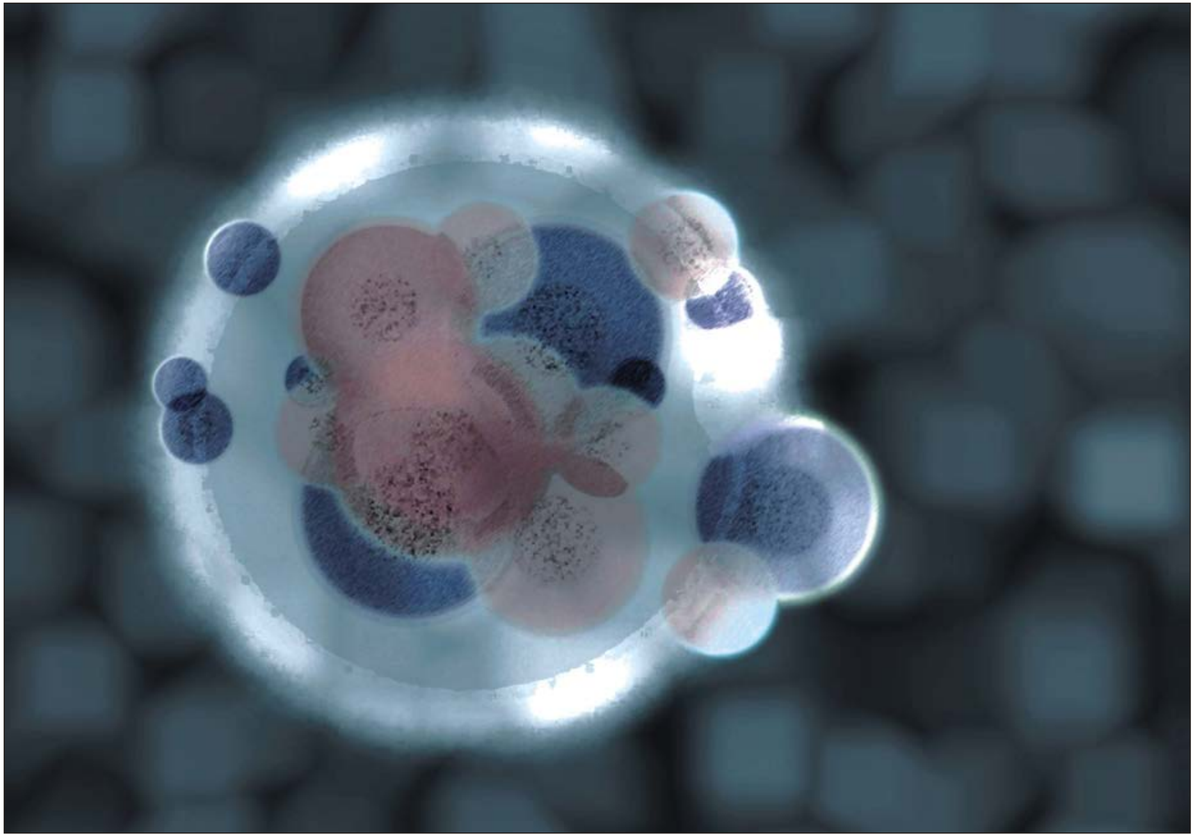

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Salutary Effect of Ferulic Acid Against D-Galactosamine Challenged Liver Damage

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Abstract: In the present study, we evaluated the effect of Ferulic Acid (FA) supplementation (20 mg kg^{-1} body weight) on GalN provoked hepatotoxicity in male Wistar rats. Hepatotoxicity was induced by a single intraperitoneal injection of GalN (500 mg kg^{-1} body weight) and manifested by an increase in the levels hepatospecific marker enzymes like Aspartate Transaminase (AST), Alamine Transaminase (ALT), Alkaline Phosphatase (ALP) and Lactate Dehydrogenase (LDH). In contrast, pretreatment with FA significantly ameliorated all these alterations. GalN intoxicated rats visualize a remarkable oxidative stress, as evidenced by a significant elevation in Lipid Peroxidation (LPO) with a concomitant decrease in the glutathione (GSH) activity. These changes were coupled with a marked decline in the activities of enzymic antioxidants [superoxide dismutase (SOD), catalase (CAT), Glutathione Peroxidase (GPx) and Glutathione Reductase (GR)] in the liver tissue of GalN administered rats. FA pretreated rats exhibit a significant inhibition of LPO and augmentation of endogenous antioxidants. Further, there was an increase in the levels of cholesterol, triglycerides and free fatty acids followed by a decrease in the levels of phospholipids in serum and liver. Pretreatment with FA reversed these alterations to near normal. Results of this study revealed that FA could afford a significant protection in the alleviation of GalN induced hepatocellular injury.

Key words: Ferulic acid, D-galactosamine, hepatotoxicity, lipid peroxidation, oxidative stress, antioxidants

INTRODUCTION

Liver damage arises via direct injurious attack by a wide variety of primary hepatotoxins such as alcohol, aflatoxin, heavy metals and drugs (Nakagiri *et al*., 2003). Among these, D-Galactosamine (GalN) is well established as a suitable experimental model of liver injury. Its toxicity is of clinical importance because there is a close resemblance between the multifocal necrosis produced by GalN and the lesion of viral hepatitis in humans (Shi *et al*., 2008). The mechanism of GalN induced hepatotoxicity appear to inhibit the synthesis of RNA and protein and through a decrease in the cellular UTP concentration (Wills and Asha, 2006). The GalN has great liver specificity because hepatocytes have high levels of galactokinase and galactose-1-uridyl transferase (Lim *et al*., 2001). Reports have shown that singlet oxygen plays a role in GalN induced liver cell injury (Sakaguchi and Yokata, 1995). GalN has been proposed to be hepatotoxic due to its ability to destruct liver cells, possibly by a free radical mechanism (Quintero *et al*., 2002) and peroxidation of endogenous lipids is a major factor in the cytotoxic action of GalN (Hu and Chen, 1992).

Recently, there is a great deal of interest in the health benefits of phenolic compounds because of their antioxidant potential (Rice-Evans *et al*., 1998). Ferulic acid (FA) is a ubiquitous plant phenolic constituent that arises from the metabolism of phenylalanine and tyrosine (Castelluccio *et al*., 1996). It occurs primarily in rice, wheat, oat, roasted coffee, rapeseed, tomatoes and many other plants. FA occurs mainly in various esters form with polar compounds such as sugar (Saulnier *et al*., 1995) and non polar ones such as sterols in plants (Nystrom *et al*., 2005). Reports has shown that FA is a potent antioxidant (Graf, 1992; Scott *et al*., 1993) and also act as a strong membrane antioxidant in humans and is known to be effective against skin disorders, cancer, ageing, fatigue, muscle wasting, cold, flu and influenza (Deuster). It readily forms a resonance stabilized phenoxy radical

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which accounts for its potent antioxidant potential (Graf, 1992). FA protected membranes from lipid peroxidation and neutralized alkoxyl and peroxyl radicals (Trombino *et al*., 2004) and also scavenges hydroxyl radical (Ogiwara *et al*., 2002), nitric oxide (Wenk *et al*., 2004), peroxynitrite (Pannala *et al*., 1998; Dinis *et al*., 2002) and superoxide radical (Kaul and Khanduja, 1999; Kikuzaki *et al*., 2002). It has been shown that long term administration of FA protects the β-amyloid peptide induced oxidative stress (Yan *et al*., 2001). FA derivatives are receiving greater attention nowadays in the research world because of its wide range of therapeutic effects. FA 15, a hydrophobic derivative of ferulic acid suppresses the skin tumor promotion in mice (Murakami *et al*., 2002). Recent studies has shown that NCX 2057 a nitric oxide-releasing derivative of natural antioxidant ferulic acid, modulated the iNOS expression in raw 264.7 macrophages (Ronchetti *et al*., 2006). Reports have shown that geranylated derivative of FA inhibited the azoxymethane induced colon carcinogenesis in rat (Han *et al*., 2001). To our knowledge, so far no other biochemical investigation has been carried out on the effect of FA against GalN induced liver injury. In this scenario, the present investigation was carried out to study the protective effects of FA on GalN induced liver injury.

MATERIALS AND METHODS

Drugs and chemicals: D-galactosamine and Ferulic acid were purchased from Sigma Aldrich Limited, USA. All other chemicals and solvents used were of the highest purity and analytical grade.

Animal model: This study was conducted in the month of November 2008. Male albino wistar rats weighing (120-150 g) were obtained from the Animal House, Vel's College of Pharmacy, The Tamilnadu Dr. M.G.R. Medical University, Chennai, India. Animals were fed with commercially available standard rat pelleted feed from M/s Hindustan Lever Limited, Bangalore, India. The feed and water were provided *ad libitum*. The animals were deprived of food for 24 h before experimentation but allowed free access to tap water throughout. 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 were randomized into 4 groups of 6 rats each as follows:

Group 1: Control rats received normal saline (1 mL kg^{-1}, body weight) orally for 7 days

Group 2: Toxic control rats received normal saline (1 mL kg^{-1}, body weight) orally for 7 days

Group 3: Drug control groups received FA (20 mg kg^{-1}, body weight) by oral gavage for 7 days

Group 4: Rats pretreated with FA by oral gavage (20 mg kg^{-1}, body weight) for 7 days

Groups 2 and 4 also received GalN intraperitoneally (500 mg kg^{-1}, body weight) on 7th day. After the 7 days experimental period (i.e., on the 8th 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 assays. Blood was collected and serum was separated for analysis of biochemical parameters.

Enzyme indices of cellular integrity: Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) and Lactate Dehydrogenase (LDH) were estimated by the method of King (1965 a, b, c). Protein content was estimated by the method of Lowry *et al*. (1951).

Lipid peroxidation: Tissue lipid peroxide level was determined as MDA (Ohkawa *et al*., 1979). The absorbance was measured photometrically at 532 nm and the concentrations were expressed as nmol malondialdehyde (MDA) min/mg/protein.

Assay of antioxidants: SOD was assayed by the method of Misra and Fridovich (1972). Catalase (CAT) level was estimated by the method described by Sinha (1972). Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al*. (1973). Glutathione Reductase (GR) was assayed by the method of Staal *et al*. (1969). Total reduced glutathione (GSH) was determined by the method of Ellman (1959).

Estimation of lipid parameters: The extraction of serum and tissue lipids was done according to the procedure of Folch *et al*. (1957). The estimation of total cholesterol was carried out by the method of Zlatkis *et al*. (1953) and triglycerides by the method of Foster and Dunn (1973). Free fattyacids was estimated by the method of Falholt *et al*. (1973) and phospholipids by the method of Zilversmit and Davis (1950).

Statistical analysis: The results were expressed as Mean±SD for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS 13.0 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 the present study, intraperitoneal administration of a single dose of GalN (500 mg kg^{-1} body weight) induced severe biochemical changes as well as oxidative injury in liver tissue. There was a significant ($p<0.05$) rise in the levels of hepatic marker enzymes (AST, ALT, ALP and LDH) in the serum of GalN administered rats (Group2) as compared to that of (Group 1) control rats (Table 1). The administration of FA to Group 4 rats restored the levels of these enzymes to near normalcy ($p<0.05$) as compared to those GalN-injected rats (Group 2). In FA alone administered rats (Group 3) versus controls (Group 1), no significant changes were observed.

Injection of GalN induced a significant ($p<0.05$) increase in the level of lipid peroxidation (Fig. 2), which was paralleled by significant ($p<0.05$) reduction in the level of GSH (Fig. 1) in the liver tissue of Group2 rats as compared to normal control rats (Group 1). Glutathione plays an important role in the regulation of variety of cell functions and in cell protection from oxidative injury. Depletion of GSH results in enhanced lipid peroxidation and excessive lipid peroxidation can cause increased GSH consumption, as observed in the present study. In this study, treatment with FA (Group 4) significantly ($p<0.05$) counteracted the GalN-induced lipid peroxidation and restored the level of GSH to near normal level in Group 4 rats as compared to that of Group2 rats.

Activities of glutathione-dependent antioxidant enzymes (Gpx and GR) and anti-peroxidative enzymes (SOD and CAT) were significantly ($p<0.05$) lower in the liver tissue of GalN-injected rats (group 2) as compared to control rats (group 1). In the present study, the treatment of Group 4 rats with FA, significantly ($p<0.05$) reversed all these GalN-induced alterations in the activities of antioxidant enzymes (SOD, CAT, GPx and GR) to a near normal status. The normal rats receiving FA did not show any significant changes as compared to that of (Group 1) normal control rats (Table 2). The observed reduction in the activities of GPx, GR in

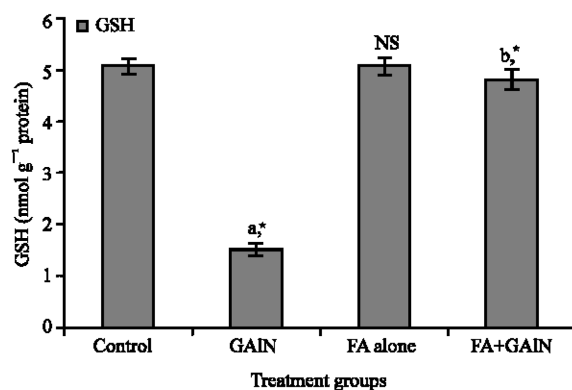

Fig. 1: Levels of GSH in the liver of the experimental animals. Results are given as Mean±SD for 6 rats. Comparisons are made between: (a) Group 1 and Group 2, (b) Group 2 and Group 4 (NS) Group 1 and Group 3. *Statistically significant ($p<0.05$), NS-non-significant

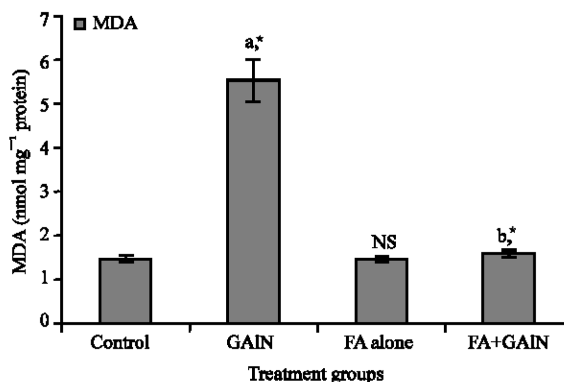

Fig. 2: Levels of MDA in the liver of the experimental animals. Results are given as Mean±SD for 6 rats. Comparisons are made between: (a) Group 1 and Group 2, (b) Group and Group 4 and (NS) Group 1 and Group 3. *Statistically significant ($p<0.05$), NS-non-significant

Table 1: Effect of ferulic acid and D-galactosamine on the activities of liver marker enzymes in serum

Groups	AST (IU L^{-1})	ALT (IU L^{-1})	ALP (IU L^{-1})	LDH (IU L^{-1})
Group 1 (Control)	74.34±5.64	59.26±4.06	78.18±3.84	219.15±8.44
Group 2 (GalN)	614.16±48.41^{a,*}	542.50±53.08^{a,*}	166.65±13.79^{a},*	376.53±31.44^{a,*}
Group 3 (FA)	74.79±5.24^{NS}	60.61±3.74^{NS}	79.89±3.77^{NS}	221.93± 8.11^{NS}
Group 4 (GalN+FA)	80.00±7.34^{b,*}	63.70±4.10^{b,*}	86.67±4.72^{b,*}	230.46±9.33^{b,*}

Results are expressed as Mean±SD for 6 rats. Comparisons are made between: (a) Group 1 and Group 2; (b) Group 2 and Group 4. NS- Group 1 and Group 3. *Statistically significant ($p<0.05$); NS-non-significant

Table 2: Effect of Ferulic acid and D-galactosamine on the activities of liver enzymic antioxidants

Groups	SOD (U mg^{-1} of protein)	CAT (U mg^{-1} of protein)	GPx (U mg^{-1} of protein)	GR (nmoles min^{-1} mg^{-1} of protein)
Group 1 (Control)	10.18±0.32	80.04± 3.78	19.65±0.48	28.12±0.79
Group 2 (GalN)	5.14±0.35[a,*]	44.09±3.58[a,*]	9.26±0.75[a,*]	80.90±1.48[a,*]
Group 3 (FA)	10.61±0.39[NS]	80.71±3.90[NS]	20.10±0.77[NS]	28.52±1.12[NS]
Group 4 (GalN+FA)	8.50± 0.41[b,*]	72.36±5.38[b,*]	16.51±0.71[b,*]	25.83± 1.25[b,*]

Results are expressed as Mean±SD for six rats. Units-SOD: U mg^{-1} protein, one unit is equal to the amount of enzyme that inhibits auto-oxidation of epinephrine. CAT: U mg^{-1} protein, μmoles H_2O_2 consnmed min^{-1} mg^{-1} protein. GPx: U mg^{-1} protein, μmoles GSH oxidized min^{-1} mg^{-1} protein. GR: nmoles NADPH oxidized min^{-1} mg^{-1} protein. Comparisons are made between: (a) Group 1 and Group 2; (b) Group 2 and Group 4. NS-Group 1 and Group 3. *Statistically significant ($p<0.05$); NS non-significant

Table 3: Effect of Ferulic acid and D-galactosamine on Serum cholesterol, triglycerides, free fatty acids and phospholipids

Groups	Cholesterol (mg dL^{-1})	Triglycerides (mg dL^{-1})	Free fatty acids (mg dL^{-1})	Phospholipids (mg dL^{-1})
Group 1 (Control)	75.64±7.1	47.22±4.1	19.32±1.24	107.40±7.92
Group 2 (GalN)	104.62±9.1[a,*]	111.21±9.42[a,*]	35.04±2.73[a,*]	75.21±6.1[a,*]
Group 3 (FA)	78.11±6.92[NS]	47.38±3.9[NS]	20.16±1.97[NS]	109.81±9.1[NS]
Group 4 (GalN+FA)	81.04±7.1[b,*]	58.13±4.93[b,*]	25.37±2.18[b,*]	98.40±8.53[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. NS- Group 1 and Group 3. *Statistically significant ($p<0.05$); NS non-significant

Table 4: Effect of Ferulic acid and D-galactosamine on Liver cholesterol, triglycerides, free fatty acids and phospholipids

Groups	Cholesterol (mg/g wet tissue)	Triglycerides (mg/g wet tissue)	Free fatty acids (mg/g wet tissue)	Phospholipids (mg/g wet tissue)
Group 1 (Control)	11.20±0.96	23.80±1.28	0.70±0.04	25.30±1.70
Group 2 (GalN)	19.58±1.20[a,*]	42.14±1.69[a,*]	2.20±0.10[a,*]	16.94±1.43[a,*]
Group 3 (FA)	11.62±0.88[NS]	24.07±1.79[NS]	0.71±0.03[NS]	24.68±2.19[NS]
Group 4 (GalN+FA)	14.00±0.89[b,*]	30.01±2.56[b,*]	1.27±0.09[b,*]	21.47±1.79[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. NS- Group 1 and Group 3. *Statistically significant ($p<0.05$); NS non-significant

GalN-induced (Group 2) rats might be due to the decreased availability of their substrate, GSH. FA administered (Group 3) rats did not show any significant change when compared with control rats, indicating that it does not *per se* have any adverse effects. The levels of cholesterol, triglycerides and free fattyacids (Table 3, 4) were significantly ($p<0.05$) increased while that of phospholipids was decreased in rats intoxicated with GalN (Group 2). These altered levels were brought back to near normal when pretreated with FA (Group 4). The normal rats receiving FA alone (Group 3) did not show any significant change when compared with control rats (Group 1) indicating that it does not have any adverse effects .

DISCUSSION

The present study determined the protective effects of ferulic acid against GalN induced liver damage in male wistar rats. GalN is an indirect hepatotoxicity inducing chemical, whose action might be related to its metabolism in liver and the subsequent effects on protein synthesis since it depletes uracil nucleotides. It was reported that the liver function and morphological changes of liver tissue induced by GalN were similar to those of viral hepatitis (Shi *et al*., 2008). Liver damage induced by GalN commonly reflects turbulence of liver cell metabolism, which leads to characteristic changes in the serum marker enzyme activities (Mitra *et al*., 1998). AST, ALT, ALP and LDH are the most sensitive markers employed in the diagnosis of hepatic damage because these are cytoplasmic in location and are released into the circulation after cellular damage (Sallie *et al*., 1991). The increased activities of these hepatospecific marker enzymes were observed in our present investigation which is in accordance with previous reports (Han *et al*., 2006). Pretreatment with FA significantly decreased the activities of AST, ALT, ALP and LDH suggesting that it offer protection by preserving the structural integrity and stabilizing the hepatocellular membrane against GalN induced oxidative damage which is in line with previous reports (Srinivasan *et al*., 2005).

Reactive oxygen species can be classified into oxygen-centered radicals and oxygen-centered non radicals. Oxygen-centered radicals are superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH), alkoxyl radical (RO•) and peroxyl radical (ROO•). Oxygen-centered non radicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other reactive species are nitrogen species such as nitric oxide (NO•), nitric dioxide ($NO_2\bullet$) and peroxynitrite ($OONO^-$) (Halliwell *et al*., 1995; Simon *et al*., 2000). Previous reports has revealed that GalN provoke serious oxidative insult due to the ROS in the rat liver leading to apoptosis and necrosis (Sun *et al*., 2003).

The ROS in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation (Boff and Min, 2002). Lipid peroxidation refers to the reaction of oxidative deterioration of polyunsaturated lipids because the cell membrane is highly composed of lipids. Lipid Peroxidation involves the direct reaction of oxygen and lipid to form radical intermediates and to produce semi stable peroxides, which in turn damage the enzymes, nucleic acids, membranes and proteins (Sivalokanathan *et al*., 2005). Thus Lipid peroxidation has been implicated as a major process in cellular damage (Plaa and Hewitt, 1989). It has been stated that the mechanism of hepatic injury induced by GalN is due to the instability of cellular membranes as a result of lipid peroxidation (Sakaguchi and Yokota, 1995). In the present study the increased levels of MDA in liver of rats treated with GalN reflected the lipid peroxidation as the consequence of oxidative stress caused by GalN which is in corroboration with earlier reports (Wong *et al*., 2007).

Scavenging of free radicals is one of the major antioxidant mechanisms to inhibit the chain reaction of lipid peroxidation. The free radical scavenging activity of ferulic acid has been well documented (Ju *et al*., 1990). In our study pretreatment with FA reduced the elevated levels of MDA in rats intoxicated with GalN. This may be attributed relatively to the structure of FA, which is a phenolic compound that acts by scavenging free radicals and quenching lipid peroxidative chain. The hydroxyl and phenoxyl groups present in this phenolic compound donate their electrons to the free radicals and quench them. Furthermore, FA, due to its phenolic nucleus and unsaturated side chain, readily forms a resonance-stabilized phenoxy radical which accounts for its potent antioxidant activity. In addition, the carboxylic acid group also acts as an anchor of ferulic acid by which it binds to the lipid bilayer providing protection against lipid peroxidation (Kanski *et al*., 2002).

It has been demonstrated that lipid peroxidation induced by GalN is concurrent with GSH depletion (Seckin *et al*., 1993; Korda, 1998). The tripeptide gamma-glutamylcysteinylglycine or GSH is the major non enzymatic regulator of intracellular redox homeostasis, ubiquitously present in all the cell (Meister and Anderson, 1983). It exists in equilibrium with its disulfide form (GSSG) and the ratio of GSH to GSSG could be used as an indicator of the redox status of the cell. Glutathione disulfide can also be reduced back to GSH by the action of GR utilizing NADPH as a reductant (Argyrou and Blanchard, 2004). Present observation shows that pretreatment with ferulic acid refurbish the decreased levels of hepatic GSH and the diminished activities of GR induced by GalN which was concurrent with the previous reports (Manna *et al*., 2007). Earlier studies shows that ferulic acid concomitantly increases the activities of GSH and GR in oxidative stress conditions (Rukkumani *et al*., 2004).

Cells orchestrate an extensive repertoire of antioxidant defensive system (Fang *et al*., 2002) which encompass Superoxide Dismutase (SOD), catalase (CAT), Glutathione Peroxidase (GPx) and Glutathione Reductase (GR). SOD, a metaloprotein has been reported as one of the most important enzymes in the enzymic antioxidant defence system. It scavenges the superoxide anion to form hydrogen peroxide, hence diminishing the toxic effect caused by this radical (Gupta *et al*., 2003). CAT is a hemeprotein an enzyme predominantly located in peroxisomes, catalyzes the dismutation of toxic hydrogen peroxide (Masaki *et al*., 1998). GPx is a selenoenzyme which plays an important role in the reduction of H_2O_2 in the presence of reduced GSH. The detoxifying action of GPx against H_2O_2 protects cell membrane against oxidative damage (Jung *et al*., 2005). In this study diminished activities of SOD, CAT and GPx were observed in the GalN intoxicated rats suggesting the increased lipid peroxidation. Several reports had shown the beneficial effect of FA on oxidative stress in the liver with an increase in antioxidant enzymes content particularly glutathione peroxidase, superoxide dismutase and catalase (Sudheer *et al*., 2005; Balasubashini *et al*., 2004). Pretreatment with ferulic acid increased the activity of SOD, CAT and GPx which may be due to the scavenging of the radicals generated by GalN induced lipid peroxidation thereby decreasing the utilization of these antioxidant enzymes to reduce the GalN induced oxidative insult. This might be responsible for the increased activities of antioxidant enzyme on administration of FA which is in line with the previous reports that FA is an effective scavenger of free radicals (Toda *et al*.,1991).

In the present study FA also showed protection against serum and liver lipid changes caused by GalN evidencing a broad spectrum of hepatoprotective property. Any liver disease will show an elevated blood cholesterol level (McIntyre and Rosalki, 1992). The significant increase of cholesterol noted in the present study might be due to the inability of the diseased liver to remove cholesterol from circulation. This finding could be correlated with the results of the previous studies (Dhanabal *et al*., 2006). Hepatocellular damage due to alcohol, virus and drug induced hepatitis causes a modest hypertriglyceridemia (Glickman and Sebesin, 1982), which is due to the biochemical changes inferring with the transport of triglycerides out of liver. This study also showed an increased accumulation of triglycerides in GalN intoxicated rats which is in agreement with previous

reports (Dwivedi *et al*., 1992). Accumulation of free fatty acids is a consequence of changes in hepatic lipid metabolism. This is well correlated in our study with the increased levels of free fatty acids due to the administration of GalN, which is responsible for the increment of phospholipase A2 activity as a consequence of the high level of intracellular calcium causing the hydrolysis of liver membrane phospholipids and release of arachidonic acid (Kramer and Sharp, 1997). This might account for the decreased levels of phospholipids observed in the serum and liver of GalN challenged rats. Rats pretreated with FA prior to the induction of hepatic damage showed a restoration of the altered lipid levels induced by GalN towards near normalcy thereby showing the modulating effect of FA against GalN induced changes in the lipid levels in rats which is in line with the previous report (Balasubashini *et al*., 2003).

In conclusion, the present finding shows that FA effectively inhibits lipid peroxidation and improves the antioxidant status in the tissues. It also prevent the leakage of liver marker enzymes into circulation by protecting the membrane and inhibition of lipid peroxidation caused by GalN toxicity. Hence, in present study, FA was found to be effective against GalN induced hepatotoxicity.

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