Original Article

Hepatoprotective Role of Silybum Marianum against Azithromycin-Induced Histological and Biochemical Changes in Albino Wistar Rats

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ABSTRACT

Objective: To observe the harmful effects of azithromycin on the liver of albino Wistar rats and analyze the protective effect of silybum marianum against azithromycin-induced histological and biochemical changes in their liver.

Methodology: This experimental study was conducted at the Baqai Medical University, Karachi from December 2023 to March 2024. After ethical approval, 30 male albino Wistar rats were categorized into three groups with 10 rats each. Group A was kept as a control, group B was given azithromycin 200 mg/kg/day for 7 days orally, group C received azithromycin 200 mg/kg/day for 7 days, and silybum marianum (seed extract) 100 mg/kg/day for 21 days orally. After the experiment, all the rats were sacrificed and 3 ml blood was drawn out by cardiac puncture for estimation of liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST) & alkaline phosphatase (ALP) and superoxide dismutase (SOD). The liver of the sacrificed rats was removed and stained with hematoxylin and eosin (H&E). Data was analyzed using the Statistical Package for the Social Sciences (SPSS) version 24.

Results: The mean levels of liver enzymes were significantly raised and SOD levels were significantly reduced in group B as compared to group A and C rats. The liver parenchyma of group B rats exhibited significant histopathological changes such as inflammation, hemorrhage, and steatosis, whereas, all group A and most of the group C rats showed no histopathological changes with statistical significance.

Conclusion: Azithromycin-treated rats showed significant biochemical and histopathological changes in the liver parenchyma as compared to control group rats. In azithromycin and silybum marianum-treated rats, the biochemical and histopathological changes were significantly reduced as compared to azithromycin-treated rats alone, indicating the hepatoprotective effects of silybum marianum.

Keywords: *Silybum marianum. Azithromycin. Superoxide dismutase. Aspartate aminotransferase. Oxidative stress.*

INTRODUCTION

Hepatotoxicity is an injury to the liver caused by various xenobiotics such as food additives, antifungal medicines, radioactive elements, environmental toxins, and drugs. It can be hepatocellular, cholestatic, and mixed, resulting in raised levels of liver enzymes including alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase.[1] Drugs that cause hepatic injury include chlorpromazine, amoxicillin, macrolides, tetracycline, metoclopramide, antiepileptic drugs, anti-tuberculous drugs, and chemotherapeutic drugs.[2]

Azithromycin is an effective, semisynthetic broad-spectrum macrolide, a derivative of erythromycin, that has been used to treat infections over the last 50 years.[3] It is used in the treatment of pulmonary infections, enteric fever, gastrointestinal diseases, infections of the genitourinary system, pharyngitis, and tonsillitis.[4] Azithromycin acts by preventing the growth of bacteria. It binds with the 23S rRNA of the 50S bacterial ribosomal subunit and inhibits their protein synthesis.[5] It is adequately absorbed orally and remains stable in gastric acid. It penetrates almost all body tissues and has the largest volume of distribution. Azithromycin is concentrated and excreted in the bile as an active drug and eliminated from the body through feces, and partially it is excreted in the urine.[6]

Hepatocellular injury induced by azithromycin appears in 1-3 weeks after commencement of treatment in 1-2% of cases. This results in the release of free radicals, these free radicals produce oxidative stress causing increased levels of liver enzymes and infiltration of neutrophils and phagocytes (Kupffer's cells). It results in cholestatic jaundice, hepatic failure, and even death. The histopathological changes that appear in the liver are inflammation, steatosis, congestion, and hemorrhage.[7]

Silymarin is a natural compound derived from the silybum marianum (milk thistle) family, Asteraceae Compositae. It inhibits the activity of free radicals because of its antioxidant properties. The extract of its fruit and seeds is used worldwide to treat liver diseases such as cirrhosis, alcoholic liver disease, viral hepatitis, and drug-induced hepatotoxicity.[8] Its anticancer, anti-fibrinolytic, anti-inflammatory,

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antiviral, and antibacterial effects are proven. It promotes protein synthesis and facilitates the reconstruction of damaged liver tissue.[9] Silymarin is quickly absorbed after oral administration and has good tissue distribution. It is excreted partly through the biliary system and partly by the kidneys.[10]

Post-COVID-19 pandemic, azithromycin has become the most widely used drug globally. Recently, complications caused by azithromycin have been reported more frequently. This drug can induce oxidative stress on the liver, potentially leading to hepatotoxicity. The current study aims to determine the antioxidative effects of silymarin seed extract along with their possible protective effects against azithromycin-induced hepatic injury, by utilizing histological and biochemical parameters.

METHODOLOGY

This experimental study was conducted from December 2023 to March 2024 in the animal house of Baqai Medical University, Karachi after approval from the Board of Advanced Study and Research, Baqai Medical University, and the ethical committee (Letter No. BMU-EC/02-2022, 25-04-2022). A sample size of 30 was collected through the Resource Equation method.[11] A total of 30 healthy, adult, male albino rats aged 10-12 weeks, and weighing 150- 200 grams were included. Any sick rats were excluded from the study. The rats were marked using a permanent marker and separated randomly into three groups (10 per group): A, B, & C. Group A was kept as a control group. Group B was given azithromycin 200 mg/kg/day for 7 days.[12] Group C was given silybum marianum seed extract 100 mg/kg/day orally in the morning for 21 days, and azithromycin 200 mg/kg/day for 7 days. After the weight measurement, the animals were kept in plastic cages in natural 12-hour day or light and 12-hour night or dark cycles. The room temperature was kept constant at 21-24°C, with humidity ranging from 60-70%. They were given a laboratory pellet diet and water ad libitum. Acclimatization was conducted one week before the study to assess their physical condition based on their behaviour, weight changes, and activities.

To produce hepatocellular injury, Zyto (HIGH-Q) tablets (Generic Name Azithromycin) were bought from a local drugstore. The seeds of silybum marianum were ordered from the Shaheen Chemist in Islamabad. The Department of Pharmacognosy, University of Karachi, verified its botanical identification, and a voucher number (SMS-07-22) was generated. Seed extract of silybum marianum was prepared for the study after mixing it with ethanol. The 1000 g seeds of silymarin were crushed into fine powder and soaked in 1.5 litres of 99.8% ethanol. After three days, filtered through filter paper, the extract was evaporated to dryness in a rotary evaporator and again re-soaked in ethanol and filtered, and dried. The 1000 g of silymarin seed powder yielded 46 g of ethanol extract, which is 4.6% of the dry weight of silymarin seeds. Silybum marianum seed extract 100 mg/kg/day was given orally to rats for 21 days in group C.[13] Rats of groups A, B, & C were sedated and sacrificed on the 22nd day of the study. The blood was drawn by cardiac puncture and stored in ethylenediaminetetraacetic acid (EDTA) tubes and gel-containing tubes for liver function tests and superoxide dismutase (SOD). The livers were dissected out and fixed in 10% formalin. Then it was processed through fixation, embedding, dehydration, and clearing into tissue blocks. The tissue was cut into 5-micron-thick slices with the help of a rotary microtome and then stained with Hematoxylin and Eosin (H&E). A light microscope was used to detect the histopathological changes, i.e., inflammation, hemorrhage, and steatosis.

The grading system ranged from 0 to 3, with each value representing a specific degree of histopathological change. The grades were determined as a percentage to measure the extent of the detected histopathological changes. Grade 0 (zero) signified a negative outcome, while grades 1(0-30%), 2(31-50%), and 3(51-100%) denoted mild, moderate, and severe changes, respectively.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS) version 24 was used to analyze the data. The normality of the data was assessed using Shapiro-Wilk. The data was found to be normally distributed ($p > 0.05$). The biochemical parameters i.e. lipid profile (ALP, AST, and ALT) and SOD were expressed as mean±standard deviation (SD) and all groups were compared using a one-way ANOVA and Post-Hoc Tukey's test. For the analysis of histopathological parameters, Fisher's exact test was used. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The results revealed that the mean levels of hepatic enzymes were significantly raised in group B as compared to groups A and C. The mean SOD levels were significantly decreased in group B rats as compared to groups A and C (Table 1).

The comparison among the groups exhibited that the levels of hepatic biomarkers in the group B rats were remarkably raised ($p < 0.05$) than the mean AST, ALT, and ALP levels of the group A and group C rats. The mean AST, ALT, and ALP levels of the group C rats were not notably different ($p > 0.05$) from those of the group A rats. The mean serum SOD level of the group B rats was significantly lower ($p < 0.05$) than the mean serum SOD level of the group A and group C rats as displayed in Table 2. The microscopic examination of H&E-stained liver sections of groups A & C showed normal architecture of the liver. It is shown in Figures 1 and 2, respectively. The liver parenchyma of group B rats exhibited significant histopathological changes such as inflammation, hemorrhage, and steatosis as shown in Figures 3, 4, & 5.

All rats in group A showed no histopathological changes. In azithromycin-treated group B, 10(100%) rats exhibited moderate inflammation. Mild steatosis was seen in 4(40%) and moderate steatosis in 4(40%) of group B rats. Five (50%) showed mild congestion and hemorrhage was observed in 5(50%) and moderate in 4(40%) of the rats in group B. Absence of inflammation, steatosis, congestion & hemorrhage was observed in 6(60%), 8(80%) and 7(70%) of group C rats, respectively. The histopathological changes of group C rats were mostly of mild severity. All these results were statistically significant (Table 3).

Table 1: Comparison of Biomarkers (ALT, AST, ALP & SOD) among the Groups (one-way ANOVA)

Variables	Group A (Mean±SD)	Group B (Mean±SD)	Group C (Mean±SD)	p-value
AST Levels (IU/L)	86.2±4.2	113±5.8	91.6±9.7	<0.05*
ALT Levels (IU/L)	50.7±4.0	174.7±5.7	52±9.1	
ALP Levels (IU/L)	42.3±3.6	169±5.6	45.5±4.0	
SOD Levels (U/mL)	3.16±0.9	0.54±0.4	3.03±1.1	

**Significant p-value*

Table 2: Differences in Mean Levels of AST, ALT, ALP & SOD between Different Groups (Post-Hoc Tukey's Test)

Groups Comparison	AST (IU/L)		ALT (IU/L)		ALP (IU/L)		SOD (U/mL)	
	Differences of Mean	p-value	Differences of Mean	p-value	Differences of Mean	p-value	Differences of Mean	p-value
A & B	26.80000	0.000*	124.00000	0.000*	126.70000	0.000*	-2.60000	0.001*
B & C	21.40000	0.000*	122.70000	0.000*	123.50000	0.000*	-2.49600	0.001*
A & C	5.40000	0.125	1.30000	0.990	3.20000	0.992	-0.12900	0.997

**Significant p-value*

Table 3: Association of Histopathological Changes in the Liver among Groups A, B, & C Rats

Parameters		Groups (Frequency & Percentage)			p-value
		A (n=10)	B (n=10)	C (n=10)	
Inflammation	**None**	10(100%)	0(0%)	6(60%)	0.001*
	Mild	0(0%)	0(0%)	3(30%)	
	Moderate	0(0%)	10(100%)	1(10%)	
Steatosis	**None**	10(100%)	2(20%)	8(80%)	0.001*
	Mild	0(0%)	4(40%)	2(20%)	
	Moderate	0(0%)	4(40%)	0(0%)	
Congestion & Hemorrhage	**None**	10(100%)	1(10%)	7(70%)	0.001*
	Mild	0(0%)	5(50%)	3(30%)	
	Moderate	0(0%)	4(40%)	0(0%)	

**Significant p-value*

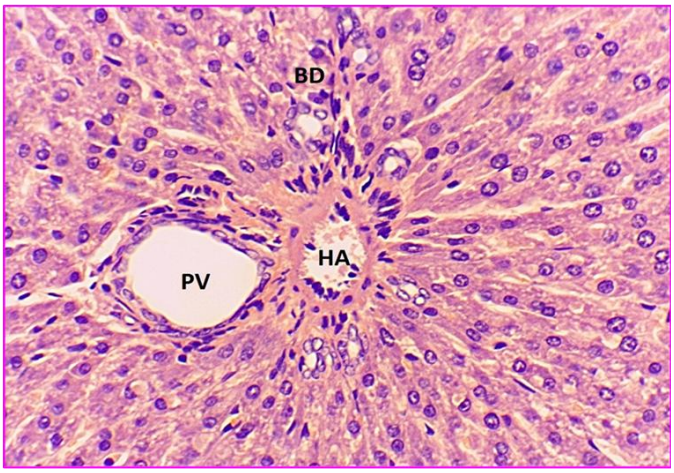

Figure 1: Liver Section of All Group A Rats shows the Normal Portal Area with Bile Duct (BD), a Branch of the Hepatic Artery (HA), and Portal Vein (PV), Radiating Sinusoids (H&E stain, 400X magnification)

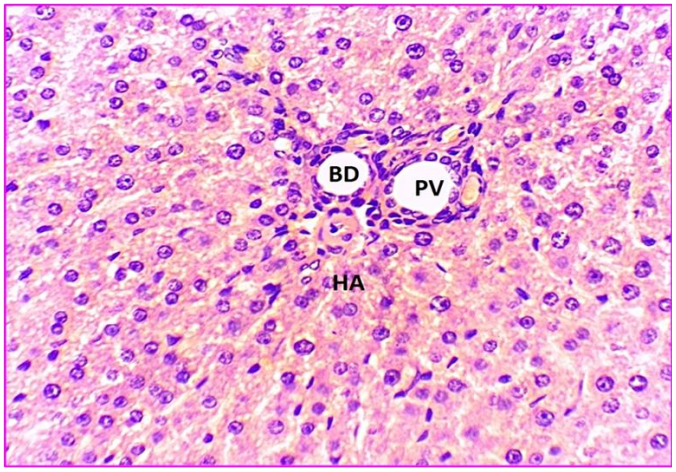

Figure 2: Microscopic Picture of a Section of the Liver of Group C (silymarin-treated) Rats showing a Normal Portal Area, no Inflammatory Cells Seen. Bile Duct (BD), and a Branch of the Hepatic Artery (HA), the Portal Vein (PV) (H&E stain, 400X magnification)

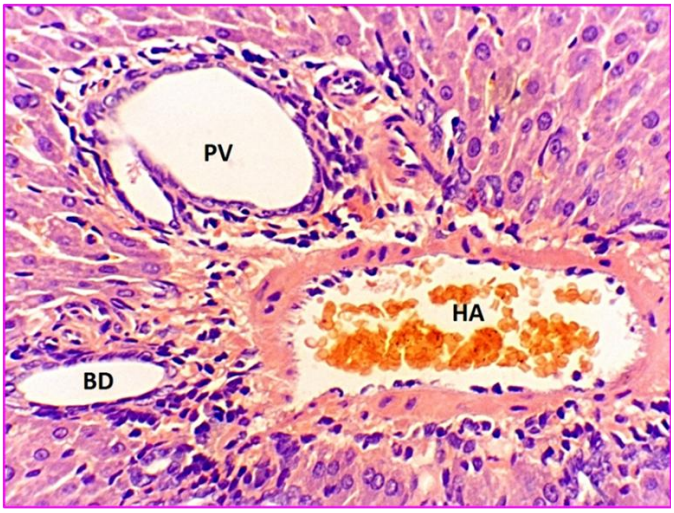

Figure 3: Microscopic Image of the Liver of Group B Rat Displaying Marked Infiltration in the Portal Area by Inflammatory Cells, Portal Vein (PV), Congested Hepatic Artery (HA), and Bile Duct (BD) (H&E stain, 400X magnification)

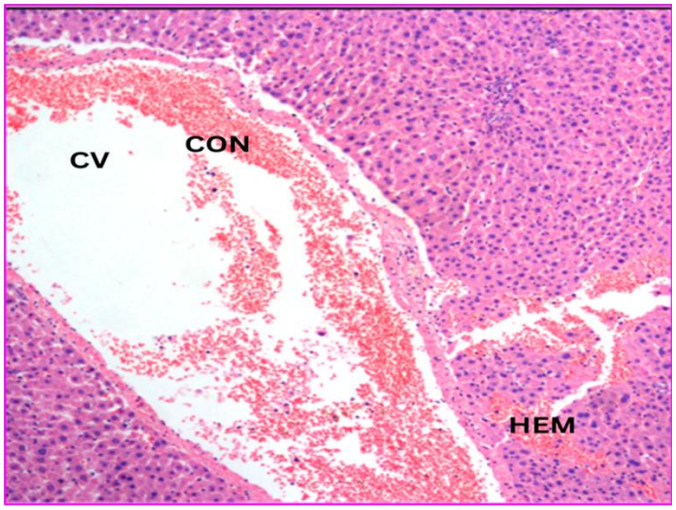

Figure 4: Photomicrograph from a Section of Hepatic Tissue of Group B Rats showing Congestion (CON) in the Central Vein (CV), and Hemorrhage (HEM) between the Hepatocytes (H&E stain, 100X magnification)

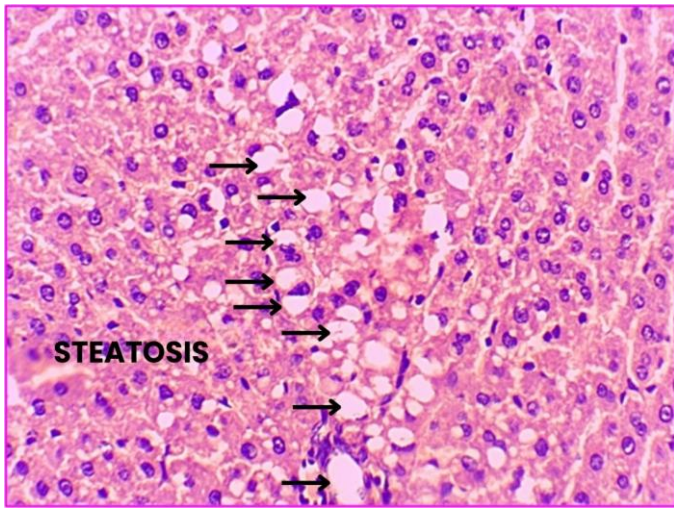

Figure 5: Photomicrograph of a Section of Group B Rat Liver showing Mild Steatosis (Arrow) (H&E stain, 400X magnification)

DISCUSSION

Our results showed significantly increased levels of liver enzymes (ALT, AST and ALP) and decreased SOD levels in group B azithromycin-treated rats as compared to group A control group. Similar to our results, Omara et al. showed that azithromycin-treated rats had a significant increased ALT & AST and decreased SOD levels. This is attributed to damage to liver cells caused by azithromycin resulting in cellular leakage, dysfunction, and liberation of free radicals.[14] Ali et al. also reported that azithromycin-induced hepatotoxicity is indicated by a significant increase in hepatic enzymes and decreased SOD levels. The levels of ALT, AST, and ALP were increased by 131.8%, 75.2%, and 153.6%, respectively in azithromycin-treated rats as compared to controls. The SOD levels were decreased by 607.4% in azithromycin-treated rats. The study linked these findings to oxidative stress, mitochondrial dysfunction, and bile transport inhibition leading to cell death due to azithromycin.[15] In another study conducted by Dadoub et al. in 2022, ALT and AST levels of the control group (21.5±2.5, 41.87±2.98) were significantly lesser as compared to those of azithromycin-treated rats (ALT: 76.29±3.6, AST: 80.7±1.9). The antioxidant SOD levels were also significantly reduced in azithromycin-treated rats. The antioxidative enzyme superoxide dismutase controls oxidative stress by preventing the liberation of free radicals. Cells rely on SOD as their primary intracellular defense mechanism against oxidative stress caused by free radicals. Superoxide dismutase acts as a catalyst in the conversion of superoxide radicals ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2).[16] In a study conducted in 2022, Hamza et al. also observed decreased level of SOD in hepatotoxicity.[17] These results are consistent with our findings, in which decreased SOD levels were observed in group B compared to group A.

The liver enzymes in group C rats of our study were significantly decreased as compared to group B. The levels of SOD were also increased due to the antioxidant effects of silymarin. Silymarin reduces oxidative stress and inhibits the liberation of free radicals, thus enhancing the healing process. A review done by Karimian et al. showed that silymarin is the preferred natural chemical for the treatment of liver diseases in rats due to its antioxidant and free radical scavenging effects.[18] Radwan et al. also showed a significant increase in liver enzymes in azithromycin-treated rats as compared to controls. The ALT, AST & ALP levels (99.17±3.9, 136.17±4.7 and 395.8±13.7, respectively) were significantly reduced in azithromycin-treated rats as compared to silymarin and azithromycin-treated rats (ALT: 59.83±2.7, AST: 104.50±4, ALP: 148.6±4.9). A significant increase in SOD levels was also observed in silymarin and azithromycin-treated rats.[19]

In this study, microscopic examination of the liver sections of rats of all three groups revealed that the rats of group B underwent significant histological changes as compared to the other two groups. We observed a marked distortion in the hepatic parenchyma. Inflammation of hepatocytes indicated by swelling and ballooning with the disturbance in the arrangement of the hepatic cords were seen. In addition, steatosis of microvacuoles with small droplets of fat showed hyperchromatic nuclei in the cytoplasm of the hepatocytes. A similar study was conducted by Ortiz et al. in 2021, which showed similar findings of steatosis and ballooning of hepatocytes due to the liberation of free radicals and oxidative stress. The study also observed dilated portal vein, mononuclear cell infiltration, congestion, and hemorrhage in the periportal area.[20] Fotouh et al. in 2023 described that after administering Azithromycin, areas of coagulative necrosis were observed. These changes were characterized by intense sinusoidal congestion and the appearance of new blood vessels. Researchers linked these changes to mitochondrial dysfunction resulting from raised reactive oxygen species and reduction of adenosine triphosphate.[21]

Dilatation of the central vein and hepatic sinusoids, along with hepatocellular infiltration around the central vein with lymphocytes and eosinophils and infiltration of neutrophils was also observed in our study. Similar findings were also described by Shiri Aghbash et al., who observed that the infiltration of neutrophils and Kupffer's cells occurred due to oxidative stress and liberation of free radicals in azithromycin-induced hepatotoxicity.[22] Other studies demonstrated that silymarin reduces liver damage by neutralizing free radicals and inhibiting lipid peroxidation in membrane-bound fatty acids after exposure to hepatotoxic substances.[23,24]

A randomized controlled trial conducted by Jin et al. demonstrated that silymarin supplementation significantly decreased liver stiffness and improved liver function in patients with metabolic dysfunction-associated steatotic liver disease, highlighting its potential in managing liver conditions associated with metabolic dysfunction.[25] Similarly, another clinical trial evaluated silymarin's efficacy in preventing anti-tuberculosis drug-induced liver injury, finding that patients receiving silymarin

exhibited lower levels of liver enzymes and a reduced incidence of hepatotoxicity compared to the control group.[26]

CONCLUSION

Azithromycin-treated rats showed significant biochemical and histopathological changes in the liver parenchyma as compared to control group rats. In azithromycin and silybum marianum-treated rats, the biochemical and histopathological changes were significantly reduced as compared to azithromycin-treated rats alone, indicating the hepatoprotective effects of silybum marianum.

LIMITATIONS & RECOMMENDATIONS

The study determined exclusively the effects of acute exposure to azithromycin, potentially neglecting essential effects related to chronic use or drug accumulation. Further studies should be conducted to observe the long-term effects of azithromycin on the liver by administering azithromycin for weeks or months. It is also suggested that a human trial is recommended to suggest the hepatoprotective effect of silymarin with the administration of azithromycin.

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Authors' Contributions:
S.A: Conception, design, data collection, analysis, and interpretation
M.T: Statistical analysis and interpretation of data
U.B: Analysis of histological slides
S.I.A: Critical revision and final approval of the article
T.K: Drafting of the article
M.M.H: Contribution to the methodology (silymarin seeds extract formation)

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