

ANTIOXIDANT AND HEPATOPROTECTIVE EFFECT OF *Andrographis paniculata* LEAF EXTRACT ON DICLOFENAC INDUCED HEPATOTOXICITY IN RATS

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Summary

Many plant products are known to exert antioxidative effects by quenching various free radicals and singlet molecular oxygen. *Andrographis paniculata* (Kalmegh) is used extensively in the Indian traditional system of medicine as a hepatoprotective and hepatostimulative agent and has been reported to have antioxidant effects against different hepatotoxins. The aim of the present study was to evaluate the hepatoprotective effect of *Andrographis paniculata* (AP) against single dose of Diclofenac (DIC) induced hepatotoxicity in rats. Hepatoprotective activity of aqueous ethanol extract of AP was evaluated against repeated dose of Diclofenac (150 mg/kg, i.p) for 30 days in rat. Aqueous extract of AP significantly protected the hepatotoxicity. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (GGT) and alkaline phosphatase (ALP) levels were significantly ($p < 0.01$) elevated in the DIC alone treated animals. Antioxidant status in liver tissue such as activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione s-transferase (GST), a phase II enzyme, and levels of reduced glutathione (GSH) were declined significantly ($p < 0.01$) in the DIC alone treated animals. Hepatic lipid peroxidation (MDA content) was enhanced significantly ($p < 0.01$) in the DIC treated group. AP also significantly decreased the lipid peroxidation in liver. Administration of AP (200 and 400 mg/kg) prior to Diclofenac significantly declines the activities of serum transaminases, GGT and ALP levels. Furthermore the hepatic antioxidant status i.e. SOD, CAT, GPx, GST and GSH were enhanced in the *Andrographis paniculata* plus Diclofenac treated group than the DIC alone treated group. The results of the present study concluded that the hepatoprotective effect of aqueous ethanol extract of AP against DIC -induced acute toxicity is mediated either by preventing the decline of hepatic antioxidant status or due to its direct radical scavenging capacity.

Keywords: Antioxidant; Diclofenac; *Andrographis paniculata*.

Running Title:- Hepatoprotective effect of *Andrographis paniculata*

Introduction

Andrographis paniculata Nees (Acanthaceae) (AP) is a medicinal plant traditionally used for the treatment of cold, fever, laryngitis and several infectious diseases ranging from malaria to dysentery and diarrhea in China, India and other south east Asian countries [1]. It is also known as “King of Bitters” of Acanthaceae family. This plant has been used through out the centuries against different diseases [2] especially as hepatoprotective agent [3]. *Andrographis paniculata* has demonstrated a number of different pharmacological actions in in-vitro and/or animal studies. Anticancer [4], immunomodulatory [5], anti-inflammatory [6], antipyretic [7], hepatoprotective [8-10], hypotensive [11], hypoglycemic [12], antiplatelet [13-15] and antithrombotic activity have all been reported.

Diclofenac (DIC), is a nonsteroidal anti-inflammatory drug, which has analgesic and anti-inflammatory effects and widely used for treatment of a variety of rheumatoid disorders [16]. DIC causes a rare but potentially sever liver injury in humans [17-18]. The hepatotoxicity of DIC was also documented in experimental animal studies [16,19,20].

The mechanism of DIC hepatotoxicity involves covalent protein modification by reactive metabolites [21-22], oxidative stress generation by peroxidase-catalyzed reaction [23,24] and mitochondrial injury propagation by reactive oxygen species [25,26]. This DIC-induced hepatic toxicity was prevented by antioxidants and also cytochrome P-450 inhibitors [27,16]. It is logical to consider antioxidants as primary candidates to counteract such toxic effect. In recent years, accumulating evidence supported the protective effects of phenolic antioxidants from medicinal plants against oxidative stress-mediated disorders [28]. Although several natural extractions from plants have been shown to protect against chemical-induced liver toxicity, a consensus on the protective effects of natural substances for the treatment of DIC- induced hepatic toxicity however has not yet been reached.

The aim of this work was to establish the antioxidant and hepatoprotective effect of *Andrographis paniculata* on an animal model of diclofenac induced liver damage.

Materials and methods

Animals

Twenty four adult male white Wister albino rats, weighing 122 ± 4.5 g 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, $25 \pm 2^\circ\text{C}$ 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.

Chemicals

Diclofenac was obtained as a gift sample from Dey's Medical Stores (Mfg.) Ltd., 62, Bondel Road, Kolkata-700019, India. Pyridine (C₅H₅N), ethanol (C₂H₅OH), n-butanol (CH₃(CH₂)₃OH), disodium hydrogen phosphate (Na₂HPO₄), hydrogen peroxide (H₂O₂), dihydrogen potassium phosphate anhydrous (KH₂PO₄) and thiobarbituric acid were purchased from Merck India Ltd., Mumbai, India. Sodium azide (NaN₃), reduced glutathione (GSH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), nitroblue tetrazolium (NBT) and riboflavin were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals and reagents used were analytical reagent grade.

Collection of Plant material

Fresh matured leaves of *Andrographis paniculata* (Acanthaceae) were collected from our Institute's (Jadavpur University, Kolkata, India) garden and were identified by a pharmacognosy expert. At the time of collection standard herbarium record sheets were completed with the name of the collector, collection number, date, locality and local name.

Extraction of Plant material

Air dried powder (1kg) of fresh mature *Andrographis paniculata* (AP) leaves were extracted by percolation at room temperature with 70% ethanol. Leaf extract of *Andrographis paniculata* 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 experiment [29].

Experimental design

The rats were randomly divided into 4 groups of 6 animals each as follows:

Group I: Normal rats fed normal diet and water only for 28 days.

Group II: Experimental rats treated with Diclofenac (150mg/kg/day, i.p) only for 28 days.

Group III: Animals treated with Diclofenac (150mg/kg/day, i.p) along with *Andrographis paniculata* (200mg/kg, orally) for 28 days.

Group IV: Animals treated with Diclofenac (150mg/kg/day) along with *Andrographis paniculata* (400mg/kg, orally) for 28 days.

The extract was administered by oral gavage 1 h before Diclofenac administration.

Biochemical Estimation

The animals were sacrificed 24 h after the administration of Diclofenac using ether anesthesia; blood was collected directly from the heart of each animal. Serum was separated for the estimation of the activities of serum aspartate aminotransaminase (AST), glutamate pyruvate transaminase (ALT), alkaline phosphatase (ALP) and γ -glutamyl transpeptidase (GGT). Liver was dissected out for the determination of antioxidant status.

Serum ALT, AST, GGT and ALP were determined by kinetic method using the kit obtained from Merck, Germany in a double beam spectrophotometer (Elico SL-164, Elico Limited, Hyderabad, India). The transaminases activities were determined as change in absorbance/min at 340 nm. Serum ALP activity was determined from the rate of release of paranitrophenol at 405 nm.

Preparation of tissue homogenate

Livers were excised, washed thoroughly in ice-cold saline to remove the blood. They were then gently blotted between the folds of a filter paper and weighed in an analytical balance. Ten percent of homogenate was prepared in 0.05 M phosphate buffer (pH 7) using a polytron homogenizer at 4°C. The homogenate was centrifuged at 3000g for 20 min to remove the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities and the levels of reduced glutathione (GSH), lipid peroxidation, and total protein.

Determination of liver antioxidant enzymes

SOD activity was determined from the ability of the tissue homogenate to scavenge the superoxide anion generated from the photo-illumination of riboflavin according to the method of Mc Cord and Fridovich (1969) [30]. Tissue CAT activity was determined from the rate of decomposition of H₂O₂ (Beers and Sizer, 1952) [31]. GPx activity was determined by measuring the decrease in GSH content after incubating the sample in the presence of H₂O₂ and NaN₃ (Hafemann et al., 1974) [32]. Reduced GSH was determined according to the method of Moron et al. (1979) [33]. based on the formation of a yellow colored complex with DTNB. GST activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB (Habig et al., 1974) [34]. The level of lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1¹/₃/₃-tetramethoxypropane as standard (Ohkawa et al., 1979) [35]. Protein content in the tissue was determined (Lowry et al., 1951) [36]. using bovine serum albumin (BSA) as the standard.

Histopathological examination

Portions of the liver were fixed in 10% formalin and then embedded in paraffin. Microtome sections 5 µm thickness were prepared from each liver samples and stained with hematoxylin-eosin (H&E). The sections were examined for the pathological findings of hepatotoxicity such as centrilobular necrosis, fatty infiltration, fibrosis, lymphocyte infiltration, etc.

Statistical analysis

Data were expressed as mean ± SEM Kruskal-Wallis non parametric two way 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

Serum biochemical study

Serum activities of transaminases, AST, ALT, GGT and ALP were given in Table 1&2. Single dose of DIC significantly elevated AST, ALT and GGT activities when compared to the normal animals. Treatment of aqueous ethanol extract of *Andrographis paniculata* 1 h prior to DIC (500mg/kg) administration significantly protected the elevation of transaminases and ALP activities. The activities of AST, ALT and GGT in the high dose *Andrographis paniculata* (400 mg/kg) plus DIC treated group were 69.70 ± 5.48 and 83.11 ± 9.40 IU/L, respectively. Similarly the activity of ALP was significantly ($p < 0.01$) decreased in the *Andrographis paniculata* (400 mg/kg) plus DIC treated group (163.91 ± 5.18 IU/l) than the DIC treated group (242.85 ± 11.81 IU/l).

Table 1 Effect of ethanol extract of *Andrographis paniculata* (AP) on serum ALT and AST activities in rats with acute diclofenac (DIC) administration

Groups	Treatment	ALT (IU/L)	AST (IU/L)
Normal	Vehicle	36.97 ± 2.83	72.86 ± 8.35
DIC	150	$108.12 \pm 6.52^*$	$122.96 \pm 10.93^*$
AP + DIC	200	$82.64 \pm 3.57^{**}$	$95.61 \pm 6.28^{**}$
AP + DIC	400	$69.70 \pm 5.48^{**}$	$83.11 \pm 9.40^{**}$

Values are mean \pm S.D, n=6 animals. * $p < 0.01$ (Dunnett's t-test) significantly different from normal group. ** $p < 0.01$ (Dunnett's t-test) significantly different from DIC group.

Table 2 Effect of ethanol extract of *Andrographis paniculata* (AP) on serum GGT and ALP activities in rats with acute diclofenac (DIC) administration

Groups	Treatment	GGT IU/L)	ALP (IU/L)
Normal	Vehicle	2.36 ± 0.92	119.23 ± 7.29
DIC	150	$7.04 \pm 0.41^*$	$242.85 \pm 11.81^*$
AP + DIC	200	$3.65 \pm 0.38^{**}$	$195.28 \pm 7.02^{**}$
AP + DIC	400	$2.87 \pm 0.81^{**}$	$163.91 \pm 5.18^{**}$

Values are mean \pm S.D, n=6 animals. * $p < 0.01$ (Dunnett's t-test) significantly different from normal group. ** $p < 0.01$ (Dunnett's t-test) significantly different from DIC group.

Liver antioxidant study

Activities of hepatic SOD, CAT and GPx were provided in the Table 3. SOD and GPx activities were significantly ($p < 0.01$) enhanced only in the high dose of *Andrographis paniculata* (400 mg/kg) plus DIC treated group. However the hepatic CAT activity was found to be non-significant when compared to the DIC treated group. Moreover, the activities of CAT and GPx and level of GSH (Fig. 1) in the 200 mg/kg *Andrographis paniculata* plus Diclofenac treated group were non-significantly differ from the DIC treated group. Hepatic MDA level was significantly ($p < 0.01$) elevated in the DIC treated group (172.19 ± 6.2 nmol/g tissue) than the normal animals (79.38 ± 5.2 nmol/g tissue) (Fig. 2). Treatment of *Andrographis paniculata* prior to DIC significantly prevented the elevation of MDA. Further the activity of GST was enhanced and normalized in the *Andrographis paniculata* (400 mg/kg) plus DIC treated animals (Fig. 3). The activities of GST in the normal and *Andrographis paniculata* (400 mg/kg) plus DIC treated groups were 72.18 ± 4.6 and 69.27 ± 6.8 μ mol CDNB-GSH conjugate formed/min/mg protein, respectively.

Table 3 Effect of ethanol extract of *Andrographis paniculata* (AP) on hepatic SOD, CAT and GPx activities in rats with acute diclofenac (DIC) administration

Groups	Treatment	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein)
Normal	Vehicle	18.60 ± 1.29	54.61 ± 2.15	24.38 ± 0.92
DIC	150	12.92 ± 1.58^b	32.19 ± 7.32^a	16.29 ± 1.18^a
AP + DIC	200	$20.11 \pm 2.52^*$	45.62 ± 1.68^{NS}	22.23 ± 1.32^{NS}
AP + DIC	400	$19.65 \pm 1.91^{**}$	49.21 ± 3.29^{NS}	$23.14 \pm 2.18^{**}$

Values are mean \pm S.D, n=6 animals.

** $p < 0.01$, * $p < 0.05$ (Dunnett's t-test) significantly and NS non-significantly different from DIC group.

^a $p < 0.01$ and ^b $p < 0.05$ (Dunnett's t-test) significantly different from normal group.

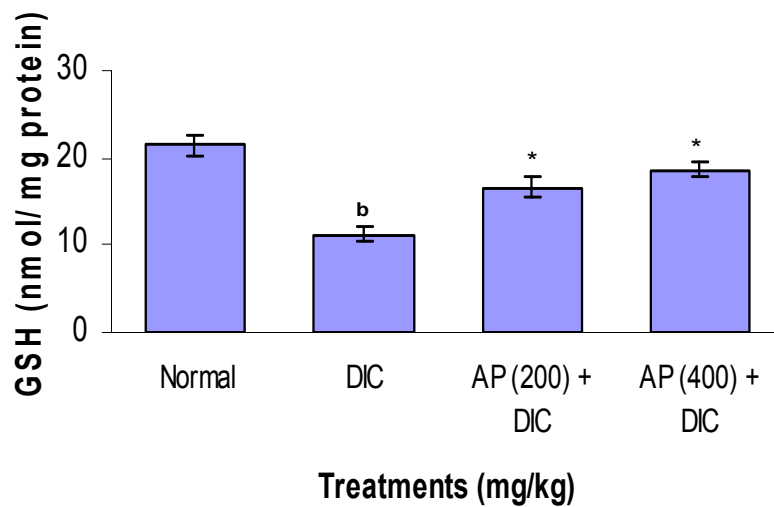


Fig. 1. Effect of *Andrographis paniculata* (AP) leaf extract on hepatic GSH level in rat treated with Diclofenac (DIC). Values are mean \pm S.D, n=6 animals. *P<0.05 significantly different from DIC treated group. ^bP<0.05 significantly different from normal group.

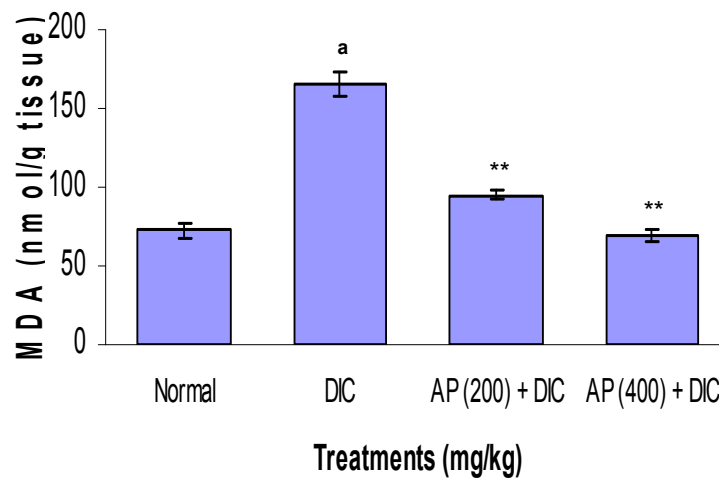


Fig. 2. Effect of *Andrographis paniculata* (AP) leaf extract on hepatic MDA level in rat treated with Diclofenac (DIC). Values are mean \pm S.D, n=6 animals. **P<0.01 significantly different from DIC treated group. ^aP<0.01 significantly different from normal group.

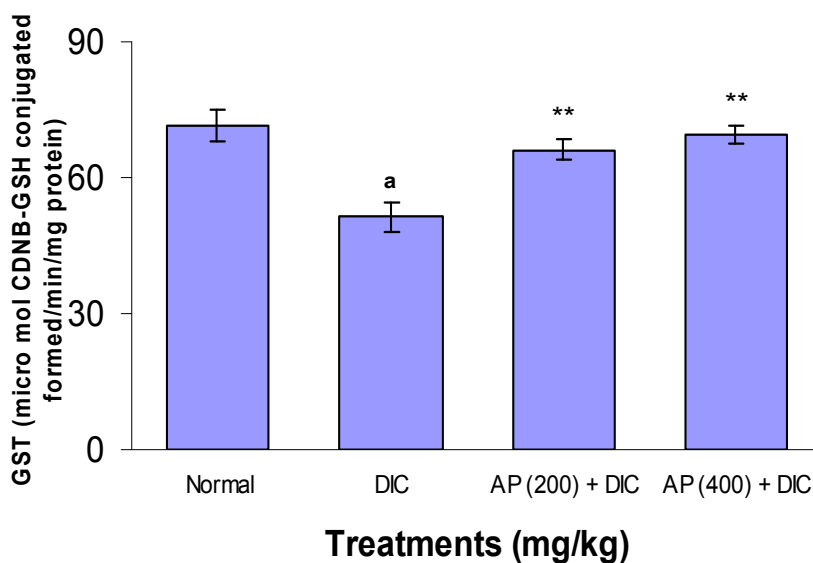


Fig. 3. Effect of *Andrographis paniculata* (AP) leaf extract on hepatic GST level in rat treated with Diclofenac (DIC). Values are mean \pm S.D, n=6 animals. **P<0.01 significantly different from DIC treated group. ^aP<0.01 significantly different from normal group.

Histopathological study

Histopathological analysis of the diclofenac alone treated animal showed severe centrilobular necrosis, fatty infiltration and lymphocytes infiltration (Fig. 5) as compared with normal untreated animals (Fig. 4). The findings were significantly decreased in the *Andrographis paniculata* plus Diclofenac treated groups (Fig. 6&7).

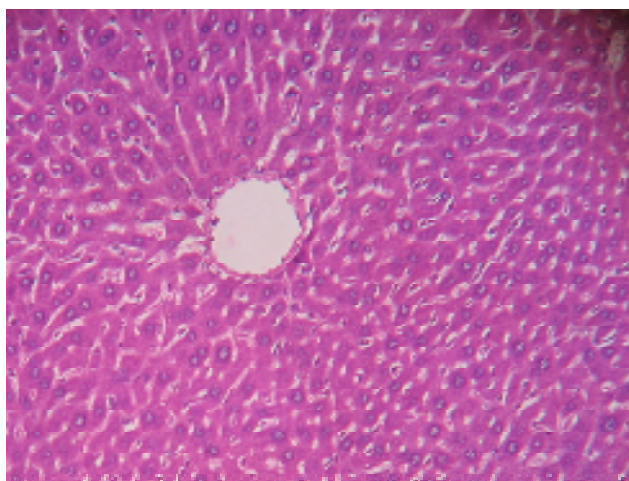


Fig. 4. Liver section of control rats showing normal hepatic structure (H&E, x 400).

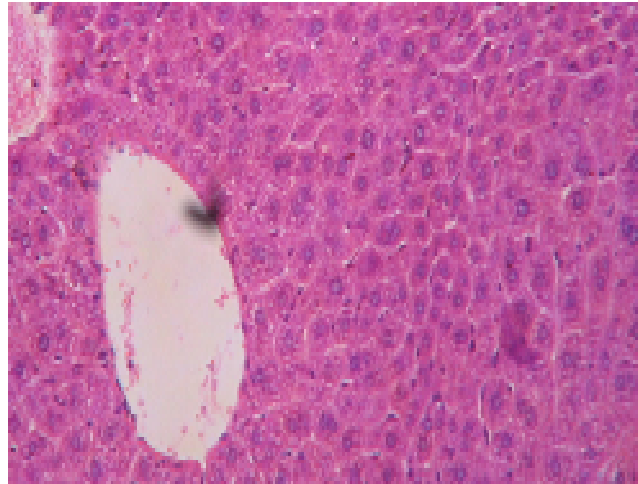


Fig.5. Liver section of Diclofenac (DIC) treated rats showing vacuolations of hepatocytes, karyomegaly and sinusoidal leucocytosis (H&E, x 400).

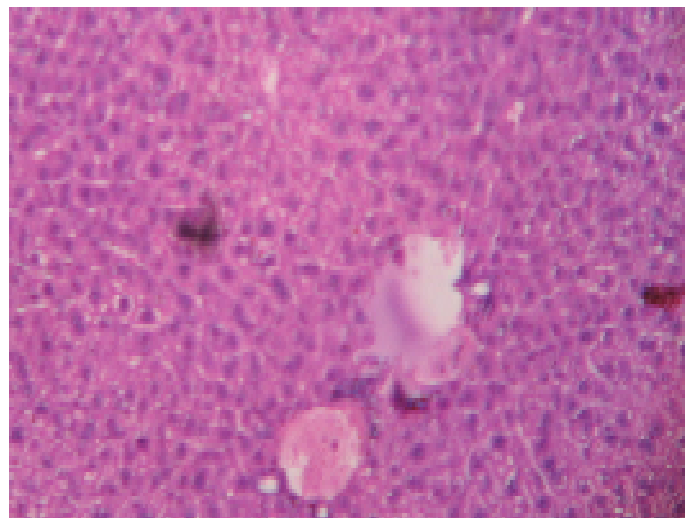


Fig. 6. Liver section Diclofenac (DIC) + *Andrographis paniculata* (AP) (200 mg/kg) supplemented rats showing recovered normal hepatocytes (H&E, x 400).

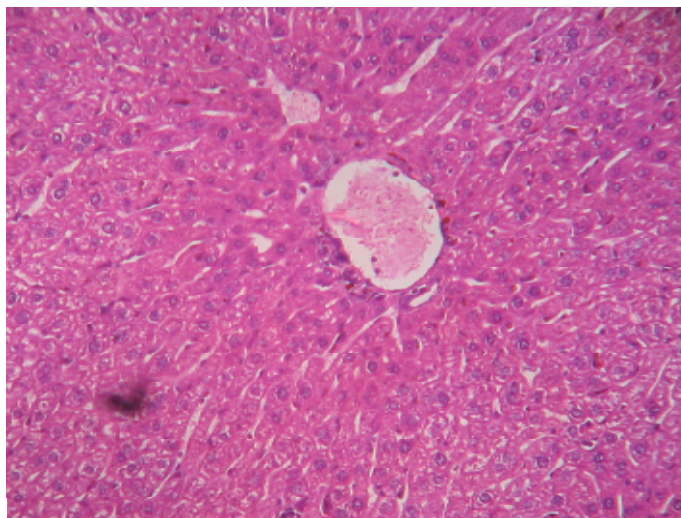


Fig.7. Liver section Diclofenac (DIC) + *Andrographis paniculata* (AP) (400 mg/kg) supplemented rats showing almost normal hepatic structure as compared to control rats (H&E, x 400).

Discussion and conclusion

Administration of a single high dose of Diclofenac significantly ($p < 0.01$) elevated the serum transaminase, γ -glutamyl transpeptidase (GGT) and ALP activities compared to the normal animals. This indicated necrosis of hepatocytes that results in the leakage of transaminases, GGT and the elevation of serum ALP from a possible cholestasis. The significantly decreased serum transaminases, GGT and ALP activities in the *Andrographis paniculata* administered groups prior to DIC demonstrated its hepatoprotective effect. However, a single high dose of aqueous ethanol extract of *Andrographis paniculata* could produce only a partial protection. Hence, more prophylactic doses of extract of *Andrographis paniculata* are required to render a complete protection.

Cytochrome P-450 enzymes are the major catalysts involved in the metabolism of drugs. NSAIDs is mainly metabolized by cytochrome P-450 to form an electrophilic metabolite, N-acetyl-p-benzoquinonimine, which is primarily inactivated by conjugation with glutathione (Orecnius and Moldeus, 1984; Dahlin et al., 1984) [37,38]. A large number of the metabolites produced by NSAIDs are found to generate superoxide anion and other free radicals in the biological systems (Vries, 1984) [39]. However, at a higher dose of DIC (150 mg/kg), intermediate metabolites accumulate and cause liver damage. Depletion of glutathione beyond certain critical level can lead to oxidative stress and development of overt hepatotoxicity (Mitchell et al., 1973) [40].

GSH is the most important endogenous antioxidant marker for chemical-induced toxicity to help eliminating the over produced ROS. The reduced hepatic antioxidant status is related to oxidative stress and elevation of lipid peroxidation that resulted in the leakage of hepatic enzymes to serum in the DIC alone treated animals. Treatment of *Andrographis paniculata* at 400 mg/kg plus DIC significantly enhanced the hepatic antioxidant activity including the hepatic GSH level compared to the DIC alone treated animals. The elevated hepatic reduced GSH level

could partially explain the hepatoprotective mechanism of the *Andrographis paniculata* at 400 mg/kg dose. Reduced GSH can function as a reductant in the metabolism of hydrogen peroxide and various organic peroxides. The GPx present in the cells can catalyze this reaction. Cighetti et al. (1993) [41]. reported that depletion of GSH below a threshold value was associated with a significant conversion of xanthine dehydrogenase to reversible xanthine oxidase, a superoxide radical generation reaction catalyzing enzyme. Therefore the enhanced hepatic GPx and SOD activities in the high dose *Andrographis paniculata* plus DIC treated group further support its hepatoprotective effect. The elevated antioxidant status in the liver of *Andrographis paniculata* (400 mg/kg) plus DIC treated group is related to the decreased MDA level could maintain the membrane integrity and prevented the leakage of hepatic enzymes to serum. The histopathological analysis of liver section indicates a moderate centrilobular necrosis, fatty infiltration and lymphocytic infiltration in the high dose *Andrographis paniculata* plus DIC treated animals with respect to the DIC alone treated animals. In the present study decreased hepatic GST activity of the DIC alone treated animals in the present study could support the enhanced lipid peroxidation. Administration of *Andrographis paniculata* plus DIC significantly and dose dependently elevated the hepatic GST activity and protected the liver toxicity. The enhanced GST activity, a phase II enzyme, can also explain the increased detoxification of the reactive metabolites generated from the DIC metabolism in the liver of *Andrographis paniculata* treated animals.

DIC-induced liver lesion was associated with massive elevation in liver MDA level. The MDA elevation has been well accepted as a reliable marker of lipid peroxidation (Packer, 2002) [42]. MDA elevation is a result of oxidative stress demonstrated here through the decrease of total antioxidant capacity, GSH level, and antioxidant enzyme activities (SOD & CAT) in liver. The oxidative stress occurs when the generation of ROS overrides the ability of the endogenous antioxidant system to remove excess ROS. In our study low dose of *Andrographis paniculata* used in this study did not prevent the DIC -induced decline of hepatic activity of CAT, GPx and GSH level. However, this dose was effective to render hepatoprotection as evident from the decreased activities of serum transaminases; ALP and hepatic MDA level when compared to DIC alone treated animals. Further hepatic SOD and GST activities were significantly enhanced. Hence the protection at low dose of *Andrographis paniculata* may be partially related to the significantly elevated hepatic GST and SOD activities.

Conclusion

The results of the present study concluded that aqueous ethanol extract of *Andrographis paniculata* significantly and dose dependently prevented the DIC-induced acute hepatotoxicity by enhancing the hepatic antioxidant activity. However, further detailed studies are required to establish its clinical application.

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