



Ameliorative Effect of Piperine from *Piper Nigrum* on D-Galactosamine - Induced Hepatotoxicity in Mice

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ABSTRACT

The purpose of this study was to evaluate the hepatoprotective and antioxidant properties of piperine against D- galactosamine induced hepatotoxicity in mice. Hepatotoxicity was induced in mice by a single dose of galactosamine (700 mg/kg, i.p.) activities of serum glutamate oxaloacetate transferase (SGOT), serum glutamate pyruvate transferase (SGPT), alkaline phosphatase (ALP) and tumour necrosis factor- alpha (TNF- α) were estimated in serum while lipid peroxidation and antioxidant status were determined in liver homogenate of control and experimental mice. Galactosamine administration (700 mg/kg, i.p.) significantly ($p < 0.05$) increased the levels of SGOT, SGPT, ALP, bilirubin, TNF- α and lipid peroxidation and caused depletion in antioxidant status. Piperine and silymarin treatment to D- galactosamine treated mice resulted in decreased SGOT, SGPT, ALP, bilirubin, TNF- α and lipid peroxidation levels along with an increase in antioxidant status. The study suggests that the hepatoprotective activity of piperine is significant at the dose of 25mg/kg as compared to the standard drug silymarin.

Keywords: Antioxidant, Hepatoprotective, Hepatotoxicity, Piperine.

INTRODUCTION

Hepatitis is a common disease and occurs at a high incidence around the world. It can be induced by viruses, alcohol and various drugs and chemicals and may lead jaundice, carcinoma and cell death.¹ As such there is no dependable liver protective agent for the treatment for hepatitis and modern medicines that are used have adverse side effects and are quite expensive. So, there is a need to evaluate the protective ability of some natural compounds to develop a safe and effective treatment for this disease. D-Galactosamine (D-GalN) can be used to assess the hepatoprotective activity^{2,3} of various compounds. Hepatic injury caused by D-GalN results in changes in morphological and biochemical characteristics similar to those as observed in virus induced hepatitis.⁴

Piperine is primarily found in the fruit of the pepper vine, *Piper longum* and *Piper nigrum* (Piperaceae family).⁵ Piperine possesses diverse pharmacological actions such as Immunomodulatory,⁶ antitumour,⁷ antioxidant⁸ and antidepressant⁹ activities. However, to the best of our knowledge, the hepatoprotective effect of piperine against D- galactosamine induced liver injury has not yet been reported.

MATERIALS AND METHODS

Animals

Male Swiss albino mice weighing about 25 -30grams, were purchased from the Karigiri hospital, Vellore, Tamilnadu, India. The animals used in this study were taken care in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals

(CPCSEA), Ministry of culture, Government of India, Chennai.

Drug and chemicals

The commercially available piperine (light yellow powder, >98% purity by HPLC) was purchased from Natural Remedies Ltd., Bangalore, India and stored at -20°C . Silymarin was obtained from Microlabs, Goa, India. A suspension of piperine and silymarin was made with saline. Fresh solution was prepared before each experiment. All other reagents used were standard laboratory reagents of analytical grade and were purchased locally.

Experimental protocol

Animals were divided into 5 groups and each group consisted of six animals. All animals were made to fast 24 hr before the commencement of the study. Group I rats, received saline (0.89 % NaCl, i.p.) and served as control; in group II rats (D-GalN induced test group), toxicity was induced by single dose of galactosamine (700 mg/kg, i.p.), in group III rats or the drug treated group (piperine + galactosamine), piperine (25mg/kg, i.p.) was administered before the single injection of galactosamine (700 mg/kg, i.p.). Group IV rats served as the positive control group (silymarin + galactosamine) and silymarin (25 mg/kg, i.p.) was administrated after the single injection of galactosamine (700 mg/kg, i.p.); group V rats received piperine (25mg/kg, i.p.) alone. Animals were decapitated after 18hrs of galactosamine injection and blood and liver tissue samples were collected and used for further analysis.



Biochemical Parameters

The activities of liver marker enzymes namely, SGOT,¹⁰ SGPT,¹⁰ ALP¹¹ and bilirubin¹² were determined in serum. Assays of antioxidant enzymes i.e., superoxide dismutase(SOD),¹³ catalase(CAT),¹⁴ glutathione peroxidase(GPX),¹⁵ glutathione reductase(GR),¹⁶ glutathione-s-transferase (GST),¹⁷ total reduced glutathione(RG)¹⁸ and lipid peroxidation (LPO)¹⁹ were analyzed in liver tissue homogenate.

Effect of piperine and Silymarin on TNF- α production

TNF- α level in plasma of control and experimental mice was determined by enzyme-linked immunosorbent assay (ELISA, Cayman Chemicals, USA), according to the manufacturer's instructions.

HPLC Analysis

The extract piperine (from Black pepper) was cleaned up by passing through 2 μ m membrane filter in HPLC consisting of a photodiode array detector, binary pump and manual sample injector. Chromatographic separation

was accomplished using C18 10- μ m column. Piperine analysis was performed on reverse-phase column with an isocratic elution at 345nm (Joe, 1996). A quantity of 10 μ l of piperine sample was eluted with a mobile phase containing a mixture of acetonitrile-water (50:50 v/v) at a flow rate of 1.0ml/min.

Histopathological Studies

Immediately after sacrifice, a portion of the liver was fixed in 10% formalin, washed and then dehydrated in descending grades of isopropanol and finally with xylene. The tissue was then embedded in molten paraffin wax. Sections of 5 μ m thicknesses were cut, stained with haematoxylin and eosin and then observed under microscope.

Statistical Analysis

The data obtained was computed to calculate the mean, standard deviation (S.D.) and ANOVA to find out the statistical significance between control and experimental groups.

Table 1: Effect of Piperine on liver antioxidant enzymes specific activities and lipid peroxidation levels

Parameters	Group-I	Group II D-GalN	Group III DGalN+Piperine	Group IV DGalN+Silymarin	Group V (Piperine)
Lipid Peroxidation(LPO) (nmol of MDAformed/mg protein)	1.316 \pm 0.117	2.716 \pm 0.117a*	1.50 \pm 0.141b*	1.70 \pm 0.179c*	1.283 \pm 0.147
Superoxide dismutase(SOD) (Units/mg protein)	235.67 \pm 2.16	118.0 \pm 1.10a*	217.50 \pm 2.43b*	213.83 \pm 3.43c*	224.14 \pm 9.30
Catalase(CAT) (μ mol of H ₂ O ₂ consumed/min/mg protein)	12.35 \pm 0.321	7.166 \pm 0.427a*	9.83 \pm 0.532b*	9.816 \pm 0.445c*	11.00 \pm 0.66
Glutathione peroxidase(GPx) (μ g of GSH utilized/min/mg protein)	75.25 \pm 0.689	33.417 \pm 1.93a*	64.51 \pm 1.96b*	63.51 \pm 1.67c*	73.25 \pm 1.64
Glutathione reductase (GR) (nmol of NADPH oxidized/min/mg protein)	121.27 \pm 1.66	68.33 \pm 0.876a*	110.93 \pm 5.69a*	100.43 \pm 1.10c*	117.27 \pm 3.72
Glutathione-S-transferase(GST) (nmol of 1-chloro-2,4- dinitrobenzene-GSH conjugate formed/min/mg protein)	91.98 \pm 1.12	68.83 \pm 0.931a*	83.18 \pm 1.28b*	81.517 \pm 0.72c*	88.35 \pm 0.61
Total reduced glutathione(nmol/mg/protein)	45.85 \pm 1.43	23.41 \pm 0.86a*	41.05 \pm 0.66b*	38.88 \pm 1.30c*	43.35 \pm 0.98

Each column represents mean \pm SD (n=6). Treatment of groups are as follows: Group I- Control mice; Group II- D-galactosamine (700mg/kg/b.wt); Group III- D-galactosamine+Piperine(25mg/kg/b.wt); Group IV- D-galactosamine + Silymarin (25mg/kg/b.wt); Group V- Piperine(25mg/kg/b.wt). Comparisons were made as follows: a-control vs D-GalN; b-D-GalN vs Piperine+D-GalN; c-Silymarin vs. D-GalN. The symbols represent statistical significance at: * p < 0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.

RESULTS AND DISCUSSION

D-GalN induced liver injury has been found to cause liver damage that closely resembles human viral hepatitis.²⁰ The mice treated with D-Galactosamine alone (D-GalN control group) developed severe hepatocellular injuries which can be seen as significant (p<0.05) elevation in serum SGOT, SGPT (Fig.1) and ALP (Fig.2) activities when it is compared with the normal control group. By contrast, treatment with piperine (25mg/kg) prevented the elevation of serum enzymes suggesting that its

hepatoprotective activity might be due to its effect against cellular leakage and loss of functional integrity of the cell membrane.

The damage of liver induced by D-GalN can be observed by the significant(p<0.05) reduction of liver antioxidant enzymes like SOD, CAT, GPX, GR, GST and RG and a significant increment of LPO in the liver homogenate of D-GalN control group as compared to the normal control group. In the piperine treated groups, the activity of antioxidant enzymes (SOD, CAT, GPx, GR, GST, RG) were

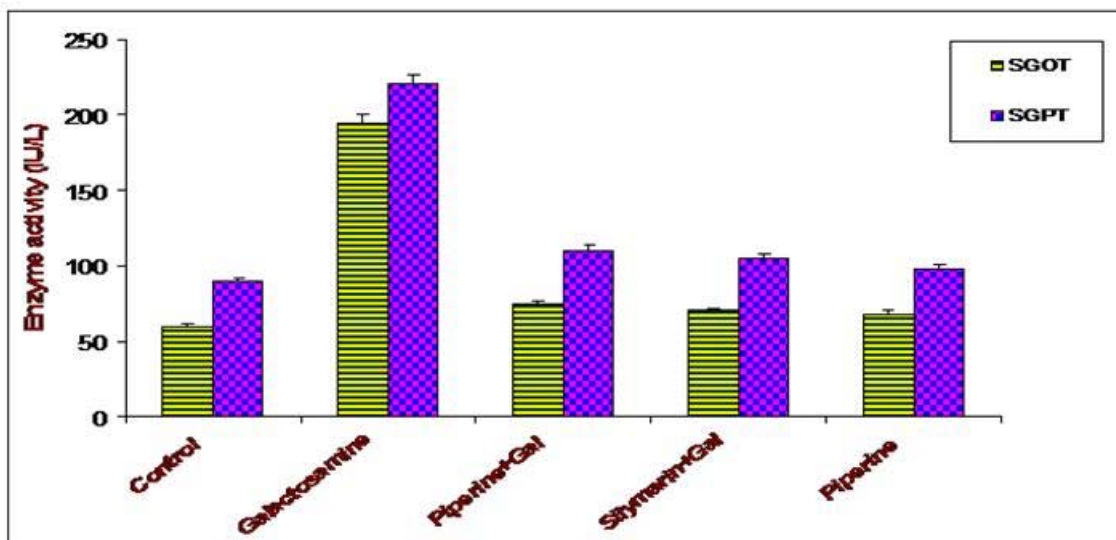
increased ($p < 0.05$) significantly (Table.1) along with reduction in the LPO levels which can be suggested due to its potent free radical scavenging activity and the antioxidant property. Also, it has been reported that piperine treatment decreases lipid peroxidation *in vivo* and beneficially influenced cellular thiol and antioxidant status against chemically induced oxidative stress in various pathological diseases.^{21,22}

Liver injury induced by D-GalN provoked significant increased levels of bilirubin in the D-galactosamine treated group. Whereas, the levels are reduced in the piperine treated group which is near to that of the control group (Figure 3). Silymarin also prevented the liver from elevating levels of serum bilirubin. Any unusual increase

in the levels of bilirubin in serum indicates hepatobiliary disease and severe disturbances of hepatocellular function.²³ Piperine mediated suppression of the increased bilirubin level suggests the possibility of this alkaloid being able to stabilize biliary dysfunction.

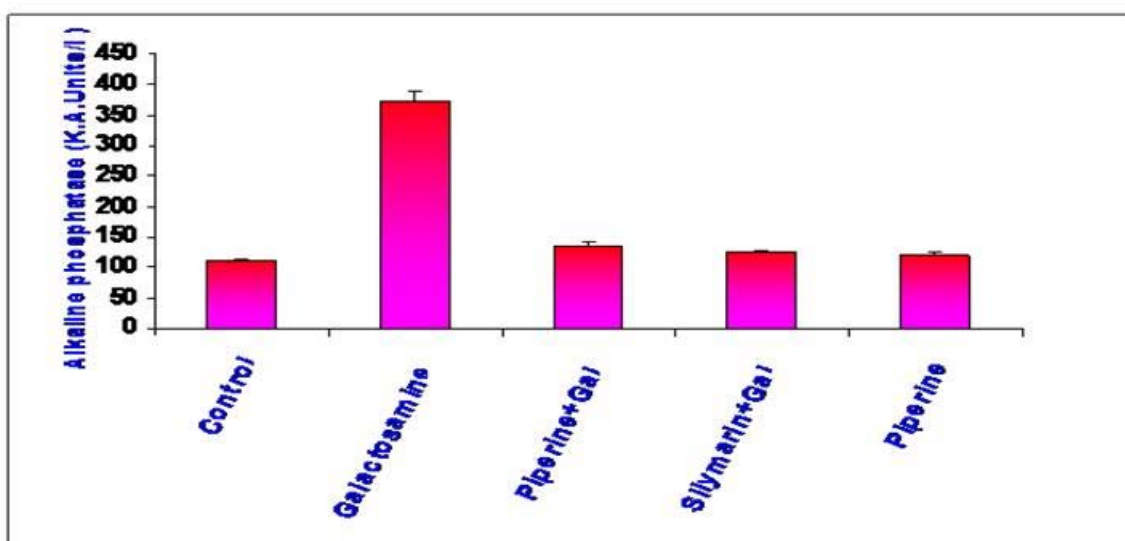
The increased levels of TNF- α in serum samples in the galactosamine induced group have been observed. The results show that the levels of TNF- α were decreased significantly ($p < 0.05$) in the piperine (25mg/kg) treated group (Figure 4).

Also, quantitation of piperine was made from peak area ratio, which was based on a calibration curve generated from standard piperine. HPLC elution profile is given in Figure 5 (a) & 5(b).



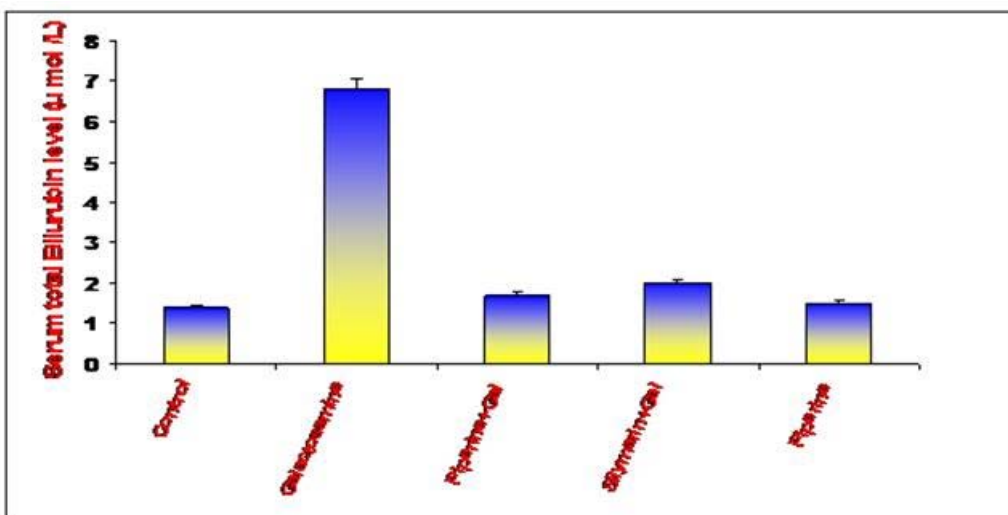
Each column represents mean \pm SD, (n=6). Comparisons were made as follows: Group I vs Group II, III, IV, V. The symbols represent statistical significance at: * $p < 0.05$. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.

Figure 1: Effect of Piperine on the activity of SGOT and SGPT in the serum of control and experimental mice



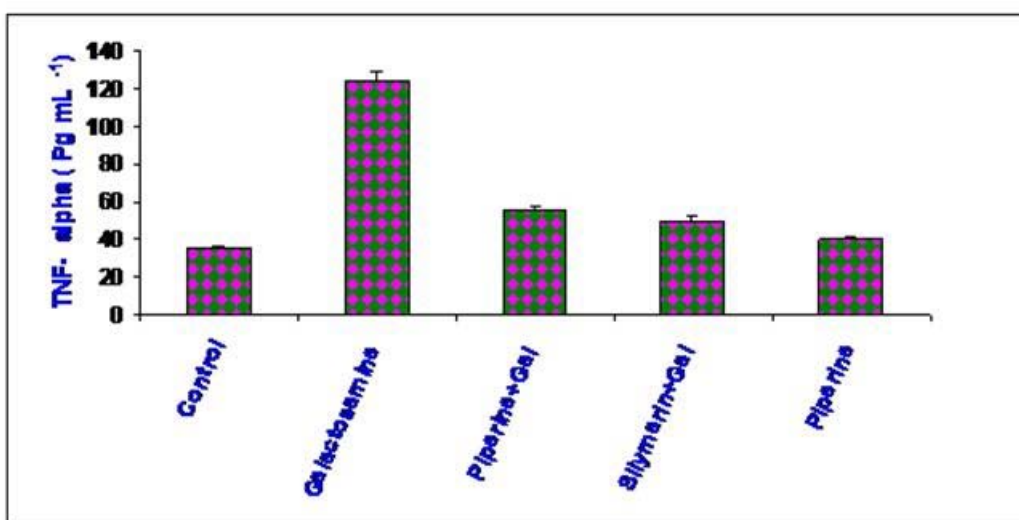
Each column represents mean \pm SD, (n=6). Comparisons were made as follows: Group I vs Group II, III, IV, V. The symbols represent statistical significance at: * $p < 0.05$. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test

Figure 2: Effect of piperine on the activity of alkaline phosphatase in the serum of control and experimental mice



Each column represents mean± SD, (n=6). Comparisons were made as follows: Group I vs Group II, III, IV, V. The symbols represent statistical significance at: * p < 0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test

Figure 3: Effect of piperine on serum bilirubin levels in control and experimental mice



Comparisons were made as follows: Group I vs Group II, III, IV, V. The symbols represent statistical significance at: * p < 0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.

Figure 4: Effect of Piperine on TNF α levels in the serum of control and experimental mice

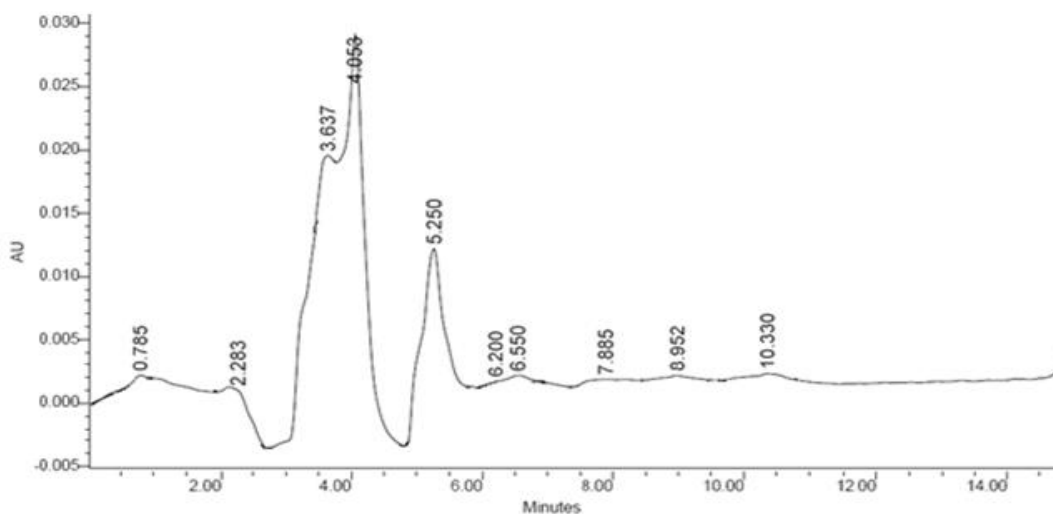


Figure 5(a): Piperine (Black pepper)

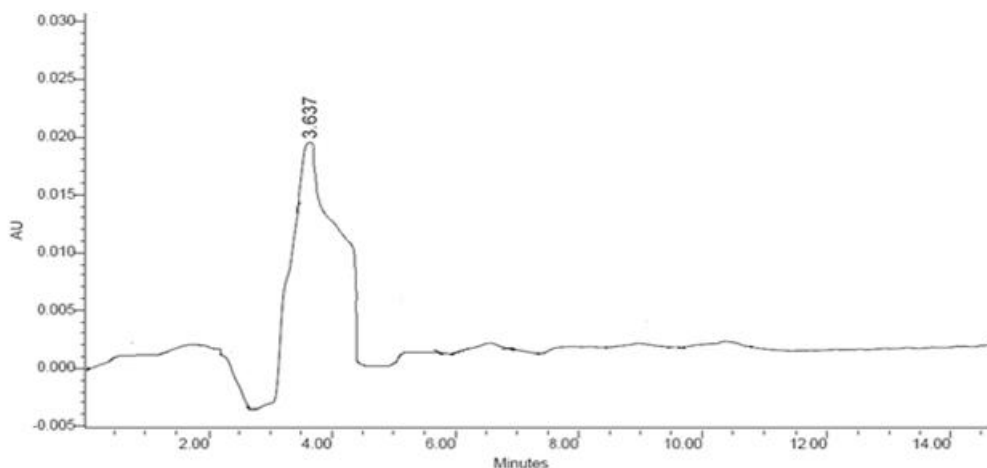
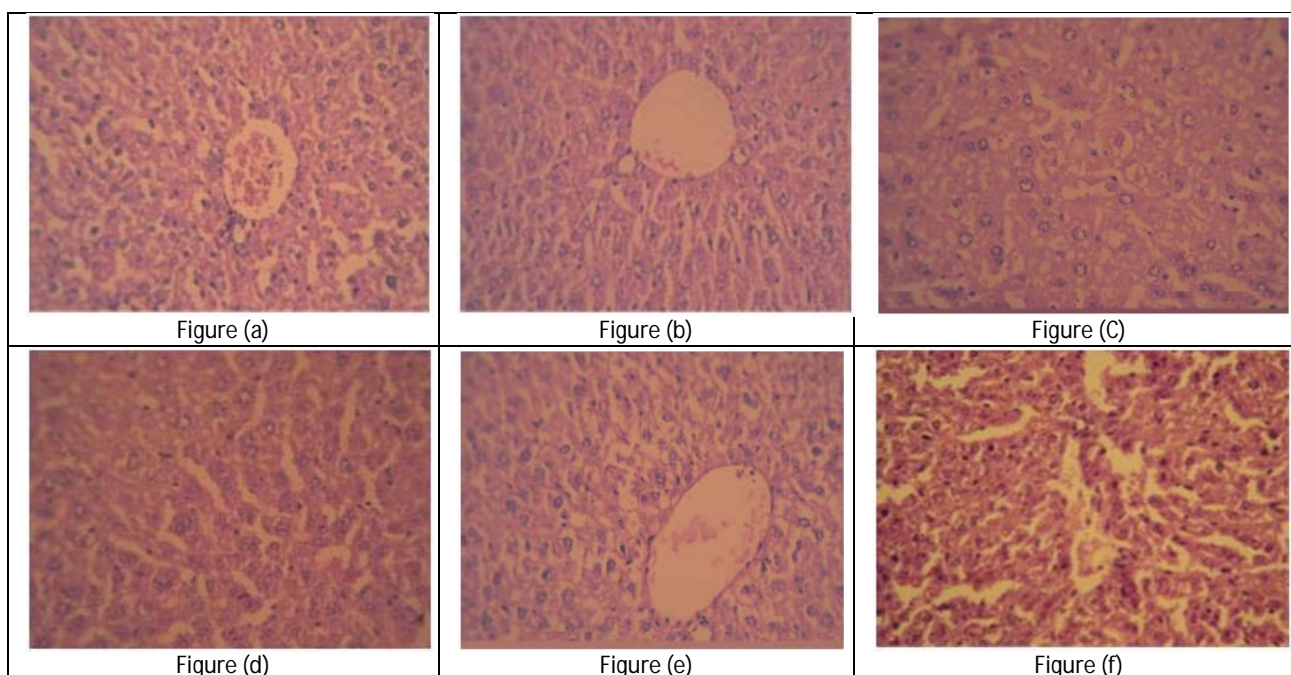


Figure 5(b): Piperine Standard

Figure 5: HPLC Analysis of Piperine



Liver sections of **Fig.6 (a)**. Group I: normal control mice showing normal liver lobular architecture with central vein. **Fig. (b)**. Group II: galactosamine (700mg/kg, i.p.) induced mice. **Fig. (c)**. Acute D-GalN, liver section of galactosamine (700mg/kg, i.p.) induced. **Fig. (d)**. Group III treated with piperine (25mg/kg, i.p.) + galactosamine (700mg/kg, i.p.) **Fig. (e)**. Group IV: treated with silymarin (25 mg/ kg, i.p.) + galactosamine (700mg/kg, i.p.). **Fig. (f)**. Group V: Piperine (25mg/kg, i.p.) treated mice. (H & E staining, original magnification 400x).

Figure 6: Histopathological Observations

Histology of the liver sections of normal control mice (Group I) showed central vein surrounded by hepatocytes with clear granular cytoplasm (Figure 6a). The liver sections of galactosamine treated animals (Group II) showed hepatic cells with toxicity characterized by damage where we observe enlarged cell, vacuolated cytoplasm and large nuclei with condensed chromatin (Figure 6b). The liver sections of acute D-GalN (700mg/kg) shows damage with enlarged cell, vacuolated cytoplasm and large nuclei with condensed chromatin (Figure 6c). Piperine treated group appears to prevent the galactosamine toxicity as revealed by the central vein and hepatocytes with ballooning clear cytoplasm (Figure 6d). Silymarin (Group IV) also exhibited protection from

galactosamine induced changes in the liver (Figure 6e) where reactive hepatocytes (binucleate) and normal degenerative hepatocytes were seen. Piperine (Group V) exhibits protection revealed by the normal hepatocytes and hepatocytes with condensed nuclei (Figure 6f).

CONCLUSION

Piperine is a powerful scavenger of oxygen free radicals. The present study, thus demonstrated the protective role of piperine against the D-galactosamine induced liver damage through its strong antioxidant, anti-inflammatory and hepatoprotective effects.

The hepatoprotective activity of piperine is significant at the dose of 25mg/kg. Piperine inhibits the acute liver toxicity which might be due to its effect on antioxidants and TNF- α . Further studies are necessary for better understanding of the mechanism of action and evaluating the efficacy of this compound on liver that are possibly damaged during intoxication.

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