

Increased acetylcholine esterase activity produced by the administration of an aqueous extract of the seed kernel of *Thevetia peruviana* and its role on acute and subchronic intoxication in mice

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ABSTRACT

Background: The real mechanism for *Thevetia peruviana* poisoning remains unclear. Cholinergic activity is important for cardiac function regulation, however, the effect of *T. peruviana* on cholinergic activity is not well-known. **Objective:** To study the effect of the acute administration of an aqueous extract of the seed kernel of *T. peruviana* on the acetylcholine esterase (AChE) activity in CD1 mice as well its implications in the sub-chronic toxicity of the extract. **Materials and Methods:** A dose of 100 mg/kg of the extract was administered to CD1 mice and after 7 days, serum was obtained for ceruloplasmin (CP) quantitation and liver function tests. Another group of mice received a 50 mg/kg dose of the extract 3 times within 1 h time interval and AChE activity was determined for those animals. Heart tissue histological preparation was obtained from a group of mice that received a daily 50 mg/kg dose of the extract by a 30-days period. **Results:** CP levels for the treated group were higher than those for the control group (Student's *t*-test, $P \leq 0.001$). AChE activity in the treated group was significantly higher than the control group (Tukey test, control vs. *T. peruviana*, $P \leq 0.001$). Heart tissue histological preparations showed leukocyte infiltrates and necrotic areas, consistent with infarcts. **Conclusion:** The increased levels of AChE and the heart tissue infiltrative lesions induced by the aqueous seed kernel extract of *T. peruviana* explains in part the poisoning caused by this plant, which can be related to an inflammatory process.

Key words: Acetylcholine esterase, ceruloplasmin, *Thevetia peruviana*, yellow oleander toxicity

INTRODUCTION

Poisonings caused by *Thevetia sp.* are reported widely.^[1,2] A wide variety of bradyarrhythmias and tachyarrhythmias may occur following ingestion of the plant, where *Thevetia* cardiac glycosides seemed to be responsible for the intoxication. Despite those findings, important epidemiological and clinical difference exists between

poisoning due to yellow oleander and digoxin^[3] making the mechanism of *Thevetia* intoxication not yet clear.

Na-K-ATPase inhibition and hyperkalemia are important hallmarks of cardiac glycoside poisoning. Digoxin antibodies had been used as a safe and effective antidote for *Thevetia* poisoning as well-Fructose-1, 6-diphosphate, because of its Na-K-ATPase activity stimulation and potassium efflux inhibition from myocardial cells.^[4] Cardiac glycosides cause Na-K-ATPase inhibition, the high doses of digoxin decrease the acetylcholine (ACh) concentration in the rat heart with no changes in the activity of choline acetyltransferase, acetylcholine esterase (AChE), and the concentration of choline.^[5] On the other hand, it has been reported that the latex of *Thevetia peruviana* caused

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significant reduction in AChE activity in nervous tissue of the freshwater air breathing fish *Channa marulius* in a time and dose dependent response.^[6]

The aim of this work was to study the effect acute administration of an aqueous extract of the seed kernel of *T. peruviana* on the AChE activity in CD1 mice as well its implications on the sub-chronic toxicity of the extract.

MATERIALS AND METHODS

Plant material

The *T. peruviana* fruit kernel used for the study was collected in April of 2010 at the community of Izúcar de Matamoros, Puebla, Mexico. The “Herbarium of Medicinal Plants” from the Mexican Institute of Social Security identified and authenticated the plant samples, and a specimen voucher with the number 15545 was deposited and registered.

Aqueous extract preparation for toxicological studies

T. peruviana seed kernel (130 g) was grounded by an electric grinder, extracted in a Soxhlet apparatus using distilled water, and filtered. The resulting solution (100 mL) was concentrated under reduced pressure at 40°C to yield 4 g of a solid reddish-brown residue, yielding 3.07% w/w.

Animals

Male CD1 mice (25-30 g) were used for this study. The animals were fed with Rodent Lab Chow 5001 (Agribands, Purina, Mexico) and water *ad libitum*. Animals were fasted for 16 h prior to their use for the assays.

Animal care and experimental procedures were carried out according to the Mexican Official Standard (NOM-062-Z00-1999) for the use and care of laboratory animals, which is in accordance with the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Toxicity assays

Acute-one dose assay

A unique 100 mg/Kg dose (0.2 mL/30 g) of aqueous seed kernel extract was administered to 20 mice using an oral cannula (Animal feeding needles, 20G, X1-11/2” Poper and Sons, Inc. Newhyde Park, NJ, USA). The mice were observed daily over 7 days to identify toxic effects: death, diarrhea, salivation, irritability, loss of weight, ataxia, and anesthesia. The control group (20 mice) received isotonic saline solution. On the 7th day, the animals were weighed, anesthetized in an ether chamber, and terminally bled via an armpit incision. Serum was obtained and frozen at -20°C until final use for ceruloplasmin (CP) quantitation and liver function tests.

Acute-three doses assay

A first group of 18 mice received a dose of 50 mg/Kg (0.2 mL/30 g) of the aqueous extract 3 times within 1 h time interval. A second group of 18 mice received a dose of 80 mg/Kg in a 0.2 mL/30 g of methyl parathion (Dragon Micro Foley, Mexico, 40%) three times with 1 h interval. Parathion suspension was prepared in saline-gati gum 1%. A third control group of 18 mice received saline-gati gum 1% (0.2 mL/30 g), three times within 1 h time interval. All animals were administered using an oral cannula. The animals were anesthetized 15 min after the last dose in an ether chamber and after that terminally bled via an armpit incision. A 1 ml of blood sample was collected in a Vacutainer tube with EDTA as anticoagulant. Acetyl cholinesterase activity was determined in whole blood samples.

Sub chronic assay

A group of 20 mice received a daily dose of 50 mg/Kg (0.2 mL/30 g) of the aqueous extract by a 30-days period. A control group of 20 mice received only isotonic saline solution (0.2 mL) by the same period of time. The last day animals were weighed, anesthetized in an ether chamber and terminally bled by an armpit incision; serum was obtained and frozen at -20°C until used. Animal hearts were extracted, immediately weighed on an analytical balance, and the relative weight of the organs was calculated. Hearts were placed in a 10% formaldehyde- Phosphate Buffer Solution (PBS), paraffin embedded, cut in to 5 µm-thick sections using a rotating microtome and stained with H and E.

Liver function tests

Alkaline phosphatase (ALP), gamma glutamyltranspeptidase (gamma-GT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatin kinase (CK), and creatin kinase-MB (CK-MB) were determined using a Instrumental Laboratory (IL) ILab 600 Chemistry analyzer (Diamond Diagnostics, Holliston, MA, USA).

Ceruloplasmin

Values of CP were assessed by radial immuno-diffusion (RID). Rabbit serum anti-mouse CP Immunoglobulin G (IgG) was obtained immunizing New Zealand rabbits with mouse CP purified according to the method of Ehrenwal.^[7] The purity of the CP preparation was further established by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunization of rabbits was carried out. Animals were blood drawn on 45th day and the IgG anti-CP was obtained by Diethylaminoethyl (DEAE) cellulose. Specificity of IgG against CP was evaluated by immuno-electrophoresis were two parallel precipitating bands appeared in α globulin region that correspond to CPI and CII. Plates for RID were prepared with 2.0 mL of melting 1% agarose (Beckton Dickinson) in PBS, cooled at

45°C and 150°C L of rabbit IgG anti-mouse CP was added. The mixture was poured into 35 mm Falcon plates. Mouse CP was used as standard (25, 50, 75 and 100 mg/dL) in a volume of 5 µL. Diameters of standards and samples were measured at 48 h. Samples values were interpolated in a calibration curve. In the intra-assay for serum CP, the variation coefficient was under 5%.

Determination of whole blood AChE

A kinetic method for the determination of mouse erythrocytes AChE, which entails the use of 6,6'-Dithiodinicotinic acid (DNNTA), instead the use of 5,5'-Ditihiobis (2-nitrobenzoic acid), DTNB, as a chromogen was used in the Ellman's reaction.^[8] In this procedure, the hydrolysis of acetyl cholinesterase liberates thiocholine, which reacts with DNNTA to yield thionicotinic acid, this compound has an optimal absorption wavelength at 340 nm. The increase in absorbance at 340 nm is proportional to enzyme activity. The interference from plasma cholinesterase was eliminated by the inclusion of quinidine.^[9] The procedure was carried out as follows: A working solution containing 61.66 mg of DNNTA (Aldrich, 373605), 25.8 mg of quinidine hydrochloride monohydrate (Aldrich, Q0750) and 1 mL of Triton X-100 contained in 1 L of phosphate buffer 100 mmol/L, pH 7.6 was prepared. Acetylthiocholine iodide (Sigma Chemical Company, A5751) 10.5 mmol/L was used as substrate. First, 2.0 mL of the working solution were poured in a screw cap cuvette, 5 µL of whole blood sample was added, mixed and pre-incubated at 30°C for 2 min. Then, 100 µL of the substrate were added and the absorbance increase per minute at 340 nm and at 30°C was determined.

Statistical analysis were conducted using the SPSS (v. 11.0) program.

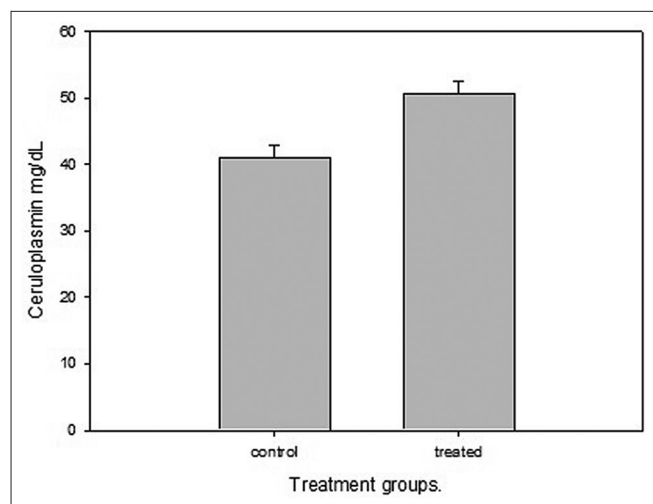


Figure 1: Ceruloplasmin (CP) levels for control and treated animals. Graphic represents the mean and standard error of CP concentrations in mg/dL at column width (Student's *t*-test, $P \leq 0.001$)

RESULTS

The acute-one dose assay (100 mg/kg of weighing) produced important early poisoning symptoms (10 min after treatment). Animals suddenly became quiet and withdrew to the cage corner in a crouching position, showing tachycardia. After that, fine body tremors and pilomotor erection appeared. The animals recovered of tachycardia 20 min after treatment and became very tired. Treated animals showed a significant weight loss. Figure 1 shows the CP levels; the treated group had a significant higher level than the control (Student's *t*-test, $P \leq 0.001$). Control and treated animals showed no statistical difference in ALP, gamma-GT, ALT, AST, CK and CK-MB levels (data not shown).

For the acute-three doses assay (50 mg/Kg of weighing), animals showed poisoning signs from the second dose, which included loss of grip in the hind limbs, muscle twitching became pronounced, tachycardia, and after that a complete paralysis of the hind limbs. The recovery became after 30 min, and animals were observed very tired. After the third dose, the animals developed the previous signs and ataxia in the first 2 min. At this stage, animals became moribund and arrhythmic, and some suffered bradycardia and died. On the other hand, parathion intoxicated animals showed excessive salivation and complete paralysis of the hind limbs, since the second dose. Figure 2 shows the AChE activity levels in the different treatment groups after the third dose. The ANOVA analysis shows a significant difference among groups (Tukey HSD test, control vs. parathion, $P \leq 0.001$; control vs. *T. peruviana*, $P \leq 0.001$; parathion vs. *T. peruviana*, $P \leq 0.001$).

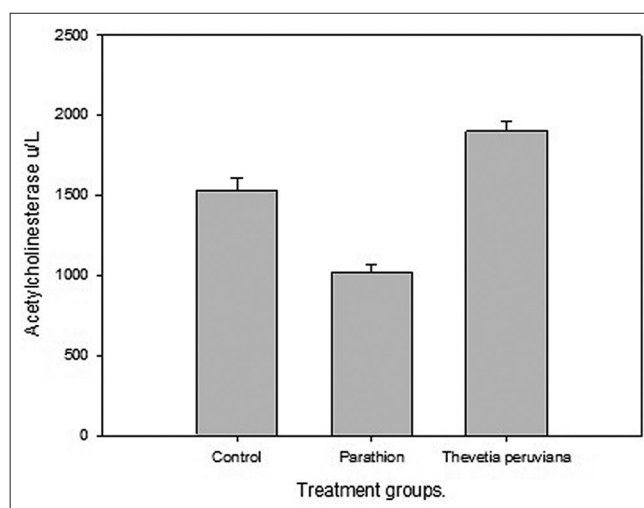


Figure 2: Acetylcholine esterase activity (AChE). Graphics represent the mean and standard error of AChE in U/L at column width (Tukey HSD test $P \leq 0.001$ control vs. parathion; $P \leq 0.001$ control versus *Thevetia peruviana*, $P \leq 0.0001$ parathion versus *T. peruviana*)

For the sub-chronic assay (daily doses of 50 mg/Kg of weighing for 30 days), treated animals showed an important weight loss during the study (data not shown). Figures 3 and 4 show the heart tissue histological observation, treated animals showed important leukocyte infiltrates and necrotic areas in myocardium, consistent with infarcts.

DISCUSSION

For the acute-three dose assay the treated animals showed ataxia after the second dose, and their levels of AChE were higher than in the control and parathion intoxicated groups, leading to a diminution of ACh levels. ACh is the principal vagus neurotransmitter and the receptors that recognize ACh are acetylcholine receptors (AChRs). The two major AChRs in the body are nicotinic (nAChRs) and muscarinic receptors (mAChRs). The mAChR subtype 2 receptor and its signaling cascade has shown to have predominant effects on nodal function (heart rate and conduction block) as well as contractility, and recent evidence suggests that the mAChR subtype 3 receptor may also play an important role in initiation and perpetuation of atrial fibrillation.^[10] The enhanced AChE activity showed as result of the administration of the extract may thus, explain in part previous findings where most of the young patients intoxicated with *T. peruviana* had conduction defects affecting the sinus or atrioventricular nodes and relatively few had atrial or ventricular tachyarrhythmias or ventricular ectopic beats that are typical of digoxin poisoning.^[11]

In addition to its role in cardiac control, the AChRs modulate interactions between the nervous system and the immune system. For instance, AChR agonist, nicotine, dampens inflammation. The cholinergic anti-inflammatory pathway signal through the efferent vagus nerve is mediated primarily by nicotinic Ach receptors on tissue macrophages. This cholinergic anti-inflammatory pathway, mediated by Ach, acts inhibiting the production of TNF, IL-1, Macrophage migration inhibitory factor (MIF), and High-mobility group protein B1 (HMGB1), suppresses the activation of

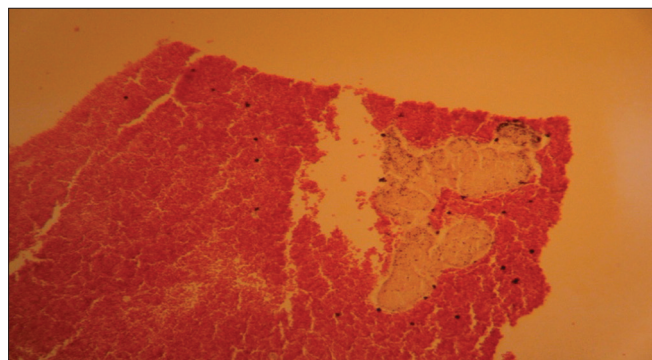


Figure 3: Necrosis areas in myocardium at column width (H and E, $\times 100$)

nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and enhances eNO production.^[12] ACh has a regulatory role on serotonin, dopamine and neuropeptides levels, suggesting that close interactions exists between immunity and neurotransmission.^[13] By the other hand, all AChE inhibitors enhance the activity of cholinergic neurons in the brain and thus, may suppress inflammation. Since ACh is an anti-inflammatory molecule, when AChE concentrations increased ACh levels decrease, and this leads to absence or reduction in the anti-inflammatory actions exerted by ACh. Thus, increased concentration of AChE in plasma CSF, leukocytes, RBC, platelets and other tissues indirectly reflects a reduced concentration of ACh, and an increase in the local and systemic inflammation.^[14] Hence, it is reasonable to propose that AChE activity is increased in all the inflammatory conditions even when plasma, CSF and tissue concentrations of CRP, IL-6, TNF- α , and other markers of inflammation are not appreciably elevated. Thus, increase in the AChE activity in plasma, CSF RBC, leukocytes (including macrophages and T cells), platelets and other tissues is a reliable, unique and specific marker to detect acute, chronic, and low-grade systemic inflammation.^[15]

In the sub-chronic assay, the aqueous extract of *T. peruviana* increased AChE levels, which suggests that one of the plant poisoning mechanisms is the generation of pro-inflammatory cytokines and that response explains in part the high levels of CP in the treated animals. CP is a serum α_2 -globulin considered as an acute phase reactant, where its serum concentration increases during infection, tissue injury, and other pathological states.^[16] The physiological role of CP may include extracellular antioxidant activity by promoting Iron mobilization and preventing metal-catalyzed free radicals tissue damage.^[17] Despite the unknown function of CP, it is likely that the protein is involved in host defense and repair processes mediated by the immune system, namely during

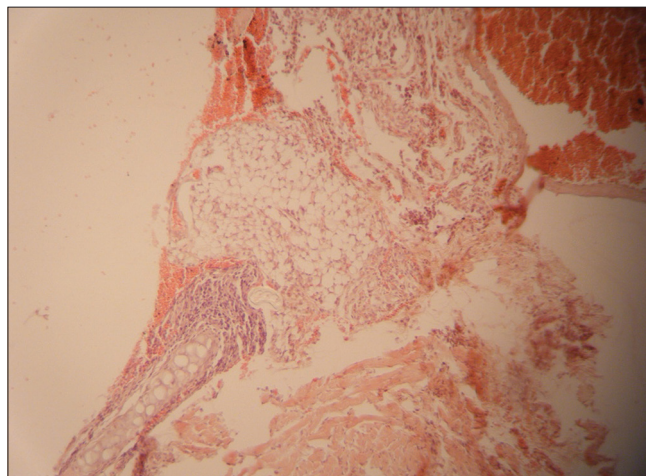


Figure 4: Infiltration of leukocytes in myocardium at column width (H and E, $\times 100$)

inflammation,^[18] and hyperoxia.^[19] In addition, several cytokines and other factors are known to induce CP synthesis by hepatic cells including interferon-gamma,^[20] interleukin-6,^[21] and lipopolysaccharides,^[22] suggesting a strong link between this protein and immune function.^[23]

The results of this study showed also that the administration of the aqueous extract of the seed kernel of *T. peruviana* induces weight loss in mouse models in acute and sub-chronic assays. It is reported that, in addition to the cardiogenic effect of *T. peruviana*, there is an important diuretic effect that may explain the weight loss of the treated groups.^[24]

The increased levels of AChE and the heart tissue infiltrative lesions induced by the aqueous seed kernel extract of *T. peruviana* explains in part the poisoning caused by this plant, which can be related to an inflammatory process.

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