

HEPATOPROTECTIVE AND INHIBITION OF OXIDATIVE STRESS IN LIVER TISSUE OF *OXYSTELMA ESCULENTUM* ON PARACETAMOL INDUCED HEPATIC DAMAGE IN RATS

Ashok Kumar Durairaj, Thamil Selvan Vaiyapuri, Upal Kanti Mazumder*, Malaya Gupta

Department of Pharmaceutical Technology, Jadavpur University, Kolkata. 700 032, India.

Summary

The protective effects of methanol extract of *Oxystelma esculentum* R. Br. (MEOE) on paracetamol induced hepatotoxicity and the possible mechanism involved in this protection were investigated in rats. The MEOE at dose level of 200 and 400 mg/kg and standard drug silymarin 25mg/kg b.w/day p.o for 7 days were administered to the paracetamol (750mg/kg) induced liver injury in rats. The degree of protection was measured by using biochemical parameters such as serum transaminase (SGOT and SGPT), alkaline phosphatase (ALP), bilirubin, total protein, albumin, globulin, cholesterol and phospholipids. Further the effects of MEOE on lipid peroxidation (LPO), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were estimated. The methanol extract of *Oxystelma esculentum* (MEOE) at the doses of (200mg/kg and 400mg/kg) produced significant hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin and lipid peroxidation while it significantly increased the levels of GSH, SOD and CAT in a dose dependent manner. The effects of MEOE were comparable to that of standard drug silymarin. These results suggest that MEOE could protect from paracetamol-induced lipid peroxidation perhaps by its antioxidative effects. Hence eliminating the deleterious effects of toxic metabolites from paracetamol.

Key words: Antioxidants, biochemical parameters, hepatoprotective activity, *Oxystelma esculentum*, paracetamol.

Introduction

Liver is an important organ for detoxification and liver diseases are of serious health problem. Most of the hepatotoxins induce tissue injury after having been metabolized to free radicals and cause subsequent cell damage through mechanism of covalent binding and lipid peroxidation. In fact, biochemical damage produced by reactive oxygen has emerged as a fundamental final common pathway of tissue injury in a wide variety of disease processes. Normally host cells are protected from oxygen derived radical injury by naturally occurring free radical scavengers and antioxidants¹. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species. When the protective mechanisms are overwhelmed however, host tissues become susceptible to damage by oxygen radicals. The agents that scavenge these free radicals are considered increasingly important in therapeutic intervention. Thus the protective role of antioxidant enzymes in the treatment and prevention of various diseases have gained significant importance².

Oxygen species such as hydroxy radicals, superoxide anion radicals and singlet oxygen are agents that attack polyunsaturated fatty acids in cell membranes and give rise to lipid peroxidation in living systems. Lipid peroxidation is also strongly associated with aging and carcinogenesis³. However, living systems are protected from active oxygen species by enzymes such as superoxide dismutase, glutathione peroxidase and catalase. These living systems have also been reported to receive non-enzymatic protection by endogenous antioxidants such as α -tocopherol, ascorbic acid, β -carotene, and uric acid⁴.

Over the past 25 years, epidemiological studies have shown a diminished risk of chronic diseases in populations consuming diets high in fruits and vegetables⁵. It has been suggested that antioxidants found in large quantities in fruits and vegetables may be responsible for this protective effect⁶. Generally, food antioxidants act as reducing agents, reversing oxidation by donating electrons and hydrogen ions⁷. Much attention has been focused on natural antioxidants and some antioxidants isolated from natural sources with high activity have been reported^{8,9}.

In the absence of reliable liver protective drugs in allopathic medical practices, naturally occurring compounds have been found to have major role in the management of various liver diseases. Numerous medicinal plants and their formulations are used for liver disorders in ethno medical practices and in traditional systems of medicine in India. However a satisfactory remedy for serious liver diseases is not still available, so search for effective hepatoprotective drugs are continued.

Oxystelma esculentum R. Br. is a perennial twining herb with milky juice. It is distributed throughout the plains, on hedges and among bushes usually near water and lower hills of India, Ceylon and Java^{10,11}. In Tamil it is called as usippalai, in Kannada as dugdhike, Telugu in dudipala and Hindi, Bengali & Oriya in dudhialata¹². The decoction of the plant used as gargle in aphthous ulcerations of mouth and in sore throat. Root considered specific for jaundice and milk sap used as a wash for ulcers^{13,14}. In Ayurveda, the plant is a diuretic, aphrodisiac, anthelmintic and bronchitis, useful in leucoderma and the fruit is expectorant, anthelmintic; the juice is used in gonorrhoea, pain in muscles¹⁵.

However, no work has been reported on the hepatoprotective properties of this plant. Keeping this in view, the present study has been undertaken to investigate hepatoprotective activity and antioxidant role of the methanol extract of *Oxystelma esculentum* (MEOE) on paracetamol induced liver damage in rats.

Materials and Methods

Plant material

The aerial part of the plant *Oxystelma esculentum* R. Br. (Family: Asclepiadaceae) was collected in the month of November 2004 from Srirangapatnam, Near Mysore, Karnataka, India. The plant material was taxonomically identified by the Prof. Revenna, H.O.D, Department of Botany, Kuvempu First Grade Degree College, Channapatna, Karnataka, India, and the voucher specimen (No:

DAKJU-02/2005) were preserved in our departmental laboratory for future reference. The plant material were dried under shade with occasional shifting and then powdered with a mechanical grinder and stored in an airtight container. The dried powdered material (480 g) was defatted with petroleum ether (60-80⁰ C) in a soxhlet apparatus. The defatted powdered material thus obtained was further extracted with methanol for 72 hours in the soxhlet. The solvent was removed by distillation under suction and the resulting semisolid mass was vacuum dried using rotary flash evaporator to yield (14.60%w/w) a solid residue. Phytochemical screening of the extract revealed that the presence of glycosides, triterpenes, tannins, flavonoids and steroids. The dried MEOE was suspended in distilled water and used for the further studies.

Drugs and chemicals

1-Chloro-2, 4-dinitrobenzene (CDNB), Bovine serum albumin (Sigma chemical St. Louis, MO, USA), Thiobarbituric acid, Nitro blue tetrazolium chloride (NBT) (Loba Chemie, Bombay, India), 5,5'-dithio *bis*-2-nitrobenzoic acid (DTNB) (Sisco Research Laboratory, Mumbai, India), Silymarin (Sivylar) was purchased from Ranboxy laboratories, Indore was used as standard drug. All the other reagents obtained were used of analytical grade.

Animals

Studies were carried out using Male Wister albino rats (150-180g). They were obtained from the animal house, Indian Institute of Chemical Biology (IICB), Kolkata, India. The animals were grouped and housed in polyacrylic cages (38x23x10cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25±2⁰C) with dark and light cycle (14/10h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Paracetamol- induced liver damage in rats (Acute model)

Five groups (I-V) each containing of six male Wister albino rats weighing in the range of 150-180g were selected. Group I served as control and was fed orally with normal saline 5ml/kg daily for seven days. Groups II rats were similarly treated as group I. Group III and IV were treated with 200mg and 400mg/kg methanolic extract/day/orally respectively for seven days. While group V were fed silymarin 25mg/kg as standard for seven days.

On the seventh day, 750mg/kg paracetamol suspension was given by oral route in a dose of to all rats except rats in group I. The biochemical parameters were determined 24 hours after the last dose¹⁶.

Biochemical studies

Blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 g at 30⁰ C for 15 min and used for the estimation of various biochemical parameters namely SGOT, SGPT¹⁷, SALP¹⁸, Serum bilirubin¹⁹ were estimated, total protein, albumin and albumin/globulin (A:G) ratio²⁰ was measured and Concentrations of cholesterol and phospholipids were determined^{21,22}. Cholesterol/phospholipids ratio was calculated. After collection of blood samples the rats were sacrificed and their liver exercised, rinsed in ice cold normal saline followed by 0.15 M Tris-Hcl (pH 7.4) blotted, dry and weighed. A 10% w/v of homogenate was prepared in 1.15% KCl solution and processed for the estimation lipid peroxidation²³. A part of homogenate after precipitating proteins with Trichloro acetic acid (TCA) was used for estimation of glutathione²⁴. The rest of the homogenate was centrifuged at 1500 g for 15 min at 4⁰ C. The supernatant thus obtained was used for the estimation of superoxide dismutase (SOD)²⁵ and catalase (CAT) activity²⁶.

Determination of thiobarbituric acid reactive substances (TBARS)

The tissues were then homogenized in 1.15% KCl (pH 7.4) with a Teflon-glass homogeniser. TBARS in tissues was determined (Ohkawa et al, 1979). 0.2ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 8% Thiobarbituric acid (TBA) were added. The volume of the mixture was made up to 4ml with distilled water and then heated at 95⁰ C on a water bath for 60min using glass balls as condenser. After incubation the tubes were cooled to room temperature and final volume was made to 5ml in each tube. 5.0 ml of butanol: pyridine (15:1) mixture was added and the contents were vortexed thoroughly for 2 min. After centrifugation at 3000 g for 10 min, the upper organic layer was taken and its OD read at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides were expressed as μ moles of thiobarbituric acid reactive substances (TBARS)/ g protein using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Determination of reduced glutathione (GSH)

A 30% w/v liver homogenate was prepared in 1.15% KCl (pH 7.4) and TCA was added to precipitate proteins. The samples were centrifuges at 15,000 rpm at 4⁰ C for 1 hr. The supernatant was analyzed for content of reduced glutathione and expressed as μ g/g of liver tissue.

Assay of superoxide dismutase (SOD)

One milliliter of nitroblue tetrazolium (NBT) solution (156 μ g NBT in 100 mM Phosphate buffer, pH7.4), 1ml NADH solution (468 μ g in 100 mM phosphate buffer, pH7.4) and 0.1ml of liver homogenate were mixed. The reaction started by adding 100 μ g of phenazine metho sulphate (PMS) solution (60 μ g NBT in 100mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25⁰ C for 5min, and the absorbance at 560nm was measured against blank sample without liver homogenate.

Assay of catalase (CAT)

The estimation catalase activity was done spectrophotometrically following the decrease in absorbance at 230 nm. The tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1-4⁰ C and centrifuged at 5000rpm. The reaction mixture contained 0.01M phosphate buffer (7.0), 30mM H₂O₂ and the enzyme extract. The specific activity of catalase was expressed in terms of units/mg protein. Absorbance values were compared with a standard curve generated from known CAT.

Statistical analysis

Results were reported as mean±SEM for 6 rats in each group. The biochemical parameters were analyzed statistically using one-way ANOVA, followed by Dunnett's test. The minimum level of significance was fixed at $P < 0.001$.

Results

Changes in the activities of serum enzymes (GOT, GPT, ALP), bilirubin, and total protein content are in the paracetamol-induced liver damage in rats are summarized in the Tzable 1. Administration of paracetamol (750mg/kg, p.o) after, 24 hours intoxication caused a marked and significant ($P < 0.001$) elevation of enzyme levels in paracetamol treated group, in comparison with the control. There were significant ($P < 0.001$) restorations of enzyme levels on administration of MEOE at both doses (200mg and 400mg/kg) and silymarin (25mg/kg) as compared with paracetamol treated group. It was noted that the size of the liver was enlarged in paracetamol treated rats but it was normal in MELN treated groups. A significant reduction in liver weight supports this result ($P < 0.001$) (Table 1).

The enhanced concentration of serum albumin, nonsignificant changes in serum globulin level and abnormal albumin: globulin (A: G) ratio was observed. Moreover treatment of rats with paracetamol produced

an increase in the serum level of cholesterol, decrease in the level of phospholipids and a subsequent increase in the cholesterol to phospholipids ratio from 0.55 ± 0.02 in normal rats to 1.26 ± 0.05 in paracetamol treated rats. This result is an indication of membrane rigidity caused by paracetamol. However, administration of MEOE significantly prevented changes in membrane lipids and fluidity. Cholesterol/ phospholipids ratio of 0.71 ± 0.01 , 0.58 ± 0.02 and 0.54 ± 0.01 were observed for MEOE at 200, 400mg/kg and standard drug silymarin respectively (Table 2).

The effect of MEOE on rat liver tissue lipid peroxidation, glutathione, and antioxidant enzyme levels such as SOD and CAT are summarized in Figure 1-4. Lipid peroxidation level (expressed in term of malondialdehyde (MDA) formation) was significantly increased in the paracetamol control intoxicated rats when compared with the normal rats. Treatment with MEOE at the doses of 200mg and 400mg/kg significantly ($P < 0.001$) prevented the increase in MDA levels and brought back near to normal level. Glutathione, SOD and CAT levels were significantly ($P < 0.001$) increased in MEOE treated groups. The effects of MEOE were compared with the standard drug silymarin.

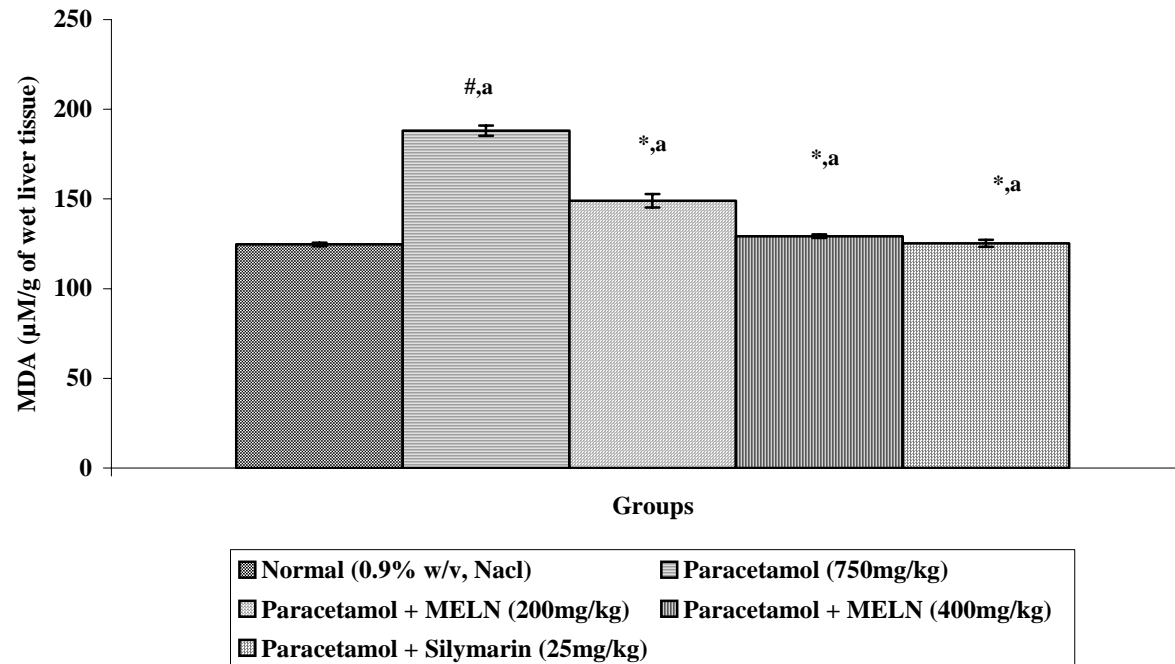


Figure 1. Effect of methanol extract of *Oxystelma esculentum* (MEOE) on Lipid peroxidation in paracetamol intoxicated rats. Values are mean±SEM, n= 6, # compared with normal group, * compared with paracetamol control, ^a $P < 0.001$ is considered statistically significant

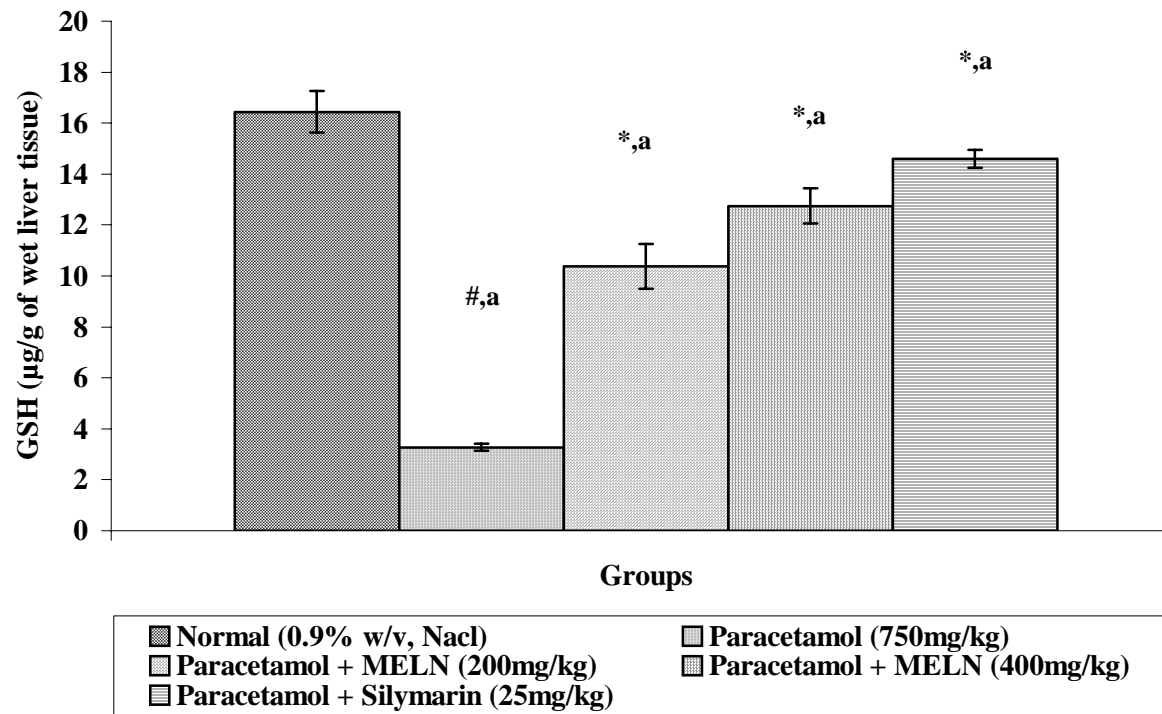


Figure 2. Effect of methanol extract of *Oxystelma esculentum* (MEOE) on Reduced glutathione level in paracetamol intoxicated rats. Values are mean±SEM, n= 6, # compared with normal group, * compared with paracetamol control, ^a $P < 0.001$ is considered statistically significant

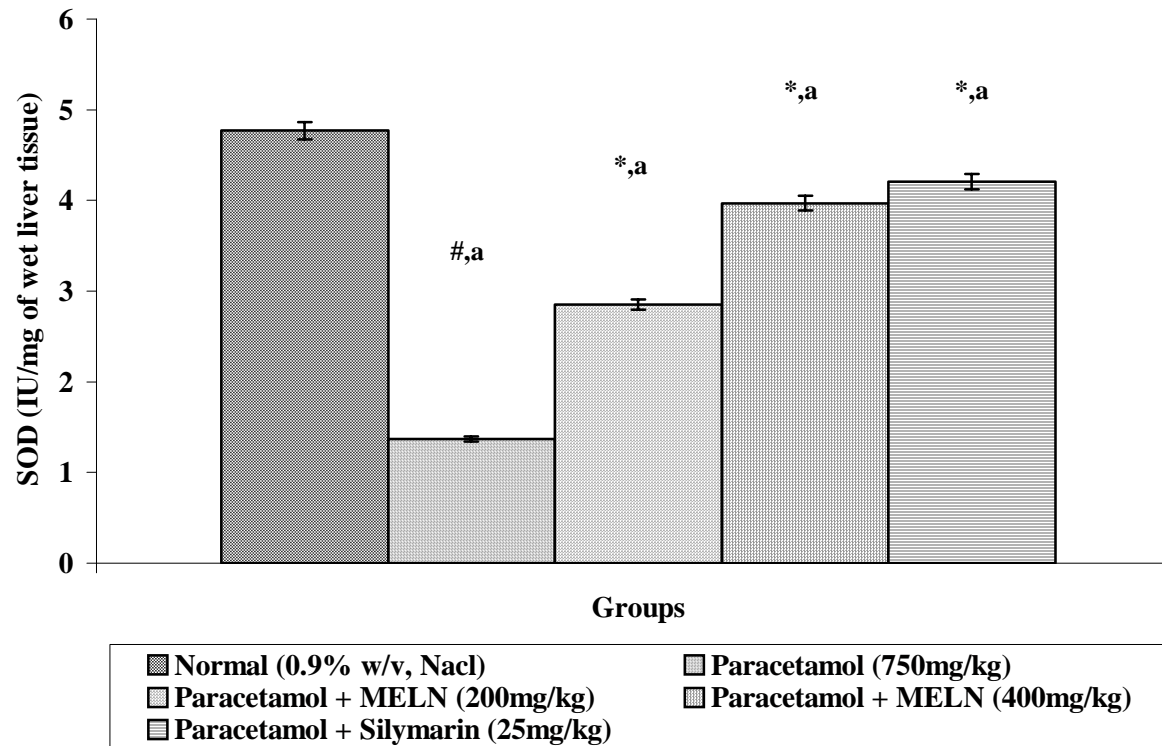


Figure 3. Effect of methanol extract of *Oxystelma esculentum* (MEOE) on Superoxide dismutase in paracetamol intoxicated rats. Values are mean±SEM, n= 6, # compared with normal group, * compared with paracetamol control, ^a $P < 0.001$ is considered statistically significant

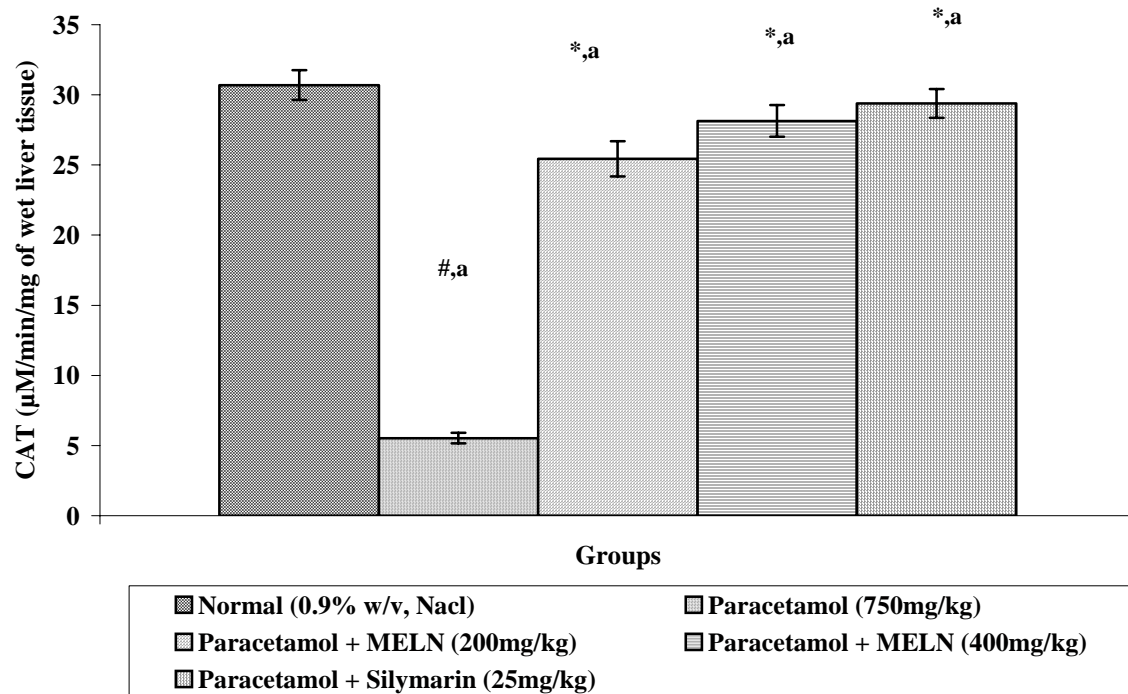


Figure 4. Effect of methanol extract of *Oxystelma esculentum* (MEOE) on Catalase level in paracetamol intoxicated rats. Values are mean±SEM, n= 6, # compared with normal group, * compared with paracetamol control, ^a $P < 0.001$ is considered statistically significant

Table 1. Effect of methanol extract of *Oxystelma esculentum* (MEOE) on liver weight and serum biochemical parameters. Values are mean \pm S.E.M, (n= 6). # compared with normal group, * compared with paracetamol control, ^a $P < 0.001$ is considered statistically significant

Parameters	Group I	Group II	Group III	Group IV	Group V
Liver weight (Wt/100g b.w)	3.23 \pm 0.06	6.49 \pm .26 ^{#,a}	5.25 \pm 0.07 ^{*,a}	4.30 \pm 0.12 ^{*,a}	3.38 \pm 0.15 ^{*,a}
SGOT (U/L)	51.00 \pm 1.78	117.50 \pm 2.55 ^{#,a}	77.83 \pm 2.99 ^{*,a}	61.50 \pm 2.29 ^{*,a}	64.66 \pm 2.40 ^{*,a}
SGPT (U/L)	41.33 \pm 1.90	133.33 \pm 2.26 ^{#,a}	85.33 \pm 2.26 ^{*,a}	74.83 \pm 1.66 ^{*,a}	52.66 \pm 2.41 ^{*,a}
ALP (U/L)	59.66 \pm 1.42	120.50 \pm 3.91 ^{#,a}	79.83 \pm 1.79 ^{*,a}	64.16 \pm 1.72 ^{*,a}	65.66 \pm 2.32 ^{*,a}
Bilirubin (mg %)	1.05 \pm 0.11	2.91 \pm .001 ^{#,a}	1.73 \pm .011 ^{*,a}	1.45 \pm 0.11 ^{*,a}	1.31 \pm 0.01 ^{*,a}
Total proteins (g/dL)	7.38 \pm 0.14	4.16 \pm 0.01 ^{#,a}	5.00 \pm 0.19 ^{*,a}	6.61 \pm 0.11 ^{*,a}	7.10 \pm 0.10 ^{*,a}

Table 2. Effect of methanol extract of *Oxystelma esculentum* (MEOE) on serum lipid profiles. Values are mean ± S.E.M, (n= 6). # compared with normal group, * compared with paracetamol control, ^a P < 0.001 is considered statistically significant

Parameters	Group I	Group II	Group III	Group IV	Group V
Cholesterol (mg/dL)	32.02±0.83	52.35±1.00 ^{#,a}	39.42±0.92 ^{*,b}	34.46±0.92 ^{*,a}	32.59±0.84 ^{*,a}
Phospholipids (mg/dL)	58.30±0.84	41.66±1.14 ^{#,a}	55.06±1.05 ^{*,a}	59.38±1.14 ^{*,a}	59.73±1.58 ^{*,a}
Cholesterol / Phospholipids (C: P ratio)	0.55±0.02	1.26±0.05 ^{#,a}	0.71±0.01 ^{*,a}	0.58±0.02 ^{*,a}	0.54±0.01 ^{*,a}
Albumin (g /dL)	4.53±0.10	3.04±0.10 ^{#,a}	4.10±0.04 ^{*,a}	4.21±0.05 ^{*,a}	4.42±0.10 ^{*,a}
Globulin (gm/dL)	2.72±0.11	5.06±0.06 ^{#,a}	3.55±0.13 ^{*,a}	2.92±0.12 ^{*,a}	2.84±0.10 ^{*,a}
Albumin / Globulin (A: G ratio)	1.67±0.04	0.60±0.02 ^{#,a}	1.16±0.04 ^{*,a}	1.45±0.04 ^{*,a}	1.56±0.06 ^{*,a}

Discussion

Paracetamol is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats and mice with toxic doses²⁷. Protection against paracetamol-induced toxicity has been used as a test for potential hepatoprotective activity by several investigations^{28,29,30}. The covalent binding of N- acetyl-p-benzoquinoneimine, an oxidation product of paracetamol, to sulfhydryl groups of protein resulting in cell necrosis and lipid peroxidation induced by decrease in glutathione in the liver as the cause of hepatotoxicity have been reported earlier^{31,32}, which is one of the most important natural antioxidants of the hepatocytes, renders the cell remarkably susceptible to oxidative stress³³.

In the assessment of liver damage by paracetamol, the determination of enzyme levels such as SGOT and SGPT is largely used. Necrosis or membrane damage releases the enzyme in to circulation; therefore, it can be measured in serum. A high level of SGOT indicates liver damage such as that due to viral hepatitis as well as cardiac infarction and muscle injury. SGPT catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury³⁴. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver³⁵. Serum ALP and bilirubin level on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary presence³⁶.

This present study evaluated the hepatoprotective activities of MEOE in paracetamol induced liver toxicity. Acute administration of paracetamol produced a marked elevation of the serum levels of SGOT, SGPT, ALP, Serum bilirubin, total proteins, cholesterol and phospholipids in treated animals (Group II to V) when compared with that of control group (Group I). Treatment with MEOE at a dose of 200mg/kg and 400mg/kg significantly reduced the elevated levels of the enzymes.

Treatment with MEOE decreased the serum levels of GOT, GPT towards the respective normal value that is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by paracetamol. The above changes can be considered as an expression of the functional improvement of hepatocytes, which may be caused by an accelerated regeneration of parenchyma cells. Effective control of alkaline phosphatase (ALP) and bilirubin levels points towards an early improvement in the secretory mechanism of the hepatic cell.

Cells have a number of mechanisms to protect themselves from the toxic effects of ROS. SOD removes superoxide (O_2^-) by converting it to H_2O_2 , which can be rapidly converted to water by CAT and glutathione peroxidase (GPx)³⁷. In addition, a large reserve of reduced glutathione is present in hepatocytes and red blood cells for detoxification of xenobiotics or free radicals. However, oxidative stress results in toxicity when the rate at which the ROS are generated exceeds the cell capacity for their removal.

Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. MDA is one of the end products in the lipid peroxidation process³⁸. In order to elucidate the protection mechanism of MEOE, paracetamol induced rat liver, after MEOE injection was examined at lipid peroxidase levels and antioxidative enzyme activities. In our study, elevations in the levels TBARS in liver of rats treated with paracetamol were observed. The increase in MDA level in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with MEOE significantly reversed these changes. Hence the hepatoprotection of MEOE is due to its antioxidant effect.

Glutathione is the natural antioxidant in our body system and it is protective chemically induced hepatic damage and oxidative stress by antioxidant mechanism³⁹. GSH level decreased with increased level of lipid peroxidation in paracetamol treated rats. MEOE significantly increased the level of GSH in a dose dependent manner.

Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes such as superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) system⁴⁰. The SOD dismutates superoxide radicals O_2^- into H_2O_2 plus O_2 , thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. The observed increase of SOD activity suggests that the MEOE have an efficient protective mechanism in response to ROS. And also, these findings indicate that MEOE may be associated with decreased oxidative stress and free radical –mediated tissue damage.

Catalase is a key component of the antioxidant defense system. Inhibition of these protective mechanism results in enhanced sensitivity to free radical –induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of super oxide radicals and hydrogen peroxide⁴¹. Administration of MEOE increases the activities of catalase in paracetamol-induced liver damage rats to prevent the accumulation of free radicals and protects the liver from paracetamol intoxication.

The Phytochemical analysis shows that MEOE contains flavonoids, triterpenoids and steroids. A number of scientific reports indicated certain flavonoids, triterpenoids and steroids have protective effect on liver due to its antioxidant properties^{42,43,44}. Presence of these compounds in MEOE may be responsible for the protective effects on paracetamol-induced liver damage in rats.

In conclusion, the results of this study demonstrate that MEOE has a potent hepatoprotective action on paracetamol-induced hepatic damage in rats. Our results show that the hepatoprotective effects of MEOE may be due to its antioxidant and free radical scavenging properties. Further, investigation is underway to determine the exact phytoconstituents that is responsible for its hepatoprotective effect.

Acknowledgement

One of the author ASHOKKUMAR.D, Senior Research Fellow, grateful to AICTE, New Delhi, India for providing financial support to this work.

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***Corresponding Author**

Address for Correspondence:

Prof. (Dr). Upal Kanti Mazumder,
Department of Pharmaceutical technology,
Jadavpur University,
Kolkata,
West Bengal,
India-700 032.
Phone: +91-9433435247
E-mail: ashokkumardurairaj@yahoo.co.in