

Protective Effects of p-CA Against Acute Liver Damage Induced by LPS/D-GalN in Wistar Albino Rats

Seerat Mehdi¹, Fiaz-ud-Din Ahmad¹, Arslan Hussain Lodhi¹, Umair Khurshid², Ahmed Awais Khalid¹, Sheikh Safeena Sidiq¹, Liaqat Hussain³, Mirza Shaharyar Baig¹

¹Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan; ²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan; ³Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University Faisalabad, Faisalabad, Pakistan

Correspondence: Fiaz-ud-Din Ahmad, Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur, Khawaja Fareed Campus, Railway Road, Bahawalpur, 63100, Pakistan, Tel +92-320-8402376, Email fiaz.ahmad@iub.edu.pk

Aim: Liver regulates metabolism of biomolecules and injury of liver causes distortion of metabolic functions. This injury may be oxidative or inflammatory induced by numerous factors including alcohol, pathogens and xenobiotics. This scientific study was planned to investigate the anti-inflammatory and anti-oxidant potential of p-coumaric acid (p-CA) on Lipopolysaccharide/D-Galactosamine (LPS/D-GalN) induced liver injury.

Methods: DPPH analysis, reducing power assay and HPLC analysis were performed during in-vitro studies of p-CA. Similarly, in-vivo experiments were performed using Wistar Albino rats. Normal control and intoxicated group received (5mL/kg normal saline p.o), standard treatment groups received ascorbic acid (100mg/kg p.o) and silymarin (25mg/kg p.o), while p-CA treatment groups received (100mg/kg p.o) for 28-days. After completion of 28-days, LPS/D-GalN injection (300 mg D-GalN/kg and 10 µg LPS/kg i.p.) was given at 6th, 12th and 24-hours to all groups except normal control group. Animals were sacrificed; serum and liver samples were harvested and subjected to biochemical and histological examinations, respectively.

Results: The results revealed that p-CA possess strong antioxidant activity. Increased levels of leukocyte infiltration (TLC), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), lipid panel (eg TG, TC, LDL-C, VLDL-C), whereas decreased HDL-C levels noticed in LPS/D-GalN groups as compared to normal control groups. Pro-inflammatory markers (eg TNF- α , IL-6, IL-1 β) and lipid peroxidation marker, eg malondialdehyde (MDA) increased while superoxide dismutase (SOD) and reduced glutathione (GSH) levels were decreased significantly in groups treated with LPS/D-GalN. ANOVA with Bonferroni post hoc analysis was used for statistical analysis of. H&E staining was done to assess architectural abnormalities among liver cells.

Conclusion: In conclusion, p-CA could ameliorate LPS/D-GalN induced hepatic injury via regulation of immune responses, liver function enzymes, lipid profile, oxidative stress and pro-inflammatory markers.

Keywords: p-coumaric acid, lipopolysaccharide, D-galactosamine, oxidative stress, inflammation

Introduction

The liver is the largest parenchymal organ in the body which performs essential metabolic regulation and biotransformation of most of the chemicals, drugs and toxins. It is a critical hub for macronutrient metabolism, immune system homeostasis, blood volume modulation, endocrine system control, cholesterol homeostasis, and the breakdown of xenobiotics, including many current drugs.¹ However, liver is more susceptible to many drugs/toxins which lead towards precipitation of liver injury. Liver injury is frequently accompanied by endotoxins which causes severe damage to the hepatocytes. Its pathogenesis directly involves oxidative damage and immune mediated inflammatory reactions.² A surfeit of high-fat diet,³ high-fructose diet,⁴ high-cholesterol diet,⁵ alcohol consumption,⁶ radiation,⁷ and some

chemicals or drugs including Concanavalin A,⁸ Thioacetamide,⁹ CCl₄,¹⁰ acetaminophen¹¹ and Lipopolysaccharide/D-Galactosamine can cause liver damage.¹² Liver transplantation is a well-known cost effective option available throughout the world, followed by a limitation due to the shortage of donors. Therefore, it is essential to find an optimized and effective drug to treat liver failure. Lipopolysaccharide (LPS) consist of hydrophobic domain known as lipid A (endotoxin), oligosaccharide (non-repeating core), and distal polysaccharide (O-antigen)¹³ It is major structural toxic component of the cell membranes of Gram-negative bacterium. It plays an principle role in endotoxic injury and destroy healthy tissues, eventually causing cancer or atherosclerosis.¹⁴ According to previous reports LPS/D-GalN induced liver injury result from the overproduction of cytokines.¹⁴ In resident macrophages of liver, LPS triggers the biosynthesis of diverse mediators of inflammation, such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β),^{15,16} and activates the production of co-stimulatory molecules required for the adaptive immune response.¹⁷ In mononuclear and endothelial cells, LPS also stimulates tissue factor production.^{18,19} Tissue factors are needed for clearing local infections and inflammations, and they also act in synergy. When overproduced in systemic circulation leads to the onset of severe sepsis. However, various inflammatory mediators and clotting factors can destroy small blood vessels and precipitate gram-negative bacterial septic shock, accompanied by disseminated intra-vascular coagulation and finally multiple organ failure.^{20,21} D-Galactosamine (D-GalN) induces liver damage by interfering with the synthesis of RNA, protein and DNA. It is metabolized via the Leloir pathway, which leads to the production of uridine derivatives of d-galactosamine. The two enzymes in Leloir pathway, galactokinase and UDP-galactose uridyl transferase have low specificity for substrate so these enzymes convert galactosamine into galactosamine-1-phosphate and UDP-galactosamine, respectively. UDP-galactosamine blocks UDP-galactose-4' epimerase enzyme which results in the accumulation of UDP-galactosamine in the liver cells. These reactions deplete uridine triphosphate (UTP), uridine diphosphate (UDP), uridine monophosphate (UMP), and sugar derivatives of uridine such as UDP-Galactose and UDP-glucose which are essential for the synthesis of RNA and proteins.²² d-Galactosamine is a potent toxin which sensitize animals to the lethal effects of LPS in the acute liver injury model and basically it is a fundamental way to amplify the endotoxin induced TNF- α and other pro-inflammatory interleukins.²³ p-Coumaric acid (p-CA) is a polyphenolic compound which acts as secondary metabolite in plants and present in mushrooms, cereals (rice, corn, oats and wheat), fruits (apples, olives and grapes) and vegetables (carrots, beans, potatoes, tomatoes and onions).²⁴ It has been proved to have anti-oxidative,^{25,26} antimicrobial, anti-inflammatory and immunomodulatory,^{27,28} antiviral,²⁹ fungicidal,³⁰ anti-mutagenic,³¹ neuroprotective,³² anticancer,³³ antidiabetic and anti-hypercholesterolemic properties.³⁴ In addition, recent studies have reported its protective effects against oxidative stress and lipid peroxidation.³⁵ p-CA is a vital molecule showing great hepatoprotection against various toxic chemicals, eg acetaminophen,³⁶ ethanol,³⁷ and fipronil.³⁸ For all that, p-CA has been suggested to have potential anti-inflammatory and antioxidant effects in LPS/D-GalN induced liver injury model. Thus, the current scientific research was come up with the main objectives to determine (1) in-vitro antioxidant activity of p-CA using DPPH chemical; (2) scrutinize the protective effects of p-CA against LPS/D-GalN induced inflammatory and oxidative stress-induced liver injury.

Materials and Methods

In vitro Assay

DPPH Free Radical Neutralizing Assay

Free radical scavenging activity of p-CA was measured by using a stable free radical DPPH (1,1-diphenyl-2-picrylhydrazyl). 50 mL of DPPH (0.2 mg/mL) was prepared in ethanol. 2 mL of DPPH solution was added into 1mL of p-CA (0.5 mg/mL) dissolved in methanol at different concentrations. The mixture was then shaken and allowed to stand for 30 minutes at room temperature. Absorbance was monitored at 517-nm using spectrophotometer. The standard compound was ascorbic acid and experiment was done in a triplicate. Low absorbance value of the reaction mixture indicated higher free radical scavenging activity of the sample. The percent DPPH scavenging effect of standard ascorbic acid and p-CA was calculated separately by using the following formula.³⁹ All the above-mentioned protocol was performed in thrice and results were calculated by the following formula:

$$\text{Inhibition \%} = \left(\frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Control}}} \right) \times 100$$

$$A_{\text{control}} = \text{Absorbance of Control} \quad A_{\text{sample}} = \text{Absorbance of Sample}$$

Reducing Power

Reducing power of p-CA was measured by direct reduction of Fe³⁺ to Fe²⁺ which imparts change in colour of the reaction mixture due to formation of Perl's Prussian Blue complex. Absorbance of reaction mixture is directly proportional to the reduction potential of compounds. Ferric reducing antioxidant power (FRAP) method with slight modification⁴⁰ was used to measure the reduction potential of p-CA. Different concentrations of p-CA (5–30 µg/mL) in 0.75 mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 PBS and 1.25 mL of 1% potassium ferricyanide [K₃Fe(CN)₆]. The reaction mixture was incubated at 50 °C for 20 min. After 20-minutes, the reaction mixture was acidified with 1.25 mL of 10% trichloroacetic acid. Finally, 0.5 mL of FeCl₃ (0.1%) was added to this solution. Absorbance was measured at wavelength of 700 nm in a spectrophotometer. Increased absorbance of reaction mixtures indicates greater reduction capability.⁴¹

High Performance Liquid Chromatography

Samples were kept at 4 °C before analysis. Stock solutions of standard and test compounds were prepared with ethanol in the range of 0.1–400 µg/mL. 20 µL of sample was injected into the column. The HPLC analysis was performed using column Shim-pack CLC-ODS (C18) 25 cm × 4.6 mm, 5 µm with 100 mm of column length (Shimadzu Scientific Instruments, Kyoto, Japan) and detector model (SPD-10AV, UV-Visible). The flow rate was 1 mL/min and detection was performed at 280 nm. 20 µL of sample was injected into shim-pack C18 column using the gradient elution. Two solvents used were 1% (v/v) acetic acid in water; pH = 2.27 (solvent A) and 100% ACN (solvent B). The gradient elution started with following conditions: 15% B for 0–15 minutes, 45% B for 15–30 min and 100% B for 30–45 min (end analysis). Identification and quantitative analysis were conducted in comparison with reference standard compounds.⁴²

In vivo Assay

Animals and Ethical Decision

Wistar Albino rats (150 ± 10 g; n: 6 per group) were used in this experimentation. All animals were placed in room with the temperature (22 ± 1 °C), humidity (50% ± 5%), 12h day/night cycle, free access of diet and tap water ad libitum in their respective cages. The protocol of this experimental study was approved by the Pharmacy Animal Ethics Committee (PAEC/22/75), Department of Pharmacology, Faculty of Pharmacy, The Islamia University of Bahawalpur. All the procedures of this study were done in accordance with Care and Use of Laboratory Animals Guide.

Drugs and Experimental Models

p-CA and LPS (Cat. No. L2630) were obtained from Sigma-Aldrich, USA. D-Galactosamine was obtained from Acros organics. Animals were randomly divided into 15 groups comprises of 6 animals each; Group 1, 2, and 3 consist of control animals received NS vehicle (5mL/kg b.w.; i.p), for consecutive 28 days and sacrificed at 6h, 12h and 24h respectively. Group 4, 5 and 6 consist of intoxicated groups received normal saline (NS) vehicle (5mL/kg b.w.; i.p), group 7, 8 and 9 consist of standard treatment groups received ascorbic acid (100 mg/kg) in NS vehicle (5mL/kg b.w.; i. p), group 10, 11 and 12 consist of standard treatment groups received silymarin (25 mg/kg) in NS vehicle (5mL/kg b.w.; i.p.), group 13, 14 and 15 consist of treatment groups received p-CA (100mg/kg) in NS vehicle (5mL/kg b.w.; i.p) for consecutive 28 days³² followed by the subjected treatment with the total volume of combination of LPS (10µg/kg) and D-GalN (300mg/kg) dissolved in NS and sacrificed at 6 h, 12h and 24h respectively. All animals were kept fasted with water ad libitum for 12 hours prior to experimental procedures. Animals were given anesthesia with a mixture of ketamine and xylazine (10:1) i.p. surgical procedure was performed and blood samples were collected by procedure of cardiac puncture and sera was separated by centrifugation for 15 minutes at 3000-rpm. The liver tissues were harvested immediately, preserved in 10% formalin and subjected to homogenization⁴³ for antioxidant enzymes tests and histopathological analysis.

Biochemical Investigation and Protein Determination

Freshly collected blood samples were diluted with Turk's fluid in WBC pipette, in which red cells get lysed without affecting the leukocyte. Leukocyte count was done using a Neubauer's chamber.⁴⁴ Markers of hepatic damage; serum alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin (TB) levels and lipid panel (TG, TC, HDL, LDL and VLDL) were measured in spectrophotometer by using commercial kits following the supplier's protocol.⁴⁵ Enzyme-linked immunosorbent assay (ELISA) kits (BT Lab, England) were used to quantify the levels of inflammatory markers; TNF- α , IL-6 and IL-1 β , and oxidative markers; MDA, SOD and GSH in the supernatants. The absorbance was read at 450 nm wavelength by a microplate reader (Bio-Rad Laboratories). A standard curve was constructed using serial dilutions of standards provided within the kits and sample concentrations were calculated by interpolation method.^{46,47}

Histopathology of Liver Tissues

After dissection, livers were excised and preserved in 10% formalin solution. Microtome sections (5 μ m thick) of the samples were taken and stained with hematoxylin and eosin. The images of the stained slides were viewed on an optical microscope at 10X resolution power.⁴⁸

Statistical Evaluation

The results obtained were calculated at GraphPad Prism and MS excel and interpreted as mean \pm SEM. All the values obtained from different groups were statistically analyzed by one-way ANOVA followed by Bonferroni's post hoc test. $p < 0.05$ was considered significantly different.⁴⁶

Results

In vitro Assay

DPPH Free Radical Neutralizing Assay

The DPPH free radical quenching capacity of ascorbic acid was 94.44% with an IC₅₀ value of 80.33 μ g/mL while that of p-CA was 74.31% with an IC₅₀ value of 392.94 μ g/mL as shown in Figure 1.

Reducing Power

The reduction potential of a compound is a significant indicator of its antioxidant potential activity.^{40,41} p-CA displayed powerful reducing ability at different dilutions (5–40 μ g/mL) as compared to standard compounds. Reduction potential of p-CA and standard compounds presented the following sequence: α -tocopherol > BHT > p-CA as shown in Figure 2. The results illustrate that p-CA can neutralize free radicals by its reducing power. In this way, it is capable of terminating

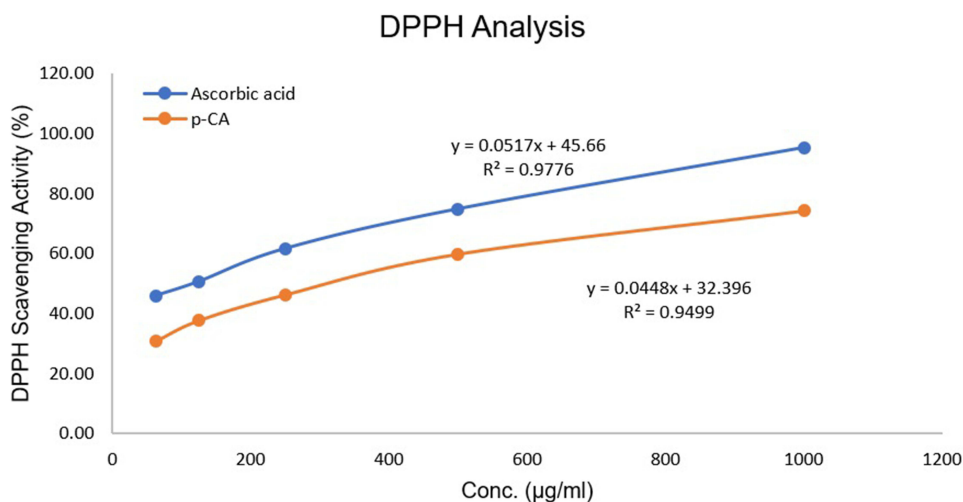


Figure 1 The graph representing free radical scavenging capacity of Ascorbic acid and p-CA in DPPH assay. The results were expressed as Mean \pm SEM of triplicate and expressed as percent inhibition.

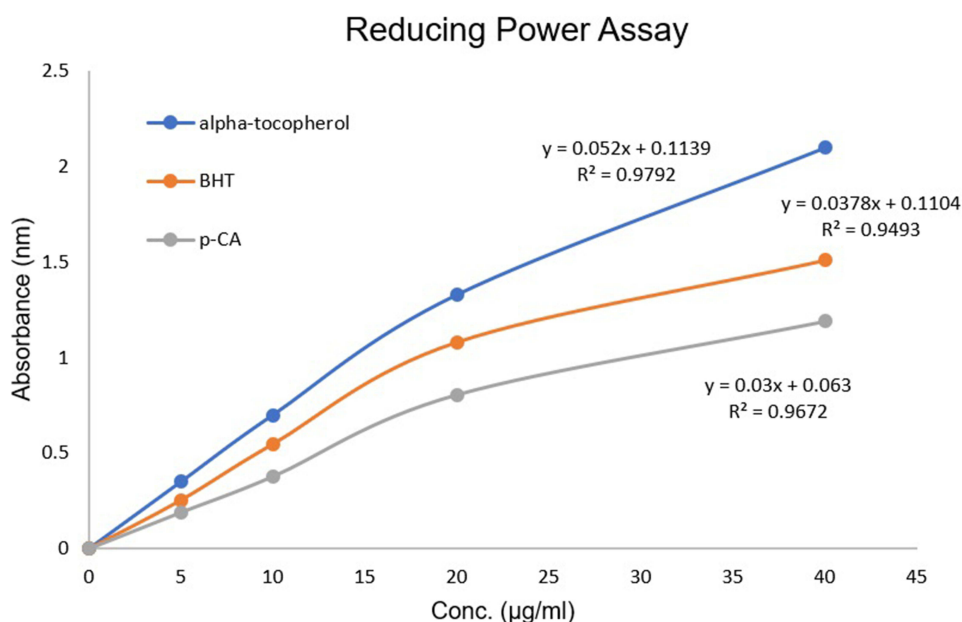


Figure 2 The graph representing $Fe^{3+} \rightarrow Fe^{2+}$ reducing power of different concentrations (5–40 µg/mL) of p-CA and reference anti-oxidants α -tocopherol and BHT using spectrophotometric detection. Control samples contain all the reagents except test compound. The data represented as absorbance of sample and standard compounds. **Abbreviation:** BHT, butylated hydroxytoluene.

radical chain reactions that may be very damaging in the living system. The electron donating properties of p-CA for quenching free radicals by forming stable products.

High Performance Liquid Chromatography

The peak of p-CA was detected at retention time of 16.500 minutes, while the retention time of gallic acid 4.533 min and of quercetin is 2.973 min as shown in Figure 3. The retention time (min) and peak area of standards and sample are tabulated in Table 1. The longer retention time may be due to higher affinity of the compounds towards the stationary phase and less affinity for the mobile phase. Xu and Howard⁴⁹ supported that p-CA is a less polar compound in comparison to other standard phenolic acids such as gallic acid, and quercetin thus leading to increased elution time for the compound.

In vivo Assay

Evaluation of Immune Response

Leukocyte infiltration was the prominent phenomena during inflammatory reactions results from the effects of LPS and D-Galactosamine.⁵⁰ The effects of standard drugs and p-CA treatment on serum TLC levels are shown in Figure 4. TLC levels in groups pretreated with p-CA decreased significantly ($p < 0.05$) in serum at 6h, 12h and 24h after LPS/D-GalN coinjection as compared to groups receiving only LPS/D-GalN treatment.

Evaluation of Serum Liver Enzymes

Aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin (TB) levels among serum are important markers of liver cell damage.⁴⁵ As shown in Tables 2 and 3, these factors increased significantly ($p < 0.05$) in rats administered with LPS/D-GalN. Pre-treatment with p-CA significantly reduced ($p < 0.05$) liver enzymes in serum in comparison to the LPS/D-GalN treated groups.

Evaluation of Serum Lipid Profile

The liver plays central role in the lipids and lipoproteins metabolism and homeostasis. Due to hepatocellular damage, cholesterol biosynthesis and elimination with bile are both disturbed and serum levels fluctuate from normal values. Serum levels of total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL-C), very low-density

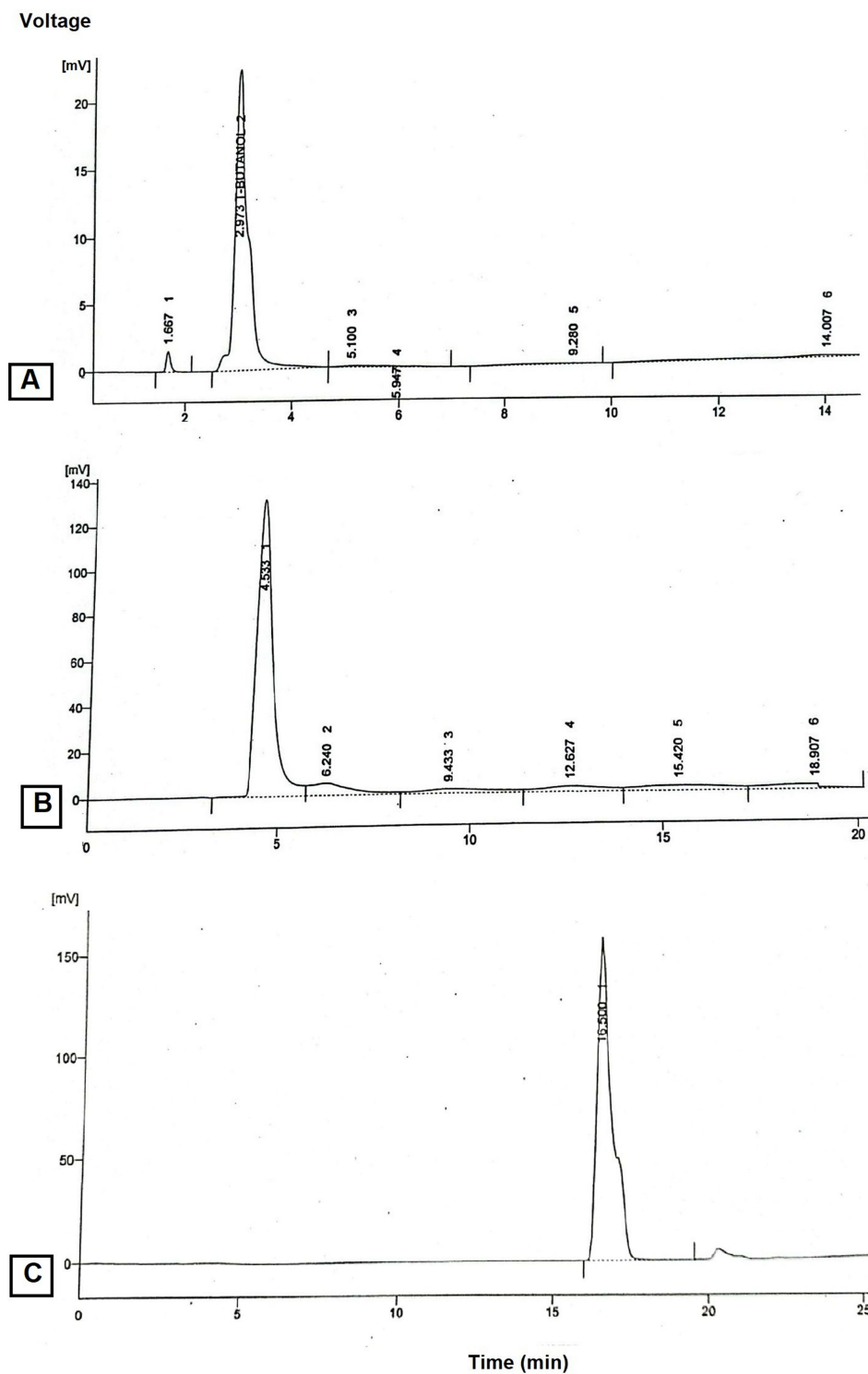


Figure 3 HPLC chromatogram of (A) Quercetin, (B) Gallic acid and (C) p-coumaric acid are shown, using C18 Shim-pack CLC-OD analytical column (25 × 4.6 mm i.d.), with particle size of 5 μm and thermostatic at 30 °C. The flow rate was 1 mL/minute and absorbance was detected at 280 nm.

lipoproteins (VLDL-C) were elevated significantly ($p < 0.05$) as well as high density lipoproteins (HDL-C) levels were reduced significantly ($p < 0.05$) observed in rats of the LPS/D-GalN groups as shown in Figure 5A-. Pre-treatment with standard drugs and p-CA significantly reduced TG, TC, LDL and VLDL levels as well as significantly raised HDL levels in serum when compared with LPS/D-GalN treated groups ($p < 0.05$).

Table 1 Retention Time (Min), Height [mV] and Peak Area [mV.s] of Sample and Standards

Compound	Retention Time (Min)	Height [mV]	Peak Area [mV.s]
Quercetin	2.973	24.564	376.434
Gallic acid	4.533	122.981	3778.773
p-Coumaric acid	16.500	160.239	5173.158

Evaluation of Inflammatory Markers

LPS trigger the release of pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β which potentially deteriorate hepatic cells and lead towards cellular necrosis.⁴⁶ The LPS/D-GalN administration significantly increased levels of TNF- α , IL-6 and IL-1 β in supernatants as compared to the control groups ($p < 0.05$). Pre-treatment with standard drugs and p-CA significantly decreased the levels of TNF- α , IL-6 and IL-1 β in supernatants as compared to the LPS/D-GalN treated group ($p < 0.05$) as shown in Figure 6A–).

Evaluation of the Antioxidant Enzymes

LPS/D-GalN challenge elevated oxidative stress due to un-controlled and un-balanced redox reactions and enervating antioxidant enzyme system in liver tissue (eg super-oxide dismutase and glutathione) and can lead to extensive liver damage. MDA is produced at the end of lipid peroxidation reactions. So, it is important biomarker of lipid oxidation.⁴⁷ Therefore, we investigate the protective effects of p-CA on the levels of oxidative stress markers. Accordingly, rats that received LPS/D-GalN had significantly higher concentrations of MDA ($P < 0.05$), while significantly reduced levels of SOD and GSH ($p < 0.05$) in comparison to the normal control group as shown in Figure 7A–. p-CA pre-treatment significantly reduced the level of MDA ($P < 0.05$), while improved the levels of SOD and GSH ($p < 0.05$) in liver tissues when compared to the LPS/D-GalN group.

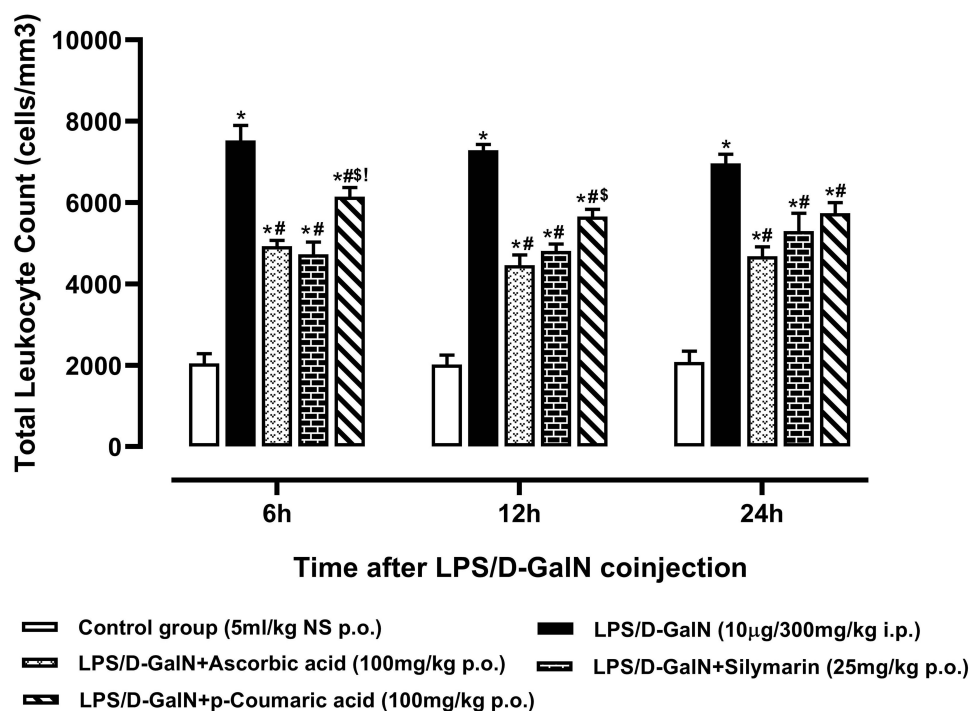


Figure 4 Total Leukocyte Count of normal control, LPS/D-GalN and LPS/D-GalN + treatment groups. The values are mean \pm SEM ($n=6$). Statistical analysis was done one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for all groups in respective hours. The results are considered significant *If $p < 0.05$. *Indicates $p < 0.05$ vs normal control, #Indicates $p < 0.05$ vs LPS/D-GalN, \$Indicates $p < 0.05$ vs LPS/D-GalN + Ascorbic acid, !Indicates $p < 0.05$ vs LPS/D-GalN + Silymarin (25mg/kg).

Table 2 Effect of Treatment on Alanine Transaminase (ALT) and Aspartate Aminotransferase (AST) of LPS/D-GalN-Induced Liver Injury among Wistar Albino Rats

Groups	ALT (U/L)			AST (U/L)		
	6 Hours	12 Hours	24 Hours	6 Hours	12 Hours	24 Hours
Control	35.43±3.45	31.84±4.31	30.18±6.00	57.14±3.52	57.65±3.56	58.27±3.63
LPS/D-GalN	163.15±3.40*	178.03±3.28*	161.41±3.44*	232.31±5.05*	253.56±2.99*	251.32±3.32*
LPS/D-GalN+AA	123.99±4.27* [#]	126.52±3.61* [#]	130.13±5.32* [#]	131.64±6.01* [#]	130.92±5.49* [#]	131.50±5.53* [#]
LPS/D-GalN+Sily	126.80±3.36* [#]	125.62±5.09* [#]	135.23±5.02* [#]	134.44±6.19* [#]	131.40±5.89* [#]	132.40±5.78* [#]
LPS/D-GalN+p-CA 100mg/kg	133.79±5.21* [#]	132.11±5.25* [#]	130.59±3.76* [#]	168.48±3.71* [#] ! [§]	158.41±4.01* [#] ! [§]	159.53±3.74* [#] ! [§]

Notes: n=6 Wistar-Albino rats per group, each value expressed as mean±SEM. One-way ANOVA was applied for statistical analysis followed by Bonferroni post hoc test for all groups in respective days. *p < 0.05 versus normal control; [#]p < 0.05 versus LPS/D-GalN; [§]p < 0.05 versus LPS/D-GalN+AA; [!]p < 0.05 versus LPS/D-GalN+Silymarin (25mg/kg).

Abbreviations: LPS, Lipopolysaccharides; D-GalN, D-Galactosamine; AA, Ascorbic acid; p-CA, p-Coumaric acid; Sily, Silymarin.

Table 3 Effect of Treatment on Alkaline Phosphatase (ALP) and Total Bilirubin (TB) of LPS/D-GalN-Induced Liver Injury among Wistar Albino Rats

Groups	ALP (U/L)			Total Bilirubin (mg/dl)		
	6 Hours	12 Hours	24 Hours	6 Hours	12 Hours	24 Hours
Control	64.65±1.85	66.86±2.42	66.08±1.59	0.44±0.03	0.46±0.05	0.52±0.09
LPS/D-GalN	144.23±3.93*	167.31±2.26*	159.64±3.08*	1.70±0.07*	1.79±0.08*	1.68±0.07*
LPS/D-GalN+AA	101.19±2.81* [#]	98.94±3.52* [#]	99.79±3.99* [#]	0.91±0.03* [#]	0.85±0.02* [#]	0.77±0.03* [#]
LPS/D-GalN+Sily	103.16±3.37* [#]	106.49±3.63* [#]	113.48±4.90* [#]	0.93±0.04* [#]	0.92±0.05* [#]	1.05±0.08* [#] !
LPS/D-GalN+p-CA 100mg/kg	126.75±4.33* [#] ! [§]	128.89±2.74* [#] ! [§]	127.43±3.58* [#] ! [§]	1.17±0.02* [#] ! [§]	1.19±0.02* [#] ! [§]	1.14±0.04* [#] ! [§]

Notes: n=6 Wistar-Albino rats per group, each value expressed as mean±SEM. One-way ANOVA was applied for statistical analysis followed by Bonferroni post hoc test for all groups in respective days. *p < 0.05 versus normal control; [#]p < 0.05 versus LPS/D-GalN; [§]p < 0.05 versus LPS/D-GalN+AA; [!]p < 0.05 versus LPS/D-GalN+Silymarin (25mg/kg).

Abbreviations: LPS, Lipopolysaccharides; D-GalN, D-Galactosamine; AA, Ascorbic acid; p-CA, p-Coumaric acid; Sily, Silymarin.

Histopathology

In this study, we evaluated the effects of p-CA on liver injury. The LPS/D-GalN administration led to liver tissue damage of the liver tissue. Treatment with p-CA greatly reduced liver injury which was accessed by Haematoxylin and Eosin staining. The normal control groups (a, b, c at 6h, 12h and 24h) showed the normal structure of liver cells. On the other hand, sections from the LPS/D-GalN treated groups (d, e, f at 6h, 12h and 24h) showed chronic inflammatory cells with immense neutrophils infiltration, derangement of hepatocyte architecture, blood congestion in central vein and hemorrhagic patches. (g-o) All the treatment groups showed significant protection against LPS/D-GalN induced liver damage as shown in Figure 8.

Discussion

There are numerous models for exploration of the role of inflammation and oxidative stress in liver injury caused by xenobiotics. LPS/D-GalN induced liver injury is a well-known experimental model that has been widely used for the induction of liver injury to elucidate pathogenesis.⁵¹ It has been validated that LPS activates resident Kupffer cells, stimulates inflammatory cascade followed by production of pro-oxidative markers. Moreover, D-GalN, HPC selective inhibitor and hepatotoxic agent, sensitizes to LPS and augments its toxic effects. It can consume uridine monophosphate in the liver, inhibiting the nucleic acid, glycoprotein and cholesterol synthesis, damaging the hepatocytes and causing liver injury within a few hours. It is remarkable that D-GalN can intensify the toxic effects of LPS.⁵² In present study, LPS/D-GalN injection was

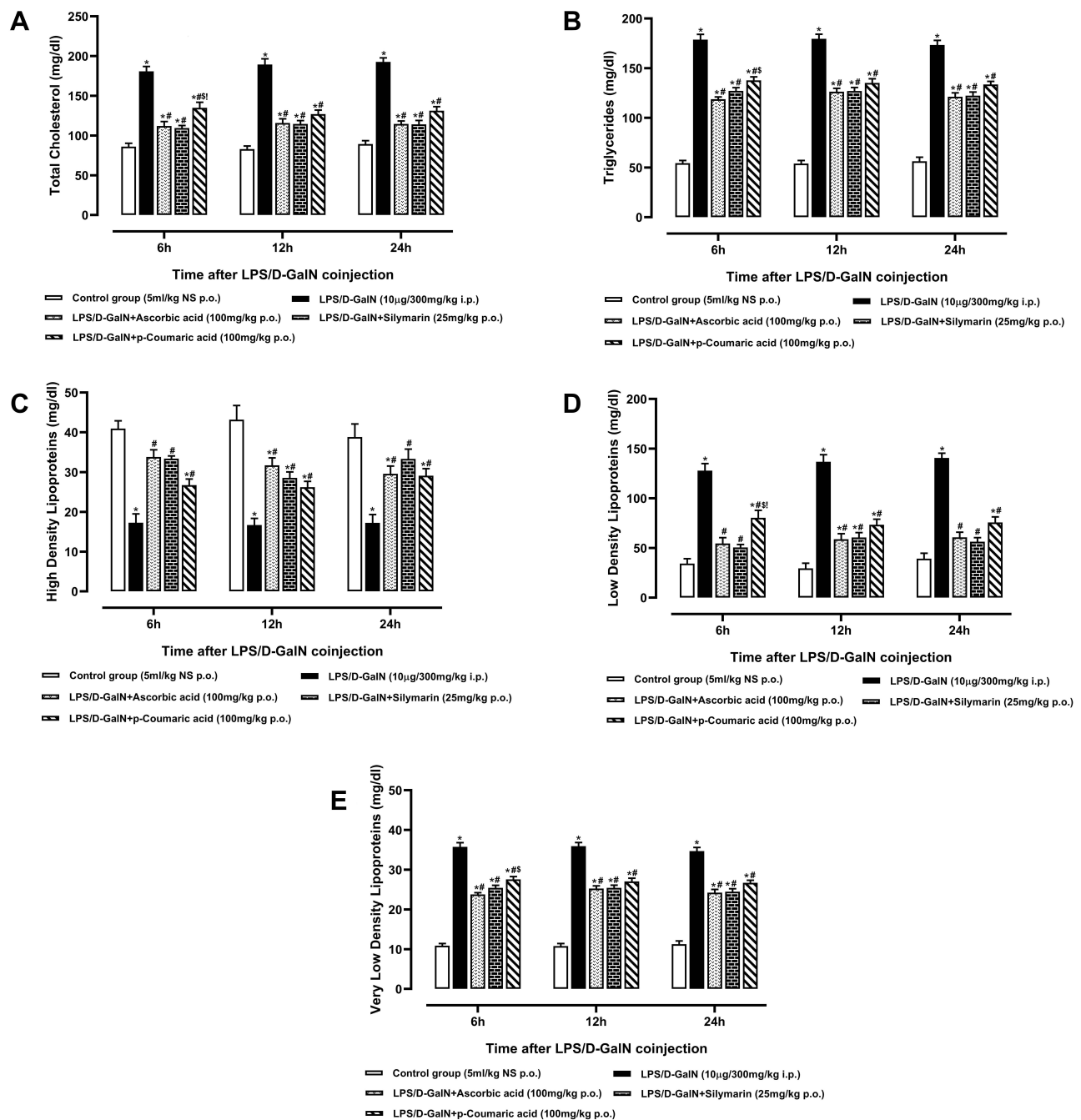


Figure 5 Serum Levels of (A) Total Cholesterol, (B) Triglycerides, (C) High Density Lipoproteins, (D) Low Density Lipoproteins and (E) Very Low-Density Lipoproteins of normal control, LPS/D-GalN and LPS/D-GalN + treatment groups. The values are mean \pm SEM (n=6). Statistical analysis was done one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for all groups in respective hours. The results are considered significant (*) if $p < 0.05$. * indicates $p < 0.05$ vs normal control, # indicates $p < 0.05$ vs LPS/D-GalN, \$ indicates $p < 0.05$ vs LPS/D-GalN + Ascorbic acid, ¹Indicates $p < 0.05$ vs LPS/D-GalN + Silymarin (25mg/kg).

used to induce liver injury in male albino rats. LPS/D-GalN-induced liver injury is a well-known experimental model which presented substantial liver injury with profound changes in biochemical and histopathological and parameters.^{14,53} Following the challenge of LPS/D-GalN, levels of liver function enzymes: ALT, AST and ALP, which are indicators of hepatic damage, increased in the serum.^{45,54} The levels of bilirubin were also increased in response to LPS/D-GalN treatment.^{52,55} Bilirubin plays an important role as a physiologic antioxidant by scavenging peroxy radicals and preventing oxidation of fatty acids and proteins. Its activity is augmented in oxidative stress as an adaptive mechanism.^{56,57} Our results in the present study revealed that p-CA significantly ameliorated the increased levels of liver function enzymes. LPS activates NF- κ B by binding to the

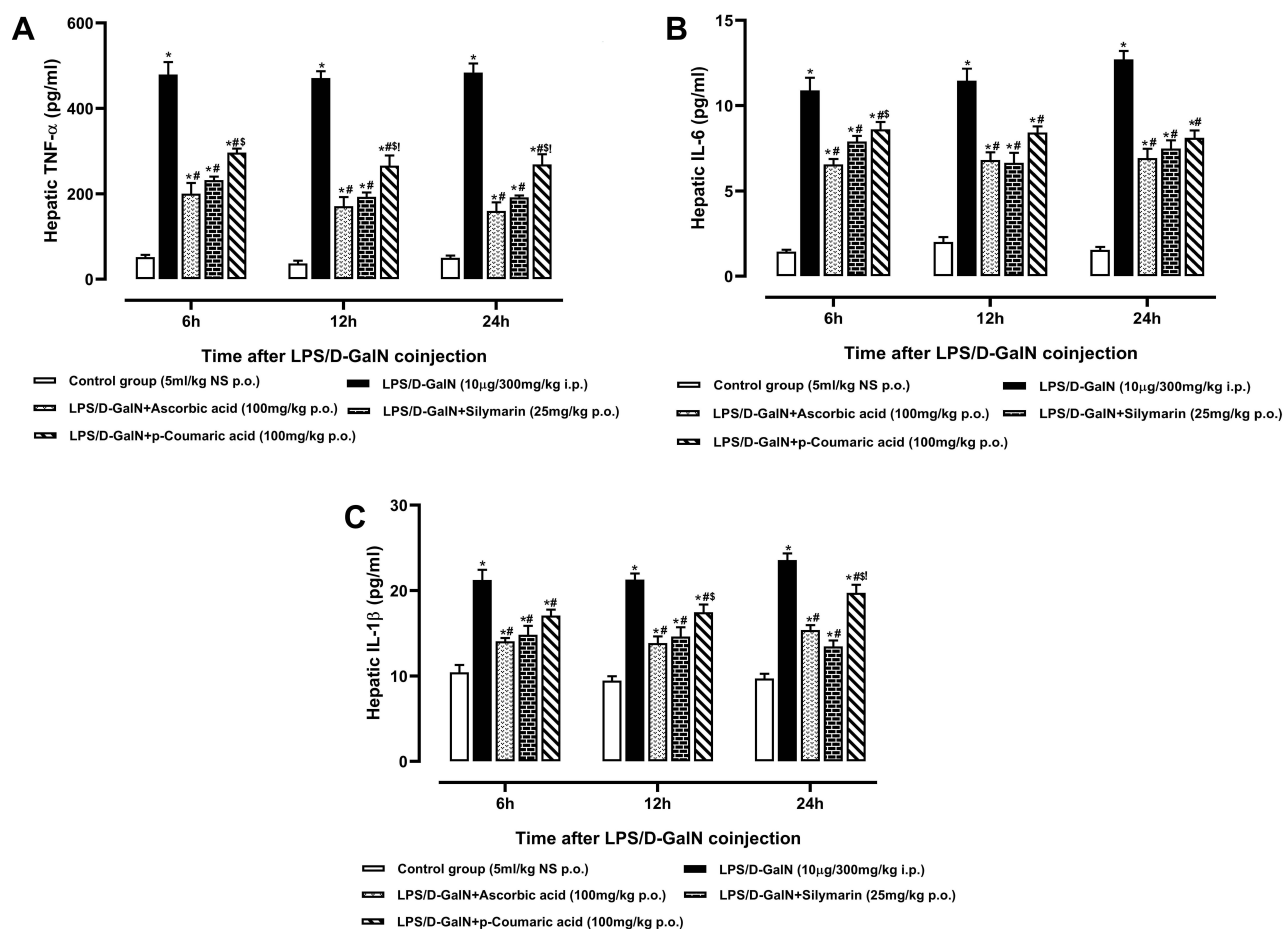


Figure 6 Hepatic (A) Tumor necrosis factor- α , (B) Interleukin-6 and (C) Interleukin-1 β of normal control, LPS/D-GalN and LPS/D-GalN + treatment groups. The values are mean \pm SEM (n=6). Statistical analysis was done one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for all groups in respective hours. The results are considered significant *if $p < 0.05$. #Indicates $p < 0.05$ vs normal control, #Indicates $p < 0.05$ vs LPS/D-GalN, \$Indicates $p < 0.05$ vs LPS/D-GalN + Ascorbic acid, #Indicates $p < 0.05$ vs LPS/D-GalN + Silymarin (25mg/kg).

TLR4 complex in Kupffer cells.⁵⁵ The activated NF- κ B enters into the nucleus and causes the transcription of various inflammatory genes such as TNF- α , IL-1 β , IL-6 etc.⁵⁵ D-GalN, on the other hand, metabolized in the liver and selectively depletes uridine nucleotides, inhibits RNA synthesis, inhibits the transcription of hepatocytes and potentiates toxic effects of LPS.^{23,58} The combined effects of these two agents produce a severe hepatocyte necrosis and inflammatory reactions.⁴⁸ TNF- α is a key pathogenic factor in liver injury which produce primarily in liver macrophages. It induces apoptosis and necrosis in hepatocytes by inducing other inflammatory mediators eg IL-6 and IL-1 β that orchestrate inflammatory reactions.⁵⁹ p-CA inhibits activation and transfer of NF- κ B to nucleus and inhibits the production of inflammatory cytokines.^{36,55} Following the progression of liver damage, TNF- α increases extravasation of leukocytes from sinusoids into liver parenchyma by different mechanisms, which exacerbates the liver injury.⁶⁰ Increased accumulation and infiltration of inflammatory cells may induce apoptosis.⁶¹ p-CA pre-treatment during this study clearly elaborated the protective effects against LPS/D-GalN induced inflammatory response while improving hepatic necrosis and increasing the level of anti-oxidants.⁶² p-CA had alleviated the overproduction of inflammatory mediators eg TNF- α , IL-6 and IL-1 β in liver tissue, which suggested its anti-inflammation activity in liver injury.³⁶ Neutrophils infiltration is the important factor which is responsible for the liver damage during systemic inflammation. These cells also produce inflammatory mediators and ROS and exacerbate liver injury.⁶³ Our study elaborated that using p-CA led to reduce total leukocyte count in liver tissue. Thus, it seems that p-CA attenuates liver damage by the alleviation of neutrophils infiltration. The liver is an important organ which plays a significant role in metabolism of drugs, de-toxification of toxic chemicals and biomolecule's synthesis. Alteration in metabolic functions of liver cells leads to imbalance in cellular antioxidant defense which results in ROS production and tissue injury.⁴⁷ Injection of LPS/D-GalN in rats

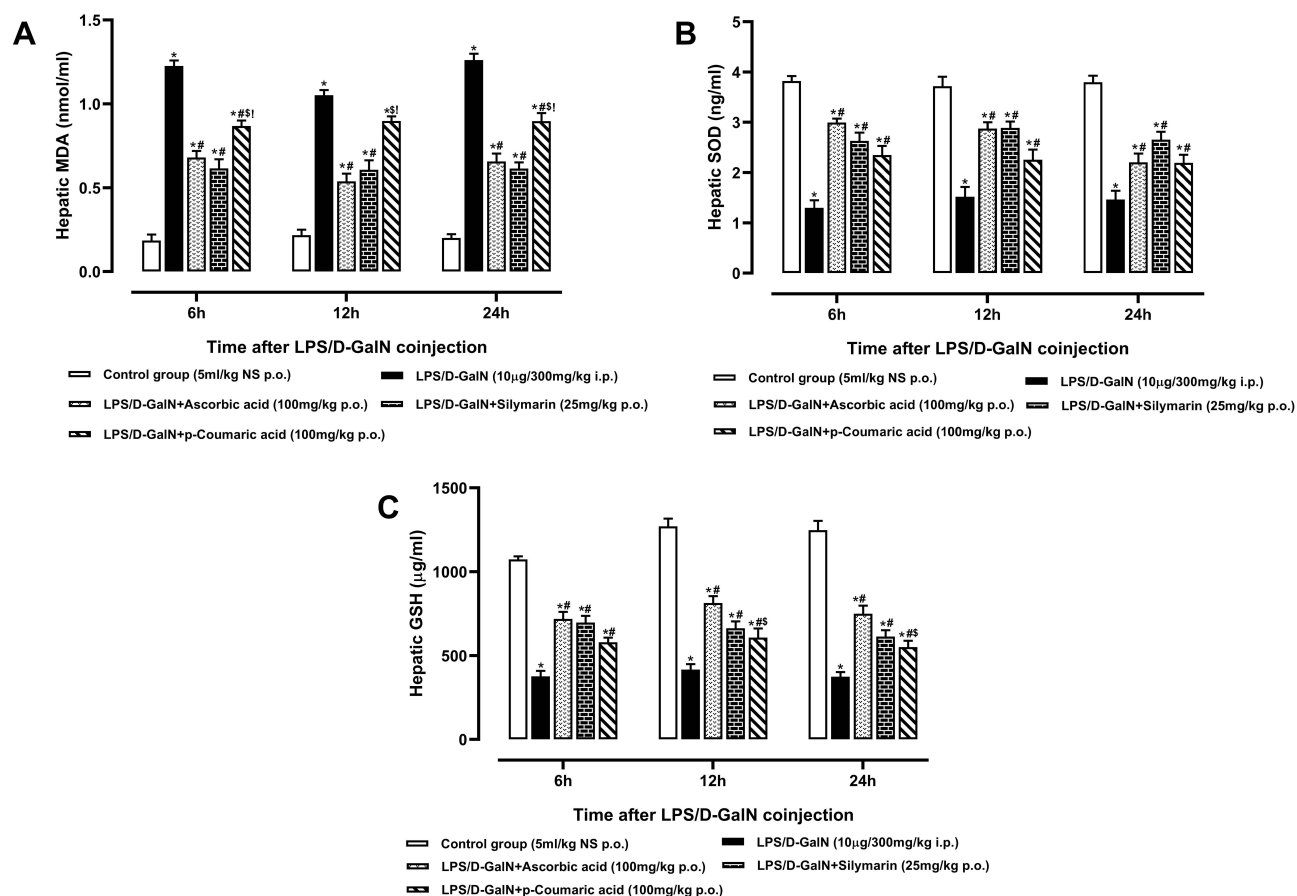


Figure 7 Hepatic (A) Malondialdehyde, (B) Superoxide Dismutase and (C) Reduced Glutathione levels of normal control, LPS/D-GalN and LPS/D-GalN + treatment groups. The values are mean \pm SEM (n=6). Statistical analysis was done one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test for all groups in respective hours. The results are considered significant *If $p < 0.05$. *Indicates $p < 0.05$ vs normal control, #Indicates $p < 0.05$ vs LPS/D-GalN, §Indicates $p < 0.05$ vs LPS/D-GalN + Ascorbic acid, †Indicates $p < 0.05$ vs LPS/D-GalN + Silymarin (25mg/kg).

augmented lipid peroxidation reactions due to metabolic disturbances. D-GalN is an amino sugar that when metabolized by hepatocytes produces ROS.⁵² Oxidative stress is actually redox imbalance in which levels of ROS exceed the magnitude of antioxidants as shown by increased hepatic malondialdehyde (MDA) levels. Lipid peroxidation⁶⁴ alters the cell membranes structure and fluidity resulting in cytolysis and cellular destruction.⁶⁵ LPS/D-GalN treatment significantly elevated total cholesterol levels which may be due to inability of necrotic liver to remove or synthesize cholesterol.⁶⁶ Pretreatment of p-CA showed protection against lipid changes caused by LPS/D-GalN induced oxidative insult. These findings could be correlated with the previous studies.⁶⁷ Hepatocellular damage due to alcohol, virus and drug-induced hepatitis causes modest hypertriglyceridemia⁶⁸ which is due to the biochemical changes interfering with the transport of triglycerides out of liver. Our study showed an increased accumulation of triglycerides, low density lipoproteins and very low-density lipoproteins in LPS/D-GalN induced rats as reported in previous research.^{67,69} Reduced glutathione (GSH) is ubiquitous antioxidant, constantly synthesized intracellularly from cysteine, glutamate, and glycine. GSH plays a major role in regulation of intracellular redox balance by detoxifying ROS. Lower levels of GSH and reduced GSH to oxidized GSH ratio (GSH:GSSG), is the measure of redox balance.⁷⁰ Glutathione plays a major role in the immune regulation, DNA synthesis, and cellular detoxification⁷¹ by combating with oxidative stress through scavenging free radicals reactive oxygen/nitrogen species.⁷² In present study, the GSH levels were reduced significantly in LPS/D-GalN challenged group, indicating that LPS/D-GalN induced oxidative reactions converted glutathione to its oxidized form.^{12,72} Pretreatment with p-CA significantly elevated glutathione levels in hepatocytes ($p < 0.05$). In addition to GSH, superoxide dismutase (SOD) counteracts oxidation of biomolecules (proteins, lipids and DNA) by quenching ROS. SOD reduces superoxide ion to less toxic product, namely hydrogen peroxide, which further reduced to water by the action of catalase and glutathione peroxidase.⁷³ In this experimental

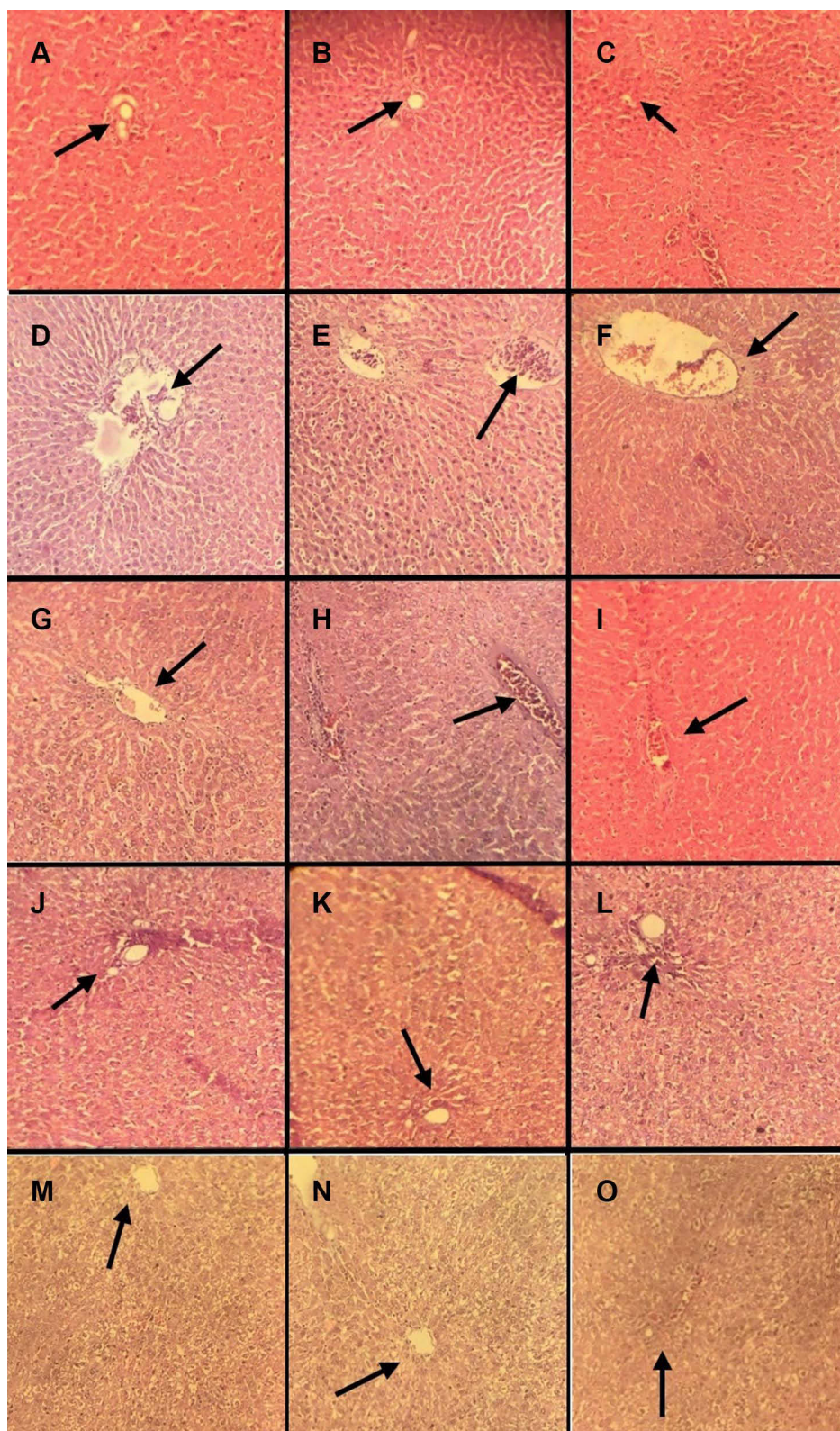


Figure 8 Photomicrographs of sections of liver tissues. (A–C at 6h, 12h and 24h) Control group. (D–F at 6h, 12h and 24h) LPS/D-GalN. (G–I at 6h, 12h and 24h) LPS/D-GalN + Ascorbic acid. (J–L at 6h, 12h and 24h) LPS/D-GalN + Silymarin. (M–O at 6h, 12h and 24h) LPS/D-GalN + p-CA 100mg/kg. Arrow heads indicate the blood congestion in central vein in the hepatic lobule and accumulation of polymorphonuclear cells at the site of inflammation in the liver tissues.

work, hepatic levels of SOD decreased significantly in LPS/D-GalN treated groups while p-CA tried to overcome this depletion in comparison to LPS/D-GalN treated groups. LPS/D-GalN treatment significantly increase total cholesterol levels which may be due to the inability of the injured liver to remove cholesterol from the circulation.⁶⁶ Pretreatment of p-CA also showed protection against serum and liver lipid changes caused by LPS/D-GalN evidencing a broad spectrum of hepatoprotective property. This finding could be correlated with the results of the previous studies.⁶⁷ Hepatocellular damage due to virus, alcohol and drugs causes hypertriglyceridemia which may be due to interference of oxidative stress with transport of triglycerides.^{66,68} Our study showed that LPS/D-GalN treatment increased serum levels of triglycerides, low density lipoproteins and very low-density lipoproteins in rats as reported in previous research.^{67,69} H&E staining was used in current study to detect the histological features of p-CA treated liver tissues in comparison to LPS/D-GalN-induced liver damage. Liver tissues of the control group showed normal structure of livers. LPS/D-GalN injected animals showed the histopathological changes, including extensive blood congestion in central vein and loss of hepatic architecture and massive inflammatory cells infiltration.^{14,64} These liver histopathological changes were restored by the treatment with p-CA. By summarizing the outcomes, p-CA inhibited LPS/D-GalN-induced liver injury by suppressing pro-inflammatory cytokines, maintaining oxidative balance and homeostasis in the liver and regulating hepatic enzymes and lipid profile in serum. Thus, p-CA could be used as an antioxidant and anti-inflammatory agent in the management of liver injury.

Limitations of the Study

Lipid metabolizing enzymes, eg lipoprotein lipase (LPL), lecithin-cholesterol acyl transferase (LCAT) and hepatic triglyceride lipase (HTGL) are limitations of this study.

Conclusion

The present study showed LPS/D-GalN-induced liver injury followed by hepatic dysfunction and morphologic pathology among experimental rats. In conclusion, our study elaborated that the use of p-CA in the treatment of LPS/D-GalN-induced liver injury can alleviate the damage to liver by ameliorating inflammation, oxidative stress and leukocytes infiltration.

Provision of Data

All the data is provided within the manuscript submitted.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

All authors declared that they had no competing interests.

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