Antioxidant activity and hepatoprotective potential of *Terminalia pallida*

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Abstract

Terminalia pallida (TP) Brandis is one of the oldest medicinal herb of India, is an ingredient of Indian Ayurvedic drug 'triphala' used for the treatment of digestion and liver disorders. In Indian traditional system of medicine, the fruits are also used in the treatment of hepatic disorders and treatment of diabetes by tribal people. Acetaminophen (APAP) is used as an analgesic which produces liver and kidney necrosis in mammals at high doses. The aim of the present study was to investigate the hepatoprotective and antioxidant activities of the ethanol extract of *Terminalia pallida* at two doses level of 250 mg/kg & 500 mg/kg B/W on acetaminophen- induced hepatotoxicity in rats. The results of study showed that APAP significantly increased serum levels of GOT & GPT, ALP and total bilirubin. In addition, the ethanol extract of TP significantly ($p<0.01$) elevated the decreased level of antioxidant enzymes such as superoxide dismutase (SOD) & catalase (CAT), glutathione peroxidase (GPX), glutathione-s-transferase (GST) and reduced glutathione (GSH). Histological analysis of the liver of these rats revealed marked necro-inflammatory changes by APAP and ethanol extract of TP attenuated the necro-inflammatory changes in the liver. The activity of ethanol extract of *Terminalia pallida* at 500 mg/kg B/W was comparable to the standard drug silymarin (25mg/kg B/W). This study reveals that ethanol extract of TP showed significant hepatoprotective and antioxidant properties from APAP induced liver damage & oxidative stress.

Key words: Hepatoprotective, Terminalia pallida, acetaminophen, silymarin, antioxidant

Introduction

Herbal medicines have recently attracted much attention as alternative medicines useful for treating or preventing life style related disorders and relatively very little knowledge is available about their mode of action. There has been a growing interest in the analysis of plant products which has stimulated intense research on their potential health benefits. APAP is an antipyretic analgesic drug that is available over-the-counter, and an overuse of APAP can cause overproduction of ROS during formation of N-acetyl-p-benzoquinoneimine (NAPQI) by cytochrome P450 [1]. Many studies have demonstrated that overproduction of Reactive oxygen species (ROS) [such as super oxide anion, hydroxyl radical and hydrogen peroxide] can further aggravate the oxidative stress and the result is a unifying mechanism of injury that occurs in many developments of clinical disease processes, such as heart disease, diabetes, liver injury, cancer, aging, etc. [2-6]. Maintaining the balance between ROS and antioxidant enzymes (especially superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) is therefore crucial, and could serve as a major mechanism in preventing damage by oxidative stress. This balance has been suggested to play an important role in drug toxicity, such as from acetaminophen (APAP) [7]. This mechanism has been suggested to participate in the development of oxidative stress and injury in APAP-induced hepatotoxicity [8]. Hepatotoxicity arises from infectious diseases and oxidative damages, etc.
Terminalia pallida Brandis. (Family: Combretaceae) is a small evergreen endemic tree mainly distributed in the Tirupathi Hills, Andhra Pradesh, India [9]. The fruits of the plants are widely used in the treatment of ulcers[10], diarrhea, venereal diseases, diabetes mellitus[11-13] peptic ulcers[14], antibacterial & antifungal [12], hepatic disorders [10]and skin disease[15]. The bark has mild diuretic property [16] This hypothesis was tested here by evaluating the hepatoprotective and antioxidant effects of *Terminalia pallida* against acetaminophen -induced liver injury. The histopathological changes from liver biopsy, enzymatic alterations, and change of liver ingredients content were examined.

Materials and Methods

Plant material
The whole plant of *Terminalia pallida* was collected from Nilgiri hills, Ooty, Tamilnadu region and authenticated through Government Arts College, Ooty. Voucher specimen (AECBT-01/2007-2008) has been retained in the Anna bioresearch foundation Arunai engineering college, Tiruvannamali,Tamilnadu, India.

Extraction
The whole 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^{o}C [17]. The solvent was completely removed by rotary vacuum evaporator. The extract was freeze dried and stored in a 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 x10 cm) and maintained under standard laboratory conditions (temperature 25 ± 20C) 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 Animal's Ethical Committee (NO.1011/C/06/CPCSEA).

Acute toxicity study

The safety study was carried out using OECD guide lines No. 423. Three rat of same age group and weight were taken in a single dose of ethanol extract of *Terminalia pallida* up to the highest dose of 2000 mg/kg orally. The animals were observed for 1 h continuously and then hourly for 4 h and finally after every 24 h up to 15 days for any mortality or gross behavioural changes [18]

Drugs and Chemicals

Silymarin was purchased from Micro labs, Tamilnadu. India. Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin and total protein kits were procured from Span Diagnostics, Surat, India, and the rest of the chemicals utilized were of analytical grade and were obtained from Ranbaxy Research Laboratory, Hyderabad, India.

Experimental treatments

Animals were divided into five groups of six animals each. Group I treated with vehicle (distilled water) was kept as normal. Group II treated with a single dose of acetaminophen (AAP) of 750mg/kg body weight was kept as toxin control. Group III and IV were treated with ethanol extract of *Terminalia pallida* 250 mg/kg and 500 mg/kg body wt plus AAP and Group V were fed with standard drug silymarin 25 mg/kg daily for seven days. The extract was administered by oral gavages 1 h before AAP administration [19].

Preparation of serum from blood

After 24 h, animals were sacrificed by chloroform anesthesia. Blood was collected by heart puncture. The blood samples of each animal were taken and allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 600×*g* for 15 min and analyzed for various biochemical parameters including serum glutamate oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminases (SGPT) [20], alkaline phosphatase (ALP) [21], bilirubin [22] and total protein [23].

Preparation of liver homogenate

Hepatic tissues were homogenized in KCl [10 mM] phosphate buffer (1.15%) with ethylene-diamine tetra acetic acid (EDTA; pH 7.4) and centrifuged at 12,000×g 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.

Biochemical estimation of markers of oxidative stress

MDA content was Measured according to the earlier method reported [24]. SOD activity was determined according to previous report [25]. CAT activity was determined from the rate of decomposition of H_2O_2 by the reported method [26]. GPx activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3 [27]. Glutathione reductase activity was assayed according to previous reports [28]. Protein content in the tissue was determined by earlier method reported [29-30], using bovine serum albumin (BSA) as the standard.

Histopathological study

On completion of closing regimen animals were sacrificed and the liver dissected out. Paraffin sections were prepared for histological examination and following standard procedure [31]. 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 acetaminophen treated group. $P < 0.05$ was considered as significant.

Results and Discussion

Acute toxicity study

Rat when fed with ethanol extract of *Terminalia pallida* up to 2000 mg/kg, po exhibited no mortality or any sign of gross behavioral changes when observed initially for 72 h and finally up to 15 days.

The effect of *Terminalia pallida* on serum marker enzymes is presented in table 1 and fig 1, 2 & 4. The serum levels of GOT & GPT, ALP and total bilirubin were markedly significantly ($p< 0.01$) elevated and that of protein levels significantly ($p< 0.01$) decreased in acetaminophen treated animals, indicating liver damage. Administration of ethanol extract of *Terminalia pallida* at the doses of 250 and 500 mg/kg remarkably significantly ($p< 0.05$; $p< 0.01$) prevented hepatotoxicity induced by acetaminophen.

Analysis of MDA levels by thiobarbituric acid reaction showed a significant ($P<0.01$) increase in the acetaminophen treated rats. Treatment with *Terminalia pallida* (250 mg/kg & 500 mg/kg) significantly ($P<0.01$; $P<0.01$) prevented the increase in MDA level which was brought to near normal (fig 1). Acetaminophen treatment caused a significant ($P<0.01$) decrease in the level of SOD, catalase, GPX and GST in liver tissue when compared with control group. The treatment of *Terminalia pallida* at the doses of 250 and 500 mg/kg resulted in a significant ($P<0.05$; $P<0.01$) increase of SOD, catalase, GPX and GST when compared to Group II (Table 2; Fig 2 & 3). The standard drug, silymarin treated animals also showed a significant ($P<0.01$) increase in antioxidant enzymes levels compared to Group II. Morphological observations showed an increased size and enlargement of the liver in acetaminophen treated groups. These changes were reversed

by treatment with silymarin and also *Terminalia pallida* 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 5 (a). The acetaminophen treated animals showed severe centrilobular necrosis and fatty infiltration (Fig 5 b). Treatment with different doses of ethanol extract of *Terminalia pallida* and silymarin produced mild degenerative changes and absence of centrilobular necrosis when compared with control [Fig 5 (c), 5 (d) & 5 (e)]. All these results indicate a hepatoprotective potential by the ethanol extract of *Terminalia pallida*.

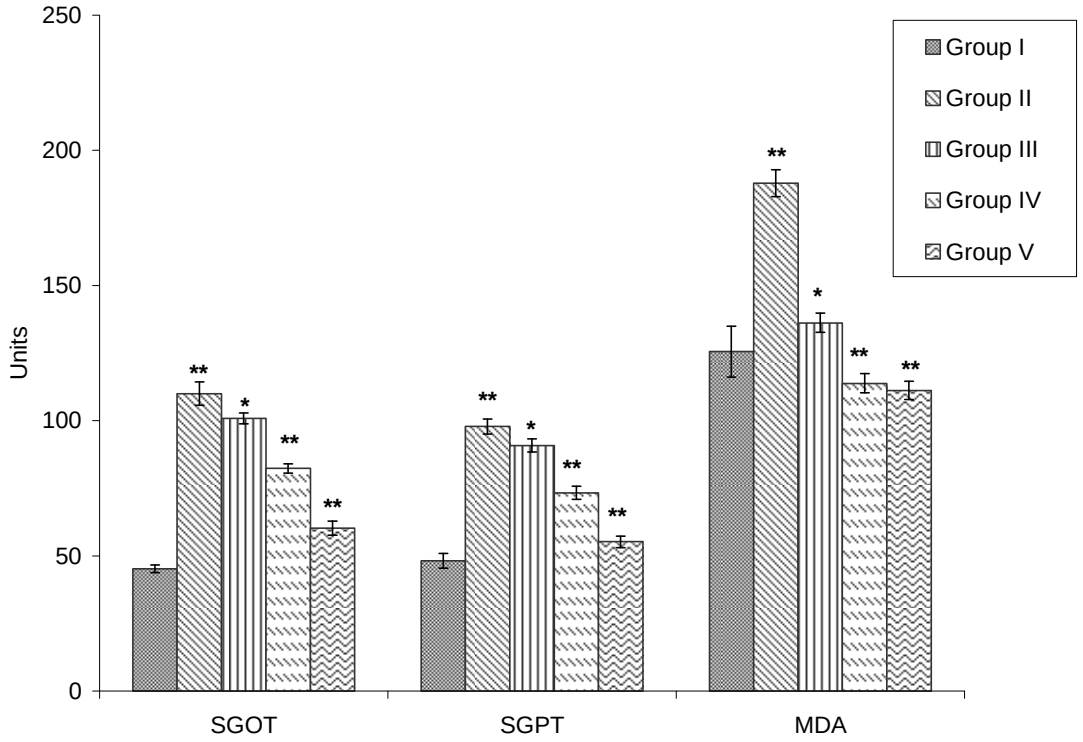

Fig.1. Effect of ethanolic extract of *Terminalia pallida* and silymarin (standard drug, (25 mg/kg)) on serum levels of SGOT (IU/L), SGPT (IU/L) and MDA (nM/mg of protein) [Lipid peroxidation (LPO)] level of hepatic tissue during acetaminophen treated hepatotoxicity and oxidative stress in rats. (Values are mean ± S.D. (*n* = 6). **p < 0.01, *p < 0.05, respectively)

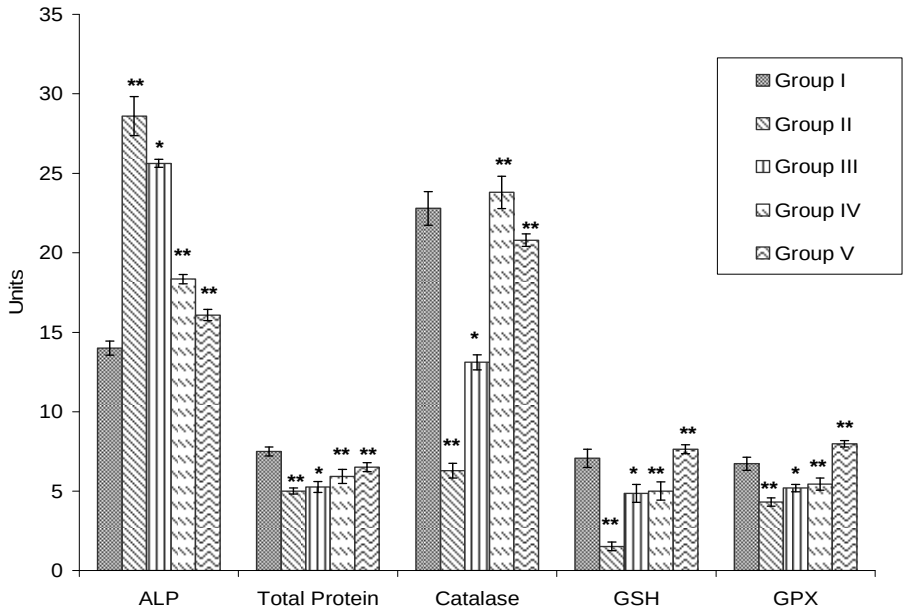

Fig.2. Effect of ethanolic extract of *Terminalia pallida* and silymarin (standard drug, (25 mg/kg)) on serum levels of alkaline phosphotase (ALP) (IU/L) & total protein and hepatic levels of CAT (U/mg protein), GSH (U/mg protein) and GPX (micrograms of glutathione utilized/min/mg protein) during acetaminophen treated hepatotoxicity and oxidative stress in rats. (Values are mean ± S.D. (*n* = 6). **p < 0.01, *p < 0.05, respectively)

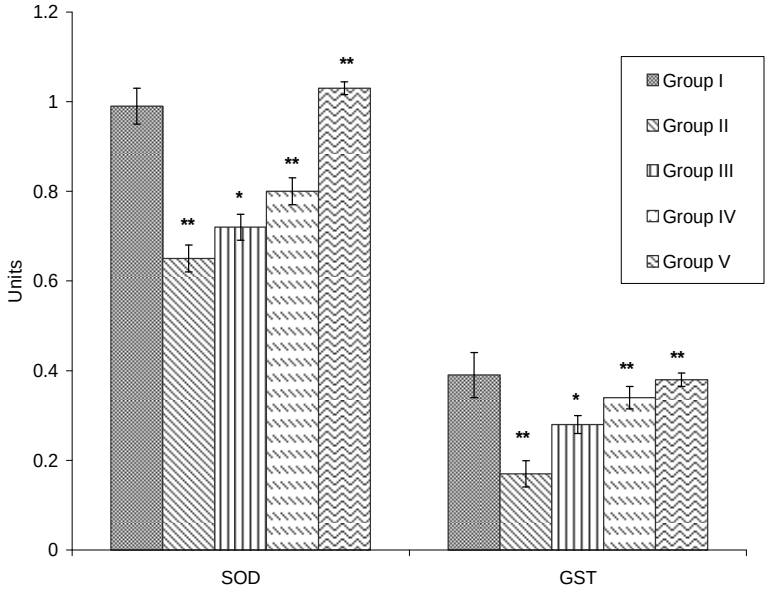

Fig.3. Effect of ethanolic extract of *Terminalia pallida* and silymarin (standard drug, (25 mg/kg) on hepatic levels of SOD(units of activity/mg protein) & GST (Units/mg protein) during acetaminophen treated hepatotoxicity and oxidative stress in rats. (Values are mean ± S.D. (*n* = 6). **p < 0.01, *p < 0.05, respectively)

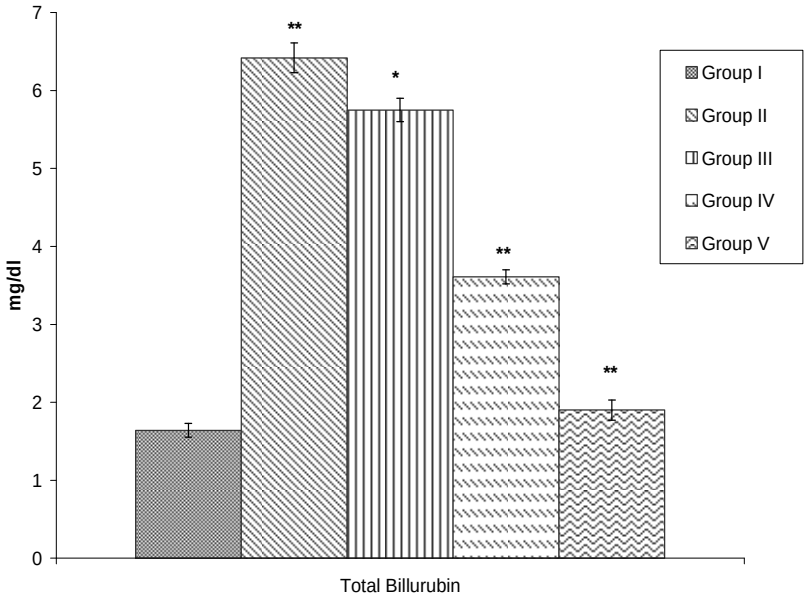

Fig.4. Effect of ethanolic extract of *Terminalia pallida* and silymarin (standard drug, (25 mg/kg)) on serum levels of total bilurubin (mg/dl) during acetaminophen treated hepatotoxicity and oxidative stress in rats. (Values are mean ± S.D. (n = 6). **p < 0.01, *p < 0.05, respectively)

The safe evaluation study of ethanol extract of *Terminalia pallida* showed that no mortality of rat occurred, at a limit dose of 2000 mg/kg body weight given per os. This is an indication the extract has low acute toxicity when administered per os. According to[32], substances with LD_{50} of 1000 mg/kg body weight/oral route are regarded as being safe or of low toxicity.

Acetaminophen a widely used antipyretic analgesic drug produces acute hepatic damage on accidental over dosage. The hepatic damage is established that, a fraction of acetaminophen is converted via the cytochrome P450 pathway to a highly toxic metabolite; N–acetyl–p–benzoquinamine (NAPQI) [1] which is normally conjugated with glutathione and excreted in urine. In overdose situations, however, glutathione levels are exhausted and NAPQI can directly modify susceptible protein residues in what is widely believed to be the first step in a cascade of biochemical events leading to hepatocyte death [33 -40]

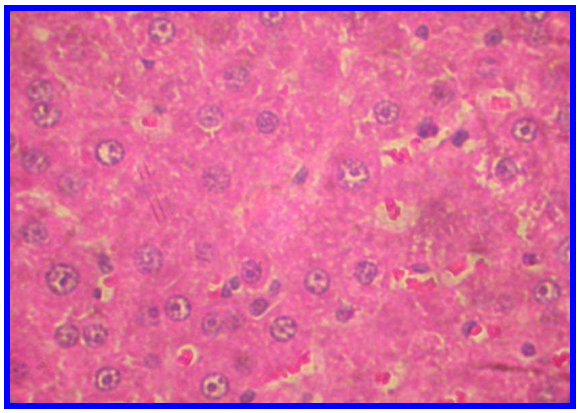

Fig. 5(a)

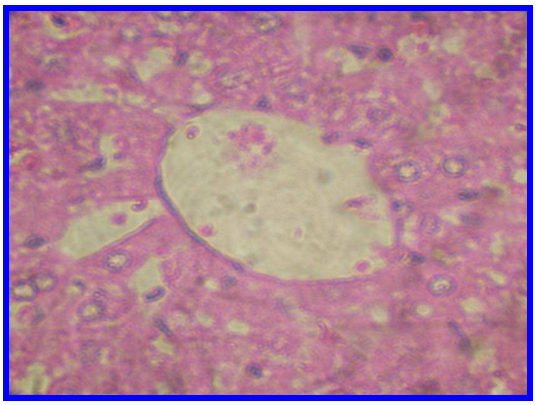

Fig. 5(b)

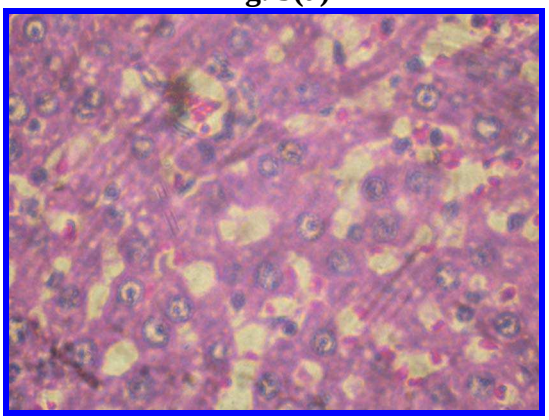

Fig. 5(c)

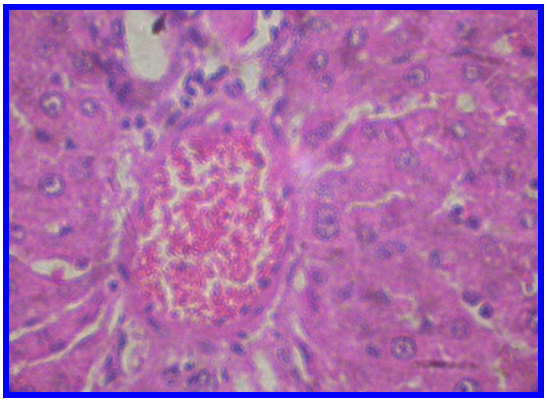

Fig. 5(d)

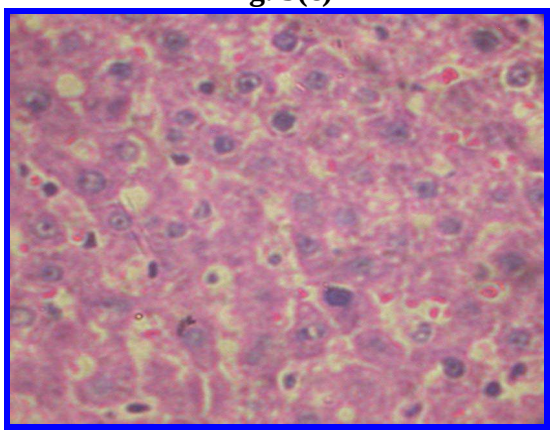

Fig. 5(e)

Fig.5. Hepatoprotective effect of ethanol extract of *Terminalia pallida* against acetaminophen (AAP) induced acute hepatotoxicity in rats. Liver sections were stained with H&E 100X. (a) Normal; (b) AAP; (c) *Terminalia pallida* (250 mg/kg body wt) + AAP ; (d) *Terminalia pallida* (500 mg/kg body wt) + AAP and (e) Silymarin (25 mg/kg body wt) + AAP

In the present study, rat treated with single dose of AAP treated animals developed a **s**ignificant hepatic damage and oxidative stress, resulted in a marked increase in serum SGOT, SGPT, SALP and total bilirubin levels. 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 ethanol extract in a dose dependent manner and also by silymarin at a dose of 25 mg/kg. The reversal of increased serum enzymes in acetaminophen induced liver damage by the extract 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 hepatocytes [42-43]. Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretary mechanism of the hepatic cells, as well as repair of hepatic tissue damage caused by AAP. This indicates the anti-lipid per oxidation and/or adaptive nature of the systems as brought about by plant extract against the damaging effects of free radical produced by AAP.

The increase in MDA level in liver induced by acetaminophen suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with *Terminalia pallida* significantly reverses these changes. Hence it is likely that the mechanism of hepatoprotection of *Terminalia pallida* is due to its antioxidant effect.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury [44]. SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. In *Terminalia pallida* causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [45].

sTherefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. A higher dose (500 mg/kg) of *Terminalia pallida* and silymarin increases the level of CAT.

Both reductions of GPX &GSH activity AAP-treated rats as observed in this study indicate the damage to the hepatic cells. Administration of *Terminalia pallida extract* promoted the reactivation of hepatic glutathione reductase enzyme in AAP-treated rats. The restoration of GSH level after the administration of plant extract to such AAP treated rats due to the protective effect of the ethanol extract.

Severe centrilobular necrosis and fatty infiltration in in hepatocytes was produced by acetaminophen. Treatment with different doses of ethanolic extract of *Terminalia pallida* produced only mild degenerative changes and absence of centrilobular necrosis, indicating *Terminalia pallida* treatment significantly rescured these signs of inflammation and necrosis. Suggesting that *Terminalia pallida* treatment conferred hepatoprotectivity.

Conclusion

The ethanol extract of *Terminalia pallida* significantly protects against liver injuries as well as oxidative stress, resulting in improved serum biochemical parameters such as SGOT, SGPT and SALP. The reduced levels of parameters of SOD, CAT, GSH, GPX, and GST in acetaminophen-treated rats were significantly increased by treatment with ethanol extract of *Terminalia pallida*. Further studies to characterize the active principles and to elucidate the mechanism are in progress.

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