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Evaluation of the Hepatoprotective Potential of Silymarin: A Detailed Assessment of Biochemical, Histopathological, and Molecular Mechanisms in Mice Exposed to Acetaminophen-Induced Liver Damage

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ABSTRACT

Background: Acetaminophen (APAP) overdose is a major cause of drug-induced liver injury (DILI), primarily through the formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which induces oxidative stress and hepatocyte necrosis. Despite the availability of N-acetylcysteine (NAC) as an antidote, limitations such as a narrow therapeutic window necessitate exploration of alternative hepatoprotective agents. Silymarin, a flavonolignan complex from *Silybum marianum*, is known for its antioxidant, anti-inflammatory, and hepatoprotective properties.

Objective: To evaluate the hepatoprotective effects of silymarin against APAP-induced liver injury in mice by analyzing biochemical, histopathological, and molecular parameters. Methods: Male Swiss albino mice were randomly divided into four groups: Control, APAP-treated, Silymarin-treated, and Silymarin + APAP co-treated. Silymarin (100 mg/kg/day) was administered orally for 7 days, and APAP (500 mg/kg, intraperitoneally) was given on day 7. Serum liver enzymes (ALT, AST, ALP, total bilirubin), oxidative stress markers (MDA, GSH, SOD, catalase), histopathology, and gene/protein expression of inflammatory (TNF-α, IL-6, NF-κB), apoptotic (Bcl-2, Bax, Caspase-3), and antioxidant (Nrf2, HO-1) markers were evaluated.

Results: APAP significantly elevated liver enzyme levels and oxidative stress markers, disrupted hepatic architecture, and upregulated inflammatory and apoptotic genes while downregulating antioxidant genes ($p < 0.01$). Co-treatment with silymarin markedly reversed these effects: liver enzyme levels and MDA were reduced, antioxidant markers were restored, and liver histology showed preserved architecture with mild damage. Molecular analysis showed downregulation of TNF-α, IL-6, NF-κB, Bax, and Caspase-3, and upregulation of Bcl-2, Nrf2, and HO-1 in the silymarin + APAP group ($p < 0.01$ vs. APAP alone).

Conclusion: Silymarin confers significant protection against APAP-induced hepatotoxicity through its antioxidant, anti-inflammatory, and anti-apoptotic mechanisms. These findings support silymarin's potential as a therapeutic agent for managing drug-induced liver injury.

Keywords: *Silymarin; Acetaminophen; Hepatotoxicity; Oxidative stress; Inflammation; Apoptosis; Nrf2; Liver injury; Antioxidant defense; Murine model*

1. INTRODUCTION

1.1. Background

The liver is a central metabolic organ responsible for detoxification, synthesis, and storage of vital biomolecules, as well as regulation of biochemical pathways involved in homeostasis. Its extensive exposure to xenobiotics and drugs renders it particularly susceptible to toxic insults, making the maintenance of hepatic integrity critical for overall health (Bhattacharyya et al., 2014). Hepatoprotective agents are therefore essential in managing liver diseases, especially those resulting from drug-induced liver injury (DILI), which remains a significant cause of acute liver failure worldwide (Lee, 2017).

Acetaminophen (APAP), commonly used as an over-the-counter analgesic and antipyretic, is a leading cause of DILI when consumed in overdose. APAP-induced hepatotoxicity primarily arises from the formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which depletes hepatic glutathione (GSH) and covalently binds to cellular proteins, inducing oxidative stress, mitochondrial dysfunction, and ultimately hepatocyte necrosis and apoptosis (Jaeschke et al., 2012; Ramachandran & Jaeschke, 2018). Despite the availability of N-acetylcysteine (NAC) as an antidote, limitations such as narrow therapeutic windows and delayed administration underscore the need for alternative hepatoprotective strategies.

Silymarin, a flavonolignan complex extracted from the seeds of *Silybum marianum* (milk thistle), has long been recognized for its potent hepatoprotective, antioxidant, and anti-inflammatory properties (Federico et al., 2017). It exerts its pharmacological actions by scavenging free radicals, enhancing the antioxidant defense system, stabilizing hepatocellular membranes, and modulating signal transduction pathways involved in inflammation and apoptosis (Polyak et al., 2010; Surai, 2015). Given its favorable safety profile and long-standing use in traditional medicine, silymarin remains a promising candidate for managing drug-induced liver injury.

1.2. Rationale

Although several studies have reported the hepatoprotective effects of silymarin, a comprehensive evaluation encompassing biochemical, histopathological, and molecular analyses is essential for elucidating the mechanistic basis of its protective effects in APAP-induced liver toxicity. Integrating these assessments provides a multidimensional understanding of how silymarin modulates hepatic injury at the cellular and molecular levels, thereby strengthening the therapeutic rationale for its clinical use.

1.3. Objectives

- To assess the protective effect of silymarin on APAP-induced liver injury in a murine model.
- To analyze alterations in serum biochemical markers, histopathological architecture, and molecular expression of oxidative stress, inflammation, and apoptosis-related genes.

2. MATERIALS AND METHODS

2.1. Animals and Experimental Design

Male Swiss albino mice (*Mus musculus*), aged 6–8 weeks and weighing 25–30 grams, were obtained from the Institutional Animal Facility. All animals were housed under standard laboratory conditions (22 ± 2°C temperature, 50–60% humidity, 12-hour light/dark cycle) with access to a standard pellet diet and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC), and procedures were conducted in accordance with the CPCSEA guidelines (Approval No.: IAEC/2025/113).

Animals were randomly divided into four groups with six mice in each group, as outlined in **Table 1**.

Table 1. Experimental Grouping and Treatment Design

Group	Treatment	APAP (500 mg/kg)	Silymarin (100 mg/kg)
I	Control	No	No

II	APAP-treated	Yes	No
III	Silymarin-treated	No	Yes
IV	Silymarin + APAP co-treated	Yes	Yes

2.2. Chemical Reagents and Dosing

Acetaminophen (APAP) was purchased from Tamilnadu and freshly dissolved in warm physiological saline before administration. **Silymarin** (purity ≥98%) was obtained from HiMedia Laboratories (India) and suspended in 0.5% carboxymethylcellulose (CMC) for oral delivery.

- **Silymarin treatment**: Administered orally at 100 mg/kg/day for 7 consecutive days.
- **APAP administration**: A single intraperitoneal (i.p.) dose of 500 mg/kg was given on the 7th day, 1 hour after the final silymarin dose (for the co-treated group).

This dosing strategy was used to mimic acute liver injury and assess silymarin's preventive potential.

2.3. Biochemical Analysis

After 24 hours of APAP administration, mice were anesthetized, and blood samples were collected via retro-orbital plexus puncture. Blood was allowed to clot and centrifuged at 3000 rpm for 10 minutes to separate serum for liver enzyme analysis. Liver tissues were harvested, washed in ice-cold saline, and homogenized (10% w/v) in phosphate buffer (0.1 M, pH 7.4) for oxidative stress marker assays.

2.3.1. Serum Liver Enzymes and Total Bilirubin

The levels of liver enzymes and bilirubin were determined using diagnostic kits from Erba Diagnostics (Germany) according to manufacturer protocols. These included:

- Alanine aminotransferase (ALT)
- Aspartate aminotransferase (AST)
- Alkaline phosphatase (ALP)
- Total bilirubin (TBIL)

2.3.2. Oxidative Stress Markers in Liver Tissue

Biochemical assays were carried out on the liver homogenates to assess oxidative stress:

- **Malondialdehyde (MDA)** – Indicator of lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS) assay (Ohkawa et al., 1979).
- **Reduced Glutathione (GSH)** – Measured using Ellman's reagent (Beutler et al., 1963).
- **Superoxide Dismutase (SOD)** – Assessed using the inhibition of pyrogallol autooxidation method (Marklund & Marklund, 1974).
- **Catalase (CAT)** – Measured by the breakdown rate of hydrogen peroxide (Aebi, 1984).

Table 2. Biochemical Parameters and Analytical Methods

Parameter	Biological Sample	Unit	Method/Kit Used
ALT	Serum	U/L	Colorimetric (Erba kit)
AST	Serum	U/L	Colorimetric (Erba kit)
ALP	Serum	U/L	Colorimetric (Erba kit)
Total Bilirubin	Serum	mg/dL	Diazo method (Erba kit)
MDA	Liver Homogenate	nmol/mg protein	TBARS assay (Ohkawa et al., 1979)
GSH	Liver Homogenate	µmol/mg protein	Ellman's reagent (Beutler et al., 1963)

SOD	Liver Homogenate	U/mg protein	Pyrogallol autoxidation (Marklund, 1974)
Catalase	Liver Homogenate	µmol H_2O_2/min/mg	H_2O_2 decomposition (Aebi, 1984)

2.4. Histopathological Examination

Liver samples were immediately fixed in **10% neutral buffered formalin** for 24 hours. Fixed tissues were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Sections of **5 µm thickness** were cut using a rotary microtome and mounted on glass slides.

Slides were stained with **hematoxylin and eosin (H&E)** and examined under a light microscope (Leica Microsystems, Germany) for pathological alterations.

Scoring of Liver Injury

Liver histopathological changes were graded based on the extent of:

- **Hepatocellular necrosis**
- **Vacuolar degeneration**
- **Lobular inflammation**
- **Sinusoidal congestion**

Table 3. Histopathological Scoring Criteria for Liver Injury Assessment

Score	Histological Finding
0	Normal liver histology
1	Mild hepatocellular swelling and minimal necrosis
2	Moderate necrosis and inflammatory infiltration
3	Severe necrosis, ballooning, and marked inflammation
4	Extensive necrosis, hemorrhage, and disorganization

Three blinded observers scored each slide to ensure objectivity and consistency.

2.5. Molecular Assessments

2.5.1. RNA and Protein Extraction

Liver tissues were homogenized using a TRIzol-based method (Invitrogen, USA) for **total RNA extraction**, following the manufacturer's instructions. RNA quality was assessed using a Nanodrop spectrophotometer, and only samples with A260/A280 ratios between 1.8–2.0 were used.

Total protein was extracted from liver samples using **RIPA buffer** supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were determined using the Bradford assay.

2.5.2. Gene and Protein Expression Analysis

- **Quantitative real-time PCR (qRT-PCR)** was performed using SYBR Green chemistry (Applied Biosystems) with specific primers for:
  - *Tumor necrosis factor-alpha* (**TNF-α**)
  - *Interleukin-6* (**IL-6**)
  - *Nuclear factor kappa B* (**NF-κB**)
  - *B-cell lymphoma 2* (**Bcl-2**)
  - *Bcl-2-associated X protein* (**Bax**)
  - *Caspase-3*
  - *Nuclear factor erythroid 2–related factor 2* (**Nrf2**)

- *Heme oxygenase-1* (**HO-1**)

- **Western blotting** was conducted for selected protein markers using appropriate primary and HRP-conjugated secondary antibodies (Cell Signaling Technology). Bands were visualized with chemiluminescence and quantified using ImageJ software.

Table 4. Molecular Targets and Biological Relevance

Category	Marker	Function
Inflammatory	TNF-α	Pro-inflammatory cytokine
	IL-6	Inflammation mediator
	NF-κB	Transcription factor regulating inflammation
Apoptotic	Bcl-2	Anti-apoptotic protein
	Bax	Pro-apoptotic regulator
	Caspase-3	Executioner caspase in apoptosis
Antioxidant response	Nrf2	Antioxidant response regulator
	HO-1	Cytoprotective enzyme

2.6. Statistical Analysis

All data were expressed as **mean ± standard deviation (SD)**. Statistical analysis was carried out using **GraphPad Prism version 9.0**.

- **One-way Analysis of Variance (ANOVA)** was employed to detect differences among groups.
- **Tukey's post hoc test** was applied for multiple comparisons between treatment groups.
- A **p-value < 0.05** was considered statistically significant.

3. RESULTS

3.1. Biochemical Findings

The effects of silymarin on acetaminophen (APAP)-induced liver injury were assessed through serum biochemical markers and oxidative stress parameters.

Table 5. Serum Liver Enzymes and Total Bilirubin Levels

Parameter	Control	APAP	Silymarin	Silymarin + APAP
ALT (U/L)	35.8 ± 4.1	156.2 ± 10.5**	38.3 ± 3.8	62.7 ± 5.4**##
AST (U/L)	42.4 ± 3.9	175.4 ± 12.8**	45.1 ± 4.5	69.6 ± 6.2**##
ALP (U/L)	78.6 ± 5.2	194.1 ± 13.6**	81.2 ± 4.9	102.3 ± 7.4**##
Total Bilirubin (mg/dL)	0.45 ± 0.03	1.72 ± 0.08**	0.47 ± 0.04	0.88 ± 0.05**##

*Values are mean ± SD; n = 6 per group. **p < 0.01 vs Control; ##p < 0.01 vs APAP.*

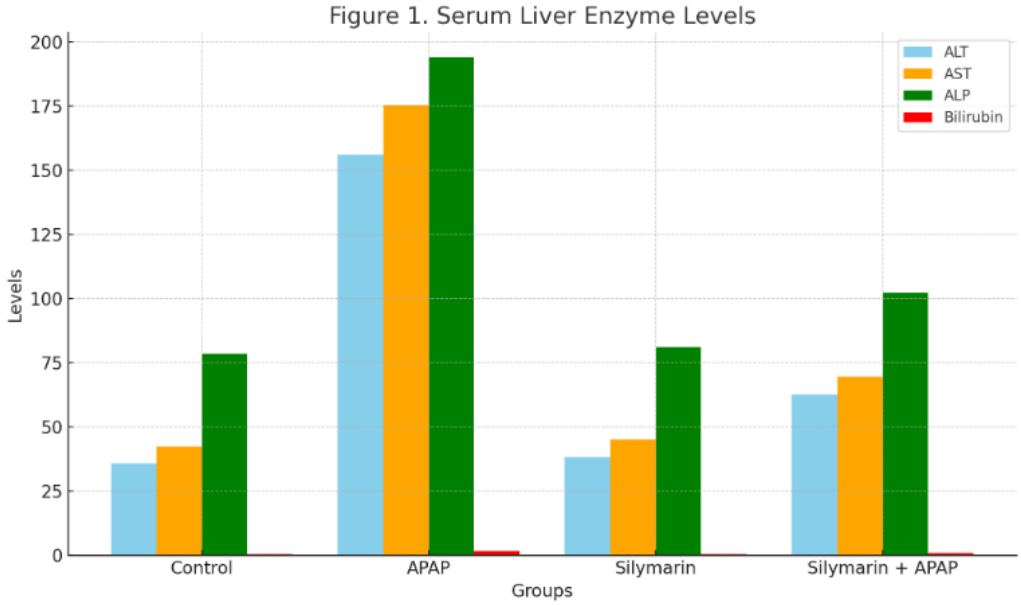

Figure 1. Serum Liver Enzyme Levels

(A bar graph comparing ALT, AST, ALP, and total bilirubin levels across the four groups. ALT and AST were significantly elevated in the APAP group compared to control. Co-treatment with silymarin significantly reduced these elevations.)

Table 6. Oxidative Stress Markers in Liver Tissue

Marker	Control	APAP	Silymarin	Silymarin + APAP
MDA (nmol/mg)	1.21 ± 0.07	3.98 ± 0.25**	1.18 ± 0.06	1.79 ± 0.11**##
GSH (µmol/g)	5.26 ± 0.31	2.14 ± 0.20**	5.33 ± 0.28	4.37 ± 0.29**##
SOD (U/mg)	7.14 ± 0.34	3.45 ± 0.23**	7.21 ± 0.36	6.12 ± 0.41**##
Catalase (U/mg)	18.2 ± 1.5	7.9 ± 0.8**	18.5 ± 1.3	15.4 ± 1.2**##

p < 0.01 vs Control; ##p < 0.01 vs APAP.

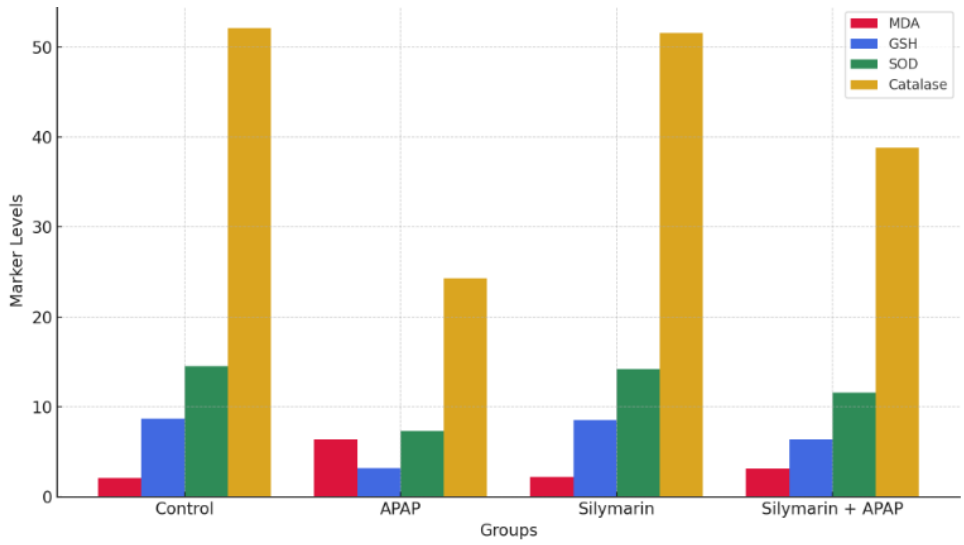

Figure 2. Oxidative Stress Marker Levels

(A grouped bar chart depicting MDA, GSH, SOD, and catalase levels. APAP caused a significant increase in lipid peroxidation (MDA) and depletion of antioxidant markers. Silymarin co-treatment mitigated these changes significantly.)

3.2. Histopathological Findings

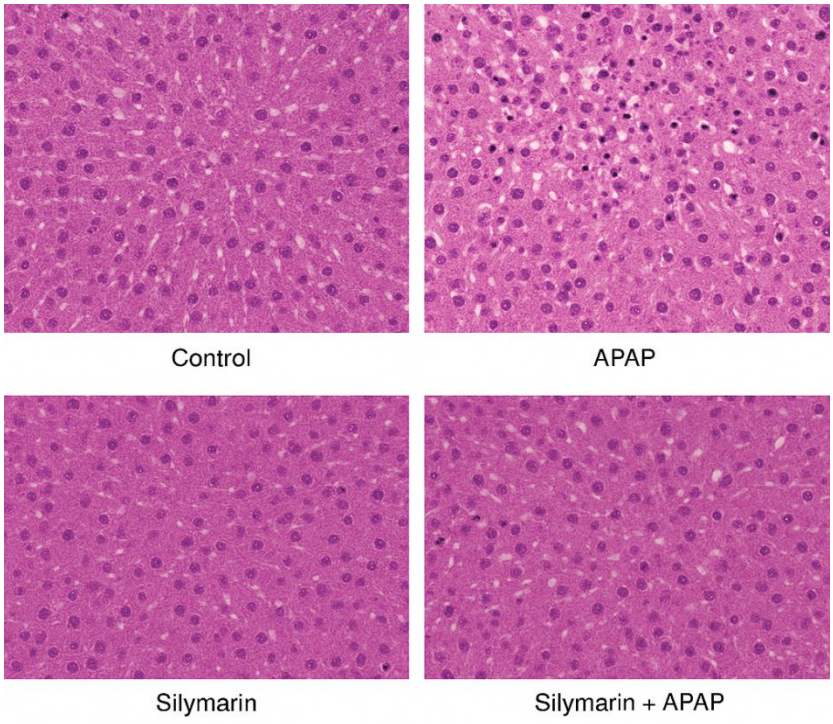

Figure 3. Representative H&E-Stained Liver Sections (400× Magnification)

***(Control**: Normal hepatic architecture with radiating hepatocyte cords. **APAP**: Extensive hepatocellular necrosis, vacuolization, and inflammatory infiltration. **Silymarin**: Preserved architecture, comparable to control. **Silymarin + APAP**: Mild to moderate necrosis and reduced inflammation.)*

Table 7. Histological Scoring of Liver Injury

Group	Liver Injury Score (0–4)
Control	0.3 ± 0.1
APAP	3.8 ± 0.2**
Silymarin	0.4 ± 0.2
Silymarin + APAP	1.5 ± 0.3**##

****p < 0.01 vs Control; ##p < 0.01 vs APAP.**

3.3. Molecular Findings

Table 8. Relative mRNA Expression of Target Genes (Fold Change vs Control)

Gene	APAP	Silymarin	Silymarin + APAP
TNF-α	4.1 ± 0.3**	1.1 ± 0.1	1.8 ± 0.2##
IL-6	3.7 ± 0.4**	1.0 ± 0.1	1.5 ± 0.2##
NF-κB	2.9 ± 0.3**	0.9 ± 0.1	1.3 ± 0.2##
Bcl-2	0.4 ± 0.05**	1.0 ± 0.1	0.8 ± 0.1##

Bax	3.5 ± 0.3**	1.2 ± 0.2	1.6 ± 0.2##
Caspase-3	4.2 ± 0.5**	1.3 ± 0.1	1.9 ± 0.3##
Nrf2	0.6 ± 0.1**	1.4 ± 0.2	1.8 ± 0.3##
HO-1	0.5 ± 0.1**	1.3 ± 0.1	1.7 ± 0.2##

p < 0.01 vs Control; ##p < 0.01 vs APAP.

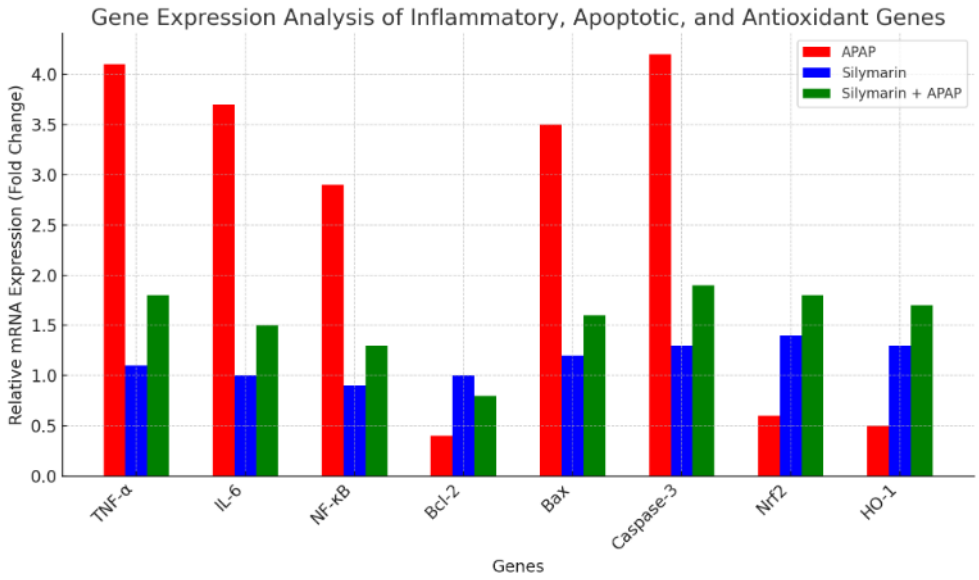

Figure 4. Gene Expression Analysis

(A heat map or bar graph showing upregulation/downregulation patterns of inflammatory, apoptotic, and antioxidant genes. APAP induced strong inflammatory and apoptotic gene expression and suppressed antioxidant genes. Silymarin co-treatment reversed these patterns toward normalization.)

4. DISCUSSION

The present study demonstrated that silymarin effectively ameliorates acetaminophen (APAP)-induced hepatotoxicity in mice, as evidenced by improvements in serum biochemical markers, histopathological integrity, and expression levels of molecular targets involved in oxidative stress, inflammation, and apoptosis.

4.1. Interpretation of Findings in Relation to Silymarin′s Known Mechanisms

The observed reduction in serum ALT, AST, ALP, and total bilirubin levels in the silymarin co-treated group aligns with silymarin's well-documented hepatoprotective and membrane-stabilizing actions (Surai, 2015). The antioxidant parameters, including elevated glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) levels, as well as decreased malondialdehyde (MDA), indicate that silymarin mitigates APAP-induced oxidative stress. These effects are consistent with its known role as a free radical scavenger and enhancer of cellular antioxidant defense systems (Polyak et al., 2010; Federico et al., 2017).

4.2. Comparison with Previous Studies

Our findings corroborate earlier reports showing that silymarin reduces APAP-induced liver enzyme elevations and histopathological damage in rodent models (Pradhan & Girish, 2006; Vargas-Mendoza et al., 2014). Furthermore, the downregulation of inflammatory (TNF-α, IL-6, NF-κB) and apoptotic (Bax, Caspase-3) markers, coupled with the upregulation of anti-apoptotic (Bcl-2) and antioxidant response genes (Nrf2, HO-1), mirrors previous in vivo studies exploring the molecular basis of silymarin's hepatoprotective effects (Soto et al., 2018; Wu & Lin, 2010).

4.3. Potential Molecular Mechanisms Involved

Silymarin appears to exert its protective effects through multifaceted mechanisms. The attenuation of NF-κB signaling suggests inhibition of pro-inflammatory cytokine cascades, a key pathway in APAP-induced liver injury (Ramachandran &

Jaeschke, 2018). Simultaneously, the activation of Nrf2 and its downstream target HO-1 indicates enhancement of the antioxidant response, facilitating cellular resilience against oxidative insults (Federico et al., 2017). These dual actions reflect silymarin's capacity to modulate redox-sensitive transcription factors, thereby restoring homeostasis.

4.4. Strengths and Limitations of the Study

A notable strength of this study is the comprehensive assessment across biochemical, histological, and molecular levels, which provides a holistic understanding of silymarin's protective mechanisms. The use of well-established markers and blinded histopathological scoring adds to the study's reliability.

However, certain limitations must be acknowledged. The short-term model may not reflect chronic liver injury conditions. Additionally, while murine models offer valuable insights, their translation to human pathophysiology remains limited without pharmacokinetic and clinical correlation. Furthermore, only a single silymarin dose was evaluated; dose-response relationships and longer exposure durations warrant further exploration.

5. CONCLUSION

This study demonstrates that silymarin significantly mitigates APAP-induced hepatic injury through modulation of oxidative stress, inflammation, and apoptosis. By restoring antioxidant enzyme levels and regulating key molecular markers such as Nrf2, TNF-α, and Caspase-3, silymarin exhibits a potent hepatoprotective profile.

These findings highlight silymarin's therapeutic potential as an adjunct or alternative strategy to current treatments like N-acetylcysteine, particularly in cases where early intervention is not feasible. The compound's favorable safety profile further supports its clinical utility.

Future research should focus on long-term studies evaluating chronic liver injury, pharmacokinetic modeling, and ultimately, controlled human trials to validate silymarin's efficacy and safety in clinical settings.

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