INTRODUCTION
Liver is the main organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation. Herbs have recently attracted attention as health beneficial food and as source materials for drug development. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases including liver diseases with relatively little knowledge regarding their modes of action.

Butea monosperma Lam. is commonly known as Palash in Hindi and widely distributed in India. Its flowers are used to treat leprosy, gout, skin, eye diseases and has been reported to be associated with various remedial properties such as antihypertensive, antistress, antiestrogenic and chemopreventive. In the present study, hepatoprotective effects of B. monosperma (800 mg/kg, p.o.) was studied against biochemical, light and ultrastructural and genotoxic changes induced by carbon tetrachloride (CCl\textsubscript{4}, 0.15 ml/kg, i.p.). Subchronic exposure to CCl\textsubscript{4} for 3 weeks caused sharp elevation in the activity of liver marker enzymes (serum transaminases, alkaline phosphatase and lactate dehydrogenase), albumin, bilirubin, creatinine and urea level in serum. Tissue biochemistry revealed significant reduction in antioxidant status i.e. glutathione reductase, glutathione peroxidase, super oxide dismutase and catalase. Oxidative stress was measured by estimating reduced glutathione level and amount of thiobarbituric acid reactive substances. CCl\textsubscript{4} administration significantly decreased aniline hydroxylase activity and increased microsomal lipid peroxidation. A 5-day treatment of B. monosperma extract after toxicant administration reversed alterations in blood and tissue biochemical variables almost as same as in silymarin treated positive control. In conclusion, data suggest aqueous extract of B. monosperma prevent hepatic damage and improved cellular architecture through its antioxidant activity.

Keywords: Hepatoprotective; carbon tetrachloride; oxidative stress, Butea monosperma

MATERIALS AND METHODS
Preparation of plant extract
Flowers of Butea monosperma (B. monosperma) was generously obtained from Central Council for Research in Unani Medicine, India and identified by the Dr. A.K. Jain, Professor, School of Studies in Botany, Jiwaji University, Gwalior (M.P.). Flowers were dried in shade, powdered and extracted with distilled water (250 g/4 l) for 18 h with concomitant shaking. Filtrate was evaporated in vacuum to yield a yellow powder, which was administered i.p.

Preparation of doses and treatments
The CCl\textsubscript{4} was administered at the dose of 0.15 ml/kg, i.p. with vehicle (olive oil)\textsuperscript{10}. An aqueous suspension of extract was prepared in distilled water and selected optimum dose was administered (800 mg/kg, p.o.) to the animals on previous study. Silymarin (50 mg/kg), a known hepatoprotective agent was administered as positive control.
Experimental procedure

Female rats were divided into five groups of six animals each. Group 1 received olive oil as vehicles and served as normal control. Groups 2-4 were administered CCl₄ (0.15 ml/kg, i.p.) for 3 weeks. Animals of group 2 treated as experimental control. Groups 3 and 4 were orally administered *B. monosperma* aqueous extract (800 mg/kg) and silymarin (50 mg/kg) respectively for 5 days after CCl₄ administration. Animals of all the groups were sacrificed after 48 h of the last treatment.

Liver marker enzymes

Blood was withdrawn by puncturing retro-orbital venous sinus, centrifuged and serum was isolated to determine aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ¹¹, serum alkaline phosphatase (Kit No.11767700011730) and lactate dehydrogenase (Kit No. 11760200011730) were assessed by kit methods as per instructions provided by the company E - Merck (Germany).

Markers of oxidative stress

Lipid peroxidation (LPO) was determined by measuring thiobarbituric acid reactive substances (TBARS) ¹². Reduced glutathione (GSH) level was determined by dithionitrobenzoic acid (DTNB) ¹³.

Metabolic enzymes

The activities of adenosine triphosphatase (ATPase) ¹⁴ and glucose-6-phosphate (G-6-Pase) ¹⁵ were determined in liver.

Antioxidant defense system

The activities of glutathione peroxidase (GPx) ¹⁶, glutathione reductase (GR) ¹⁷, super oxide dismutase (SOD) ¹⁸ and catalase (CAT) ¹⁹ were determined in liver.

Micromosomal fraction

Liver microsomes were prepared by calcium precipitation method ²⁰ for the estimation of MDMEs. Activity of AH was assayed by measuring the intensity of blue colored conjugate of phenol and p-amino phenol (PAP) at 4630 nm ²¹ and expressed as nM PAP/min/g liver. Malondialdehyde (MDA) contents, a measure of LPO, were assayed in the form of thiobarbituric acid reactive substance (TBARS) in micromosomal fraction ¹².

Genotoxicity evaluation: Comet assay

Comet assay was performed according to Singh et al., 1988 ²²

Histopathological observations

Liver samples were fixed in Bouin’s fixative and processed to obtain 5 μm thick paraffin sections and stained with hematoxyline and eosine (H&E) and observed under photo micrographic attachment.

Transmission electron microscopy

1 mm³ pieces of liver were fixed in Karnovsky’s fixative (3.2% glutaraldehyde prepared in 0.1 M phosphate buffer) at 4°C for 18 h followed by washing the samples with phosphate buffer. Osmium tetraoxide (1%) was used for post fixation, then tissues were dehydrated in acetone series and subsequently embedded in epon resin and polymerized at 70°C for 24 h. Ultrathin sections were cut on Reichert Jung Ultra cut-E Microtome using glass knives. The sections were placed on uncoated grids, stained with uranyl acetate and lead citrate and examined under JEOL JEM 1200 EX transmission electron microscope at 80 KV ²³

Statistical analysis

Data were subjected to statistical analysis through one-way analysis of variance (ANOVA) significant at 5 % followed by student’s t-test at P < 0.05. Results are presented as mean ± S.E. of six animals used in each group.

RESULTS

Liver marker enzymes

Liver marker enzymes studies represented hepatoprotective efficacy of *B. monosperma* aqueous extract against subchronic injury induced by CCl₄. *B. monosperma* treatment for five days reversed varying degree of changes in serological alteration. CCl₄ elicited toxic response, thereby; a significant enhanced release of AST, ALT, SALP and LDH was observed (Fig 1A-D). *B. monosperma* aqueous extract protected against altered enzymatic activities and prevented their leakage, conferring its hepatoprotective efficacy (P<0.05). On the basis of % protection, *B. monosperma* showed more than 50% recoupment in diagnostic enzymes of liver dysfunction. It exhibited its better hepatoprotective effect in recouping activities of ALP.

Serological estimation

Toxicant exposure caused significant increase in albumin, bilirubin, urea and creatinine (P<0.05). On the basis of % protection, *B. monosperma* aqueous extract showed more than 80% protection in bilirubin and creatinine level. Treatment showed 60% and 50% protection in albumin and urea respectively (Figure 2A-D).

Markers of oxidative stress

Effect of CCl₄ and *B. monosperma* on LPO and GSH level are presented in figure 3A-B. CCl₄ administered in experimental group showed increased oxidative stress as assessed by increased MDA production and diminished GSH contents (P<0.05). Treatment with *B. monosperma* LPO was significantly decreased with the increase in GSH contents. More than 60% protection was observed in LPO and GSH level with therapy.

Metabolic enzymes

Treatment of *B. monosperma* aqueous extract for 5 consecutive days significantly decreased G-6pase and ATPase activity as compared to CCl₄ intoxicated groups (P<0.05). *B. monosperma* therapy proved to be effective in these variables and significantly recovered the activity of metabolic enzymes. Analysis of variance showed significantly improved activities of G-6pase and ATPase with *B. monosperma* therapy (P≤0.05; Figure3C-D).

[1] Neetu Sharma & Sangeeta Shukla/ Int. J. Res. Ayurveda Pharm. 9 (2), 2018
Antioxidant defense system

Figure 4A-D present influence of B. monosperma aqueous extract on tissue non-enzymatic antioxidative status. CCl₄ exposure significantly lowered the GR, GPx, SOD and CAT (P<0.05). B. monosperma treatment increased GR, GPx, SOD and CAT status in target organ showing recoupment as compared to silymarin treated positive control (P<0.05). % protection showed 56%, 35%, 50% and 30% recovery in antioxidant status with B. monosperma treatment.

Microsomal fraction

Activity of AH was significantly diminished and level of microsomal LPO was increased by CCl₄ administration (P<0.05). B. monosperma therapy showed improvement in the AH activity and microsomal LPO respectively at 5% level of probability. Percent protection showed 39% recovery in AH and 62% in LPO. B. monosperma therapy proved to be effective in these variables and significantly recovered the activity of microsomal AH and LPO level (Figure 4E-F).

Genotoxicity evaluation

In all CCl₄ treated groups (0.15 ml/kg), hepatocytes showed an increase of 2 folds percent damage (23.8%) and tail length 17.3 μm. Therapy with BM showed 79% protection in tail length, 86% in tail moment and 56% in tail DNA as compared to that of control DNA (Figure 5A-G).

Light microscopic study

Light microscopic studies of control liver showed normal parenchymal architecture with well formed cords of hepatocytes and sinusoidal spaces (Figure 6A). Administration of CCl₄ produced enlargement in the whole liver with obvious pathological changes. Heavy lymphocytic infiltration around the central vein, vacuolation, swelling in hepatocytes and disturbed cord arrangement due to hepatocytic degeneration were observed (Figure 6B). Therapy with B. monosperma aqueous extract at 800 mg/kg showed well formed cord arrangement of polygonal hepatocytes and almost normal sinusoids (Fig. 6C). Silymarin treatment improved the histoarchitecture of liver same as control with well formed hepatocytes arranged in cords with remarkable nuclei (Figure 6D).

Ultra-structural studies:

Ultrastructure of control liver showed uniformly distributed mitochondria with smooth and rough ER at proximity to the nucleus (Figure 7A). The CCl₄ administration showed extensive cellular damage with marked dilation in ER and loss of cell cytoplasm at several places as a result of which a number of vacuoles appeared with poor distribution of glycogen. Loss in organization of rough ER was seen with detached ribosomes and heavy lipid accumulation on CCl₄ administration (Figure 7B). B. monosperma aqueous extract treatment provided overall improvement in the ultrastructure when compared with experimental control group as seen the nucleus has normal appearance with intact nuclear envelop and nucleolus (Figure 7C). After silymarin treatment, rough ER showed linear attachment of ribosomes with small glycogen rosettes and well formed mitochondria in cytoplasm (Figure 7D).

DISCUSSION

Hepatic cells participate in a variety of metabolic activities and contain a host of enzymes. In liver injury; the transport function of the hepatocytes is disturbed, resulting in the leakage of plasma membrane, thereby causing an increased enzyme level in serum. The elevated activities of AST and ALT in serum are indicative of liver injury 23,26. Alkaline phosphatase mainly arises from the lining of canaliculi in hepatocytes and also brush border of the renal tubules. It is excreted normally via bile through liver and involves in active transport across the capillary wall. Increased activity of alkaline phosphatase, which occurs due to de novo synthesis by liver cells, is a reliable marker of hepatobiliary dysfunction due to damage 27. More than 10 fold increase in the activity of this enzyme after CCl₄ intoxication clearly indicated severity of damage in liver. LDH is an intracellular enzyme and also is used as a marker of liver injury. In this investigation, leakage of LDH from injured hepatocytes was obvious after subchronic administration of CCl₄. B. monosperma therapy attenuated increased level of these enzymes and caused a subsequent recovery towards normalization that might be due to recoupment in cell membrane. Stabilization of AST, ALT, SALP and LDH activities by B. monosperma treatment improved the histoarchitecture of liver same as control hepatocytes and almost normal sinusoids (Fig. 6C). Silymarin therapy showed improvement in the functional status of liver cells, which may be due to free radical scavenging action of active constituents of B. monosperma i.e. butin and isobutrin. Investigators have previously demonstrated antioxidative and hepatoprotective effect of the extract of Bauhinia racemosa 28 and Emblica officinalis 29 against CCl₄ induced toxicity.

Generation of reactive oxygen species via LPO is one of the mechanisms involved in tissue damage 30. Significant increase in hepatic and microsomal LPO confirms damage in these organs due to increased oxidative insult 31. Administration of B. monosperma at 500 mg/kg doses inhibited LPO as an effective chain breaking antioxidant and helped in preventing CCl₄ induced peroxidative damage. Decrease in GSH level might be due to its increased utilization by the hepatocytes in scavenging toxic radicals of CCl₄. It has been reported that most covalent binding of toxicant to hepatic protein occurs only after depletion of GSH, and the severity of hepatic necrosis is related to the degree of covalent binding 32. Several flavonoids are found in B. monosperma that are known to increase the expression of e-glutamyl cysteine synthetase resulting increased production of glutathione. A possible mechanism of the protective effects of B. monosperma in maintaining the GSH towards control might be due to several bioactive compounds and active constituents i.e. butin and isobutrin present in it that might protect oxidative damage by directly neutralizing reactive oxidants, increase the capacity of endogenous antioxidant defense and increase the steady state of GSH and/ or its rate of synthesis that confers enhanced protection against oxidative insult.

ATPase is a mitochondrial lipid-dependent membrane-bound enzyme. Any alteration in membrane lipid leads to change in membrane fluidity, which in turn alters ATPase activity and subsequently energy dependent cellular function. CCl₄ exposure provoked significant loss of ATPase activity in liver, which might be due to dysfunctional changes in mitochondria and cell membrane permeability. Inhibition of ATPase after CCl₄ exposure has also been confirmed in other studies 34. B. monosperma aqueous extract prevented membrane lesion to a large extent with concomitant recovery in enzymatic activity by maintaining cell membrane permeability. The G-6-Pase is a crucial enzyme of glucose homeostasis and plays an important role in the regulation of the blood glucose level. Administration of B. monosperma restored this enzyme activity of G-6-Pase due to membrane stabilization and improvement in metabolism. Our findings substantiated the therapeutic effect of Rhicicissus tridentate 29.
Figure 1 (A–D): Activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase (SALP) and lactate dehydrogenase (LDH). C = Carbon tetrachloride (CCl₄), BM = Butea monosperma (800 mg/kg), S = Silymarin (50 mg/kg). CCl₄ vs normal at *P≤0.05; treatments vs CCl₄ at †P≤0.05. * Significant for ANOVA at P≤0.05; †AST = 136, †ALT = 180, †SALP = 306; †LDH= 60.1
**Figure 2 (A–D):** Activities of albumin, bilirubin, urea and creatinine. C = Carbon tetrachloride (CCl4), BM = *Butea monosperma* (800 mg/kg), S = Silymarin (50 mg/kg). CCl4 vs normal at *P*≤0.05; treatments vs CCl4 at *P*≤0.05. *Significant for ANOVA at *P*≤0.05; *albumin = 4.39, *bilirubin = 114, *urea = 141; *creatine = 47.4.

**Figure 3 (A–D):** Activities of Lipid peroxidation (LPO), Reduced glutathione (GSH), Adenosine triphosphatase (ATP) and Glucose-6-phosphatase (G-6-pase). C = Carbon tetrachloride (CCl4), BM = *Butea monosperma* (800 mg/kg), S = Silymarin (50 mg/kg). CCl4 vs normal at *P*≤0.05; treatments vs CCl4 at *P*≤0.05. *Significant for ANOVA at *P*≤0.05; *LPO = 141, *GSH = 32.9, *ATP = 141; *G-6-pase = 47.4.
Figure 4 (A–F): Activities of Glutathione reductase (GR), Glutathione peroxidase (GPx), Superoxide dismutase (SOD), Catalase (CAT), Aniline hydroxilase (AH) and microsomal lipid peroxidation (MLPO). C = Carbon tetrachloride (CCl₄), BM = Butea monosperma (800 mg/kg), S = Silymarin (50 mg/kg).

CCl₄ vs normal at *P<0.05; treatments vs CCl₄ at *P<0.05. * Significant for ANOVA at P<0.05; * GR = 43.7, * GPx = 27.5, * SOD = 18, * CAT = 21.3, * AH = 8.62; * MLPO = 133
Figure 5 (A-G): CCl₄ = Carbon tetrachloride (CCl₄), BM = Butea monosperma (800 mg/kg), S = Silymarin (50 mg/kg). CCl₄ vs normal at *P≤0.05; treatments vs CCl₄ at *P≤0.05. @ Significant for ANOVA at P≤0.05; @ Tail length=258, @ Tail DNA=122, @ Tail movement=318.

A: Photomicrographs of liver of control rats showing normal hepatic cells with well prominent nucleus; well-formed central vein; preserved cytoplasm (x-100).
B: Liver section of CCl₄ treated rats showing massive fatty changes, loss of cellular boundaries and collapsed cell, membrane with hyperchromatic nuclei (x-100).
C: Therapy with BM showing well-formed central vein with chord arrangement (x-100).
D: Therapy with silymarin showing better formed hepatocytes (x-100).
Figure 7:
A: TEM of control liver showing prominent nucleus (N), along with intact nuclear envelop, well-formed extensive endoplasmic reticulum (ER) and mitochondria (M) with distinct cristae (x 5600).
B: Micrographs showing CCl₄ intoxication. Swelling with fragmentation of mitochondrial cristae (M), endoplasmic reticulum is disarrayed and shows bizarre arrangement (ER) (x 5600).
C: Micrographs showing treatment with BM. Well-formed intact nucleus (N) was visible, mitochondria with clear cristae (M) (x 5600), endoplasmic reticulum (ER) were persist (x 5600).
D: Therapy of Silymarin showing improved structure of hepatocyte, mitochondria (M), endoplasmic reticulum (ER) and prominent nucleus (N) (x 5600).

Hypoalbuminemia and reduction in hepatic protein content due to CCl₄ intoxication threw the light on cellular damage, which decreased functional efficiency of liver. It is assumed that B. monosperma stimulated protein synthesis, which eventually induced the repair of tissue damage and the replacement of enzymes and structural components damaged by toxic reactions. Bilirubin is one of the most useful clinical parameters to know the severity of hepatic necrosis. It is an important degradation product of hemoglobin and is normally excreted into the bile. If hepatic parenchymal damage is severe, less bilirubin will be excreted and hyperbilirubinemia is observed that reflects pathophysiology of liver damage. Increase in total serum bilirubin concentration after CCl₄ administration might be attributed to the failure of normal uptake, conjugation and excretion by the damaged hepatic parenchyma. A noticeable observation with B. monosperma was that this compound profoundly decreased the elevated level of serum bilirubin, which suggests that it can be used in the condition of jaundice. In the present investigation there was a significant rise in serum urea and creatinine concentration after toxicant administration. It may be due to dysfunctional and dystrophic changes in the liver and kidney. Due to severe renal impairments, urea excretion falls and its concentration in serum rises rapidly. Accumulation of the urea has been reported in liver diseases like cirrhosis and encephalopathy. Our experiment revealed that B. monosperma significantly provided protective effects on serum urea and creatinine, leading to normal hepatic physiology as well as improved glomerular filtration rate.

To protect themselves against free radicals, cells have developed antioxidant defense and repair systems, which prevent accumulation of oxidatively damaged molecules. The antioxidant defense system includes enzymes i.e. glutathione peroxidase, catalase, glutathione reductase, glutathione-S-transferase, superoxide dismutase. These radical scavenging enzymes provide the first-line of defense against oxidizing species. Excessive production of free radicals might result in alterations in the biological activity of cellular macromolecules. Therefore,
reduction in the activity of these enzymes might result in a number of deleterious effects due to accumulation of superoxide radicals, hydrogen peroxide and depletion in GSH level. Administration of *B. monosperma* thus, reduced the generation of free radicals significantly in the liver. Similar findings were also reported with plant extracts of *Momordica dioica* Roxb. 38.

During the process of evolution, the liver developed DMEs, which are different from the enzymes of intermediate metabolism 39. CYP2E1 metabolizes and activates many toxicologically important substrates including CCl4 to more toxic product in endoplasmic reticulum 40. Activities of AH in hepatocytes were significantly decreased after CCl4 administration, which clearly indicated damage in endoplasmic reticulum. *B. monosperma* restored this enzymatic activity more towards control by modulating MDMEs and indicated its hepatoprotective efficacy. This is probably due to the fact that flavonoids, which are the main constituents of *B. monosperma* have membrane permeation properties that might influence CYP450 enzyme system by modulating the DMEs 41,42.

Comet assay has been found highly effective in biomonitoring of natural compounds. Natural compounds in herbal extracts possess varied antioxidative efficiency to reserve the DNA damage. Damaged DNA strands were broken into fragments and migrated towards the anode during electrophoresis 43. The extent of DNA damage was then quantified using normalized tail length. This normalized tail length of the comet indicated the amount of damage as longer tails indicated that the strand breaks were frequent and that the DNA was fragmented to several smaller molecules. One hundred comets on each slide were scored according to the length and relative intensity of fluorescence in the tail was compared to the head. Lower ratio suggested lower level of DNA damage or higher antioxidant activity 43. Subchronic exposure to CCl4 could produce a genotoxic response with the metabolic activation. The protective effects against DNA damage was very clear when compared with the damage level of the positive control; *B. monosperma* depicted over 60% reduction in the level of DNA damage. These results might suggest that there may be more than one compound that exhibited antioxidant activity and these compounds have different polarity. *B. monosperma* aqueous extract suggesting its genoprotective effect on DNA damage.

Light and ultrastructural observations were also consistent with the biochemical findings of this study. Electron micrographs of liver after CCl4 administration exhibited fatty degeneration, dilated endoplasmic reticulum and inconsistency in nuclear envelop. Damage in ER and inhibition of AH activity evidenced that CCl4 inactivated CYP system. The reversal of alterations after treatment with *B. monosperma* might be due to the presence of OH functionality in its structure that inhibited free radical formation or propagation of lipid peroxyl radicals in biomembranes preventing mitochondria and other cellular damage along with normalization of metabolizing enzymes.

*B. monosperma* aqueous extract was found to possess butrin and isobutrin as its major active constituents. Butrin and isobutrin has been reported to act as strong antipatotoxic agents. It is reported that approximate yield 5mg of isobutrin and 4 mg butrin from 24 mg butanol fraction. This corresponds to 20-83 % isobutrin and 16.6 % butrin in the methanolic extract 44. Since the active constituents of *B. monosperma* extract have known protective effects in other studies, so the observed hepatoprotective activity of *B. monosperma* extract in our study may be suggested due to the presence of these compounds.

CONCLUSION

Thus, it can be concluded that *B. monosperma* aqueous extract possesses strong potential as an emerging hepatoprotective agent by reversing hepatic oxidative insult, CYP enzymatic activity and ultrastructural changes may be due to the presence of its active constituents, viz., butrin and isobutrin in addition with other minor constituents and by the antioxidant effect of flower aqueous extract.

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