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Exploring the hepatoprotective effects of silymarin in experimentally induced- diclofenac sodium toxicity in rabbits

[a] Shahbaz Ul Haq, [b] Adeel Khalid, [c] Khush Hal Meghwar, [d] Arsalan Khan, [e] Shahrood Ahmed Siddiqui, [f] Rafiq Ullah, [g]Muhammad Sadeeq, [h] Muhammad Wasim Usmani, [b] Khurram Ashfaq *

[a] Department of Pharmacology, Shantou University Medical College, Shantou, Guangdong, China,
[b] Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, Pakistan,
[c] Department of Food Animal Metabolism and Management in Circular Economy, University of Bologna, Via Zamboni, Italy,
[d] Veterinary Research and Disease Investigation Center, Dera Ismail Khan, Pakistan,
[e] Vaccine Production Unit, Tandojam, Sindh, Pakistan
[f] Department of Zoology, Abdul Wali Khan University, Mardan, Pakistan
[g] University of Veterinary and Animal Sciences, Swat, Pakistan,
[h] Department of Veterinary Pathology, Faculty of Veterinary and Animal Sciences, Ziauddin University, Karachi, Pakistan.

Authors' Contribution

Haq, S., **A. Khalid** & **K.H. Meghwar** proposed and designed the study as well as analyzed the data. **A. Khurram**, **M.W. Usmani, S. A. Siddiqui**, conducted the research on the rabbits. **A. Khan**, prepared the drug dosages, **R. Ullah** & **M. Sadeeq**, monitored the research parameters during investigation and analyzed the data.

*Corresponding Author's Email Address: drkhurramashfaq33@gmail.com

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ABSTRACT

The study was aimed to investigate the hepatoprotective effects of varying doses of silymarin against liver damage caused by diclofenac sodium in rabbits. Silymarin, extracted from the *Silybum marianum* plant, is known for its antioxidant properties and is used to treat liver diseases. The rabbits (n=40) were divided into 4 groups. Group A served as the control (with vehicle solution), group B, as toxin control with diclofenac sodium (50mg/kg intra-peritoneally), group C received both diclofenac sodium and protective dose of silymarin (50mg/kg orally), while group D was administered diclofenac sodium along with higher dose of silymarin (100mg/kg orally). At the study's conclusion, rabbits were humanely euthanized and blood samples were collected for analysis. Enzymes such as Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST), direct bilirubin, serum urea, and total bilirubin were measured. The study revealed that higher dose of silymarin (100mg/kg) was more effective in reducing serum enzyme levels compared to lower dose, indicating stronger hepatoprotective effect. This suggests silymarin's potential as the therapeutic agent against liver damage and toxicity induced by substances like diclofenac sodium.

Keywords: Hepatotoxicity, hepatoprotective effect, liver enzymes, herbal drugs.

INTRODUCTION: Diclofenac sodium-induced liver damage is significant because diclofenac is a widely used medication with the potential to harm the liver. Understanding and mitigating this risk is crucial for patient safety and optimizing pain and inflammation management. It is classified as an aryl acetic acid derivative. Antipyretic, analgesic, and anti-inflammatory in nature, it exerts these effects via the inhibition of physiological and inflammatory prostaglandin synthesis (Ertekin *et al.*, 2019), used in managing various pain-related conditions, such as ankylosing spondylitis, spondylosis, rheumatoid arthritis, and osteoarthritis, in addition to all forms of muscular pain. Additionally, certain patients develop chronic renal and gastric toxicity (Klomjit and Ungprasert, 2022). The hepatic metabolism of diclofenac sodium results in the development of hepatic disorders (Jeon *et al.*, 2021). By inhibiting cyclooxygenase, diclofenac sodium hinders the biological synthesis of prostaglandin from arachidonic acid; this activity impacts physiological functions including osmoregulation, reproduction, water transport, and immune defense in organisms (Sun *et al.*, 2022). Furthermore, chronic and acute toxicity of diclofenac have detrimental effects on marine life, disrupting their regular physiological processes such as nutrition, reproduction, swimming, development, and growth, ultimately threatening their survival. Histopathological examination reveals the presence of hypersensitive reactions, which are characterized by severe hepatocyte damage caused by immune responses.

Antioxidant properties are among the potential health benefits of herbal remedies that are acquiring popularity and used globally (Abdeta *et al.*, 2020), with increasing popularity today, especially for hepatic diseases, due to their accessibility, affordability, and low side effects. Milk thistle (Silybum marianum) has been utilized for centuries as a reliable herbal remedy for liver disorders. The preclinical research has extensively documented the antioxidant and liver-protective properties of silymarin, which is derived from milk thistle (Gillessen and Schmidt, 2020). It is a complex mixture of plant-based compounds with known biological effects, predominantly flavonolignans, flavonoids (such as taxifolin and quercetin), and polyphenols (Surai, 2015). Silybin, which comprises approximately 50–60% of the complex, is the most abundant and biologically active of the flavonolignan isomers found in silymarin. The remaining 35% is composed of the following isomers: silicenin (20%), silidianin (10%), and isosilibinin (5%) (Gillessen and Schmidt, 2020). Poor bioavailability results from the hydrophobic and nonionizable characteristics of silybin, which contribute to its limited water solubility (Bijak, 2017). However, the bioavailability of silybin can be influenced by adjacent molecules such as flavonoids, phenol derivatives, and amino acids (Abenavoli *et al.*, 2018). Consequently, the active component, silibinin, is present in varying concentrations, solubilities, and oral bioavailability across commercially available silymarin products (Mukhtar *et al.*, 2021). In addition, silymarin and silibinin have the potential to interact with statins by inhibiting transport proteins (organic anion transporting polypeptide and breast cancer resistance protein) in the liver, which could have an effect on the metabolism of statins (Abenavoli *et al.*, 2018). Silibinin, a constituent of silymarin, functions as a highly effective free radical scavenger, safeguarding hepatic cells against oxidative harm induced by substances such as acetaminophen and CCl4 (Teschke, 2018). By stimulating glutathione synthesis, it enhances the liver's antioxidant capacity and inhibits numerous reactive oxygen species (ROS) (Esmaeil *et al.*, 2017). Silifinin functions as a lipid peroxidation inhibitor, cell membrane stabilizer, and protective barrier against toxic compounds such as carbon tetrachloride (Zhao *et al.*, 2017). In its early phases, it can also impede the progression of liver fibrosis (Clichici *et al.*, 2016). Silymarin demonstrates anti-inflammatory, antioxidant, antifibrotic, and regenerative properties in the context of non-alcoholic fatty liver disease (NAFLD), in addition to ameliorating metabolic factors such as insulin resistance and hyperlipidemia (Sun *et al.*, 2023). Furthermore, silymarin and its constituent silybin have exhibited inhibitory effects on the proliferation of liver cancer cells; furthermore, their synergistic effects with specific pharmaceutical agents amplify these effects (Gu *et al.*, 2015). When combined with doxorubicin, silymarin has the potential to inhibit telomerase activity in hepatic carcinoma cells, according to additional research (Yurtcu *et al.*, 2015). Utilized as a natural remedy for ailments affecting the nervous system, kidneys, prostate, lungs, and liver, among others (Abenavoli and Milic, 2017). Sillymarin possesses a range of protective activities, including immunomodulatory, antifbrutic, and membrane-stabilizing properties, antioxidant, anti-apoptotic, and anti-inflammatory properties (Yassin *et al.*, 2022). The potential antitumor properties of this botanical agent have been evaluated in a variety of malignancies, including prostate, lung, liver, cervical, breast, and bladder cancers (Koltai and Fliegel, 2022). The different mechanisms for the antitumor activities of silymarin have been reported by previous studies (Delmas *et al.*, 2020). As a result of the spread of hepatitis, milk thistle is an increasingly in-demand herb on an international scale (Jahan *et al.*, 2016). The silymarin content of milk thistle exhibits a range of 1.5% to 3.5%, with a quality standard of 3% to 6%. Although the silymarin constituents remain constant among samples, variations in the flavonolignan content

and geographical origin indicate the possibility that particular ecotypes have been domesticated (Arampatzis *et al.*, 2019). Despite its economic and pharmaceutical value, milk thistle domestication and breeding efforts have been limited (Hamouda, 2019). Silymarin content and composition vary genetically among milk thistle ecotypes; these variations are influenced by environmental conditions, harvesting methods, fruit maturation, and sowing depth (Tayoub *et al.*, 2018).

OBJECTIVES: The current investigation was formulated with the following aims in mind: The objective of this study is to assess the hepatoprotective potential of silymarin against liver injury induced by diclofenac sodium in rabbits. Additionally, the anti-fibrotic and anti-cirrhosis effects of silymarin in diclofenac sodium-induced liver toxicity will be investigated.

MATERIAL AND METHODS: For this experiment, 40 rabbits of both sexes weighing between 350 and 900 grams were purchased from the local market of Faisalabad. , The rabbits were acclimated to their environment with free access to both food and water throughout the acclimation period. The rabbits were maintained autonomously in enclosures throughout the duration of the research, following a 12-hour light-dark cycle. Diclofenac sodium and silymarin were utilized and. Prior to administration, all medications were dissolved in an isotonic saline solution containing 0.9% NaCI. The rationale for selecting rabbits as experimental animals in the current study was based on the similarity of biochemical changes observed in rabbits to those observed in other animal species, their accessibility, ease of handling, and low cost (Qamar *et al.*, 2011). Throughout the duration of the investigation, rabbits were housed autonomously in cages in a controlled environment with a light-dark cycle and climate (23±2°C and 50-60% relative humidity). The administration of study drugs to each group of ten rabbits followed the subsequent outline. Group A: The control group received the vehicle alone (10 ml/kg body weight P/O) for 15 days while conducting normal operations. In the toxicity control group (Group B), diclofenac sodium (50 mg/kg I/P) was administered twice daily. Group C consisted of rabbits that were administered diclofenac sodium (50mg/kg I/P) and a moderate dose of silymarin (50mg/kg P/O) herbal medication twice daily. Group D: A high dose of the herbal remedy (100 mg/kg) and 50 mg/kg diclofenac sodium were administered to the rabbits. All rabbit groups were administered these medications for a duration of 15 days. Approximately 3 mL blood were aseptically collected from both the marginal ear vein and the jugular vein, using sterile test vials, centrifuged for 3 min. at 5000 RPM (Alhassan *et al.*, 2012). The concentrations of alanine transaminases (ALT) and aspartate transaminases (AST) in serum were determined using commercially available kits, whereas the calorimetric determination of alkaline phosphatase (ALP) was performed using commercially available kits in accordance with the method of Belfield and Goldberg. The measurement of total bilirubin and direct bilirubin was performed on an automatic blood biochemical analyzer at 37°C using standard reagent packages, within three hours of sample collection. Urea nitrogen in the blood was quantified utilizing commercially accessible instruments. The mean value was reported along with the standard error (SE). The data were analyzed using version 16.0 of the SPSS program. A one-way analysis of variance (ANOVA) was conducted, and Tukey's test was utilized to compare the variables between the groups. Significant differences were identified at a significance level of $P<0.05$.

RESULTS AND DISCUSSION: Significant alterations are observed in various biochemical parameters of rabbits that are administered diclofenac sodium. Induced hepatotoxicity in all groups was a high dose of diclofenac sodium (50 mg/kg body weight) (figure 1). Enzymes such as ALT, ALP, and AST serve as indicators of the liver's overall health. Their elevated level indicates damage and injury to the liver. Additionally, significant increases were observed in the levels of total bilirubin, direct bilirubin, and blood urea nitrogen. The statistical analysis unequivocally demonstrates these alterations. The results of ANOVA for ALP, ALT, AST, Direct Bilirubin, Total Bilirubin, and Blood Urea Nitrogen are presented in table 1. The results indicate that all of these parameters increased significantly when only diclofenac sodium was administered to each group. Moreover, their concentrations decreased substantially following treatment with silymarin. The observed reduction in each of the aforementioned parameters provides evidence for silymarin's hepatoprotective properties. In table 2, group A has the lowest values of ALP, ALT, and AST (12.55 ± 1.28, 45.21 ± 1.14, and 61.85 ± 1.48, respectively). These values are close to normal, given that group A serves as the control and does not receive diclofenac sodium or silymarin treatment. Group B has significantly elevated levels of ALP (33.50 ± 1.81), ALT (90.50 ± 4.01), and AST (118.45 ± 6.38) in comparison to Group A, due to the fact that it is administered diclofenac sodium at a dose rate of 50 mg/kg body weight, I/P. Group C exhibits reduced levels of ALP (29.65 ± 1.90), ALT (82.30 ± 5.03), and AST (103.45 ± 2.96) due to the administration of silymarin at a protective dose of 50mg/kg body weight (I/P) and diclofenac sodium (50mg/kg body weight, I/P).

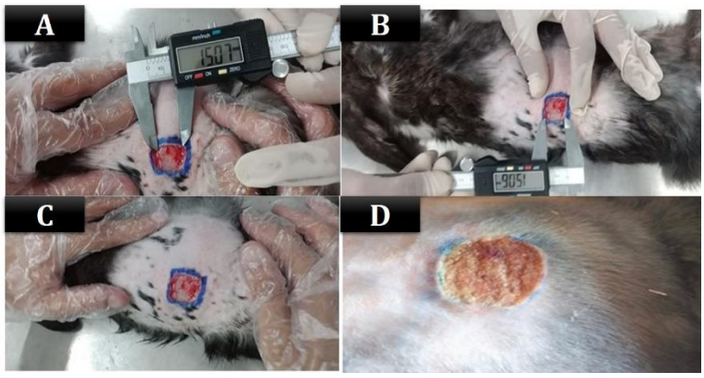

Figure 1: Step-wise demonstration on the experimental animals. Measurement of wound diameter (A), wound depth (B), marked appearance of the wound (C) and healing at a predetermined time point post-injury(D).

Parameters	Source of variation	Degree of freedom	Sum of squares	Mean squares	F-value
	Treatment	3	2487.72	829.24	283.03**
ALP	Error	36	105.48	2.93	
	Total	39	2593.19		
	Treatment	3	11647.4	3882.5	204.74**
ALT	Error	36	682.7	19.0	
	Total	39	12330.0		
	Treatment	3	17493.2	5831.1	308.90
AST	Error	36	679.6	18.9	
	Total	39	18172.7		
	Treatment	3	20.4017	6.8006	167.07**
Direct Bilirubin	Error	36	1.4654	0.0407	
	Total	39	21.8671		
	Treatment	3	18.3090	6.1030	541.19**
Total Bilirubin	Error	36	0.4060	0.0113	
	Total	39	18.7150		
	Treatment	3	2358.07	786.02	226.34**
Blood Urea Nitrogen	Error	36	125.02	3.47	
	Total	39	2483.09		

Table 1: ANOVA for all respective parameters. Aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST).

Group	ALP (IU/L)	ALT (IU/L)	AST (IU/L)
A	12.55 ± 1.28	45.21 ± 1.14	61.85 ± 1.48
B	33.50 ± 1.81	90.50 ± 4.01	118.45 ± 6.38
C	29.65 ± 1.90	82.30 ± 5.03	103.45 ± 2.96
D	24.95	72.9	86.3

Table 2: Representation of aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) values in the experimental groups.

The observed decrease in levels of ALP, ALT, and AST in this cohort indicates that silymarin may have a hepatoprotective impact. In a similar vein, Group D demonstrates a decrease in ALP (24.95), ALT (72.9), and AST (86.3), in addition to being administered a hepatoprotective dose of silymarin (100 mg/kg body weight, per oral). Significantly, the reduction in ALP, ALT, and AST levels within this cohort demonstrates that silymarin has a substantial effect on hepatocyte recovery.

As shown in table 3, the group A, which serves as the control group, has the lowest values of direct bilirubin (0.146 ± 0.043), total bilirubin (0.429 ± 0.035), and blood urea nitrogen (17.12 ± 1.98). These values are in close proximity to the normal range, given that Group A has not been subjected to any treatment involving diclofenac sodium or silymarin. On the other hand, Group B exhibits significantly higher values for direct bilirubin (1.958 ± 0.280), total bilirubin (2.126 ± 0.152), and blood urea nitrogen (38.00 ± 2.16), all of which can be attributed to the intraperitoneal administration of diclofenac alone at a dose of 50mg/kg body weight. On the other hand, Group C exhibits a decrease in blood urea nitrogen value (29.30 ± 1.77), direct bilirubin value (1.670 ± 0.221), and total bilirubin value (1.630 ± 0.142) due to the administration of silymarin at a protective dose of 50mg/kg body weight orally and

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intraperitoneally.

Group	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	DB (mg/dL)		TB(mg/dL)		BUN (mg/dL)	
A	12.55 ± 1.28	45.21 ± 1.14	61.85 ± 1.48	0.146 ± 0.043	±	0.429 ± 0.035	±	17.12 ± 1.98	±
B	33.50 ± 1.81	90.50 ± 4.01	118.45 ± 6.38	1.958 ± 0.280	±	2.126 ± 0.152	±	38.00 ± 2.16	±
C	29.65 ± 1.90	82.30 ± 5.03	103.45 ± 2.96	1.670 ± 0.221	±	1.630 ± 0.142	±	29.30 ± 1.77	±
D	24.95	72.9	86.3	0.820 ± 0.183	±	0.754 ± 0.024	±	23.55 ± 1.48	±

Table 3: The confirmation of direct bilirubin (DB), total bilirubin (TB) and blood urea nitrogen (BUN) in the groups.

DISCUSSION: Patients have utilized herbal medicines since antiquity to treat a wide range of conditions, from trivial ailments to severe chronic conditions, as self-prescribed by themselves and others in their vicinity (El-Dahiyat *et al.*, 2020). Herbs with medicinal properties are frequently utilized by patients; therefore, physicians should be aware of both the positive and negative effects of these herbs (Rashrash *et al.*, 2017), Consequently, the primary objective of the current investigation is to assess the hepatoprotective properties of herbal remedies that are commonly used in society, notwithstanding their pharmacological effects and in the absence of pharmacological evaluations. The research utilized animals that were administered diclofenac sodium at a high dose (50mg/kg body weight I/P). Throughout the duration of drug administration, no gross toxicity was observed in any of the groups; however, weight loss was observed in response to diclofenac administration. Environmental stress may have contributed to the rabbits' weight loss, given that they were confined in enclosures.

Liver function tests are land mark to evaluate the liver dysfunction (Umicevic *et al.*, 2022). The liver serves numerous essential functions within the animal body; therefore, it is impossible for a single test to comprehensively evaluate the liver's correct functioning (Umicevic *et al.*, 2022). Due to the abundance of drug-metabolizing enzymes in the liver, it is the primary site of drug-induced toxicity. These conversions typically increase the solubility of substances in water; they facilitate the formation of xenobiotics and bioactivators, which in turn generate reactive metabolites with detrimental effects (Yamazaki *et al.*, 2016). Diclofenac-induced liver injury is characterized by the activation of immune-mediated reactions, as evidenced by the histopathological appearance of hypersensitive reactions. These immune reactions cause significant damage to hepatocytes (Bindu *et al.*, 2020).

Spondylitis is treated with diclofenac sodium, a non-steroidal anti-inflammatory medication. The current investigation demonstrated noteworthy alterations in the concentrations of ALP, AST, ALT, total bilirubin, direct bilirubin, and blood urea nitrogen total and direct bilirubin in all rabbits administered diclofenac doses. Nevertheless, a substantial rise in ALP was attributed to a disruption in bone metabolism. An increase in ALP blood serum level is initially initiated by both hepatic and bone disturbances (Oh *et al.*, 2017). All liver enzyme tests were performed on the four groups of rabbits used in the experiments, including ALP, ALT, AST, direct bilirubin, total bilirubin, and blood urea. Group A served as the control group and received only vehicles. As the toxicity control group, Group B was administered a high dose of diclofenac sodium (50 mg/kg body weight, I/P). In addition to a protective dose of silymarin (50mg/kg body weight, P/O), Group C is administered diclofenac sodium (50mg/kg body weight, I/P). A hepatoprotective dose of silymarin (100mg/kg body weight, P/O) and a dose of diclofenac sodium (50mg/kg body weight, I/P) are administered to Group D. All parameters exhibited an increase in the groups that received diclofenac administration, whereas their levels decreased considerably in the groups that received silymarin administration. The findings of these studies indicate that silymarin possesses hepatoprotective properties. The study's limitations include its reliance on a rabbit model, limited treatment duration, single toxin exposure, small sample size, and narrow range of silymarin doses. The clinical significance of the study's findings lies in the potential use of silymarin, particularly at higher doses, as a protective agent against liver damage caused by diclofenac sodium and potentially other medications. This suggested the practical application in healthcare where silymarin supplementation could be considered to reduce the risk of drug-induced liver injury, enhancing the safety of pain and inflammation management. However, clinical trials and guidelines are necessary to confirm and implement these findings in patient care. To advance this research, future studies should consider clinical trials in large populations of laboratory animals, elucidate the mechanisms of silymarin's hepatoprotective effects, explore combination therapies, assess long-term safety, improve bioavailability, examine dietary interactions, study diverse populations, conduct comprehensive toxicity evaluations and establish clinical guidelines aiming to enhance our understanding and utilization of silymarin for liver protection and treatment.

CONCLUSION: The study confirms the sustained efficacy of silymarin in alleviating diclofenac sodium-induced toxicity in rabbits. Elevated levels of liver enzymes, including ALT, AST, ALP, total bilirubin, direct bilirubin, and blood urea nitrogen, indicated liver toxicity and injury. Silymarin, known for its hepatoprotective properties as a plant extract, was selected as the investigational drug, demonstrating a significant and favorable impact in reducing elevated liver enzyme concentrations—a key indicator of hepatic injury. In contrast to previous investigations utilizing rodents as research models, our study employed rabbits, representing a broader spectrum of species for a more comprehensive understanding. In summary, silymarin exhibited the capability to decrease ALT, AST, and ALP levels. Notably, concentrations of total bilirubin, direct bilirubin, and blood urea nitrogen were significantly higher at a dosage of 100 mg/kg of silymarin after 50 days of oral administration compared to 50 mg/kg. This conclusion highlights the potential of silymarin in ameliorating liver damage, emphasizing the importance of dosage considerations in therapeutic applications

CONFLICT OF INTEREST: The authors declared no conflict of interest.

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