International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

International Journal of Pharmacology 4 (5): 376-381, 2008
ISSN 1811-7775
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Ficus hispida Linn. Leaf Extract Possesses Antioxidant Potential and Abrogates Azathioprine Induced Prooxidant and Antioxidant Imbalance in Rat Liver

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Abstract: The present study was set out to explore the antioxidant effect of methanolic leaf extract of *Ficus hispida* Linn. (FH) against azathioprine (AZA) induced liver injury in male Wistar rats. *In vitro* antioxidant activity of FH was examined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide radical inhibition assays, the extract exhibited IC_{50} values of 29.33±1.14 and 21.51±0.96 μg mL^{-1}, respectively. The *in vivo* experiments revealed that AZA (50 mg kg^{-1} body weight; single intraperitoneal injection) caused a severe oxidative insult in the liver, which was depicted by a substantial drop in the enzymic [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST)] and non-enzymic antioxidants [glutathione (GSH), Vitamin C (Vit C), Vitamin E (Vit E)]. In contrary, pretreatment with FH (400 mg kg^{-1} body weight; pretreated orally for 21 days) maintained the antioxidant status at near normalcy. In addition, AZA induced lipid peroxidation (LPO) was also significantly alleviated by FH administration. These results underscore that *Ficus hispida* leaves possess remarkable antioxidant potential and hence it could be evaluated as an effective supplement to attenuate azathioprine induced oxidative stress.

Key words: Azathioprine, *Ficus hispida*, oxidative stress, DPPH, antioxidants

INTRODUCTION

Azathioprine (AZA), an immunosuppressant is widely administered in the clinic for the prevention of rejection in organ transplantations, autoimmune diseases such as autoimmune hepatitis, rheumatic arthritis or chronic inflammatory bowel diseases like Crohn's disease and ulcerative colitis (Heneghan and McFarlane, 2002; Maltzman and Koretzky, 2003). Besides, AZA also finds its use in dermatology for treatment of immunobullous diseases, generalized eczematous disorders and photodermatoses (Bardek *et al*., 2007). Despite these advantages, its therapeutic potential is limited by adverse effects such as hepatotoxicity, myelotoxicity, hypersensitivity and gastrointestinal disturbances (de Boer *et al*., 2005; La Mantia *et al*., 2007).

Azathioprine *per se* is a prodrug and it is bioactivated to 6-mercaptopurine (6-MP) through the reduction by glutathione (GSH) and then subsequently metabolized to the pharmacologically active 6-thioguanine nucleotides (Maltzman and Koretzky, 2003). Consumption of GSH during AZA metabolism decreases the activities of other antioxidants which predispose the cells to oxidative stress, thereby leading to liver injury (Rajasekaran *et al*., 2002). Literature data reveal that the hepatocytic damage caused by AZA-induced oxidative stress could be counteracted by the supplementation of antioxidants (Lee and Farrell, 2001; Raza *et al*., 2003).

The toxicity profile of conventional therapy impelled the search for novel agents to thwart the noxious effects of the former. There is an overwhelming weight of evidence which suggest that plant-based remedies are more safe and effective in the treatment of numerous liver ailments (Baek *et al*., 2008; Chaung *et al*., 2003; Rathi *et al*., 2007; Shahjahan *et al*., 2005). Fascinatingly, recent studies show that plants and their products have hepatoprotective potential against azathioprine induced hepatotoxicity (Amin and Hamza, 2005; Wu *et al*., 2006).

Ficus hispida Linn. (Moraceae), a medicinal plant, has been used by the ethnic healers in India, for its hepatoprotective (Mandal *et al*., 2000), anti-diarrhoeal (Mandal and Kumar, 2002), anti-inflammatory, antitussive, antipyretic, astringent, vulnerary, anti-ulcer and homeostatic effects. Almost all parts of *Ficus hispida* are used in Indian folklore medicine for the treatment of various ailments like leucoderma, skin diseases, jaundice and as anti-poisonous. Earlier reports reveal that *F. hispida* leaves contain active phytoconstituents such as oleanolic acid, bergapten, β-amyrin, β-sitosterol, hispidine and phenanthroindolizidine alkaloids

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(Peraza-Sánchez *et al*., 2002; Shanmugarajan *et al*., 2008). An investigation by Mandal *et al*. (2000) revealed the protective potential of *F. hispida* leaf extract on paracetamol-induced hepatotoxicity. Besides, previous studies also documented the effective antioxidant potential of *F. hispida* leaves against cyclophosphamide induced abnormalities in rat liver (Shanmugarajan *et al*., 2008). Based on these evidences, we hypothesized that treatment with methanolic extract of *F. hispida* leaves might attenuate azathioprine-induced oxidative stress in rat liver.

MATERIALS AND METHODS

Drugs and chemicals: Azathioprine was purchased from Sigma Aldrich, USA and all other chemicals and solvents used were of the highest purity and analytical grade.

Plant material: The leaves of *Ficus hispida* Linn. (Moraceae) were collected during the month of March, 2006 from the herbal garden of Anna Siddha Hospital and Research Centre, Chennai, India and was authenticated by Dr. Sasikala, Research Officer, Department of Botany, Anna Siddha Hospital and Research Centre, Chennai, India. Then, the leaves were dried under shade and pulverized in a mechanical grinder and stored in a closed container for further use.

Preparation of extract: The powdered leaves were defatted with petroleum ether (B.P. 60-80°C) and then extracted with methanol in a Soxhlet extractor. On evaporation of methanol from the methanol extract *in vacuo*, a greenish coloured residue was obtained (yield 4.5% (w/w) with respect to the dry starting material) and was stored in a desiccator.

Phytochemical screening: On preliminary screening, the methanol extract showed positive reaction for triterpenoids, Shinoda test for flavonoids, steroids, tannins, saponins and alkaloids (Shanmugarajan *et al*., 2008).

Animal model: The study was conducted on male Wistar rats (120-150 g). Animals were obtained from Tamilnadu University of Veterinary and Animal Sciences (TANUVAS), Madhavaram, Chennai, India. Animals were fed with commercially available standard rat pelleted feed (M/s Hindustan Lever Limited, Bangalore, India) and water was provided *ad libitum*. The animals were deprived of food for 24 h before experimentation but allowed free access to tap water. The rats were housed under conditions of controlled temperature (25±2°C) and were acclimatized to 12 h light: 12 h dark cycles. Experimental animals were used after obtaining prior permission and handled according to the University and Institutional legislation as regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Experimental protocol: The experimental animals (24 rats) were randomized into four groups of 6 rats each as follows:

Group 1: Control rats received normal saline (5% Tween-80 in 5 mL kg^{-1} body weight), orally for 21 days.

Group 2: Rats were injected intraperitoneally with a single dose of AZA (50 mg kg^{-1} body weight) suspended in saline (containing 5% Tween-80), on the 21st day of the experimental period.

Group 3: Rats received FH extract suspended in saline (containing 5% Tween-80) by oral gavage (400 mg kg^{-1} body weight for 21 days).

Group 4: Rats were administered with FH extract as in Group 3 followed by AZA treatment as in Group 2.

After the 21 days experimental period (i.e., on the 22nd day), all the animals were anesthetized and decapitated. Liver tissues were immediately excised and rinsed in ice cold physiological saline. The tissues were homogenized in 0.01 M Tris-HCl buffer (pH 7.4) and aliquots of this homogenate were used for the analysis of biochemical parameters.

***In vitro* assays:** The antioxidant activity of the plant extract and the standard were assessed on the basis of the radical scavenging effect of the stable DPPH free radical (Hwang *et al*., 2001). Nitric oxide radical inhibition assay was performed by using Griess reagent (Garrat, 1964).

Lipid peroxidation and antioxidants: Tissue lipid peroxide level was determined by the method of Ohkawa *et al*. (1979). SOD was assayed by the method of Misra and Fridovich (1972). Catalase (CAT) level was estimated by the method described by Beers and Sizer (1952). Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*. (1973). Glutathione-S-transferase (GST) was assayed by the method of Habig *et al*. (1974). Total reduced glutathione (GSH) was determined by the method of Ellman *et al*. (1959). Protein content was estimated by the method of Lowry *et al*. (1951).

Statistical analysis: The results were expressed as mean±standard deviation (SD) for 6 animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS 10.0 software package for Windows. Post hoc testing was performed for inter-group comparisons using the Least Significance Difference (LSD) test. p-values < 0.05 have been considered as statistically significant.

RESULTS

***In vitro* assays:** The methanolic extract of *Ficus hispida* exhibited strong antioxidant activity in the DPPH and the nitric oxide radical inhibition assays as evidenced by the low IC_{50} values (Table 1). The IC_{50} values obtained were 29.33±1.14 and 21.51±0.96 μg mL^{-1}, respectively for DPPH and nitric oxide radical inhibition assays. These values were found to be less than those obtained for the reference standard rutin.

Antioxidant status: Table 2 and 3 represent the activities of enzymic antioxidant (SOD, CAT, GPx and GST) ands the levels of non-enzymic antioxidants (GSH, Vit C and Vit E) in liver tissue of control and experimental groups of rats. Highly significant reduction in the antioxidant status was observed in rats induced with AZA (Group 2) when compared with control rats (Group 1) These adverse changes were reversed to near normalcy and an improvement in antioxidant status was noticed in AZA intoxicated rats pretreated with *Ficus hispida* extract (Group 4). However *Ficus hispida* extract alone treated rats (Group 3) did not show any significant changes when compared to control.

Table 1: *In vitro* free radical scavenging effect of the methanolic leaf extract of *Ficus hispida*

	IC_{50} (μg mL^{-1}) ±SE[a]	
Tested material	DPPH method	Nitric oxide radical inhibition assay
Methanolic extract	29.33±1.14	21.51±0.96
Rutin	41.23±1.27	36.32±3.18

[a]Data are expressed as mean±SE (n = 6)

Lipid peroxidation: Table 4 shows the extent of lipid peroxidation (measured in terms of MDA) in the liver of experimental groups. Lipid peroxide levels were found to be markedly elevated ($p<0.05$) on AZA injection to rats (Group 2) when compared to control rats (Group 1). These elevated levels were maintained at near normal status on pretreatment with *Ficus hispida* extract (Group 4). Non-significant variations were observed in *Ficus hispida* extract alone treated rats (Group 3) when compared to control (Group 1).

DISCUSSION

Azathioprine induced hepatotoxicity is a very well-documented phenomenon in humans (King *et al*., 1995; David-Neto *et al*., 1999; Pol *et al*., 1996; De Boer *et al*., 2005) as well as in different animal models (Raza *et al*., 2003). An ample literature show that the biochemical mechanism involved in the development of AZA toxicity in liver is proceeding via the extensive generation of ROS (Tapner *et al*., 2004; Raza *et al*., 2003). Free radical scavengers are believed to be protective against liver disorders (Ulicná *et al*., 2003). The DPPH radical scavenging and nitric oxide radical inhibiting ability of *Ficus hispida* suggest that the extract exerts a beneficial action against free radical mediated oxidative changes (Matsuura *et al*., 2003). In this milieu, the antioxidant that scavenges the free radicals is expected to suppress lipid peroxidation (Aniya *et al*., 1999).

In the present study, significant elevation of malondialdehyde (MDA) was observed in AZA intoxicated rats, which is indicative of the activation of lipid peroxidation (LPO) system resulting in excessive

Table 2: Effect of azathioprine and *F. hispida* on the activities of liver enzymic antioxidants

Groups	SOD (Units mg^{-1} protein)	CAT (μmoles H_2O_2 consnmed min^{-1} mg^{-1} protein)	GPx (μmoles GSH oxidized min^{-1} mg^{-1} protein)	GST (nmoles CDNB (1-chloro-2,4-dinitrobenzene) conjugated min^{-1} mg^{-1} protein)
Group 1 (Control)	6.81±0.41	73.63±4.27	96.36±5.41	211.51±12.36
Group 2 (AZA)	3.76±0.31[a]	52.27±4.29[a]	53.67±4.18[a]	141.84±10.81[a]
Group 3 (FH)	6.88±0.47[NS]	73.89±4.52[NS]	97.18±6.20[NS]	216.23±13.24[NS]
Group 4 (FH+AZA)	5.93±0.42[b]	67.56±4.82[b]	86.93±6.22[b]	195.36±12.22[b]

Results are expressed as mean±SD for six rats. Comparisons are made between: [a]Group 1 and Group 2; [b]Group 2 and Group 4. *Statistically significant ($p<0.05$); NS: Non-Significant

Table 3: Effect of azathioprine and *F. hispida* on the activities of liver non-enzymic antioxidants

Groups	GSH (nmol g^{-1} wet tissue)	Vit C (mg g^{-1} wet tissue)	Vit E (mg g^{-1} wet tissue)
Group 1 (Control)	7.11±0.37	1.73±0.08	4.12±0.23
Group 2 (AZA)	2.53±0.18[a]	0.49±0.04[a]	2.17±0.16[a]
Group 3 (FH)	7.16±0.41[NS]	1.75±0.09[NS]	4.16±0.26[NS]
Group 4 (FH+AZA)	6.68±0.41[b]	1.31±0.08[b]	3.76±0.23[b]

Results are expressed as mean±SD for six rats. Comparisons are made between: [a]Group 1 and Group 2; [b]Group 2 and Group 4. *Statistically significant ($p<0.05$); NS: Non-Significant

Table 4: Levels of MDA in the liver of the experimental animals

Groups	LPO (nmoles of MDA formed min^{-1} mg^{-1} protein)
Group 1 (Control)	1.62±0.11
Group 2 (AZA)	3.37±0.18[a]
Group 3 (FH)	1.64±0.13[NS]
Group 4 (FH+AZA)	1.93±0.08[b]

Results are given as mean±SD for six rats. Comparisons are made between: a-Group 1 and Group 2; b-Group 2 and Group 4. *Statistically significant ($p<0.05$); NS: Non-Significant

generation of free radicals. It may be noteworthy that these observations are consistent with the findings of Amin and Hamza (2005). However upon pretreatment with FH, the MDA levels were significantly decreased. The supposition was that the phytoconstituents present in FH functioned as antioxidants/anti-lipid peroxidants in the current study by protecting membrane lipids from propagating oxidative damage through termination of peroxyl radical mediated reactions. This idea is evidently rooted in the reports of various investigators on the phytoconstituents like oleanolic acid, hispidin, bergapten and β-sitosterol (Balanehru and Nagarajan, 1991; Jung *et al*., 2008; Ng *et al*., 2000; Yokota *et al*., 2006).

Antioxidant enzymes such as SOD, CAT, GPx and GST play an important role in the elimination of reactive oxygen species and protects against oxidative stress. In accordance with the previous findings, the significant decrease in SOD and CAT activity was due to exhaustion of the enzymes as a result of oxidative stress caused by AZA (Amin and Hamza, 2005). Besides, the diminished activity of GPx and GST might be owing to the decreased availability of the substrate, GSH in AZA administered rats. Nevertheless, FH treatment bolstered the antioxidant defense system as depicted by the increased tissue levels of SOD, CAT, GPx and GST, which could be attributed to the presence of antioxidants like oleanolic acid, β-sitosterol and hispidin (Jung *et al*., 2008; Kim *et al*., 2005; Li *et al*., 2007).

Non-enzymatic antioxidants such as GSH, vitamin C and vitamin E are closely interlinked to each other and play an excellent role in protecting the cell from oxidative damage. Glutathione is a ubiquitous tripeptide which functions as a free radical scavenger in the oxidative stress conditions (Wu and Cederbaum, 2004). Depletion of GSH impairs the ability of the cells to protect against the free radicals and results in enhanced lipid peroxidation. It has been well documented that metabolism of azathioprine involves the depletion of GSH (Amin and Hamza, 2005; Raza *et al*., 2003). Vitamin E (α-tocopherol) is a lipid-soluble antioxidant which is present in cellular membranes where it plays an important role in the suppression of free radical-induced lipid peroxidation (Dutta-Roy, 1999). Vit C is a free radical scavenger and functions as an antioxidant in recycling the Vit E radical back to Vit E. Both the enzymatic and the nonenzymatic regeneration of the active form of Vit C by GSH have been described in previous studies (Henning *et al*., 1991). These data clearly exemplify the reduced activities of the vitamins C and E, as pronounced GSH depletion is observed in the present study. The biologically active antioxidant phytoconstituents found in *Ficus hispida* extract spared the antioxidant activity and reduced the consumption of endogenous antioxidants, which could be responsible for the reduction of AZA induced oxidative stress plausibly through hepatic GSH restorative effect (Jeong, 1999; Oliveira *et al*., 2005). Therefore, the study described here demonstrates that *Ficus hispida* Linn. leaves possess definite antioxidant and antiperoxide activities against the prooxidant-antioxidant imbalance elicited by azathioprine and also the present study seems to support the claims of a traditional medicine practitioner about the use of *Ficus hispida* in liver ailments.

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