

PROTECTIVE EFFECT AND ANTIOXIDANT ROLE OF *ACHYRANTHUS ASPERA* L. AGAINST ETHANOL-INDUCED OXIDATIVE STRESS IN RATS

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ABSTRACT

Alcoholic liver disease is one of the most serious consequences of chronic alcohol abuse and the oxidative stress plays an important role in the development of the disease. The objective of this study was to investigate the protective activity of methanol extract of leaves of *Achyranthus aspera* against ethanol-induced oxidative stress. Administration of ethanol (5g/kg/day) for 21 days, resulted in liver injury and tested animals were treated orally with plant extract (100 and 200mg/kg), prior to ethanol administration. Hepatic marker enzymes like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total proteins and total bilirubin were analysed in serum. Lipid peroxidation marker mainly thiobarbituric acid reactive substances (TBARS) and antioxidant enzymes namely superoxide dismutase (SOD), reduced glutathione (GSH), glutathione reductase (GR) and glutathione-S-transferase (GST) activities were studied. The extract produced significant ($p < 0.05$) protective effect by decreasing the elevated liver marker enzymes, total bilirubin, lipid peroxidation marker and ameliorated the diminished serum total protein as well as antioxidant levels in a dose dependent manner. The present study suggest that leaf extract of *A. aspera* offers protection against oxidative stress and its ability might be attributed to its antioxidant potential.

Keywords: *Achyranthus aspera*, Ethanol-induced oxidative stress, Aspartate aminotransferase, Superoxide dismutase, Lipid peroxidation.

INTRODUCTION

Liver is the key organ of metabolism and excretion and is continuously, variedly exposed to xenobiotics because of its strategic placement in the body. The toxins absorbed from the intestinal tract gain access first to the liver, resulting in a variety of liver ailments. So many reactive intermediates could form in the liver during the detoxifying mechanisms, for example, alcohol-induced oxidative stress. The close relation between ethanol and liver damage is mainly due to the fact that about 80% of ingested alcohol is metabolized in the liver. Acute ethanol administration produces lipid peroxidation in the liver, as indicator of oxidative stress¹. Increased oxidative stress occurs directly due to ethanol and its oxidation products². Ethanol is extensively metabolized into cytotoxic acetaldehyde by alcohol dehydrogenase enzyme in the liver and acetaldehyde is oxidized to acetate by aldehyde oxidase or xanthine oxidase giving rise to reactive oxygen species (ROS) via cytochrome P450 2E1 (CYP 2E1)³. One factor that plays a central role in the aetiopathology of alcohol-induced liver disease and which has been the focus of much research is the excessive generation of ROS⁴. Thus, numerous interventions have been put forward to counteract the vulnerability of the liver to oxidative challenges during alcohol consumption by reinforcing the endogenous antioxidant defence system^{5, 6}.

Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparations which are employed for their treatment of liver disorders^{7, 8}. But, there is meager drug available for the treatment of liver disorders. Therefore, many folk remedies from plant origin are tested for its potential antioxidant and hepatoprotective effects against different chemical-induced liver damage in experimental animals.

The plant, *A. aspera* L. (Amaranthaceae) commonly known as Rough chaff tree in English and it is an indigenous medicinal plant of Asia, South America and Africa. It is commonly used by traditional healers for the treatment of malarial fever, dysentery, asthma, hypertension and diabetes^{9, 10}. The root extract is well reputed for its pronounced insect molting hormonal activity¹¹. A decoction of the whole plant is described to have diuretic properties and the aqueous extract is given for pneumonia. The dried herb is used to treat children for colic and also as an astringent in gonorrhoea treatment. The roots of *A. aspera* are reported to have application in infantile diarrhoea and cold¹² while dry leaves are employed against asthma¹³. The alcoholic extract of the plant is reported to have anti-inflammatory activity¹⁴.

Leaf extracts are reported to possess hypoglycemic, thyroid-stimulating and antiperoxidative activities^{15, 16}.

The seed of the plant is reported to enhance the immunity of catla¹⁷. From the shoots of *A. aspera*, an aliphatic dihydroxyketone (36, 47-dihydroxyheptacosan-4-one) has been isolated and characterized¹⁸. The ethanol extract of the root was found to have spermicidal action in vitro and in vivo studies¹⁹. Alcohol-induced hepatotoxicity model is frequently used for the investigation of hepatoprotective effects of drugs and plant extracts. Accordingly, the current study was undertaken to evaluate the possible effect of *A. aspera* to protect liver from oxidative stress during chronic ethanol exposure in *albino* rats.

MATERIALS AND METHODS

Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, bovine serum albumin (BSA), 1,2-dithio-bisnitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), picric acid, sodium tungstate, sodium hydroxide, trichloroacetic acid (TCA) and silymarin were purchased from Sigma Chemicals Co., Mumbai and all other chemicals used in this study were analytical grade with high purity.

Plant collection and preparation of plant extract

Fresh leaves of *A. aspera* were collected from Trichy district, Tamil Nadu, India during March 2009. The collected leaves were washed, shade dried, coarsely powdered and 500g were extracted with 1000ml of 80% methanol in a soxhlet extractor for 72 hrs. The extract was concentrated to dryness under reduced pressure and controlled temperature (25–35°C) to yield a green gummy residue.

Animals

Male *albino* Wistar rats (150-180g) were used for the experiment. The animals were procured from National Institute of Nutrition, Hyderabad, India. The animals were housed in polypropylene cages with sterile, inert husk materials as bedding. The experimental animals were maintained under controlled conditions (12 hrs, light and dark cycle, temperature $22 \pm 10^\circ\text{C}$ and relative humidity 40-70%). The animals were fed with a balanced commercial diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The animal experiments were performed according to the ethical

guidelines suggested by the Committee for the purpose of Control and Supervision of experiments on animals, Government of India.

Experimental protocols

After 2 weeks of acclimatization, the rats were allocated randomly to five experimental groups of six animals each.

Group-I (normal control): received 0.9% saline (5 ml/kg, b.w.).

Group-II (ethanol treated control): received 0.5 ml of 20% ethanol (5g/kg body weight).

Groups III and IV Received methanolic extract of *A. aspera* at 100 and 200mg/kg respectively, 2 hrs before administration of 20% ethanol.

Group-V (positive control): received silymarin, the known hepatoprotective compound at 25mg/kg, 2 hrs before administration of 20% ethanol.

The administration was done repeatedly on a daily basis for three weeks using metal oropharyngeal cannula. At the end of the experimental period of 3 weeks (i.e. on 22nd day), all rats from each group were sacrificed by cervical dislocation. Fresh blood was collected immediately by cardiac puncture in fresh sterilized tubes, allowed to clot and the serum was removed by centrifugation at 2500g at 30°C for 10 min. All serum samples were sterile, haemolysis-free and were kept at 4°C before determination of the biochemical analysis. The livers were removed immediately, washed with ice-cold saline and a 10% of homogenate was prepared in 0.15M Tris-HCl buffer and processed for the estimation of biochemical parameters.

Estimation of biochemical parameters

The activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed by the method of Reitman and Frankel²⁰. Serum bilirubin level was estimated by the method of Malloy and Evelyn²¹. Serum total protein was estimated by Biuret method²² and alkaline phosphatase (ALP) activities were determined by the method of Henry²³. The tissues MDA concentration was determined using the method of Jain et al.²⁴ based

on TBA reactivity. The tissues glutathione (GSH) concentration was measured using the method described by Beutler²⁵. Glutathione-S-transferase (GST) was assayed by following the method of conjugation of glutathione with 1-chloro-2, 4-dinitrobenzene (CDNB) at 340nm²⁶. Glutathione reductase (GR) activity was assayed according to Carlberg and Mannervik²⁷ as the decrease in absorbance of NADPH at 340 nm. Superoxide dismutase (SOD) activity was measured at 505nm by calculating inhibition percentage of formazan dye formation²⁸.

Statistical analysis

The experimental results were expressed as the mean \pm S.D. Student's t -test was used to analyze statistical significance followed by ANOVA. P values <0.05 were considered to be significant.

RESULTS

The pre-treatment of *A. aspera* extract on ethanol induced liver damage based on some liver marker enzymes (ALT, AST and ALP), and levels of total bilirubin were shown in Table 1. Animals in group II (ethanol only) developed hepatic damage when compared with animals in group I (saline only). This was indicated by a significant increase in the levels of the hepatic enzyme markers studied.

Pre-treatment of the animals with two different doses of plant extract (100 and 200 mg/kg) before ethanol administration (groups III and IV) ensured protection of the liver as shown by the significant decrease in the levels of all the hepatic enzyme markers studied under concern. The extract at the doses of 100 and 200mg/kg also reduced the level of bilirubin in group III and IV than the ethanol treated group in a dose dependent manner. The total protein levels were significantly (P<0.05) decreased in ethanol intoxicated rats compared to control group I. The plant extract given orally at the doses of 100 and 200mg/kg significantly reversed the altered total protein to almost normal level. The effects of pre-treatment with silymarin, a known hepatoprotective compound (group V), on all parameters are also shown in Table 1. Silymarin also produced a significant reduction in ALT, AST, ALP, serum bilirubin concentration and increase in total proteins, when compared with ethanol treated group.

Table 1: Effect of methanolic extract of *A. aspera* on hepatic markers in the serum of control and ethanol-administered rats

Group	Treatment	AST(IU/L)	ALT(IU/L)	ALP(IU/L)	Total Bilirubin (μ mol/l)	Total Proteins(g/dl)
I	Control (0.9% Nacl, 5ml/kg)	70.04 \pm 10.18 ^a	45.26 \pm 5.62 ^a	62.04 \pm 2.10 ^a	4.25 \pm 1.05 ^a	9.42 \pm 1.04 ^{ba}
II	Ethanol (5g/kg)	136.24 \pm 6.00 ^b	72.24 \pm 12.16 ^c	156.6 \pm 8.70 ^b	10.55 \pm 3.10 ^b	4.91 \pm 0.42 ^b
III	<i>A.aspera</i> extract (100mg/kg)+ethanol	93.33 \pm 8.08 ^{da}	60.72 \pm 5.24 ^{bac}	85.6 \pm 9.20 ^{da}	6.45 \pm 2.04 ^a	7.81 \pm 0.11 ^a
IV	<i>A.aspera</i> extract (200mg/kg)+ethanol	86.24 \pm 10.04 ^{da}	54.68 \pm 3.26 ^{bac}	79.6 \pm 9.40 ^{da}	6.02 \pm 1.26 ^a	8.01 \pm 1.04 ^{ba}
V	Silymarin (25mg/kg)+ethanol	106.42 \pm 15.08 ^c	48.32 \pm 5.78 ^{ba}	98.03 \pm 3.10 ^c	6.00 \pm 1.45 ^a	8.81 \pm 1.50 ^{ba}

Values are expressed as mean \pm S.D. for six rats in each group; Values not sharing a common superscript differ significantly at p < 0.05.

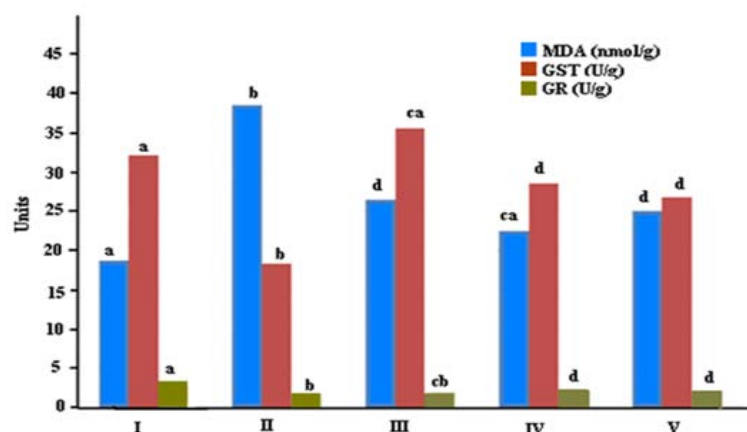


Fig. 1: Effect of methanolic extract of *A. aspera* on lipid peroxidation (MDA), glutathione-S-transferase (GST) and glutathione reductase (GR) in rats with ethanol induced oxidative stress. All values are mean \pm S.D., (n = 6). Values not sharing a common superscript differ significantly at p < 0.05.

MDA level was increased to 38.53 ± 5.25 nmol/g, while GST and GR levels were decreased to 18.34 ± 3.12 U/g, 1.72 ± 0.06 U/g respectively, in the liver tissue of rats given only ethanol. These levels were measured as 18.75 ± 2.99 nmol/g, 32.12 ± 5.32 U/g, 3.45 ± 1.03 U/g in control rat tissue. While in rats given 100 and 200mg/kg of plant extract, MDA levels were decreased to 26.52 ± 4.15 nmol/g and 22.48 ± 4.69 nmol/g, as well as GST and GR levels were increased somehow near to normal levels as 35.62 ± 4.40 U/g, 1.95 ± 0.08 U/g and 28.54 ± 4.12 U/g, 2.32 ± 0.60 U/g. These levels were also altered in silymarin pre-treated group as 25.03 ± 2.12 nmol/g, 26.80 ± 5.00 U/g, 2.17 ± 1.07 U/g (Fig 1).

Moreover, GSH and SOD levels were also decreased to 52.34 ± 2.30 mg/g and 46.32 ± 6.18 U/g, respectively in the ethanol treated rats compared to the control group, where these levels were 66.72 ± 1.32 mg/g and 72.60 ± 8.40 U/g respectively. GSH and SOD levels were increased compared to ethanol group and measured as 58.64 ± 3.12 mg/g, 67.26 ± 7.40 U/g and 60.69 ± 1.17 mg/g, 69.03 ± 6.72 U/g respectively in both group III and IV. These parameters were determined as 62.42 ± 4.18 mg/g and 64.34 ± 7.03 U/g in silymarin pre-treated group (Fig 2), which examines some increase in the antioxidant levels than ethanol treated rats alone.

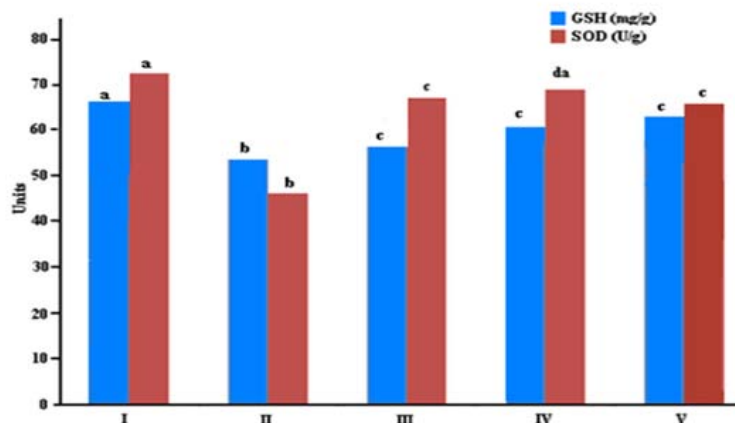


Fig. 2: Effect of methanolic extract of *A. aspera* on glutathione (GSH) and superoxide dismutase (SOD) in rats with ethanol induced oxidative stress. All values are mean \pm S.D., (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$.

DISCUSSION

The damage provoked by free radicals to macromolecule plays an essential role in the pathophysiological process of atherosclerosis, inflammation, carcinogenesis, aging, drug reaction and toxicity. Alcohol-induced hepatic tissue damage is mediated by acetaldehyde and reactive oxygen species²⁹. The removal and neutralization of these noxious toxic metabolites are considered to be vital initial steps in the prevention of alcohol-related liver diseases³⁰. The excess consumption of alcohol has been well associated with distorted damage and metabolism in liver along with leakage of cytoplasmic liver enzymes into the blood³¹. AST and ALT are considered among the most sensitive markers of hepatocellular injury. ALP, which is secreted from the lysosomes, is also a marker enzyme for assessing liver damage³². When the integrity of the lysosomal membrane changes and/or the membrane of the lysosome are ruptured by deleterious influences, this acid hydrolase enters the blood stream, producing transient increase in the activity of lysosomal enzymes in the serum. The increased levels of these enzymes (AST, ALT and ALP) in the serum have been observed in alcohol administered rats, which indicate increased permeability, damage and necrosis of hepatocytes³³. Pretreatment with the extract of *A. aspera* significantly decreased levels of serum enzyme markers, thus suggesting that the extract possessed compounds that protected the hepatocytes from alcohol induced liver injury and subsequent leakage of the enzymes into the circulation.

Serum bilirubin is one of the most sensitive tests employed in the diagnosis of hepatic diseases. It provides useful information on how well the liver is functioning³⁴. The abnormal levels of serum bilirubin are also related to the status and function of hepatic cells. In the present study, the extract has been found to reduce serum bilirubin in the treated groups compared with untreated groups. The results also showed that rats exposed to ethanol significantly decreased serum total protein levels. Hence the decline in total protein content can be deemed useful index of the severity of cellular dysfunction in chronic liver diseases. Stabilization of serum protein levels in the pre-treatment groups administered with *A. aspera* is further a clear indication of the improvement of the functional status of the liver cells.

Lipid peroxidation mediated by free radicals is considered to play a pivotal role in the mechanism by which ethanol may exert its toxic effects on the liver and other extra hepatic tissues³⁵. Increased LPO (an index of oxidative stress) and decreased antioxidant status are reflection of the liver's susceptibility to ethanol-induced oxidative damage. Oxidative damage has been reported to play a pathogenic role by promoting collagen production in the liver.³⁶ Impairment in the non-enzymatic and enzymatic antioxidant defence systems has been reported in alcoholics and experimental animals^{37, 38}. In our study, we observed an increase in TBARS and a decline in antioxidant status in alcohol fed rats. Pre-treatment with *A. aspera* (100 and 200mg/kg) significantly lower lipid peroxidation of liver tissues in dose dependent manner and the results are comparable with that of the silymarin treated group.

SOD, GST, GR and GSH, plays an important role in the biological systems to act against oxidative stress³⁹. The antioxidant enzymes such as GR, SOD and GST, take part in maintaining GSH homeostasis in tissues⁴⁰. GST represents one of the major cellular defence mechanisms against electrophilic xenobiotics and their metabolites. GR is a glutathione regenerating enzyme that permits the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by the oxidation of NADPH to NADP+. In the present study GR, GST activities were significantly decreased in the liver of rats exposed to ethanol as compared to control rats. The decline in the activities of GR and GST on ethanol exposure may be due to the involvement of these enzymes in the detoxification and possibly repair mechanism in liver. Pre-administration of *A. aspera* to the alcohol exposed rats normalized the activities of GR and GST. Induction of these enzymes has been evaluated as a means for determining the potency of many antioxidant substances⁴¹. In this context *A. aspera* is known to suppress reactive oxygen species and enhance these enzymes activities. Thus the ameliorated activities of GR and GST in alcohol exposed rats on *A. aspera* supplementation may be due to the antioxidant constituents which can scavenge free radicals.

Glutathione being an important cellular reductant, involved in protection against free radicals, peroxides and toxic compounds⁴². GSH depletion is one of the chief factors that lead to lipid peroxidation⁴³. In our present study, the GSH levels were decreased

in the liver of rats exposed to ethanol as compared to control rats. The decreased GSH level may be due to increase level of lipid oxidation products which may be associated with the less availability of NADPH required for the activity of glutathione reductase (GR) to transform oxidized glutathione to GSH⁴⁴ due to the increased production of ROS at a rate that exceeding the ability to regenerate GSH for long term ethanol exposure. The decreased GSH level in association with decreased GR activity may support the explanation as evidence.

Decreased level of superoxide dismutase is a perceptible manifestation in the hepatocellular damage⁴⁵. It scavenges the superoxide anion to form hydrogen peroxide, hence reducing the toxic effects caused by this radical. In this present study, it was observed that the extract caused a significant increase in the hepatic SOD activity to prevent free radical accumulation and thus protect the liver from injury. The active components of this plant may up regulate free radical and reactive metabolite scavenging systems. In the absence of consistent liver-protective drugs in modern medicine, a large number of plant derived natural products such as flavonoids, terpenoids and steroids have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant and hepatoprotective activity⁴⁶. In the present study, both doses of extract did not produce any damage to the system but elicit protective effects. The observed antioxidant property of *A. aspera* may enable it mop up noxious toxic metabolites released when alcohol is abused and this may explain the observed protection of the liver cells from damage and the improvement in the functional status of the cells after damage.

CONCLUSION

On the basis of the results obtained in the present study, it is concluded that ethanol-induced oxidative stress in rat liver is amenable to attenuation by *A. aspera* extract. The protective effect of *A. aspera* extract can be correlated directly with its ability to reduce the rate of lipid peroxidation as well as it restored the liver marker enzymes and enhance antioxidant defense status. The potency of *A. aspera* extract compares well with silymarin with respect to the hepatic markers observed. The findings of this study suggest that *A. aspera* can be used as a safe, cheap, and effective alternative chemopreventive and protective agent in the management of alcohol-related liver diseases.

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