Exploring Antioxidant and Anti-Inflammatory Responses in CCL4-Induced Liver Injury Mouse Model

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In the Name of ALLAH, the Most Gracious, the Most Merciful.

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CERTIFICATE

This thesis, submitted by **Mr. Raza Sufyan** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

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Dated: **April 2, 2024**

Declaration

I hereby declare the authenticity and originality of the work presented in this thesis. It is an honest embodiment of my scholarly research. It is written and composed by me. No part of this thesis has ever been published or submitted for another degree or certificate.

RAZA SUFYAN

Dedicated to my beloved family and friends

for their continuous support, love,

compassion, and blessings.

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RAZA SUFYAN

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LIST OF ABBREVIATIONS

ATF-6	Activating transcription factor 6
ASC	Apoptosis-associated speck-like protein containing a CARD
DAMPs	Damage-associated molecular patterns
ER	Endoplasmic Reticulum
GSDMD	Gasdermin
GSH	Glutathione
H & E staining	Hematoxylin and eosin
IKB	I-kappa B
IRE-1	Inositol-requiring enzyme 1
JAK	Janus Kinase
JNK	Jun N-terminal Kinase
LRR	Leucine rich Repeat
MCP-1	Macrophage chemoattractant protein-1
MAMs	Mitochondria-associated ER membranes
MYDD88	Myeloid differentiation primary response 88
NEK7	NIMA Related Kinase 7
COX-2	Cyclooxygenase-2
mTORC1	Mammalian Target of Rapamycin complex 1
NLRP3	NLR family Pyrin domain containing 3
NFKB	Nuclear factor kappa B
PAMPs	Pathogen-associated molecular patterns

PBS	Phosphate Saline Buffer
PI3K	Phosphoinositide 3 kinase
PCR	Polymerase Chain Reaction
PKB/AKT	Protein Kinase B
TXNIP	Thioredoxin-interacting protein
PKC	Protein Kinase C
ATF5	Activating Transcription Factor 5
PERK	Protein Kinase R-like ER Kinase
PTPs	Protein tyrosine phosphatases
ROS	Reactive oxygen species
SD	Standard Deviation
TLR4	Toll like Receptor 4
TLRs	Toll-like receptors TLRs
TNF-α	Tumor Necrosis Factor α
UPR	Unfolded Protein Response
FGF	Fibroblast Growth Factor
HSC	Hepatic Stellate Cells
α-SMA	Alpha Smooth Muscle Actin
ECM	Extracellular matrix
MMP	Matrix Metalloproteases
TIMP	Tissue Inhibitor Metalloproteases
KC	Kupffer Cells

APAP	Acetaminophen
TAG	Triacyl Glyceride
CYP2E1	Cytochrome P450 Isomerases
NRF2	Erythroid 2-related Factor 2
KEAP1	Kelch-like ECH-associated Protein 1
TASO	Thioacetamide S-Oxide
NK	Natural Killer Cells

ABSTRACT

Liver injury is a major global health concern that is caused due to the damage of parenchymal and non-parenchymal cells of liver. It is characterized by oxidative stress and inflammation that accelerates to fibrosis, cirrhosis and hepatocellular carcinoma. In the liver injury major intracellular signaling pathways are activated. The inhibition of these pathways is a therapeutic target for treatment the of liver injury. Several studies have reported that juglone extracted from *Reynoutria japonica* has antioxidant, anti-inflammatory, anti-fibrotic and antidiabetic activity. In our study, we developed CCL4 liver injury mice model through multiple doses of 30% CCL4 on alternative days that mimics human liver injury pathogenesis. Hepatoprotective activity of juglone (5-hydroxy-1,4-naphthoquinones) and silymarin as a positive control was evaluated for hepatoprotection. Our compound treated group showed regain in the body weight and improved AST, ALT, and ALP profile in the serum. Histological analysis of liver confirms hepatoprotective activity of compound. Real time-PCR data shows overexpression of genes involved in inflammatory pathways, oxidative stress, liver fibrosis and cell death due to apoptosis. The genes were downregulated in compound treated group and silymarin group. Therefore, we concluded that our compound has hepatoprotective activity.

Keywords: Liver injury, Oxidative Stress, Inflammation, Liver fibrosis, Juglone, CCL4

1. Introduction

1.1 Liver

The liver, a crucial organ present below diaphragm behind the lower ribs (Arslan, 2005), normally has a brown color and weighs approximately higher in male, having about 2% of total body weight. The liver is supplied with 75% of its blood from the hepatic portal vein and 25% from the hepatic artery. Interacting with the digestive and endocrine systems, the liver performs significant functions in the human body. Physiologically, it is divided into four lobes (two larger and two smaller). Two larger lobes (right and left) are separated with falciform ligament which attaches it to the abdominal cavity (Sibulesky, 2013). The hexagonal lobule is the functional unit, containing a portal triad at each corner. Hepatocytes, with basolateral and apical membranes, form the structural basis of lobules, which are further divided into three zones. Zone I, the periportal region, is the first to regenerate due to its abundant oxygen and nutrients, with cells involved in various metabolic processes. Zone II, the pericentral region, lies between zones I and III. The perivenous region is the Zone III that has the least oxygen and is primarily involved in functions such as ketogenesis, lipogenesis, and detoxification. Blood and bile move in opposite directions within the liver, facilitated by its dual blood supply. Blood from both supplies drains into the hepatic vein, located at the center of the hexagonal lobule (Saxena, Theise, & Crawford, 1999). The Space of Disse, present in the sinusoidal lining of the lobule, contains microvilli extending from basolateral membranes, facilitating communication with blood capillaries and aiding in blood supply to hepatocytes. Additionally, tissue-resident macrophages (Kupffer cells) and hepatic stellate cells (HSC) are found in the Space of Disse.

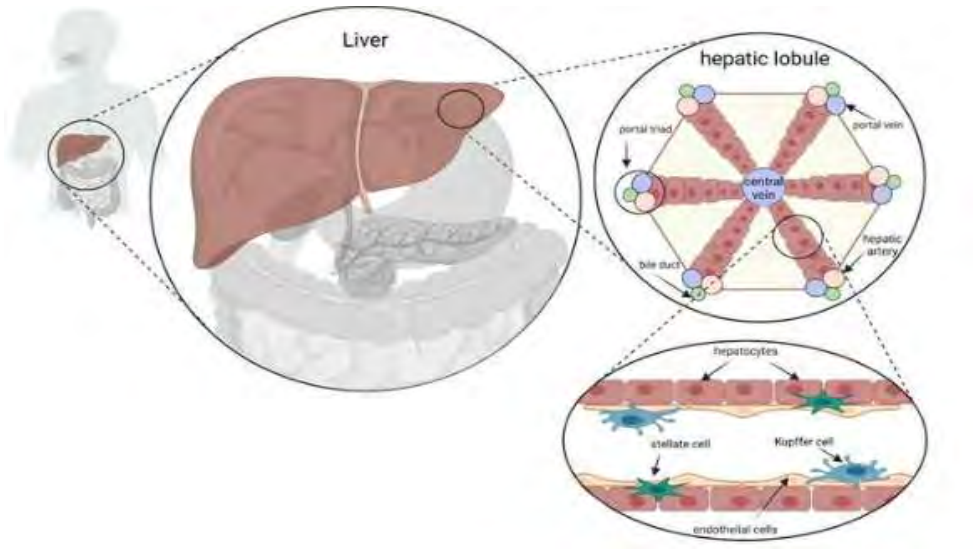


Figure 1.1 Liver lobule having parenchymal and non-parenchymal cells (Paradiso et al., 2023)

blood supply to hepatocytes. Additionally, tissue-resident macrophages (Kupffer cells) and hepatic stellate cells (HSC) are found in the Space of Disse. The liver, originating from the endoderm of the zygote, develops as part of the foregut, with development commencing after the fourth week of gestation. The fibroblast growth factor released by fetal cardiac cells and Wnt/β-catenin signaling pathways play an important role in development of liver.

1.1.1. Functions of Liver

Hepatocytes are pivotal in several essential functions including bile formation, metabolism of carbohydrates, proteins, and lipids, storage of fat-soluble vitamins, and detoxification of drugs (Dutta et al., 2021). Bile, synthesized by hepatocytes, serves crucial roles in excretion of waste materials, digestion, and lipid absorption. Stored in the gall bladder, bile is released into the duodenum via the common bile duct. Bile components are reprocessed into bile acids by microbiota of gut and reabsorbed through the ileum for reuse. Liver metabolism involves synthesizing glycogen

through glycogenesis and glucose through gluconeogenesis during carbohydrate metabolism. It also manages protein metabolism, synthesizing and degrading proteins. Liver is responsible for producing blood clotting proteins like fibrinogen and prothrombin. Additionally, it plays an important role in metabolism of lipids, synthesizing cholesterol, lipoproteins, and triacylglycerides (TAG). Ito cells also known as HSC in the liver are involved in storing fat-soluble vitamins. Vitamin K is not stored or metabolized in the liver. Liver's critical function in drug metabolism involves biotransformation of harmful substances like CCL4, Acetaminophen (APAP), and xenobiotics like bromobenzene. This process occurs in three phases: the first phase entails enzymes in the smooth endoplasmic reticulum (SER), such as cytochrome P450, converting lipophilic compounds into hydrophilic ones containing oxygen. In the second phase, conjugation reactions occur with antioxidants like glutathione. Overdosing of xenobiotics can deplete antioxidants such as glutathione, leading to liver injury, inflammation, fibrosis, and Hepatocellular carcinoma (HCC) (Almazroo et al., 2017).

1.1.2. Parenchymal and Non-Parenchymal Cells of Liver

The liver is composed of various cell types, with hepatocytes constituting 60% of its cellular composition. Alongside hepatocytes, other essential cells include Kupffer cells, hepatic stellate cells, and pit cells. Hepatocytes are primarily responsible for metabolic functions, detoxification, and immune activation within the liver. They enhance nutrient absorption by extending microvilli into the space of Disse. Kupffer cells, comprising 30% of liver cells, are tissue-resident macrophages crucial for pathogen phagocytosis. Derived from liver monocytes, they reside in the liver sinusoid and alter their phenotype upon activation by various stimuli. Hepatic stellate cells, also known as Ito cells, are non-parenchymal cells found in the space of Disse. In their inactive state, they store fat-soluble vitamins. However, during pathophysiological conditions, they become activated, contributing to extracellular matrix release and transformation into myofibroblast-like cells (Liaskou et al., 2012). Pit cells, specific natural killer cells in the liver, play a crucial role in inborn immune response, pathogen defense, and immune balance maintenance. Cholangiocytes, specialized non-parenchymal cells, aid in bile formation, modification, secretion, and

flow maintenance, facilitating fat digestion in the duodenum. Liver sinusoidal endothelial cells, comprising 15-20% of liver cells, form a permeable barrier between blood and liver cells. Their unique fenestrations and absence of a basement membrane facilitate their role in maintaining the inactivated state of Kupffer cells and hepatic stellate cells, as well as antigen presentation and immune cell recruitment during liver injury (Poisson et al., 2017).

1.2. Epidemiology of Liver Diseases

Liver diseases pose a significant global health concern, with over 25,000 deaths annually attributed to liver injury and cirrhosis for the past two decades. Projections suggest this number may rise to 40,000 in the future. Specific liver ailments such as liver injury, hepatocellular carcinoma, and age-adjusted death rates are well-documented. However, reliable data on the incidence of drug-induced hepatotoxicity is lacking (Higuchi & Gores, 2003). According to 2020 WHO data, liver diseases accounted for 3.76% of total deaths in Pakistan (World Life Expectancy). Several factors contribute to liver injuries, including chemical exposure in industries, environmental pollution, medication misuse, aflatoxin contamination, and inadequate safety standards in industrial and agricultural settings.

1.3. Liver injury

Liver injury is indeed a significant global health concern, often resulting from exposure to various environmental toxins, xenobiotics, and other chemicals. These substances can exacerbate inflammation, fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC), highlighting the critical importance of understanding liver pathophysiology and developing effective interventions. Xenobiotics, which are foreign substances not produced by the human body, can enter the body through different routes such as ingestion, inhalation, or skin absorption. Once inside the body, these chemicals can exert hepatotoxic effects, causing damage to liver tissue. Examples of hepatotoxic xenobiotics include thioacetamide, acetaminophen (APAP), carbon tetrachloride (CCl4), and carbon disulfide (Hochstein et al., 2007). The biotransformation of xenobiotics occurs primarily in hepatocytes, where lipophilic compounds are converted into more hydrophilic forms to facilitate their excretion from the body. This process involves various enzymatic reactions, including

hydrolysis, reduction, oxidation, and conjugation. Among these, oxidation is a crucial step mediated by enzymes such as cytochrome P450 (CYP450), which play a crucial role in the metabolism of numerous xenobiotics. During biotransformation, reactive metabolites may be generated, which can potentially damage cellular components. To prevent the adverse effects of these reactive intermediates, hepatocytes possess antioxidant defense mechanisms. These antioxidants scavenge free radicals and conjugate with reactive metabolites, rendering them less harmful and facilitating their excretion from the body. Understanding the mechanisms underlying xenobiotic metabolism and liver injury is essential for developing strategies to mitigate liver damage and improve patient outcomes. By elucidating these processes, researchers can identify potential targets for therapeutic intervention and develop novel treatments for liver diseases caused by exposure to environmental toxins and xenobiotics.

1.3.1. Risk Factors of Liver injury

Drug-induced liver injury (DILI) is the combined effect of hepatotoxic drugs, genetic and non-genetic factors. The drug related risk factors of liver injury including daily dose, lipophilicity of drugs and reactive metabolites such as ROS and RNS are produced through drugs. Host-related factors including genetic and non-genetic factors. The non-genetic factors includes unhealthy lifestyle such as alcohol intake, smoking, and genetics factors are mitochondrial DNA mutation and variation in metabolic enzymes. The following figure 1.2 shows the main risk factors of DILI.

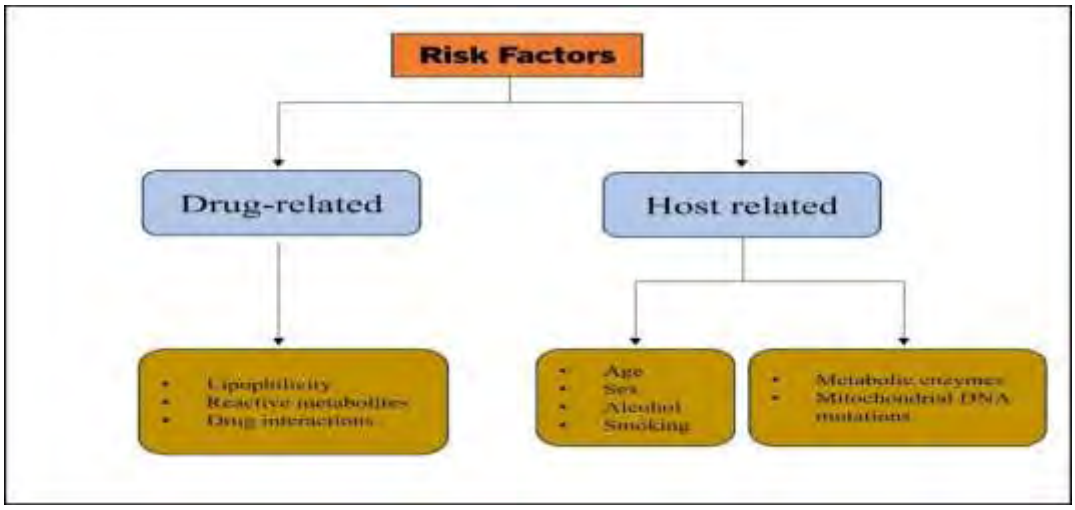


Figure 1.2 Risk factors of liver injury

1.3.2. Chemical-Induced Liver Injury Models

Animal models play a crucial role in scientific experimentation as they provide valuable insights into the functioning of the human body and allow researchers to study various diseases and conditions in controlled settings. When it comes to drug-induced liver injury models, certain chemicals are commonly used to induce liver injury in animals, including thioacetamide (TAA), acetaminophen (APAP), and carbon tetrachloride (CCl4). Among these, APAP and CCl4 are particularly popular choices. Acetaminophen (APAP) is extensively employed to cause liver injury in animal models due to its clinical relevance and well-understood mechanism of toxicity. APAP is metabolized in the liver by cytochrome P-450 enzymes, leading to the formation of a highly reactive metabolite called N-acetyl-p-benzoquinoneimine (NAPQI). Excessive production of NAPQI can deplete glutathione (GSH) levels in the liver, accelerate to oxidative stress and hepatocellular injury. This process ultimately results in cell death mediated by caspase activation (Mitchell et al., 1973). Thioacetamide (TAA) is other hepatotoxin that was previously utilize as a fungicide. In animal models, TAA is typically administered orally or via intraperitoneal injection. Once inside the body, TAA is metabolized by cytochrome (P4502E1), and formreactive metabolites, such as thioacetamide S-oxide (TASO) and thioacetamide S-dioxide (TAASO2). These metabolites induce liver injury and oxidative stress, contributing to the development of liver pathology (McGill & Jaeschke, 2019). Overall, these chemical-induced liver injury models provide researchers with valuable tools to study the underlying mechanisms of liver toxicity and explore potential therapeutic interventions for liver diseases.

1.4. Pathophysiology of CCL4 Liver Injury

Carbon tetrachloride (CCl4) is a colorless volatile liquid formed by the mixture of chlorine with chloroform in the presence of light. It is known to cause hepatic damage by elevating levels of Alanine transaminase (ALT), Alkaline phosphatase (ALP), and Aspartate aminotransferase (AST). Historically, it was used as a cleaning agent, in industrial manufacturing, and for synthesizing refrigerants. Human toxicity can occur through inhalation, dermal absorption, or ingestion, leading to cell damage in multiple organs such as the liver, kidneys, and lungs (Al Amin & Menezes, 2020). CCl4 is

highly useful as an experimental model for hepatotoxic effects and produces liver injury in both humans and other non-human mammals (Yoshida et al., 1999). The toxicity induced by CCl4 depends on the dose and duration of exposure. Low doses can lead to impairment of calcium homeostasis, release of cytokines, apoptosis, and regeneration, while high doses and prolonged exposure can cause more serious permanent damage to the liver, including inflammation, fibrosis, cirrhosis, nonspecific solvent toxicity, depression of the central nervous system, respiratory failure, and hepatocellular carcinoma (Berger et al., 1986). The liver is the principal site for CCl4 metabolism, although there are no specific receptors present on hepatocytes. The toxicity of reactive compounds involves primary and secondary bond formation with targeted biomolecules including lipids, proteins, and DNA leading to hepatocyte damage. The endoplasmic reticulum, mitochondria, and Golgi apparatus are the main organelles affected by CCl4 induction (Reynolds, 1963). CCl4 belongs to the first group of hepatotoxins metabolized by the isoenzyme of cytochrome P450 named CYP2E1, which forms reactive oxygen species (ROS) leading to lipid peroxidation and destruction of hepatocytes by forming free radicals (CCl3*, CCl2*, CCl3-OO*). The major cytochrome enzyme involved in the biotransformation of CCl4 is CYP2E1, although CYP2B1 and CYP2B2 are also capable of metabolizing CCl4. In mammals, CYP2E1 is dominant over the other isoforms (Gruebele et al., 1996). Both parenchymal and non-parenchymal cells of the liver are involved in the molecular events of CCl4 intoxication. Non-parenchymal cells include Kupffer cells and hepatic stellate cells, which are activated by the release of different cytokines from hepatocytes, causing damage to the liver. Major cytokines involved in their activation include TNF-alpha, IL-1, IL-6, IL-10, and TGFβ. When hepatic stellate cells become activated, they transform into fibroblast-like cells that release extracellular matrix (ECM). Their activation can be assessed by the expression of alpha-smooth muscle actin (α-SMA) (Hellerbrand et al., 1999). The release of TNF-alpha, interleukins, and other signals leads to liver inflammation, oxidative stress, apoptosis, and liver fibrosis through crosstalk between parenchymal and non-parenchymal cells.

1.5. Oxidative Stress in Liver Injury

In mammals, a sophisticated antioxidant system has evolved to counteract oxidative stress induced by reactive oxygen species (ROS). However, excessive ROS production can lead to oxidative stress, damaging hepatocytes. Various factors including alcohol, environmental pollutants, and chemicals like CCL4 can induce oxidative stress in the liver (Li et al., 2015). Oxidants bestowing to oxidative stress can be categorized into two types: endogenous and exogenous. Exogenous oxidants, like free radicals, are highly reactive molecules with unpaired electrons (Finkel et al., 2000). Among free radicals, oxygen-containing radicals, including superoxide, peroxyl radicals, and hydroxyl radicals, known as ROS, are particularly significant (Apel et al., 2014). ROS can induce lipid peroxidation and damage proteins, nucleic acids, and other biomolecules, leading to tissue and organ injury (McCord et al., 2000). During physiological conditions, ROS are produced and serve as defensive mechanisms against pathogens and signaling molecules regulating cell growth and death. However, elevated ROS levels cause oxidative stress, known as an imbalance between oxidant and antioxidant agents, resulting in liver injury through apoptotic and necrotic cell death pathways (McCord et al., 2000). The liver is a major target of ROS in mammals, primarily affecting liver parenchymal cells (Sánchez-Valle et al., 2012). Several subcellular structures, including mitochondria, endoplasmic reticulum (ER), microsomes, and peroxisomes, contribute to ROS production, regulated by (PPARα), that is associated with fatty acid gene expression. Non-parenchymal cells in the liver, such as Kupffer cells (KC) and hepatic stellate cells (HSC), are sensitive to oxidative stress. KCs can induce inflammation and apoptosis by releasing various cytokines like TNFα, while HSCs can contribute to liver fibrosis by releasing extracellular matrix (ECM). Oxidative stress is a major pathological change underlying various liver disorders (Feng et al., 2011).

1.5.1. Role of Nrf2/Keap1 Signaling Pathway in Oxidative Stress

The Nrf2-Keap1 signaling pathway plays a crucial role in regulating oxidative stress within hepatocytes. Nrf2 (Erythroid 2-related factor 2) controls the gene expression of antioxidant enzymes such as NADPH, NQO1, GST, GSH-Px, and HO1. There are 605 amino acids present in Nrf2 and have seven domain named as Nrf2-ECH, which

are essential for its stability and regulation of transcriptional activity (Bellezza et al., 2018). Keap1 acts as inhibitor of Nrf2. It comprises 624 amino acids and consists of five domains. Under normal conditions, Keap1 binds to the N-terminal of Nrf2, inhibiting its activity and preventing its translocation into the nucleus. The inactivation of Nrf2 is facilitated by Keap1 through the Cullin3-Ring box protein (Cul3-Rbx) complex, leading to Nrf2 breakdown by ubiquitination proteosome. (Bryan et al., 2013). However, during oxidative stress, ROS can modify Keap1, preventing its interaction with Nrf2. As a result, Nrf2 is no longer targeted for degradation and can accumulate in the nucleus. Once inside the nucleus, Nrf2 enhances the expression of Phase II antioxidant enzymes, thereby increasing defense mechanisms in response to oxidative stress. This activation of Nrf2 by ROS-modified Keap1 represents a critical adaptive response to oxidative stress within hepatocytes.

1.5.2. Role of Oxidative Stress in Hepatocytes

The liver is continuously exposed to various toxic reactive metabolites, including (ROS), which shift the internal environment towards oxidative stress, often considered the initial step in liver pathogenesis. Liver cells are particularly susceptible to factors such as insulin resistance, alcohol abuse, high-fat diets, chemicals, drugs, and environmental toxins, leading to a rapid increase in ROS concentration within cells. Parenchymal liver cells, in particular, are vulnerable to ROS-induced damage, resulting in cell injury, inflammation, scar formation, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). With 1000 to 2000 mitochondria present in hepatocytes, ROS-mediated lipid peroxidation alters membrane permeability and disrupts the respiratory chain, exacerbating oxidative stress (Li et al., 2015). The accumulation of ROS within hepatocytes induces cell death, releasing Damage-Associated Molecular Patterns (DAMPs) from the cells. In response, non-parenchymal liver cells such as Kupffer cells (KCs), hepatic stellate cells (HSCs), and other immune cells are recruited, leading to the release of proinflammatory and profibrogenic mediators (Tanaka et al., 2016). The activation of cause secretion of cytokines such as IL-6, IL-18, TNFα, Fas, and TGFβ, which promote inflammation, Fas/FasL-dependent apoptosis of hepatocytes, and the activation of HSCs, leading to the deposition of extracellular matrix (ECM). However, the specific changes in Pit cells (NK cells) in the liver mediated by oxidative stress remain unclear.

1.6. Oxidative Stress in Liver Fibrosis

Liver fibrosis is a improvable natural wound healing process that occurs in response to hepatocyte damage, characterized by the deposition of extracellular matrix (ECM). In the progression of hepatic fibrogenesis, changes related to scar formation and liver fibrosis arise due to the lack of inhibition and the inability to eliminate harmful agents (Acharya et al., 2021). There are five stages of liver fibrosis. F0 represents the initial stage with no scar formation, while F1 indicates minimal scar formation. As the fibrosis progresses, it advances to F2, where scars extend outside liver tissue, followed by F3, involving spreading to other parts of the liver, and finally F4, which leads to cirrhosis. During hepatic fibrosis, activated hepatic stellate cells release extracellular matrix proteins, while other cells such as Kupffer cells release proinflammatory and profibrotic mediators, contributing to fibrosis. The extracellular matrix mainly comprises collagen type I and other non-collagenous proteins such as proteoglycans, fibronectin, and laminin (Arriazu et al., 2014). Molecular pathways involved in hepatic fibrosis include the TGFβ/SMAD signaling pathway, Wnt/β-catenin pathway, and Hedgehog signaling (Zhanget al., 2022). Among these, the TGFβ/SMAD signaling pathway is the most profibrogenic. Activation of this pathway occurs in hepatic stellate cells through the ligand TGFβ, which binds to type-I and type-II receptors, forming dimers. The activated extracellular domain phosphorylates a specific region known as the GS domain, initiating an intracellular signaling pathway that recruits SMAD proteins to transport information from the receptor to the nucleus. Dimerization of SMAD3/4 results in translocation into the nucleus, where they act as transcription factors, increasing gene expression involved in fibrosis, including collagen type I and α-SMA *(ACTA2).* (Bissell et al., 2001).

1.7. Inflammation and Its Significance in Liver Injury

The human liver, primarily recognized as a non-immunological organ, serves vital functions in detoxification, metabolism, and nutrient storage. Nonetheless, inflammation in the liver is a crucial response mechanism aimed at combating infections and eliminating external toxins or xenobiotics. Failure to effectively remove these substances can lead to liver injury, which may progress to more severe conditions such as liver fibrosis, cirrhosis, and liver failure (Robinson et al., 2016).

Inflammation in the liver is a complex process involving the response to pathogens and xenobiotic chemicals, ultimately resulting in cell damage and apoptosis (Pegoretti et al., 2020). Chronic inflammation is closely associated with liver fibrosis, often accompanied by necrosis and apoptosis of hepatocytes (Lee et al., 2011). The release of apoptotic bodies from liver cells plays a role in activating hepatic stellate cells and Kupffer cells, contributing to hepatic fibrosis (Cichoz et al., 2008). Inflammatory chemicals such as cytokines (e.g., IL-1, IL-6, IL-1β, IL-18) and TNF-α are secreted by leukocytes and macrophages in response to tissue injury (Schmid-Schönbein, 2006). These inflammatory mediators subsequently activate various intracellular signaling pathways, orchestrating the inflammatory response in the liver.

1.7.1. Inflammatory Cascades in Liver Injury

Inflammation of liver is brought about by the activation of immune cells within the liver, including Kupffer cells, monocytes, neutrophils, and dendritic cells. When hepatocytes are damaged, they release molecules known as damage-associated molecular patterns (DAMPs). These DAMPs are recognized by PRRs present on various cells within the liver. PRRs come in different structural forms, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs) (Martinon et al., 2002). Upon recognition of DAMPs, PRRs become activated, cause the activation of caspases, which, in turn, release more pro-inflammatory cytokines and induce inflammation. Among the NLRs, NLRP3 is the most well-characterized and is associated with a wide range of inflammatory responses. The NLRP3 inflammasome complex is formed by the combination of apoptosis-associated speck-like protein containing a CARD (ASC), leading to the activation of pro-inflammatory cytokines such as IL-1β and IL-18. This process plays an important role in the propagation of liver inflammation.

1.7.2. NLRP3 Inflammasome Signaling Pathway

There are two steps including activation of NLRP3. Toll-like receptors (TLRs) detect endogenous danger signals, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs), including proinflammatory cytokines like TNF-α and IL 6. TLR activation activates NF-κB, which translocates to the nucleus and begins transcription of inflammasome components like inactive

NLRP3, proIL-1β, and proIL-18. (Takeuchi et al., 2010; Franchi et al., 2012). In the second step, the assembled inflammasome components, including NLRP3, ASC, and procaspase 1, form a multimeric complex structure. This leads to the transformation of procaspase-1 into caspase-1, which undergoes autocleavage and becomes activated. Activated caspase-1 then cleaves IL-1β, IL-18, and Gasdermin D. Gasdermin D, once activated, creates pores in the plasma membrane, resulting in the release of IL-1β and IL-18, triggering inflammatory responses and pyroptosis-mediated cell death (Ozaki et al., 2015; Shao et al., 2015).

There are three models that explain the activation of the NLRP3 inflammasome (Kate et al., 2010). The first model involves ionic flux, particularly ATP-dependent K+ ion efflux through P2X7 receptors. Decreased K+ levels activate NIMA Related Kinase 7 (NEK7) inside the cell, which directly binds to NLRP3, signaling for NLRP3 inflammasome activation (Shi et al., 2016). Additionally, ER stress-mediated elevation of intracellular Ca2+ ions influx through TRPM7 and CaSR plays a crucial role in NLRP3 activation (Horng, 2014). The second model highlights the essential role of reactive oxygen species (ROS), as all NLRP3 activators increase ROS levels inside the cell, such as those induced by CCL4 liver injury. Elevated ROS promote the interaction between thioredoxin-interacting protein (TXNIP) and the NLRP3 inflammasome, leading to its activation (He et al., 2016). The third model focuses on lysosomal disruption-mediated inflammasome activation. When cells endocytose large particulates or crystalline activators (such as cholesterol crystals, uric acid crystals, and ceramides), phagolysosome instability releases cathepsin B (CSTB) into the cytoplasm, further promoting NLRP3 inflammasome activation (Sheedy et al., 2013). Moreover, NLRP3 inflammasome can be activated by secondary factors like mitochondrial dysfunction, autophagic dysfunction, ER stress, and the action of TXNIP (W. Liu et al., 2015).

1.7.3. Interaction of Molecular Pathways between ER Stress and NLRP3 Inflammasome Activation

Numerous studies have identified that the NLRP3 inflammasome can be activated by intermediary molecules involved in ER stress and ROS. Of particular importance is ROS-stimulated thioredoxin-interacting protein (TXNIP), a critical signaling node

that bridges ER stress and NLRP3 activation. Various stress signaling pathways of the unfolded protein response (UPR), such as IRE1α and PERK-eIF2α pathways, converge at TXNIP to activate the NLRP3 inflammasome (Oslowski et al., 2012). The PERK-eIF2α signaling pathway phosphorylates eIF2α, leading to the attenuation of translation of NF-κB inhibitor (IκB), which results in the activation of NF-κB. NF-κB activation subsequently triggers the activation of NLRP3 and other pro-inflammatory signaling pathways. This pathway also promotes the expression of C/EBP homologous protein (CHOP), which helping opening of VDAC, allowing for the transport of Ca2+ ions from mitochondria. This influx of Ca2+ ions activates the NLRP3 inflammasome. Dysregulated PERK activity in ER stress can induce uncontrolled mTOR signaling, further contributing to NLRP3 inflammasome activation (X. Li et al., 2018). Additionally, IRE1α and ATF6 also play roles in triggering NLRP3 inflammasome activation through the unconventional splicing of X-box binding protein 1 (XBP1) (Talty et al., 2019). These pathways collectively underscore the intricate interplay between ER stress signaling and the activate the NLRP3 inflammatory responses.

1.7.4. Mitochondrial Dysfunction and NLRP3 Inflammasome Activation

Various pieces of evidence support the activation of the NLRP3 inflammasome through pathological events arising from mitochondrial damage. Under physiological conditions, NLRP3 primarily resides in the endoplasmic reticulum (ER), but upon stimulation, it translocates to mitochondria, indicating a crucial connection between mitochondria and NLRP3. Mitochondrial damage can occur due to calcium (Ca2+) overload, which increases mitochondrial membrane potential (ΔΨm), leading to heightened production of reactive oxygen species (ROS) and the opening of mitochondrial membrane permeability transition (MPT) pores (Sivitz et al., 2010). Components such as Cytochrome C, oxidized mitochondrial DNA (mtDNA), and cardiolipin (CL) are released into the cytosol through these pores, acting as DAMPs. These DAMPs are recognized by cytosolic PRRs, including NOD-like receptors, and directly bind to NLRP3, promoting its activation (Iyer et al., 2013). Moreover, mitochondrial dynamics, such as fission and fusion, also play a significant role in NLRP3 inflammasome activation. Excessive ROS levels enhance the activation of the mitochondrial fission machinery, particularly dynamin-related protein 1 (DRP1),

which is interacted with NLRP3 activation (A. Li et al., 2016). Additionally, defective mitophagy, the process by which damaged mitochondria are removed, can potentiate proinflammatory responses due to the accumulation of damaged mitochondria, which act as signals for further activation of the NLRP3 inflammasome and cytokine production (Zhang et al., 2019). These mechanisms collectively underscore the intricate interplay between mitochondrial dysfunction and NLRP3 inflammasome activation in driving inflammatory responses.

1.8. NLRP3 Mediated Liver Fibrosis

Liver fibrosis refers to the accumulation of extracellular matrix components such as collagen, glycoproteins, and proteoglycans, ultimately leading to cirrhosis and HCC. (Jiang & Duan, 2018). It poses a significant global mortality risk and is primarily caused by the activation of hepatic stellate cells (HSCs). The inflammasome, a multiprotein complex expressed in innate immune cells and other liver cells, becomes activated by binding to nucleotide oligomerizing domain (NOD) receptors. One of the most investigated families is the NLR family pyrin domain-containing 3 (NLRP3). NLRP3 activation occurs through various stimuli, including ROS generated during the metabolization of CCL4 in hepatocytes. NLRP3 forms a trimeric structure composed of a sensor protein, an adapter molecule called apoptosis-associated speck-like (ASC), and caspase activation. Different stimuli such as ROS, mitochondrial dysfunction, and ER stress activate NLRP3, leading to the recruitment of pro-caspase 1 and the maturation of interleukin-1β (IL-1β) and interleukin-18 (IL-18) via the recruitment domain (CARD). Inflammasome activation and the release of IL-1β

contribute to the development of liver fibrosis. A central feature of fibrotic changes involves the excessive production of extracellular matrix (ECM) by activated hepatic stellate cells (HSCs). These activated HSCs differentiate into myofibroblast-like cells, leading to scar formation in the liver (Alegre et al., 2017).

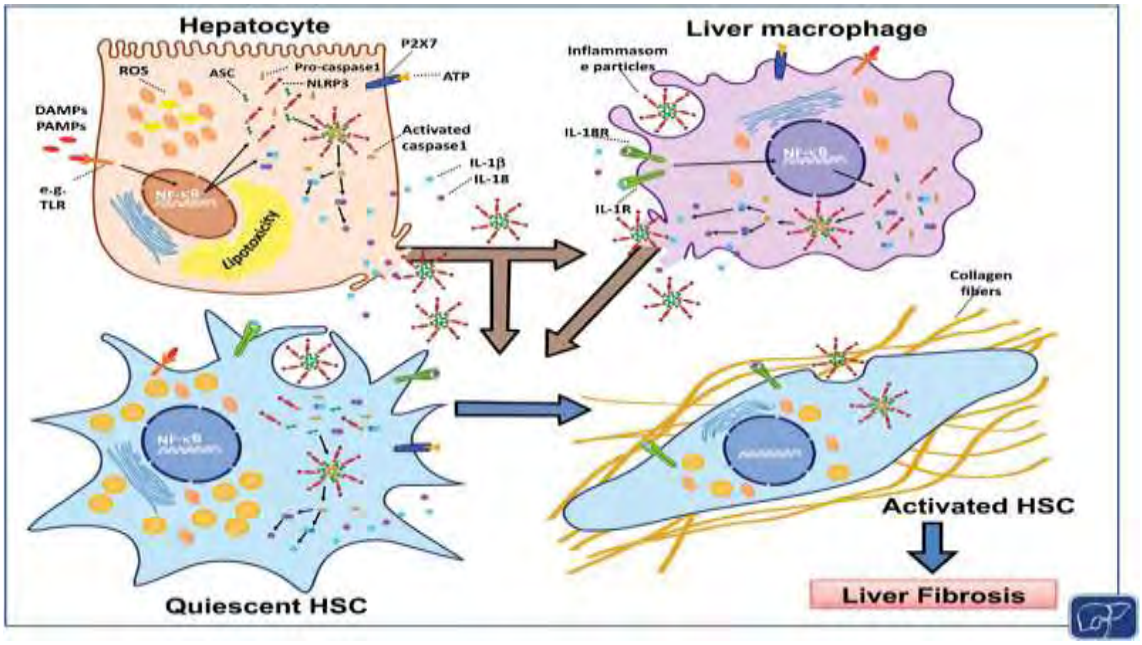


Figure 1.3 Activation of Hepatic stellate cells through NLRP3 that cause liver fibrosis.

1.8.1. Endoplasmic Reticulum Stress and Its Significance in Liver Pathology

The Endoplasmic Reticulum (ER) is an important sub-cellular structure involved in protein synthesis, protein modification, and protein maturation. Additionally, it plays roles in cholesterol biosynthesis, drug metabolism, and calcium storage (Dara & Kaplowitz, 2011). ER can experience physiological stress, caused by increased secretory activity, as well as pathological stress, resulting from an accumulation of unfolded proteins within the ER. During ER stress, several signal transduction pathways are activated, collectively termed the unfolded protein response (UPR) (Lin, Walter, & Yen, 2008). External stimuli can stimulate ER stress and trigger the UPR response (Zhang et al., 2022). In cases of liver injury, two potential outcomes exist:

- Short-term ER stress can enhance the ER's ability to process proteins, promoting ER homeostasis.
- Long-term ER stress, however, can lead to cell death through multiple signaling pathways associated with cell death. (Hu et al., 2019).

1.8.2. The Role of Endoplasmic Reticulum Stress in Liver Damage Induced by CCL4

CCl4 triggers ER stress in hepatocytes, resulting in liver cell dysfunction. Once inside liver cells, CCL4 is metabolized by the enzyme CYP450 2E1, which generates ROS, leads to oxidative stress. This oxidative stress subsequently induces ER stress in hepatocytes. During ER stress (ERS), there's an elevation in the levels of unfolded proteins (UPR), which can lead to apoptosis through various signaling pathways. Apoptosis, or programmed cell death, is a significant process occurring during ER stress. The membrane of the ER contains three transmembrane receptors, which become activated during ER stress.

1.8.3 Interplay between ER Stress and Authophagy Signaling Pathways

Autophagy is a highly conserved process in eukaryotes, acting as a protective mechanism to remove excessive proteins from cells. Three types of autophagy is known: chaperone-mediated autophagy, microautophagy, and macroautophagy, with endoplasmic reticulum stress (ERS)-mediated autophagy falling under the latter category. During the unfolded protein response (UPR) signaling pathway, C/EBP homologous protein (CHOP) is activated, inhibiting mTORC1 through AMP-activated protein kinase (AMPK) and tribble homolog 3 (TRB3). IRE1α activates TRAF, which in turn activates apoptosis signal-regulating kinase (ASK), leading to the activation of c-Jun N-terminal kinase (JNK). JNK then phosphorylates Bcl2, disrupting its association with Beclin-1, thus promoting autophagy (Fernández et al., 2015). The PERK-eIF2-ATF4 pathway activates Sestrin2, which inhibits mTORC and regulates autophagy. Additionally, the ATF6-Beclin pathway can induce autophagy by activating CHOP or Death-associated protein kinase 1 (DAPK1). The influx of calcium through Calcium/calmodulin kinase 2 enhances the process of autophagy.

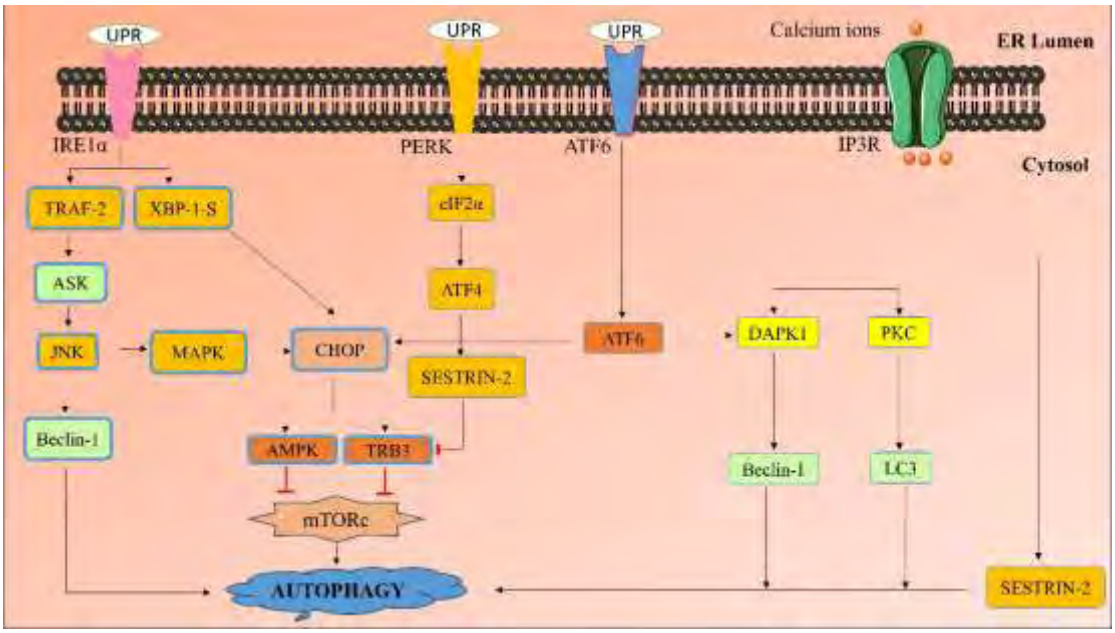


Figure 1.4 ER Stress mediated Autophagy in liver Cells

1.8.4. Interplay between ER Stress and Apoptosis Signaling Pathways

The IRE1α, PERK, and ATF6 signaling pathways are primarily involved in apoptosis due to ER stress. IRE1α becomes activated and interacts with CHOP either through the XBP1 or ASK/MAPK pathway. CHOP enhances the expression of ERO1α, which induces oxidative stress by releasing H2O2 into the cytoplasm. This, in turn, opens calcium ion channels and activates GADD34, leading to increased expression of BCL-2 and apoptosis. Additionally, through TRB3, IRE1α inhibits AKT, further promoting apoptosis. PERK, upon activation and dimerization, phosphorylates eIF2, which inhibits translation. ATF4 then translocates into the nucleus and increases the expression of CHOP, resulting in apoptosis through the upregulation of GADD34 and BCL2 expression. Furthermore, activation of IP3R by PERK releases calcium ions into the cytoplasm, activating caspase pathways (Caspase 12, 9, 3) and ultimately leading to apoptosis (Wu et al., 2020).

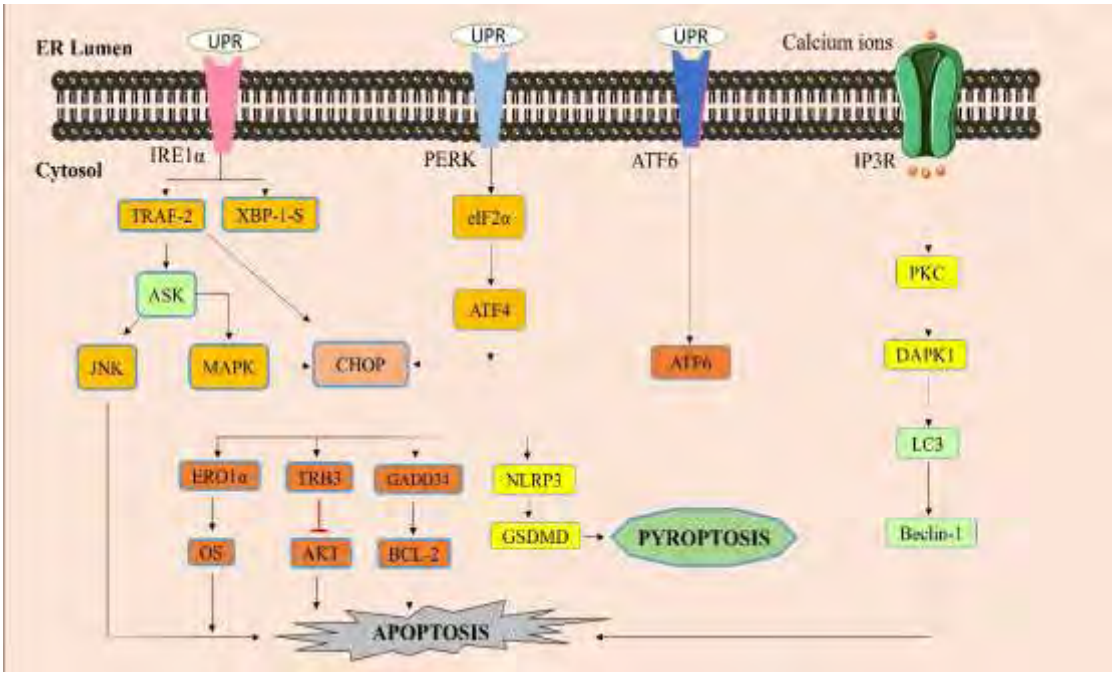


Figure 1.5 ER stress mediated Apoptosis and Pyroptosis

1.8.5 Interplay between ER Stress and Pyroptosis Signaling Pathways

Pyroptosis is a pore-forming or proinflammatory activity attributed to the Gasdermin protein family. It's characterized by the formation of pores in the plasma membrane, nuclear condensation, and degradation of genetic material, leading to an inflammatory response and eventual cell rupture. The key process in pyroptosis involves the recruitment of caspase-1, which converts inactive Gasdermin D (GSDMD) into its active form. Activated GSDMD then attacks the cell membrane, forming pores that disturb membrane permeability, alter osmotic pressure, and result in the release of cytokines, eventually leading to cell rupture. In the context of ER stress, pathways such as IRE1α-TXNIP activation leading to NLRP3 recruitment and subsequent caspase-1 activation, as well as pathways involving IRE1α-TRAF2-JNK-CHOP and PERK-ATF4-CHOP, can mediate the activation of NLRP3 and induce pyroptosis (Lebeaupin et al., 2015).

1.9. Mitochondrial Dysfunction and Hepatocellular Injury

Mitochondria are double-membraned intracellular organelles with a prokaryotic origin, playing crucial roles in energy production, calcium homeostasis, and beta-

oxidation (Zhang et al., 2019). During normal aerobic respiration, numerous electrons are transferred to produce ATP, while some are reduced to form ROS. (Wallace et al., 2005). Upon entry into hepatocytes, CCL4 damages the cell membrane of mitochondria, leading to excessive ROS production. This imbalance between antioxidants and ROS within the cell results in oxidative stress. Excessive ROS damage nucleic acids, proteins, and cell membranes, contributing to hepatocyte damage, oxidative stress, apoptosis, and organ dysfunction (Greaves et al., 2012). Mitochondrial dysfunction stands as a major mechanism underlying CCL4-induced liver injury.

1.9.1. Role of Mitochondria in Inflammation and Oxidative Stress

Mitochondria contain double-stranded circular loop DNA, housing 37 genes including those responsible for synthesizing 13 respiratory chain subunits involved in complexes I, III, IV, and V (De et al., 2008). When hepatocytes undergo damage, mitochondrial membrane permeability increases, cause the release of mitochondrial DNA (mtDNA) into the cytosol. This mtDNA acts as a damage-associated molecular pattern, activating various inflammatory pathways within the cell. The release of mtDNA relies on mitochondrial permeability transition (MPT) pores, as well as proteins like B-cell lymphoma 2 homologous antagonist killer (BAK) and Bcl-2-associated X protein (BAX) (Halestrap, 2009). BAK and BAX are implicated in Fas-mediated apoptosis in hepatocytes. Upon activation, they oligomerize on the outer mitochondrial membrane, enhancing permeability and facilitating the release of mtDNA into the cytosol (McArthur et al., 2018). The released mtDNA acts as a damage-associated molecular pattern, triggering inflammation by activating the NLRP3 inflammasome and Toll-like receptor (TLR) pathways. Activation of the NLRP3 inflammasome leads to caspase-1 activation, which converts pro-IL1β to IL-1β and pro-IL18 to IL-18, inducing inflammation in liver cells and activating non-parenchymal liver cells.

1.10. Introduction to Juglone: Hepatoprotective Compound

Juglone is a medicinal plant belongs to family Polygonaceae distributed in Southeastern Europe, Central Asia, Western Asia, Mediterranean region, and South-Western China (Bonhomme et al., 2019. It is native plant of South America, China,

South Korea and it is 1-2 meter long. It can be extracted from different parts of the tree, including the roots, leaves, husks, and bark (Khalil et el., 2019). Pharmacological research has revealed that juglone, a staple in traditional medicine, is utilized to combat a range of ailments including diabetes, liver fibrosis, hypertension, hyperlipidemia, skin infections, diarrhea, intestinal pain, and vomiting (Miara et al., 2019). Its composition comprises various bioactive and nutritive compounds such as flavonoids, phenolic acids, fatty acids, proteins, monounsaturated fatty acids, polyunsaturated fatty acids (PUFAs), and vitamins (Yan et al., 2019). Juglone exhibits various biological activities including antibacterial, antifungal, antioxidant, anti-fibrotic, and anti-inflammatory properties. The imbalance between antioxidant and ROS within a living cell, is one of the areas in which juglone's effects are observed.

Living cells are equipped with various antioxidant enzymes such as Superoxide dismutase (SOD) and Catalase (CAT), which help reduce the levels of reactive oxygen species (ROS) within the cell. ROS are primarily generated by the mitochondrial respiratory chain and the entry of xenobiotics into the cell. These ROS molecules are highly unstable and can target different cellular components including DNA, cell membranes, and organelles, leading to the development of various diseases such as liver injury, diabetes, atherosclerosis, and Parkinson's disease. In vitro experiments were conducted to assess the antioxidant properties of the ethanolic extract of juglone. The extract was tested for its ability to scavenge free radicals, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and for its ferric reducing and iron (II) chelating abilities. The reducing power capacity of the green hull of J. regia was evaluated in an ethanolic extract at a concentration of 500 mg/ml, with an absorbance determined to be 5.2. Furthermore, the ethanolic extract's reduction power activity was assessed using its absorbance at 700 nm at a concentration of 400 mg/mL, which was similar to the standard BHT. This suggests that the extract has antioxidant effects due to the presence of polyphenols, which can efficiently reduce free radicals by donating electrons. Higher levels of reducing power indicate that specific components function as electron donors and react with free radicals. (Jaiswal & Tailang, 2017). Classification of Juglone is in table 1.1.

Table 1.1: Taxonomic classification of Juglone

Classification	
Kingdom	Plantae
Phylum	Tracheophyte
Class	Magnoliopsida
Family	Polgonaceae
Order	Polygonales
Genus	Reynoutria
Specie	Japonica

1.11. Hepatoprotective Effects of Juglone

Juglone, also known as 5-hydroxy-1,4-naphthalenedione, is naturally present in *Reynoutria japonica* plants, specifically in their leaves, roots, and husks, and it demonstrates hepatoprotective activity. This compound acts as an allopathic agent, effectively restoring the levels of liver injury markers such as ALT, AST, and ALP, which are significantly elevated during liver injury. Additionally, juglone reduces the expression of alpha-Smooth muscle actin (α-SMA), Hyaluronic acid (HA), Pro-Collagen type III, as well as antioxidant enzymes like superoxide dismutase (SOD) and Glutathione (GSH) (Zhou et al., 2015).

1.12. Aim and Objectives

In this study we assessed the antioxidant, anti-inflammatory, and hepatoprotective effect of juglone in CCL4 liver injury model in mice. Previous studies have already documented its anti-inflammatory, antioxidant, and anti-fibrotic activity in vitro.

Specific objectives of this study were:

- To evaluate the anti-diabetic, anti-inflammatory, and hepatoprotective activity of juglone in a CCL4-induced liver injury mice model.
- To investigate the molecular mechanisms activated during CCL4 administration that cause oxidative stress and inflammation in the liver.

2. Materials and Methods

2.1. Animals

Mice *(Mus musculus)* were used as experimental animals where adult male mice having mean body weight (22 to 30g) and age (3 to 4 weeks) were bought from primate facility center of National institute of health (NIH) Islamabad. All the mice were acclimatized in customary cages for 1 week before the start of experiment in primate house of Quaid-i-Azam University, Islamabad.

2.2. Ethical Approval

This study was approved by the Institutional review board committee IRB of Quaid-i-Azam University, Islamabad. All the experiments were done under the standard protocols of university and all requirements were filled.

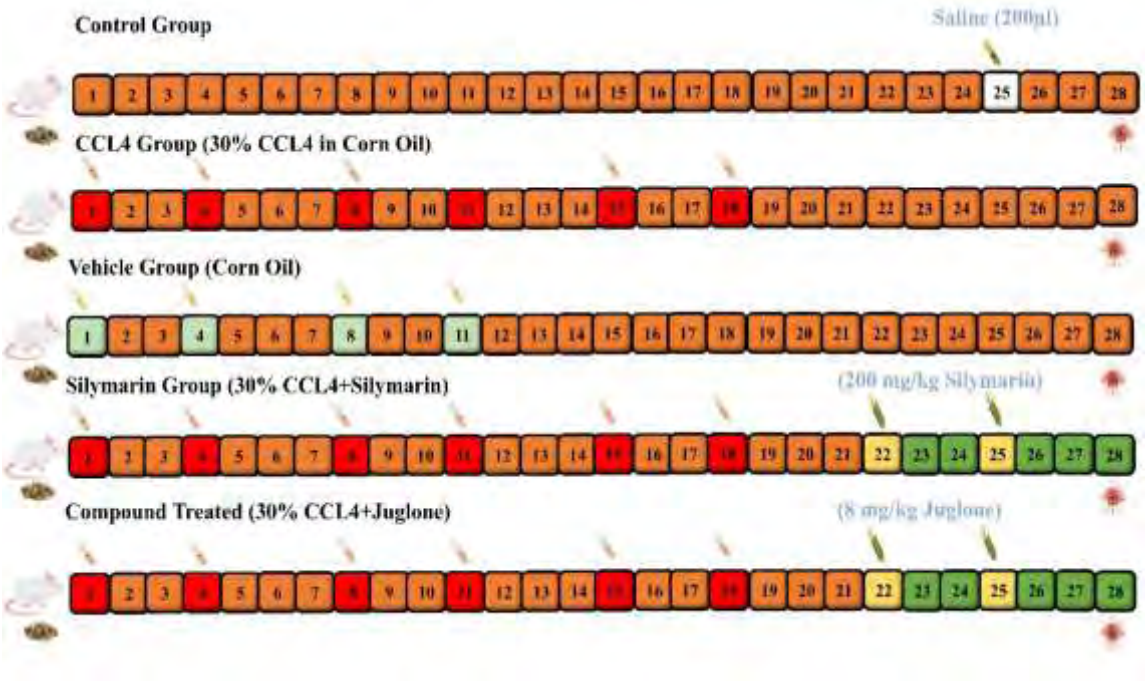


Figure 2.1: Study design and Animal grouping

2.3. Groups Division

All the animals were divided into five groups with 3 mice in each group. Group 1 was control group in which mice were provided with normal food and tap water. Control group was given only single intraperitoneal (IP) injection of saline (200µl). Group 2 was CCL4 group in which mice were given intraperitoneal injection of 30% CCL4 dissolved in corn oil on alternative days in a week (Monday, Friday) for three consecutive weeks. Group 3 was vehicle group in which mice were injected only corn oil (200 µl) intraperitoneally on alternative days for 3 consecutive weeks. Group 4 consisted of mice treated with silymarin. They were administered 30% CCL4 on alternate days for three weeks, followed by four intraperitoneal injections of silymarin (200 µl) dissolved in distilled water on alternate days for two weeks. Group 5 comprised mice treated with a compound (juglone). They received 30% CCL4 for three weeks, inducing liver injury. Subsequently, the mice were intraperitoneally injected with juglone, a plant extract (8 mg/kg) dissolved in 1% DMSO, for two weeks (two injections per week). All groups were maintained on a normal diet consisting of 4.1% fat, 22.2% protein, and 12.1% carbohydrates, as a percentage of total kcal.

Table 2.1: Groups division of experimental mice

Groups	**Name of Group**	**Treatment/Conditions**
Group 1	Control Group	Normal saline (200 µl)
Group 2	CCL4 Group	30% CCL4 dissolved in Corn oil
Group 3	Vehicle Group	Corn oil (200 µl)
Group 4	Silymarin Group	30%CCL4+Silymarin (200 mg/kg)
Group 5	Compound treated Group	30%CCL4+Juglone (8 mg/kg)

2.4. Induction of Liver Injury and Compound Treatment

The weights of all groups were measured using a weight balance. Mice in group 1 received a single intraperitoneal injection of saline (200 ul) before being euthanized for dissection. For groups 2, 4, and 5, liver injury was induced by administering 30% CCL4 (200µl dissolved in corn oil) intraperitoneally on alternate days (Monday, Friday) for three consecutive weeks. Prior to injection, the animals' feed was removed for 4 to 6 hours. Liver injury took three weeks to develop in these mice. After the three-week period, mice in the diseased group were euthanized and dissected. In the silymarin group, mice received four injections of silymarin (200 mg/kg) dissolved in distilled water on alternate days. Mice in the compound treated group were given two intraperitoneal injections of juglone, a plant-extracted compound (8 mg/kg) dissolved in 1% Dimethyl sulfoxide (DMSO). Following the injections of silymarin and the compound, the mice's weights were continuously monitored. Subsequently, mice from both the silymarin and compound treated groups were euthanized and dissected for further analysis. In group 3, mice were injected with four doses of corn oil (200µl) on alternate days. Following the injections, mice in this group were euthanized and dissected for additional analysis. The study encompassed serum and organ homogenate biochemical analysis, H&E staining, and RT-qPCR analysis for comprehensive evaluation.

2.5. Blood Collection and Serum Separation

All mice across all experimental groups were anesthetized. Blood was then collected through cardiac puncture using a 1ml syringe and transferred into 4ml gel and clot activator vacutainers (Xinle). Serum was separated by centrifuging the tubes at 6000 rpm for 10 minutes using a centrifugation machine (Hermile Labortechnit GmbH Siemensstr-25 D-78564, Wehingen). The resulting supernatant was collected in Eppendorf tubes and stored at -20°C for subsequent use.

2.6. Mice Dissection and Organ Collection

The surgical kit and glassware used were autoclaved, while other materials were washed with 70% ethanol to minimize contamination. Mice were fasted for 3 to 4

hours before dissection, and their body weights were measured. Dissections were performed in a chilled environment to preserve RNA and protein integrity. Mice were euthanized, fixed on a clean dissection board, and incised from the belly to the neck to collect visceral organs. Organs were washed in PBS, then chilled double distilled water, followed by preservation for histological and mRNA analysis.

2.7. Biochemical Analysis

Serum and liver homogenate were utilized for biochemical analysis, focusing on liver injury enzymes including AST, ALT, and ALP. AMP diagnostic kits were employed, following the provided instructions. Readings were obtained at specific wavelengths using a spectrophotometer (Multiskan Go, Type 1510, Thermo Fischer Scientific), and results were analyzed according to kit guidelines for accurate interpretation.

2.8. Histological Analysis by H&E Staining

Histological analysis, particularly using Hematoxylin and Eosin (H&E) stain, is crucial for assessing various diseases, including CCL4-induced liver injury. After organ collection and cleaning with PBS and distilled water, sections were fixed in 10% formalin for subsequent histological examination. For histological studies, liver tissues were isolated from the formalin and then dehydrated. Then these dehydrated tissues were embedded in paraffin wax. Using microtome (KD202, China), section (4μm) of these tissues were fixed on the slides and stained with hematoxylin and eosin stains (H & E staining). Then using bright field microscopy, the slides were examined at different resolutions (10x and 40x).

2.9. RNA Extraction

All tissues were initially stored at -80°C. RNA extraction from the experimental mice models was performed using an RNA Invitrogen kit (Thermofisher Scientific, Cat No # 1218301 8A). For RNA isolation, approximately 50-100 mg of tissue stored at -80°C was taken and ground in a pestle and mortar using liquid nitrogen. Subsequently, lysis buffer (0.6-0.8 ml) containing β-mercaptoethanol (10 μl) was added to the lysed tissues, followed by transfer into RNase-free Eppendorf tubes. The

lysate was then homogenized using a 21 G×1/4 (0.8 mm×32mm) gauge-syringe needle, followed by the addition of 100-200 µl of chloroform. The tube was vigorously shaken by hand for 15 seconds and then incubated at room temperature for 3 minutes before centrifugation at 12,000g for 15 minutes at 4°C.

The supernatant layer was carefully transferred to a new RNase-free tube, and an equal volume of 70% chilled ethanol was added. After vortexing, the mixture was transferred to a spin cartridge, and centrifuged for 15 seconds at 12,000g. The flow-through liquid was discarded, and the spin cartridge was reinserted for subsequent passes until all the supernatant plus ethanol mixture had passed through. Next, 700 µl of wash buffer 1 was added, and the tube was centrifuged at 12,000g for 15 seconds. The flow-through was discarded, and wash buffer II was added up to 500 µl. The tube was centrifuged again under the same conditions, and the flow-through was discarded. This process was repeated to ensure the quality of the RNA. After the addition of wash buffer II, a spin of 15 seconds was given, followed by an empty spin at 12,000g for 2 minutes to properly dry the spin cartridge membrane. Elution buffer (Nuclease-free water) was then added up to 35 µl, directly onto the center of the spin cartridge membrane. The spin cartridge was transferred to a new RNase-free collection tube and incubated for one minute at room temperature before centrifugation for 2 minutes at 12,000g, at room temperature. Finally, the quality of the eluted RNA was checked using a nanodrop machine (Colibri Spectrophotometer, Berthold Detection System GmbH 75173 Pforzheim, Germany), and the RNA was stored at -80°C until further use.

2.10. Complementary DNA (cDNA) Synthesis

After the quantitative and qualitative analysis cDNA was synthesized from total purified RNA (1 µg). For making 500-1000 ng of the cDNA from the total purified RNA, a total of 20µl of the reaction mixture was made for each reaction. Following is the table of master mix of cDNA synthesis.

Table 2.2: Master mix of cDNA synthesis

Components	**Volume/Concentration**
RNA	1.0 µg
Oligo dT Primer	0.8 µl
dNTPs	2.0 µl
Reverse Transcriptase	1.0 µl
RNA Inhibitor	1.0 µl
RT Buffer	2.0 µl
Nuclease free water	remaining volume

After preparing the reaction mixture, the tubes were incubated for 1 hour at 37 °C in a PCR machine (T3 Thermoblock, Biometra, Germany). During the PCR reaction the last phase, the tubes were heated at a high temperature (70°C) for 5 minutes to denature the activity of the reverse transcriptase enzyme Synthesized cDNA was then stored at -20°C for further use.

2.11. RT-qPCR

This is the most common and effective method to amplify the cDNA. Real time PCR (RT-qPCR) was performed by using MyGo Pro PCR system (MyGo PCR systems, IT-IS life sciences) to analyze the relative expression of different targeted genes with master mix SYBR Green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2x)).

2.11.1. Working Protocol of RT-qPCR

The reaction mixture was prepared in a biosafety cabinet to minimize the risk of contamination. Each reaction had a total volume of 10 µl. The optimized protocol for

the composition of the reaction mixture included 2 µl of SYBR Green (0.4X), 1 µl of forward primer, 1 µl of reverse primer, and 6 µl of diluted cDNA (1:10 ratio). For normalization, tissue-specific housekeeping gene Beta-actin was utilized for liver samples. Each tube received 6 µl of cDNA and 4 µl of the master mix. The 8-well RT tubes (0.1ml 8-tube strips) were carefully placed into THE qPCR machine (MyGo PCR System). Recipe of master mix is following in table 2.3.

Table 2.3: Composition and Calculation of reaction mixture

Serial No	**Composition**	**Volume (µl)**
1	SYBR Green	2
2	Forward Primer (1:10)	1
3	Reverse Primer (1:10)	1
4	cDNA (1:10)	6

2.11.2. Optimized Conditions of MyGO Pro System

The profile settings for the RT-qPCR program consisted of a total of 40 cycles. In the initial step, the temperature was maintained at 95°C for 1 minute. Subsequently, the second step involved a three-step amplification process comprising denaturation at 95°C for 10 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 17 seconds. Following this, the third step involved a pre-melt hold at 95°C for 10 seconds. The fourth step entailed high-resolution melting, which included an initial stage at 60°C for 1 minute followed by a final stage at 95°C for 15 seconds with a ramp rate of 0.05°C/s. After the completion of the RT-qPCR, the Cq values were imported into Excel for data analysis. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method to determine the fold changes in the expression of the targeted genes by using primers. Primer table is 2.4.

2.12. Statistical Analysis

To evaluate the significance of our data, we expressed our data as mean ± SEM and one way ANOVA was used for the comparison between the groups. For the pairwise multiple comparisons, we performed Tukey's Test. Differences were considered significant at a *p*-value <0.05. All experiments were performed in triplicates.

Table 2.4: Primers list for RT-qPCR

Gene	5' to 3' sequence	S. length	TM (°C)	Amplicon size
mTXNIP	**F**: TTCCTGTCCAGTGTTGGGA	19	60. 1	115bp
	R: CTGCACAGTTCTCAGGTGGA	20	60. 0	
mACTA2	**F:** GCCTCCAGTTCCTTTCCAA	19	60.2	121 bp
	R: ATCAGTGTTGCTAGGCCAGG	20	60.3	
mATF-6α	**F:** TTAGAGTGCCCGAAGCCA	18	60.5	99 bp
	R: CCGATCTTCCCACTTCCAC	19	60.5	
mMMP-2	**F:** TGGTGCTCCACTCTTCTGG	19	60.0	90 bp
	R: GCCCTCCTAAGCCAGTCTCT	20	60.0	
mGSDMD	**F:** CCCTTCCCACAACATCTCC	19	60. 3	77 bp
	R: CTTGGCTTCCCAAAGGCT	18	60. 3	
mPERK	**F:** ACTTCAAGGAAAGGGCTGTGT	21	60.2	62 bp
	R: AGTCTTGGGACACCGACAAG	20	60.2	
mNF-KB 1	**F:** GCGTCCTTTCTTGGTTCTGA	20	60. 4	114 bp
	R: GCTCAAGACACTGCACCTGA	20	60. 2	
mJNK	**F:** GGGTGCTGATGCTTTCAGAT	20	60.2	98 bp

	R: CAGAGGGTACACGGCTTCC	19	60.7	
mIL-1B	**F:** AGGGGACATTAGGCAGCAC	19	60. 1	78 bp
	R: AGTGCGGGCTATGACCAA	18	60. 2	
mERN1	**F:** GCAGCCTTATCCACACTGCT	20	60. 4	64 bp
(IRE1)	**R**: AACACACAGGGGAACAGGAG	20	60. 0	
mIL-18	**F:** GGGAGGGTTTGTGTTCCAG	19	62.3	90 bp
	R: GCAGCCTCGGGTATTCTGT	19	62.3	
mIL-6	**F:** CGGCAAACCTAGTGCGTTAT	20	60. 2	63 bp
	R: TCTGACCACAGTGAGGAATGTC	22	60. 2	
mIL-10	**F:** CCAGGGATCTTAGCTAACGGA	21	62.6	61 bp
	R: TTCGGAGAGAGGTACAACGAG	22	62.7	
mCYP2E1	**F:** TATCGACCTCAGCCCTGTTAC	21	59.2	102 bp
	R: GGATAATGATGGGCAGCAG	19	59.0	
mCOX-2	**F:** TTAGAGTGCCCGAAGCCA	18	59.0	107 bp
	R: CCGATCTTCCCACTTCCAC	19	60.1	
mBCL-2	**F:** GGCTCCCTTCATGAAATCCT	20	60.4	101 bp
	R: AGAACCCCTGTCTCCAAAGG	20	60.5	
mTGF-B1	**F:** GGAGAGCCCTGGATACCAA	19	60. 0	99 bp
	R: ACTTCCAACCCAGGTCCTTC	20	60. 3	
mNLRP3	**F:** AGCCCTCCTTCACCATCAG	19	60. 2	68 bp
	R: CACAAGCCTTTGCTCCAGA	19	60. 1	
mCASPASE-	**F:** AATAGCCCTGCAGCCCAT	18	60.6	64 bp
3	**R:** GAGCACAGTCTCCCTGAGGAT	21	60.8	
mmTORC1	**F:** GATAATTGCTGCTCCTGTGC	20	60.2	68 bp
	R: AGATCTGCTCCAAGGTGGAC	20	60.8	

mBIM	**F:** CACCTGCTGTGTGCTTCCTA	20	60.0	89 bp
	R: GCTGGCCTAAAGCAGTGAAC	20	60.0	
mBeta-Actin	**F:** GATCATTGCTCCTCCTGAGC	20	60.0	83 bp
	R: ACATCTGCTGGAAGGTGGAC	20	60.0	

3. RESULTS

3.1. Body Weight

Decrease in body weight is a common symptom observed in liver injury. To assess this, we measured the body weight of mice periodically throughout all experimental days. It was observed that the CCL4 group, silymarin group, and compound-treated group experienced a decrease in body weight during the induction of liver injury via CCL4, compared to the control and vehicle groups. However, following treatment with juglone, compound, and silymarin, these groups showed a regain in body weight (Fig. 3.1). This suggests that the compound effectively mitigated the liver injury caused by CCL4.

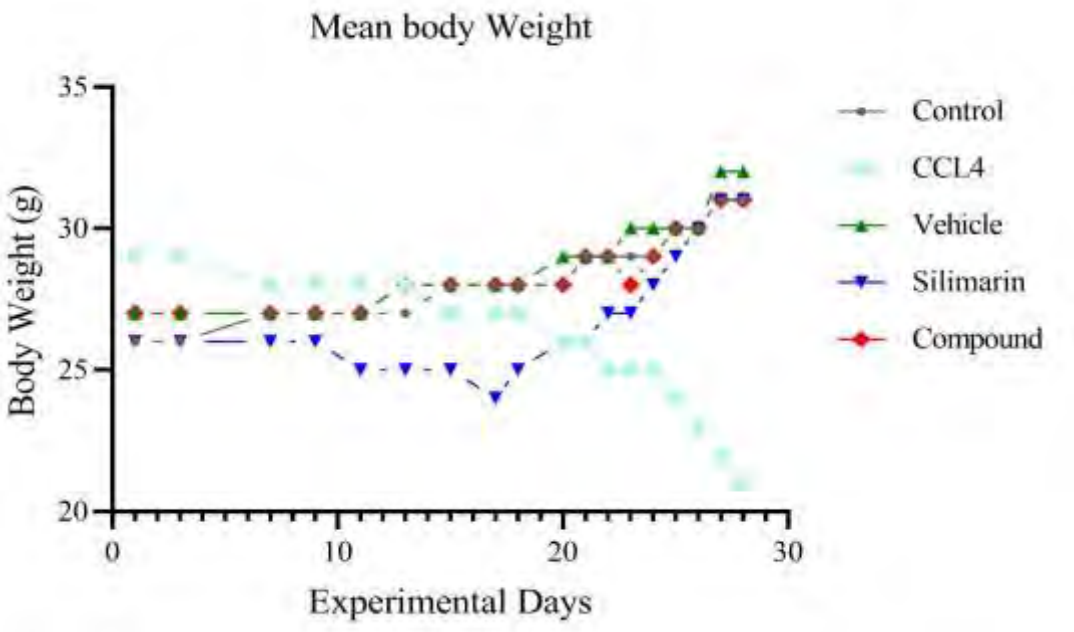


Figure 3.1: Mean body weight (g) of all groups of mice against experimental days. The groups include control, vehicle, compound treated, CCl4, and silymarin. The control group received a standard diet only, while the other groups underwent specific treatments as described. Ordinary two-way ANOVA was used, and results showed, $^{****}p < 0.0001$.

3.2. Morphological Assessment

After dissection, the livers from all experimental groups were removed and placed on glassware for observation. The morphology of the control group exhibited a normal and smooth surface. In contrast, the livers from the CCL4 group displayed lesions and scars on their surface compared to the vehicle, silymarin, and compound treated groups (Fig. 3.2). Additionally, noticeable color differences were observed among the experimental groups. The control group liver exhibited a reddish-brown color, while the change in color observed in the CCL4 group indicated significant liver injury.

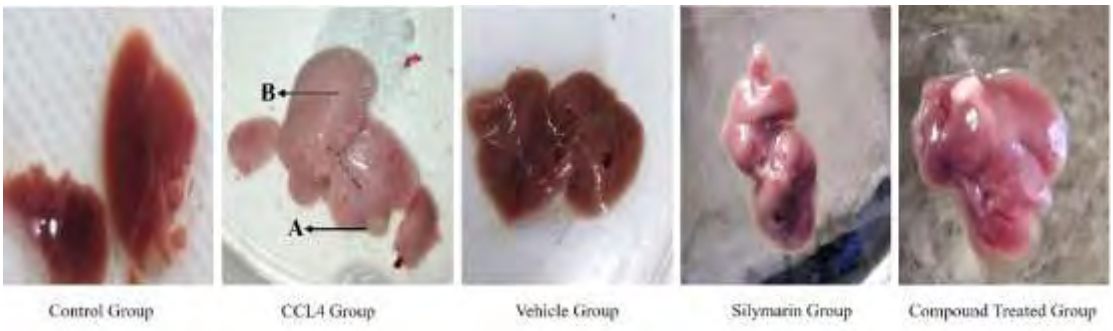


Figure 3.2: The livers of mice from all experimental groups were examined. Figure (A) displays lesions observed on the liver, while Figure (B) illustrates liver scars, indicative of liver injury.

3.3. Biochemical Parameters

Blood was collected from mice, and serum was subsequently separated. Biochemical tests were conducted to analyze liver enzymes in the serum.

3.3.1. Liver Profiling

Upon induction of CCL4 in mice, liver damage occurs, leading to alterations in liver-specific enzymes such as AST, ALT, and ALP. Liver profiling was conducted from serum to measure the levels of AST, ALT, and ALP.

3.3.1.1. AST Assay

AST, also known as serum glutamic oxaloacetate transaminase (SGOT), plays a crucial role in the breakdown of amino acids in the body. Elevated levels of AST in blood serum are associated with liver injury, as AST is released into the bloodstream when liver cells are damaged. The level of AST in serum is directly related to the decrease in the ratio of NAD to NAD+. The experiment was conducted under normal conditions, absorbance at 340 nm to determine AST level. The data were analyzed, and a graph (Fig. 3.3) was plotted, illustrating the upregulation of AST in the CCL4 group compared to other groups.

3.3.1.2. ALT Assay

During CCL4-induced liver injury, the level of ALT was found to increase. The experiment was conducted under normal conditions using kit reagents (R1+R2). Statistical analysis of the data revealed a significant elevation in the level of ALT in the CCL4 group compared to other groups, as illustrated in Fig. 3.3.

3.3.1.3. ALP Assay

ALP, an enzyme crucial for protein breakdown, is present in various forms in the body and is found in the blood. The liver is primarily responsible for synthesizing ALP, and elevated levels of ALP typically indicate liver damage. In the conducted experiment, data analysis revealed increased expression of ALP in the CCL4 group compared to other groups. This finding was further depicted in the plotted graph, as shown in Fig. 3.3.

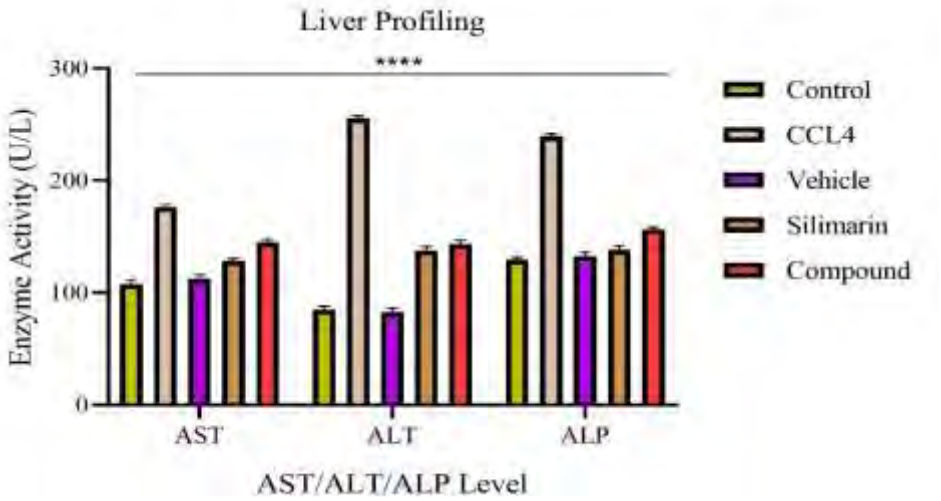


Figure 3.3 Serum enzymatic activities (U/L) of liver function (AST, ALT, and ALP) in mice. Elevated level of AST, ALT, ALP in CCL4 group observed while downregulated in all other groups. Two-way ANOVA was used for calculating the statistical significance; ****$p < 0.0001$.

3.4. Histological Analysis

The hepatoprotective effect of juglone in the CCL4 liver injury model was evaluated through histopathological observations. The histology of liver tissues in the control group reveals hepatocytes arranged normally in the centrilobular area, with no infiltration of inflammatory cells observed (Figure 3.4). Conversely, in the CCL4 group, there is a noticeable change in the architecture of liver tissues, characterized by infiltration of lymphocytes toward the centrilobular area, inflamed hepatocytes, and cell death. Results from other groups such as the vehicle group, silymarin group and compound-treated group are comparable to the control group. This suggests that our compound, juglone, exhibits hepatoprotective activity in the CCL4 liver injury model.

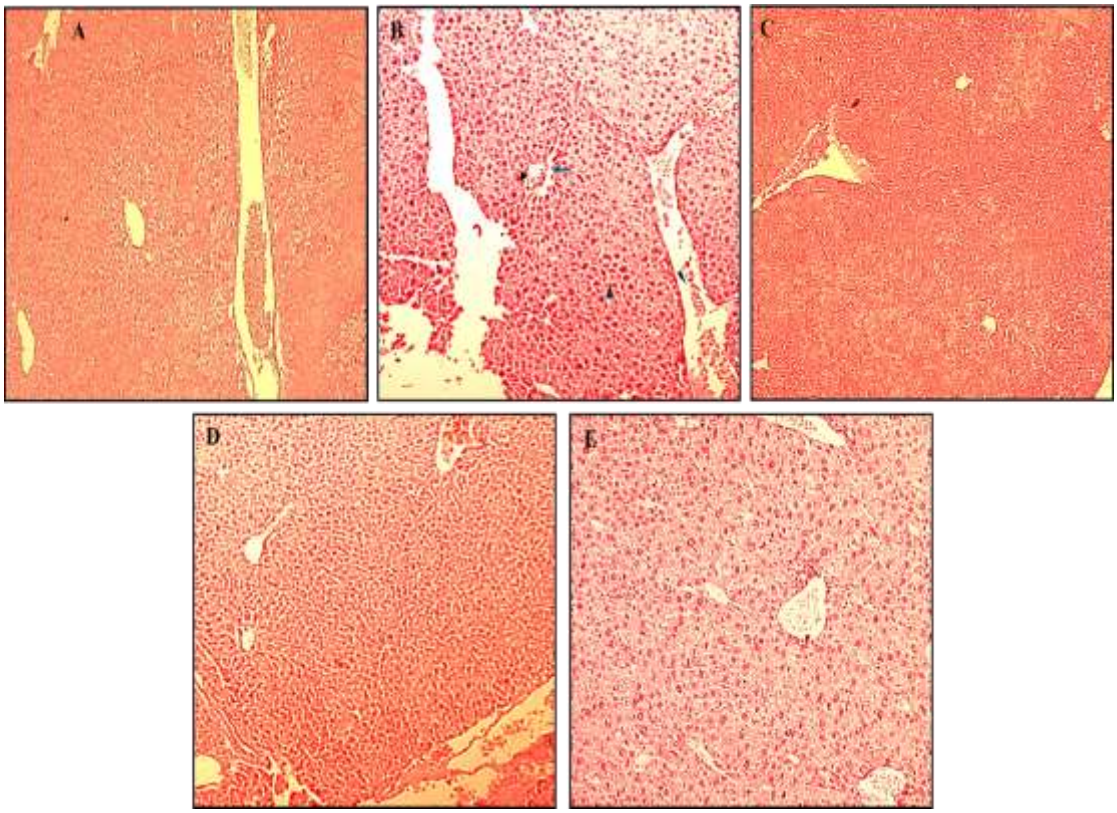


Figure 3.4: H & E staining of liver tissues of mice obtained at 10x. (A) Control group that shows no significant change in morphology of liver. (B) CCL4 group that shows destruction in architecture in liver tissues representing damage to hepatocytes, infiltration of inflammatory cells towards centrilobular area, inflamed hepatocytes and hyperammonemia. Here, in (B) ↑and ↙represents infiltration of inflammatory cells towards centrilobular area (▲) represents inflamed hepatocytes and represents hyperammonemia. Morphology of other groups in C (Vehicle group), D (Silymarin group) and E (Compound group) are comparable with control group with less infiltration of inflammatory cells and tissue regeneration.

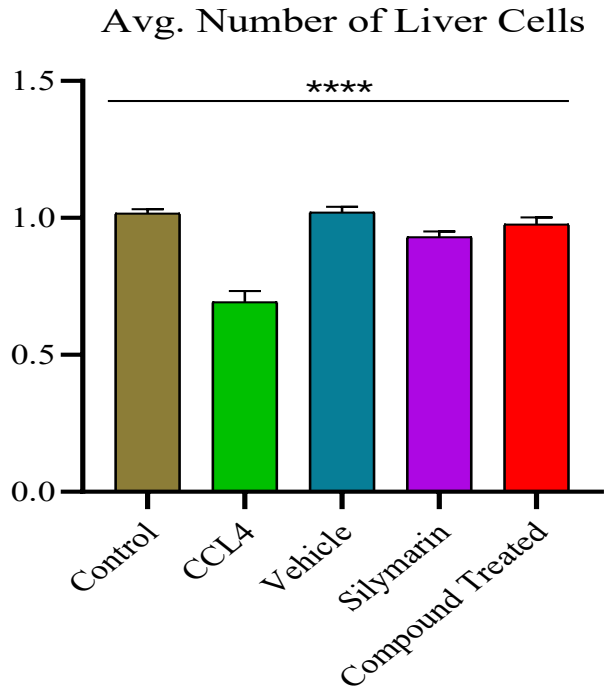


Figure 3.5 Average number of liver cells in specific area. A specific area was selected to determine the number of liver cells using Image J. The number of liver cells were reduced in CCL4 group as compared to other groups. Data was analyzed with one way ANOVA and results were significant ($p<0.0001$).

3.5. Real-Time PCR

3.5.1. Relative mRNA Expression Analysis via Real-time Polymerase Chain Reaction (RT-qPCR)

This study was designed to determine the hepatoprotective effect of juglone on the expression of various inflammatory and fibrotic genes at mRNA level through RT-qPCR by using *β-actin* as a housekeeping gene in the liver. cDNA of each experimental group was run in triplet and then data was analyzed by using the $2^{-\Delta\Delta CT}$ method to calculate the mRNA fold change. The differences between groups were analyzed by using one-way ANOVA, Tukey's test and a p-value <0.05 was considered statistically significant.

3.5.2. Relative mRNA Expression of Targeted Genes in Liver

3.5.2.1. Relative mRNA Expression of Genes Involved in Inflammation

In CCL4-induced liver injury, hepatocyte damage triggers liver inflammation. Consequently, we analyzed the relative mRNA expression of inflammation-related genes across all five groups (Control, CCL4, Vehicle, Silymarin, and Compound-treated). Our findings revealed statistically significant results. Specifically, the CCL4 group exhibited upregulation of genes such as *IL-6*, *IL-18*, *NLRP3*, *NF-kB*, and *IL-1β*. Relative mRNA expression levels showed increases in *IL-6* (1.7-fold), *IL-18* (1.7-fold), *NLRP3* (8-fold), *NF-kB* (4-fold), and *IL-1β* (32-fold), accompanied by decreased expression of the anti-inflammatory *IL-10*, as depicted in Figure 3.6. Notably, results from the normal and vehicle groups were comparable (Fig. 3.6). However, in the silymarin and compound-treated groups, expression levels of respective markers were significantly reduced. These findings suggest that the compound effectively mitigates liver inflammation induced by CCL4.

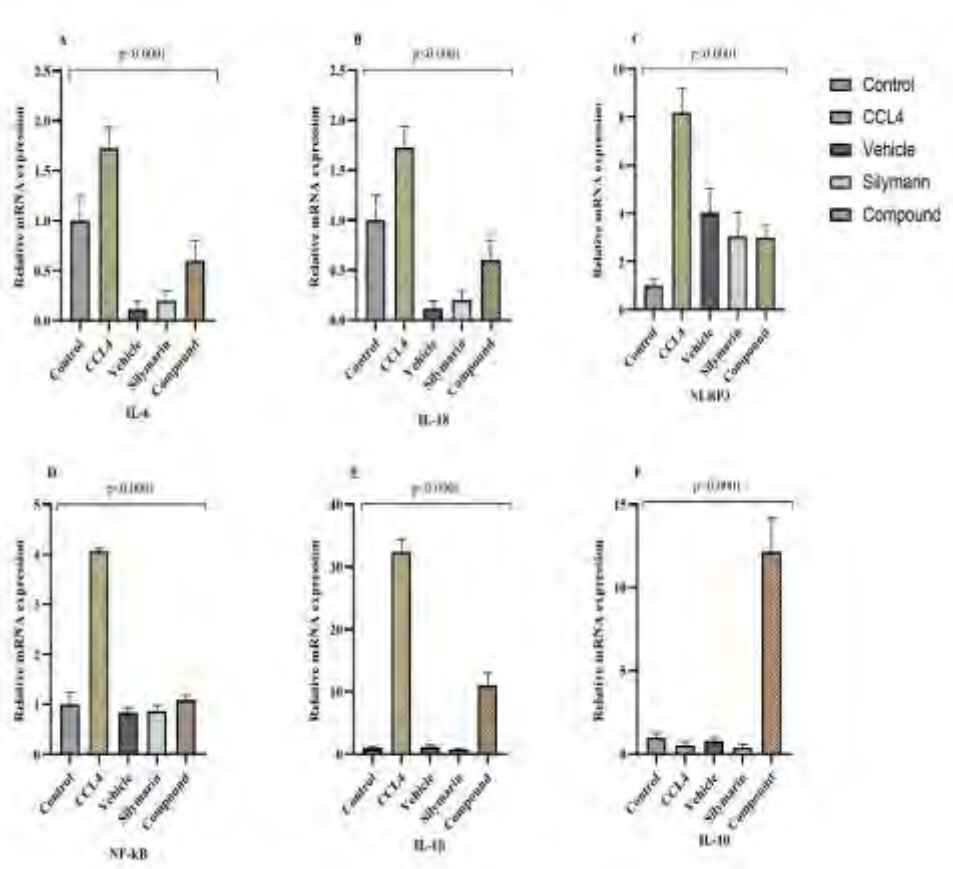


Figure 3.6: Relative mRNA expression of targeted genes involved in inflammation in liver. (A) Relative mRNA expression of *IL-6* is increased in CCL4 group but decreased in compound treated group. (B) Relative mRNA expression of *IL-18* was elevated in CCL4 group while decreased in compound treated group. (C) Relative NLRP3 expression was upregulated in CCL4 group but decreased in compound treated group. (D) Relative mRNA expression of *NF-kB* was increased in CCL4 group while decreased in compound treated group. (E) Relative mRNA expression analysis of *IL-1β* in all groups. (F) Relative mRNA expression of *IL-10* was decreased in CCL4 group while upregulated in compound treated group. Overall, the results were found significant by using ordinary one-way ANOVA and p-value<0.0001.

3.5.2.2. Relative mRNA Expression of Mediators Involved in Liver Fibrosis

Liver fibrosis develops as a consequential response to liver injury, primarily triggered by the activation of hepatic stellate cells, which subsequently release extracellular

matrix proteins. The accumulation of these proteins results in the progression of liver fibrosis. Consequently, our study focused on assessing the relative mRNA expression of genes implicated in this fibrotic process. Our findings revealed a notable increase in the relative mRNA expression of alpha smooth muscle actin *(ACTA2)* in the CCL4 group, exhibiting a 17-fold rise compared to other experimental groups. This upregulation of *ACTA2* is indicative of the activation of hepatic stellate cells, a critical step in liver fibrosis development. Similarly, the expression of *TGF-β*, recognized as a damage-associated molecular pattern (DAMP), was significantly upregulated by 2.42-fold in the CCL4 group, indicating its role in activating hepatic stellate cells through intracellular signaling pathways. Notably, this expression was remarkably reduced in the compound-treated group, suggesting a potential therapeutic effect in mitigating liver fibrosis progression. Matrix metalloproteases (MMPs) play a crucial role in degrading extracellular matrix proteins, thus alleviating fibrosis. Consistent with this function, the expression of *MMP2* was found to be upregulated (3.6 folds) in the CCL4 group, whereas it exhibited a notable downregulation in the compound-treated group, implying a potential mechanism for fibrosis resolution. Furthermore, *NLRP3,* activated within hepatocytes and subsequently released, has been implicated in the activation of hepatic stellate cells. Our study demonstrated a significant upregulation of *NLRP3* mRNA expression by 8.17-fold in the CCL4 group compared to other groups, underscoring its involvement in liver fibrosis progression (Fig. 3.7).

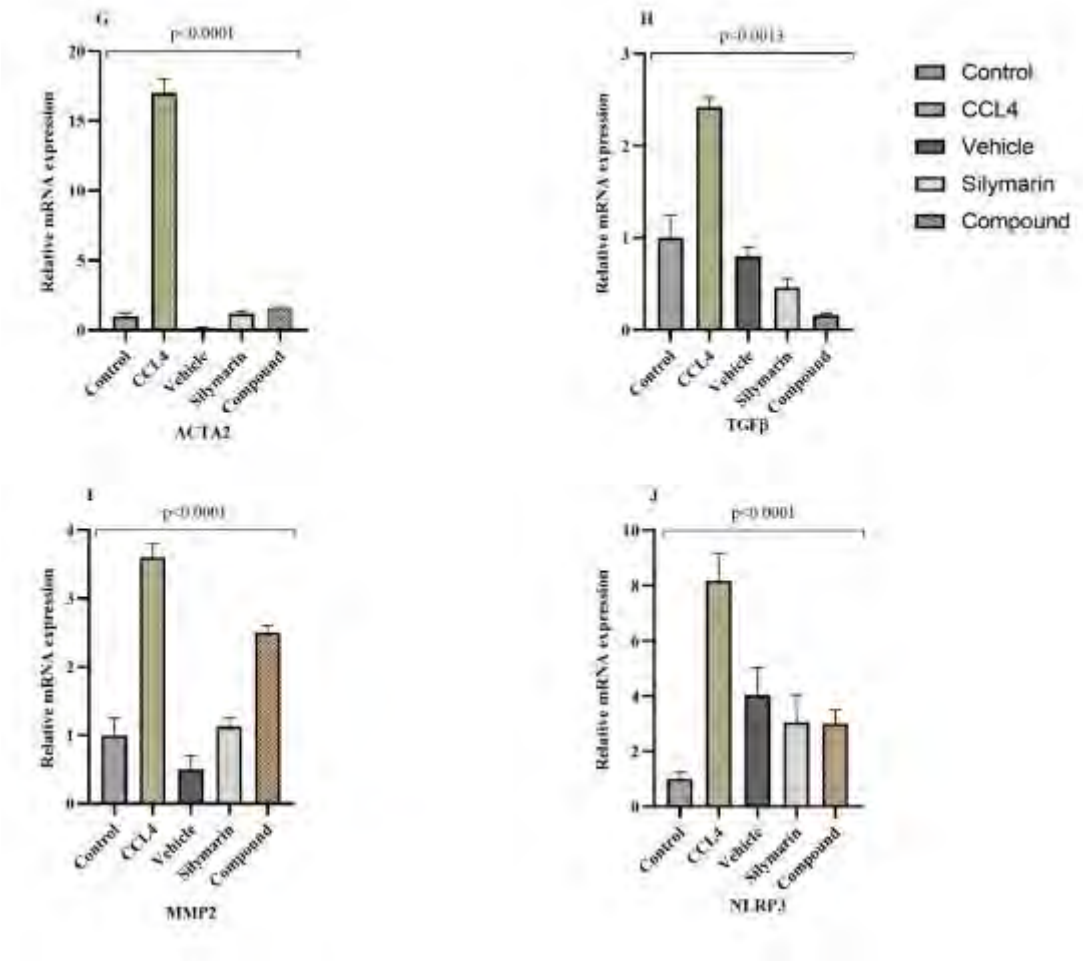


Figure 3.7: Relative mRNA expression of genes involved in liver fibrosis. Relative mRNA expression of *ACTA2* (G) was upregulated in CCL4 group as compared to other groups. *TGF-β* expression was increased in CCL4 group (H) while downregulated in other groups. (I) *MMP2* expression was upregulated in CCL4 group while downregulated in compound treated group. (J) Relative mRNA expression of *NLRP3.* Overall data was significant with ($p<0.0001$).

3.5.2.3. Relative mRNA Expression ER Mediated Pyroptosis in Liver

During CCL4-induced liver injury, an imbalance between reactive oxygen species (ROS) and antioxidants precipitates endoplasmic reticulum (ER) stress. This ER stress triggers signaling pathways that ultimately lead to pyroptosis, a form of programmed cell death. Consequently, the expression of genes such as *IRE1α, NLRP3, JNK*, and *GSDMD* becomes upregulated, contributing to the initiation of pyroptosis. In the CCL4 group, the upregulation of IRE1α was observed by 2.34-fold, while it was downregulated in other experimental groups. This suggests a specific association between *IRE1α* expression and CCL4-induced liver injury. Furthermore, *NLRP3*, activated by CHOP within the ER stress pathway, exhibited a notable upregulation of 8.17-fold in the CCL4 group compared to other experimental groups. This heightened expression underscores the pivotal role of *NLRP3* in mediating pyroptosis during liver injury induced by CCL4. Similarly, *JNK*, activated by *ASK*, *TRAF*, and *IRE1α*, displayed a 2.34-fold elevation in the CCL4 group but was downregulated in other experimental groups. This upregulation further implicates the involvement of *JNK* signaling in the progression of CCL4-induced liver injury. Moreover, *GSDMD*, which facilitates the formation of pores in the cell membrane and is activated through the *NLRP3*-caspase activation mechanism, exhibited heightened expression in the CCL4 group relative to the control and other compound-treated groups (Fig. 3.8). This suggests a direct association between *GSDMD* upregulation and the severity of liver injury induced by CCL4.

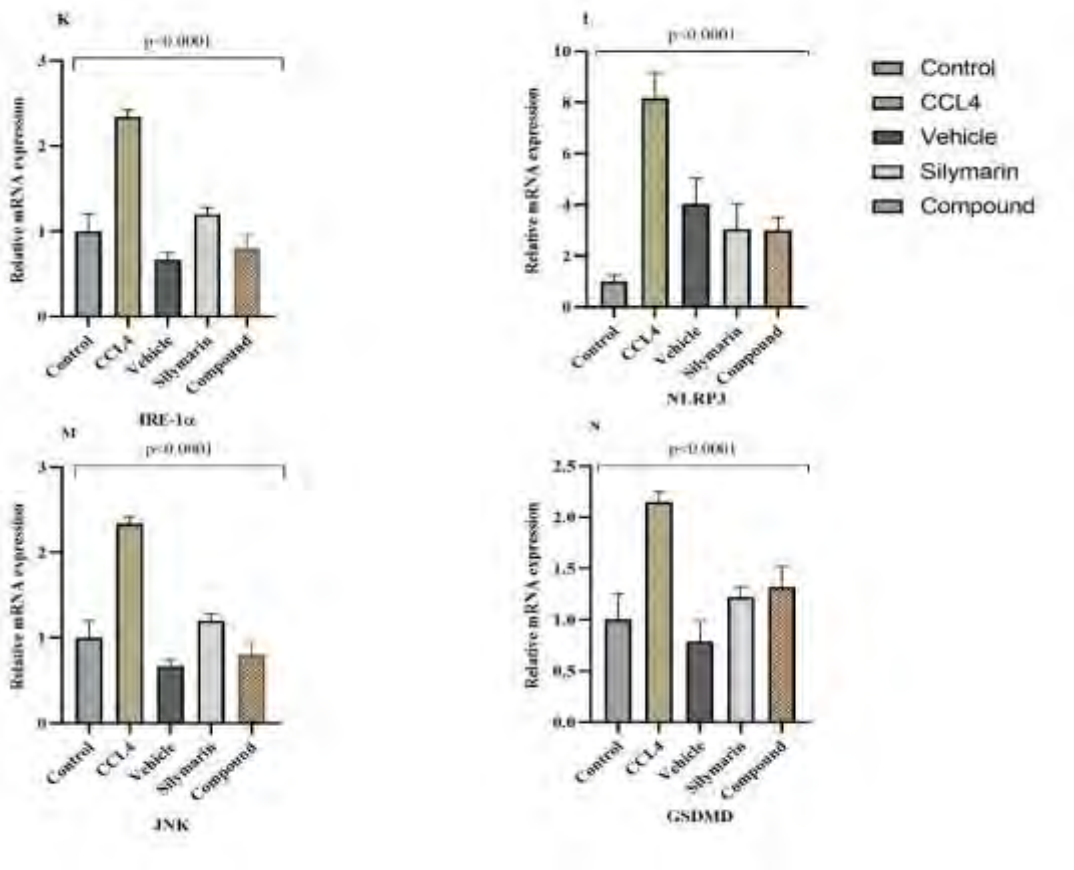


Figure 3.8: Relative mRNA expression of genes involved in Pyroptosis in the liver. (K) Relative mRNA expression of *IRE1α* in ER response in Liver cells. (L) Relative mRNA expression of *NLRP3* gene. (M) relative mRNA expression of *JNK* in all groups. (N) Quantitative mRNA expression of *GSDMD* was elevated in the CCL4 group in hepatocytes relative to other experimental groups. Overall data was significant with ($p<0.0001$).

3.5.2.4. Relative mRNA Expression Genes Involved in ER Stress-Mediated Autophagy

During liver injury, ER stress-mediated autophagy inhibition exacerbates inflammation in hepatocytes. Increased expression of genes associated with *mTORC1* activation, including *ATF6α, EIF2K, IRE1α*, and *JNK*, was observed. *ATF6α* mRNA expression

was notably elevated (3.94-fold) compared to other groups. *EIF2K* mRNA expression showed a significant upregulation (3.49-fold) in the CCL4 group. *IRE1α* mRNA expression exhibited a clear increase (2.34-fold) in the CCL4 group, with minimal expression in other groups. *JNK* expression also increased (2.34-fold), while *mTORC1* mRNA expression was elevated (3.94-fold) but downregulated in compound-treated groups, suggesting a cytoprotective mechanism against damage-associated molecular patterns (DAMPs) (Fig. 3.9).

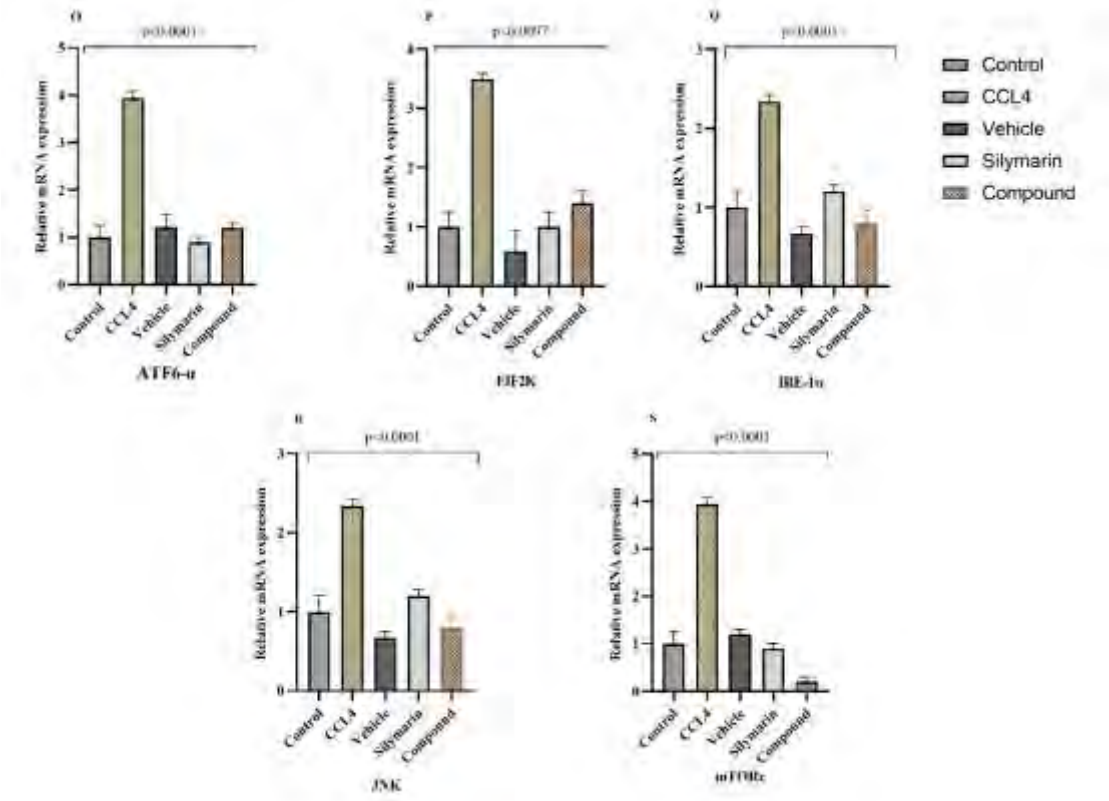


Figure 3.9: Relative mRNA expression of ER stress-mediated Autophagy genes in liver cells. (O) Relative mRNA expression of *ATF6α* in all experimental groups. (P) Quantitative mRNA expression of *EIF2K* (Q) Relative mRNA expression of IRE1α (R) mRNA expression analysis of *JNK* (S) Relative mRNA expression of *mTORC1* that shows elevated expression in the CCL4 group while reduced expression in other groups. Ordinary one-way ANOVA was performed, and Overall data was significant with ($p<0.0001$).

3.5.2.5. Relative mRNA Expression of ER Stress and Mitochondrial Dysfunction Mediated Apoptosis Markers

In the hepatocytes of mice relative mRNA expression of *BIM* (7.43 folds), *ATF6α* (3.94 folds), *IRE1α* (2.34 folds), *JNK* (2.34 folds) and EIF2k (3.49 folds) increased. The quantitative mRNA expression of *BIM* was downregulated in CCL4 group while upregulated (23.34 folds) in compound treated group (Fig. 3.10). All the results were significant with ($p<0.0001$).

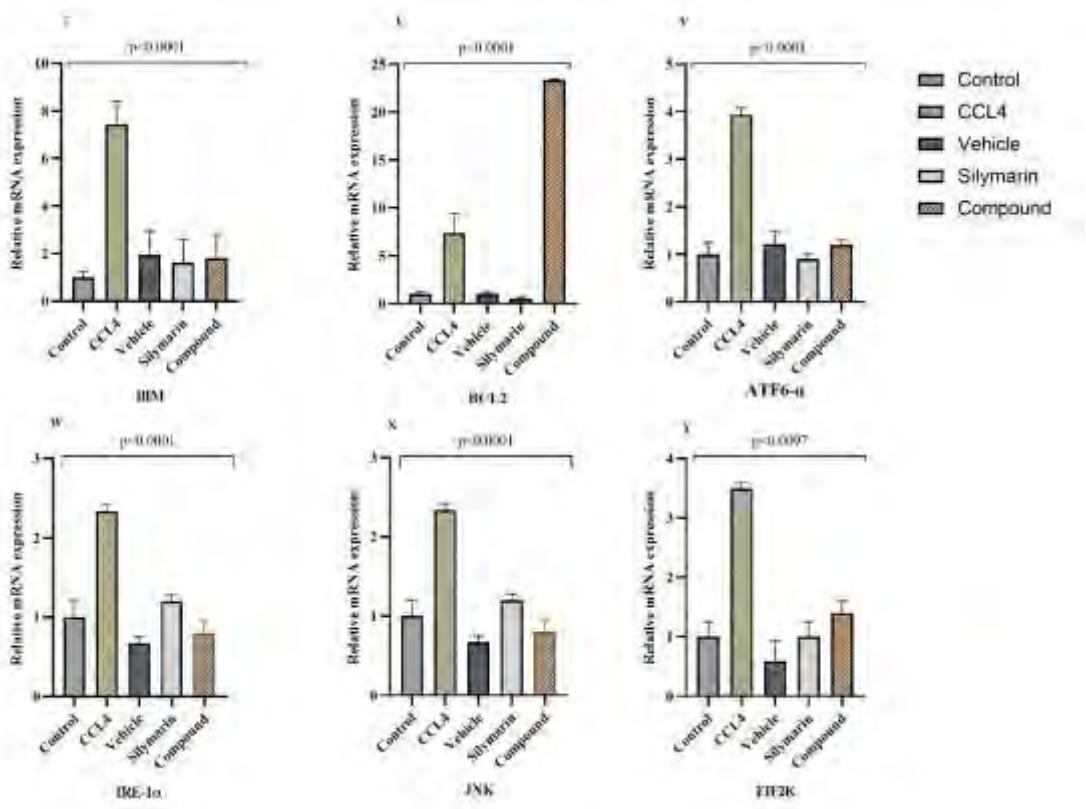


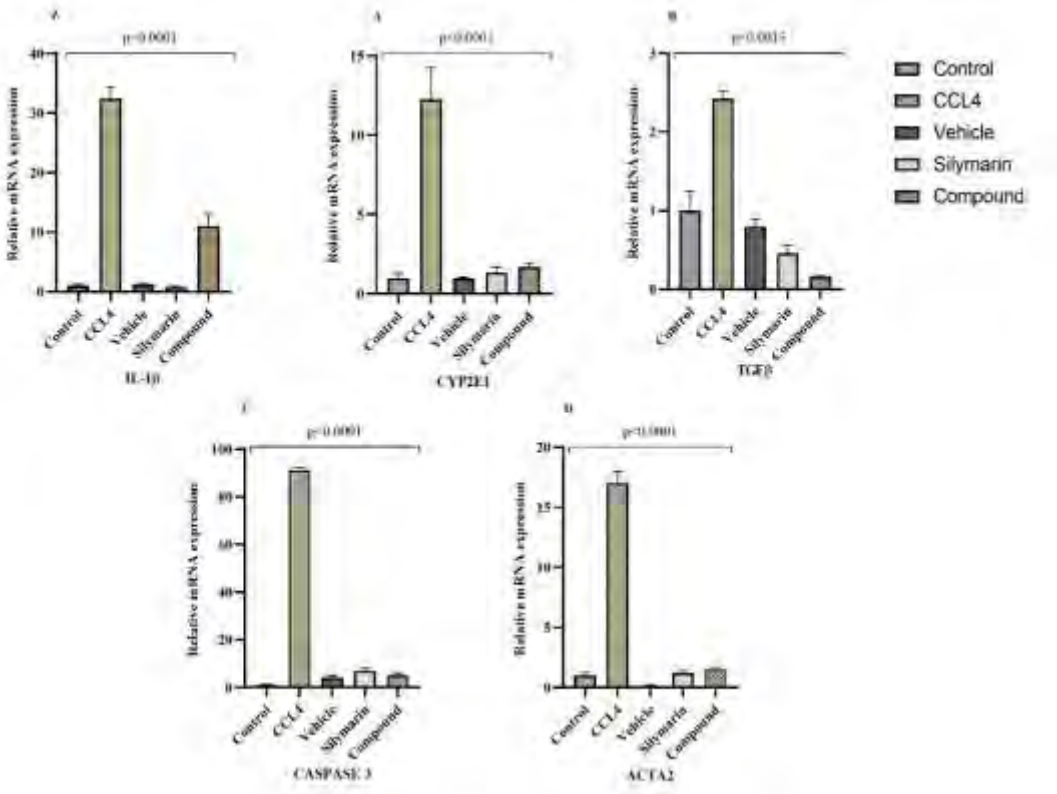
Figure 3.10: Relative mRNA expression of Apoptotic and ER stress-mediated Apoptotic Markers. (T) Relative mRNA expression of BIM upregulated in CCL4 group and downregulated in other groups. (U) Relative mRNA expression of *BCL-2* was downregulated in CCL4 group as compared to other groups. (V) *ATF6α* expression was highly increased while minimal expression in relative groups. (W) *IRE1α* expression analysis in all other groups. (X) Relative mRNA expression

analysis of *JNK* in all groups. (Y) Relative mRNA expression of *EIF2K*. Ordinary one-way ANOVA was performed, and Overall data was significant with ($p<0.0001$).

3.5.2.6. Relative mRNA Expression of Genes Involved in Oxidative Stress

Oxidative stress caused due to entry of xenobiotics entry. When CCL4 enters into cells it undergoes conversion into ROS through enzymes like CYP2E1, resulting in oxidative stress in hepatocytes. This stress induces cell death, cytokine release, and hepatic stellate cell activation. Thus, we assessed the expression of *IL-1β*, *CYP2E1, TGF-β, Caspase-3*, and *ACTA-2* using RT-qPCR. In the CCL4 group, we observed significantly increased expression levels of *IL-1β* (32.43-fold), *CYP2E1* (12.25-fold), *TGF-β* (2.42-fold), *Caspase-3* (91-fold), and *ACTA-2* (17-fold). Conversely, expression levels of these genes were notably reduced in all other groups (Fig. 3.11).

Figure 3.11: Relative mRNA expression analysis of genes involved in oxidative



stress. (Z) Relative mRNA expression analysis of *IL-1β* in all mice groups. (A) Relative expression analysis of *CYP2E1* in the liver of all mice groups. (B) Relative expression analysis of *TGF- β* in all mice groups. (C) Relative expression of *Caspase-*

3 transcripts. (D) Relative expression analysis of gene *ACTA2* involved in Hepatic stellate activation. Ordinary one-way ANOVA was performed, and Overall data was significant with ($p<0.0001$).

4. DISCUSSION

Liver injury, a pervasive global health concern, is characterized by hepatocyte damage and elevated levels of liver-specific enzymes in the bloodstream (Zimmerman et al., 1999). Its pathogenesis entails the metabolism of toxic metabolites, which can trigger specific immune responses or induce cellular damage via various signaling pathways. The liver, the largest internal gland situated below the diaphragm in the abdominal cavity, comprises four lobes—two larger and two smaller (Mish et al., 2010). Comprising predominantly parenchymal cells and non-parenchymal cells, including hepatic stellate cells, Kupffer cells, Natural Killer cells, and liver sinusoidal endothelial cells, the liver plays a pivotal role in metabolic processes (Misdrajj et al., 2010). Liver injury can result from the ingestion of chemicals orally or their absorption through the skin. These chemicals undergo metabolism by enzymes housed within subcellular organelles such as the Endoplasmic Reticulum, Mitochondria, and Golgi Apparatus. Xenobiotics like CCL4, Thioacetamide, and carbon disulphide are known culprits behind liver injury (Mitchell et al., 1973). Upon entry into cells, these chemicals are converted into reactive oxygen species, which induce lipid peroxidation, oxidative stress, inflammation, and ultimately cell death through diverse signaling pathways (McGill & Jaeschke, 2019), including ER stress-mediated autophagy (Fernández et al., 2015), pyroptosis, apoptosis (Wu et al., 2020), and the NF-kB signaling pathway.

In the current study, liver injury was induced in mice using CCL4, which enters hepatocytes and contributes to ROS production. ROS production induces oxidative stress in the liver, resulting in cellular damage, the release of pro-inflammatory and inflammatory cytokines, and the activation of non-parenchymal cells, ultimately leading to inflammation, liver fibrosis, cirrhosis, and hepatocellular carcinoma (Recknagel et al., 1989). Our study focuses on investigating the role of inflammation and oxidative stress in the development of liver injury. To evaluate the hepatoprotective effects of juglone, we designed a liver injury model (Ahmad et al., 2019), with another group administered Silymarin (Mendoza et al., 2014) as a strongly hepatoprotective positive standard control. Among various methods to induce

liver injury, we selected CCL4 as the chemical agent due to its ability to mimic human liver injury characteristics. Scholten et al. have reported CCL4 as the best agent for inducing liver injury in mice. We administered repeated doses of CCL4 intraperitoneally to induce liver injury. Consistent with previous findings (Ullah et al., 2020), we observed a significant reduction in the whole-body weight of mice during CCL4-induced liver injury. Throughout the experiment, we monitored the body weight of mice in all experimental groups. Remarkably, the body weight of the control and vehicle groups slightly increased, while a reduction was observed in the CCL4 group. However, mice in the compound-treated group and silymarin group regained body weight post-liver injury upon treatment with the compound and silymarin.

Under normal circumstances, the liver exhibits a reddish-brown color. However, intraperitoneal administration of CCL4 alters its morphology, manifesting as granules, liver enlargement, lesions, and scars (Zheng et al., 2022). In our experimental model, livers from the CCL4 group displayed changes in color, lesions, and scarring, contrasting with the livers of other groups, which resembled normal liver morphology. This observation suggests that both silymarin and our compound possess hepatoprotective effects.

Liver function biomarkers such as ALT, AST, and ALP play pivotal roles in assessing various liver diseases, including liver injury. ALT, also known as serum glutamic pyruvic transaminase (SGPT), is synthesized solely by hepatocytes and is involved in glucose and protein catabolism. ALP, primarily found in the liver and bones, facilitates the hydrolysis of monophosphoric acid esters. AST, on the other hand, is particularly abundant in the liver and is involved in amino acid catabolism. Normally confined to the cytoplasm of hepatocytes, the release of these enzymes into the bloodstream indicates severe liver injury (Wills et al., 2006). In our experiment, administration of silymarin and juglone led to reduced levels of liver-specific enzymes, indicative of their hepatoprotective activity.

Furthermore, histopathological analysis using H&E staining revealed distinct alterations in liver architecture among the experimental groups. In normal liver tissue,

hepatocytes are arranged around the central vein within hepatic lobules, with no infiltration of inflammatory cells or evidence of apoptotic or necrotic cell death. However, during CCL4-induced liver injury, significant changes occur, including apoptosis, lymphocyte infiltration, and disruption of liver architecture (Ko et al., 2020). Our results corroborate these findings, showing destruction of liver architecture, inflammatory cell infiltration, dilation of the space of Disse, and apoptosis in the CCL4 group compared to other experimental groups. Additionally, a decrease in cell number indicative of cell death was observed in the liver injury group.

To further explore our hypothesis, we analyzed the mRNA expression of cytokines implicated in inflammation during liver injury, as cytokines play a central role in the inflammatory response. During CCL4-induced liver injury, there is an excessive production of ROS, leading to oxidative stress in both parenchymal and non-parenchymal liver cells. ROS, a well-known metabolic byproduct of redox reactions facilitated by liver enzymes, contributes to hepatocyte damage and subsequent release of pro-inflammatory cytokines such as *IL-1β*, *IL-6*, and *IL-18* (Khan et al., 2011). These cytokines, in turn, exacerbate liver damage through a feedback mechanism, activating *NF-kB*, which acts as a transcription factor for further pro-inflammatory cytokine production. Thus, mRNA expression of genes encoding *mIL-1β*, *mIL-6*, *mIL-18*, and *mNF-kB* was found to be upregulated in the CCL4 group compared to other experimental groups, suggesting a heightened inflammatory response. Our experimental results indicate that our compound exhibits anti-inflammatory activity, as evidenced by the downregulation of pro-inflammatory cytokines and *NF-kB*. Moreover, the robust ROS production during oxidative stress triggers the activation of *NLRP3*, which in turn activates procaspase-1 into caspase-1, leading to the conversion of *Pro-IL-1β* and *Pro-IL-18* into their active forms, *IL-1β* and *IL-18*, respectively, which are subsequently released from cells (Tschopp et al., 2010). Notably, the relative mRNA expression of the *mNLRP3* gene was elevated in the CCL4 group but reduced in the other groups. *IL-10* functions as an anti-inflammatory agent during CCL4-induced liver injury (Huang et al., 2006). Interestingly, the expression of the *mIL-10* gene was downregulated in the CCL4 group but upregulated in the compound-treated group, indicating a significant hepatoprotective effect of juglone.

Additionally, we investigated the progression of liver injury leading to fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Hepatic stellate cells (HSCs) play a crucial role in liver fibrosis, transitioning from an inactive to an activated state in response to inflammatory mediators released by damaged hepatocytes. These activated HSCs release extracellular matrix, leading to fibrosis and, ultimately, HCC (Galun et al., 2002). Liver fibrosis represents a wound healing process involving the synthesis and degradation of structural components, necessitating the activation of HSCs and the involvement of fibrogenic factors such as *MMP-2*, *α-SMA*, and ACTA2, as well as inflammatory mediators like NLRP3 and TGF-β. *TGF-β*, for instance, acts as a mediator by binding to TGF-β receptors, thereby increasing the expression of the *mACTA2* gene, which is involved in the activation of HSCs. Accordingly, we evaluated the mRNA expression of genes such as *mACTA2*, *mTGF-β*, *mMMP-2*, and *mNLRP3*, which were found to be upregulated in the CCL4 group but downregulated in the silymarin and compound-treated groups. This observation suggests that our compound possesses anti-fibrotic activity and represents a potential therapeutic target against fibrosis by inhibiting HSC activation (Tipoe et al., 2010).

Pyroptosis, a form of cell death characterized by pore formation through the protein *GSDMD*, is triggered by ER stress mediated oxidative stress (Yu et al., 2021). ROS activate *TXNIP,* leading to *NLRP3* activation and subsequent conversion of pro-caspase 1 into caspase-1. Caspase-1 activation converts inactive *N-GSMD* into activated *GSDMD*, which forms pores in the cell membrane, disrupting its integrity and releasing pro-inflammatory cytokines. Unfolded protein response activates *IRE1α*, which, via the *JNK-CHOP* mediated pathway, activates *NLRP3*, culminating in pyroptosis (Zhou et al., 2021). Our study analyzed the expressions of genes *mIRE1α*, *mNLRP3*, *mJNK*, and *mGSDMD*. Elevated expression of these genes in the CCL4 group indicates that CCL4 induces pyroptosis during liver injury, while downregulation of these genes in the compound-treated group suggests that our compound inhibits the ER stress pathway leading to pyroptosis.

Cell death due to ER stress and mitochondrial dysfunction can lead to apoptosis and autophagy through different signaling pathways. *mTORc*, an autophagy inhibitor, was upregulated in the CCL4 group compared to other experimental groups. Inhibition of

autophagy results in impaired clearance of damage-associated molecular patterns associated with liver injury (Piccirillo et al., 2014). The homeostatic balance in the liver, crucial for normal liver function, is maintained through cell proliferation and apoptosis (Schattenberg et al., 2006). Apoptosis can occur through ER stress-mediated pathways, including the unfolded protein response (UPR), which activates *ATF6α*, *IRE1α*, and *PERK*. Activation of *IRE1α* leads to interaction with *JNK* via *XBP1*, contributing to apoptosis. PERK activation recruits eIF2α, which synthesizes ATF4, migrating to the nucleus and inducing CHOP-mediated apoptosis. Additionally, *ATF6α* triggers *CHOP*-mediated apoptosis (Wanget al., 1996). In our experiment, we measured the expression of genes such as *mIRE1α*, *mATF6α*, *mJNK*, and *meIF2α* (Tabas et al., 2011). Upregulation of these genes in the CCL4 group suggests induction of cell death through apoptosis, while downregulation in the compound-treated and silymarin groups indicates antiapoptotic activity of our compound.

Furthermore, *mBCL2*, an antiapoptotic gene inhibiting Bax-induced apoptosis (Czabotar et al., 2014), was found to have decreased mRNA expression in the CCL4 group compared to other groups, indicating the antiapoptotic activity of our compound. On the other hand, *BIM*, a pro-apoptotic gene, was upregulated in the CCL4 group but downregulated in the compound and silymarin treated groups, further supporting the antiapoptotic activity of our compound.

The imbalance between oxidants and antioxidants results in oxidative stress. Antioxidant enzymes such as GST, GSH, catalase, and superoxide dismutase present in hepatocytes mitigate oxidative stress, but excessive production of reactive oxygen species (ROS) through enzymes such as *CYP2E1*, which is more dominant in mammals, can cause oxidative stress. These ROS then attack the cell membrane, leading to lipid peroxidation (LPO) and damage to other biomolecules within the cells, resulting in cell damage (Ullah et al., 2020). The damage to liver cells accelerates liver injury, inflammation, fibrosis, and ultimately, end-stage hepatocellular carcinoma (Ritesh et al., 2015). Damaged hepatocytes release pro-inflammatory cytokines, leading to inflammation and activation of hepatic stellate cells. Activated hepatic stellate cells transform into myofibroblast-like cells, releasing extracellular matrix (ECM) and promoting liver fibrosis. Oxidative stress can also

lead to ER stress and mitochondrial dysfunction, resulting in cell death through different pathways (Fu et al., 2008). To investigate oxidative stress in liver tissues, we quantitatively measured the mRNA expression of *mCYP2E1*, *mIL1-β*, *mACTA2*, *mCaspase3*, and *mTGFβ*. Elevated expression of *mCYP2E1* indicates a strong signal of ROS production causing oxidative stress in liver cells, while downregulation in other experimental groups suggests the absence of oxidative stress in those groups. Oxidative stress leads to the release of pro-inflammatory cytokines, as confirmed by the elevated expression of *IL-1β* in the CCL4 model group compared to other groups. Furthermore, oxidative stress can activate hepatic stellate cells, leading to liver fibrosis. The enhanced expression of *ACTA2* and *TGF-β* indicates activation of hepatic stellate cells.

In our present investigation, we have successfully established a murine model of CCL4-induced liver injury that closely mimics the pathogenic manifestations observed in humans. Notably, our model exhibited characteristic features such as elevated levels of AST, ALT, and ALP, alongside a decline in body weight. For our experimental interventions, we administered juglone (extract from *Reynoutria japonica*) to the compound-treated group, while employing silymarin as a positive control for hepatoprotection. Morphological evaluations, along with assessments of body weight recovery and the restoration of liver-specific enzymes in the compound-treated cohort, underscored the hepatoprotective efficacy of our intervention. Additionally, histological scrutiny of liver specimens from all experimental groups revealed a restoration of hepatic architecture specifically in the compound-treated group. Moreover, our investigation was reinforced by analyzing the relative mRNA expression of genes associated with inflammation, liver fibrosis, and oxidative stress, as well as genes implicated in cell death mechanisms such as apoptosis, pyroptosis, and autophagy. We observed an upregulation of mRNA expression in the CCL4-induced injury group, which was notably downregulated in the compound-treated group, indicative of a reversal of liver injury mediated by our compound.

Looking ahead, there remains a considerable scope for further exploration using this model. Given that CCL4 administration via intraperitoneal injection affects not only the liver but also extrahepatic tissues such as the kidney, lungs, brain, and heart.

Future investigations could delve into examining mRNA expression patterns or conducting histological analyses of these organs. Such endeavors would shed light on the broader systemic effects of our compound beyond its hepatoprotective activity.

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