving OP-induced ChE dysfunction.

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