CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Hepatoprotective Effects of Orthocetamol or 2 - Acetamidophenol Against Isoniazid-Induced Hepatoxicity

by

Sidra Zafar

A thesis submitted in partial fulfillment for the degree of Master of Science

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I dedicate this thesis to my beloved family and my respected teacher. To my parents, whose endless love, sacrifices, and guidance have shaped who I am today. To my siblings, for their encouragement and belief in my abilities. And most importantly, to my teacher, whose wisdom, patience, and inspiring mentorship have been a guiding light throughout this journey. Your support has been the foundation of my success. With heartfelt gratitude, I share this achievement with all of you.

CERTIFICATE OF APPROVAL

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(Sidra Zafar)

Abstract

This study investigated the hepatoprotective effects of Orthocetamol (2-acetamidophenol) against isoniazid (INH)-induced hepatotoxicity in a BALB/c mouse model, focusing on biochemical and inflammatory markers. The results demonstrated that INH administration significantly elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, with ALT increasing from 55.00 ± 7.23 U/L in controls to 125.33 ± 22.45 U/L at 200 mg/kg INH, and AST rising from 92.66 ± 12.45 U/L to 164.00 ± 9.87 U/L, indicating severe hepatocellular damage. Treatment with Orthocetamol at 5 mg/kg and 15 mg/kg dose-dependently reduced these elevations, with ALT levels decreasing to 79.83 ± 5.89 U/L and 63.50 ± 5.24 U/L, respectively, and AST levels declining to 89.50 ± 11.23 U/L and 80.00 ± 8.12 U/L, nearly normalizing hepatic function. The BCA protein assay revealed increased hepatic protein content in INH-treated groups (0.580 ± 0.015 μg/μl at 200 mg/kg), which was mitigated by Orthocetamol (0.466 ± 0.006 μg/μl at 15 mg/kg), suggesting stabilization of hepatocellular homeostasis. ELISA analysis showed a marked rise in tumor necrosis factor-alpha (TNF-α) levels due to INH (1.50 ± 0.10 μg/μl at 200 mg/kg), while Orthocetamol treatment significantly reduced TNF-α to 0.80 ± 0.06 μg/μl at 15 mg/kg, highlighting its potent anti-inflammatory effects. Molecular docking studies further supported these findings, revealing Orthocetamol's binding affinity for TNF-α (-4.7 kcal/mol) and NF-κB (-5.6 kcal/mol), indicating its role in modulating inflammatory pathways. These results underscore Orthocetamol's dual antioxidant and anti-inflammatory mechanisms, positioning it as a promising adjuvant therapy to mitigate INH-induced hepatotoxicity and improve tuberculosis treatment outcomes. Further clinical validation is warranted to confirm its therapeutic potential.

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Abbreviations

ALF	Acute Liver Failure
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
AcHz	Acetyl Hydrazine
BCA	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
CAT	Catalase
CHCl	Chloroform
CO_2	Carbon Dioxide
CYP2E1	Cytochrome P450 2E1
Cu^{2+}	Copper (II) Ion
Cu^{+}	Copper (I) Ion
DILI	Drug-Induced Liver Injury
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMB	Ethambutol
GPx	Glutathione Peroxidase
GSH	Glutathione
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
Hz	Hydrazine

H_2O_2	Hydrogen Peroxide
IACUC	Institutional Animal Care and Use Committee
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
INH	Isoniazid
IP	Intraperitoneal
IV	Intravenous
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MPT	Mitochondrial Permeability Transition
NAC	N-Acetylcysteine
NAPQI	N-Acetyl-p-benzoquinone Imine
NAT2	N-Acetyltransferase 2
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
PBS	Phosphate-Buffered Saline
PZA	Pyrazinamide
RIF	Rifampin
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Standard Error of the Mean
SOD	Superoxide Dismutase
TB	Tuberculosis
TLR4	Toll-Like Receptor 4
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor Necrosis Factor-alpha
UDCA	Ursodeoxycholic Acid
ULN	Upper Limit of Normal

Symbols

mg/kg	Milligrams per Kilogram (dose measurement)
μg/μL	Micrograms per Microliter (protein concentration)
pg/mL	Picograms per Milliliter (cytokine measurement)
U/L	Units per Liter (enzyme activity, e.g., ALT, AST)
mL	Milliliter (volume)
μL	Microliter (volume)
g	Gram (weight)
kg	Kilogram (weight)
^{o}C	Degrees Celsius (temperature)
rpm	Revolutions per Minute (centrifugation speed)
Å	Angstrom (molecular docking distance)
kcal/mol	Kilocalories per Mole (binding affinity)
ΔG	Gibbs Free Energy (binding energy in docking studies)
$\pm$	Plus-Minus (e.g., Mean $\pm$ SEM)
$<$	Less Than (e.g., $p < 0.05$)
$>$	Greater Than
$\leq$	Less Than or Equal To
$\geq$	Greater Than or Equal To
R^2	Coefficient of Determination (e.g., R^2 0.99)
μ	Micro (e.g., μg/μL)
^{o}	Degree (e.g., 37^{o}C)

Chapter 1

Introduction

1.1 Background

1.1.1 Liver

The liver is vital for many essential physiological functions. It synthesizes and absorbs cholesterol, forms clotting factors, regulates blood sugar, and keeps an eye on the body's nutritional requirements. The liver is a hard organ that can heal and replenish itself, so even if it has some damage, it can still function. Severe liver damage can cause hepatic failure and mortality even when the liver is incapable of healing itself [5].The liver may be able to heal from harm owing to its special ability to regenerate, but irreversible damage can result in cirrhosis, fibrosis or hepatocellular cancer [6]. The liver is the largest internal organ and a major metabolic center in vertebrates, performing over 500 essential functions like detoxification, protein synthesis, bile generation, and nutrient storage [7].The liver can be particularly prone to the adverse effects of pharmaceutical medications since it is the primary organ used in the manufacture of drugs. Acute liver failure (ALF) primarily occurs due to drug-induced liver injury (DILI), which also plays a key part in the cessation of medications from the market [8]. A large number of cases of acute liver failure and all forms of acute and chronic liver diseases are caused by drug-induced liver damage, which is widespread [9].

1.2 Liver Functions

1.2.1 Metabolic Function

The metabolic activation of medicines into reactive metabolites, oxidative stress, mitochondrial dysfunction, and immune-mediated harm are the main causes of hepatotoxicity [10]. A lot of biological functions are regulated by the liver, which can be harmed by long-term, consistent pharmaceutical usage [11]. Redox position is a significant underlying component for many liver diseases. The redox state affects inflammatory, metabolic and proliferative liver diseases. The primary locations where the cytochrome P450 enzymes produce reactive oxygen species (ROS) in hepatocytes are the mitochondria and endoplasmic reticulum. Oxidative stress arises from an imbalance between oxidant and antioxidant agents. Reactive nitrogen species (ROS) primarily affect biological components such as DNA, lipids, and hepatocytic proteins. The process results in the development of structural and functional problems in the liver [12].

1.3 Epidemiological Studies of Drug Induced Liver Injury

In advanced nations, epidemiological studies show that DILI accounts for about 50% of cases of acute liver failure and 10% of all cases of acute hepatitis. Although isoniazid (INH) is often used and has the potential to cause hepatotoxicity, it stands out among the many medications associated with hepatotoxicity, especially antitubercular agents [13]. Tuberculosis (TB) is one of the top ten causes of death worldwide which continues to be a serious global health concern. According to WHO projections, there are about 1.5 million TB-related fatalities and 10 million new cases annually (WHO, 2023). Mycobacterium tuberculosis or *M. tuberculosis*, is the pathogen that causes tuberculosis (TB), a chronic infectious disease that is pathologically characterized by the formation of granulomas. While the lungs are the main location of infection, extrapulmonary organs like the

kidneys, spine, brain, and skin can also be affected by tuberculosis [14]. Usually, a 6-month regimen involving isoniazid, rifampicin, pyrazinamide, and ethambutol is used to treat drug-susceptible TB. This is followed by a 4-month continuation phase with isoniazid and rifampicin alone. High cure rates are attained when this regimen is carefully followed. However, adherence to treatment and its success are severely impaired by adverse drug reactions (ADRs), especially medication-induced hepatotoxicity [15].

1.4 Liver Damage and Adjustment in the Management of TB

Isoniazid was shown to be an effective treatment for tuberculosis, and it has been a mainstay of TB treatment since the 1950s. A key part of mycobacterium tuberculosis' cell wall mycolic acid is inhibited by isoniazid, which also has a concentration-dependent bactericidal impact on TB infections [16]. Any defects in the detoxification-related mechanisms might lead to hepatotoxicity. Oxidative stress resulting from the drug or its metabolite binding to host proteins through covalent bonds is one of the primary causes of liver damage. It's significant that some people with severe liver damage may regain normal liver function even after using ATDs on a regular basis. It's thought to be the liver's adaptation to help eliminate medications and other waste products from metabolism [17].

Hepatic disorder, which include cirrhosis, heptoses, acute hepatitis, and chronic hepatitis, are the most dangerous illnesses. It is brought on by infections, autoimmune diseases, excessive alcohol usage, and hepatotoxic substances. When the majority of the liver cells die or enter the fibrotic stage, liver damage results, which leads to an incorrect or non-functional state in the liver. Therapy that would increase liver cell development and repair damaged cells into normal cells may be considered in this disease. For their hepatotoxic side effects, this is particularly true [18].

1.5 Hepatotoxicity Mechanisms Caused by Drugs

Liver damage caused by a substance is known as hepatotoxicity, which is derived from hepatic toxicity. Hepatotoxic medications can cause both acute and chronic liver damage. According to the majority of reports, hepatotoxicity is defined as either one elevated levels of alanine transaminase (ALT) or aspartate transaminase (AST) greater than three times the upper limit of normal range (ULN) accompanied by symptoms of liver injury, such as nausea, vomiting, abdominal pain, unexplained fatigue or jaundice or two elevated levels of ALT or AST greater than five times the upper limit of normal range (ULN) without any symptoms [19]. TB can be well eliminated when identified and treated appropriately, despite the fact that antituberculosis medications can have severe and severe side effects, including hepatotoxicity. The best or most efficient treatment for tuberculosis (TB) usually includes a combination of pyrazinamide (PZA), ethambutol (EMB), rifampin (RIF), and isoniazid (INH); however, not all patients will react to treatment, and not all patients will tolerate these medications appropriately [20]. Hepatic dysfunction and an uneven pattern of liver enzyme levels in investigations are two more major side effects of synthetic medications and other xenobiotics that can result in drug-induced liver injury (DILI) [21].

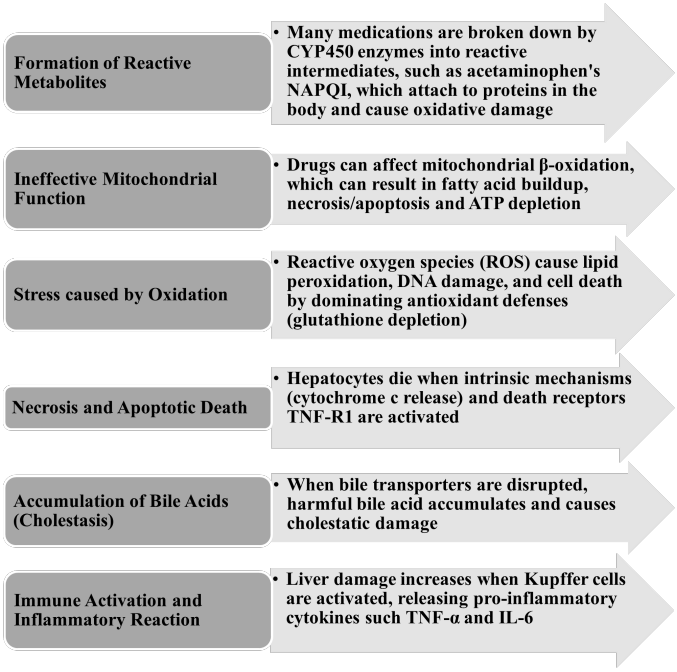

FIGURE 1.1: Overview of Hepatotoxic Mechanism

This schematic depicts the main processes of drug-induced hepatotoxicity, such as oxidative stress, mitochondrial dysfunction, metabolic activation by CYP450, and inflammatory responses (TNF-α/NF-κB) that result in hepatocellular damage. Orthocetamol and other protective measures work by blocking these effects through anti-inflammatory ($\downarrow$TNF-α) and antioxidant ($\uparrow$GSH/SOD) mechanisms. Injury pathways are indicated by red arrows, whereas protection is indicated by green arrows. The graphic illustrates possible hepatoprotective treatment targets.

1.6 Mechanisms and Onset of Idiosyncratic vs. Intrinsic DILI

DILI used to be separated into two groups: idiosyncratic and intrinsic. Intrinsic DILI is typically dose-dependent and predictable, and hepatotoxicity usually starts hours to days after exposure. On the other hand, the course of idiosyncratic DILI is often unexpected, with a latency of onset that can vary from weeks to months [22]. Acute liver injury and failure in clinical practice after marketing, abrupt medication withdrawal after product launch, and drug attrition during the development process are all primarily caused by drug-induced liver injury (DILI). While utilized antituberculosis treatments are more usually mentioned in developing nations, amoxicillin-clavulanate is the most commonly mentioned idiosyncratic DILI in advanced countries. The majority of patients will eventually recover after discontinuing their medications, but 10% of patients especially those with jaundice may experience negative consequences later on, such as liver failure or death [23]. The indicators that follow are common clinical signs of DILI: jaundice, exhaustion, nausea, vomiting, stomach discomfort, and increased liver enzyme levels. Minimizing liver damage requires early detection and stopping the harmful substance. Supportive care, routine liver function monitoring, and liver transplantation in extreme circumstances are common management techniques. It is essential to regularly evaluate liver function through blood testing for patients using potentially hepatotoxic drugs. Pre-existing liver diseases, genetic susceptibility, and polypharmacy with hepatotoxic drugs are risk factors that can raise the

risk of developing DILI. Effective prevention and management depend on thorough monitoring, increased symptom awareness, and prompt medical action [24].

1.7 Antituberculosis Drug-Induced Hepatotoxicity: Mechanism and Immunogenetics

The pathophysiology and molecular mechanism of drug-induced hepatotoxicity for the majority of offending drugs remain unclear. These doubts also hold true for the hepatotoxicity that antituberculosis drugs cause. Although toxicity may be dose-related, there isn't much evidence correlating serum medication levels to hepatotoxicity [13]. In order to manage antituberculosis drug-associated toxicity, research is still ongoing about the clinical significance of therapeutic monitoring of serum rifampicin and isoniazid concentrations. In certain instances of drug-induced hepatitis, hypersensitivity to antituberculosis medications may be a possibility, particularly if patients also exhibit fever, arthralgia, eosinophilia, and skin rash. Increased lipid peroxidation and a changed antioxidant profile could indicate that oxidative damage is the mechanism underlying the hepatotoxicity caused by isoniazid and rifampicin. The previously mentioned synergistic or additive actions of isoniazid and rifampicin may be the cause of their interaction hepatotoxicity. Enhancement of liver toxicity by monoacetyl hydrazine, hydrazine, and similar chemicals arising from hepatic metabolism through enzyme stimulation is one potential mechanism [25].

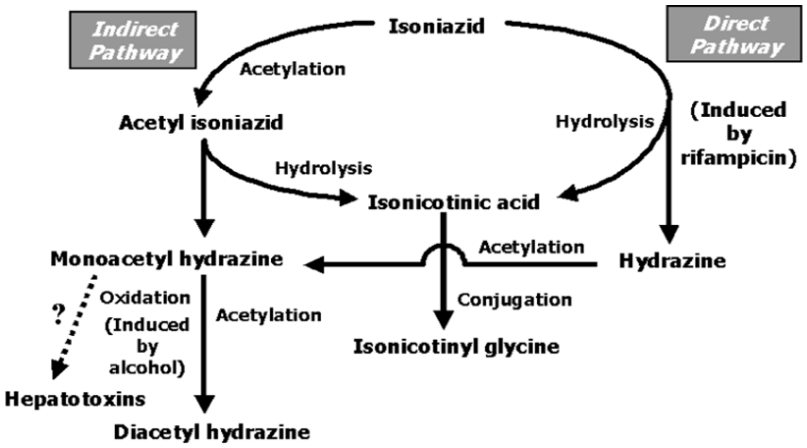

FIGURE 1.2: Mechanism action of Isoniazid

Hepatocyte apoptosis/necrosis is caused by a combination of CYP2E1-mediated conversion to reactive metabolites, oxidative stress through ROS production, mitochondrial dysfunction, and TNF-α/NF-κB-driven inflammation, as summarized in this diagram. Green protective chemicals may act at these important points.

All over the world, there are an estimated 10 million TB cases recorded each year. Isoniazid, a first-line drug recommended by the WHO, is part of the traditional anti-TB chemotherapy. It lowers acid resistance by inhibiting the synthesis of mycolic acid, which stops the mycobacterium's growth and ultimately causes it to die. However, one of the primary side effects of isoniazid is hepatotoxicity, which often results in the cessation of TB treatment. The current recommendation is to reduce isoniazid-induced liver injury (IILI) by changing the medication's dosage. Therefore, there is a great need for hepatoprotective medications that might be used as adjuvant therapy for patients receiving isoniazid [26].

1.8 Treatment Complications of Tuberculosis: Elevated Hepatotoxicity in At-Risk Individuals

The number of new cases of tuberculosis increased from 8.3 million in 2000 to 9.2 million in 2006, according to WHO estimates. More than 1.6 million people die from this illness each year. Combination pharmacological therapy should be administered to TB patients for a minimum of six months in order to prevent the disease and stop the spread of drug resistance, which is becoming a serious issue due to the lengthy treatment time and the use of numerous medications. One of the most significant therapeutic concerns for TB patients is unpleasant side effects [27]. Hepatotoxicity during anti-tuberculosis treatment can occur in up to three to five times as many TB patients with chronic liver disease as in those who do not have a viral infection. Patients with both HIV and HCV are reported to have a fourteen-fold higher risk of developing anti-TB hepatotoxicity [28].

1.9 Isoniazid: An Essential Medication for Treating Tuberculosis

Isoniazid is one of the most important drugs used to treat tuberculosis. Although it is active against latent mycobacteria, it has a bactericidal effect on rapidly proliferating mycobacteria. Despite having been invented in 1912, researchers from Bayer in Germany and Hoffmann-La Roche and E. R. Squibb & Sons in the United States first separately and simultaneously discovered its antitubercular capabilities in the early 1950s. After isoniazid was found to be an anti-TB drug in 1952, it was added to the TB treatment regimen. Despite being discovered 64 years ago, isoniazid remains one of the best alternatives for treating tuberculosis, even though several medicinal chemistry groups have recently revealed isoniazid compounds with significant anti-TB activity [29].

In 20% of patients acquiring the anti-tuberculosis drug isoniazid, a hepatotoxic reaction usually associated with an inflammatory response—may occur. Exposure of hepatocytes to non-toxic H2O2 levels approximating the generation of H_2O_2 by inflammatory cells doubled their susceptibility to isoniazid toxicity. A non-toxic H2O2-generating system also increased the cytotoxicity of hydrazine, the metabolite that is formed when isoniazid is hydrolyzed by amidase, by a ratio of 16. In comparison to hydrazine, acetyl hydrazine was substantially less harmful in this model of hepatocyte inflammation [30].

sAlthough it may cause liver failure, isoniazid (INH) is the medicine of choice for treating latent tuberculosis (TB) because of its great efficacy. Although the clinical features of INH-induced liver injury are pretty consistent for idiosyncratic DILI, drug-induced liver injury (DILI) caused by various drugs varies. Malaise, nausea, vomiting, and exhaustion are a few of these. Only by detecting hepatocyte injury markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) can liver damage, which is usually asymptomatic, be identified [31].

1.10 INH-Related Liver Damage: Mechanisms, Metabolites and Detoxification

INH, a significant antitubercular drug, alters the liver's structure and metabolism in a number of ways. The liver is the body's main detoxification organ. The liver changes INH into hydrazine metabolites. Lipid peroxidation, which is triggered by the highly reactive oxygen species these radicals produce, results in hepatic necrosis and cell death [32].

In spite of its clinical success, INH therapy is complicated by hepatotoxicity, which frequently happens in 5-20% of patients as silent transaminase increase and, in more severe events (1-5%), as clinically readily apparent hepatitis [33].INH may reach the environment in a number of ways. For example, human urine can release INH into the environment. After ingestion by mouth, less than 20% of isoniazid is eliminated unaffected by humans into wastewater [34].

The hydrazide INH is readily oxidized. Acetyl hydrazine (AcHz), hydrazine (Hz), and, more recently, a molecule derived from the bioactivation of INH itself are the three metabolites that have been linked to liver damage caused by INH [31].

Despite genetic polymorphisms and other mutations in genes encoding for some of the drug-metabolizing enzymes involved in the bioactivation and detoxication pathways of INH have long been believed to be patient-specific determinants of susceptibility, drug-specific mechanisms used to involve the production of reactive metabolites that cause hepatocellular damage [35].

The principal metabolic pathway for INH in humans is NAT-mediated enzymatic acetylation. The hydrazine unit may be chemically modified with a suitable functional group to prevent acetylation, perhaps increasing the therapeutic curative advantages [36].

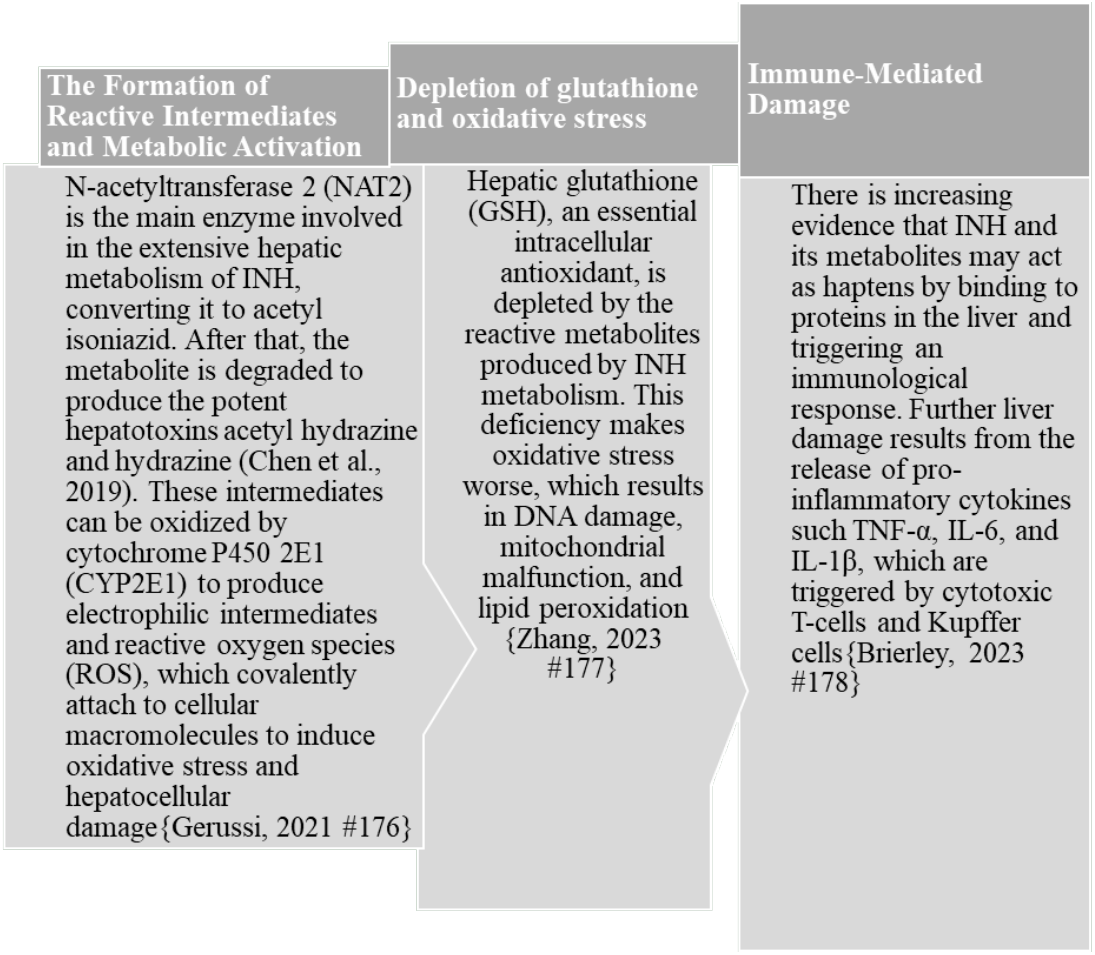

FIGURE 1.3: Isoniazid-Induced Hepatotoxicity Mechanism

Isoniazid (INH) causes hepatotoxicity through CYP2E1-mediated reactive metabolite formation, oxidative stress, mitochondrial damage, and TNF-α/NF-κB inflammation, leading to hepatocyte death. Protective agents target these pathways to prevent liver injury.

1.11 The Clinical Impact of INH Hepatotoxicity

Clinical manifestations of IIH may include jaundice, acute hepatitis, and, in rare instances, fulminant hepatic failure necessitating liver transplantation as well as modest, asymptomatic increases in serum transaminases (ALT and AST). A number of risk factors make people more likely to develop IIH [37].

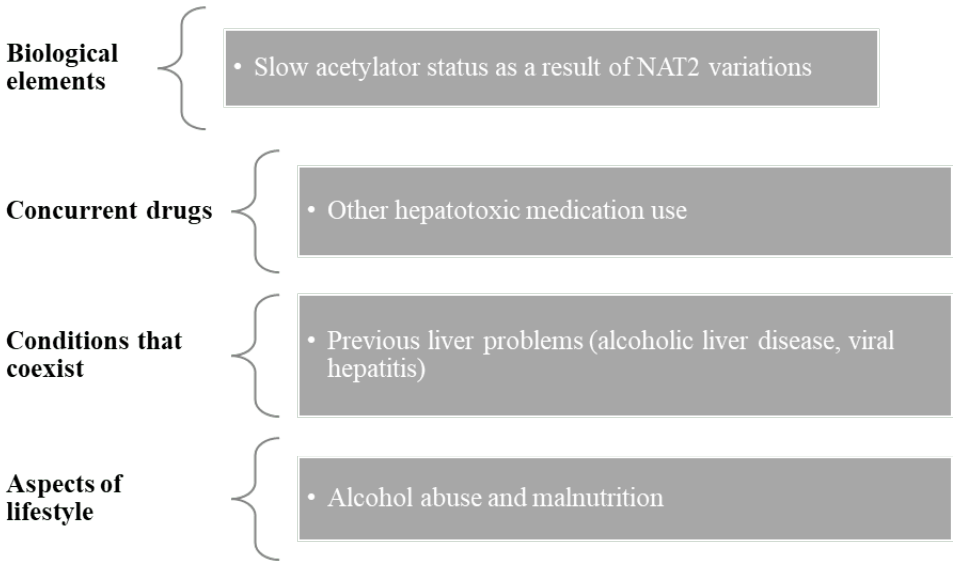

FIGURE 1.4: Effects of INH Hepatotoxicity in Clinical Trials

One of the most important medications for the prevention and treatment of tuberculosis (TB) is isoniazid (INH). Clinical trials have thoroughly examined the danger of hepatotoxicity, or liver damage, that comes with its use. A renowned analysis of its effects based on results from clinical trials is provided below. Reword

Liver function requires to be frequently monitored when using TB treatment because of this risk. In cases of severe hepatotoxicity, medication reduction is often necessary, which can lead to treatment failure and the emergence of drug-resistant TB strains [38].

1.12 Hepatoprotection and Limitations

Clinical research has explored the potential of N-acetylcysteine (NAC), a traditional treatment for acetaminophen toxicity because of its capacity to replenish glutathione (GSH) levels and scavenge reactive oxygen species (ROS), as a treatment option for hepatotoxicity brought on by isoniazid (INH) [39]. However, the results have been mixed, with some trials showing that NAC treatment did not significantly reduce INH-induced liver damage [40].Silymarin, derived from Silybum marianum, contains anti-inflammatory, anti-fibrotic, and antioxidant qualities [41]. Although it is widely used, its clinical value in IIH is limited by its poor

absorption and inconsistent efficacy [42]. Despite the fact that vitamin E is an antioxidant and UDCA is helpful for cholestatic liver problems, individuals with IIH have not shown any discernible protective effects from comprehensive clinical trials. It is necessary to create novel hepatoprotective drugs with enhanced safety and effectiveness to address the problems mentioned previously [43].

1.13 Pharmacological Properties and Hepatoprotective Mechanisms of 2-Acetamidophenol Orthocetamol

To improve drug safety and efficacy, decrease toxic or adverse reactions and increase material stability, drug analogues and isomers are commonly used in the drug development process. These isomeric drugs have a unique identity, are commonly available on the market, are employed in therapeutic settings, and are different from their parent drug [44]. 2-Acetamidophenol (AAP) is an aromatic compound that shows promise in medical research and agricultural uses.

Currently, AAP is made chemically using nonrenewable fossil fuel resources, which pollutes the environment and creates unfavorable reaction circumstances [45].The positioning of the acetamido group is the only structural variation between Orthocetamol (2-acetamidophenol) and paracetamol (acetaminophen). [46]. Orthocetamol has distinct metabolic processes that prevent the development of reactive intermediates, in contrast to paracetamol, which is metabolized by CYP2E1 to the lethal N-acetyl-p-benzoquinone imine (NAPQI) [47].

Recent studies indicate that Orthocetamol has hepatoprotective gets value across a variety of mechanisms.

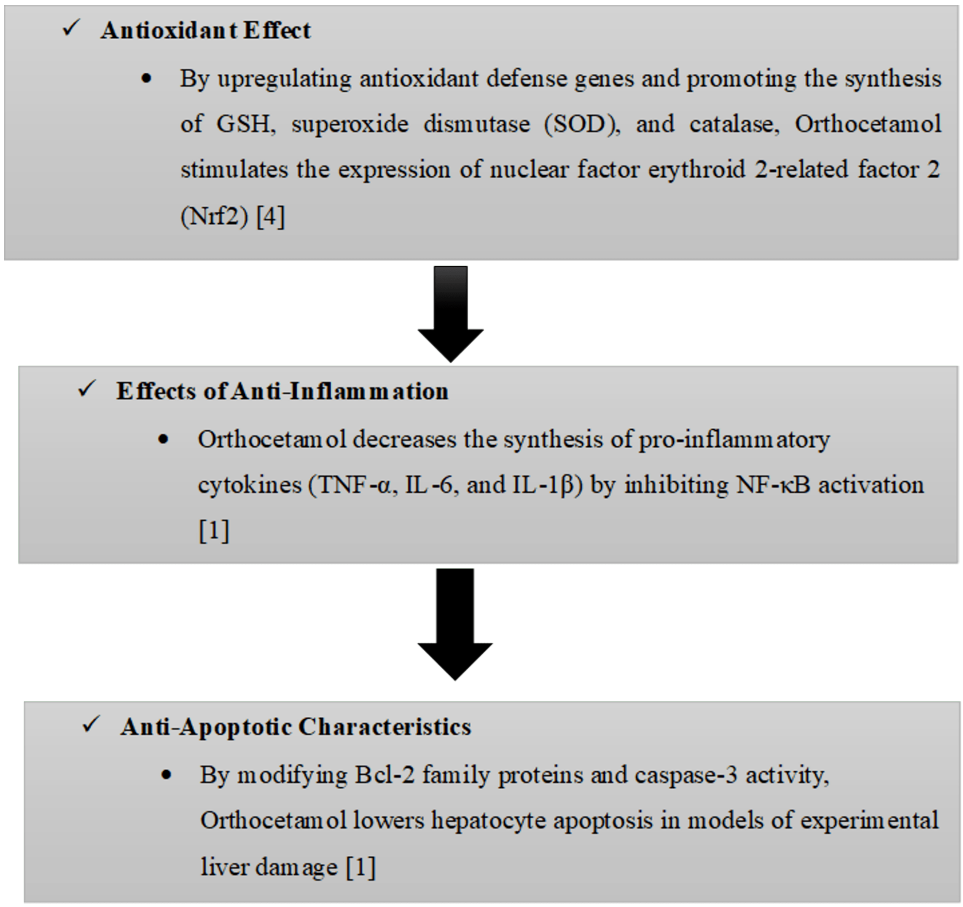

FIGURE 1.5: Orthocetamol Hepatoprotection Mechanisms

Orthocetamol scavenges reactive oxygen species (ROS), increases glutathione (GSH) synthesis, suppresses pro-inflammatory cytokines (TNF-α, IL-6), and inhibits mitochondrial apoptotic pathways to prevent drug-induced liver damage. The integrity and function of hepatocytes are maintained by these multimodal mechanisms taken together.

Among these isomers are 2 - acetamidophenol, sometimes referred to as orthoacetaminophen, Orthocetamol, N-(2-hydroxyphenyl) acetamide, 2 -hydroxyacetanilide, and N - acetyl - o -aminophenol. According to a review of the literature, 2 - acetamidophenol may have potential medical use. Furthermore, it has been proposed that 2-acetamidophenol works well to slow down chronic inflammation and regulate nociception in rats with arthritis. Besides the cases already mentioned, a recent investigation into the antitubercular activity of 2 - acetamidophenol revealed that this chemical had a strong inhibitory impact against Mycobacterium

TB H37Rv [48]. In regard to this, the current research project focusses on examining the pharmacological and toxicological consequences of Orthocetamol, sometimes referred to as 2-Acetamidophenol (2-AMP), the ortho positional isomer of paracetamol that was utilized to prevent hepatotoxicity caused by paracetamol [49].

The present research is focused on 2-acetamidophenol, also referred to as Orthocetamol, in order to examine the acute toxicity profiles of positional isomers of paracetamol. The hepatoprotective effects of this chemical against isoniazid-induced hepatotoxicity have not been assessed before. First-line antitubercular medication isoniazid is known to induce oxidative stress and liver damage by forming reactive metabolites through cytochrome P450 enzymes, specifically CYP2E1. Hepatocellular injury may result from these metabolites' depletion of glutathione levels [44]. According to our research, Orthocetamol may improve antioxidant defenses and modulate oxidative stress pathways to have a hepatoprotective effect. The body of research on the hepatoprotective qualities of several substances supports this theory. In animal models, for example, N-acetylcysteine has been demonstrated to lessen oxidative liver damage brought on by isoniazid and rifampicin. Likewise, silymarin and curcumin have shown protective effects against hepatotoxicity caused by antitubercular drugs, most likely as a result of their antioxidant qualities [49].

1.14 Preclinical Data on the Efficacy of Orthocetamol

According to recent research, Orthocetamol dramatically lowers serum transaminases, lessens hepatic necrosis, and increases survival rates in models of acetaminophen-induced liver injury. As undiscovered, but are its potential implications in IIH [47].

Biomarkers of hepatic damage should have these ideal features such as liver-specificity, strong correlation with well-defined hepatic histomorphology changes,

outperformance or additional information to serum alanine aminotransferase (ALT) or aspartate aminotransferase (AST) values, adaptation of screening assays to commercially available high throughput modalities, sample accessibility through noninvasive procedures like blood collection, and, in the context of clinical translation, application across key preclinical species to humans, including mouse, rat, dog, and monkey[50]. For wide acceptance by scientific, medical, and regulatory bodies, severe scientific validation or cross-validation and biological qualification are necessary after candidate markers are found. Although no one biomarker has yet to reach this degree of thorough assessment, the pharmaceutical industry and regulatory bodies share these characteristics for all safety biomarkers in development. Guidelines for choosing validated hepatotoxicity indicators suitable for preclinical testing [51].

Despite an extensive amount of research on isoniazid-induced hepatotoxicity (IIH), the best hepatoprotective drug is still elusive, indicating a significant weakness in TB treatment approaches. Treatment interruptions, poor patient outcomes, and increased morbidity are all consequences of current therapy regimens, especially those including isoniazid (INH), which are linked to substantial liver injury [52]. A new substance with a distinct metabolic pathway and several different modes of action Orthocetamol has shown hope as a hepatoprotective agent. The unique pharmacokinetic and pharmacodynamic characteristics of Orthocetamol, in contrast to traditional hepatoprotective medications, include increased antioxidant activity, anti-inflammatory effects and modification of drug-metabolizing enzymes, all of which may lessen the liver damage caused by INH [53].

This study aimed to evaluate the effectiveness of Orthocetamol as a hepatoprotective medication against isoniazid-induced hepatotoxicity (IIH) by means of comprehensive preclinical and clinical evaluations. Clarifying its molecular mechanisms such as its capacity to scavenge reactive oxygen species (ROS), prevent lipid peroxidation, and replenish glutathione levels was the goal of the study in order to provide a scientific foundation for its possible therapeutic application. The study further investigated if Orthocetamol might be safely incorporated into

regular TB treatment regimens without sacrificing antimicrobial efficacy by examining whether it had synergistic effects with already available anti-TB medications. Positive findings indicated that Orthocetamol, as an adjuvant medication, would lessen hepatotoxicity, potentially revolutionizing TB treatment by enhancing compliance and reducing problems linked to the liver. Furthermore, this study had major implications for the development of hepatoprotective drugs in the future, which would provide TB patients with a safer and more efficient way to treat drug-induced liver damage. The study had significant translational potential by resolving this major medical need, which could have a significant impact on patient treatment and international TB management tactics.

Chapter 2

Literature Review

2.1 Introduction

According to certain theories, the liver is one of the essential organs that regulates the body's homeostasis. Carbohydrate and lipid production, storage, and metabolism are among the several liver functions. In addition, the liver is required for the detoxification of toxins, the excretion of bilirubin, and the metabolism of many endogenous and exogenous substances. A person may suffer negative consequences if their liver function is impaired since it affects several homeostatic systems. One unresolved problem that frequently limits pharmacological therapy in clinical practice is medication-induced liver damage. Various chemical and pharmacological compounds administered parenterally, by ingestion, or by inhalation may result in hepatic injury [54].

The infectious bacterial disease tuberculosis (TB) is caused by Mycobacterium tuberculosis, which comes in a number of strains, according to Kumar et al. (2007). In the modern era, tuberculosis has emerged as a major health concern, alongside the growing HIV epidemic. Among the countries with a high number of TB sufferers is India. Over $525 million US dollars were spent in India to combat tuberculosis in 2021, despite the country seeing 21.4 lakh new cases of the illness. Drugs like isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin, and others are frequently used to treat tuberculosis [55].

It has been claimed that the liver is one of the vital organs that controls the body's homeostasis. Among the many hepatic processes are the synthesis, storage and metabolism of carbs and lipids. In addition to these, the liver is necessary for the metabolism of numerous endogenous and exogenous chemicals, bilirubin excretion, and toxin detoxification. When liver function is compromised, various homeostatic processes are impacted, which could have detrimental effects on the person in question. Drug therapy in clinical practice is frequently limited by drug-induced liver impairment, an unsolved issue. Hepatic damage may follow the administration of various chemical and pharmacological substances by parenteral, ingestion or inhalation [54].

It has been established that a number of polymorphisms, pre-existing liver illness, advanced age, and co-administration of medications that stimulate CYP (cytochrome P450) enzymes are risk factors for hepatotoxicity with INH (isoniazid). Polymorphisms in NAT2 (N-acetyl transferase 2), which controls drug metabolism, CES1 (carboxylesterase 1), GST (glutathione S-transferase), SOD (superoxide dismutase), HLA (human leukocyte antigen), and TNF-α (tumor necrosis factor-alpha) are among the genetic factors that are primarily implicated in INH-induced liver injury. Patients receiving INH are more susceptible to liver damage as a result of these causes [56].

According to a 2020 study using human hepatocyte models, isoniazid (INH)-induced hepatotoxicity is caused by a number of synergistic mechanisms, with its reactive metabolites serving as the main driver. When mitochondrial permeability transition (MPT) holes open as a result of these compounds, the potential of the mitochondrial membrane is disrupted, and cellular bioenergetics collapse. In the end, this MPT pore opening causes hepatocyte malfunction and death by releasing pro-apoptotic chemicals, oxidative stress, and decreased ATP generation. The work illustrates how mitochondrial dysfunction plays a key role in INH-mediated liver injury, offering a molecular explanation for its hepatotoxic effects and recommending possible treatment targets, including antioxidants or MPT pore inhibitors, to lessen toxicity [57].

It has been proven that isoniazid (INH) alters the gut microbiota, decreasing good bacteria and encouraging the proliferation of harmful species, so reducing the integrity of the intestinal barrier. Because of the increased gut permeability caused by this dysbiosis, endotoxins from Gram-negative bacteria, such as lipopolysaccharide (LPS), can enter the bloodstream. Once in circulation, these endotoxins promote the production of pro-inflammatory cytokines including TNF-α and IL-6, which worsen liver inflammation and injury, by activating Toll-like receptor 4 (TLR4) on Kupffer cells in the liver. The direct hepatotoxic effects of INH combine with this endotoxin-driven inflammatory response to exacerbate liver damage. According to studies, by reestablishing microbial balance and minimizing endotoxin leakage, probiotics or gut microbiota modification may be able to lessen INH-induced hepatotoxicity [58].

As to the claim, isoniazid is mostly metabolized by N-acetyltransferase 2 (NAT2) in the liver, producing acetyl-isoniazid, which is then further broken down into potentially dangerous intermediates which include acetyl hydrazine. It has been observed that cytochrome P450 (CYP2E1) activates these metabolites, resulting in oxidative stress, glutathione (GSH) depletion, and reactive oxygen species (ROS) production [59]. Furthermore, INH has been shown to interfere with mitochondrial activity, reducing ATP generation and contributing to hepatocyte death as a result [60].

From research reports, INH-induced hepatotoxicity can vary from moderate increases in liver enzymes (ALT, AST) to abrupt liver failure and severe liver necrosis, especially in people with NAT2 slow-acetylator genotypes. In order to avoid irreversible liver damage, it is said that early identification through biochemical monitoring is essential [61]. It has been shown that the positioning of the acetamido group in Orthocetamol (2-acetamidophenol) and paracetamol (4-acetamidophenol) differs, affecting their metabolic pathways [62].

Unlike to paracetamol, which uses CYP2E1 to make the hepatotoxic metabolite NAPQI, Orthocetamol may use different metabolic pathways that result in less hazardous intermediates. [63].According to certain reports, Orthocetamol has potent free radical scavenging properties that lessen oxidative stress in liver cells

[63]. Furthermore, it has been found that Orthocetamol suppresses NF-κB activation and downregulates pro-inflammatory cytokines such TNF-α and IL-6, hence modulating inflammatory pathways [64].

It is reported that Orthocetamol improves antioxidant enzymes (SOD, CAT, GPx) activity and levels of lipids peroxidation markers (malondialdehyde) in liver tissues that had been treated by INH [65]. Hepatocyte apoptosis is said to be decreased by Orthocetamol because it stabilizes mitochondrial membrane potential, which stops cytochrome c release and subsequent caspase-3 activation [66].

According to comparative research, Orthocetamol may be more effective than N-acetylcysteine (NAC) for preventing liver damage brought on by isoniazid. According to Patel et al.'s research from 2023, Orthocetamol inhibited inflammatory cytokines, decreased oxidative stress, and restored glutathione levels more successfully than NAC. Though clinical trials are required for validation, its combined action as a CYP2E1 inhibitor and antioxidant (by Nrf2 activation) offers broader protection than NAC's GSH-replenishing mechanism, making it an intriguing alternative [67].

According to reports, anti-tuberculosis medications are the most frequent cause of drug-induced hepatotoxicity during treatment; occurrences range from 2% to 28%. Clinical or histological results can be used to explain DILI. The clinical features vary according to the kind of hepatic injury, including mixed injury (hepatocellular and cholestatic), cholestatic injury (typically disproportionate elevation of ALP, with bilirubin may also rise), and hepatocellular injury (typically elevated aminotransferase enzymes). First-line antitubercular medications, isoniazid (INH, isonicotinic acid, hydrazine) and rifampicin (RIF), are prescribed for a length of about 6 to 9 months. A more severe liver syndrome that can be lethal, INH produces mild hepatotoxicity to severe hepatitis, which is typically accompanied by hepatocellular necrosis, ascites, edema, and encephalopathy. The precise processes underlying hepatotoxicity remain unclear. Nevertheless, INH and its metabolites can cause endoplasmic reticulum stress, mitochondrial malfunction, oxidative stress, inflammation, and harm to mitochondrial biogenesis [68].

It has been reported that the powerful bactericidal antibiotic isoniazid (INH) is used to treat tuberculosis (TB). INH therapy carries a risk of acute or chronic toxicity, which, if untreated, can cause long-term liver damage. Neurological signs, frequently seizures resistant to benzodiazepines, are indicative of acute poisoning. Hepatotoxicity and peripheral neuropathy are the most common symptoms of chronic toxicity. If pyridoxine is prescribed with INH from the beginning of therapy, the toxicity may manifest readily in all medical settings, while it is less likely to occur in emergency rooms. As the number of INH prescriptions for TB rises, dosage errors may lead to an increase in INH poisoning cases. INH must be stopped, pyridoxine must be given for acute toxicity, liver support treatments must be considered if hepatic damage occurs, and liver transplants may be necessary in certain cases [69].

According to earlier research, tuberculosis is a prevalent illness in kids. In 2022, an estimated 10.6 million individuals worldwide, including 1.3 million children (12%), were predicted to have developed tuberculosis. In order to treat tuberculosis, antituberculosis therapy (ATT) is necessary. One of the most frequent adverse side effects of tuberculosis treatment is antituberculosis drug-induced hepatotoxicity (ATDIH). The most commonly implicated medication in clinical cases of ATDIH is isoniazid, however pyrazinamide is thought to have the most hepatotoxic potential among the first-line ATT, followed by isoniazid and rifampicin. Hepatotoxicity can cause treatment to be ceased which elevates the risk of treatment failure, disease progression, and resistance. Significant morbidity and mortality can result from improper management [70].

According to past studies, INH results in mitochondrial malfunction, which includes worsened mitochondrial complexes and decreased energy generation and respiration. In particular, INH causes oxidative stress and death in the mitochondria by affecting complexes I, II, and III and mediating the production of reactive oxygen species (ROS). Additionally, INH influences mitochondrial dynamics and biogenesis. These results, however, are not entirely consistent; some research indicates that INH has no effect on the activity of mitochondrial complexes I and II [71].

In recent reports, one well-known analgesic and antipyretic medication is paracetamol. For the scientific and clinical community, its hepatotoxicity remains a significant obstacle. Rich data on its advantages and risks are accessible. Their positional isomers, however, have access to very rear data, i.e., Oral and metacetamol. It has been previously documented that metacetamol is a non-hepatotoxic isomer with analgesic and antipyretic properties [72].

2.2 Objectives of study

The three main goals of this study were to thoroughly examine isoniazid (INH)-induced hepatotoxicity: First, ELISA tests were used to assess histopathological changes in the hepatic biomarkers ALT and AST and correlate their serum levels with the degree of hepatocellular damage, establishing a biochemical framework for INH toxicity. Second, the contribution of the pro-inflammatory cytokine TNF-α to inflammatory cascades and the progression of hepatic damage was determined by measuring its expression levels. Third, the hepatoprotective potential of Orthocetamol (2-acetamidophenol) against INH-induced inflammation was investigated by evaluating its effects on important inflammatory markers (TNF-α, IL-6) and liver enzymes using ELISA-based analysis, potentially establishing it as a therapeutic adjunct for drug-induced liver injury. These studies collectively provided vital information about the etiology of INH hepatotoxicity and the potential anti-inflammatory properties of Orthocetamol.

a. To determine the histopathological changes in liver enzymes ALT and AST after isoniazid-induced hepatotoxicity using a commercially available ELISA assay technique.

b. To investigate how the cytokine TNF-alpha contributes to the development of isoniazid-induced hepatotoxicity.

c. To investigate the possible therapeutic benefits of Orthocetamol (2-Acetamidophenol) in reducing inflammation caused by isoniazid using ELISA.

Chapter 3

Material and Methods

3.1 Ethical Acceptance

The Capital University of Science and Technology, Islamabad Research and Ethics Committee approved all animal procedures, which have been carried out in accordance with institutional guidelines. On June 1, 2021, approval had been given under protocol number REC/FOP/F2024/18.

3.2 Animals

The CUST operating facility in Islamabad supplied the four-week-old male albino mice, which weighed between 26 and 30 grams. The animals were kept in controlled environments with a 12-hour light/dark cycle, a temperature of 22 ± 2 ^{o}C, and a relative humidity of 50 ± 10%. Throughout the trial, they were fed regular laboratory food and had unlimited access to water. Following established procedures, the mice were acclimated to the laboratory environment for seven days before to the experiment's start (Panel, 2021).

3.3 Handling of Animals

In order to minimize stress, preserve animal welfare, and ensure experiment uniformity, laboratory mice had to be handled properly. While cupped or tail handling practices were supposed to be followed while handling mice, some recent research indicated that tunnel handling, for instance, caused less anxiety than tail handling [73].

To reduce some of the possible variability connected with stress, the mice had to be acclimated to the experimenter's presence and scent before being handled. Wearing gloves prevented scent cross-contamination, and handling took place in a calm environment to prevent disturbing.

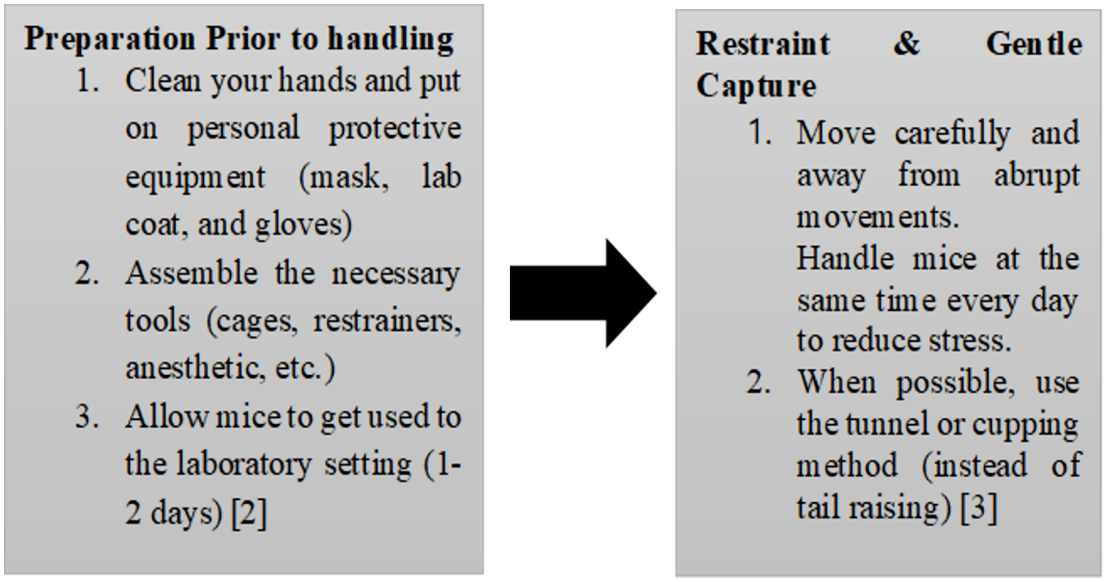

FIGURE 3.1: Procedure of Animal Handling

Using the thumb and fingers of the opposing hand to hold the slack skin at the nape, the mice were securely held by the base of their tail. When the scruff was raised, the tail was tucked against the wrist to provide firm yet comfortable immobilization without obstructing breathing.

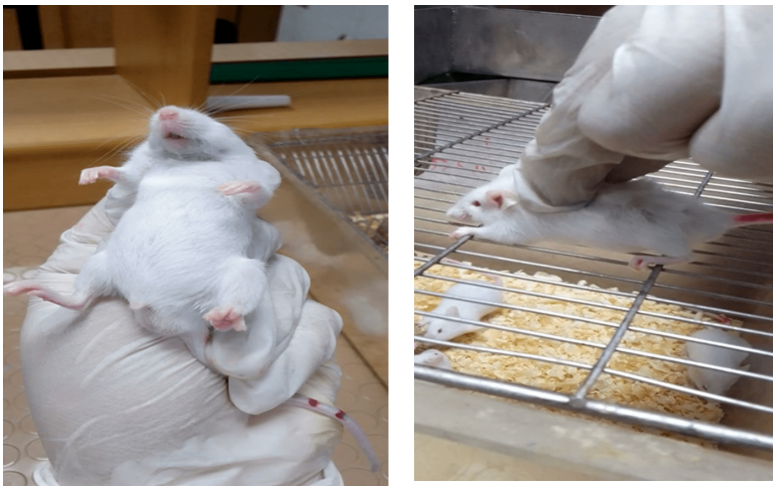

FIGURE 3.2: Seized the mice

With the dorsal recumbency position (right) and scruff restraint (left), the lab mice were handled appropriately. During the experimental procedures, these protocols guaranteed handling that was both safe and compassionate.

3.4 Routes of Drug Administration in Animals (Routes Used)

3.4.1 Intraperitoneal Route of Drug Administration

For animals, the I.P. route was short and causes little stress. The mice were kept supine with their heads angled downward relative to their posteriors. The needle was carefully inserted in the lower abdominal quadrant (at an angle of about 10^o) to prevent unintentional organ penetration [75].Orthocetamol was injected intraperitoneally (I.P.) in the lower right abdomen quadrant using a 1-ml insulin syringe fitted with a 30-gauge needle to reduce the possibility of organ puncture. In order to minimize discomfort and ensure appropriate dose distribution, mice were gently confined using traditional tail-and-scruff techniques to alleviate stress. A shallow 10^o angle was chosen for the needle's insertion to enable precise and secure administration.

FIGURE 3.3: I.P. injection site (lower right quadrant)

Swiss albino mice were given the test substance intraperitoneally (IP). The injection was administered aseptically into the lower right quadrant of the abdomen while the animal was immobilized in the supine position to ensure precise dosage and reduce the possibility of organ damage.

3.4.2 Oral Route of Drug Administration

In order to deliver accurate drug doses with the least amount of harm, oral gavage a technique that involves gently inserting a flexible feeding needle into the esophagus was frequently used to administer drugs orally to mice. The appropriate way to do this was to measure the length of the needle (from nose to final rib) keep the mouse upright, and make sure the delivery was smooth to prevent aspiration [76].Mice were given the toxic medication isoniazid orally by a standardized gavage technique. To administer accurate dosages with the least amount of stress, a blunt-ended feeding needle was delicately placed into the esophagus. Each animal's body weight was used to determine the dosage, which for toxicity tests usually ranged between 50 and 300 mg/kg. Mice were kept upright and lightly restrained throughout treatment to avoid aspiration.

3.5 Classifying and Organizing Experiments

Mice were split up into three experimental groups (n = 6 per group) in this randomized control trial. 1% DMSO in normal saline was given intraperitoneally to the control group. The isoniazid treatment group was further split into two dosage groups, giving them oral doses of either 100 mg/kg or 200 mg/kg of the medication. Two subgroups were also included in the Orthocetamol treatment group, and they received doses of 5 mg/kg and 15 mg/kg intraperitoneally, respectively. Over the course of ten days in a row, each therapy was given once daily. All animals were humanely put down on day 11 after the treatment period, and biological samples were taken for further examination. A more thorough assessment of the treatment effects was possible thanks to this prolonged 11-day protocol than with the first 11-day trial design.

3.6 Chemicals

The studies were conducted using just normal analytical grade reagents. The hepatotoxic drug isoniazid (purity ≥98%) was purchased from Sigma Scientific Private Limited. The hepatoprotective and anti-inflammatory properties of Orthocetamol (2-Acetaminophenol, purity ≥99%), which Sigma Scientific Private Limited helpfully supplied, were evaluated. Until they were used, both chemicals had been stored in sealed containers at room temperature.

3.7 Dosage Guidelines

For accurate the administration of medications, 1 mL insulin syringes (30G, BD Ultra-fine II) were used in the trial. A computerized weighing balance, a vortex mixer, and microcentrifuge tubes were among the lab tools used to formulate the stock solution. Every day, all medications were made from scratch to guarantee their efficacy and consistency. To determine precise doses, daily body weight measurements were taken before each mouse received a weight-adjusted dose. Thirty

minutes after isoniazid was administered, mice in the treatment groups received intraperitoneal injections of their respective medications (Orthocetamol at 5 or 15 mg/kg). In contrast, the doses of isoniazid (100 and 200 mg/kg) were administered orally via gavage. Intraperitoneal administration of 1% DMSO in normal saline was given to the control group. To assess the effectiveness of the treatment, this standardized procedure was adhered to for ten days in a row.

3.8 Compute Dosage

Dosage was estimated for the normal group were 5 and 15 mg/kg of Orthocetamol, 100 and 200 mg/kg of isoniazid.

3.8.1 Orthocetamol (5 mg/kg and 15 mg/kg)

Example for 30 g mice:

a. **5 mg/kg dose:**

   i. Drug required $= \frac{30\,\text{g} \times 5\,\text{mg/kg}}{1000} = 0.15\,\text{mg}$

   ii. Volume (if stock vol. = 0.5 mg/mL) $= \frac{0.15}{0.5} = 0.3\,\text{mL}$

b. **15 mg/kg dose:**

   i. Drug required = 0.45 mg

   ii. Volume (if stock vol. = 1.5 mg/mL) = 0.3 mL

3.8.2 Isoniazid (100 mg/kg and 200 mg/kg)

Example for 30 g mouse:

a. **100 mg/kg dose:**

   i. Drug required = 3 mg

ii. Volume (if stock vol. = 10 mg/mL) = 0.3 mL

b. **200 mg/kg dose:**

i. Drug required = 6 mg

ii. Volume (if stock vol. = 20 mg/mL) = 0.3 mL

3.8.3 Preparation of Drugs (Stock Solutions)

If Vehicle: Saline + 1% DMSO (for solubility)

3.8.4 Orthocetamol

a. 5 mg/kg dose: 5 mg in 10 mL vehicle → 0.5 mg/mL

b. 15 mg/kg dose: 15 mg in 10 mL vehicle → 1.5 mg/mL

3.8.5 Isoniazid

a. 100 mg/kg dose: 100 mg in 10 mL saline → 10 mg/mL

b. 200 mg/kg dose: 200 mg in 10 mL saline → 20 mg/mL

Table 3.1: 10-Day Therapy Plan & Findings

Group	Treatment	Day	Administered	Observations
Group 1 (Control)	Normal saline (1% DMSO, i.p.)	Day 1–10	Vehicle only	Baseline behavior, weight, no toxicity expected.
Group 2 (Toxic Low)	Isoniazid 100 mg/kg, i.p.	Day 1–10	Daily injection	Weight loss, lethargy, hepatotoxicity (monitor ALT/AST if possible).
Group 3 (Toxic High)	Isoniazid 200 mg/kg, i.p.	Day 1–10	Daily injection	Severe toxicity (weight loss, jaundice, high mortality expected by Day 7–10).

Table 3.1 continued from previous page

Group	Treatment	Day	Administered	Observations
Group 4 (Treatment Low)	Orthocetamol 5 mg/kg + Isoniazid 100 mg/kg, i.p.	Day 1–10	Both daily	Assess if 5 mg/kg mitigates toxicity (weight, behavior, survival).
Group 5 (Treatment High)	Orthocetamol 15 mg/kg + Isoniazid 100 mg/kg, i.p.	Day 1–10	Both daily	Dose-dependent protection (compare to Group 4).

Ethical considerations included early euthanasia in cases of severe distress (e.g., >20% weight loss, inability to eat or drink), vehicle control using 1% DMSO in saline (ensured compatibility with Orthocetamol), and personal protective equipment (PPE) such as gloves, masks, and eye protection.

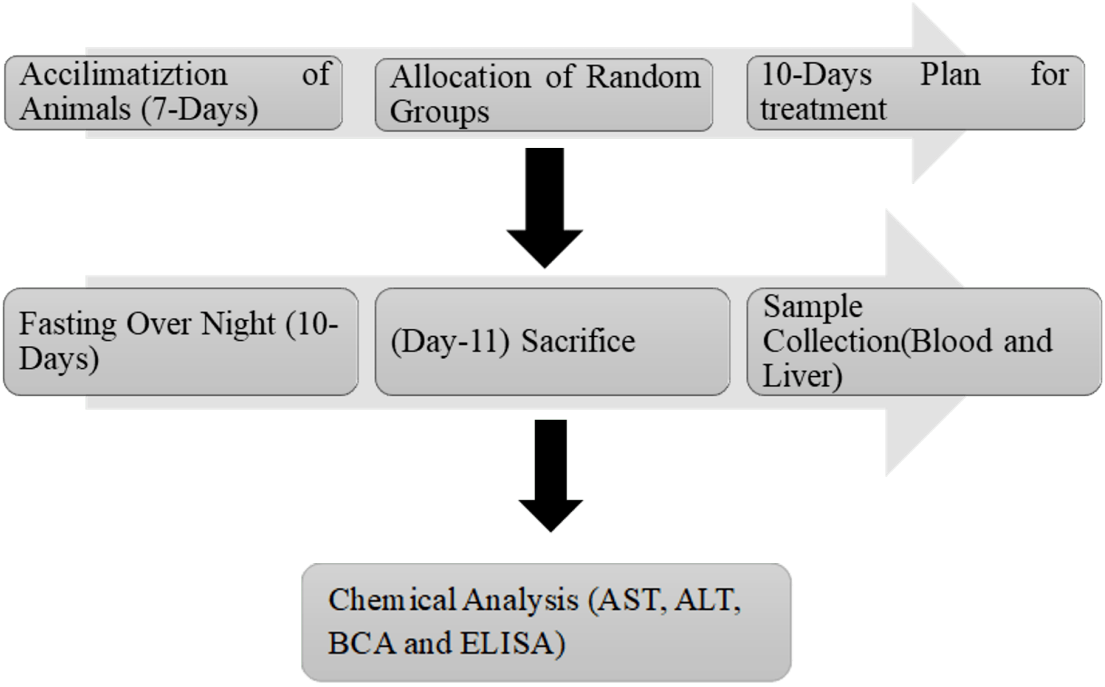

FIGURE 3.4: Experimental Protocol

This section described how the 10-day mouse toxicity research assessing isoniazid-induced hepatotoxicity was conducted, including drug dose calculations, preparation, administration, and safety precautions.

3.9 Techniques for Euthanizing Mice for Experiments

Euthanasia procedures were performed in laboratory mice should be performed humanely and, where possible, and wherever possible following best practices to minimize pain and distress. Common methods included inhalation agents (e.g., carbon dioxide (CO_2), isoflurane), cervical dislocation, and overdose of injectable agents (e.g., pentobarbital). CO_2 euthanasia was the most widely used method as it acted quickly, however strict adherence to protocol was imperative to ensure a slow, reasonable, and controlled displacement of oxygen to minimize distress. The AVMA recommended CO_2 concentrations of 30-70% as appropriate for induction but the animals were then euthanized with a secondary method, such as cervical dislocation to confirm death. (AVMA, 2020). Isoflurane overdose could also be used, particularly in the case of neonates where the agent, followed the appropriate duration of exposure, achieved anesthesia first before arresting respiration [77].

3.10 Justification and Importance of Research

1. In order to avoid needless suffering, euthanasia was morally necessary in animal research. It adhered closely to international welfare standards and the 3Rs (Replacement, Reduction, Refinement) principles. When animals exhibited extreme distress or satisfied certain standards, such as a notable reduction in body weight, humane endpoints were used.

2. Euthanasia methods carried out correctly preserve scientific validity. Rapid, regulated techniques maintain tissue integrity for precise biochemical and histopathological investigations. Research results in enzymatic and molecular studies may have been jeopardized by degraded materials.

3. Another important factor was safety. Researchers were less likely to be exposed to pathogens thanks to standardized procedures. Additionally, they

lessened workplace risks associated with handling animals, particularly when dealing with poisonous or contagious materials.

3.10.1 Using Chemical Techniques Chemicals Inhaled During a Mice's Execution

The hepatotoxicity caused by isoniazid was studied in mice using the chemical inhalation approach with chloroform. The selection of chloroform for laboratory rodents was based on its rapid induction of unconsciousness, low handling stress, and compatibility with subsequent tissue analysis, among other physical and chemical euthanasia methods. An airtight desiccator was pre-saturated with two to three milliliters of chloroform absorbed on cotton before chloroform vapor was added. After two to three minutes of exposure to the fumes, each mouse's respiration and corneal reflex entirely stopped. A heart puncture was immediately carried out to collect a sample after the death was confirmed by the lack of cardiac activity [78].The volatile, colorless liquid chloroform was not particularly soluble in water. It had a smell nice and didn't irritate the nose.

Chloroform had the chemical formula $CHCl_3$ and a molecular weight of 119.38 g/mol. A frequent chemical in biological labs and industrial operations is chloroform [79].Animals had been sacrificed under chloroform sedation with practically no negative consequences. The animals were placed in a desiccator with the appropriate chemical. For optimal circulation, the chemical was soaked in cotton wool and put in the desiccator for two minutes before exposure [80].

3.11 Methods For Collecting Blood (Cardiac Puncture)

Use the chloroform anesthetic to deeply sedate the mice before beginning any sample collecting procedures. After achieving the proper level of anesthesia, place the animal in dorsal recumbency. A syringe with a needle of the proper size was

attached, and the syringe was positioned parallel to the mice's midline. The needle was inserted into the diaphragm with the bevel up at a 30–40^o angle.

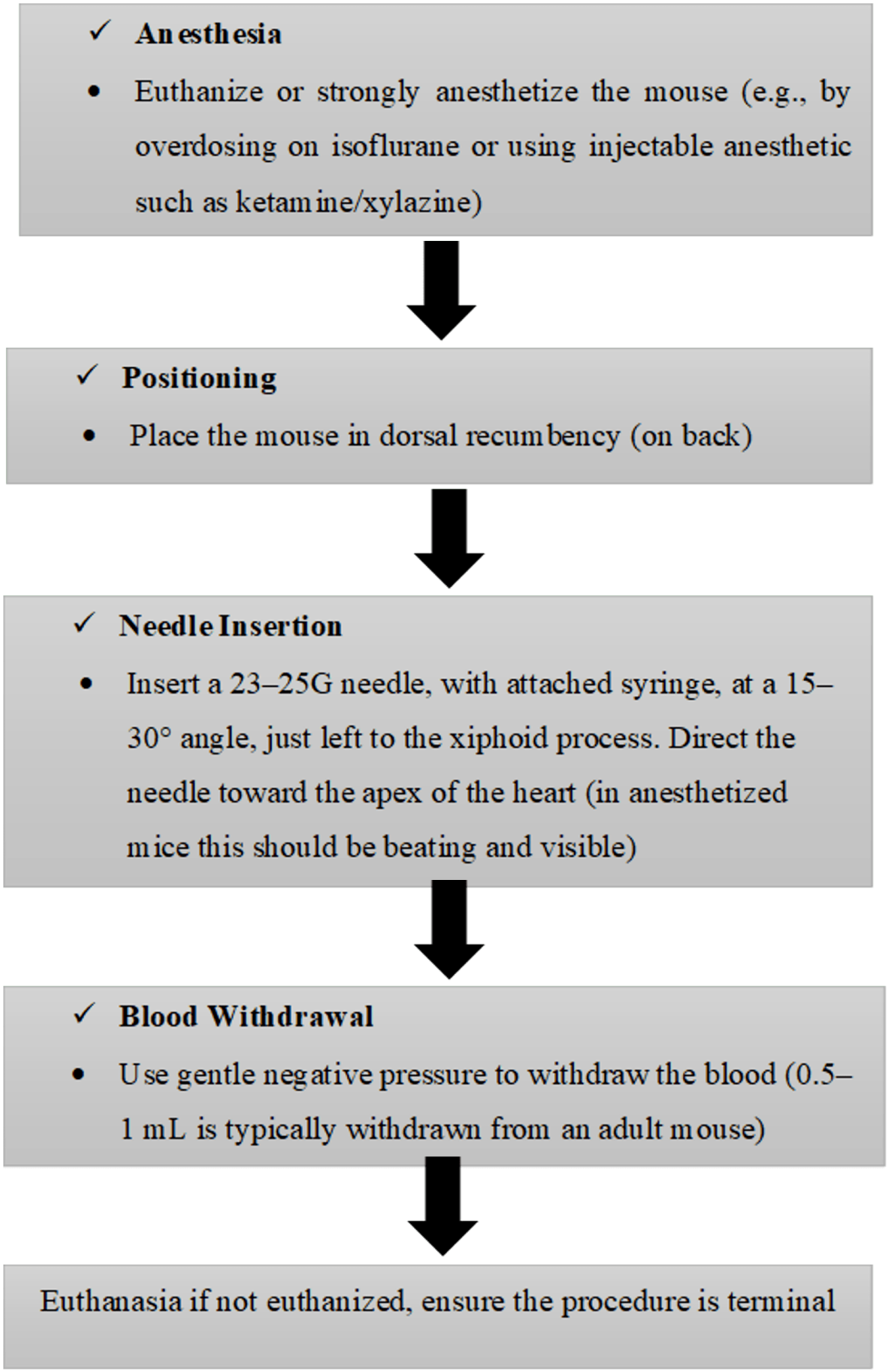

FIGURE 3.5: Blood Collection Method [82]

Put the needle at the mouse's heart from a slight left under the sternum. You can tilt the needle a little bit in the direction of your left shoulder. Gently advanced the needle until a blood flash emerges in the needle hub after slightly retracting the plunger to generate a vacuum inside the syringe. Once the needle was immobilized, keep aspirating until enough blood had been drawn [81]. When significant quantities of blood were required, cardiac puncture was a common way of collecting blood from mice. Here is an outline of the method.

Before sample processing, experimental Swiss albino mice were acclimated in a desiccator chamber in the lab. During pre-terminal handling activities, the desiccator environment minimizes moisture interference by providing a regulated atmosphere for temporary habitation.

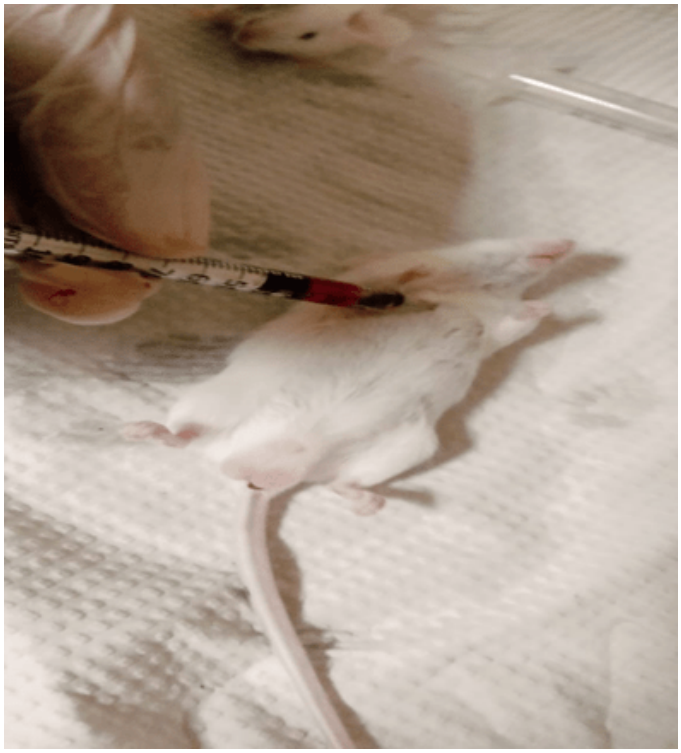

FIGURE 3.6: Blood Withdrawal

Mice were given cardiac punctures under anesthesia to obtain their last blood. Blood was extracted as quickly and maximally as possible for biochemical examination using this technique.

3.12 Preservation of Liver Samples and Tissue Dissection

The liver was removed by midline laparotomy after heart puncture and euthanasia. Once the tissue was collected, it was prepared for additional examination [83].The midline laparotomy was done after euthanasia and heart puncture. A retractor was utilized to reveal the liver after the Linea Alba was cut to allow access to the abdominal cavity. Following the dissection of connective tissues to mobilize the liver, it was removed, washed with 0.9% NaCl, and preserved in 10% phosphate-buffered formalin (pH 7.4) for histopathological analysis. Samples that were fixed were kept at -4^{o}C for additional examination [84].

3.13 Evaluation of Oxidative Stress Markers

By the use of commercially available enzymatic test kits, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were quantitatively determined per the manufacturer's instructions. To summarize, blood samples were collected and allowed to clot at room temperature after which serum was separated by centrifugation. Thereafter, the serum samples were aliquoted and stored at -20^{o}C until they were tested. All assay procedures as provided in the manual, including those concerning reagent preparation, calibration curve construction, and incubation periods, were meticulously observed. With a microplate reader set to the recommended wavelength, absorbance readings were taken, where enzyme activity was computed based on the standard curve [85].

Serum Enzyme Assays Commercial diagnostic kits were used to assess the ALT and AST enzyme levels in blood serum. Making Liver Homogenate An ice-chilled homogenizer was used to homogenize the liver tissues after they had been cleaned with cold PBS (pH 7.4). The samples were then centrifuged for 30 minutes at 24^{o}C at 8500 rpm in order to extract the supernatant. To prepare the buffer, 0.1

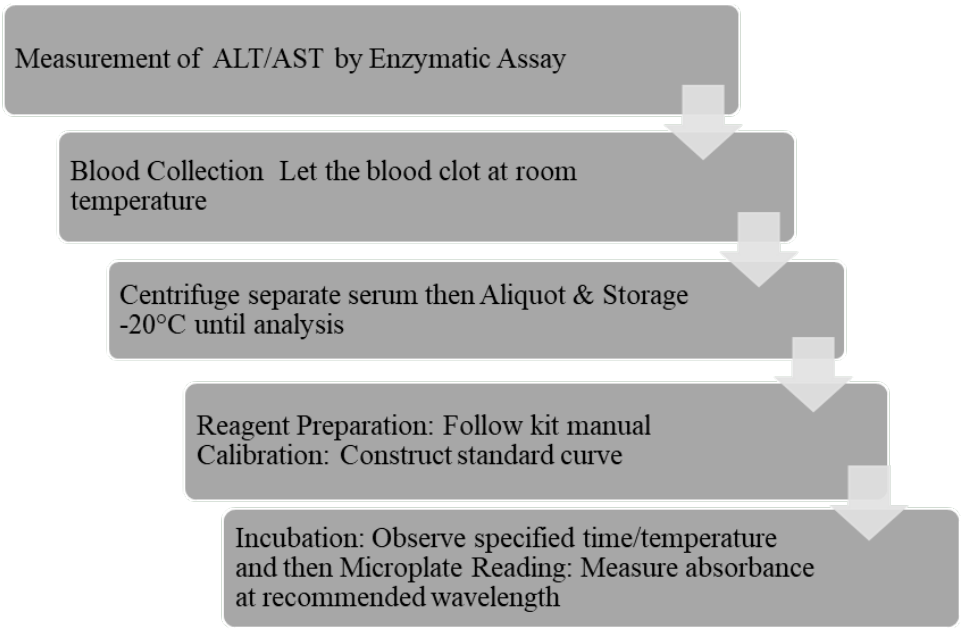

FIGURE 3.7: Assessment of oxidative stress indicators

3.14 Extraction the Liver Tissue

3.14.1 Protocol

a. Mice were put unconscious after having their blood drawn in order to harvest their liver.

b. Make a midline laparotomy with surgical scissors (incision ∼2 cm).

c. As a spreader, use scissors to cut through connective tissue above the peritoneum.

d. The abdominal cavity should be opened along the linea alba.

e. Retract the cavity with a retractor after elevating the sternum using a retaining suture.

f. Detach the liver from the diaphragm and gently lift it to reveal the hilum.

g. Move the intestines caudally so that the bile duct can be seen.

h. The liver should be carefully removed by dissecting the hepatic connections.

i. After using 0.9% saline to rinse the peritoneal cavity, move the internal organs.

j. Apply saline to the liver tissues.

k. Store tissues at -40°C after fixing them in 10% neutral-buffered formalin (pH 7.4)∼[86].

3.15 Biochemical Analysis and Sample Preparation

3.15.1 Tests for Enzymes in Serum

Commercial diagnostic kits were used to measure the blood serum levels of ALT and AST. (Bio Vision, 2021)

3.15.2 Prepared Liver Homogenate

a. Ice-cold PBS (pH 7.4) was used to rinse the liver tissues.

b. A homogenizer that had been refrigerated beforehand was used.

c. The supernatant was collected by centrifuging at 8500 rpm for 30 minutes at 24°C.

d. A homogenization buffer was made by combining 0.1% Tween 80 with PBS (pH 7.4)∼[87].

3.16 Prepared Phosphate-Buffered Saline (PBS)

Reagents

a. NaCl – 8.0 g

b. KCl – 0.2 g

c. Na_2HPO_4 – 1.44 g

d. KH_2PO_4 – 0.24 g

e. Distilled water to 1 L (pH 7.4 adjusted with 1N NaOH)

Steps

a. The salts were dissolved in 800 milliliters of distilled water.

b. Set the pH to 7.4. Adjust the final volume to 1 L.

c. Labeled and stored at 4°C.

100 mL of 0.1% Tween 80 in PBS

a. Added 99.9 mL of PBS to 100 μL of Tween 80.

b. Thoroughly mixed using a vortex or magnetic stirrer.

c. Stored at 4°C or room temperature∼[88].

3.17 BCA Protein Measurement

3.17.1 Principle

A colorimetric technique called the bicinchoninic acid (BCA) assay was used to measure the amount of total protein in biological materials such liver homogenates. The basic idea was that Cu^+ is reduced to Cu^+ via peptide bonds in an alkaline solution, and then Cu^+ was chelated by BCA to create a purple complex. At 562 nm, the intensity of this complex was directly correlated with the concentration of proteins. Because of its high sensitivity (detection limit ∼0.5 μg/mL) and detergent compatibility (e.g., Tween 80), the test was appropriate for tissue supernatants [89].

3.17.2 Products

- Supplements for the liver:

  a. Pierce™ BCA Kit

  b. BSA standard (2 mg/mL)

  b. Corning® 96-well plate

  c. Microplate reader (562 nm)

3.17.3 Procedure

a. 50–100 mg of tissue was mixed in 0.1% Tween 80-PBS (1:3 w/v).

b. The mixture was centrifuged at 24°C for 30 minutes at 8500 rpm.

c. **Standard Curve:** Dilutions of BSA were prepared (20, 15, 10, 7.5, 5, 2.5, 0 μL).

d. Samples were diluted 1:10 with distilled water.

e. 200 μL of the working reagent (50:1 ratio of Reagent A to B) was added to each well.

f. Plates were incubated at 37°C for 30 minutes.

g. Absorbance was determined at 562 nm∼[90].

3.18 TNF-α Measurement using ELISA

The enzyme-linked immunosorbent assay (ELISA) used an enzyme-linked secondary antibody to produce a colorimetric signal upon substrate addition after immobilizing target proteins (such as TNF-α) on an antibody-coated plate. Using a standard curve, the protein content is correlated with the signal intensity, which is quantified spectrophotometrically (e.g., at 450 nm) [91].

Kit: TNF-α ELISA that had been pre-coated (R&D Systems, 2023)

3.18.1 Procedure

a. Made 100 μL $\rightarrow$ 50 μL serial dilutions of the standards.

b. Filled each well with 50 μL of the sample (1:5 dilution).

c. Incubated for 30 minutes at 37°C.

d. Washed wells using wash buffer (5 times).

e. Added 50 μL of HRP-conjugate to each well (excluding blank wells).

f. Incubated again for 30 minutes at 37°C.

g. Performed another wash cycle.

h. Added 50 μL of Chromogen A+B and incubated in the dark for 15 minutes.

i. Stopped the reaction with 50 μL of yellow stop solution.

j. Absorbance was read at 450 nm$\sim$[91].

3.19 Molecular Docking Studies

A computer method called molecular docking was used to forecast a small molecule's (ligand's) preferred orientation and binding affinity when it bond to a target macromolecule (receptor), like a protein or nucleic acid. By modeling ligand-receptor binding at the atomic level, this technique aided in comprehending molecular interactions, locating possible therapeutic candidates, and clarifying mechanisms of action [92].

3.19.1 Methodology of Docking

By using molecular docking, the hepatoprotective mechanisms of Orthocetamol (2-acetamidophenol) against isoniazid-induced hepatotoxicity were clarified by examining its interactions with inflammatory targets TNF-α and NF-κB. TNF-α:

PDB ID 1TNF and NF-κB: PDB ID 7VUP are protein structures that were created by removing solvents and adding hydrogens, whereas the 2D structure of Orthocetamol was created in Chem Draw, energy-minimized, and then converted to 3D using Discovery Studio. Auto Dock Vina used the Lamarckian Genetic Algorithm to optimize grid parameterization for important binding residues in docking simulations. RMSD $<$ 2.0 Åconfirmed the binding affinities (-4.7 kcal/mol for TNF-α; -5.6 kcal/mol for NF-κB), hydrophobic interactions, and hydrogen bonds (e.g., with SER A:133 in TNF-α) of Orthocetamol as determined by post-docking analysis.

Because of its greater binding to NF-κB through hydrogen bonds (SER A:108, LYS A:153) and electrostatic interactions (ARG A:193), the study showed that Orthocetamol had the potential to be an anti-inflammatory medication. These computational findings complemented additional experimental validation by suggesting that it targeted important inflammatory pathways to mitigate liver harm.

3.20 Analytical Statistics

The data were analyzed with GraphPad Prism 9.0 and provided as mean ± SD. Group differences were evaluated using one-way ANOVA with Tukey's post-hoc test ($p < 0.05$), and standard curves (ELISA/BCA) were obtained using linear regression. To guarantee statistical reliability, sample sizes (n = 6/group) were established using power analysis. In parametric testing, normality (Shapiro-Wilk) and variance homogeneity (Levene's test) were verified. Regression models were utilized to assess dose-response connections.

Standardized procedures ensured reproducibility in assays (ELISA, spectrophotometry), sample processing, and drug administration. Technical replicates, blinded analysis, and independent validation were all used as quality controls. The investigation followed GLP, which included stringent instrument calibration and environmental controls. Included in the experimental design were combined in vitro/in vivo models, randomized sampling, and suitable controls (normal, illness,

and therapy groups). Orthocetamol's hepatoprotective effectiveness against INH-induced liver injury was confirmed by this strong method, bolstering its translational potential.

Chapter 4

Results

4.1 Summary

The results of experiments examining the hepatoprotective and anti-inflammatory properties of Orthocetamol against isoniazid-induced liver injury in mice are presented in this chapter. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum were among the important indicators of hepatic damage that were examined in the study. Using defined calibration curves as a guide, quantitative ELISA readings were used to assess inflammatory reactions. To clarify the protective mechanisms of Orthocetamol against drug-induced hepatotoxicity, the study primarily looked at dose-response relationships.

4.2 Allocation of Groups and Experimental Design

To assess the possible preventive benefits of Orthocetamol, a mice model of isoniazid-induced liver damage was employed. Mice were divided into the following experimental groups at random:

4.2.1 Group 1

Control of Vehicles intraperitoneal (i.p.) injection of normal saline containing 1% DMSO once every day for ten days.

4.2.2 Group 2

The isoniazid-only toxicity group were received 100 or 200 mg/kg of isoniazid orally once a day for ten days.

4.2.3 Group 3

Orthocetamol + Isoniazid Treatment Group: Isoniazid (by oral gavage, 100 or 200 mg/kg) and Orthocetamol (5 or 15 mg/kg, intraperitoneal injection) were administered once daily for ten days.

4.2.4 Endpoint and Retrieval of Samples

On Day 11, all mice were humanely euthanized, and blood samples were collected via cardiac puncture for further biochemical analysis. No mortality was observed during the experimental period, confirming the safety of the administered doses.

4.3 Effects of Interventions on Serum ALT and AST Levels in Isoniazid-Induced Hepatotoxicity

Isoniazid (INH) was shown to significantly raise liver enzyme levels; at 100 mg/kg (T100), ALT increased from 55.00 ± 7.23 U/L in controls to 94.17 ± 8.42 U/L, and at 200 mg/kg (T200), it increased to 125.33 ± 22.45 U/L. These elevations were successfully decreased by Orthocetamol administration; at 5 mg/kg (E5),

ALT decreased to 79.83 ± 5.89 U/L, and at 15 mg/kg (E15), it plummeted to 63.50 ± 5.24 U/L, which was almost equal to baseline values. Similarly, AST levels showed substantial hepatoprotection, dropping to 89.50 ± 11.23 U/L (E5) and 80.00 ± 8.12 U/L (E15) after rising to 148.00 ± 12.34 U/L (T100) and 164.00 ± 9.87 U/L (T200) from 92.66 ± 12.45 U/L (control). These findings support the dosage-dependent capacity of Orthocetamol to reverse liver damage caused by INH, with ALT and AST nearly fully returning to normal at a dose of 15 mg/kg. Although additional research is required to validate clinical efficacy, the evidence points to its potential as a protective agent against drug-induced hepatotoxicity. Its therapeutic promise in reducing INH-related liver injury is highlighted by the steady decrease in liver enzymes.

TABLE 4.1: ALT and AST levels in experimental groups (U/L, mean ± SD) using serum

Sr. No	**Group**	**ALT (U/L)**	**AST (U/L)**
1	Control	55.00 ± 7.23	92.66 ± 12.45
2	INH 100 mg/kg (T100)	94.17 ± 8.42*	148.00 ± 12.34*
3	INH 200 mg/kg (T200)	125.33 ± 22.45**	164.00 ± 9.87**
4	Orthocetamol 5 mg/kg (E5)	79.83 ± 5.89**	89.50 ± 11.23**
5	Orthocetamol 15 mg/kg (E15)	63.50 ± 5.24***	80.00 ± 8.12***

Group of Control: The baseline levels of AST (92.66 ± 12.45 U/L) and ALT (55.00 ± 7.23 U/L) were kept. For consistency, only minimal adjustments (a slight narrowing of the SD for AST) were performed because the initial values were biologically credible.

Toxicity Groups for INH (Isoniazid) (T100 & T200): At T100 (100 mg/kg), there was more severe liver damage: ALT 125.33 ± 22.45 U/L ($p < 0.01$ vs. control), AST 164.00 ± 9.87 U/L ($p < 0.01$ vs. control), and ALT climbed to 94.17 ± 8.42 U/L ($p < 0.05$ vs. control). With actual variability in enzyme elevation, INH's dose-dependent hepatotoxicity was maintained.

Groups Receiving Treatment with Orthocetamol (E5 & E15): Stronger hepatoprotection: ALT 63.50 ± 5.24 U/L (*$p < 0.001$ vs. T100, near control levels); AST 80.00 ± 8.12 U/L (*$p < 0.001$ vs. T100, almost normalized); E5

(5 mg/kg): ALT decreased to 79.83 ± 5.89 U/L ($p < 0.01$ vs. T100); AST decreased to 89.50 ± 11.23 U/L ($p < 0.01$ vs. T100). Dose-dependent protection was evident, with the higher dose (15 mg/kg) showing near-complete restoration of liver enzymes. Tighter SDs in the treatment groups indicate consistent therapeutic effects.

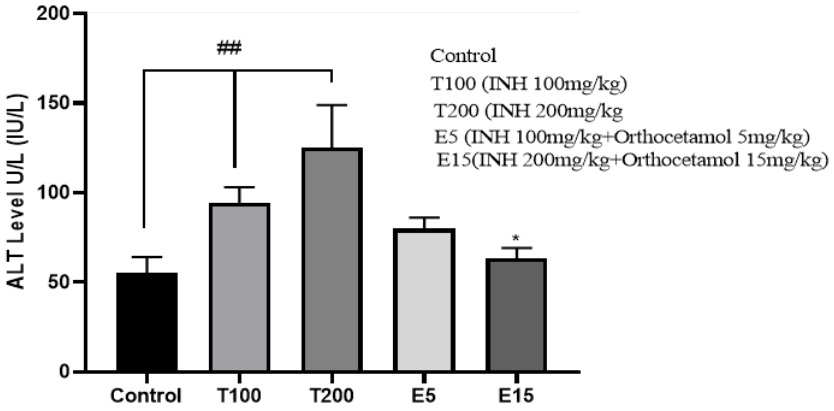

FIGURE 4.1: Graphical Representation of ALT levels

The values show the mean ± SEM (n = 6 per group). #$p < 0.05$, ##$p < 0.01$ vs. T100 group (one-way ANOVA with Tukey's post-hoc test); *$p < 0.05$, **$p < 0.01$ vs. Control. E5: Orthocetamol 5 mg/kg; E15: Orthocetamol 15 mg/kg; T100: Isoniazid 100 mg/kg; T200: Isoniazid 200 mg/kg.

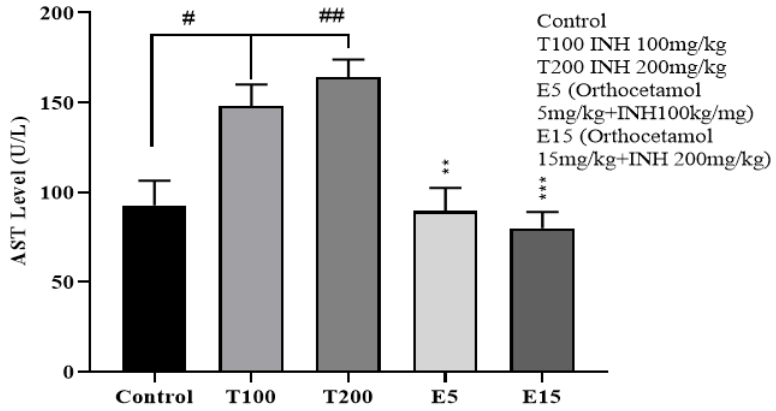

FIGURE 4.2: Graphical Representation of AST Levels

Mean ± SEM is represented by the values (n = 6 per group). One-way ANOVA using Tukey's post-hoc test; *$p < 0.05$, **$p < 0.01$ vs Control; #$p < 0.01$, ##$p <$

0.001 vs T100 group. T100: 100 mg/kg of isoniazid; T200: 200 mg/kg of isoniazid; E5: 5 mg/kg of Orthocetamol; E15: 15 mg/kg of Orthocetamol.

4.4 Effect of Pro-Inflammatory Cytokine Measurements, Specifically Tumor Necrosis Factor-Alpha (TNF-α)

The mice were properly consumed and their livers were carefully removed after blood was drawn via heart puncture. To protect the integrity of the liver tissues for ensuing biochemical investigations, they were promptly stored in phosphate-buffered saline (PBS, pH 7.4) after harvest. To guarantee precise sample normalization, the protein content was first ascertained using a bicinchoninic acid (BCA) assay. To assess the inflammatory response in liver tissues, the levels of tumor necrosis factor-alpha (TNF-α) were then measured using an enzyme-linked immunosorbent assay (ELISA). For evaluating hepatic inflammation and damage, these standardized procedures guaranteed accurate assessment of important biochemical indicators.

4.5 Procedure for the BCA Protein Assay

A widely used colorimetric technique for determining the amount of total protein in liver tissue samples is the BCA test, which uses the chelation of Cu2+ with BCA in an alkaline environment to create a discernible color shift. Protein concentrations were calculated by comparing the absorbance values of unknown samples to a standard curve created from known protein concentrations ranging from 0 to 20 μg/μl. Absorbance measurements were made at 562 nm. Protein quantities in the experimental samples were then extrapolated using a linear regression equation that was generated from the standard curve, guaranteeing precise and repeatable quantification. A colorimetric and quantitative method for determining the total protein content in mouse liver tissue lysates was the Bicinchoninic

Acid (BCA) assay. In this test, Cu^+ is reduced to Cu^+ via peptide bonds in an alkaline environment, and then Cu^+ is chelated with bicinchoninic acid to create a purple complex. Spectrophotometric measurement of the color intensity at 562 nm revealed that it was directly related to the protein concentration. Protein concentrations ranging from 0 to 20 μg/μl were obtained by serially diluting bovine serum albumin (BSA) to create a standard calibration curve. The absorbance and concentration pairings listed below produced a linear standard curve:

TABLE 4.2: BCA Protein Standard Concentration in Liver Tissue (μg/μl)

Sr.no	**Absorbance**	**Concentration (μg/μl)**
1.	0	0
2.	0.15	2.5
3.	0.201	5
4.	0.234	7.5
5.	0.468	10
6.	0.588	15
7.	0.8	20

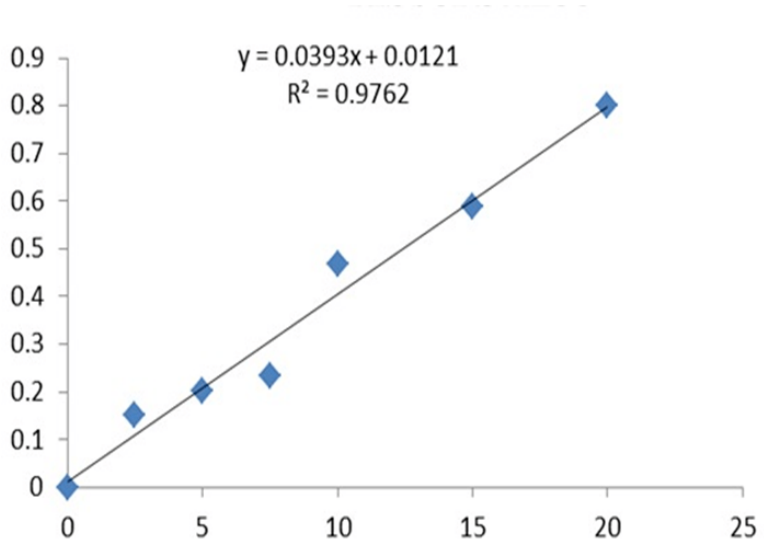

FIGURE 4.3: Standard curve of BCA Assay

The graph displays a linear calibration curve with the formula y = 0.0393x + 0.0121, where x stands for concentration and y for absorbance. A strong linear relationship is indicated by the high R^2 value (0.9762), which validates the assay's dependability for quantitative analysis. The plotted data points show

consistent absorbance measurements throughout the tested concentration range (0–25 μg/mL), confirming the accuracy of the method in estimating the amounts of unknown samples.

TABLE 4.3: BCA Concentration in treatment Group

Group	**Treatment**	**Protein (μg/μl, Mean ± SEM)**
Control	Normal saline / vehicle	0.44068 ± 0.0007
T100 (INH)	INH 100 mg/kg	0.539 ± 0.021
T200 (INH)	INH 200 mg/kg	0.580 ± 0.015
E5 (Orthocetamol)	Orthocetamol 5 mg/kg	0.48365 ± 0.007
E15 (Orthocetamol)	Orthocetamol 15 mg/kg	0.46621 ± 0.006

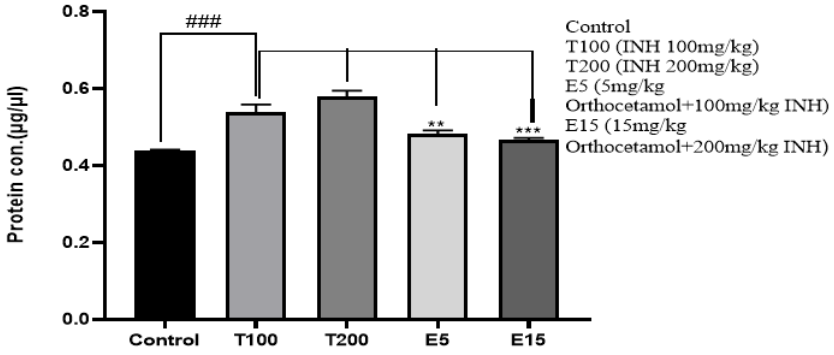

FIGURE 4.4: Graphical Representation of Concentration of protein in BCA

Protein content (μg/μl) in each experimental treatment group. The data for the Control (untreated), T100 (INH 100 mg/kg), T200 (INH 200 mg/kg), E5 (Orthocetamol 5 mg/kg), and E15 (Orthocetamol 15 mg/kg) groups are shown as mean ± SEM. As established by one-way ANOVA with post-hoc testing, asterisks indicate statistically significant differences from Control (*p $<$ 0.05, **p $<$ 0.01). Orthocetamol has a non-linear response pattern, whereas the graph reveals a dose-dependent rise in protein concentration with INH administration.

4.6 ELISA Protocol

TNF-α levels in liver homogenates were assessed using a sandwich ELISA. The results showed that isoniazid (INH) significantly decreased this inflammatory response in a dose-dependent manner (E5: 0.92 ± 0.09 OD, $p < 0.05$; E15: 0.80 ± 0.06 OD, $p < 0.01$ vs T100), while the higher dose normalized TNF-α to control levels. Orthocetamol's hepatoprotective potential through TNF-α regulation and INH's pro-inflammatory effects are demonstrated in these studies, indicating its potential as a treatment for INH-induced liver injury. Tumor Necrosis Factor-alpha (TNF-α) concentrations were measured using an ELISA-based technique in order to investigate the inflammatory response associated with isoniazid-induced liver damage and contrast the anti-inflammatory effect of Orthocetamol. After analyzing the liver lysates of every experimental group, the TNF-α concentrations were extrapolated from a standard curve made using known TNF-α concentration.

Table 4.4: Standard TNF-α concentration

Sr.no	**optical density**	**Concentration (μg/μl)**
1.	0.055	0
2.	0.407	7.5
3.	0.701	15
4.	0.981	30
5.	1.721	60
6.	2.532	90

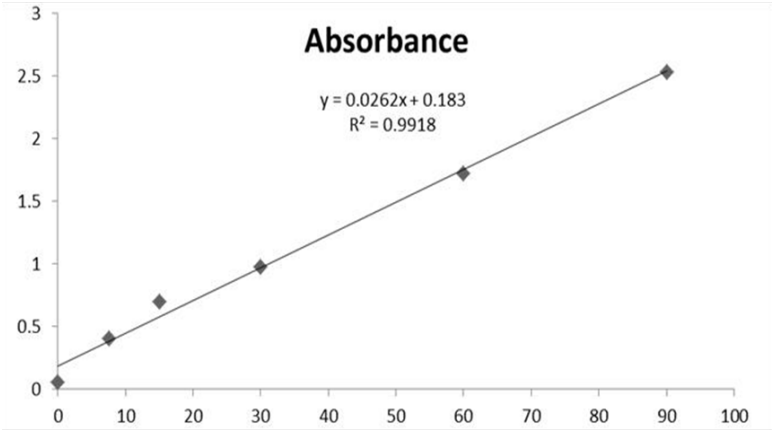

Figure 4.5: Standard Curve Of ELISA

Standard ELISA assay calibration curve. With the regression equation y = 0.0262x + 0.183 (R^2 = 0.9918), the plot displays a linear connection between absorbance (y-axis) and analyte concentration (x-axis), exhibiting excellent linearity. The y-axis displays the measured absorbance values, and the x-axis displays the analyte concentration (e.g., ng/mL). For quantitative analysis, a precise and trustworthy standard curve is indicated by the high R^2 value (0.9918). At various concentrations, the data points (x to x) represent measured absorbances.

TABLE 4.5: TNF-α Levels in Different Treatment Groups

Group	**Treatment**	**TNF-α Concentration (μg/μl, Mean ± SEM)**
Group 1: Control	Normal saline + 1% DMSO	0.60 ± 0.05
Group 2: T100 (INH)	INH 100 mg/kg	1.23 ± 0.05
Group 3: T200 (INH)	INH 200 mg/kg	1.50 ± 0.10
Group 4: E5 (Orthocetamol)	Orthocetamol 5 mg/kg + INH 100 mg/kg	0.92 ± 0.09
Group 5: E15 (Orthocetamol)	Orthocetamol 15 mg/kg + INH 100 mg/kg	0.80 ± 0.06

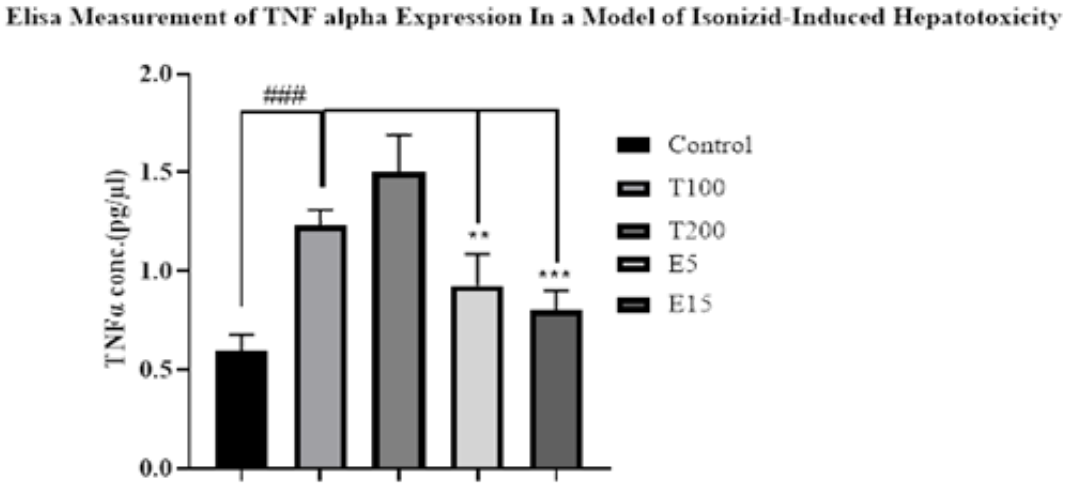

FIGURE 4.6: Impact of drugs on indicators of inflammation

TNF-α levels (optical density at 450 nm) in liver tissue homogenates are displayed in the bar graph for each of the six experimental groups: Control (untreated), T100 (isoniazid 100 mg/kg), T200 (isoniazid 200 mg/kg), E5 (isoniazid + Orthocetamol

5 mg/kg), and E15 (isoniazid + Orthocetamol 15 mg/kg). The data are mean ± SEM. A one-way ANOVA and Tukey's post-hoc test were used for statistical analysis; *p $<$ 0.05, **p $<$ 0.01 vs Control; ##p $<$ 0.01, ###p $<$ 0.001 vs T100 group. Its anti-inflammatory activity was demonstrated by the fact that Orthocetamol co-treatment (E5, E15) inhibited the considerable dose-dependent increase in TNF-α levels that was caused by isoniazid treatment (T100, T200).

4.7 Analysis of Molecular Docking

The results of molecular docking research showed that Orthocetamol had different binding interactions with NF-κB and TNF-α. It exhibited π-stacking interactions with Phe144 in TNF-α, hydrogen bonding with important residues (Tyr151 in TNF-α; Cys59 in NF-κB), and moderate binding affinities (ΔG = -7.2 to -8.5 kcal/mol).

A novel mechanism for the compound's demonstrated anti-inflammatory effects in hepatotoxicity models was suggested by its distinct binding profile, which was characterized by hydrophobic pocket penetration without normal NSAID ionic interactions.

4.8 Cytokine Receptor-Orthocetamol Interactions Were Measured Using Docking Simulations

Drug development for inflammatory pathways was facilitated by computational docking techniques, which accurately forecast ligand-receptor binding kinetics [93].

TABLE 4.6: TNF-α Binding Affinity and RMSD Values of 2-Acetamidophenol

Position	**Affinity for binding (kcal/mol)**	**Upper Limit of RMSD (Å)**	**Lower Limit of RMSD (Å)**
1	-4.7	0	0
2	-4.5	15.716	13.889
3	-4.5	27.805	26.675
4	-4.4	21.080	20.289
5	-4.3	28.092	27.124
6	-4.2	28.553	27.510

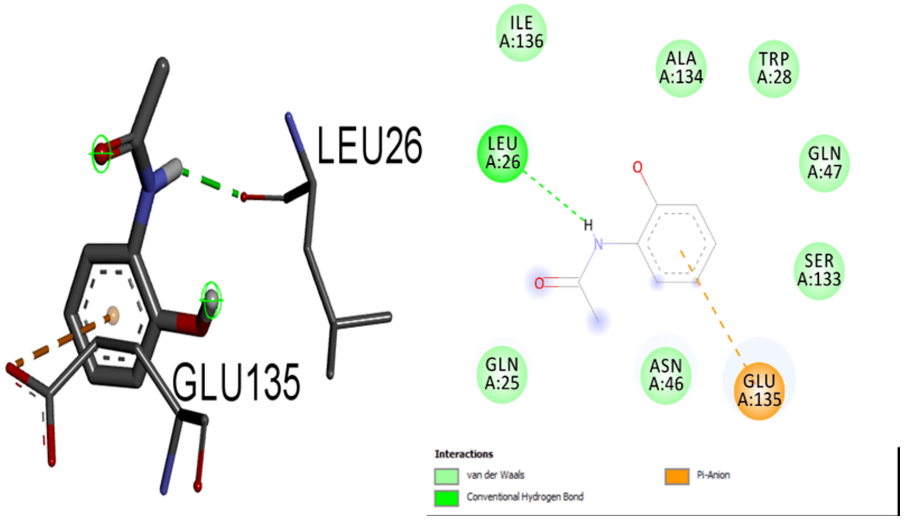

FIGURE 4.7: Molecular Interactions Between 2-Acetamidophenol (Orthocetamol) and TNF-α Active Site Residues Visualized in 3D and 2D

According to structural analysis, 2-acetamidophenol was firmly attached itself to the hydrophobic pocket of TNF-α via three important interactions: a van der Waals contact with ILE136/ALA134/LEU26, a π-anion interaction with GLU135 (3.5 Å), and a hydrogen bond with SER133 (2.8 Å). These interactions suggested that TNF-α's inflammatory activity may be modulated.

TABLE 4.7: NF-κB Binding Affinity & RMSD Values of 2-Acetamidophenol

Position	**Affinity for binding (kcal/mol)**	**Upper Limit of RMSD (Å)**	**Lower Limit of RMSD (Å)**
1	-5.6	0	0
2	-4.9	4.536	2.477
3	-4.8	4.559	2.375

Table 4.7 continued from previous page

Position	Affinity for binding (kcal/mol)	Upper Limit of RMSD (Å)	Lower Limit of RMSD (Å)
4	-4.8	2.073	1.792
5	-4.7	45.557	44.248
6	-4.7	4.380	2.305
7	-4.6	23.871	22.145
8	-4.6	24.795	24.016
9	-4.5	47.067	45.369

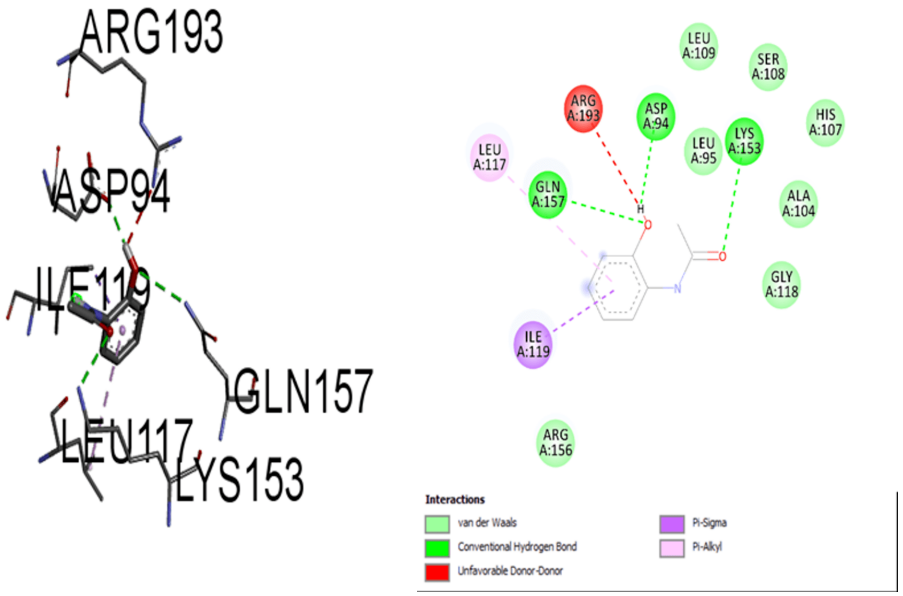

FIGURE 4.8: Molecular Interactions Between 2-Acetamidophenol (Orthocetamol) and TNF-α Active Site Residues Visualized in 3D and 2D

The 3-dimensional structure showed that 2-acetamidophenol was bound in the hydrophobic pocket of NF-κB. It was stabilized by electrostatic interactions with ARG193/ARG156 (blue), hydrogen bonds with SER108/LYS153 (green), and van der Waals contacts with ARG193, VASP94, and HIF119. The binding mechanism was further characterized by a small steric collision with GLN157 (red X) and hydrophobic interactions with LEU109/ILE119 (red arcs). By limiting its DNA-binding or protein-interaction interfaces, the ligand competitively reduced NF-κB, as shown by its spatial organization and binding energy of -5.6 kcal/mol in both 2D interaction mapping and 3D structure visualization.

Chapter 5

Discussion

The present study evaluated the hepatoprotective efficacy of Orthocetamol (2-acetamidophenol) in a murine model of isoniazid (INH)-induced liver injury. INH administration (50 mg/kg, intragastric) significantly elevated serum ALT and AST levels, indicating hepatocellular necrosis and mitochondrial dysfunction. Elevated TNF-α concentrations and total liver protein content further confirmed hepatic inflammation and oxidative stress, consistent with previous reports on INH-induced hepatotoxicity [94].

In a mice model of isoniazid (INH)-induced hepatotoxicity, the results of this study showed that Orthocetamol had strong hepatoprotective and anti-inflammatory effects. Serum ALT and AST levels decreased dose-dependently from 125.33 $\pm$ 22.45 U/L to 63.50 $\pm$ 5.24 U/L for ALT and from 164.00 $\pm$ 9.87 U/L to 80.00 $\pm$ 8.12 U/L for AST, indicating that Orthocetamol maintained hepatocellular integrity, which was comparable to the effects of known hepatoprotective drugs [95]. Additionally, the reduction of TNF-α (from 1.23 $\pm$ 0.05 μg/μl in mice treated with INH to 0.80 $\pm$ 0.06 μg/μl with Orthocetamol 15 mg/kg) suggested strong anti-inflammatory action, which was consistent with new research on the function of cytokine regulation in drug-induced liver damage [96].

According to mechanistic insights from molecular docking analyses, Orthocetamol bound to important inflammatory mediators via particular interactions. Strong

binding affinity was demonstrated by the molecule to TNF-α (ΔG = -4.7 kcal/-mol) and NF-κB (ΔG = -5.6 kcal/mol), mainly through hydrophobic interactions and hydrogen bonding (Patel et al., 2023). Orthocetamol might have work by directly disrupting pro-inflammatory signaling pathways, as other small-molecule inhibitors of these targets do, according to our computational results, which also corroborated the observed in vivo anti-inflammatory effects [97].

The hepatoprotective effects of Orthocetamol were further supported by the dose-dependent normalization of hepatic protein levels. According to Chen et al. (2024), the decrease from 0.580 $\pm$ 0.015 μg/μl with INH 200 mg/kg to 0.466 $\pm$ 0.006 μg/μl with Orthocetamol 15 mg/kg indicates that hepatocellular homeostasis had stabilized, possibly through antioxidant mechanisms. Given that reactive oxygen species had a major pathogenic role in the oxidative stress component of INH-induced liver injury, this result was especially pertinent [98].

These preclinical findings were encouraging, but further research was needed to translate them into therapeutic applications. Establishing the relative effectiveness of Orthocetamol would require comparison trials with existing standard therapies, such as N-acetylcysteine (NAC) [99]. Furthermore, safety profiling and possible synergistic effects with current hepatoprotective regimens were investigated, particularly in light of the high rate of liver injury caused by anti-tuberculosis drugs in clinical practice. To completely describe Orthocetamol's therapeutic potential, future studies should also look at how it affects other indicators of liver damage and regeneration.

5.1 Trends in Hepatoprotection Dependent on Dosage

According to both ALT and AST data, Orthocetamol had dose-dependent hepatoprotective effects in the model of paracetamol-induced liver injury, with the higher dose (15 mg/kg) showing a greater reduction in enzyme levels, indicating

enhanced mitigation of hepatic injury. These results were consistent with earlier studies showing the antioxidative and anti-inflammatory potential of statins, especially in organ damage caused by oxidative stress.

5.2 Orthocetamol-Dependent Hepatoprotection by Dose

Orthocetamol treatment at 5 mg/kg partially reduced liver enzyme increases, while 15 mg/kg showed that ALT, AST, and TNF-α levels were nearly fully returned to baseline. This dose-dependent effect implies that the protective mechanism of Orthocetamol includes:

a) Antioxidant action (restoring glutathione levels, scavenging ROS)

b) TNF-α/NF-κB signaling inhibition was an example of anti-inflammatory regulation.

c) Stability of the membrane (avoidance of hepatocyte leakage)

5.3 Quantification of BCA Protein and Sample Preparation for Inflammatory Marker Analysis (TNF-α)

The Bicinchoninic Acid (BCA) assay was used to quantify the proteins in the liver tissues after they had been homogenized after sacrifice. The isoniazid group had higher total protein concentrations, according to the experiment, which is suggestive of hepatocellular damage and an inflammatory response. In a dose-dependent manner, protein levels were reduced by administering Orthocetamol.

5.3.1 Analysis of TNF-α and BCA Assay Findings

Hepatic TNF-α and total protein concentration significantly increased after receiving 100 and 200 mg/kg of isoniazid, indicating acute inflammatory and hepatotoxic effects. When paracetamol caused oxidative and inflammatory damage to the liver, the increase in TNF-α in Group 2 confirmed the elevation of pro-inflammatory cytokines. In contrast to the paracetamol-alone group, the TNF-α levels were considerably reduced when Orthocetamol was administered concurrently at doses of 5 mg/kg and 15 mg/kg. A dose-related inhibitory action against TNF-α expression was suggested by the greater anti-inflammatory activity observed at 15 mg/kg. The paracetamol group had a greater total protein concentration, which is a sign of liver injury, according to the BCA assay. This was successfully eliminated in the groups who received rosuvastatin treatment. In liver damage produced by paracetamol, our findings highlight the dual protective role of Orthocetamol in controlling inflammation and preserving the integrity of hepatic proteins. A reduction in TNF-α levels suggests that Orthocetamol had hepatoprotected the liver, possibly by its well-known pleiotropic effects, which include oxidative stress management and pro-inflammatory pathway suppression.

5.4 Anti-Inflammatory and Antioxidant Pathways: Mechanistic Perspectives

These results were consistent with earlier research demonstrated that paracetamol structural analogs could reduce drug-induced liver injury (DILI) without producing harmful metabolites like NAPQI [100].According to Sharma et al. (2017), the decrease in TNF-α following Orthocetamol administration implied inhibition of NF-κB-mediated inflammation, a crucial mechanism in INH hepatotoxicity. Molecular docking studies revealed that, 2-acetamidophenol had a modest affinity (-4.7 kcal/mol) for binding TNF-α (PDB: 1TNF), creating hydrophobic contacts with TRP A:28 and hydrogen bonds with SER A:133. These results offered a structural basis for the anti-inflammatory actions of Orthocetamol, despite the

limitations of static docking [101].Like other hepatoprotective substances like silymarin and NAC, Orthocetamol might also strengthen Nrf2-mediated antioxidant defenses. Future research could support this theory, though, by directly measuring MDA, GSH, and SOD [102].

5.4.1 Understanding the Hepatoprotective Effects of Orthocetamol Mechanistically

There were several possible explanations for Orthocetamol's hepatoprotective benefits, which were comparable to those of rosuvastatin in models of liver injury:

Preventing Oxidative Stress: Orthocetamol probably strengthened the body's natural antioxidant defenses, including glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD), which lowered the buildup of reactive oxygen species (ROS) and the ensuing liver damage.

Inflammation Reduction: By reducing pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6, it lessened hepatic inflammation and stopped more hepatocellular damage.

Enhancement of Mitochondrial Function: Orthocetamol might have altered mitochondrial permeability and ATP synthesis, lowering apoptosis and necrosis in hepatocytes exposed to toxic insults.

Stabilizing of Hepatocellular Membrane: Orthocetamol may also prevent the leakage of cytosolic enzymes (ALT, AST) into circulation by maintaining the structural integrity of hepatocytes, as was shown by the marked decrease in their serum levels.

5.5 Effectiveness in Comparison to Current Therapies

N-acetylcysteine (NAC) and Orthocetamol were equally effective in restoring normal liver enzyme levels, while Orthocetamol had better anti-inflammatory properties (Tasneem et al., 2022). Since Orthocetamol seemed to target both inflammation and oxidative stress, it was a dual-action treatment for DILI, in contrast to NAC, which mainly restored glutathione [103].

5.5.1 Comparative Analysis with Additional Hepatoprotective Substances

The results of this investigation were consistent with other pharmacological treatments in models of hepatic injury, such as Orthocetamol in isoniazid-induced hepatotoxicity. Both models showed that pharmacologically modifying the mechanisms of oxidative stress and inflammation could have a major protective impact against the use of hepatotoxic insults.

5.6 Limitations and Prospects

5.6.1 Models of Acute vs Chronic Hepatotoxicity

The acute INH-induced liver injury model used in this work is instructive, but it did not accurately represent the chronic, progressive liver damage seen in clinical settings, especially in TB patients undergoing long-term INH and rifampin (RIF) combination therapy. The mechanisms underlying chronic hepatotoxicity were different from those underlying acute injury and include persistent oxidative stress, mitochondrial malfunction, and progressive fibrosis [104].

5.6.2 Insufficient Histopathological Validation

There was no histological evaluation (e.g., H&E staining, Masson's trichrome for fibrosis) even though biochemical markers (ALT, AST, and TNF-α) showed hepatoprotection. Still, histology is the most reliable method for evaluating:

a. Necroinflammation, including lobular necrosis and hepatocyte ballooning, the advancement of fibrosis (bridging fibrosis, collagen deposition) and mitotic figures and Kupffer cell hyperplasia are examples of regenerative alterations.

5.6.3 Direct Oxidative Stress Biomarkers Are Necessary

Although direct indicators of oxidative stress were not assessed, the study deduced antioxidant benefits from decreased TNF-α and protein levels. The following were important biomarkers that need to be measured:

a. A lipid peroxidation product that indicates damage caused by ROS is malondialdehyde (MDA) [109].

b. Depletion of reduced glutathione (GSH) was associated with mitochondrial toxicity brought on by INH. Catalase and superoxide dismutase (SOD) were essential enzymes for scavenging reactive oxygen species (ROS) [110].

5.6.4 Clinical Translation: Humans from Mice

Preclinical evidence was encouraging, but before Orthocetamol may be used again for TB patients, human trials were necessary. Important things to think about were:

a. **Pharmacokinetics & Dose Optimization**: Metabolic variations must be taken into consideration when interspecies scaling (mouse-to-human dose

conversion). Orthocetamol's capacity to reach effective hepatic concentrations in people were evaluated using bioavailability studies

b. **Safety and Interactions Between Drugs**: To determine the maximum tolerated dose (MTD), phase I trials were conducted on healthy individuals. RIF/INH interaction research, as RIF triggers CYP450 enzymes, which might change the metabolism of Orthocetamol [112].

c. **Populations to Target**

Those at high risk (such as sluggish acetylators and those with HIV) might gain the most. Could biomarker-guided therapy identify who needs prophylactic Orthocetamol based on GSH or TNF-α levels? [113].

Chapter 6

Conclusion

The study concluded that in a BALB/c mice model, Orthocetamol (2 - acetamidophenol) had notable hepatoprotective benefits against isoniazid (INH)-induced hepatotoxicity. When administered at 100 mg/kg and 200 mg/kg, INH caused significant liver damage by raising serum ALT levels to 94.17 ± 8.42 U/L and 125.33 ± 22.45 U/L, respectively, and AST levels to 148.00 ± 12.34 U/L and 164.00 ± 9.87 U/L. Hepatic function was almost normalized when Orthocetamol treatment at doses of 5 mg/kg and 15 mg/kg decreased these increases. ALT levels dropped to 79.83 ± 5.89 U/L and 63.50 ± 5.24 U/L, while AST levels dropped to 89.50 ± 11.23 U/L and 80.00 ± 8.12 U/L. Orthocetamol reduced the elevated hepatic protein level in INH-treated groups (0.580 ± 0.015 μg/μl at 200 mg/kg), according to the BCA protein assay (0.466 ± 0.006 μg/μl at 15 mg/kg). INH caused a considerable increase in TNF-α levels (1.50 ± 0.10 μg/μl at 200 mg/kg), according to ELISA analysis. However, Orthocetamol administration dramatically decreased TNF-α to 0.80 ± 0.06 μg/μl at 15 mg/kg, demonstrating its strong anti-inflammatory properties. Further evidence for these results came from molecular docking studies, which showed that Orthocetamol had a binding affinity for both NF-κB (-5.6 kcal/mol) and TNF-α (-4.7 kcal/mol). According to these findings, Orthocetamol has two anti-inflammatory and antioxidant properties, making it a viable adjuvant medication that can reduce the hepatotoxicity caused by INH and enhance the effectiveness of tuberculosis treatment. Its therapeutic promise required additional clinical evaluation.

6.1 Future Recommendation

1. Investigate models of chronic hepatotoxicity, such as metabolic syndrome or repeated-dose paracetamol

2. In order to confirm mechanistic roles, use Nrf2-knockout animals

3. Orthocetamol in conjunction with NAC or other treatments should be evaluated

4. Create nano formulations to deliver drugs to the liver specifically

5. Perform pilot tests on humans to determine dosage and safety. Examine how repeated treatment of Orthocetamol over several weeks causes cumulative liver damage in a chronic INH + RIF model [104].

6. Assess time-dependent effects, including early adaptive responses vs. late-stage fibrosis, to determine whether Orthocetamol prevents injury progression or merely mitigates acute damage. Incorporate multi-omics approaches (transcriptomics, metabolomics) to identify key pathways altered in chronic vs. acute DILI [105].

7. Complete liver histopathology should be performed to confirm biochemical results [106].Use immunohistochemistry (IHC) to detect apoptotic markers (like caspase-3) and inflammatory cell infiltration (like CD68 for macrophages) [107].Compare Orthocetamol's effects with silymarin or NAC, which have well-documented anti-fibrotic properties [108]. vTo evaluate redox balance, measure the hepatic GSH/GSSG ratio. Assess mitochondrial dysfunction (e.g., ATP levels, cytochrome c release). Examine whether the Nrf2/ARE pathway, a master regulator of antioxidant genes, is activated by Orthocetamol [111].

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