

ANTIOXIDANT AND HEPATOPROTECTIVE EFFECT OF *Azadirachta indica* LEAF EXTRACT ON ACECLOFENAC INDUCED HEPATOTOXICITY IN RATS

Darbar Soumendra¹, Bose Anirbandeep¹, Bhaumik Uttam, Chatterjee Nilendra¹, Roy Bikash¹, Chattaraj Tapas Kumar¹, Das Anjan² and Pal Tapan Kumar^{1*}

¹Bioequivalence Study Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India

²Department of Pathology, Chittaranjan National Medical College and Hospital, Kolkata-700 014, India

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ABSTRACT

Hepatotoxicity is the most serious adverse effects of Aceclofenac. In this study, the effect of Aceclofenac (ACE) induced liver damage in rats was investigated. Administration of ACE (90mg/kg/day) for 28 days produced severe liver injury, as demonstrated by dramatic elevation of serum hepatospecific markers like serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (ALP) and serum α -glutamyl transpeptidase (GGT). In addition, the level of plasma and hepatic thiobarbituric acid reactive substances (TBARS) was elevated in ACE treated rats as compared to those of the experimental control rats. A remarkable reduction in hepatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activity and reduced glutathione (GSH) content were also observed on ACE administration as compared with experimental control rats. However, simultaneous treatments with *Azadirachta indica* (AI) leaf extract (both 250 and 500mg/kg) significantly attenuated ACE induced hepatotoxicity. The results showed that serum AST, ALT, ALP and GGT ($p < 0.05$), and hepatic TBARS ($p < 0.01$) were reduced dramatically, and hepatic SOD ($p < 0.05$), CAT ($p < 0.05$), GPx ($p < 0.01$) activity and GSH ($p < 0.05$) content were restored remarkably by AI supplementation. It is therefore suggested that *Azadirachta indica* can provide a definite hepatoprotective and antioxidant effect against hepatic injury caused by Aceclofenac.

Keywords: Aceclofenac; Antioxidant enzymes; *Azadirachta indica*; Hepatotoxicity

INTRODUCTION

Azadirachta indica (AI) (Common name: Neem; Family: Meliaceae) is an evergreen tree known for its potent insecticidal and medicinal properties that grows throughout the greater parts of India and Burma. The leaf extract has shown immunomodulatory, anti-inflammatory, anti-hyperglycemic, anti-ulcer, anti-malarial, anti-fungal, anti-bacterial, anti-viral, anti-oxidant, anti-mutagenic and anti-carcinogenic properties. Studies revealed that the water soluble portion of alcoholic extract of *Azadirachta indica* leaves possessed significant hepatoprotective activity¹⁻⁶.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain and inflammation. NSAIDs produced their therapeutic effects by inhibiting the cyclooxygenase (COX) enzymes, which are involved in the biosynthesis of prostaglandins (PGs). Two different forms of cyclooxygenase are effected to varying degree by different NSAIDs. Conventional NSAIDs inhibit both COX-1 and COX-2 at therapeutic doses. Nonsteroidal anti-inflammatory drugs are among the most common drugs associated with drug induced liver injury, with an estimated incidence of between 3

and 23 per 100,000 patient years. Drug induced liver injury (DILI) encompasses a spectrum of clinical disease ranging from mild biochemical abnormalities to acute liver failure. Antibiotics and NSAIDs are the most common cause of DILI⁶⁻¹².

Hepatotoxicity is one of the common side effects of nonsteroidal anti-inflammatory drugs (NSAIDs)¹³. Aceclofenac sodium is chemically designed as [[2-(2', 6'-dichlorophenyl) amino] phenylacetoxycetic acid] is a prodrug in the aryl-acetic acid class, is a commonly used NSAID in several countries. Aceclofenac is an oral non-steroidal anti-inflammatory drug (NSAID) that is effective in the treatment of painful inflammatory diseases and has been used to treat more than 75 million people worldwide¹⁴. Chronic used of aceclofenac, damages gastrointestinal mucosa by irritant action, causing alteration in mucosal permeability and/or suppression of prostaglandin synthesis. Aceclofenac has antipyretic, analgesic, and anti-inflammatory effects, is an inhibitor of arachidonic acid level. The use of oral nonsteroidal anti-inflammatory drugs is associated with upper gastrointestinal complications, particularly perforated

*Correspondence : darbar_soumen@yahoo.co.in

and bleeding peptic ulcer¹⁵. Our recent work established that, aceclofenac at a dose of 90 mg/kg produce severe GI and hepato toxicity in experimental animals^{16,17}. The aim of this work was to establish the antioxidant and hepatoprotective effect of *Azadirachta indica* on an animal model of aceclofenac induced liver damage.

EXPERIMENTAL

Chemicals and reagents

Aceclofenac Sodium was obtained from Dey's Medical Stores (Mfg) Ltd, 62 Bondel road. Kolkata-700019, India, as a gift sample. ALT, AST and ALP kits were obtained from Merck, Germany, and GGT kit was obtained from LABKIT, Spain. TBA, TCA and HCl were obtained from Merck, Germany. Chemicals for SOD, GSH, and CAT were obtained from Sigma Chemical Co. U.K. All other reagents used for the experiments were of analytical grade.

Collection of Plant material

Fresh green leaves of *Azadirachta indica* (AI) were collected from our Institute's (Jadavpur University, Kolkata, India) garden in the month between July – Sept'08 and were identified by an expert. A voucher specimen (No. AD-44/08-09) of the plant has been preserved in our department. The authenticity of the sample was identified by taxonomist Dr. Nanda Dulal Paria, HOD, Department of Botany, University of Calcutta.

Extraction of Plant material

Air dried powder (1kg) of fresh mature *Azadirachta indica* (AI) leaves were extracted by percolation at room temperature with 70% ethanol. Leaf extract of *A. indica* was concentrated under reduced pressure (bath temp. 50°C) and finally dried in a vacuum desiccator. The residue was dissolved in distilled water and filtered. The filtrate was evaporated to dryness. The dried mass (yield=50.2g) was suitably diluted with normal saline water and used in experiment¹⁸.

Animals

Fifty adult male albino wistar rats, weighing 122±4.5g were used as experimental animals in this study. The animals were housed in the animals care centre of faculty of Pharmacy, Jadavpur University. They were kept in wire-floored cages under standard laboratory conditions of 12h/12h light/dark, 25±2°C with free access to food and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India and approved by the Institutional Animal Ethics Committee (IAEC) of Jadavpur University.

Experimental design

The rats were randomly divided into 5 groups of 10 animals each as follows:

Group I: Normal control untreated rats fed with normal diet and water for 28 days.

Group II: Normal rats treated with AI leaf extract (250mg/kg, orally) only for 28 days.

Group III: Experimental rats treated with ACE (90mg/kg/day, i.p) only for 28 days.

Group IV: Animals treated with ACE (90mg/kg/day) along with AI (250mg/kg, orally) for 28 days.

Group V: Animals treated with ACE (90mg/kg/day) along with AI (500mg/kg, orally) for 28 days.

Serum and tissue preparation

After the experimental period animals were subjected to light ether anesthesia and killed by cervical dislocation. The blood sample was collected in heparinised centrifuge tube and centrifuge to obtain serum. The abdomen was excised and the liver was removed immediately by dissection, washed in ice-cold isotonic saline and blotted between two filter paper. The liver was wrapped in aluminum foil and stored at -80°C. A 10% liver homogenate was prepared in ice-cold 0.1 M potassium phosphate buffer, pH 7.5 using Branson sonifier (250, VWR Scientific, USA)¹⁹.

Estimation of liver marker enzymes

Serum aspartate aminotransferase (AST) and Serum alanine aminotransferase (ALT) were assayed using the standard diagnostic kit based on the method of Reitman and Frankel (1957)²⁰. Serum alkaline phosphatase (ALP) was estimated using the diagnostic kit based on the method of King (1965)²¹. The serum α -glutamyl transpeptidase (GGT) was assayed according to the method of Rosalki and Rau (1972)²².

Estimation of Thiobarbituric acid reactive substances (TBARS)

Assay for lipid peroxidation was carried out by measuring TBARS in the tissues by the method of Ohkawa et al (1979) and Yagi (1978)²³. The pink chromogen produced by the reaction of malondialdehyde, a secondary product of lipid peroxidation, with thiobarbituric acid was estimated at 532 nm.

Assay of Super Oxide Dismutase (SOD)

SOD was assayed by the method of Kakkar et al (1984). The assay was based on the 50% inhibition of the formation of NADH-phenazinemethosulfate-nitrobluetetrazolium (NBT) formazan at 520 nm²⁴.

Assay of catalase (CAT)

The activity of CAT was assayed by the method of Sinha (1992) based on the conversion of dichromate in acetic acid to perchromic acid and then chromic acetate, when heated in the presence of hydrogen peroxide. The chromic acetate formed was measured at 620nm²⁵.

Assay of glutathione peroxidase (GP_x)

GP_x in liver was assayed by the method of Rotruck et al (1973)²⁶. A known amount of enzyme preparation

was incubated with H_2O_2 in the presence of GSH for a specified time period. The amount of H_2O_2 utilized was determined by the method of Ellman et al. The enzyme activity was expressed as μmol of GSH consumed per min per mg protein.

Estimation of reduced glutathione (GSH)

GSH in the tissues was assayed by the method of Ellman (1959)²⁷. GSH estimation was based on the development of yellow colour when 5,5-dithiobis (2-nitro benzoic acid) di-nitrobenzoic acid was added to compounds containing a sulfhydryl group.

Estimation of total protein

Protein was estimated by the method of Lowry et al (1951) using bovine serum albumin as the standard²⁸.

Histopathology

A small portion of the liver tissue from all the groups was excised immediately after sacrifice. Tissue was fixed in 10% formalin in phosphate buffer (pH 7.0) for 24 hr. at room temperature for histology. Tissue were embedded in paraffin and sections were cut at 3-5 micron slices and were stained with haematoxyline and eosin (H&E) and observed under light microscope²⁹.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Kruskal-Wallis non parametric ANOVA test was performed to find whether or not scores of different groups differ significantly. To test inter-group significant difference, Mann-Whitney U multiple comparison test was performed. SPSS 10.0 software (SPSS Inc, 1999) was used for statistical analysis. Differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the average weight gain, food intake and liver body weight ratio of control and experimental rats during the experimental period. The food intake and weight gained were significantly reduced in ACE administered rats and the liver-body weight ratio was significantly increased as compared with control rats. Rats co-administered AI (both 250 & 500mg/kg) along with ACE showed significant weight gain, increased food intake and decreased liver-body weight ratio ($p < 0.05$) as compared with untreated ACE treated rats.

Table 1. Effect of *A. indica* on body weight and liver weight to body weight ratio of control and aceclofenac administered rats.

Groups	Body weight Day 1	Body weight Day 28	Net gain (g)	Average food Intake (g)	Liver wt $\pm$ 100 body wt
Control	122.11 $\pm$ 6.34	163.58 $\pm$ 0.93	41.47 $\pm$ 4.61	6.78 $\pm$ 0.25	2.77 $\pm$ 0.14
AI (250)	121.49 $\pm$ 5.80	158.27 $\pm$ 7.03	36.78 $\pm$ 1.96	6.12 $\pm$ 0.32	2.74 $\pm$ 0.18
ACE (80mg/kg)	121.06 $\pm$ 7.33	132.48 $\pm$ 6.53	11.42 $\pm$ 0.96	4.25 $\pm$ 0.26	5.19 $\pm$ 0.38
ACE + AI (250)	120.32 $\pm$ 6.51	155.46 $\pm$ 5.14	35.14 $\pm$ 1.26	5.16 $\pm$ 0.46	4.01 $\pm$ 0.38
ACE + AI (500)	122.15 $\pm$ 3.94	161.79 $\pm$ 3.37	39.64 $\pm$ 3.36	5.96 $\pm$ 0.45	3.30 $\pm$ 0.09

Data are expressed as mean $\pm$ S.E., $n=10$ per group. * $p < 0.05$, compared with the normal control group; # $p < 0.05$, compared with the Aceclofenac group.

Administration of ACE produced severe liver damage, as indicated by marked increase in the activity of AST, ALT, ALP and GGT (Fig.1-4). However, as AI

co-administration, the activity was significantly decreased ($p < 0.05$) as compared with rats treated with ACE.

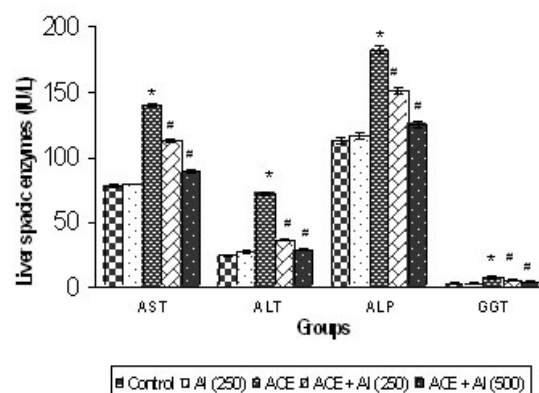


Fig. 1: AST, ALT, ALP and GGT of Control and experimental groups.

Data are expressed as mean $\pm$ S.E., $n=10$ per group. * $p < 0.05$, compared with the normal control group; # $p < 0.05$, compared with the Aceclofenac (ACE) group. AI= *A. indica*

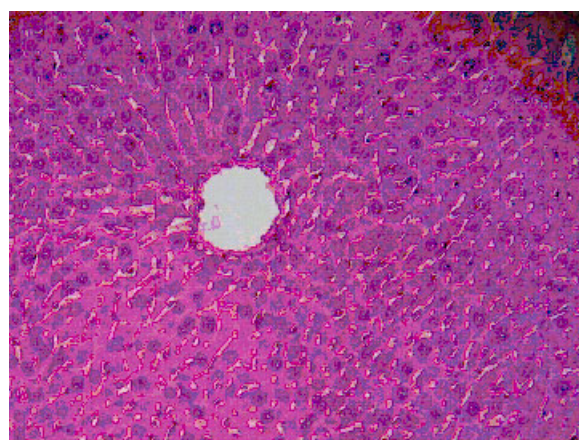


Fig. 2: Liver section of control rats showing normal hepatic structure (H&E, x 400).

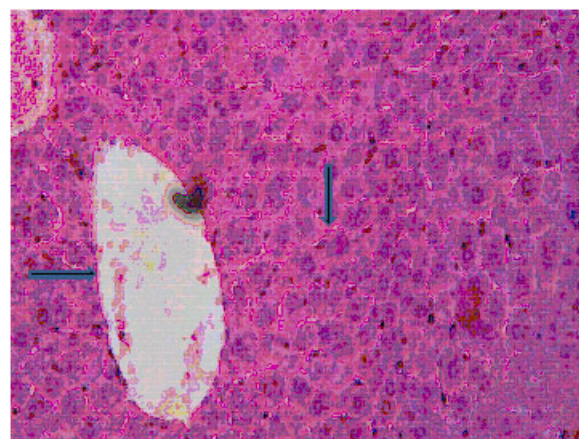


Fig. 3: Liver section of Aceclofenac treated rats showing vacuolations of hepatocytes, karyomegaly and sinusoidal leucocytosis (H&E, x 400).

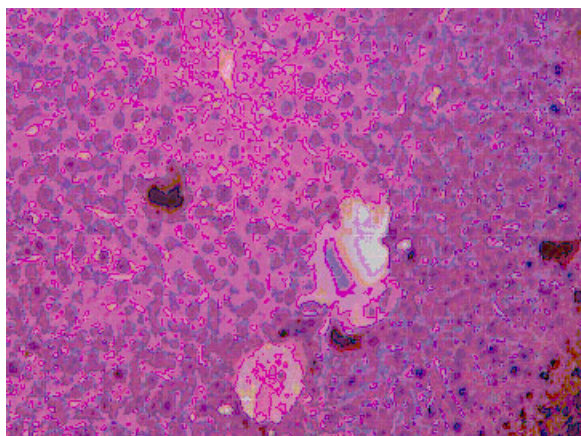


Fig. 4: Liver section Aceclofenac + *A. indica* (250 mg/kg) supplemented rats showing recovered normal hepatocytes (H&E, x 400).

Table-2 and Table-3 shows the levels of TBARS and the activity of SOD, CAT, GSH and GPx of control and experimental rats in plasma and liver cells. Lipid peroxidation, indicated by TBARS was significantly higher in plasma and liver of ACE administered rats as compared with normal control rats. TBARS level was lowered significantly in the plasma as well as in the liver cells of ACE administered rats treated with AI ($p < 0.01$). In Group II, no such variation of results observed which indicate administration of AI alone have no toxic effect on animals.

Table 2. Effect of *A. indica* on plasma TBARS, SOD, CAT, GPx and GSH of control and aceclofenac treated rats.

Group's	TBARS [nmol mg protein]	SOD [U mg protein]	CAT [U mg protein]	GPx [U mg protein]	GSH [nmol mg protein]
Control	1.45 ± 0.03	2.51 ± 0.07	3.74 ± 0.32	22.17 ± 1.43	35.12 ± 0.61
AI (250)	1.38 ± 0.02	2.52 ± 0.09	3.94 ± 0.14	21.35 ± 0.82	37.14 ± 0.64
ACE (50 mg/kg)	3.43 ± 0.25	1.39 ± 0.08	1.72 ± 0.03	13.15 ± 0.49	15.15 ± 0.71
ACE + AI (250)	2.54 ± 0.02	2.35 ± 0.03	2.96 ± 0.35	17.95 ± 0.42	25.38 ± 0.58
ACE + AI (500)	2.02 ± 0.05	2.58 ± 0.22	2.91 ± 0.38	19.38 ± 1.30	30.66 ± 1.38

TBARS thiobarbituric acid reactive substances, SOD superoxide dismutase, CAT catalase, GPx glutathione peroxidase, GSH reduced glutathione.

Data are expressed as mean ± S.E., $n=10$ per group. * $p < 0.05$, ** $p < 0.01$ compared with the normal control group; # $p < 0.05$, ## $p < 0.01$ compared with the Aceclofenac group.

Table 3. Effect of *A. indica* on liver TBARS, SOD, CAT, GPx and GSH of control and aceclofenac treated rats.

Groups	TBARS [nmol mg protein]	SOD [U mg protein]	CAT [U mg protein]	GPx [U mg protein]	GSH [nmol mg protein]
Control	0.73 ± 0.05	5.72 ± 0.15	15.13 ± 1.72	13.22 ± 1.06	17.31 ± 0.37
AI (250)	0.69 ± 0.03	6.02 ± 0.12	14.19 ± 1.67	12.70 ± 1.02	18.45 ± 0.34
ACE (50 mg/kg)	1.91 ± 0.06	2.07 ± 0.13	7.02 ± 2.23	6.91 ± 0.48	10.55 ± 0.39
ACE + AI (250)	1.10 ± 0.02	4.23 ± 1.25	10.58 ± 2.25	11.65 ± 0.25	14.56 ± 0.48
ACE + AI (500)	0.95 ± 0.02	5.68 ± 0.16	13.77 ± 2.68	12.46 ± 1.01	16.91 ± 0.34

TBARS thiobarbituric acid reactive substances, SOD superoxide dismutase, CAT catalase, GPx glutathione peroxidase, GSH reduced glutathione.

Data are expressed as mean ± S.E., $n=10$ per group. * $p < 0.05$, ** $p < 0.01$ compared with the normal control group; # $p < 0.05$, ## $p < 0.01$ compared with the Aceclofenac group.

The activity of SOD, CAT and GPx in the plasma and liver of rats on ACE administration (group-III) were significantly lowered than the control rats. Treatment with AI to ACE administered rats (group-IV & V) significantly elevated SOD, CAT and GPx activity

($p < 0.0$) as compared to those rats receiving ACE treatment along (group-III).

The concentration of GSH was significantly lower in plasma and liver cells of rats treated with ACE (group-III) as compared with control rats (group-I). Treatment with AI to aceclofenac administered rats (group-IV & V) significantly elevated GSH levels ($p < 0.05$) as compared with those receiving ACE along (group-III). Histological observations basically supported the results obtained from serum enzymes assays. The liver of ACE-intoxicated rats showed lymphocytes infiltration, large central vein, kupffer cells around the central vein, loss of cellular boundaries and tissue necrosis (Fig. 3) as compared to normal animals (Fig. 2). ACE (250 & 500 mg/kg) supplementation in rats suppressed the overt histological alterations and led to normal liver tissues (Fig.4&5).



Fig. 5: Liver section Aceclofenac + *A. indica* (500 mg/kg) supplemented rats showing almost normal hepatic structure as compared to control rats (H&E, x 400).

Liver is a versatile organ in the body concerned with regulation of internal chemical environment. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences³⁰. Earlier it has been well documented that both ALT and AST are considered among the most sensitive markers to assess hepatocellular damage leading to liver cell necrosis^{31,32}. ALP, which is secreted from the lysosomes, is also a marker enzyme for assessing liver damage³³. Slight to moderate increases in ALP (1-2 times normal) occurred in liver disorders³⁴. GGT has been claimed to be an extremely sensitive test and marker of hepatic damage. Estimating the activities of serum marker enzymes, like AST, ALT, ALP, and GGT, can make assessment of liver function. When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released in to the blood stream. Their estimation in the serum is a useful quantitative marker of the extent and type of hepatocellular damage³⁵. The enhanced activities of these serum marker enzymes observed in ACE treated rats in our study correspond to the extensive liver damage induced by ACE. Results indicate that AI leaf extract (both 250 & 500 mg/kg)

administration could blunt ACE -induced increase in activities of different marker enzymes of hepatocellular injury, viz. AST, ALT, ALP and GGT (Fig. 1) suggesting that AI possibly has a protective influence against ACE induced hepatocellular injury and degenerative changes.

Oxidation of polyunsaturated fatty acids (lipid peroxidation) of membranes is a common process in living organism, since they are the target of oxygen-derived free radicals produced during mitochondrial electron transport³⁶. Products of lipid peroxidation formed in the primary site reaching the other organs and tissues via the blood stream provoke lipid peroxidation there and consequently cause cellular and tissue damage³⁷. Increased accumulation of lipid peroxidation products in cells can result in cellular dehydration, whole cell deformity and cell death³⁸. Co-administration of AI leaf extract rats showed significantly decreased levels of these lipids peroxidation markers as compared with ACE treated rats. The inhibition of lipid peroxidation by AI leaf extract therefore, may be one of the mechanisms by which AI exerts its protection against ACE mediated tissue injury.

Free radical scavenging enzymes, such as SOD and CAT, are the first line of defense against oxidative injury. SOD is ubiquitous cellular enzyme that dismutates superoxide radical to H₂O₂ and oxygen, is one of the chief cellular defense mechanisms. The catalase enzyme may also be released into the extracellular environment in which it has the potential to function as a potent antioxidant, and thereby regulated cell survival³⁹. In light of these considerations, it seems plausible that extracellular catalase might function as an important autocrine antioxidant and survival factor⁴⁰. The second line of defense consists of the non-enzymic scavengers reduced Glutathione. GSH is an important naturally occurring antioxidant as it prevents the hydrogen of sulfhydryl group to be abstracted instead of methylene hydrogen of unsaturated lipids. It has been reported that determination of GSH, can serve as a key to know the amount of antioxidant reserve in the blood and probably in the organism and also, contribute in evaluating the possibilities available for the recuperation of alcoholic patients^{41,42}.

As shown in Table 2 and 3, ACE treatment decreased SOD, CAT, reduced Glutathione and increased lipid peroxidation both plasma and liver cell. Pretreatment with AI leaf extract (250 and 500mg/kg) improved the SOD, CAT, Glutathione and peroxidase levels significantly and reduced lipid peroxidation. Our study focused that AI possesses antioxidant activity, in treatment of ACE induced hepatic cell injury. Glutathione peroxidase (GPx) is a critical antioxidant enzyme in the detoxification of peroxides. Its high affinity and relatively low substrate specificity for peroxides renders GPx more effective than CAT in the removal of peroxides⁴³. The observed decreased activity of GPx in the study might be due to increased concentration of hydroperoxides or due to decreased concentration

of GSH in ACE intoxicated rats. Lower GPx levels would increase steady-state hydroperoxide levels due to reduced capacity of peroxide elimination, which can lead to liver damage⁴⁴. Pretreatment with AI leaf extract increases the activity of GPx in ACE treated rats. Histological findings in liver tissue showed that AI supplementation alleviated steatosis induced by ACE.

CONCLUSION

In conclusion, we determined that aceclofenac could increase the liver enzyme levels and affect some hepatospecific biochemical parameters. Increase in these parameters may occur due to peroxidation reactions, arising in aceclofenac biotransformation during drug administration and these reactions may inflict oxidative injury to cellular components. In the light of these results, *A. indica* leaf extract may play a role in the prevention of hepatic cellular injury produced by non steroidal anti-inflammatory drugs. However, there is a need for more detailed studies in order to assess the possible relationships between antioxidants and aceclofenac hepatotoxicity. Therefore, we have been presently carrying out further studies investigating the hepatotoxicity of various non steroidal anti-inflammatory drugs.

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