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Antioxidant and hepatoprotective effect of *Macrotyloma uniflorum* seed in antitubercular drug induced liver injury in rats

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Abstract

Horse gram [*Macrotyloma uniflorum* Lam. (Verdc.)] is a lesser known bean consumed as a whole seed or sprout in India. Its seeds are rich in flavonoids and phenolic acids. The protective effect of a hydroalcoholic extract of the seed of Macrotyloma uniflorum (MUSE) in anti-tubercular drug (ATD) induced liver injury and the probable mechanism involved in this protection was investigated in rats. The phenolic acids viz., ferulic and p-coumaric acid were isolated and quantified from MUSE. MUSE (250 mg/kg and 500 mg/kg) and the reference drug Liv.52 (500 mg/kg) was administered orally for 30 days to ATD (isoniazid 7.5 mg/kg, rifampicin 10 mg/kg and pyrazinamide 35 mg/kg) treated rats. Levels of marker enzymes (AST, ALT, ALP and γ-GT), albumin, total proteins and bilirubin were estimated in serum. Activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase), reduced glutathione levels and the lipid peroxidation marker malondialdehyde were determined in liver. MUSE elicited significant hepatoprotective and antioxidant activity by attenuating the ATD–elevated levels of the marker enzymes, bilirubin and malondialdehyde and restored the ATD–depleted levels of albumin, total proteins, reduced glutathione and the antioxidant enzymes. The present findings indicate that the hepatoprotective effect of GIE in ATD–induced oxidative damage may be due to its antioxidant activity.

Keywords: *Macrotyloma uniflorum* seed extract, Anti-tubercular drugs, Hepatoprotective, Antioxidant activity.

Introduction

Tuberculosis (TB) is a major health burden worldwide. In India, it is one of the foremost public health problems, causing a significant burden of morbidity and mortality. Among the various causes of anti-tubercular drug (ATD) treatment interruption, drug induced liver injury is a common one. Isoniazid, rifampicin and pyrazinamide, the first line drugs used for tuberculosis chemotherapy, are associated with hepatotoxicity and when given in combination, their toxic effect is enhanced.[1] The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8-30%) compared with that in advanced countries (2%-3%) with a similar dose schedule.2

ATD-induced hepatotoxicity is due to the biotransformation of isoniazid, rifampicin and pyrazinamide to their respective reactive metabolites which are capable of binding to cellular macromolecules and inducing toxicity by a multiple step mechanism.[3] Rifampicin is toxic to liver, this having been found both in the treatment of tuberculosis and cholestasis.[4, 5] When it was used together with pyrazinamide, a 5.8% incidence of severe liver injury was reported, 2.6% with isoniazid and 1.1% when given alone.[2]

In absence of reliable liver-protective drugs in modern medicine, the quest for herbal hepatoprotective which are thought to be safer continues.

Macrotyloma uniflorum Lam. (Verdc.) (Family: Fabaceae) is a lesser known drought resistant legume grown throughout Asia, Africa and Australia and primarily utilized as feed to animals and horses. In India it is known as the "poor man's pulse" and used as a staple food. The *Macrotyloma* seeds also known as horse gram are small, ovoid and greyish brown or light reddish brown in colour with faint mottles and small scattered black spots on them. Traditionally, horse gram has been used to treat various ailments such as haemorrhoids, tumors, bronchitis, splenomegaly, heart disease, leukoderma, urinary discharge, obesity, diabetes and asthma.[6] Recently, it has also been investigated for antimicrobial and haemolytic activities.[7, 8] The soup prepared from horse gram is considered to be a useful remedy for treating common cold, throat infections, fever and urolithiasis.[9] The seeds have been reported to have potent antioxidant activity.[10] In our present study we have selected the seed of *Macrotyloma uniflorum* which is used in traditional medicine for various liver disorders. Based on traditional claims from folk

medicine, our present study evaluates the hepatoprotective activity of seeds of *Macrotyloma uniflorum* in ATD induced liver injury in rats.

Seeds of *M. uniflorum* contain varying amounts of carbohydrates, proteins, amino acids, lipids, phenolic compounds (3,4-dihydroxy benzoic acid, vanillic acid, caffeic acid, p-cumaric acid, ferulic acid, chlorogenic acid, syringic acid and sinapic acid), flavonoids and tannins (quercetin, kaempferol and myricetin), fatty acids (hexanoic acid and hexadecanoic acid), phytosterols (stigmasterol and β-sitosterol), anthocyanidins (cyanidin, petudin, delphinidin and malvidin), saponins and minerals like iron, calcium and molybdenum.[11, 12] Phenolic acids obtained from *M. uniflorum* are considered to be potent antioxidants which act by scavenging free radicals and reactive oxygen species.[10, 12]

With this background, the present study was carried out to investigate the hepatoprotective effect and a possible underlying antioxidant activity of the hydroalcoholic extract of the seeds of *M. uniflorum* by assaying various marker enzymes, antioxidant enzymes, lipid peroxidation and GSH in rats with ATD-induced liver injury.

Materials and Methods

Plant material

The seeds of *M. uniflorum* were collected from the Pune region of Maharashtra, India and were authenticated at the Blatter Herbarium, St. Xavier's College, Mumbai after matching with the existing specimen (accession no.AD-06). The seeds were dried, powdered mechanically and defatted using petroleum ether. The dry defatted powder was extracted in a Soxhlet apparatus with 70% methanol as the solvent at 60°C. The dry extract was stored in an air-tight container for experimental use.

Drugs and chemicals

Isoniazid was a gift sample obtained from Amsal Chem. Pvt. Ltd., Mumbai, India. Rifampicin and pyrazinamide were also gift samples provided by Sandoz Pvt. Ltd, Mumbai. Epinephrine, trichloro Acetic Acid (TCA) and 5, 5′-Dithiobis (2-nitrobenzoic acid) - (DTNB) were purchased from Sigma Chemical Co., St Louis, MO, USA. Thiobarbituric acid (TBA), reduced glutathione (GSH), oxidized glutathione and nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Himedia Laboratories, Mumbai, India. All other chemicals were obtained from local sources and were of analytical grade.

Isolation of active constituents

After successful development of TLC, preparative High Performance Thin Layer Chromatography of the hydroalcoholic extract of the seeds of *Macrotyloma uniflorum* (MUSE) was carried out on the CAMAG HPTLC System for isolation, identification and quantification of its active constituents, ferulic and p-coumaric acid. The absorbance value of different bands in the crude extract after TLC separation was studied using the Desaga Scanner for the most possible wavelength absorption of ferulic acid and p-coumaric acid. Prior to MUSE application, 2 HPTLC plates (HPTLC Silica gel 60 F254, Merck) of 10 x 10 cm were activated at 110 °C for 30 min. MUSE (100 mg) was dissolved in 10 ml of methanol and 100 µL of this solution was applied as a single band of 180 mm length on the activated HPTLC plates using a Linomat V applicator (CAMAG, Switzerland). The plates were then developed with 10 ml of the solvent system comprising toluene: ethyl acetate: formic acid (6.8:2.3:0.9) in the twin trough chromatographic chamber and examined in the UV Chamber at 320 nm for ferulic acid and at 315 nm for p-coumaric acid. After development, the constituents (Rf 0.72 for ferulic acid and Rf 0.55 for p-coumaric acid) were marked and scraped out from the plates. The scraped material was mixed with methanol and eluted from silica gel by centrifugation at 3000 rpm. The supernatant was evaporated on a water bath to get ferulic acid and p-coumaric acid. Further confirmation of the isolated constituents was done by using UV–Visible Spectrophotometer for single peak and Infrared Spectrophotometer for major functional groups.

Preparation of calibration curve

Ferulic acid (10 mg) and p-coumaric acid (10 mg) were dissolved separately in 10 ml of methanol to yield solutions of 1000 µg/mL. From these stock solutions, aliquots of standard ferulic acid and p-coumaric acid (2-10µL) were applied on the silica gel 60 F_{254} plates. The plates were developed to a distance of 66 mm in the selected solvent system. The plates were dried and calibration curves plotted.

Quantification of ferulic acid and p-coumaric acid in MUSE

MUSE (50 mg) was dissolved in 10 ml methanol to yield a solution of 5 mg/mL. This solution (25 µL) was applied onto the silica gel plate and processed as outlined above. The amount of p-coumaric acid and ferulic acid in MUSE was calculated from the calibration curve.

Experimental animals

Wistar albino rats (150-200 gm) of either sex were used. They were housed in clean polypropylene cages under standard conditions of humidity (50 ±5 %), temperature (25±2°C) and light (12 h light/12 h dark cycle) and fed with a standard diet (Amrut laboratory animal feed, Pune, India) and water *ad libitum*. All animals were handled with humane care. Experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (Animal House Registration No.25/1999/CPCSEA) and conform to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research.

Preparation of test and reference drug solutions

The dry *Macrotyloma uniflorum* seed extract (MUSE) was dissolved in distilled water and the aqueous extract solution was used for administration. Isoniazid and pyrazinamide were dissolved in distilled water while rifampicin was first dissolved in dilute hydrochloric acid and later mixed with the above mixture. This mixture of anti-tubercular drugs (ATD) was immediately administered to rats. Liv. 52 tablets were powdered & suspension was made using 1% w/v carboxymethyl cellulose (C.M.C) and administered.

Experimental procedure

Animals, after acclimatization (6–7 days) in the animal quarters, were randomly divided into five groups of six animals each and treated in following manner:[13]

Group I served as Normal Control and received aqueous solution of 1% w/v C.M.C orally once daily for 30 days.

Group II served as Toxicant Control and received an anti-tubercular drug cocktail – a combined mixture of isoniazid (7.5 mg/kg), rifampicin (10 mg/kg) and pyrazinamide (35 mg/kg) orally once daily for 30 days.

Group III termed as MUSE 250 received MUSE (250 mg/kg, p.o.) and ATD treatment, both once daily for 30 days.

4. Group IV termed as MUSE 500 received MUSE (500 mg/kg, p.o.) and ATD treatment, both once daily for 30 days.

5. Group V termed as Standard and received Liv.52 tablets (500 mg/kg, p.o.) suspended in 1% w/v C.M.C and ATD treatment, both once daily for 30 days.

Rats were sacrificed 24 h after the last injection by cervical dislocation and blood was collected by cardiac puncture. The collected blood was allowed to coagulate at Room Temperature for 30

min. The serum was separated by centrifugation in a pathological centrifuge at 1,200× g at 30^{0}C for 15 min and used for the estimation of marker enzymes {aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (ɣ- GT)}, bilirubin, albumin (Alb) and total proteins (TP). The livers were dissected immediately, washed with ice-cold saline and divided into two equal parts. One part was used to prepare a 10% (w/v) homogenate in 1.15% (w/v) KCl. An aliquot was used for the determination of lipid peroxidation (LPO). The homogenates were centrifuged in a refrigerated tabletop centrifuge at 7,000× g for 10 min at 4°C, and the supernatants were used for the assays of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). The remaining part of the liver was fixed in 10% (w/v) buffered formalin and used for histological studies.

Marker enzyme assays

The liver marker enzymes AST, ALT, ALP and γ-GT were assayed in serum using standard kits supplied from Span Diagnostics (Surat, India).

Protein estimation

The levels of TP and Alb were determined in the serum of experimental animals by using the Lowry et al method and the bromocresol green method respectively.[14, 15] The results were expressed as g/dL.

Bilirubin estimation

Level of bilirubin in serum was determined by method of Malloy and Evelyn[16], using kits supplied by Span Diagnostics Ltd (Mumbai, India). The results were expressed as mg/dL for bilirubin.

Lipid peroxidation

The quantitative estimation of LPO was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in the liver using the method of Ohkawa *et al.*[17] The amount of malondialdehyde (MDA) formed was quantified by reaction with TBA and used as an index of lipid peroxidation. The results were expressed as nmol of MDA/g of wet liver using molar extinction coefficient of the chromophore (1.56×10^{-5}/M/cm) and 1,1,3,3-tetraethoxypropane as standard.

Glutathione estimation

GSH was estimated in the liver homogenate using DTNB by the method of Ellman.[18] The absorbance was read at 412 nm and the results were expressed as µmol/g of wet liver.

Antioxidant enzyme assays in liver homogenate

SOD was assayed by the method of Sun and Zigman in which the activity of SOD was inversely proportional to the concentration of its oxidation product adrenochrome, which was measured spectrophotometrically at 320 nm.[19] 1 unit of SOD activity is defined as enzyme concentration required to inhibit the rate of auto oxidation of epinephrine by 50% in 1 min at pH 10.

CAT was estimated by the method of Clairborne, which is a quantitative spectroscopic method developed for following the breakdown of H_2O_2 at 240 nm in unit time for routine studies of CAT kinetics.[20]

GPx estimation was carried out using the method of Rotruck *et al.*, which makes use of the following reaction:[21]

$$H_2O_2 + 2\,GSH \rightarrow 2H_2O + GSSG \text{ (oxidized glutathione)}$$

GPx in the tissue homogenate oxidizes glutathione and simultaneously H_2O_2 is reduced to water. This reaction is arrested at 10 min using TCA and the remaining glutathione is reacted with DTNB solution to result in a coloured compound, which is measured spectrophotometrically at 420 nm.

GR activity was determined by using the method of Mohandas et al., in which the following reaction is implicated:[22]

$$NADPH + H^+ + GSSG \rightarrow NADP^+ + 2\,GSH$$

In the presence of GR, oxidized glutathione undergoes reduction and simultaneously, NADPH is oxidized to $NADP^+$. Enzyme activity is quantified at Room Temperature by spectrophotometrically measuring the disappearance of NADPH/min at 340 nm.

Histopathological Studies

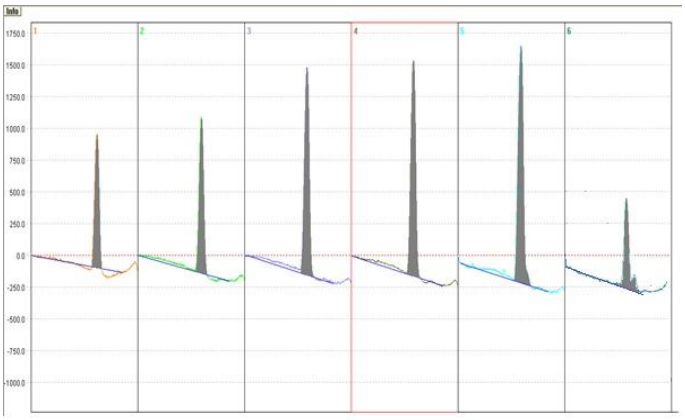
The parts of the livers which were stored in 10% (w/v) buffered formalin were embedded in paraffin, sections cut at 5 µm and stained with hematoxylin and eosin. These sections were then examined under a light microscope for histo-architectural changes.

Statistical analysis

The results of hepatoprotective and antioxidant activities are expressed as mean ± SEM. Results were statistically analysed using one-way ANOVA, followed by the Tukey–Kramer post test for individual comparisons. $P<0.05$ were considered to be significant.

Results

The Rf values of ferulic acid and p-coumaric acid were noted to be 0.72 at 320 nm and 0.55 at 315 nm respectively. The amounts of ferulic acid and p-coumaric acid were found to be 4.25 mg/g MUSE and 1.47 mg/g MUSE respectively. (Figures 1a and b are HPTLC chromatograms for ferulic acid and p-coumaric acid).



Component 1 : Component 1

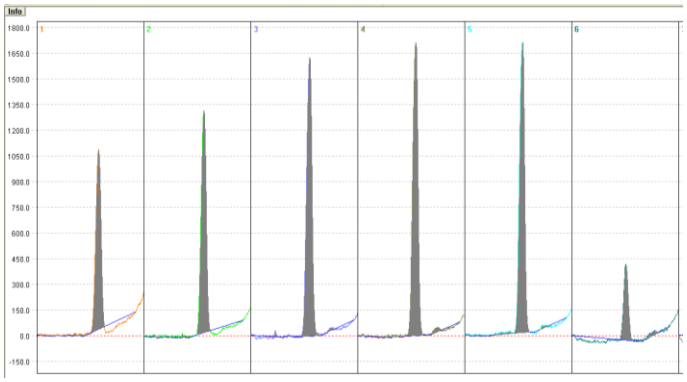
Lane type	y-Pos.[mm]	Area	Height	CV[%]	Conc.[ng]	Type
Standard 1 : FA	50.2	3244.868	1052.007		2000.0	
Standard 2 : FA	48.8	3849.759	1228.965		4000.0	
Standard 3 : FA	48.1	5574.707	1636.967		6000.0	
Standard 4 : FA	47.9	5815.260	1697.056		8000.0	
Standard 5 : FA	48.4	7053.844	1868.956		10000.0	
Sample 1 : muse	48.3	833.562	407.309		1063.7	<

Amount of substances

Sample name	Component name	Amount of substance	Ratio
Sample 1 : muse	1 : Component 1	425.5 µg	0.425 %

Note: FA- ferulic acid; muse –MUSE (*Macrotyloma uniflorum* seed extract)

Figure 1a: Chromatogram of ferulic acid



Component 1 : Component 1

Lane type	y-Pos.[mm]	Area	Height	CV[%]	Conc.[ng]	Type
Standard 1 : coumaric acid	42.3	3167.819	1029.375		2000.0	
Standard 2 : coumaric acid	41.5	3834.859	1273.486		4000.0	
Standard 3 : coumaric acid	40.9	5102.042	1599.678		6000.0	
Standard 4 : coumaric acid	40.4	5247.161	1679.419		8000.0	
Standard 5 : coumaric acid	40.4	5065.246	1666.782		10000.0	
Sample 1 : muse	38.5	1166.781	434.376		1771.3	<

Amount of substances

Sample name	Component name	Amount of substance	Ratio
Sample 1 : muse	1 : Component 1	708.5 µg	1.417 %

Note: muse –MUSE (*Macrotyloma uniflorum* seed extract)

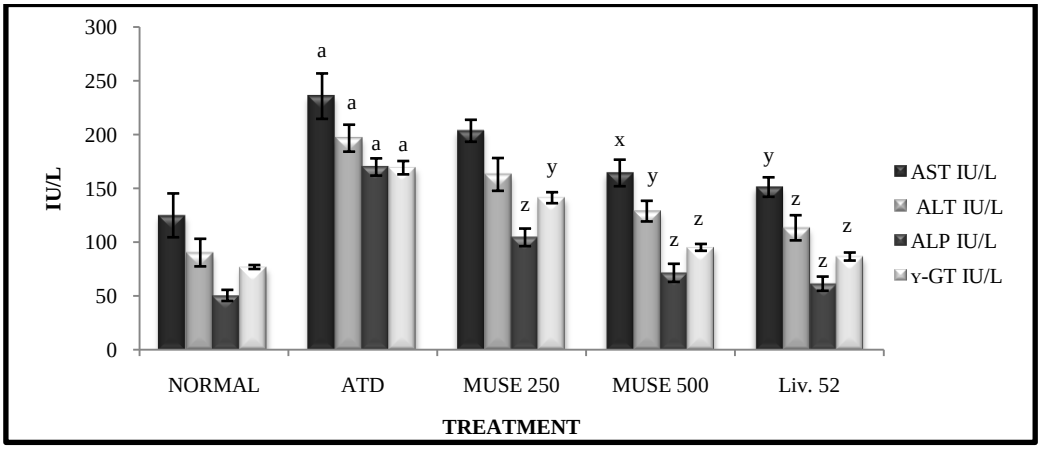
Figure 1b: Chromatogram of p- coumaric acid

Effect of MUSE on AST, ALT, ALP, γ-GT, Alb, TP and bilirubin

The effects of MUSE on serum marker enzyme activity (AST, ALT, ALP and γ-GT), albumin (Alb), total protein (TP) and bilirubin are summarized in figures 2 and 3 respectively.

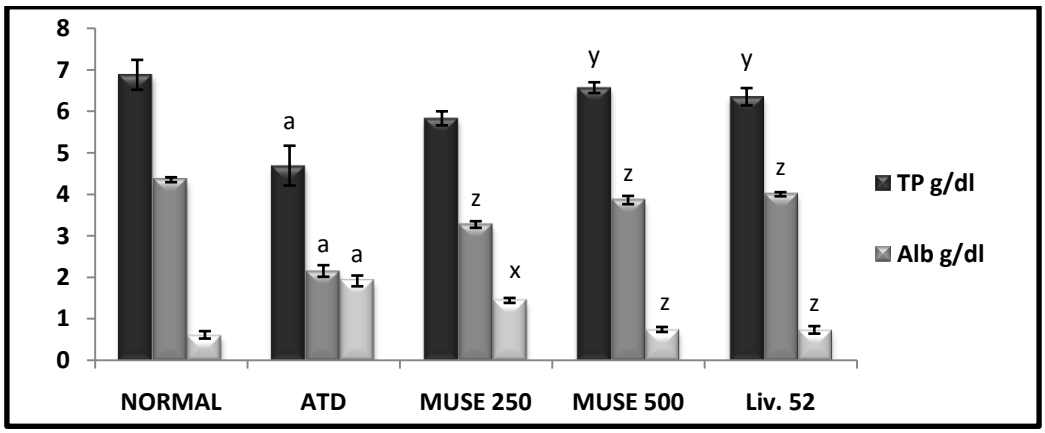
In the ATD treated group of rats there was a significant elevation in the activities of the serum marker enzymes (AST, ALT and ALP). Pre-treatment of MUSE 500 and Liv.52 to ATD intoxicated rats attenuated significantly ($p<0.05$ and $p<0.01$ respectively) the ATD elevated activities of AST and ALT ($p<0.01$ and $p<0.001$ respectively). Treatment of MUSE 250, MUSE 500 and Liv.52 to ATD fed rats decreased significantly ($p<0.001$) the ATD-elevated activities of ALP and γ-GT ($p<0.01$, $p<0.001$ and $p<0.001$ respectively).

In ATD treated rats there was a significant decrease in albumin level. The effect was reversed significantly by pre-treatment with MUSE 250, MUSE 500 and Liv.52 ($p<0.001$). The ATD depleted total protein level was significantly restored by administration of MUSE 500 and Liv. 52 ($p<0.01$ and $p<0.001$ respectively). Similarly, the serum bilirubin levels, elevated by ATD treatment were significantly attenuated by MUSE250, MUSE500 and Liv.52 treatments ($p<0.05$, $p<0.001$ and $p<0.001$ respectively).



Values are mean ± SEM; N = 6 in each group. P values: a < 0.001 when ATD compared with Normal Control; x < 0.05, y< 0.01, z < 0.001 when Experimental groups compared with ATD

Figure 2: Effect of *Macrotyloma uniflorum* seed extract on AST, ALT, ALP and γ GT in ATD intoxicated rats



Values are mean ± SEM; N = 6 in each group. P values: a < 0.001 when ATD compared with Normal Control; x < 0.05, y< 0.01, z < 0.001 when Experimental groups compared with ATD

Figure 3: Effect of *Macrotyloma uniflorum* seed extract on serum total proteins, albumin and bilirubin in ATD intoxicated rats

Effect of MUSE on LPO, GSH, SOD, CAT, GPx and GR

The effects of MUSE on liver antioxidant enzymes (SOD, CAT, GPx and GR), GSH and LPO are summarized in Table.1. MDA, the LPO marker was significantly elevated ($p<0.001$) in the ATD administered group of rats when compared with the Normal group. Treatment of MUSE 250, MUSE 500 and Liv.52 to ATD intoxicated rats attenuated significantly ($p<0.001$) the increased levels of MDA.

Significantly decline in GSH levels ($p<0.001$) was observed in ATD treated group when compared with the Normal Control group of rats. Treatment with MUSE 250, MUSE 500 and Liv.52 restored significantly ($p<0.05$, $p<0.001$ and $p<0.001$ respectively) the GSH levels depleted by ATD.

Liver SOD and CAT activities were examined to be strikingly lower ($p<0.001$) in ATD treated rats when compared with the Normal group of rats. Treatment with MUSE 250, MUSE 500 and Liv.52 significantly ($p<0.05$, $p<0.001$ and $p<0.001$ respectively) restored the ATD-depleted SOD and CAT activities.

Liver GPx and GR activities were depleted significantly ($p<0.001$) by ATD treatment. Treatment with MUSE 500 and Liv.52 restored significantly ($p<0.001$) and ($p<0.001$ and $p<0.01$ respectively) the ATD depleted GPx and GR activities respectively.

Table 1: Effect of *Macrotyloma uniflorum* seed extract on liver GSH, TBARS, SOD, CAT, GPx and GR in ATD intoxicated rats

Biochemical parameters	Group I Normal Control	Group II ATD (Toxicant Control)	Group III MUSE (250 mg/kg)	Group IV MUSE (500 mg/kg)	Group V Liv.52 (500 mg/kg)
GSH (µmol/g of wet liver)	2.23 ±0.05	0.53 ±0.04 [a]	1.07 ±0.12 [x]	1.88 ±0.19 [z]	1.99 ±0.14 [z]
TBARS (nmol MDA/ g of wet liver)	14.57 ±2.77	71.00 ±2.38 [a]	51.72 ±2.50 [z]	23.27 ±2.20 [z]	20.09 ±1.60 [z]
SOD (U/mg protein)	6.82 ±0.32	2.10 ±0.58 [a]	4.05 ±0.21 [x]	5.67 ±0.34 [z]	5.91 ±0.49 [z]
CAT (U/mg protein)	8.91 ±0.76	1.71 ±0.17 [a]	4.04 ±0.61 [x]	5.82 ±0.39 [z]	7.29 ±0.36 [z]
GPx (U/mg protein)	11.11 ±0.95	4.69 ±0.91 [a]	5.09 ±0.82	9.55 ±0.43 [z]	9.75 ±0.47 [z]
GR (U/mg protein)	397.50 ±18.4	195.40 ±39.22 [a]	284.90 ±11.29	371.60 ±17.52 [z]	347.20 ±22.85 [y]

Values are mean ± SEM; N = 6 in each group, One-way ANOVA followed by Tukey–Kramer post test is applied for statistical analysis. P values: a< 0.001 when ATD compared with Normal Control. z< 0.001, y< 0.01, x < 0.05 when Experimental groups compared with ATD
1 unit of CAT = µmol H_2O_2 consumed / min / mg protein; 1 unit of GPx = µg GSH utilized / min / mg protein; 1 unit of GR = nmol NADPH oxidized / min / mg protein

Histopathological Studies

The livers of animals in the Normal Control group showed normal cellular architecture with distinct hepatic cells with portal triad and compact lobular arrangement and sinusoidal spaces (Fig.4a).

The livers of ATD treated animals showed focal centrilobular necrosis of hepatocytes. In addition, diffuse moderate lymphocytic infiltration and dilation of sinusoids was also seen (Figure 4b).

Compared to the ATD group of animals, the Liv.52 treated animals showed minimal degenerative changes in the liver. There was minimal centrilobular fatty and granular degeneration of hepatocytes and absence of necrosis and leukocytic infiltration (Figure 4c).

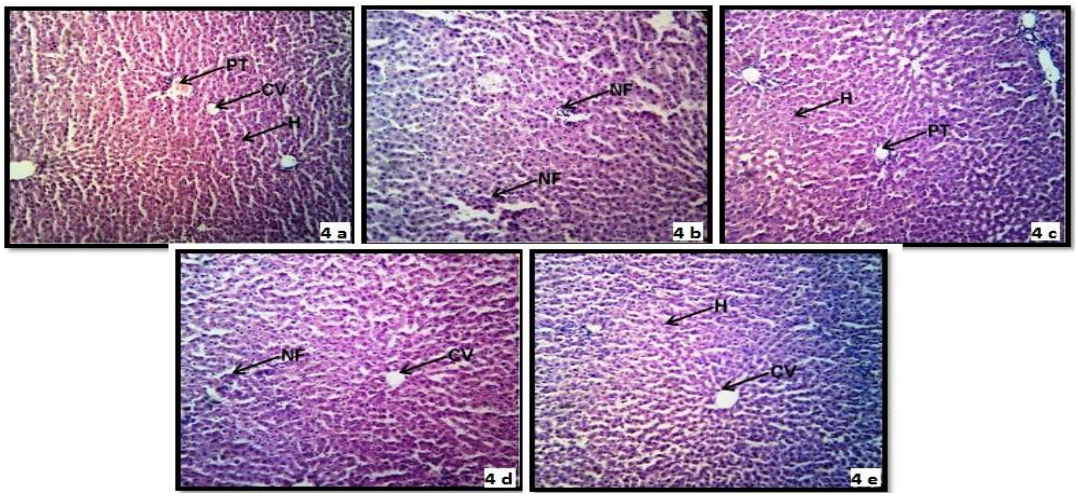


Figure 4: Histopathology of livers. **4a.** Haematoxylin and eosin staining of liver tissue of normal rats 10 X 10x = 100x. **4b.** Haematoxylin and eosin staining of liver tissue of ATD treated rats 10 X 10x = 100x. **4c.** Haematoxylin and eosin staining of liver tissue of rats treated with ATD and Liv.52 10 X 10x = 100x. **4d.** Haematoxylin and eosin staining of liver tissue of rats treated with ATD and MUSE 250 10 X 10x = 100x. **4e.** Haematoxylin and eosin staining of liver tissue of rats treated with ATD and MUSE 500 10 X 10x = 100x. [*Note:* CV- Central vein; H- Hepatocyte; NF- Necrotic foci; PT- Portal triad]

Lesions in the livers of the MUSE 250 treated animals showed mild degree of fatty and granular degeneration around the central vein with focal areas of necrosis (Figure 4d). No leukocytic infiltrate or fatty change seen.

The liver of animals in the MUSE 500 treatment group showed minimal centrilobular fatty degeneration with minimal leukocytic infiltrate but absence of necrosis comparable to the standard Liv.52 treatment group (Figure 4e).

The histopathological examination of the livers of the treatment groups showed clear signs of retrieval from ATD toxicity and provided a supportive evidence for the biochemical analysis. It was also evident that MUSE 500 showed better hepatoprotection than MUSE 250.

Discussion

Tuberculosis is one of the most common infectious diseases. In India, pulmonary tuberculosis is one of the major causes for adult deaths [2]. Treatment regimens for TB patients include five first line anti-TB drugs viz., Isoniazid (INH), Rifampicin (RIF), Ethambutol (ETM), Pyrazinamide (PZA) and Streptomycin (STP) [24]. However, the use of isoniazid, rifampicin and pyrazinamide is associated with toxic reactions in tissues, particularly in the liver, leading to hepatotoxicity and also creates difficulties in restarting the regimen.[24]

Antitubercular drugs induce hepatitis by a multiple step mechanism. This hepatitis is characterized by a fall in serum albumin concentration which is related to the severity and duration of disease. Peroxidation of endogenous lipids has been shown to be a major factor in the cytotoxic action of isoniazid and rifampicin. Antitubercular drug mediated oxidative damage is generally attributed to the formation of the highly reactive oxygen species, which act as stimulators of lipid peroxidation and source for destruction and damage to the cell membrane.[25]

ATD therapy is continued for a long period and thus, a chronic model of study is essential to mimic hepatotoxicity. Amino transferases are important class of enzymes linking carbohydrate and amino acid metabolism, thereby clearly establishing the relationship between the intermediates of citric acid and amino acids. AST, ALT and ALP are well known diagnostic indicators of liver disease. In cases of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissue into the blood stream. Observation of significant decrease in the activities of these enzymes has already been reported in the liver tissue of isoniazid, rifampicin and pyrazinamide intoxicated rats.[26, 27] Treatment of MUSE as well as Liv.52 to ATD-administered rats attenuated significantly the ATD- elevated activity of AST, ALT and ALP to near normal, showing the normalizing effect of MUSE on protein metabolism.

Alterations in protein metabolism have been considered for decades to be one of the conditions associated with hepatic dysfunction. Our results showed decreased levels of protein and albumin in the serum of ATD-administered rats when compared with the control group, which were restored by MUSE treatment, which indicates hepatoprotective activity. Stimulation of protein synthesis has been advanced as a contributory hepatoprotective mechanism that accelerates the regeneration process and the production of liver cells.[28]

Hepatotoxicity is characterised by cirrhotic liver condition which in turn increased the bilirubin release. Administration of MUSE attenuated the level of bilirubin to near normal by its cytoprotective action and also due to an inhibitory effect on cytochrome P-450.

Gamma- glutamyl transferase (γ-GT/GGT) is an enzyme found in the cell membranes of many tissues, the most notable one being the liver. GGT catalyses the transfer of the gamma- glutamyl moiety of glutathione to an acceptor that may be an amino acid, a peptide or water, leaving the cysteine product to preserve intracellular homeostasis of oxidative stress. γ-GT play a key role in the pathway for the synthesis and degradation of glutathione and drug and xenobiotic detoxification.[29] Our results showed significantly elevated activity of GGT in ATD- treated rats when compared with normal control rats. The ATD elevated GGT activity was significantly attenuated by MUSE treatment.

ATD-induced hepatitis is due to the biotransformation of isoniazid, rifampicin and pyrazinamide to their respective reactive metabolites which are capable of binding to cellular macromolecules of the liver [3]. As an alternative to inducing cellular damage by covalent binding, there is evidence that these anti-tubercular drugs cause cellular damage through the induction of oxidative stress, a consequence of dysfunction of hepatic antioxidant defence system.[30] Increase in the amount of lipid peroxidation in the liver reflected hepatocellular damage. The depletion of antioxidant defence and/or rise in free radical production deteriorates the pro-oxidant/antioxidant balance, leading to oxidative stress induced cell death.

Our findings confirm the pattern and show significant increase in the level of MDA in the liver tissue of ATD administered rats when compared with the normal control rats. The ATD- elevated MDA levels were attenuated significantly by MUSE treatment.

The mechanism of rifampin hepatotoxicity is not well known, but it is extensively metabolized by the liver and induces multiple hepatic enzymes including the CYP.[31] When used together with isoniazid (INH), it is known to increase INH toxicity. In its metabolic pathway, INH is first converted to acetyl INH which on hydrolysis yields acetyl hydrazine. Rifampicin induces the CYP to bring about quick oxidation of acetyl hydrazine to reactive metabolites.[31] Reactive metabolites of acetyl hydrazine are probably toxic to tissues through free radical generation.[32] In rats, the free radical scavenger glutathione-related thiols, and the antioxidant GPx, SOD and CAT activities are diminished by isoniazid, The combination of INH and RIF was reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation.[33]

Pyrazinamide alters nicotinamide acetyl dehydrogenase levels in rat liver, which might result in generation of free radical species.[34] There may be shared mechanisms of injury for isoniazid and pyrazinamide, because there is some similarity in molecular structure. Patients who previously had hepatotoxic reactions with isoniazid have had more severe reactions with rifampin and pyrazinamide.

The toxic metabolites of INH and pyrazinamide bind to and damage cellular macromolecules in the liver which contains GSH and the antioxidant enzymes SOD, CAT and GPx and which constitute a mutually supportive team of antioxidant enzymes that provide defence against ROS.[35]

In the present study, decreased GSH levels in ATD administered rats may be due to its increased utilization by the GSH-dependent enzymes GPx & GR. The glutathione levels were restored to normal with MUSE treatment. This may be due to an initial reduction in hepatic peroxidative activities followed by inhibition of the activities of the GSH-dependent enzymes, thereby leading to restoration of the GSH content.

SOD activity decreased significantly in ATD treated animals due to excessive formation of superoxide anions. The activities of H_2O_2 scavenging enzymes CAT & GPx also decreased significantly after ATD treatment. The decline in these enzyme activities can be explained by the fact that excessive superoxide anions may inactivate SOD, thus, resulting in an inactivation of the H_2O_2 scavenging enzymes CAT and GPx. Administration of MUSE effectively prevented the decrease in SOD, CAT and GPx activities, which may be attributed to the scavenging of radicals by MUSE, resulting in decreased formation of peroxides and protection of these enzymes.

In ATD treated rats, there was a marked depletion of GPx activity leading to a reduced availability of substrate for GR, thereby decreasing the activity of GR. Oral treatment of MUSE to ATD intoxicated rats restored the activity of GR thus, accelerating the conversion of GSSG to GSH and enhancing the detoxification of reactive metabolites by conjugation with GSH.

Conclusion

Furthermore, the histopathological findings in rat livers are in agreement with the results of biochemical studies. Histopathological findings clearly suggest that MUSE treatment reversed ATD induced architectural changes of liver tissue. The degree of hepatoprotective effect was greater in MUSE 500 mg/kg treated animal group than in MUSE 250 mg/kg treatment group. The protective effect of MUSE 500 mg/kg was comparable to that of Liv.52, a known herbal hepatoprotective, which showed near normal histology of liver.

Co-administration of MUSE with ATD for 30 days protected the rat livers from ATD induced liver injury. The two major mechanisms responsible for hepatoprotective activity of MUSE in ATD induced liver injury may thus be, i) scavenging of ROS and ii) prevention of GSH depletion. Polyphenolic compounds of MUSE stabilize the ROS by reacting with them and getting oxidized in turn to more stable less reactive radicals. Presumably, the high reactivity of OH group of polyphenols is responsible for this free radical scavenging activity.

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