



Received on 22 July, 2013; received in revised form, 05 November, 2013; accepted, 19 November, 2013; published 01 December, 2013

EFFECTS OF *PREMNA INTEGRIFOLIA* LINN. ROOTS EXTRACTS IN CCL₄ INDUCED TOXICITY IN RATS

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Keywords:

Premna integrifolia, Antioxidants, CCl₄, Oxidative stress

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ABSTRACT: *Premna integrifolia* Linn. roots are used in the Indian system of medicine for the treatment of diabetes, inflammation, liver disorders etc. The present study was aimed to investigate the antioxidant activity of *Premna integrifolia* L. (Family: Verbenaceae) in CCl₄ (carbon tetrachloride) induced oxidative stress in Sprague-Dawley rats. CCl₄ injection induced oxidative stress by a significant rise in thiobarbituric acid reactive substances (TBARS) along with reduction of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione-s-transferase (GST) and glutathione reductase (GRD). Pretreatment of rats with different doses of aqueous and methanolic extract (250 and 500 mg/kg) significantly lowered TBARS levels in CCl₄ treated rats. GSH and hepatic enzymes like SOD, CAT, GRD, and GST were significantly increased by treatment with the plant extracts, as compared to the CCl₄ treated rats. The activity of the extracts at the dose of 500 mg/kg was comparable to the standard drug, silymarin (25mg/kg). The results showed that *Premna integrifolia* extracts protect liver from oxidative stress induced by CCl₄ in rats.

INTRODUCTION: Exogenous factors such as sunlight, ultraviolet light, ionizing radiations, some chemicals and normal cellular activities lead to the production of reactive oxygen species (ROS). The major types of ROS or ROS producing species are superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl (OH) radicals¹. ROS present a paradox in the biological function: on one hand, they prevent diseases by assisting the immune system, mediating cell signaling and playing an essential role in apoptosis.

On the other hand, they can damage many biologically active molecules and hence lead to tissue damage and cell death^{2,3}.

In cells, there are several antioxidant defense mechanisms that functionally assist in preventing the destructive effects of various types of ROS.

These defense mechanisms include antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and vitamins C and E.

Deficiency or abnormality in any of these leads to over production of ROS followed by oxidation of biomolecules like proteins, lipids and DNA. At normal physiological condition, a delicate balance exists between the rate of generation and consumption of free radicals^{4,5}.

<p>QUICK RESPONSE CODE</p> 	<p>DOI: 10.13040/IJPSR.0975-8232.4(12).4697-03</p> <hr/> <p>Article can be accessed online on: www.ijpsr.com</p> <hr/> <p>DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.4(12).4697-03</p>
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In abnormal cases, particularly when the rate of production exceeds the rate of consumption, it causes potentially serious health consequences⁶.

There has been a tremendous increase in demand of herbal drugs due to their wide biological activities. They have higher safety margin and low costs than the synthetic drugs. In case of allopathic medicine, complete cure is not achieved and tolerance may be developed. Moreover they are often associated with serious side effects^{7,8}. Many natural products are in use for the treatment of liver ailments.

Polyphenolic compounds are widely distributed in plants and are known to possess excellent antioxidant activity and have the capacity to scavenge free radicals and provide antioxidant defense^{9,10}.

Premna integrifolia Linn. (Verbenaceae) also called as "Arni" is a well-known plant used in the traditional Indian system of medicine. The anti-diabetic, anti-inflammatory, hepatoprotective, immunomodulator and a cardio tonic properties have been reported in earlier studies¹¹⁻¹³. It is also used in the treatment of piles, constipation and fever. It contains alkaloids, carbohydrates, tannins, phenolic compounds and flavonoids. The alkaloids premnine, ganikarine and premnazole alkaloids are reported from roots, while flavonoids luteoline, sterols, and triterpenes are reported from the leaves¹¹. The present study was undertaken to investigate the antioxidant activity of aqueous and methanolic extracts of *P. integrifolia* roots in carbon tetrachloride induced (CCl₄) liver injury in rats.

MATERIALS AND METHODS: All chemicals used were of analytical grade. Thiobarbituric acid (TBA), n-butanol and hydrogen peroxide (H₂O₂) were obtained from Merck. Acetic acid was obtained from RFCL. Nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediamine tetra acetic acid (EDTA), bovine serum albumin (BSA), hypoxanthine, KCl, nitro blue tetrazolium (NBT), xanthine oxidase, NaOH and trichloro acetic acid (TCA) were obtained from Loba Chemie, India. 5, 5'-dithio, bis (2-nitrobenzoic acid) was obtained from HIMEDIA. Silymarin was obtained from AMSAR Pvt. Ltd. India.

Plant material: *Premna integrifolia* roots were collected from the Pharmacognosy Garden of Timba Ayurvedic Pharmacy College, Timba, Gujarat. They were identified and authenticated at Department of Botany, Govt. Agriculture College, Indore. A voucher specimen (No. SCOPE/Phcog/07-09/06) has been deposited in the museum of our department for further references.

Extraction: The roots were dried under shade, coarsely powdered and passed through Sieve No. 18. The powder was extracted using methanol and water with the help of Soxhlet and reflux respectively. The extracts were lyophilized and stored in vacuum desiccator for experimental use.

Animals: Experiments were performed on adult Sprague Dawley rats weighing 180-200g. They were kept under standardized conditions (temperature 21-24°C and a light/dark cycle of 12hours/12hours) and fed a normal laboratory diet. The institutional animal ethics committee approved the study (IAEC/SCOPE/08-09/05).

Acute toxicity study: Acute toxicity study of *P. integrifolia* (LD₅₀) was determined according to the OECD (organization of Economic Co-operation and Development) guidelines 425- Fixed Dose Procedure. The animals were divided into two groups, each consisting of five mice. The defined or fixed dose level of aqueous and methanolic extracts (2000mg/kg) was given orally to identify a dose producing evident toxicity. The animals were observed continuously for 2 hours for behavioral, neurological and autonomic profiles. The toxicity signs were observed after 24 hours till fourteen days for any lethality or death.

Experimental procedure: Seven days after acclimatization, the rats were divided into seven groups having six rats in each group. Group 1 served as normal and was given the vehicle (saline). Group 2 received a single dose of equal mixture of carbon tetrachloride and olive oil (50%, v/v, 1mL/kg i.p.) on the 7th day. Groups 3 and 4 were treated with aqueous extracts at dose levels of 250 and 500 mg/kg per day p.o., respectively, for 7 days. Group 5 and 6 were treated with methanolic extracts at dose levels of 250 and 500 mg/kg per day p.o., respectively, for 7 days. Group 7 was treated with standard drug, silymarin (25 mg/kg per day p.o.) for 7 days.

The animals of groups 3-7 were also administered with a single dose of equal mixture of carbon tetrachloride and olive oil (50%, v/v, 1mL/kg i.p.) on the seventh day¹⁴.

Preparation of Liver Homogenate: The animals were dissected after cervical dislocation under ether anesthesia and the liver was excised out. The liver was then weighed and homogenized in KCl (10mM), phosphate buffer (1.15%) with ethylenediamine tetra acetic acid (EDTA; pH 7.4) and centrifuged at $12,000 \times g$ for 60 minutes. The supernatant was used for further studies¹⁴.

Determination of Lipid Peroxidation: Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction method¹⁴. Acetic acid 1.5ml (20%, pH 3.5), 1.5ml of thiobarbituric acid (0.8%) and 0.2ml of sodium dodecyl sulphate were added to 0.1ml of supernatant and heated at 100°C for 60 minutes. The mixture was cooled and 5ml of n-butanol- pyridine (15:1) mixture and 1ml of distilled water were added and vortexed vigorously after centrifugation at $1200 \times g$ for 10 minutes. The organic layer was separated and the absorbance was measured at 532 nm using a UV-Visible spectrophotometer.

Determination of Antioxidant Enzymes: The activity of SOD was measured according to the method of Zheleva (2005)¹⁵. In glass tubes 200 μ l hypoxanthine (0.075mg/ml of PBS), 20 μ l EDTA (0.085mM), 400 μ l NBT (0.05mg/ml of PBS), 100 μ l of supernatant isolated from rat liver homogenates, 880 μ l PBS buffer (pH 7.4) and 400 μ l xanthine oxidase (0.25mg/ml of PBS) were added to a final volume of 2 ml. Tubes were stirred, stoppered and incubated at 37°C for 20 min. After incubation, samples were put on ice and absorbance was measured at 560 nm.

Catalase, Reduced glutathione, Glutathione-s-transferase, Glutathione reductase activities were estimated according to the methods reported by Mukherjee (2007)¹⁴.

For Catalase activity, 0.1ml of supernatant was added to a cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by addition of 1.0ml of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically at 240 nm.

For Reduced glutathione activity, to 0.01ml of supernatant, 2ml of phosphate buffer (pH 8.4), 0.5ml of 5, 5'-dithio, bis (2-nitrobenzoic acid) and 0.4 ml double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm.

For Glutathione-s-transferase activity, the reaction mixture consisted of 2.75ml of sodium phosphate buffer (0.1M; pH 7.4), 0.1ml reduced glutathione (1mM), 0.1ml supernatant in a total volume of 3.0ml. The changes in absorbance were recorded at 340 nm.

For Glutathione reductase activity, the assay system consisted of 1.65 ml sodium phosphate buffer (0.1M; pH 7.4), 0.1ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1ml NADPH (nicotinamide adenine dinucleotide phosphate) (0.1 mM), and 0.05 ml supernatant in a total mixture of 2 ml. The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm at 30 sec intervals for 3 minutes.

Estimation of Total Proteins: Protein concentration was estimated according to the method of Lowry *et al* (1954)¹⁶. 0.5 ml of tissue homogenate was mixed with 0.5 ml of 10% trichloro acetic acid (TCA) and centrifuged for 10 min. The precipitate obtained was dissolved in 1.0 ml of 0.1 N NaOH. From this an aliquot was taken for protein estimation. 0.1 ml of aliquot was mixed with 5.0 ml of alkaline copper reagent and allowed to stand at room temperature for 10 min. 0.5 ml of Folin's phenol reagent was added and the blue color developed was read after 20 min at 640 nm. The amount of total protein content was calculated using a standard curve of BSA.

Statistical analysis: The results obtained in the above studies were reported as Mean \pm S.E.M. The total variations present in a data were analyzed by one way analysis of variance (ANOVA) followed by Dunnett test. For this, window based STAT software was used.

Histopathology: Conventional techniques of paraffin-wax sectioning and haematoxylin-eosin staining were used for histological studies¹⁷. Slices of fresh liver tissue were cut and fixed in buffered neutral formalin fixative for 24h.

Following fixation, the tissues were washed and processed through an ascending series of alcohol (30%, 50%, 70%, 90% and 100%), cleared in methyl salicylate and infiltrated with wax at 57°C. The tissues thus cleared were embedded in paraffin. Sections of 6-8 µm thickness were cut, stained by aqueous haematoxylin alcohol-eosin and examined under a microscope at 10x magnification

RESULTS:

- 1. Acute Toxicity Study:** There was no toxic reaction found and from the results, obtained doses of 250 and 500mg/kg, b.w. were selected for further experiments.
- 2. Lipid peroxidation:** Malondialdehyde (MDA) level is widely used as a marker of free radical mediated lipid peroxidation injury. MDA levels were measured in the liver and results are shown in **Table 1**. MDA level in CCl₄ treated group was significantly increased ($p < 0.001$) when compared to normal group. Treatment with aqueous, methanolic extracts and silymarin showed significant decrease ($p < 0.001$) in

MDA levels in a dose dependent manner when compared with CCl₄ treated group.

- 3. Antioxidant enzymes activity:** SOD, CAT, GSH, GST and GR were measured as an index of antioxidant status of tissues. Significantly lower liver SOD, CAT, GSH, GST and GR were observed in rats in CCl₄ treated groups as compared to the normal group. There was a significant increase ($p < 0.001$) in the levels of SOD, CAT, GSH, GST and GR in the groups treated with aqueous and methanolic extracts at a dose of 250 and 500mg/kg, as compared to the CCl₄ treated group. The standard drug, silymarin treated rats also showed significant increase ($p < 0.001$) in the enzymes level when compared to the CCl₄ treated groups.
- 4. Histopathology:** The histology studies supported the above finding. The degenerative changes like inflammation of cells and necrosis were found in the hepatocytes of CCl₄ induced liver toxicity in rats. Administration of extracts showed recovery of the liver to near normal architecture (**figures 1-7**).

TABLE 1: EFFECTS OF *P. INTEGRIFOLIA* EXTRACTS ON ANTIOXIDANT ENZYMES IN CCl₄ INDUCED LIVER DAMAGE IN RATS

Group (n=6)	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GSH (µmol/mg protein)	GST (µmol/mg protein)	GR (nmol/mg protein)	Total protein (g/dl)
Normal	198.685±2.173**	6.177±0.202**	73.733±0.562**	6.535±0.211**	4.821±0.049**	82.162±0.668**	6.725±0.149**
Control(CCl ₄) 1ml/kg	504.702±3.471	2.510±0.225	42.385±0.495	1.888±0.151	1.204±0.137	37.508±0.552	3.192±0.147
Aq. ext. 250mg/kg	397.5±3.672**	3.597±0.133**	47.73±0.520**	2.763±0.090**	2.343±0.156**	55.950±0.323**	4.002±0.161**
Aq. ext. 500mg/kg	335.541±2.093**	4.114±0.151**	54.682±0.468**	4.017±0.097**	2.756±0.072**	65.155±0.616**	4.524±0.075**
MeOH ext. 250mg/kg	369.412±3.451**	3.896±0.091**	51.241±0.709**	3.160±0.120**	2.593±0.072**	59.012±0.642**	4.248±0.053**
MeOH ext. 500mg/kg	293.139±2.352**	4.827±0.109**	58.794±0.792**	4.554±0.107**	3.332±0.090**	68.992±0.487**	4.812±0.105**
Standard (Silymarin) 25mg/kg	236.462±1.972**	5.360±0.157**	68.187±0.444**	5.865±0.111**	4.215±0.091**	78.006±0.618**	5.538±0.089**

Values are expressed as mean ± SEM, n = No. of animals in each group. **P < 0.001 vs. control

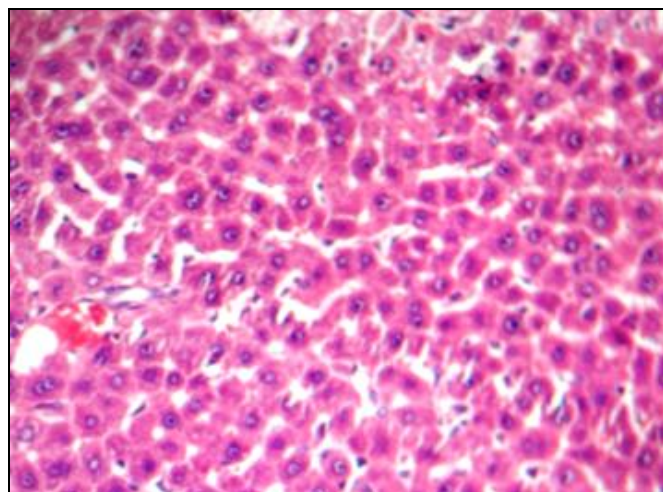


FIGURE 1: LIVER OF NORMAL RAT SHOWING NORMAL CELLULAR ARCHITECTURE

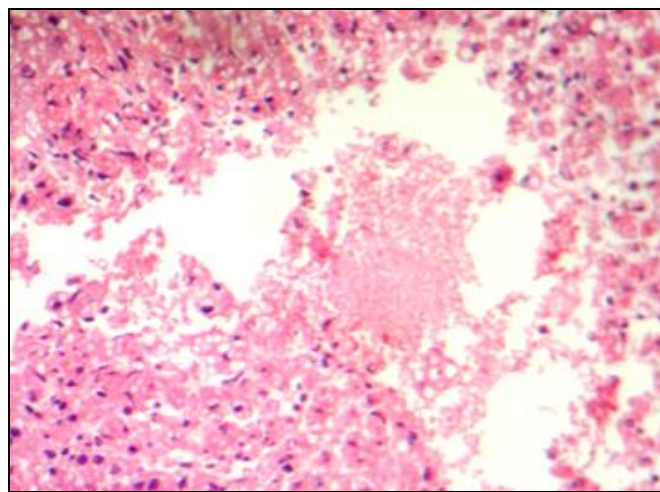


FIGURE 2: LIVER OF CCl₄ TREATED RAT SHOWING EXTENSIVE DAMAGE

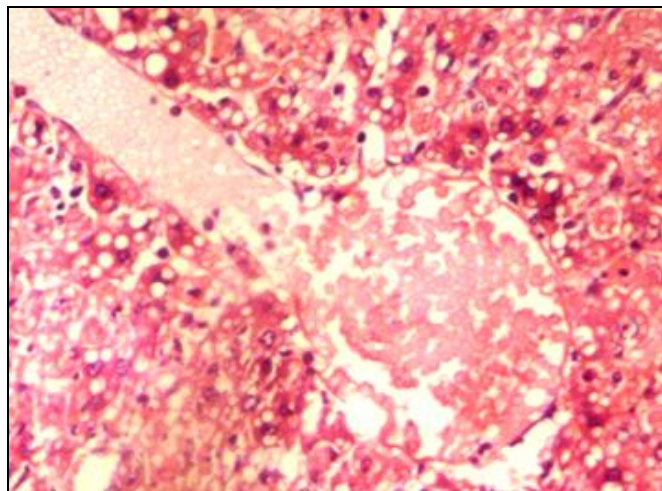


FIGURE 3: LIVERS OF RATS TREATED WITH AQUEOUS EXTRACT (250mg/kg) SHOWING RESTORATION TO NEAR NORMAL CELLULAR ARCHITECTURE

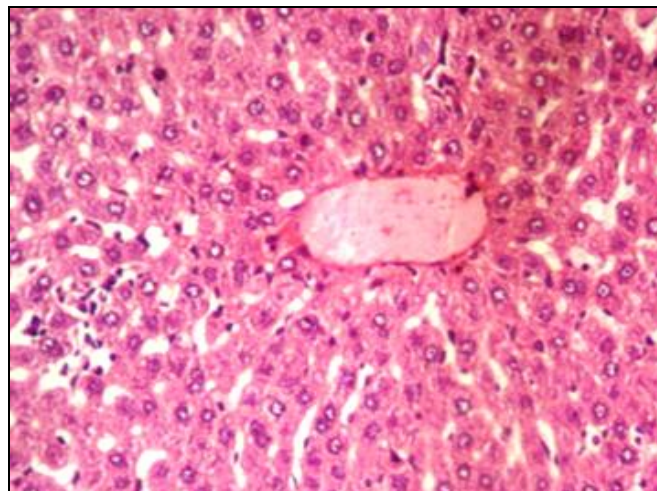


FIGURE 6: LIVERS OF RATS TREATED WITH METHANOLIC EXTRACT (500mg/kg) SHOWING RESTORATION TO NEAR NORMAL CELLULAR ARCHITECTURE

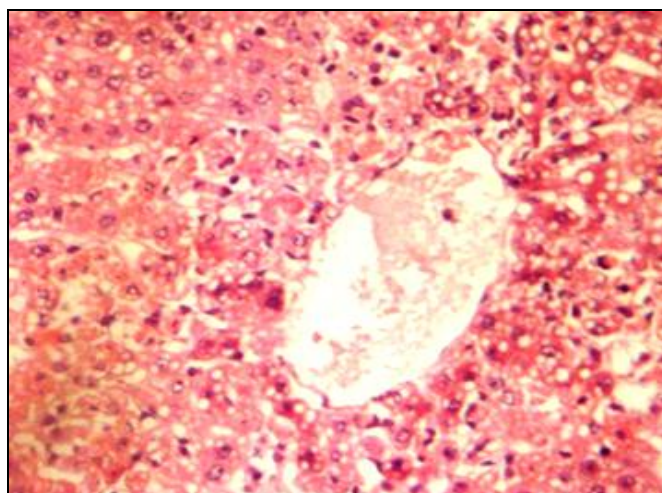


FIGURE 4: LIVERS OF RATS TREATED WITH AQUEOUS EXTRACT (500mg/kg) SHOWING RESTORATION TO NEAR NORMAL CELLULAR ARCHITECTURE

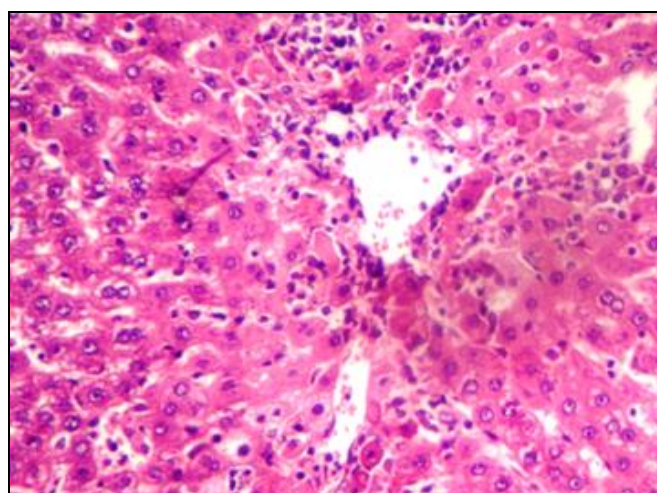


FIGURE 7: LIVER OF RAT TREATED WITH SILYMARIN (25MG/KG) SHOWING RESTORATION TO NORMAL CELLULAR ARCHITECTURE

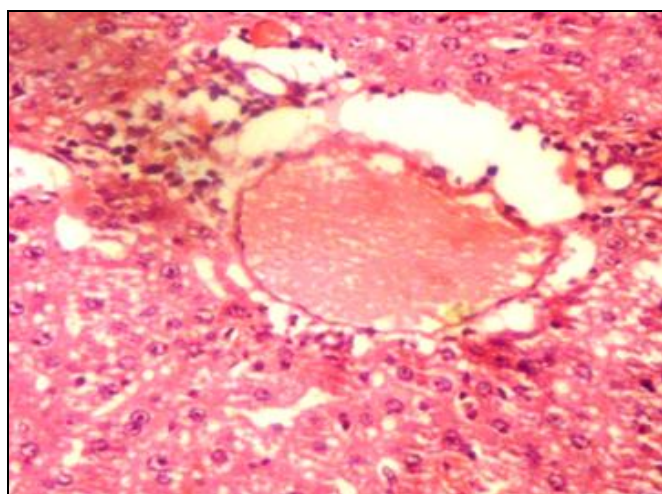


FIGURE 5: LIVERS OF RATS TREATED WITH METHANOLIC EXTRACT (250mg/kg) SHOWING RESTORATION TO NEAR NORMAL CELLULAR ARCHITECTURE

DISCUSSION: In this study, rats treated with a single dose of CCl_4 developed hepatic damage and oxidative stress, which was observed from the substantial decrease in the levels of SOD, CAT, GSH, GST and GR. It has been hypothesized that one of the principle causes of CCl_4 -induced liver injury is formation of lipid peroxides by free radical derivatives of CCl_4 (CCl_3^{\cdot})^{18, 19}.

Carbon tetrachloride (CCl_4) is a well-known hepatotoxic agent. The basis of its hepatotoxicity lies in its biotransformation by the cytochrome P_{450} system to two free radicals. The first metabolite, a trichloromethyl free radical, forms covalent adducts with lipids and proteins and interacts with O_2 to form a second metabolite, a tri chloromethylperoxy free radical, or removes hydrogen to form

chloroform. This sequence of events leads to lipid peroxidation of membranes and consequent liver injury. In response to this hepatocellular injury, "activated" hepatic Kupfer cells release increased quantity of active oxygen species and other bioactive agents. Thus the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl₄-induced hepatotoxicity^{20, 21}.

The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, catalase and GPX. These enzymes constitute a mutually supportive team of defense against ROS such as superoxide anion and H₂O₂ in cells^{19, 22}. In CCl₄-induced hepatotoxicity, the balance between ROS production and these antioxidant defenses is lost and oxidative stress results, which through a series of events deregulates the cellular functions leading to hepatic necrosis.

The reduced activities of SOD, GSH, GRD, catalase, and GST observed in the study, signify the hepatic damage in the rats administered with CCl₄. This decrease may be due to the increased production of reactive oxygen radicals that reduce the activity of these enzymes. The extracts treated groups showed significant increase in the levels of these enzymes, which indicate their antioxidant activity¹⁹.

Glutathione is a major, non-protein thiol in living organisms which performs a key role in coordinating innate antioxidant defense mechanisms. It is involved in the maintenance of normal structure and functions of cells, probably by its redox and detoxification reaction²². Among the non-enzymic antioxidants, GSH is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals, including CCl₄²³.

In the present study, a decrease in hepatic tissue GSH level was observed in the CCl₄-treated groups. An increase in hepatic GSH level in the rats treated with the extracts was observed. Both the extracts promoted the conversion of GSSG (oxidized glutathione) into GSH (reduced glutathione) by the reactivation of hepatic

glutathione reductase (GR) enzyme in CCl₄ treated group. The availability of sufficient amount of GSH thus increased the detoxification of active metabolites of CCl₄ through the involvement of GPX²⁴.

The level of lipid peroxide is a measure of membrane damage and alterations in structure and function of cellular membranes. In the present study, elevation of lipid peroxidation in the liver of rats treated with CCl₄ was observed. Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acids (PUFA)²⁵.

MDA, a secondary product of lipid peroxidation, is used as an indicator of tissue damage involving a series of chain reactions. It reacts with thiobarbituric acid, forming a red coloured product. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent the formation of excessive free radicals²⁶.

Treatment with both the extracts significantly reversed these changes. Hence, it is possible that the mechanism of hepatoprotection of methanolic and aqueous extracts of *Premna integrifolia* roots may be due to their antioxidant action. The extracts showed the dose dependent activity which is comparable with the standard drug, Silymarin.

Histopathological observation supported the results obtained from the enzymes assay¹⁰. These studies were performed to provide direct evidence of the hepatotoxicity of CCl₄, and the antioxidant effects of extracts of *P. integrifolia*¹⁸. The liver of CCl₄ intoxicated rats showed the high percentage of necrosis. Silymarin was effective in reducing the area of necrosis. Both the aqueous and methanolic extracts were found to be effective as they decreased the area of necrosis comparable to standard.

From the data of present study, it may be concluded that the altered biochemical profile due to CCl₄ exposure is reversed towards normalization by *P. integrifolia* extracts. Beneficial effects of *P. integrifolia* extracts may be due to the presence of flavonoids and phenolic components that have

proven antioxidant effects¹¹. The results obtained suggest that the compounds present in *P. integrifolia* efficiently work on the liver to keep it functioning normally and minimizing cell disturbances.

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How to cite this article:

Jain S, Singh M, Barik R and Malviya N: Effects of *Premna integrifolia* Linn. roots extracts in CCl₄ induced toxicity in rats. *Int J Pharm Sci Res* 2013; 4(12): 4697-03. doi: 10.13040/IJPSR. 0975-8232.4(12).4697-03

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