Neuroprotective potential of *Indigofera oblongifolia* leaf methanolic extract against lead acetate-induced neurotoxicity

Saleh Al-Quraishy¹, Mohamed A. Dkhil², Shaimaa R. Ibrahim³, Ahmed E. Abdel Moneim²*  

1 Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia  
2 Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt  
3 Department of Molecular Drug Evaluation, National Organization for Drug Control & Research (NODCAR), Giza, Egypt

**How to cite this article:** Al-Quraishy S, Dkhil MA, Ibrahim SR, Abdel Moneim AE (2016) Neuroprotective potential of *Indigofera oblongifolia* leaf methanolic extract against lead acetate-induced neurotoxicity. Neural Regen Res 11(11):1797-1803.  

Open access statement: This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

**Abstract**

Lead (Pb) is one of the most common environmental toxicants, exposure to which can cause significant neurotoxicity and an associated decline in brain function. This study investigated the possible neuroprotective role of *Indigofera oblongifolia* leaf methanolic extract (IOLME) against lead-induced neurotoxicity. Rats were intraperitoneally injected with lead acetate, with or without IOLME (intragastric administration for 5 days), and the neuroprotective effect of IOLME was assessed by measuring the lead concentration, redox status (lipid peroxidation, nitric oxide and glutathione), enzymatic antioxidant activities (superoxide dismutase, catalase, glutathione peroxidase and reductase), PCR assays of apoptosis markers (Bax and Bcl-2) and histopathology of the brain. The increases in the lipid peroxidation, nitric oxide, and apoptosis, the decreases in the glutathione level and the activity of antioxidant enzymes, and the altered histology of the brain induced by lead acetate were mitigated in the brain of rats pre-treated with IOLME. These findings indicate that IOLME has beneficial effects and it mitigates lead acetate-induced neurotoxicity via its antioxidant and anti-apoptotic activities.

**Key Words:** nerve regeneration; lead acetate, *Indigofera oblongifolia*; neurotoxicity; oxidative stress; apoptosis; neural regeneration

**Introduction**

Lead (Pb) is omnipresent in the environment but non-essential and, indeed, toxic. Once introduced into the body, Pb has a wide-ranging negative effect on many organs, and induces many biochemical, physiological, and behavioural alterations (Abdel Moneim et al., 2011a; Abdel Moneim, 2012). The brain is particularly susceptible to the deleterious effects of Pb, which has been reported to induce damage to the nervous system through several direct and indirect mechanisms. The direct neurotoxic effects of Pb include damage to neuronal mitochondria, leading to programmed cell death, and excitotoxicity through the over-stimulation of the N-methyl-D-aspartate (NMDA) receptor and distribution of the release of neurotransmitters (Sanders et al., 2009).

Oxidative stress has been recognized to be a major indirect mechanism of Pb neurotoxicity (Abdel-Moneim et al., 2012). The induction of oxidative stress is characterized by increased levels of reactive oxygen species (ROS) such as superoxide (O²⁻) and hydroxyl (•OH) radicals, hydrogen peroxide (H₂O₂), and lipid peroxide (Abdel Moneim, 2012). Consistently, Pb has been found on the one hand to restrain sulphydryl dependent enzymes those restrain ROS and interfere with metals such as calcium and, to a lesser extent, zinc, which play a role in the activity of antioxidant enzymes, and, on the other hand, to increase the vulnerability of cells to oxidative attack by modulating the integrity of phospholipids in the membrane and, as a result, the membrane function (Abdel-Moneim et al., 2011; Sadek, 2012).

Medicinal plants are now important targets of drug synthesis. Indeed, as Kartal (2007) has argued, much of the modern pharmaceutical industry is founded on compounds identified from medicinal plants discovered by native peoples and local societies. Various crude extracts and drugs of plant origin have shown promise in the treatment of a range of neurological diseases, and many studies have demonstrated how the antioxidant properties of natural products help to mitigate the toxicity of lead acetate (Abdel Moneim et al., 2011b; Abdou and Hassan, 2014). One plant that has attracted interest in this context is *Indigofera oblongifolia*, a perennial shrub belonging to the family Fabaceae, which is widely distributed in tropical Africa, Asia, Australia and North and South America (Lubbad et al., 2015). Phytochemical studies of *Indigofera oblongifolia* show that it contains indigin, a novel alkylated xanthene, as well as indigoferic acid, indirubin, p-hydroxy (E)-cinnamic acid, beta-sitosterol and 3-hydroxybenzoic acid (Sharif et al., 2005). Sethi et al. (2006)
has gone further to explore the scientific reasons for the anti-inflammatory and anti-cancer activities of Indigofera oblongifolia. All parts of Indigofera oblongifolia are used to treat enlargement of the liver and spleen. Indigofera oblongifolia is also used traditionally in folk medicines to treat infection of the urinary tract and skin, dissolved urinary stones and to relieve coughs (Aggarwal et al., 2011).

To date, however, no pharmacological report is available on whether Indigofera oblongifolia leaf methanolic extract (IOLME) exhibits any neuroprotective effect on Pb-induced neurotoxicity. The current study was therefore planned to utilize a rat model to elucidate whether IOLME, when pre-administered before lead acetate (LA) treatment, can ameliorate Pb-induced neurotoxicity.

Materials and Methods

Materials
Analytical grade chemicals and reagents were used in this study, with the LA trihydrate (CAS Number 6080-56-4) being purchased from Sigma (St. Louis, MO, USA). Double-distilled water was used as a solvent.

Plant material and extract procedure
As part of an ongoing research on the medicinal potential of Indigofera oblongifolia, a survey was conducted to locate Indigofera oblongifolia plants in Jazan Province in southwest Saudi Arabia, as reported by Dkhil et al. (2016). The collected leaves were identified and authenticated by Dr. Jacob Pandalayil (Botany Department, College of Science, King Saud University, Saudi Arabia), before being thoroughly cleaned, air dried under shade at room temperature and powdered using an electrical grinder. The powdered leaves were treated with 70% methanol and kept at 4°C for 24 hours with periodic mixing. After 24 hours, the mixture was filtered and a vacuum evaporator (Heidolph, Schwabach, Germany) was used to evaporate the solvent until it was dry and used in the subsequent experiments. The residues were dissolved in distilled water and stored in an airtight container at −20°C.

Animals and treatment
Nine-week-old healthy male Wistar albino rats, weighing 150–180 g were purchased from VACSERA animal facility (Cairo, Egypt) and housed in the Zoology Department, Helwan University, Cairo, Egypt. The rats were acclimatized for 7 days under standard laboratory conditions of 12 hour light/dark cycles at 25 ± 2°C with free access to pelleted rodent feed and water.

The 28 rats were randomly divided into four groups, with seven rats in each group. The first group (Group I) acted as the control, with each rat in this group receiving 0.3 mL of saline orally, and then, an hour later, an intraperitoneal injection of 100 µL of saline. Groups II (LA group) and IV (IOLME + LA group) received an intraperitoneal injection of LA at 20 mg/kg body weight to induce acute toxicity as described by Abdel Moneim (2012), and groups III (IOLME group) and IV were intragastrically administered IOLME at 0.1 g/kg body weight according to Lubbad et al. (2015). These respective doses were administered daily at the same time for 5 days. In group IV (IOLME + LA group), the IOLME treatment was given no more than an hour prior to the LA injection. All of these protocols and animal handling of this study were approved by the Research Ethics Committee of Zoology Department, Faculty of Science, Helwan University, Egypt for the Laboratory Animal Care and were performed in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals 8th edition (NIH Publication No. 85-23 revised 1985).

Within 24 hour administration of the last treatment, all the animals were killed by decapitation. The brains of the rats were dissected out and blotted free of blood by washing twice in ice-cold 50 mM Tris–HCl, pH 7.4. Each brain was then carefully divided longitudinally into two halves, one for the histopathological study and the other for the molecular and biochemical studies. The second half was divided into two equal parts, one of which was weighed and immediately homogenized in an ice-cold medium of 50 mM Tris–HCl (pH 7.4) to give a 10% (w/v) homogenate. After being centrifuged at 1,000 × g for 10 minutes at 4°C, the supernatants obtained from the homogenates were used for various biochemical investigations. The second part was wrapped in plastic film and kept at −80°C for molecular assays.

Determination of protein content and lead concentration in the rat brains
The total protein content of the homogenized brain was determined using the method of Lowry et al. (1951). Lead concentration in the brain tissue was also determined using a standard method involving drying the tissue samples at 60°C and then heating at 450°C for 24 hours. A hot nitric acid solution (1 M) was then used to dissolve the combusted samples, with the solution being made up to 50 mL with deionized water. The prepared samples were analyzed at 283.3 nm using a Perkin Elmer flame atomic absorption spectrophotometer 3100 (PerkinElmer, Inc., Waltham, MA, USA). Lead accumulation in the brain was expressed as µg/g dry weight.

Oxidative stress in rat brains
The method in Ohkawa et al. (1979) was used to estimate lipid peroxidation (LPO) in the brain by determining the amount of malondialdehyde (MDA) formed. Briefly, the homogenized brain was mixed with 1 mL of 0.67% thiobarbituric acid and 1 mL of 10% trichloroacetic acid in a bath of boiling water for 30 minutes. The absorbance at 535 nm was measured to determine thiobarbituric acid-reactive substances (TBARS), with the result representing the level of MDA. The amount of nitric oxide (NO) present in the brain was measured using the optimized acid reduction method of Green et al. (1982). In this method, the brain homogenate was immersed in an acidic medium such that, when nitrite was added, nitrous acid diazotise sulphanilamide coupled with N-(1-naphthyl) ethylenediamine was formed. The resultant azo dye with its bright reddish-purple colour, could

Figure 2 The neuroprotective effects of *Indigofera oblongifolia* leaf methanolic extract (IOLME) pre-administration on oxidative stress markers (TBARS, NO, and GSH) in the brain tissue of rats treated with lead acetate (LA) for 5 consecutive days. Values are expressed as the mean ± SEM (*n* = 7). *P* < 0.05, vs. control; #P < 0.05, vs. LA (one-way analysis of variance followed by Duncan’s test). TBARS: Thiobarbituric acid reactive substances (a byproduct of lipid peroxidation); NO: nitric oxide; GSH: glutathione.

Figure 3 The protective effects of *Indigofera oblongifolia* leaf methanolic extract (IOLME) pre-administration on the activity of antioxidant enzymes in the brain tissue of rats treated with lead acetate (LA) for 5 consecutive days. Values are expressed as the mean ± SEM (*n* = 7). *P* < 0.05, vs. control; #P < 0.05, vs. LA (one-way analysis of variance followed by Duncan’s test). SOD: Superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GRd: glutathione reductase.

Figure 1 The neuroprotective effects of *Indigofera oblongifolia* leaf methanolic extract (IOLME) on lead accumulation in the brain tissue of rats treated with lead acetate (LA) for 5 consecutive days. Values are expressed as the mean ± SEM (*n* = 7). *P* < 0.05, vs. the control; #P < 0.05, vs. LA (one-way analysis of variance followed by Duncan’s test).

be measured at 540 nm. Finally, glutathione (GSH) was assayed using the method of Ellman (1959).

**Antioxidant status of rat brains**

Superoxide dismutase (SOD) in rat brain is able to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. This ability enables the activity of the enzyme to be assayed. Catalase (CAT), meanwhile, was estimated by quantifying the H$_2$O$_2$ consumed after the enzyme reaction, measuring at 340 nm for 120 seconds at 20 second intervals. Glutathione reductase (GRd) activity was measured indirectly by measuring the ability of the enzyme to catalyse the reduction of glutathione in the presence of NADPH. The associated decline in absorbance was measured at 340 nm. Glutathione peroxidase (GPx) activity in the brain was measured indirectly based on the ability of the enzyme to oxidize GSH into GSSG (oxidized glutathione). The GSSG was recycled again to its reduced state by GRd in the presence of NADPH, the disappearance of which was measured at 340 nm.

**Quantitative real-time PCR**

Total RNA was extracted from the brain using a RNeasy Plus Minikit (Qiagen, Valencia, CA, USA). cDNA synthesis was performed using the RevertAid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fisher Scientific Inc., Ottawa,
Results

Pb2+ concentration in the brain

The data in Figure 1 shows that Pb2+ concentration in the brain tissue was significantly elevated in the LA intoxicated group (Group II) compared with the control rats (P < 0.05). However, significantly reduced Pb2+ concentration in the brain was determined in the IOLME + LA group (Group IV) compared to the LA-intoxicated group (Group II) (P < 0.05).

LA-induced oxidative stress in the brain

Injection of LA also caused a significant elevation in LPO and (NO levels in the brain (P < 0.05). Meanwhile, a significant (P < 0.05) decrease in the GSH content in the brain was found in rats treated with LA when compared to the control group. Lipid peroxidation and NO levels in brain tissue were not more significantly changed in rats treated only with IOLME compared to the controls. Interestingly, pre-treatment with IOLME significantly decreased the lipid peroxidation and NO levels and raised GSH levels in the brain tissue (Figure 2).

The results illustrated in Figure 3 demonstrate that LA injections decreased the activities of SOD, CAT, GPx and GRd (P < 0.05). Pre-treatment with IOLME, however, significantly attenuated the inhibition of antioxidant enzyme activities as compared to rats treated with lead alone. Consistent with these biochemical results, the qRT-PCR findings showed that the expression levels of SOD2, CAT, and GPx1 mRNA in the brain tissue were down-regulated after exposure to LA but when the rats were pre-treated with IOLME, expression levels of these genes were increased significantly (Figure 4).

LA-induced apoptosis in the brain

We also investigated the anti-apoptotic effects of IOLME in the brain of rats exposed to LA. The expression levels of Bcl-2 and Bax in the brain were determined. The qRT-PCR results showed that the Bcl-2 mRNA expression level was markedly down-regulated (Figure 5), unlike Bax that up-regulated in the LA-treated group (P < 0.05). The pre-administration of IOLME, however, enhanced Bcl-2 expression compared with that in the LA-treated group and restrained Bax expression in the brain tissue.

Histological examination

The brain was fixed in 10% formaldehyde/PBS for 24 hours at room temperature, and then prepared into 4–5 μm thick paraffin-embedded sections. These were stained with hematoxylin-eosin and imaged on a Nikon light microscope (Eclipse E200-LED, Tokyo, Japan).

Statistical analysis

Statistical Package for the Social Sciences (SPSS; Version 20, IBM Corporation, Armonk, NY, USA) was used for data analysis. The results were expressed as the mean ± SEM. One way analysis of variance (ANOVA) followed by Duncan’s test was applied for determining the significance. The acceptable level of significance was established at P < 0.05.

Discussion

This study aimed to investigate whether IOLME has a neuroprotective property towards the neurotoxicity introduced by LA. Our data show that, in rats, (a) IOLME does indeed have a neuroprotective property towards LA-induced neurotoxicity and neuronal apoptosis. (b) This effect can be attributed to the antioxidative and anti-apoptotic activities of IOLME, mediated by the promotion of antioxidant enzymes and induction of the anti-apoptotic protein, or by restraining the free radical production induced by LA.

Both animal and human studies have suggested that exposure to Pb is associated with increased oxidative stress and a heightened incidence of neurotoxicity. The increase in lipid peroxidation observed in our study is consistent with earlier studies (Jusko et al., 2008; Abdel Moneim, 2012; Ashafaq et al., 2016). Pb exerts some of its neurotoxic effects by promoting oxidative damage and peroxidation of the lipids in the cell membranes, thus compromising cellular functions by impairing the physicochemical properties, fluidity, and integrity of cell membranes, thereby increasing the cell vulnerability to lipid peroxidation and cell death (Dkhil et al., 2016). Pre-administration of IOLME significantly inhibited LPO production in the brain, however, and thus acted to combat lipid peroxidation. IOLME has previously been reported to restrain LPO in different body organs, including the kidney and liver (Shahjahan et al., 2005). It is, however, not clear whether such an effect could be due to the scavenging property of IOLME for reactive species or due to neutralization of free radicals.

The findings of our study also demonstrated an increased level of NO upon exposure to LA. This is probably due to the ability of Pb to promote inducible nitric oxide synthase, which serves to increase the rate of NO production. Furthermore,
Figure 4 The protective effects of *Indigofera oblongifolia* leaf methanolic extract (IOLME) pre-administration on mRNA expression of SOD2, CAT, and GPx1 genes in the brain tissue of rats treated with lead acetate (LA) for 5 consecutive days.

Values are expressed as the mean ± SEM (n = 7). *P < 0.05, vs. control; #P < 0.05, vs. LA (one-way analysis of variance followed by Duncan’s test). SOD: Superoxide dismutase; CAT: catalase; GPx: glutathione peroxidise.

Figure 5 The protective effects of *Indigofera oblongifolia* leaf methanolic extract (IOLME) pre-administration on mRNA expression of Bax and Bcl-2 genes in the brain tissue of rats treated with lead acetate (LA) for 5 consecutive days.

Values are expressed as the mean ± SEM (n = 7). *P < 0.05, vs. control; #P < 0.05, vs. LA (one-way analysis of variance followed by Duncan’s test).

Figure 6 Effect of *Indigofera oblongifolia* leaf methanolic extract (IOLME) on the histological alterations due to lead acetate (LA) injection in rat brains (hematoxylin-eosin staining, original magnification × 400).

Sections of control rats show normal architecture in the different parts of the brain. Sections from the LA-treated group, however, show extensive neuronal damage (white arrows), apoptotic neurons (red arrows) and degeneration of Purkinje neurons without basophilia (black arrows). Sections of the IOLME-treated groups show a normal structure except for a few degenerative neurons in the cerebral cortex (white arrows). Sections of brains where IOLME was administered to the animal prior to LA treatment show slight degenerated neurons (white arrows).
the increased ROS levels associated with Pb can also promote inflammation by stimulating the transcription factors such as nuclear factor kappa B, thus activating inducible nitric oxide synthase in microglial cells; this may explain the elevated levels of NO observed in the brain in our study (Shen et al., 2014). The hazards associated with NO are greatly increased when it forms the highly reactive peroxynitrite anion (ONOO−) by reacting with the superoxide radical (O2−).

The oxidative stress induced by LA in the brains of the studied rats was correlated with a significant depletion of GSH. The possible mechanism for this depletion could be connected to the central role of GSH in Pb biotransformation through bile, whereby Pb binds to the -SH group of GSH and is then excreted. This process serves to deplete GSH, however, which could then lead to oxidative damage and a consequent promotion of LPO (Ponce-Canchihuan et al., 2010). Additionally, the depletion in the GSH content in the brain reported in the current study may be due to the increased utilization of GSH-GPx in the detoxification of H2O2 generated by LA (Flora et al., 2012). Our findings are in accordance with Bokara et al. (2009), who demonstrated that Pb treatment modified the glutathione status, i.e., the formation of glutathione, reduced form (GSH); a decline associated with the accumulation of glutathione, oxidized form (GSSG). Interestingly, we found that IOLME administration markedly restored the GSH content and decreased LPO levels in the brains of LA-treated rats. This suggests that IOLME can minimize oxidative damage to some degree by restoring GSH content and decreasing the lipid peroxide level.

The current study also confirmed that intoxication of rats with LA disrupts the redox balance, as indicated by a reduction in SOD, CAT, GPx, and GRd activities. Our findings are in agreement with previous reports that have demonstrated that Pb disrupts redox homeostasis (Akande et al., 2016; Dkhil et al., 2016; Hasanein et al., 2016). Furthermore, GPx, CAT and SOD are potential targets for Pb neurotoxicity because these enzymes depend on other necessary co-factors (cations) for their proper molecular structure and function (Dua et al., 2016). Interestingly, however, IOLME partly ameliorated the disruptive effects of LA on the antioxidant enzyme system. The enhancement of antioxidant enzyme activities could be explained by either a compensatory mechanism of IOLME against oxidative stress, or antioxidant gene overexpression, or both (Hashish et al., 2015).

The primary negative effects of Pb on brain function are thought to be due to its propensity to damage the microvasculature of the nervous system (Deveci, 2006). In the present study, while all the examined regions were affected in rats exposed to LA, the cerebellum, striatum and cerebral cortex were the most affected regions. Similar results were found when new-born Long–Evans and adult Wistar rats were exposed to LA (Abdel Moneim et al., 2011b; Barkur and Bairy, 2016). The appearance of the condensed nucleus seen in our study reflects a certain phase of apoptosis since they display a markedly condensed cytoplasm and nucleoplasm (Ratan et al., 1994). Furthermore, the dark and small neurons were usually ischemic due to substantial abnormalities in the capillary wall with subsequent disorders in the structural elements of the blood–brain barrier. Lastly, the distorted shrunken neurons seemed to be a result of structural and functional dysfunction resulting from biosynthesis of cell proteins; nucleic acids, certain enzymes and various neurotransmitters (Afifi and Embaby, 2016). IOLME pre-administered to rats prevented such alterations.

Apoptosis is a physiological process described as a gene-regulated episode that is governed by a number of pro- and anti-apoptotic genes expressing homologous proteins of the Bcl-2 family, such as Bax and Bcl-2 (Dua et al., 2016). The ratio of Bax to Bcl-2 is associated with the survival or death of cells following an apoptotic insult, with an elevated ratio potentially triggering apoptosis and targeting the cells for death (Abdel Moneim, 2016). In the current study, quantitative real-time PCR analysis revealed a significant elevation in Bax and a decline in Bcl-2 levels in the LA group, suggesting oxidative stress induced intrinsic apoptosis mediated by mitochondria. Supporting our findings, Rong et al. (2008) demonstrated that LA promotes apoptosis in PC12 culture cells, which may be connected to up-regulation of nuclear factor-kappa B and p53 genes and down-regulation of the Bcl-2 gene. The apparent protection against this process afforded by IOLME may be due to suppression of intracellular ROS production/neutralization and/or promotion of anti-apoptotic gene expression.

In conclusion, our findings demonstrate that IOLME has a neuroprotective effect. The inhibition of LA-induced neurotoxicity by IOLME is due, at least in part, to its antioxidant and anti-apoptotic activities. However, the metal chelating efficacy of IOLME remains to be elucidated.

Acknowledgments: The authors would like to thank the Deanship of Scientific Research at King Saud University for funding the work through the research group project, No. PRG-1436-02.

Author contributions: SA, MAD and AEAM conceived the study. AEAM participated in study design, sample collection and statistical analysis, and drafted the paper. SRI prepared plant material and drafted the paper. All authors read and approved the final version of this paper for publication.

Conflicts of interest: The authors declare that they have no competing interests.

Supplementary information: Supplementary data associated with this article can be found, in the online version, by visiting www.nrronline.org.

Plagiarism check: This paper was screened twice using CrossCheck to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

References

1802


