PROTECTIVE EFFECT OF METHANOLIC EXTRACT OF *ANNONA SQUAMOSA* LINN IN ISONIAZID-RIFAMPICIN INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The present study was made to investigate the protective effect of methanolic extract of *Annona squamosa* on isoniazid-rifampicin-induced hepatotoxicity in rats. Rats were divided into five different groups (n=6), group 1 served as a control, Group 2 received isoniazid (100 mg/kg, i.p.) and co-administered with rifampicin (100 mg/kg, i.p.), in sterile water, group 3 and 4 served as extract treatment groups and received 250 & 500 mg/kg bw, p.o methanolic extract of *Annona squamosa* and group 5 served as standard group and received silymarin 2.5 mg/kg bw, p.o. All the treatment protocols followed 21 days and after rats were sacrificed blood and liver were used for biochemical and histological studies, respectively. Administration of isoniazid and rifampicin caused a significant elevation in the levels of liver marker enzymes and thiobarbituric acid reactive substances (TBARS, oxidative stress markers) in experimental rats. Administration of methanolic extracts of *Annona squamosa* significantly prevented isoniazid-rifampicin-induced elevation in the levels of serum diagnostic liver marker enzymes (alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP) and gamma glutamate transpeptidase (**γ-GT**)), serum bilirubin, and TBARS level in experimental groups of rats. Moreover, total protein and reduced glutathione (GSH) levels were significantly increased in treatment group. The effect of extract was compared with a standard drug, silymarin. The changes in biochemical parameters were supported by histological profile. It is to be concluded that the methanolic extract of *Annona squamosa* protects against isoniazid and rifampicin-induced oxidative liver injury in rats.

Keywords: *Annona squamosa,* isoniazid, rifampicin, hepatotoxicity, oxidative stress.

INTRODUCTION

Drug-induced liver toxicity is a common cause of liver injury. It accounts for approximately one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver disease (Kaplowitz, 2001). Different types of drugs such as acetaminophen, chloroquine and isoniazid are inducers of hepatotoxicity in world. Isoniazid and rifampicin, the first line drugs used for tuberculosis therapy are associated with hepatotoxicity (Tasduq *et al*., 2005). The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8 - 30%) compared to that in advanced countries (2- 3%) with a similar dose schedule (Sharma, 2004). Recent studies indicate the existence of a strong correlation between hepatic injury and oxidative stress in experimental animals treated with anti-tuberculosis drugs. Since all the drugs used in the treatment of tuberculosis are shown to have hepatotoxic effects, studies have been performed to prevent or reduce the toxicity by the use of natural herbal drugs and/or synthetic compounds, without interfering with the therapeutic actions of the drugs. Garlic, silymarin, N-acetylcysteine and several other herbal drugs are proved to have such effects. It is of importance to note that the inhibition of CYTP450 2E1 and antioxidant actions seem to be the common mechanism of action of herbal drugs (Sude *et al*., 2008).

The plant *Annona squamosa* (Family annonaceae) is commonly called custard apple in English, sharifa in Hindi (Morton, 1987). This plant is reputed to possess varied medicinal properties like insecticidal agent, free radical scavenging activity, hypoglycemic and anti-diabetic activity (Watt, 1972, Cheema *et al*., 1985, Shirwaikar *et al*., 2004, Gupta *et al*., 2005, Kaleem *et al*., 2006). Many bioactive components like acetogenin, flavonoids, aporphine alkaloids, glycoside and squamoline were isolated from the bark and leaves of this plant (Yang and Chi-Ming, 1972, Bhakuni *et al*., 1972, Bhaumik *et al*., 1979, Forgacs *et al*., 1980, Seetharaman, 1986, Hopp *et al*., 1988, Li *et al*., 1990, Hopp *et al*., 1997). The present study was made to investigate the protective actions of methanolic extract of leaves of *Annona squamosa* (MEAS) against hepatotoxicity caused by isoniazid-rifampicin.

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MATERIAL AND METHODS

Chemicals

Bilirubin, total protein, alkaline phosphatase (ALP), alanine transaminases (ALT), aspartate transaminases (AST), and gamma glutamate transpeptase (γ-GT) were assayed by using kits from Ranbaxy Diagnostic, New Delhi. All the drugs, chemicals and reagents used for biochemical estimation were purchased from Sigma-Aldrich, USA.

Animals

Male Wistar albino rats, weighing about 150 – 200 g were obtained from Institute Animal Center and used in the experiments. The protocol was approved by the Institute's Animal Ethical Committee. Animals were kept in the animal house at an ambient temperature of 25 ^{0}C and 45 – 55% relative humidity, with 12 h each of dark and light cycles. Animals were fed pellet diet and water *ad-libitum*.

Preparation of plant extract

Leaves of *Annona squamosa* were collected from Madurai, Tamilnadu in November 2007 and identified by Dr. D. Stephen, department of botany, American college, Madurai. The shade dried leaves were powdered to get a course granule. About 750 g of dried powder was extracted with methanol by continuous hot percolation, using soxhlet apparatus. The resulted dark – brown extract was concentrated up to 100 ml on Rota vapour under reduced pressure. The concentrated crude extract (12 g) was lyophilized into powder and used for the study.

Phytochemical screening

The methanolic extract obtained was subjected to preliminary phytochemical screening, to identify the chemical constituents. The methods of analysis employed were those described by (Trease and Evans, 1989, Harbone and Baxter, 1993).

Induction of experimental hepatotoxicity

Isoniazid and rifampicin solution were prepared separately in sterile distilled water. Rats were treated with isoniazid (100 mg/kg, ip) and co-administered with rifampicin (100 mg/kg, ip), for 21 days (Yue *et al*., 2004, Saleem *et al*., 2008). In order to study the effect of methanolic extract of *Annona squamosa* (MEAS) in rat, 250 and 500 mg/kg bw, *p.o.* were used respectively. Silymarin (2.5 mg/kg bw, *p.o.*) was used as a standard drug in this study (Parthasarathy *et al*., 2007). Rats were divided into five different groups (n=6), group 1 was served as a control, group 2 was toxic control receive isoniazid+rifampicin (100 mg/kg bw i.p), group 3 and 4 were served as extract treatment groups received 250 & 500 mg/kg bw, p.o methanolic extract of *Annona squamosa* and group 5 served as standard group received silymarin 2.5 mg/kg bw, p.o. Rats were treated as per the treatment protocol. Body weights of these rats were monitored sequentially in control and experimental animals for a period of 21 days.

Biochemical estimation

Rats were sacrificed 1 h after administration on day 21. The blood was collected by retro-orbital artery bleeding. Blood samples were centrifuged for 10 min at 3000 rpm to separate the serum. ALP, ALT, AST, γ-GT, total protein and bilirubin levels were estimated from the serum by using standard kits (Rajesh *et al*., 2005). Liver was excised immediately, quickly cooled and perfused with cold normal saline. Ten percent homogenate was prepared by homogenizing the liver tissue by using 0.3 m phosphate buffer. TBARS (Okhawa *et al*., 1979) and GSH (Ellman, 1959) levels were estimated from the liver homogenate by using spectrophotometric determination.

Histopathological studies

The livers were excised quickly and fixed in 10% formalin and stained with haemotoxylin and eosin and then observed under microscope for degeneration, fatty changes or necrotic changes as evidence of hepatotoxicity.

STATISTICS

All values were expressed as means ± SEM (n = 6 in each group). One-way ANOVA was applied to test for significance of biochemical data of the different groups. Significance is set at $P \leq 0.05$.

RESULTS

The freshly prepared methanolic extracts were subjected to preliminary phytochemical screening test for various constituents. This revealed the presence of alkaloids, tannins, saponins, flavonoids, glycosides, terpenoids and steroids.

There was no mortality in any of the groups. The body weight and relative liver weights of the experimental animals calculated at the end of the study had no statistically significant difference when compared to the control animals.

Three fold rises in biochemical parameters in toxic control group (G2) is indication for liver injury by INH + RIF treatment. Increased biochemical parameters (ALP, AST, ALT, TB & TP) were significantly reduced by co-administration of MEAS in two different doses (250 & 500 mg/kg bw) and silymarin treatment group. The results were presented in table 1.

Increased liver TBARS level in G2 group is indication for increased oxidative stress by treatment of INH + RIF. Increased liver TBARS significantly (P<0.001) reduced by co-administration of MEAS in two different doses

(250 & 500 mg/kg bw) and silymarin treatment group. The INH+RIF-administered animals exhibited significantly (P<0.01) low levels of hepatic GSH levels significantly increased by co-administration of MEAS in two different doses (250 & 500 mg/kg bw) and silymarin treatment group. The results were shown in table 2.

Hepatocytes of the normal control group (G1) showed a normal lobular architecture of the liver. In the INH + RIF treated group (G2) the liver showed cell swelling, congestion and feathery degeneration of the liver cells and portal triaditis. Co administration of MEBC (G3-250 & G4-500 mg/kg bw) showed minimal changes with

Table 1: Effect of methanolic extract of *Annona squamosa* in different biochemical parameters in INH + RIF induced-hepatotoxic rats

Groups	Total bilirubun (mg/dl)	Total protein (mg/dl)	ALP (IU/dl)	AST (IU/dl)	ALT (IU/dl)	γ-GT (IU/dl)
Normal Control (G1)	0.35 ± 0.01	6.47 ± 0.01	117.39 ± 2.1	118.39 ± 5.99	30.99 ± 0.38	90.31 ± 1.73
Toxic Control (G2)	1.06 ± 0.04♦	4.35 ± 0.04♦	243.64 ± 7.55♦	368.72 ± 7.25♦	124.91 ± 2.64♦	169.24 ± 4.29♦
MEAS 250 mg/kg (G3)	0.84 ± 0.6**	7.3 ± 0.11***	150.55 ± 11.49***	164.84 ± 1.66***	67.02 ± 2.75***	116.68 ± 1.75**
MEAS 500 mg/kg (G4)	0.62 ± 0.14***	7.8 ± 0.11***	151.99 ± 9.39***	166.96 ± 4.39***	79.46 ± 575***	128.70 ± 3.67***
SIL Group (G5)	0.42 ± 0.03***	7.85 ± 0.01***	111.29 ± 11.23***	147.88 ± 3.82***	49.52 ± 3.48***	90.18 ± 1.04***

All values are expressed as means ± SEM. Statistical analysis was done using One-way ANOVA followed by Post Test.
♦ P<0.001 vs Control (G1) *P<0.05 vs Toxic Control (G2) **P<0.01 vs Toxic Control (G2) ***P<0.001 vs Toxic Control (G2)
MEAS-methanolic extract of *Annona squamosa*, SIL-silymarin, INH-isoniazid, RIF-rifampicin

Table 2: The levels of TBARS & GSH after the treatment of rats with isoniazid-rifampicin and MEAS on 21 days Treatment (n=6)

Group	TBARS nmol/g wet wt	GSH µg/g wet wt
NORMAL CONTROL (G1)	299.24 ± 0.32	81.91 ± 0.4
TOXIC CONTROL (G2)	422.57 ± 0.15[a]	38.96 ± 2.7[b]
MEAS 250 mg/kg (G3)	213.54 ± 0.5[#]	78.20 ± 1.2[#]
MEAS 500 mg/kg (G4)	245.16 ± 0.17[#]	89.5 ± 0.16[#]
SIL GROUP (G5)	272.19 ± 0.23[#]	99.16 ± 3.25[#]

All values are expressed as means ± SEM. Statistical analysis was done using One-way ANOVA followed by Post Test.
[a] P<0.001 when compared with G1, [b] P<0.01 when compared with G1, [#] P<0.001 when compared with G2
MEAS-methanolic extract of *Annona squamosa*, SIL-silymarin, INH-isoniazid, RIF-rifampicin

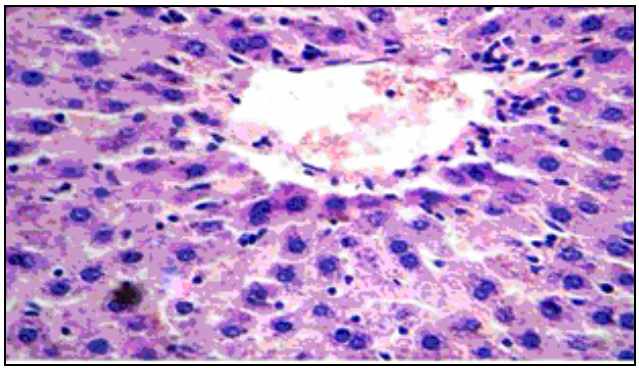

Fig. 1: Hepatocytes of the normal control group showed a normal lobular architecture, hepatic cords of the liver (H&E, x 10).

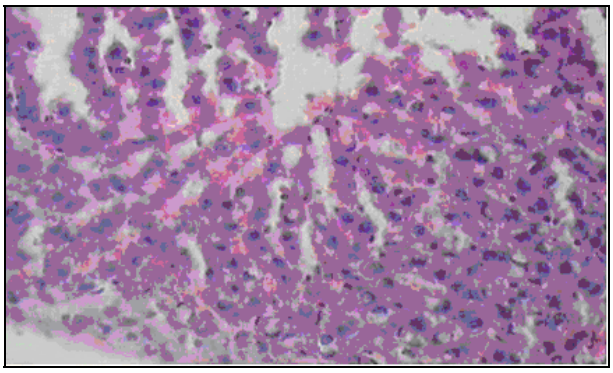

Fig. 2: Hepatocytes of the INH + RIF treated group showed cell swelling, congestion and feathery degeneration of the liver cells (H&E, x 10).

moderate portal triaditis and their lobular architecture was normal. Silymarin pretreated group (G5) showed normal hepatocytes without degeneration and their lobular architecture was normal. These above findings indicated the hepatoprotective effect of MEAS. The results were presented in figs. 1-5.

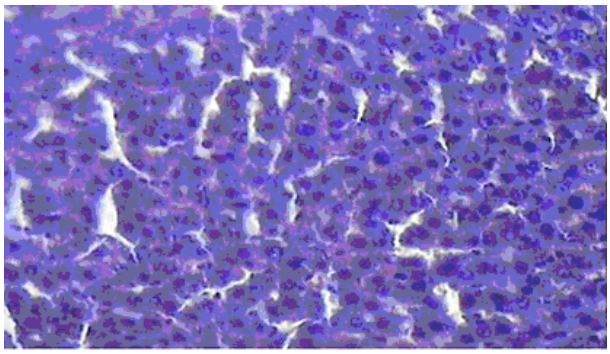

Fig. 3: Hepatocytes of the MEAS 250 mg/kg, pretreated group showed minimal congestion and their lobular architecture was normal (H&E, x 10).

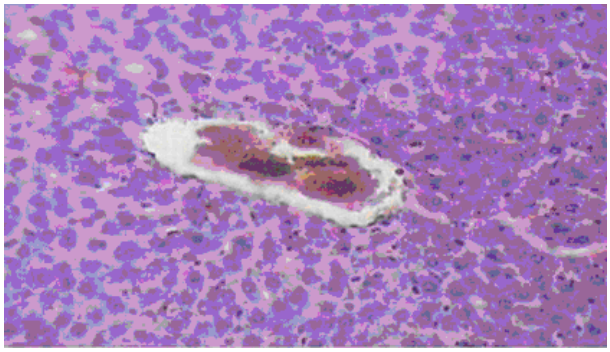

Fig. 4: Hepatocytes of the MEAS 250 mg/kg, pretreated group showed minimal changes with moderate portal triaditis and their lobular architecture was normal (H&E, x 10).

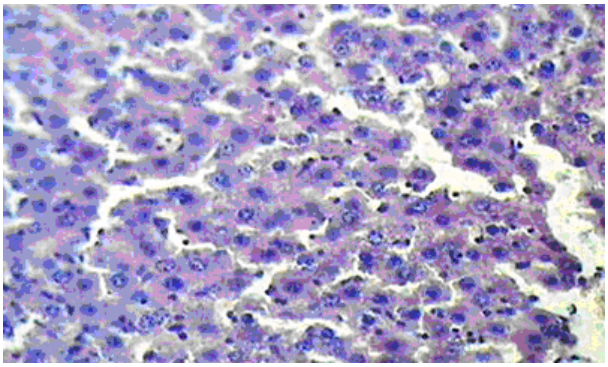

Fig. 5: Hepatocytes of the Silymarin pretreated group showed normal hepatocytes without degeneration and their lobular architecture was normal (H&E, x 10).

DISCUSSION

In the present study, hepatotoxicity model in Wistar rats was successfully produced by administering INH and RIF (100 mg/kg per day each) i.p. A marked rise above the normal upper limits in the measured serum transaminases in INH+RIF group on day 21 of the experiment was a biochemical indication of liver injury.

During the metabolism of INH, hydrazine is produced directly (from INH) or indirectly (from acetyl hydrazine). From earlier study it is evident that hydrazine plays a role in INH-induced liver damage in rats, which is consistent with the report by Sarich *et al* (Sarich *et al*., 1996, Garner *et al*., 2004). The combination of INH and RIF was reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation, and cytochrome P450 and was thought to involve the synergistic effect of RIF on INH. However, its role in INH-induced hepatotoxicity is unclarified, as INH itself is an inducer of CYP2E1. Previous reports also indicate that there did not seem to be clear evidence that INH proves much more injuries than RIF and, in this connection, they consider that it is the combination of these two drugs that confers the additive, or even synergistic potential of liver toxicity than either agent alone, as conjectured. INH is metabolized in the liver primarily by acetylation and hydrolysis, and it is these acetylated metabolites that are thought to be hepatotoxins. Previous reports in rats suggest that the hydrazine metabolite of INH which has subsequent effect on CYP2E1 induction is involved in the development of INH-induced hepatotoxicity, and also oxidative stress as one of the mechanism for INH + RIF induced hepatic injury (Yue *et al*., 2004, Saleem *et al*., 2008).

In this study the results suggest that the statistically significant different in biochemical parameters in toxic control group (G2), indicate that hepatic damage has been induced by INH + RIF. Following treatment with MEAS (250 & 500 mg/kg bw) and Silymarin (2.5 mg/kg), all the parameters were reduced and total protein and GSH restored to the normal values. Our previous report by using aqueous and ethanolic extract showed the same findings in this plant (Saleem *et al*., 2008).

Metabolism of chemicals takes place largely in the liver, which accounts for the organ's susceptibility to metabolism-dependent, drug induced injury. The drug metabolites can be electrophilic chemicals or free radicals that undergo or promote a variety of chemical reactions, such as depletion of reduced glutathione; covalently binding to proteins, lipids, or nucleic acids; or inducing lipid peroxidation (Kaplowitz, 2004). In present study in toxic control group (G2) increased level of TBARS (a marker for oxidative stress), reduction in the GSH concentration is indication for increased oxidative stress

in INH + RIF treatment group. Elevation of TBARS were significantly reduced by co-administration of MEAS (250 & 500 mg/kg bw) and Silymarin (2.5 mg/kg) and elevation of GSH level after MEAS and silymarin treatments indicate that the extracts is useful for the treatment of drug injury caused by INH + RIF.

Hepatocellular disintegrate, feathery degeneration, portal triaditis and the inflammation in the liver were observed in the centrilobular region by histopathological examination in INH-RIF treated groups. Previous studies also showed the same results in isoniazid-treated group (Yue *et al*., 2004, Ravinder *et al*., 2006, Saleem *et al*., 2008). Simultaneously administered MEAS prevented the induction of histopathological injuries in INH+RIF co-treated animals.

The hepatoprotective effect of the extract may be explained depending on the fact that *Annona squamosa* contains flavonoids which might have scavenged the free radical offering hepato protection. Previous reports also suggest that the protective role of *Annona squamosa* leaf extracts could be due to the antioxidative effect of flavonoids present in the leaf (Kaleem *et al*., 2006).

CONCLUSION

This study showed that MEAS has a significant protective action against the hepatotoxicity induced by the drugs used in the treatment of tuberculosis. The hepatoprotective role of MEAS might be due to its antioxidant potential mechanism suggesting that the extract of plant may be useful to prevent the oxidative stress induced damage. More research is required in this view-point to develop a good hepatoprotective drug from leaves of *Annona squamosa.* Purification of extracts and identification of the active principle may yield active hepatoprotective ingredients.

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