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Curative effect of *plumbago indica* root extract on thioacetamide induced hepatotoxicity in experimental rats

Eldhose Binil[1,2], Kuria Kose Jayesh[1], Latha mukalel sankunni[1]*

***Corresponding author:**

Latha Mukalel Sankunni

[1]School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala-686560, India
[2]Department of Radiation Medicine, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington DC 20057, USA.

Abstract

The root parts of *Plumb ago indicia* is widely used in the Indian Ayurvedic traditional system. The study was designed to evaluate the hepato curative activity Plumb ago *Indica* (PLBM) methanolic root extracts at different doses (100 and 200 mg/kg bw) against thioacetamide induced liver damage in albino wistar rats. Liver damage was induced by administration of Thioacetamide (100 mg/kg bw) and was assessed by evaluating the activity of liver-function marker enzymes, antioxidant enzymes and lipid peroxidation. The liver sections were evaluated for histopathological changes. PLBM significantly (p 0.05) reversed the elevation of serum liver enzymes and hepatic levels of antioxidant markers. Histopathological findings also confirmed the curative effect of *Plumb ago Indica* in a dose dependent manner. Thus this study scientifically rationale the use of *Plumbago indica* root as a promising drug against various liver disorders.

Keywords: *Plumb ago indica,* antioxidant, hepatotoxicity, curative effect, oxidative stress, thioacetamide.

Introduction

Natural dietary antioxidants are considered as therapeutic proxies to prevent oxidative stress-related disorders [1,2,3]. Tissues that undergo oxidative stress are vulnerable to free radical production which leads to critical failure of biological functions and alter the imbalance between productions of bodily antioxidant defense mechanism, resulting in tissue damage [4,5]. Thus free radicals, from both endogenous and exogenous sources, may ultimately lead to chronic ailments such as cancer, diabetes, neurodegenerative disorders and ageing [6]. Recently synthetic antioxidants are replaced by natural antioxidants due to its less side effects and toxicity [7]. Physicians and patients are thus in need of effective therapeutic agents with low incidence of side effects. Plants potentially constitute such groups and there is a worldwide trend to go back to traditional medicinal plants [8].

Plumbago indica also known as *Plumbago rosea* is one of the common plants used in Ayurveda system of medicine. Its root extract is widely used due to its medicinal property such as antibacterial [9], antifungal [10], antiparasitic [11,12], antifertility [13] and antitumor activities [14]. Our previous studies revealed the protective efficacy of *P. indica* root extract against thioacetamideinduced oxidative stress [15]. However no comprehensive evidence has yet been documented for the hepatocurative activity of *P. indica* experimentally. Here in this paper, the curative efficacy of methanolic root extract of *P. indica* (PLBM) in combating oxidative stress induced by thioacetamide is described.

Materials and Methods

Chemicals and Reagents

Thioacetamide (TAA) and Silymarin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Animals and diets

Male albino Wistar rats weighing 158 ± 6.3 g (mean ± S.D, n = 30) were used in this study. Animals were housed in polypropylene cages, given standard rat chow (Sai Feeds, Bangalore, India) and drinking water, and maintained under controlled temperature (26-28 ºC), with a 12 h light/12 h dark cycle. Animal studies were performed according to Institutional Animal Ethics Committee (IAEC) regulations approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Reg. no.B23032014-01) and conducted humanely.

Preparation of plant extract

P. indica roots were cleaned, chopped, shade-dried and powdered. Dried powder (50g) was soxhlet extracted with 400 ml of methanol for 48 h. Methanolic extracts (yield 16.83%) were concentrated

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under reduced pressure using a rotary evaporator. Extract was suspended in water to respective dosage and kept under 4 ºC refrigeration.

Preparation of doses and treatments

TAA suspended in normal saline was administered at 100 mg/kg body weight (bw) subcutaneously (sc) to induce oxidative stress in rats [16].Silymarin, at an oral dose of 100 mg/kg bw, was used as standard control in the experiment. Different doses (100 and 200 mg/kg b.w) of PLBM was suspended in distilled water were also prepared for oral administration to the animals.

Experimental design

Rats were divided into five groups with six rats in each group and that were treated as follows:

Group I: Control rats (vehicle only)
Group II: Thioacetamide control (100 mg/kg, s.c.)
Group III: Thioacetamide (as in group II) + Silymarin (100 mg/kg, p.o.)
Group IV : Thioacetamide (as in group II) + PLBM (100 mg/kg, p.o.)
Group V: Thioacetamide (as in group II) + PLBM (200 mg/kg, p.o.)
All groups except group I received a single dose of TAA (100 mg/kg; s.c.) on 1st day of the experiment. Groups III–V received Silymarin and PLBM at24 and 48 h after the TAA challenge. Group I animals were used as vehicle controls received normal saline instead of drug and TAA. All animals were sacrificed 72 h after TAA administration.

Estimation of marker enzymes

Hepatotoxicity was assessed by quantifying the serum levels of aspartate transaminase (AST) (EC.2.6.1.1), alanine transaminase (ALT) (EC.2.6.1.2), alkaline phosphatase (ALP) (EC.3.1.3.10) and lactate dehydrogenase (LDH) (EC.1.1.1.27) by a kinetic method using a kit from Agape Diagnostic Ltd., India. Activities of these serum enzymes were measured by using a semi-autoanalyser (RMS, India).

Tissue analysis

Liver was excised and washed thoroughly in ice cold saline. Ten percent of the livers weight was used to prepare homogenates in 0.1 M Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 3000 rpm for 20 min at 4ºC, and the supernatant was used for the estimation of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH) lipid peroxidation (as thiobarbituric acid reactive substance (TBARS) and conjugated dienes (CD).

Tissue CAT (EC.1.11.1.60) activity was determined from the rate of decomposition of H_2O_2[17]. GPx (EC 1.11.1.9) activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3[17]. GR (EC 1.6.4.2) activity was assayed at 37 ºC by following the oxidation of NADPH by GSSG at 340 nm[18]. GST (EC 2.5.1.18) activity was determined from the rate of increase in conjugate formation between reduced glutathione (GSH) and 1-chloro-2,4-dinitro benzene (CDNB) [19]. GSH was determined on the basis of the formation of yellow colored complexes with 5,5-Dithiobis (2-nitrobenzoic acid) DTNB[20].The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1'1'3'3' tetramethoxypropane as standard [21].

Histopathological analysis

Small pieces of liver tissues were fixed in 10% buffered formalin and processed for embedding in paraffin. Sections of 5-6 µm were cut and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany) by taking microphotographs using a Moticam 1000 camera at the original magnification of 100X. Liver sections were graded numerically to assess the degree of histological features in acute hepatic injury. Centrilobular necrosis is the necrosis around the central vein characterized by the prominent ballooning, swollen granular cytoplasm with fading nuclei. Bridging hepatic necrosis is a form of confluent necrosis of liver cells linking central veins to portal tracts or portal tracts to one another [22].

Statistical analysis

The results of the analysis were expressed as mean ± SD and all statistical comparisons were made using one-way ANOVA tests followed by Turkey's post hoc analysis, and *P*- values over than or equal to 0.05 were considered significant.

Results

Effect of PLBM and Silymarin post-treatment on changes in serum enzyme levels of rats intoxicated with TAA

A significant (p 0.05) increase in the serum levels were found in AST, ALT, ALP, and LDH in group II by the administration of a single dose of TAA, when compared to normal control. Treatment with PLBM at doses of 100 and 200 mg/kg showed a significant decrease of AST, ALT, ALP and LDH (p 0.05). Standard control drug, silymarin at a dose of 100 mg/kg also reversed the elevation of serum enzymes (Table 1). Treatment with 100 mg/kg of PLBM and silymarin exhibited a curation of 83.1% and 87.8%in AST levels, 81.1% and 75.5% in ALT levels, 68.4% and 63.1% in ALP levels and 86.5% and 85.14% in LDH levels respectively. This shows an indication of an establishment of the regeneration process. Among the two doses of PLBM, 100mg/kg has shown maximum effect which was almost comparable to those of the normal control and silymarin.

Table 1. Curative effects of *Plumbago indica* methanolic extract on changes in serum enzyme levels of rats treated with TAA. Values are mean ± S.D, n = 6 animals. † p 0.05 vs. normal control. * p 0.05 vs. Thioacetamide control.

GROUPS	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	LDH (IU/L)
Normal control	196 ± 0.12	70 ± 0.03	370 ± 0.53	1233 ± 0.04
TAA (100mg/kg b.w)	410 ± 0.66†	160 ± 0.02†	484 ± 0.43†	2431 ± 0.02†
Silymarin (100mg/kg,*sc.)*+ TAA	222 ± 0.64*	92 ± 0.04*	412 ± 0.04*	1411 ± 0.21*
PLBM (100mg/kg b.w)+ TAA	232 ± 0.19*	87 ± 0.41*	406 ± 0.05*	1394 ± 0.30*
PLBM (200mg/kg b.w)+ TAA	219 ± 0.71*	95 ± 0.07*	414 ± 0.02*	1404 ± 0.83*

Effect of PLBM on the measurement of liver antioxidant parameters

In order to find out the effect of PLBM on liver antioxidant status, the activities of non-enzymic antioxidants GSH and enzymic-antioxidant such as GR, GPx, SOD, CAT were measured (Table 2). The activities of non-enzymic and the levels of enzymic antioxidant were significantly (p 0.05) decreased in rats administrated with TAA alone. Administration of 100 mg/ kg of PLBM and silymarin significantly increased to 93.6% and 58.2% for G.R, 85% and 54.4% for GPx, 95.3% and 64% for GSH, 81.4% and 70.8% for SOD, 79.3% and 70.8% for CAT significantly. Among the two doses of PLBM, 100mg/kg has shown maximum restored activity which was almost comparable to those of the normal control and silymarin.

Table 2. Curative Effects of PLBM against TAA Induced Changes in the liver antioxidant Status. Values are mean ± S.D, n = 6 animals. † p 0.05 vs. normal control. * p 0.05 vs. Thioacetamide control.

GROUPS	GSH (nmol/mg protein)	GR (nmol of GSSG utilized/min/mg protein)	GPx (nmol of GSH oxidized/min/mg protein)	SOD(enzyme required for 50% inhibition of NBT reduction)	CAT (U/mg protein)
Control	26.4 ± .0.65	22.38 ± 0.63	34.41 ± 4.5	8.23 ±0.98	36.26± 4.46*
TAA (100mg/kg,*s.c*	13.6 ± 0.34†	8.07 ±0.37†	15.48 ± 1.56†	2.29 ±0.36†	21.99 ± 8.72†
Silymarin (100mg/kg b.w)+ TAA	21.8 ± 0.42*	16.4 ± 0.42*	25.79 ± 1.56*	6.50 ± 0.12*	34.15 ± 412*
PLBM (100mg/kg b.w)+ TAA	25.8 ±0.21*	21.56 ± 0.90*	31.62 ± 2.56*	7.09± 0.63*	33.32± 8.12*
PLBM (200mg/kg b.w)+ TAA	23.9 ±0.44*	19.11 ± 0.15*	30.71 ± 3.91*	7.13 ± 0.45*	30.31 ± 4.11*

Effect of TAA and PLBM on the levels of lipid per oxidation and conjugate dienes in the liver of control and experimental rats.

The results of hepatocurative effect of PLBM on TAA- intoxicated rats are shown in Table 3 which illustrates a significant raise (p 0.05) in the concentration of TBARS and CD during TAA treatment as compared with the vehicle control. Administration of 100 mg/kg of PLBM along with TAA resulted in a drastic decline (p 0.05) of TBARS and CD in the liver compared with the corresponding TAA treated group. Silymarin and 200 mg/kg of PLBM also lowered the level of TBARS and CD when compared with the TAA treated group (group II).

Table 3. Curative Effects of PLBM against the levels of lipid peroxidation and conjugated dienes in the liver of control and experimental rats. Values are mean ± S.D, n = 6 animals. † p 0.05 vs. normal control. * p 0.05 vs. Thioacetamide control.

GROUPS	TBARS (mmol/mg tissue)	CD (mmol/mg tissue)
Normal control	0.54 ± 0.04	51.08 ± 6.21
TAA (100mg/kg,*sc.)*	0.83± 0.05 †	74. 12± 7.72 †
Silymarin (100mg/kg b.w) + TAA	0.62±0.03 *	59.2 ± 3.46 *
PLBM (100mg/kg b.w) + TAA	0.56± 0.05*	52.83 ± 6.81 *
PLBM (200mg/kg b.w) + TAA	0.6± 0.07*	52.83 ± 6.81*

Histopathological analysis

Histopathological examination of liver sections of normal animals showed normal cellular architecture with distinct bile duct, prominent cytoplasm and central vein. (Figure 1.a). The liver sections of the rats of TAA group exhibited hepatic cell with severe toxicity with high degree of damage, characterized by the centrilobular necrosis, focal necrosis and scattered infiltration across liver parenchyma and

hemorrhage. (Figure 1.b). The sections of the rats treated with the PLBM (100 and 200 mg/kg) which is intoxicated with TAA exhibited less centrilobular necrosis and bile duct proliferation compared to the TAA control (Figure 1.d and 1.e). Among this 100 mg/kg of PLBM showed better activity than the 200 mg/kg of PLBM. The standard silymarin at 100 mg/kg intoxicated with TAA, almost restored the normal architecture of the liver with slight centrilobular fatty changes, necrosis and bile duct proliferation. (Figure 1.c).

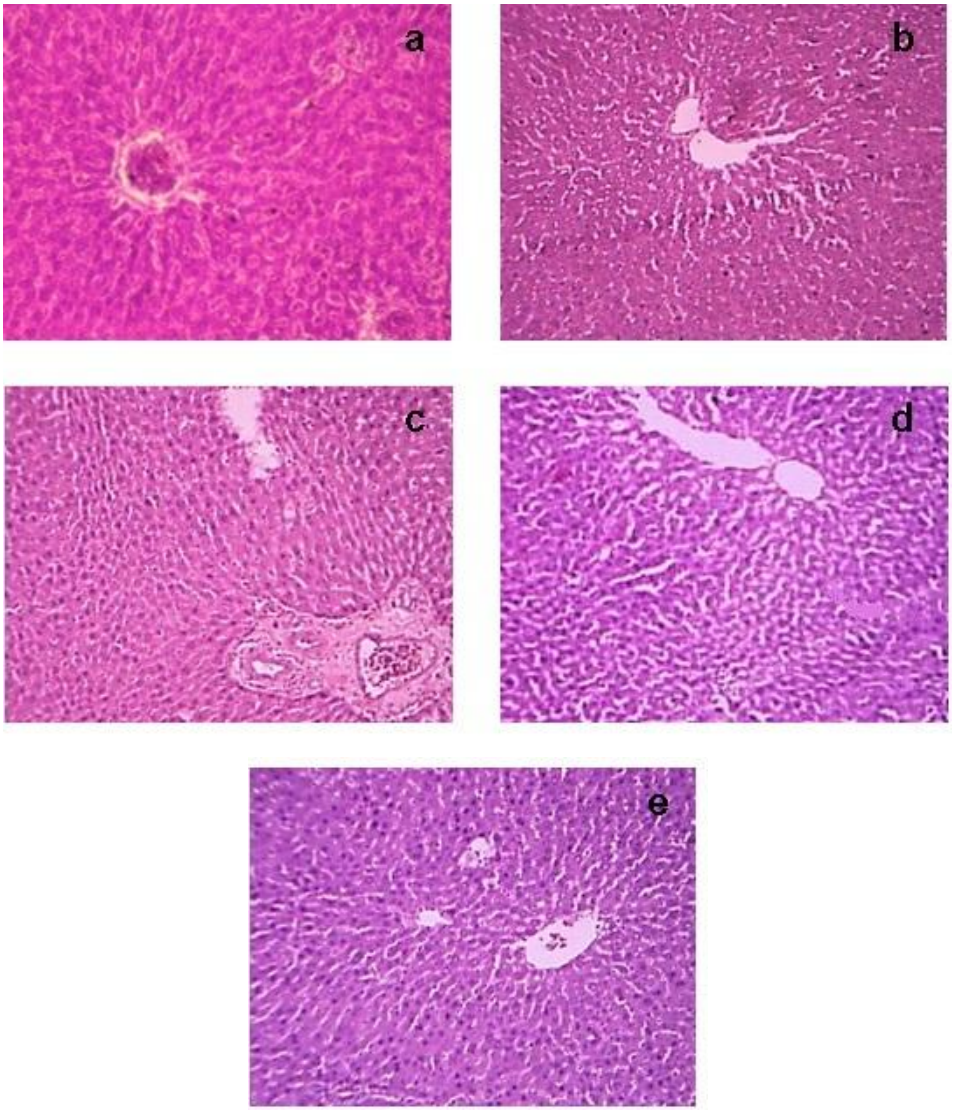

Figure 1. Histopathological changes occurred in rat liver due to post-treatment with PLBM and standard drug Silymarin (hematoxylin and eosin, 100x). (a) Normal control; (b) TAA control (100mg/*kg,sc.*); (c) Silymarin (100mg/kg) + TAA;(d) PLBM (100mg/kg) + TAA; (e) PLBM 200mg/kg) + TAA).

Discussion

Hepatotoxic injury is considered as one of the most perspective and remarkable indicators of thioacetamide administration. TAA is an organic solvent with thiono sulfur components have been used widely to induce liver cirrhosis [23]. The mechanism of TAA toxicity is due to the formation of TAA- S- oxide, which is responsible for the change in cell permeability, increased intracellular concentration of Ca^{++}, increase in nuclear volume and enlargement of nucleoli, and also inhibits the mitochondrial activity which leads to cell death [24].

In the present study, the administration of TAA significantly (P 0.05) elevated the levels of serum ALT, AST, ALP and LDH in rats treated with TAA. This is due to the increased permeability of plasma membrane or cellular necrosis, leading to the leakage of the enzymes to the blood stream [25].The administration of PLBM (100and 200 mg/kg/b.wt) resulted in the stabilization of these

enzymes, which demonstrates the curative effect of PLBM on TAA intoxication.

Free radical oxidative damage can contribute to acute hepatitis [26].To prevent oxidative damage in the cell, a variety of antioxidant scavenge free radicals are essentials. The intracellular antioxidant system comprises of different free radical scavenging antioxidant enzymes along with some non- antioxidants like GSH and other thiols. CAT, SOD, GR and GPx constitute the first line of cellular antioxidant defense system. Therefore, these antioxidants are expected to be consumed by enhanced radical reactions. When excess free radicals are produced, this equilibrium is lost and consequently oxidative imbalance is established [27]. The non-enzymatic antioxidant defense system protects the aerobic organisms from deleterious effect of the active oxygen metabolites. GSH is an important endogenous antioxidant system found to have key function in protective processes and its low concentration has been implicated in TAA induced hepatitis and its depletion closely related to the lipid peroxidation and disturbance of Ca^{2++} induced by toxic agents [28]. An observed increase in tissue GSH content in treated group show that PLBM tends to cure the tissue depletion of GSH, in post treatment groups of hepatic and renal tissues. GR activity levels in hepatic and renal tissues of post – treatment groups shows significantly (P 0.05) elevated levels, which illustrate the role of PLBM to maintain the level of GSH in tissue, since GR is essential to maintain the level of GSH. GPx catalyzes the GSH dependent reduction of H_2O_2 and other peroxides and protects the organism from oxidative damage [29]. PLBM and Silymarin significantly (P 0.05) showed significant restoration of GPx activity after post-treatment in liver and kidney. This might be due to the antioxidant activity by detoxifying endogenous metabolic peroxides generated after TAA injury in hepatic and renal tissues.

SOD which converts superoxide radicals to H_2O_2 is widely distributed in cells having oxidative metabolism and is believed to protect cells against the toxic effects of superoxide anion [30]. Superoxide anions are known to exert destructive effects on cellular components with LPO being one such consequence. CAT is a heme protein, which catalyses the direct degradation of hydrogen peroxide to water. It protects the cellular constituents against oxidative damage. Reduced activity of SOD and CAT after exposure to TAA could be correlated to increased generation of H_2O_2. PLBM and Silymarin significantly (P 0.05) maintained the SOD and CAT activity near to the normal level in both hepatic and renal tissues of post- treated rats.

There was an increase in the levels of lipid peroxidation (LPO) in tissue after TAA administration. The increase in the levels of TBARS and CD indicate enhanced LPO leading to tissue injury and failure of the antioxidant defense mechanism to prevent the formation of excess free radicals [31]. Treatment with PLBM causes significant decrease in the levels of TBARS and CD in rats intoxicated with TAA suggesting that PLBM may exert a stabilizing action on hepatic tissues, demonstrating the potent antioxidant effects on post- treated rats. Histopathological evaluation also supported the efficacy of the extract as an anti-hepatotoxic agent which is due to its antioxidant activity.

Conclusions

The present study concluded that the root extract of *Plumbago indica* is a safe and significant antioxidant which is able to reduce and counter the negative effects of increased oxidative stress produced by TAA. This antioxidant activity of the drug could be attributed to the presence of polyphenolic compounds and flavonoids in its extracts. However further molecular level of work is required for the mechanism of action of drug, which is under way.

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