INTRODUCTION

Acetaminophen is a widely used analgesic and antipyretic drug that is safely employed for a wide range of treatments (1). Overdose of APAP in human is fairly common and is often associated with hepatic (2-4) and renal damage (5). Although nephrotoxicity is less common than hepatotoxicity in APAP overdose, renal tubular damage and acute renal failure can occur even in the absence of liver injury (6-8) and can even lead to death in humans and experimental animals (9-10). Studies are going on throughout the world for the search of protective molecules that would provide maximum protection to the liver, kidney as well as other organs and practically very little or no side effects would be exerted during their function in the body (11-12). A number of herbs are traditionally used in different countries in response to drug or toxin induced hepatic and renal disorders (13).

*Corresponding author.
Mr. S. Palani
Dept. of Biotechnology, Anna Bioresearch Foundation, Arunai Engineering College, Tiruvannamalai-606603
Tamil Nadu, India.
Tel.: +91-04175 237419, 9790607796
Telefax: +91-04175 237780
E-mail: spalanitvm@gmail.com

Madhuca longifolia is a folkloric medicinal plant that is commonly used for the treatment of snakebite as antidote in Southern part of Tamilnadu, India. Stem bark is used to cure hydrocele, wounds in stomach (ulcer), scabies and rheumatism. Acetaminophen (APAP) is commonly used as an analgesic and an antipyretic agent that, in high doses, produces liver and kidney necrosis in mammals. The aim of the present study is to investigate the nephro, hepatoprotective and antioxidant activities of the ethanol extract of Madhuca longifolia (EEML) in two dose levels of 500 mg/kg & 750 mg/kg B/W on APAP induced toxicity in rats. Biochemical studies show that there is an increase in the levels of serum urea, hemoglobin (Hb), total leukocyte count, creatinine, packed cell volume, DLC, mean corpuscular volume and raised body weight along with reduced levels of neutrophils, mean corpuscular Hb content, mean corpuscular hematocrit, granulocytes, uric acid, and platelet concentrations. These values are retrieved significantly by the treatment with extracts at two different doses. The antioxidant studies reveal that the levels of renal superoxide dismutase, catalase, reduced glutathione and glutathione peroxidase in the APAP treated animals are increased significantly along with decreased MDA content in EEML treated groups. Apart from these, histopathological changes also reveal the protective nature of the Madhuca longifolia extract against APAP induced necrotic damage of hepato and renal tissues. In conclusion, these data suggest that the EEML can prevent both renal and liver damage from APAP induced toxicity in rats and it is likely mediated through its antioxidant activities.

Keywords: Madhuca longifolia, antioxidant, acetaminophen, Nephroprotective, hepatoprotective.
MATERIALS AND METHODS:

**Plant material**

*Madhuca longifolia* leaves was collected from Tirumala hills of Chittoor district, Andhra Pradesh, India and the plant material was taxonomically identified and authenticated by the botanist, Voucher specimen (AECBT-06/2007-2008) of this plant has been retained in the Anna Bioresearch foundation, Arunai engineering college, Tiruvannamalai, Tamilnadu, India.

**Extraction**

The leaves of the plant was dried under shade and then powdered with a mechanical grinder to obtain a coarse powder. Equal quantity of powder was passed through 40 mesh sieve and extracted with ethanol (90% v/v) in soxhlet apparatus at 60°C (22). The solvent was completely removed by rotary vacuum evaporator. The extract was freeze dried and stored in vacuum desiccators.

**Animals**

Studies were carried out using wistar albino male rats (150-200g), obtained from Indian veterinary preventive medicine (IVPM), Ranipet, Tamilnadu, India. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) and maintained under standard laboratory conditions (temperature 25 ± 20°C) with dark and light cycle (12/12 h). The animals were fed with standard pellet diet supplied by poultry research station, nandhanam, India and fresh water *ad libitum*. All the animals were acclimatized to laboratory condition for a week before commencement of experiment all procedures described were reviewed and approved by the university animals ethical committee.

**Paracetamol induced hepato and nephrotoxicity in rats**

Animals were randomized and divided into four groups (I - IV) of six animals in each group. Group I served as untreated control and fed orally with normal saline 5 ml/kg body weight daily for 14 days. Group II rats were similarly treated as group I. Groups III and IV animals were treated with 500 mg/kg and 750 mg/kg body weight of the EEML for 14 days, respectively. The extract was administered by oral gavages 1 h before APAP administration (23). On the 14th day, APAP suspension was given by oral route, in a dose of 750 mg/kg body weight to all rats except the rats in group I.

**Hematological study for nephrotoxicity**

After 48 h, animals were sacrificed by chloroform anaesthesia. The blood samples were collected by cardiac puncture under diethyl ether anaesthesia, using 21 gauge (21 G) needles mounted on a 5ml syringe (Hindustan syringes and medical devices ltd, Faridabad, India) and separated into two parts. The first part blood mixed with ethylene diamine tetra-acetic acid (EDTA) – coated sample bottles for analyzed Hematological parameters like full blood count (FBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet concentration (PLC) and total leucocyte count (TLC). These parameters were analyzed using automatic hematological system (Sysmex Hematology – Coagulation system, Model MO-1000 I, Trans Asia, Japan).

**Sampling and biochemical analysis for hepato toxicity study**

The second part of blood samples were mounted and centrifuged for 10min at 5000 rpm. The obtained clear sera were stored at -20 °C for subsequent measurement of blood urea, creatinine and uric acid levels using colorimetric assay kits, Bayer (Seamon) according to the manufacturer’s instructions. The remaining part of serum was analyzed for various biochemical parameters including serum glutamate oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminases (SGPT) (24), alkaline phosphatase (ALP) (25), bilirubin (26) and total protein (27).

**Preparation of hepato and renal homogenate**

Hepatic and renal tissues were individually homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-diamine tetra acetic acid (EDTA; pH 7.4) and centrifuged at 12,000g for 60 min. The supernatant was used for assay of the marker enzymes glutathione peroxidase, glutathione-s-transferase, superoxide dismutase and catalase, reduced glutathione, thiobarbituric acid reactive substances (TBARS) content, and protein estimation for oxidative stress effects in liver and kidneys.

**Biochemical estimation of markers of oxidative stress in liver and kidneys**

MDA content was measured according to the earlier method reported (28). SOD activity was determined according to previous report (29). CAT activity was determined from the rate of decomposition of H$_2$O$_2$ by the reported method (30). GPX activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H$_2$O$_2$ and NaN$_3$ (31). Glutathione reductase activity was assayed according to previous reports (32). Protein content in the tissue was determined by earlier method reported (33), using bovine serum albumin (BSA) as the standard.

**Histopathological examination**

On completion of closing regimen animals were sacrificed, the liver and kidney dissected out. Paraffin sections were prepared for histological examination and following standard procedure (34). Hematoxylin-eosin stained sections were observed.

**Statistical analysis**

The obtained results were analyzed for statistical significance using one way ANOVA followed by Dunnet test using the graph pad statistical software for comparison with control group and APAP treated group. P < 0.05 was considered as significant.
Fig. 1. Effect of EEML on serum levels of SGOT (IU/L), SGPT (IU/L) and MDA (nM/mg of protein) [Lipid peroxidation (LPO)] level of hepatic tissue during APAP treated hepatotoxicity and oxidative stress in rats. Values are mean ± S.D. (n = 6). **p < 0.01, *p < 0.05, respectively.

Fig. 2. EEML on serum levels of Alkaline phosphatase (ALP) (IU/L) & total protein and hepatic levels of CAT (U/mg protein), GSH (U/mg protein) and GPX (micrograms of glutathione utilized/min/mg protein) during APAP treated hepatotoxicity and oxidative stress in rats. Values are mean ± S.D. (n = 6). **p < 0.01, *p < 0.05, respectively.

Fig. 3. Effect of EEML on hepatic levels of SOD (units of activity/mg protein) & GST (Units/mg protein) during APAP treated hepatotoxicity and oxidative stress in rats. Values are mean ± S.D. (n = 6). **p < 0.01, *p < 0.05, respectively.

Fig. 4. Effect of EEML on serum levels of total bilirubin (mg/dl) during APAP treated hepatotoxicity and oxidative stress in rats. Values are mean ± S.D. (n = 6). **p < 0.01, *p < 0.05, respectively.

RESULTS

Effect of EEML on GOT, GPT, ALP, protein levels and total bilirubin concentrations for hepatotoxicity determination

The effect of EEML on serum marker enzymes was presented in fig 1-4. The serum levels of GOT, GPT, ALP and total bilirubin were markedly significantly (p < 0.01) elevated (Fig 1 & 2) and that of protein levels were significantly (p < 0.01) (Fig.2) decreased in APAP treated animals, indicating liver damage. Administration of EEML at the doses of 500 and 700 mg/kg remarkably significantly (p < 0.05; p < 0.01) prevented hepatotoxicity induced by APAP.

Effect of EEML on serum urea, uric acid and creatinine concentrations for nephrotoxicity determination

Serum urea and creatinine concentrations were significantly increased (p < 0.01) in the APAP treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity (Fig 6 & 8). Treatment with the EEML showed significant (p < 0.05 & p < 0.01) (Group III & IV) decrease in concentrations of serum urea and creatinine compared to the APAP treated group. However the levels of uric acid (UA) significantly decreased (p<0.01) in the APAP
Fig. 5. Effect of treatment with EEML on the renal intracellular CAT activity & blood hematological parameters (Neutrophil, MCHC & MCH), in rats with APAP (APAP)-induced nephrotoxicity. All values are mean ± S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett’s t-test.)

Fig. 6. Effect of treatment with ethanol extract of Madhuca longifolia on the renal intracellular GPX, GSH activity, blood hematological parameters (Gran,TLC & Hb) and serum urea (UR) levels, in rats with APAP (APAP)-induced nephrotoxicity. All values are mean ± S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett’s t-test.)

Fig. 7. Effect of treatment with EEML on the renal MDA level, blood hematological parameter (PLC) and serum uric acid levels, in rats with Acetaminophen (APAP)-induced nephrotoxicity. All values are mean ± S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett’s t-test.)

Fig. 8. Effect of treatment with ethanol extract of Madhuca longifolia on the blood hematological parameter (MCV, DLC, PCV) and serum creatinine levels, in rats with APAP (APAP)-induced nephrotoxicity. All values are mean ± S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett’s t-test.)

Effect of EEML on hematological parameters for nephro toxicity determination

APAP caused a significant (P<0.01) increase in the levels of Hb, PCV, DLC and MCV (Fig 6 & 8) (Group II) when compared to the treated groups (Group II, Fig.7), when compared to the control group. Treatment with EEMIL significantly (p < 0.05 & p < 0.01) (Group III & IV respectively) increased the uric acid levels, compared to the APAP treated group.
Fig. 9. Effect of treatment with ethanol extract of *Madhuca longifolia* on renal SOD activity in rats with Acetaminophen (APAP)-induced nephrotoxicity. All values are mean ± S.D., (n = 6). **p < 0.01, *p < 0.05 with respect to control. (One way ANOVA followed by Dunnett’s t-test.)

![Graph showing SOD activity](image)

Fig 10. Hepatoprotective effect of EEML against APAP (AAP) induced acute hepatotoxicity in rats. Liver sections were stained with H&E 100X. (a) Normal; (b) APAP; (c) EEML (500 mg/kg body wt) + APAP; (d) EEML (750 mg/kg body wt) + APAP

![Liver sections](image)

Antioxidant parameters of liver and kidneys

Analysis of MDA levels by thiobarbituric acid reaction showed a significant (P<0.01) increase in the APAP treated rats in both hepatic and renal tissues. Treatment with EEML (500 mg/kg & 750 mg/kg) significantly (P<0.05; P<0.01) prevented the increase in MDA level which was brought to near normal (fig 1 & 7) in both hepatic and renal tissues. APAP treatment caused a significant (P<0.01) decrease in the level of SOD, catalase, GSH, GPX, GRD and GST in liver and renal tissue when compared with control group. The treatment of EEML at the doses of 500 and 750 mg/kg resulted in a significant (P<0.05; P<0.01) increase of SOD, catalase, GSH, GRD, GPX and GST when compared to Group II (Fig 2, 3&5, 6) in both Hepatic and renal tissues.

Histopathological examination of liver tissue

Morphological observations showed an increased size and enlargement of the liver in APAP treated groups. These changes were reversed by treatment with EEML at the two different doses tested groups. Histopathological profile of the normal animal showed normal hepatocytes with well preserved cytoplasm and there was no sign of inflammation, which has been illustrated in Fig 10 (A). The APAP treated animals showed severe centrilobular necrosis and fatty infiltration (Fig 10 B). Treatment with different doses of EEML produced mild degenerative changes and absence of centrilobular necrosis when compared with control [Fig 10 (C) and 10(D)]. All these results indicate a hepatoprotective potential by the EEML.
Histopathological examination of Kidney tissue

The histological changes in kidneys and pathological manifestations are presented in Fig. 11. The histopathological observation in APAP-treated rats showed the tubular necrosis, inflammatory cell infiltration, tubular degeneration, hemorrhage, swelling of tubules and vacuolization. This could be due to the accumulation of free radicals as the consequence of increased lipid peroxidation by the renal tissues of APAP-treated rats. Our study also reported that *Madhuca longifolia* significantly reduces the histological changes induced by APAP. Normal glomeruli and tubules were observed in normal rat kidney (H&E 100X) (Fig 11 A). The APAP (750 mg/kg Bw) treated rat kidney (H&E 100X) shown that multiple foci of hemorrhage, necrosis and cloudy swelling of tubules (Fig 11 B). The treatment with APAP and *Madhuca longifolia* extract (500 mg/kg) group observed normal appearance of glomeruli and mild tubules in rat kidney (Fig 11 C). Almost normal appearance of kidney glomeruli and tubules were appeared in APAP *Madhuca longifolia* extract (750 mg/kg) treated rat kidney (Fig11 D).

DISCUSSION

APAP (N-acetyl-p-aminophenol, Paracetamol), a widely used analgesic and antipyretic drug is known to cause hepatotoxicity in experimental animals and humans at high doses (35-39). The laboratory features of hepatotoxicity induced by APAP resemble other kinds of acute inflammatory liver disease with prominent increase of GOT, GPT, and ALP levels (40). In the present study, the serum level of hepatic enzymes GOT, GPT, ALP and total bilirubin levels were increased and reflected the hepatocellular damage in the APAP-induced hepatotoxicity animal model. This is indicative of cellular leakage and loss of functional integrity of cell membrane in liver (41). However the total protein level was decreased. There was a significant (P<0.01) restoration of these enzyme levels on administration of the EEML in a dose dependent manner.

The reversal of increased serum enzymes in APAP induced liver damage by the EEML may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocyes (42-43). Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretory mechanism of the hepatic cells, as well as repair of hepatic tissue damage caused by APAP. This indicates the anti-lipid per oxidation and/or adaptive nature of the systems as brought about by EEML against the damaging effects of free radical produced by APAP.

The vital function that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication by xenobiotics, makes the hematopoietic system unique as a target organ (44). The various blood cells (erythrocytes, leucocytes, and platelets) are produced at a turnover rate of about 1 to 3 million per second in a healthy human adult and this value could be altered in certain physiological or pathological states including hemolytic anemia or suppressive inflammation (45). Certain drugs including alkylating cytotoxic agents could also affect blood formation rate and the normal range of hematological parameters (44). Treatment with APAP oral dose significantly increased the Hb, PCV, DLC & MCV levels. After administration of EEML these levels are significantly decreased compared to the APAP induced group. Whereas the levels of granulocyte, MCH, MCHC, neutrophils and PLC were decreased significantly in the APAP treated group, compared to the normal control group. However after administration of *Madhuca longifolia* extract these levels are significantly increased compared to the APAP treated. However this study shows that the plant extract could contain candidate molecules reversing the hepatotoxic effect of APAP, with ensuing improvement of hematopoiesis.

Blood urea nitrogen is found in the liver protein that is derived from diet or tissue sources and is normally excreted in the urine. In renal disease, the serum urea accumulates because the rate of serum urea production exceeds the rate of clearance (46). Elevation of urea and creatinine levels in the serum was taken as the index of nephrotoxicity (47). Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatinine breakdown (46). Thus serum urea concentration is often considered a more reliable renal function predictor than serum creatinine. In this study, APAP induced nephrotoxicity showed a significant (P<0.01) increase in the serum urea and creatinine concentrations in the Group II (APAP induced) rat when compared to the normal group (Group I). Moreover, oral administration of EEML significantly (P<0.01) decreased in group III & IV when compared to the Group II. However the level of uric acid is significantly decreased (P<0.01) in the Group II rats when compared to Group I. Oral administration of plant extract significantly (P<0.01) increases the uric acid level in Group I when compared to the APAP induced rats (Group II).

Previous studies have demonstrated that oxidative stress is a major mechanism in the development of APAP-induced hepatotoxicity (48-50). In the present study, administration of hepatotoxic and nephrotoxic doses of APAP to rats resulted in development of oxidative stress damage in hepatic and renal tissues. Thus, oxidative stress and lipid peroxidation are early events related to radicals generated during the hepatic metabolism of APAP. Also the generation of reactive oxygen species has been proposed as a mechanism by which many chemicals can induce nephrotoxicity (51). Previous studies have clearly demonstrated that acute APAP overdose increases the lipid peroxidation and suppresses the antioxidant defense mechanisms in renal tissue (52-53). However in the APAP treated animals the MDA levels are increased significantly, when compared to normal control rats. On administration of EEML, the levels of MDA decreased significantly when compared to APAP induced rats.

During liver and kidney injury, superoxide radicals are generated at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which damages kidney. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism (54). The
The present study also demonstrated that acute APAP overdose resulted in a decrease in the SOD, CAT and GST activities, when compared with normal control rats. It is due to enhanced lipid peroxidation or inactivation of the antioxidant enzymes. When rat was treated with the EEML the reduction of SOD, CAT and GST activity was increased significantly when compared with induced group (P<0.01) (Group II). Current evidence suggests that intracellular GSH plays an essential role in detoxification of APAP and prevention of APAP-induced toxicity in the liver and kidney (55-57). However, APAP was found to increase the microsomal superoxide and hydrogen peroxide production in mice. The generation of the reactive oxygen species appears as an early event which precedes intracellular GSH depletion and cell damage in APAP hepatotoxicity (58). APAP administration also caused a significant decrease in GSH content. Administration of Madhuca longifolia extract helped to uplift the GSH depletion induced by APAP.

APAP-induced hepatotoxicity and nephrotoxicity was evidenced by biochemical measurements and histopathological changes that coincide with the observations of other investigators (59-60). The biochemical results were also confirmed by the histological findings which showed preservation of the glomeruli and the surrounding Bowman’s capsule and mildly swollen tubules. Other nephroprotective medicinal plants have been reported of inhibiting xenobiotic-induced nephrotoxicity in experimental animal models due to their potent antioxidant or free radicals scavenging effects (61). In addition, alkaloids have also been reported to strongly inhibit lipid peroxidation induced in isolated tissues via its antioxidant activity (21). The protection offered by the extract could have been due to the presence of flavonoids and alkaloids (62-63). The histopathological findings in liver shows severe centrilobular necrosis and fatty infiltration in hepatocytes was produced by APAP. Treatment with different doses of EEML produced only mild degenerative changes and absence of centrilobular necrosis, indicating Madhuca longifolia treatment significantly rescured these signs of inflammation and necrosis. This result indicated that Madhuca longifolia treatment conferred hepatoprotective activity.

The activity elicited by the extract might be due to its ability to activate antioxidant enzymes. The findings suggest the potential use of the ethanolic extract of Madhuca longifolia as a novel therapeutically useful for hepato and nephroprotective agent. The protection offered by the extract might be due to the presence of flavonoids and alkaloids (62-63). Therefore, further studies to elucidate their mechanisms of action should be conducted to aid the discovery of new therapeutic agents for the treatment of hepatic and renal diseases. Further studies to characterize the active principles and to elucidate the mechanism are in progress.

REFERENCES

52. Abdel-Zaheer OA, Abdel-Rahman MM, Hafez MM, Omran FM. Role of nitric oxide and reduced glutathione in the protective effects of aminoguanidine, gadolinium chloride and oleandonic acid against APAP-induced hepatic and renal damage. Toxicology , 2007; 243: 124-34.

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