EFFECT OF METHANOLIC LEAF EXTRACT OF *CALPURNIA AUREA (DIGITA)* IN ISONIAZID AND RIFAMPICIN INDUCED HEPATOTOXICITY IN MICE

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A THESIS PAPER SUBMITTED TO THE DEPARTMENT OF BIOMEDICAL SCIENCES, FACULTY OF MEDICAL SCIENCES, INSTITUTE OF HEALTH, JIMMA UNIVERSITY IN A PARTIAL FULFILLMENT FOR REQUIREMENT OF DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOCHEMISTRY

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Approval sheet

This is to certify the thesis entitled “Effect of methanolic leaf extract of Calpurnia aurea (digita) in Isoniazid and Rifampicin induced hepatotoxicity in mice” and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medical Biochemistry is a record of original research carried out by Mulugojam Melkie (ID No. RM 3365/12) under our supervision and the part this thesis doesn’t submitted for any other degree or diploma. Therefore we recommend that it is accepted as fulfilling the thesis requirements and has been submitted for examination with my approval as advisor.

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Declaration

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This is to certify that the thesis prepared by **Mulugojam Melkie**, entitled: "Effect of methanolic leaf extract of *Calpurnia aurea (digita)* in Isoniazid and Rifampicin induced hepatotoxicity in mice" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medical Biochemistry complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Background: Globally, liver disease is the leading cause of death and 50% of liver failure is caused by drug-induced liver damage. Hepatotoxicity attributed with anti-TB drugs accounts 5–34.9% TB patients. Many drugs and other therapies are applied to treat liver disease, but the efficiency and side effect of those drugs are the other burden. Traditionally *C.aurea* is used to treat liver disease in different part of Ethiopia. This study aimed to evaluate the effects of methanolic leaf extracts of *C. aurea* in INH -RIF induced hepatotoxicity in mice.

Methods: From both sexes 30 mice were randomly divided into six groups. G-I treated with 1mL of normal saline, G-II administered INH 100mg/kg - RIF 100mg/kg, G-III treated with 100mg/kg Silymarin and INH-RIF, G-IV given *C. aurea* 200mg/kg and INH -RIF, G-V treated with *C. aurea* 400mg/kg and INH- RIF and G-VI given 600mg/kg of *C. aurea* extract with INH -RIF per day for 21 days. Mice were fasted for 12 hours on 21th day and anesthetized by ketamine/xylazine. Then blood sample were taken to determine serum level of liver enzymes, total bilirubin and albumin; whereas liver was taken for histopathological investigation. Data were entered into epidata and exported to SPSS for analysis. Statistical comparisons among groups were done by one way ANOVA followed by Tukey's test at $P< 0.05$ and expressed as mean ± SD.

Result: G-II mice showed elevated serum levels of liver enzymes and TBIL with lower albumin levels than G-I mice. G-V mice (400mg/kg *C.aurea* extract) showed significant decrease in ALT, AST, ALP, TBIL and increase in albumin which is comparable to Silymarin group; whereas G-IV & G-VI shows insignificant reduction in LFT parameters as compared to G-II. The liver sections of G-II mice displayed lobular lymphocytic inflammation, necrosis, hepatocellular & canalicular cholestasis, sinusoidal dilatation, and vacuolar degeneration, while these deformities and abnormalities were not seen in G-V mice.

Conclusion and Recommendation: According to findings *C.aurea* extract showed good hepatoprotective potential against INH-RIF-induced liver injury. For other researchers recommended conducting more studies on hepatoprotective effect of *C.aurea* on different models.

Keyword: *Calpurnia aurea*, Isoniazid, Rifampicin, Hepatotoxicity, Liver histopathology

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ABBREVIATIONS AND ACRONYMS

AADAC:	Arylacetamide deacetylase
AcHZ:	Acetylhydrazine
AcINH:	N-acetylisoniazide
ALF:	Acute liver failure
ALT:	Alanine aminotransferase
ALP:	Alkaline phosphatase
ANOVA:	Analysis Of Variance
AST:	Aspartate amino transferase
CAR:	Constitutive active receptor
CEHC:	Carboxyethyl hydroxychroman
CLD:	Chronic liver disorder
CYP:	Cytochrome P450
DB:	Direct bilirubin
DILD:	Drug induced liver damage
DILI:	Drug induced liver injury
GSH:	Reduced glutathione
JUTIDRC:	Jimma University Tropical and Infectious Disease Research Center
HB;	Hemoglobin
HBV:	Hepatitis B virus
HCV:	Hepatitis C virus
HELLP:	Hemolysis Elevated Liver enzymes and Low Platelets
INH:	Isoniazid
INA:	Isonicotinic acid
LDH:	Lactate dehydrogenase
MDH:	Malate dehydrogenase
NADH:	Nicotinamide adenine dinucleotide reduced form
NIH:	National Institutes of Health
OECD:	Organization for Economic Co-Operation and Development
P-5-P:	Pyridoxal-5-Phosphate
PO:	Per orally

PXR:	Pregnane X receptor
RfD:	The Reference Dosage
RFP:	Rifampicin
RXR:	Retinoid X receptor
SD:	Standard deviation
SGOT:	Serum Glutamic-Oxaloacetic Transaminase
SGPT:	Serum Glutamic-Pyruvic Transaminase
SST:	Serum separator tubes
TB:	Tuberculosis
TCA:	Tricarboxylic acid cycle
TNF-α:	Tumor necrosis factor-α
WD:	Wilson disease
WHO:	World Health Organization

CHAPTER ONE
INTRODUCTION

1.1 Background

Liver is the main site of nutrient metabolism and waste metabolite excretion (1,2). It is responsible for metabolic characteristic protection in addition to detoxing of exogenous and endogenous troubles consisting of xenobiotics, medications, viral infections, and chronic alcoholism (3). The liver's multifaceted features and strategic role make it susceptible to a number of illnesses (4).

Liver diseases are public health problem in developing countries. Mostly xenobiotics including toxic chemicals, anti-tubercular drugs (isoniazid, rifampicin and pyrazinamide), anticancer drugs (azathioprine, doxorubicin, cisplatin), immunosuppressive drugs (cyclosporine), analgesic and anti-inflammatory drugs (Paracetamol, thioacetamide) leads to the development of drug induced. Thus causes associated with age, gender, alcoholism, nutrition, and cytochrome P450 enzyme genetic variants to cause hepatic damage (4,5).

Drug-induced liver injury (DILI) can be either intrinsic DILI, which is dose-dependent and predictable event, or idiosyncratic DILI, which is a non-dose-dependent and unpredictable event that occurs when a person is exposed to toxic doses of commonly used substances or medications(6,7). DILI leads either chronic liver disease (CLD) or acute liver failure (ALF). CLD is a loss of hepatic function as a result of prolonged infection or injury. Cirrhosis is a common side effect of this disease(8). ALF is uncommon and serious complication of sudden hepatocyte injury that can progress to death over days or weeks. A consistent pattern of rapid-onset aminotransferases increase, abnormal mentation and disordered coagulation results from a range of assaults to liver cells (9,10).

Tuberculosis is a pandemic chronic communicable disease caused by mycobacterium tuberculosis. Isoniazid (INH) and rifampicin (RIF) are the first-line anti-tuberculosis drugs recommended by the WHO. These two drugs are metabolized within the liver, and both INH-RIF can cause hepatotoxicity. More importantly, RIF increases the hepatotoxicity of INH during a combination therapy involving INH-RIF. INH-RIF cause liver damage, including liver failure, cell necrosis, inflammation, and steatosis related to oxidative stress.

INH and its toxic metabolite hydrazine inhibit Nuclear factor-erythroid factor 2-related factor 2 (Nrf2) translocation to the nucleus and induce apoptosis via oxidative stress (11,12). Oxidative stress damages the inner membrane of mitochondria to cause mitochondrial oxidative pressure and impairment of strength homeostasis (11,13).

To mitigate INH-RIF induced liver damage, antioxidant-rich substances or pharmaceuticals may be utilized (14). Medicinal plants are major source of antioxidants that can be used for treatment of liver damage and it has become a favorable therapy internationally for pathological liver conditions. In the eastern world, herbal treatment has been used to alleviate disorders related to liver and other internal organs for many centuries (15).

In Ethiopia there is biodiversity of flora that have a range of biological activities including treatment of various liver diseases(16). *Calpurnia aurea* is a flowering plant traditionally it used for the treatment of various human and animal disease. In different parts of Ethiopia it is used to treat liver disease, syphilis, malaria, rabies, diabetes, hypertension, diarrhea, leishmaniasis, trachoma, elephantiasis, wound, amoebiasis, snake bite, tick control, fungal diseases, and various swellings. Different scientific investigations conducted on antimalarial, antihypertensive, antidiarrheal, antibacterial, antioxidant and anticancer effect of different part of *C.aurea* (17–21).

In recent years, researchers have used scientific methods to evaluate the effects of plants for the treatment of liver ailments. Therefore this study was aimed to study the effect of leaf extract of *C. aurea* on combination therapy of INH- RIF induced hepatotoxicity in mice.

1.2. Statement of the Problem

Liver disease is the most common causes of death and illness around the world. According to the Global Burden of Disease project, in 2010 more than 2 million peoples are died as a result of liver diseases such as acute hepatitis, cirrhosis and liver cancer (4,22,23).

Liver damage affects more than 10% of the world's population(24). DILI is responsible for 10% of all acute hepatitis cases, 5% of all hospital admissions, and 50% of all acute liver failures (25). More than 75% of idiosyncratic medication reactions result liver transplantation or death, which is extraordinary (26). A very prevalent cause of acute liver illness, drug-induced liver injury has a fatality rate of roughly 10% (9). In the United States, DILI is responsible for over 50% of all ALF cases(27). Over a thousand drugs and substances have been linked to liver damage(28).

Anti-TB drugs are one of the commonest group underlying idiosyncratic hepatotoxicity worldwide(29). Isoniazid and rifampicin, the first line drugs used for tuberculosis therapy are associated with hepatotoxicity. Globally, anti-TB DILI is reported in 2% to 28% and China reporting an incidence rate of 2.55%(30).

Hepatotoxicity attributed to anti-TB drugs has been reported 5–34.9% in people treated with anti-TB drugs (13). A meta-analysis of study involving several anti-tuberculosis drug regimens estimates the incidence of liver toxicity is 2-6% with co-administered isoniazid and rifampicin and 1.1% with rifampicin alone (31) . Rifampicin increased the prevalence of drug-induced liver injury in a multidrug regimen from 1.6% to 2.55% in adults. The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8 - 30%) compared to that in advanced countries (2- 3%) with a similar dose schedule (10).

In Ethiopia the prevalence of anti-tuberculosis drug-induced hepatotoxicity is high. A study done in Amhara regional state Dessie referral hospital and in South Ethiopia, Dawro Zone, Tercha District Hospital showed TB/HIV co-infected patients treated with anti-tuberculosis developed hepatotoxicity 20.2% and 8%, respectively(32,33). In All Africa Leprosy Tuberculosis Rehabilitation and Training Center in Addis Ababa in 2021, anti-TB drugs induced 56.9 % moderate hepatotoxicity, 36.9% severe hepatotoxicity and 6.2% very sever hepatotoxicity (34) .

Drug-induced hepatotoxicity would be treated in medicinal plants which possess high antioxidant activity. The majority of the population in developing countries, including 90% of African population, rely on traditional medicinal plants for their healthcare(35). In Ethiopia 80% of the population is still dependent on traditional medicine (36). The biodiversity of the Ethiopian plants offer great possibilities in the search for natural products with novel structures that have a range of biological activities including treatment of various diseases including liver disease (20,37). *C.aurea* is medicinal plant traditionally utilized throughout Ethiopia to treat distinctive afflictions including liver disease (18).

As per review of literature there is no scientific report so far on determination of preventive effect of extract of *C. aurea* in drug induced hepatotoxicity. Therefore this study was intended to evaluate the protective effect of *C. aurea* leaf extracts on INH - RFP induced liver damage.

1.3 Significance of the Study

Liver disease is the serious problem of the world, but there is no appropriate antihepatotoxic medication available in allopathic medicine. In addition to this one of the problems in controlling TB disease is that most of the first-line anti-TB drugs are hepatotoxic especially isoniazid, rifampicin and pyrazinamide.

Herbal medicine has become more accepted and their usage is prevalent. In Ethiopia many plants are being utilized as traditional medicine to treat different diseases including liver disease from which *C.aurea* is the one that is utilized to treat liver diseases, hypertension, and diabetic. Seed and leaf part of the plant is taken orally to treat liver illness in Amhara, Shinasha and southern nations in Ethiopia. But still there is lack of scientific proofs to substantiate their effective usage and immense potential to treat liver diseases. Hence, it is necessary to establish the scientific basis and knowledge for the therapeutic actions of this traditional medicinal plant that may serve as an input for developing more effective drugs.

Therefore, this study will fill the gap in information and give scientific evidences on hepatoprotective effect of *C.aurea* leaf extract on the INH and RIF liver damage. And it will provide scientific support for traditional users on the use of *C. aura* leaf as a hepatoprotective. It may also help environmental policymakers to enact policies to protect, manage and eventually restore natural resources as they have medicinal importance.

CHAPTER TWO

LITERATURE REVIEW

2.1. Liver and liver disease

Liver is the major organ in the body which is placed in the upper right part of the abdominal cavity, beneath the diaphragm, and on top of the stomach, right kidney, and intestines. The human liver begins to develop from the third week of pregnancy and does not reach full maturity until roughly 15 years of age. In adults, liver accounts for around 2% of body weight (1,2).

A variety of products which can be immunological (genetic factors) or environmental factors can cause liver disease(2). The incidence of hepatic diseases has increased worldwide because of changes in lifestyle, dietary habits, contamination in food or drinking, chemical and drug abuse, and hepatic infections. The most common hepatic diseases include hepatitis, cirrhosis, fatty liver, bile duct obstruction, and jaundice (2). In European populations risk factors like alcohol consumption, obesity, and type 2 diabetes prevalence, and high dose of drugs as well as hepatitis B and C infection were identified as key upstream of liver disease (2,4).

2.2 Drug induced liver damage

A common cause of liver damage is drug-induced liver toxicity. It causes around half of liver failure cases that resemble either acute or chronic liver disease which continue as global health issues (28). DILI occurs in roughly1/1000 to 1/10000 people taking therapeutic drug dosages (7). More than 900 drugs, toxins, and herbs have been linked to liver damage, and drugs are responsible for 20-40% of all cases of acute hepatic failure (38,39).

Some medicines are acutely toxic to the liver and cause dose-related hepatotoxic reactions within hours of exposure, whilst others may cause liver harm only in susceptible people days or weeks later. These reactions are allergic more correctly defined as idiosyncratic. some medicines by themselves are not hepatotoxic but drug-drug interactions play a role in toxicity (25)

Drug-induced hepatotoxicity has a variety of mechanisms as shown in Figure 1. Nuclear receptors activated by drugs and xenobiotics to increase the expression of cytochrome P450 enzymes. It generates reactive metabolites and free radicals which in turn bind to macromolecules, and increase cellular toxicity. In other way natural products increase the expression of phase II enzymes, the level of intracellular antioxidant (GSH), and antioxidant enzymes. The natural products may also inhibit the

activities of cytochrome P450 enzymes in order to attain dynamic homeostasis and reduce cellular toxicity.

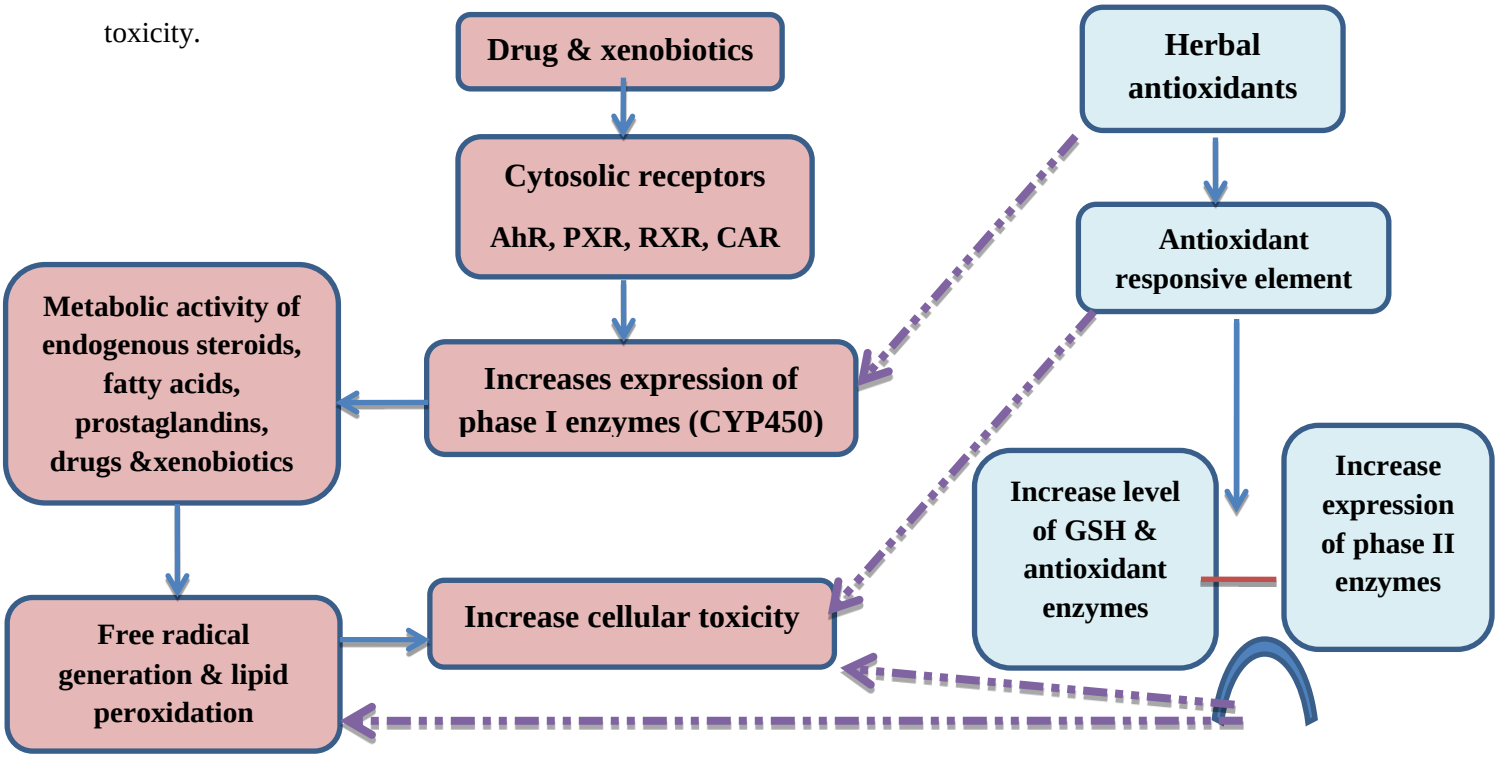


Figure 1: Drug & xenobiotic metabolic mechanisms and effects of herbal antioxidants (25).

A person's sensitivity to a potentially hepatotoxic substance is influenced by a number of factors (Figure 2) and may all have an impact on the risk of drug-mediated hepatotoxicity (5,25).

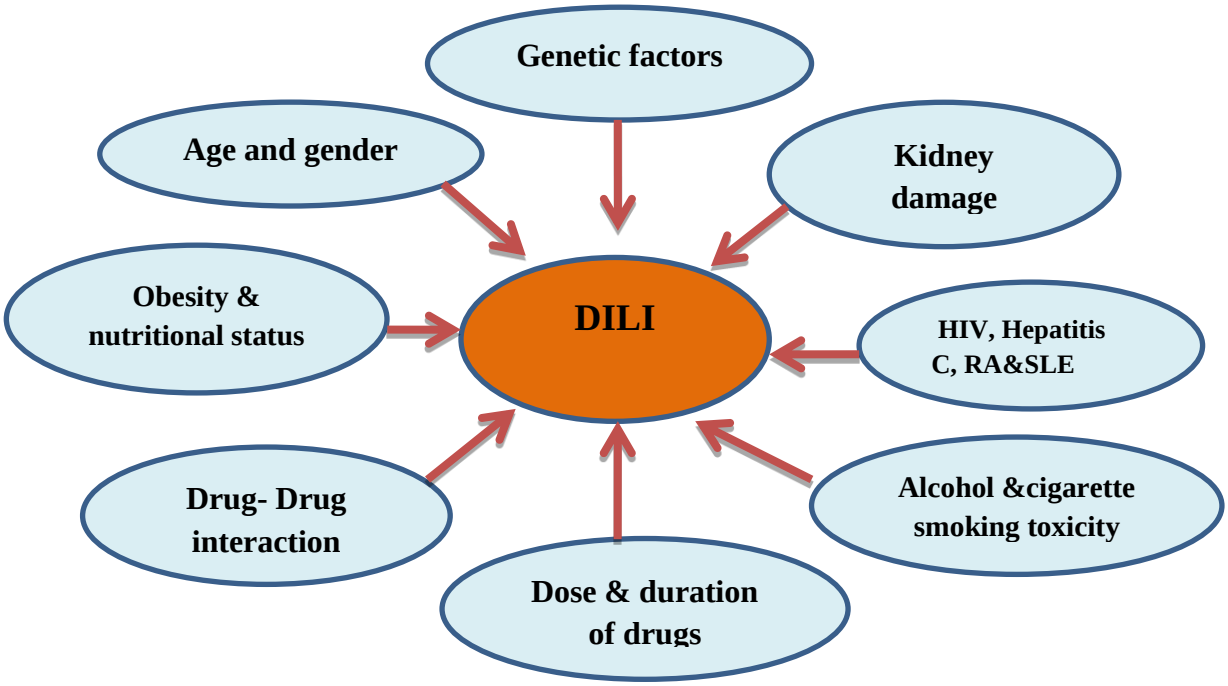


Figure 2: Factors affecting drug-induced hepatic toxicity (5,25).

Millions of people are currently suffering from hepatic damage caused by alcohol, chemicals and infections. Paracetamol, anti-TB drugs, nitrosamines, and polycyclic aromatic hydrocarbons are all known to cause liver damage (5).

2.2.1 Anti-TB drugs

The first-line anti-TB drugs are effective but their liver toxicity may lead to drug interruption; which can in turn be the cause for the development of Multidrug Resistant Tuberculosis (MDR-TB). Among the first-line anti-TB drugs, isoniazid, rifampicin, and pyrazinamide are known to cause hepatotoxicity(40). They are metabolized in the liver and the principal agents responsible for anti-TB drug-induced hepatotoxicity(41).

I. isoniazid

INH is mainly metabolized in different ways in the liver. In path 1, isoniazid is acetylated by N-acetyltransferase 2 (NAT2) to give N-acetylisoniazide (AcINH). AcINH can be enzymatically hydrolyzed by amidase to form acetyl hydrazine (AcHz) and Isonicotinic acid (INA). Then AcHz can be deacetylated to hydrazine (Hz) by hydrolysis through amidase or further acetylated to diacetylhydrazine by NAT2. Diacetylhydrazine does not have hepatotoxic effect. In route 2, INH is hydrolyzed by amidase to Hz with the simultaneous formation of INA; it can also be metabolized to oxoacid hydrazone species and other toxic metabolites with catalysis of CYP2E1. Hz can through NAT2 degraded to ammonia or acetylated to AcHz (42–44).

II. Rifampicin

After absorption from the stomach, approximately 85 % of RIF is metabolized in the liver where it is either deacetylated to form 25-deacetyl RIF via arylacetamide deacetylase, or hydrolyzed to form 3-formyl RIF (Figure 4). Although the deacetylated form of RIF does not exhibit cytotoxicity to liver. RIF activates pregnane X receptor (PXR), a member of the nuclear receptor superfamily and in turn it up regulates the transcription of drug metabolizing enzymes including that of the CYPs (45).

2.2.2 Isoniazid and Rifampicin Induced Hepatotoxicity

Drugs metabolized by phase I drug-metabolizing enzymes is often leads to generation of oxygen free radicals, which initiates lipid peroxidation to cause oxidative stress and it can be the prime cause in the genesis of liver damage(43,46,47). The attributing mechanisms anti-TB drug

induced hepatotoxicity and liver damage, oxidative stress is the major one(46,48). INH is the primary toxin and RIF potentiates its toxicity through altered kinetics of metabolites(49). The frequency of hepatotoxicity is increased when these drugs are used in combination(50). Rifampicin aggravated the hepatotoxicity due to its high amidase activity and cytochrome P450 that involved in release of large concentrations of acetyl hydrazine from isoniazid. The reactive metabolites of an acetyl-hydrazine bind with hepatic proteins causing cell injuries (29,51) Hydrazine causes acute toxicity in a concentration-dependent manner, characterized by glutathione depletion, increase in glutathione disulfide, loss of catalase activity, and lactate dehydrogenase release in hepatocytes (52,53). Acetyl hydrazine and hydrazine act as acetylating agents by binding covalently with liver cell macromolecules, causing liver necrosis(54)

According to different studies INH and RIF-induced hepatotoxicity shows alterations of various cellular defense mechanisms consisting of enzymatic and non-enzymatic components like reduced glutathione (GSH), increase lipid peroxidation, cause apoptosis (10,51); increase serum levels of ALT,AST,ALP,LDH, cholesterol, TBIL, and decrease levels of total protein and serum albumin(55) cause necrosis and liver failure (56), cause cellular inflammation (57) and antioxidant enzymes SOD, CAT, and GPx are significantly decreased(58). In order to prevent DILD induced liver damage anti-apoptotic, anti-oxidant, and anti-inflammatory effects may all play a great role (25).

2.3 Treatment of liver diseases

Liver disease affects people of all ages, genders, regions, and races around the world. Managements of hepatic disorders are endlessly challenged because of the inaccessibility of effective medicines that focus on arrest or reverse disease progression, their side effects and their prices, particularly within the developing world (24). The allopathic medical system, which is used to treat liver disorders, is sometimes insufficient and can have dangerous side effects. Herbal medications are more extensively used for the treatment of hepatic disorders than allopathic drugs due to their higher compatibility and acceptability with minimal/no side effects (59,60).

Plant-derived compounds from native cultural practices are an efficient alternative for the sources of recent hepatoprotective remedies. According to scientific research; Plants having antioxidant, anti-inflammatory and antiviral phytochemical activities have been found to have

good hepatoprotective qualities. Medicinal plants have been demonstrated to be useful in treating a variety of liver disorders. As a result, in the form of such plants, nature supplies a suitable treatment for any condition, including hepatic disorders (61,62).

2.4 Hepatoprotective Effects of Medicinal Plants

At the start of history; human beings use plants for the treatment of various ailments. About 14% - 28% plant species are used medicinally for a few ailments and diseases in the world. Nearly 50% of modern medicines are derived from natural products. Solely concerning one-tenth of the flowering plant species occurring globally are investigated for their pharmaceutical use. However, most of those plants haven't been explored with chemicals and pharmacologically. Nearly 45,000 plant species are found in Africa, however only 11% of them have documented medicinal use (59,63,64).

Traditional medicines are now used by a quarter of the world's population. The efficiencies of plant-derived medications, as well as a growing interest in natural products, have stimulated scientists' interest in medicinal plants. There is no rational treatment for liver disease, and it continues to be a challenge for modern medicine. However, Herbal items were employed for therapeutic purposes for different disease including liver disease (15,59,63).

So many findings were reported on hepatoprotective effects of different medicinal plants against anti TB drugs especially on isoniazid and rifampicin induced hepatotoxins in animal model. Some are *Adenanthera pavonina (*Linn.*)* studied by Mujahid in India(65), *Erythrina indica* leaves (66), leaf of *Lasianthera africana (*Icacinaceae) studied by Nwidu in Nigeria (67), *Anacyclus pyrethrum* Linn studied by Usmani in India (47), *Tamarindus indica* Linn studied by Meena in India(55), *Ensete Ventricosum (Welw.) Cheesman* studied by Abebe in Ethiopia(13), *Cassia fistula* studied by Jehangir in Pakistan(50), *Moringa oleifera* studied by Salama in Egypt and *Punica granatum* Studied by Yogeeta in India.

Herbal medications were utilized in the form of juice, latex, or dried powder. Phenols, coumarins, lignans, essential oils, monoterpines, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids, and xanthines are some of the chemical constituents found in liver-protecting herbal medicines (41,59). In Ethiopia a total of 276 medicinal plants from 89 families

were traditionally employed for the treatment of various liver ailments with in different preparation procedure as shown in Figure 3 (36,59).

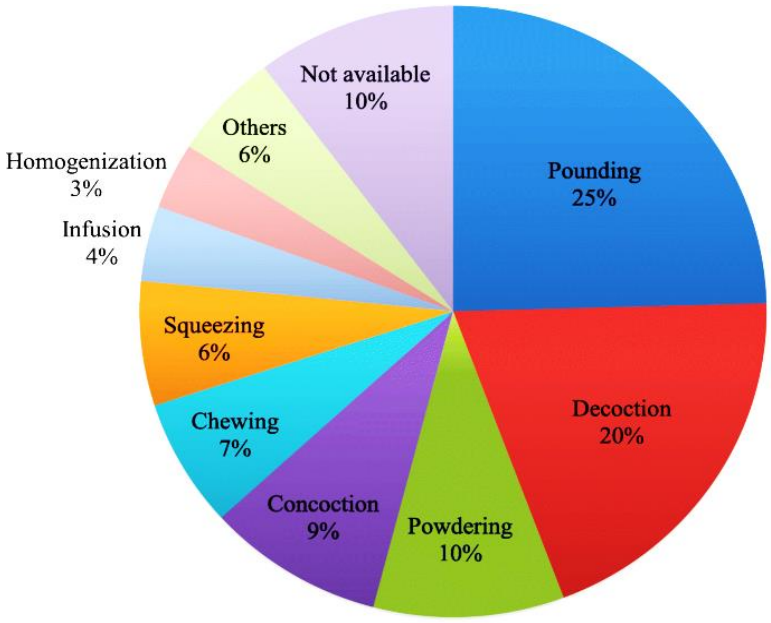


Figure 3: Methods of plants preparation used for treatment of hepatic disorders in Ethiopia(59).

2.4.1 *Calpurnia aurea*

Calpurnia aurea is a genus of flowering plants within the family of Fabaceae (Figure 4) Fabaceae family is the third biggest flowering plant family, with 730 genera and approximately 19,400 species (68,69). Fabaceae is the most frequent family found in tropical rainforests and dry woodlands of Americas and Africa. Many members of the family are utilized as medicinal herbs and sources of different medicines (70).

The *C.aurea* genus comprises shrubs or small trees in or along the margin of forests in many parts of Ethiopia and widely distributed in Africa from Cape Province to Eritrea and which also occurs in Southern India (19,71). In Ethiopia *Calpurnia aurea* is known by a local name “digita” in Amharic and “chekata” in Afaan Oromo. Different part of the plant such as bark, leaves, flowers, roots, fruits and seeds have been utilized to treat various human and animal diseases(20).

In Shinasha peoples the leaves and seed of *C.aurea* were taken orally to treat amoebiasis and giardiasis, while Amhara peoples used for the treatment of diabetes mellitus and hepatotoxicity. People in the Tigray region use the roots, leaves, and fruits to treat human/livestock tick and lice.

In the rest of the country, it is used to treat headaches, eye diseases, scabies, coughs, syphilis, leishmaniasis, tapeworm, trachoma, ringworm, elephantiasis, stomach ache, vomiting, wound, cancer, bowel, rheumatism, fibril illness (michii), kuruba, black leg, snake bite, to induce uterine contractions and as an pesticide used to destroy rice weevil, lice and ticks (20,21,37,72).

Preliminary phytochemical analysis of the plant extracts indicated presence of various phytochemicals. The hydro alcoholic leaf and seed extract of *C aurea* revealed the presence of alkaloids, terpenoids, flavonoids, steroids and phytosteroids, tannins, saponins, phenols and cardiac glycosides using standard qualitative phytochemical screening procedures (19). In the presence of these active phytochemicals *C.aurea* possess strong antioxidant, free radical-scavenging and anti-lipidemic properties (73–75). Mostly leaf of *C.aurea* were rich in flavones and polyphenols than the seeds which contains alkaloids and tannins in high proportion (73).

Figure 4: Leave of *Calpurnia aurea* (picture captured by the researcher, October, 2021)

2.5 Conceptual frame work

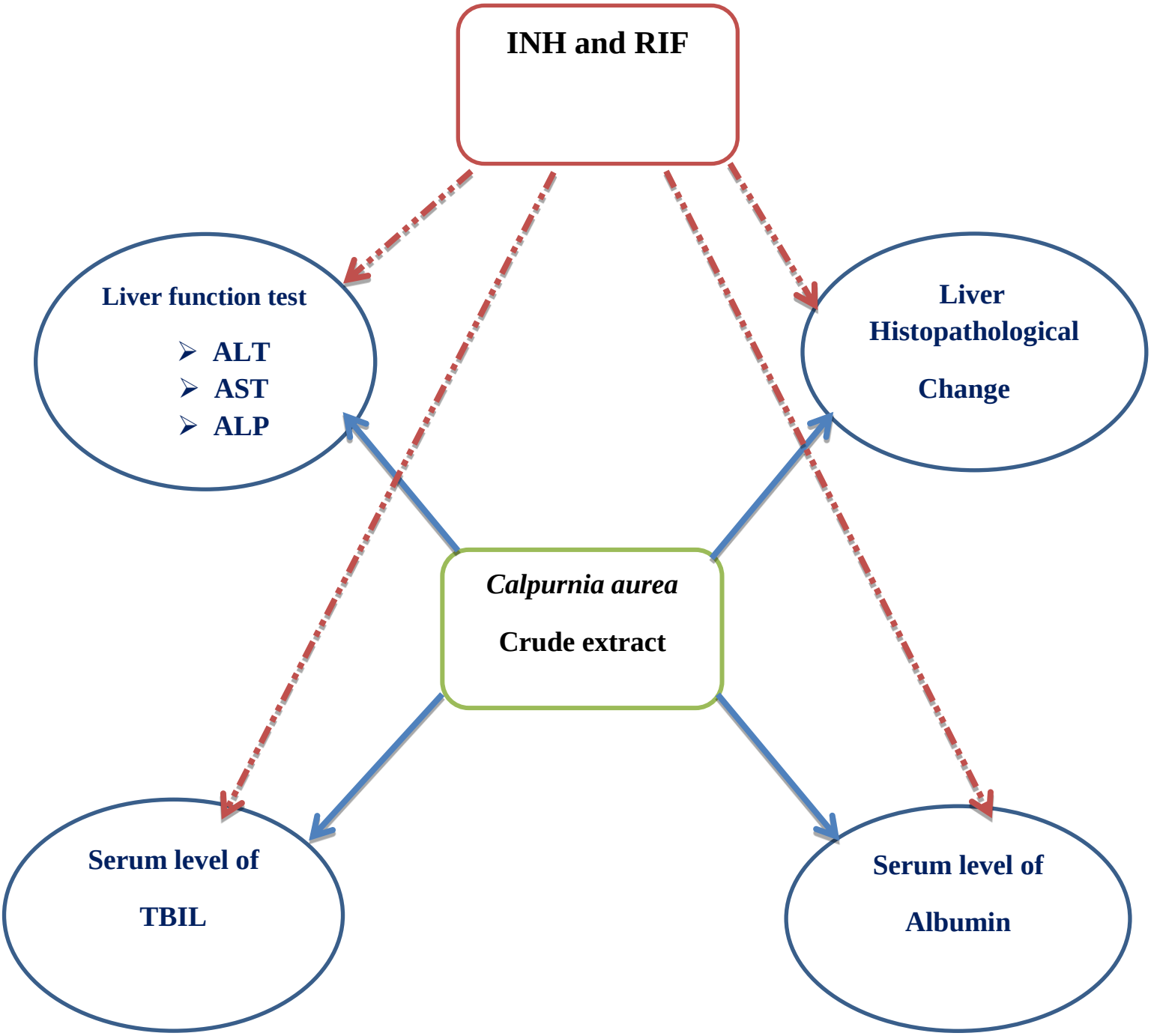


Figure 5: Conceptual frame work developed after reviewing different literature and based on the objective of the study

CHAPTER THREE

OBJECTIVE

3.1. General objective

To evaluate the effect of methanolic leaf extract of *C. aurea* in isoniazid and rifampicin induced liver injury in Mice.

3.2 Specific objectives

- ✓ To analyze the effect of *Calpurnia aurea* leaf extract on liver weight and liver index on INH - RIF induced Mice.
- ✓ To determine the effect of *Calpurnia aurea* leaf extract on liver enzymes (ALT, AST and ALP) on INH -RIF induced Mice.
- ✓ To evaluate the effect of *Calpurnia aurea* leaf extract on total bilirubin and serum albumin level on INH -RIF induced Mice.
- ✓ To investigate the effect of *Calpurnia aurea* leaf extract on liver histopathology on INH - RIF induced liver damaged in Mice.

3.3. Hypothesis

Null hypothesis (H0): Methanolic leaf extract of *Calpurnia aurea* doesn't have hepatoprotective effect in INH and RIF induced liver damage in Mice

Alternative hypothesis (H1): Methanolic leaf extract of *Calpurnia aurea* has hepatoprotective effect on INH and RIF induced hepatotoxicity in Mice.

CHAPTER FOUR
METHODS AND MATERIAL

4.1. Study area and period

The study was conducted in Jimma University Organic chemistry postgraduate laboratory and Jimma University Tropical and Infectious Disease Research Center (JUTIDRC), from December 6 to 27 2021.

4.2. Study design

A laboratory-based randomized control experimental trial was conducted on thirty mice(76).

4.3. Sample size determination

Sample size was determined based on resource equation using Degree of Freedom (E) value (77–79). The value of "E" must be between 10 and 20 and calculated by the following equation

$$E = Total\ number\ of\ mice - total\ number\ of\ groups$$

$$E = N - k = (n * k) - k \ \ where\ as\ N = \ total\ number\ of\ mice,$$

$$k = \ total\ number\ of\ group \ \ n = \ number\ of\ mice\ per\ group$$

$$E = k(n-1), value\ of\ E\ is\ between\ 10\ and\ 20\ and\ value\ of\ k\ in\ this\ study\ was\ 6$$

$$Therfore \ \ 10 = 6(n-1) \ldots\ldots\ldots\ldots 1\ eq$$

$$\frac{10}{6} = n - 1, n = 2.33 \approx 3, which\ is\ minmum\ number\ of\ mice\ in\ group$$

$$20 = 6(n-1) \ldots\ldots\ldots\ldots 2\ eq$$

$$\frac{20}{6} = n - 1 \ \ n = 4.333 \approx 5\ which\ is\ the\ optimum\ number\ of\ mice\ in\ group$$

Therefore the total number of mice in this experimental study was $30, N = (n * k) = 5 * 6 = 3$

4.4. Materials

4. 4.1. Instruments

Mechanical grinder, filter paper (Whatman number one), gauze (Nylon clothes), plastic sample holder, deep freezer, rotary evaporator (BÜCHI Rotavapor R-200), heating bath (BÜCHI Heating Bath B- 490), volumetric flasks, funnel, aluminum foil, separator funnel, beakers, measuring cylinders, test tube, dropper, spatula, Eppendrof tube, centrifuge machine (Eppendorf Centrifuge 5804R), fully automated serum analyzer (CobasR 6000), oral gavages, frost-ended

slides, labeler, markers, syringe, refrigerator, sensitive digital balance, surgical blades, scissors, glove, open tissue processor(LeicaTP 1020, Germany), Tissue embedder(Tissue-Tek), Mold(tissue cap), wax dispenser (Electrothermal Model: MH8523, China), ribbon Microtome (Leica Model: TP 1020, Germany), Slides, Light microscopy (Olympus CX21FS1, Philippines), Oven (Gallen Kamp), desiccator, tissue cassettes, digital photo camera (Camera KRUSS optronic Germany 3.0 MP USB 2.0), ice box and mice cages were used.

4.4.2. Chemicals and Reagents

INH, RIF, Silymarin, Sulfuric Acid, HCl, NaOH, Potassium Iodide, Mercuric Chloride, , Acetic Anhydride, $FeCl_3$, Chloroform Ethanol(95%), , ALT, AST, ALP, total bilirubin and albumin reagents, 10% buffered formalin, paraffin, ketamine, xylazine, Methanol (96% For HPLC-Gold-Ultra gradient), distilled water, Normal Saline (0.9% sodium chloride solution), were used.

4.5. Plant collection and Authentication

The leaf part of *C. aurea* was collected from Jiren Mountain, Jimma town, Oromiya Regional State about 353 kilometers Southwestern of Addis Ababa, Ethiopia. The plant was identified and authenticated by plant taxonomist Melaku Wondafrash and his colleagues working at Addis Ababa University National Herbarium, Addis Ababa, Ethiopia. The specimen was given voucher number MM001 and kept in the herbarium for future reference. The leaf was dried at room temperature under the shade after washing with tap water. Dried leaf was grounded into coarse powder using mechanical grinder and packed in tightly sealed container until extraction.

4.6 Plant Extraction

Extraction was done using maceration method. *C. aurea* leaf powder (250 g) was soaked with 96% methanol with ratio of 1: 8 (one gram of the powder in 8ml of 96% methanol) for three consecutive days with mechanical shaking. After 72 hours the extract was filtered using Whatman No.1 filter paper and funnel. Then the filtrate was concentrated in rotary evaporator under 40°C and reduced pressure. Then, the concentrated extract was placed in pre-weighed evaporating dish and preserved in oven under 37°C for three days to evaporate the remaining methanol. Finally, solid crystals with jelly like appearance were obtained and percent yields were calculated using the formula below (80). The extract was packed into beaker with aluminum foil cover and store in a refrigerator until use.

$$\text{Yield}=\frac{Weighted\ of\ extract\ obtained}{Weight\ of\ plant\ sample}\times 100$$

4.7 Phytochemical screening

Phytochemical screening was done using different solvent system including methanol, acetone, ethanol, petroleum ether and chloroform. Testing for alkaloid, terpenoid, flavonoid, phenol, steroid, quinone, saponins, tannin, antraquinone, amino acids and fat of leaf extract of *C.aurea* were done by following procedures obtained from previous studies (81–84).

4.7.1 Alkaloid test (Mayer's test): 2 mL plant extract was dissolved in 8 mL distilled water and filtered, then 1% HCl was added, and 2-3 drops of Mayer's reagent (1.358g mercuric chloride + 60 mL distilled water and solution of 5g potassium iodide + 10 mL distilled water) were added, resulting in a creamy white precipitate.

4.7.2 Flavonoid test (Conc. H_2SO_4 test): Conc. H_2SO_4 was added on 2ml of Plant extract and formation of orange color showed the presence of flavonoids.

4.7.3. Terpenoid test (Salkowki's test): 2mL plant extract with 8 ml distilled water solution was filtrated then 2 ml chloroform and 2 ml H_2SO_4 was added through the test tube wall respectively. Plant extracts contain Terpenoid result brown or orange rings in a layer of two solvents.

4.7.4. Steroid test (Liebermann-Burchard test): 1ml of extract was treated with drops of chloroform, acetic anhydride and conc. H_2SO_4 that leads to the formation of dark pink or red color indicted presence of steroid.

4.7.5. Saponins test (Foam test): Solution of 2mL plant extract and 8mL of distilled water was shaken and filtered it. The cold solution as shaken vigorously and the formation of stable foam indicated as presence of saponins.

4.7.6. Tannin test (Braymer's test): 0.5g of Extract was dissolved with 10 ml distilled water. Then on filtrate few drops of 5% ferric chloride was added. Black or blue-green coloration or precipitate was taken as positive result for the presence of tannins

4.7.7. Quinone test: 2ml of extract was treated with conc. HCL and observed for the formation of yellow precipitation or coloration implies positive for quinone.

4.7.8. Phenol test (Gelatin test): few ml of plant extract was dissolved in 5mL distilled water then 1% gelatin and 10% NaCl solution was added and leads to white precipitate formation.

4.7.9. Amino acid test (Ninhydrin test): 0.1 g of extract was dissolved in 10 ml of distilled water and in the 2ml filtrate 2 drops of ninhydrin solution (10 mg ninhydrin+ 200 ml acetone) was added. Appearance of purple color indicates the presence of amino acid.

4.7.10 Antraquinone test: 2 ml extract was mixed with 2 ml of benzene then3 ml of1% NH_3 solvent was added that shows violet color to indicate presence of Antraquinone.

4.7.11 Fat test (Spot test): A small quantity of extract is pressed between two filter papers and oil stain on the paper indicates the presence of fat.

4.8 Experimental Animals

A total of 30 mice from both sex of 6-9 weeks old, and weighting 29 to 35 g mice were taken from JUTIDRC, Sokoru, Jimma, Ethiopia. The experimental animals were acclimatizing the laboratory conditions for 7 days before the experiment. Free accesses of standard food pellets and tap water were supplied for animals at all times as per the National Institutes of Health (NIH) guidelines for care and use of laboratory animals (85). The animals were maintained on a 12hrs light/dark cycle in an optimum temperature (20–25°C) and humidity environment.

4.9. Administration of Extracts

Experimental mice received their corresponding dose of crude extract calculated per kilogram of their body weight. In order to separate the mice based on their body weight code mark were given using permanent marker. *C.aurea* leaf extract were administered using oral gavages and recorded on the prepared checklist to prevent missing or double administration.

4.10. Acute Oral Toxicity Test

Acute toxicity test on the leaf extracts of *C. aurea* was carried out based on the limit test of guidelines of organization for economic co-operation and development guideline-425 (OECD) and the up and down method of experimentation on female mice (86,87). At a time a single animal was taken and fasted for 3 hours before and 1 hour after dosage of 2000mg/kg of *C. aurea* extract. Then the mice were observed for 12 hours with special attention. If the animal survives four additional animals were taken and dosed 2000mg/kg of extract sequentially with time interval. Time interval between dosing is determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. A total of five mice were tested and if more than three

animals survived the LD50 is greater than 2000 mg/kg. If the animals were not survived below 2000mg/ kg dose were taken and dosed in other mice.

4.11 Drug dose for inducing hepatotoxicity

Isoniazid and Rifampicin were obtained from Jimma university medical center. In this study to induce hepatotoxicity, 100mg/kg of isoniazid and 100mg/kg of rifampicin were used. The dose of this drug was taken based on previous studies (56,57,88–90).These drugs are diluted in normal saline once daily and given by IP to induce liver damage.

4.12 Animal grouping

The total of 30 mice was randomly classified in to six groups (five mice in each group). These experimental mice were receiving their corresponding doses of *C. aurea* leaf extracts, INH and RIF that was calculated per kilogram of their body weights per day (Figure 9). Based on the acute toxicity test done yet *C.aurea* crude extract had no toxicity effect up to 2000 mg/kg(91), therefore, the dose of extract 10% , 20% and 30% of 2000mg/kg was applied.

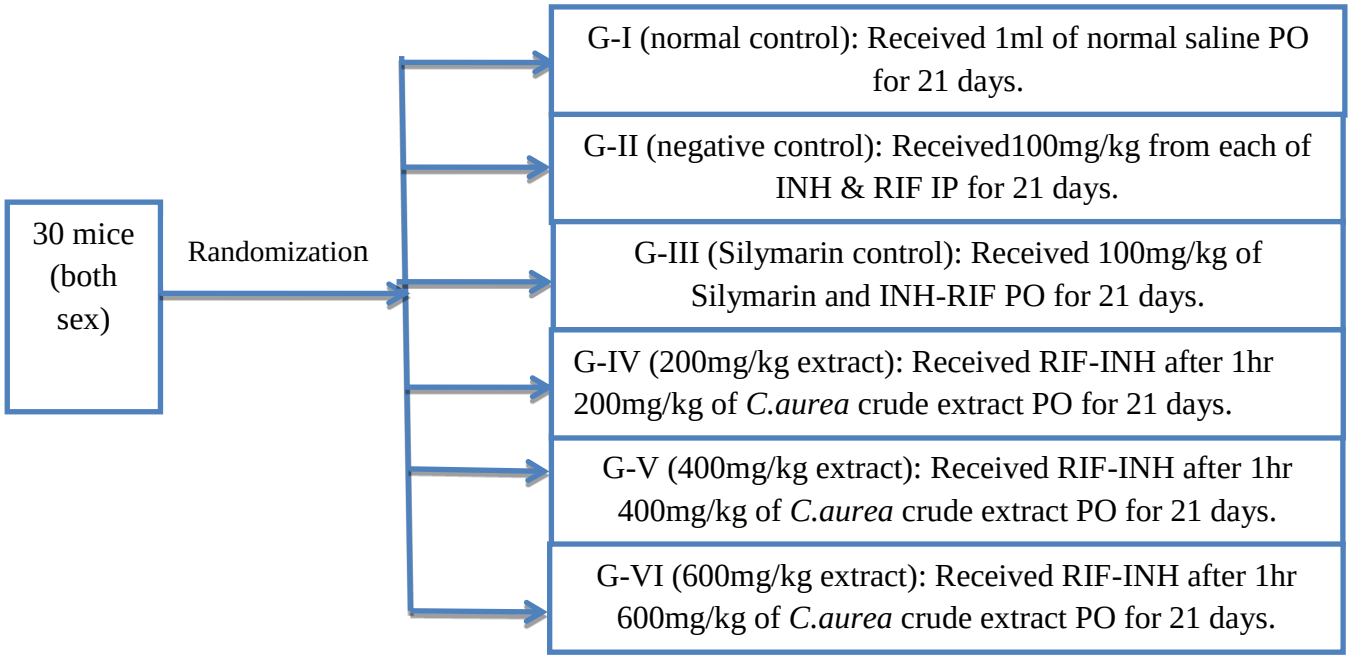


Figure 6: Animal grouping and dose protocol

4.13. Operational definitions

Normal control group: the group of mice which received normal saline.

Negative control group: the group of mice which received only Isoniazid and Rifampicin

Positive control group: the group of mice which received Silymarin with Isoniazid and Rifampicin

Experimental groups: the groups which would be treated with different doses of *calpurnia aurea* leaf extract and Isoniazid and Rifampicin

Liver Index: liver weight divide by body weight x 100 (g/g) used as the indicator of liver status

4.14 Blood and tissue collection

On 21th day animals were fasted for 12 hours then weighted and anesthetized with combination of ketamine and xylazine. The dose of 60 mg/kg ketamine and 16mg/kg xylazine was given through IP(92). After 30 min anesthetization 1-2ml of blood was collected by cardiac puncture. Then the blood was centrifuged using eppendrof centrifuge machine at room temperature for 10 minutes with speed of 3000rpm.The serum was collected in eppendrof tube and put in ice-box until serum analysis were performed for liver function test. The liver tissue was collected and fixed in 10% formalin solution for histopathological examination.

4.15 Body weight and liver index

The body weight of the mice was measured and recorded weekly to see body weight change in all groups of mice. Finally liver weight was measured then liver index was calculated(56).

$$liver\ index = \frac{liver\ weight\ (g)}{body\ weight\ (g)} \times 100\%$$

4.16 Biochemical Analysis

Serum level of ALT, AST, ALP, TBIL and Albumin were analyzed using a fully automated serum analyzer (Cobas R 6000) according principles and procedures of manufacturer manual.

4.16.1. Aspartate aminotransferase (AST)

AST, also known as serum glutamic-oxaloacetic transaminase (SGOT), that catalyze the transfer of α- amino group from aspartate to α-ketoglutarate to produce glutamate and oxaloacetic acid. The enzyme malate dehydrogenase (MDH) converts oxaloacetate to malate, and coenzyme is oxidized to NAD^+ as shown in Figure 7. The absorbance reduction at 340 nm caused by NADH oxidation is assessed, and it is directly proportional to the AST activity in the sample. The typical concentrations of AST in the blood is 5-40 U/L but increased its serum level when body tissue or organ like liver, heart were damaged (93,94).

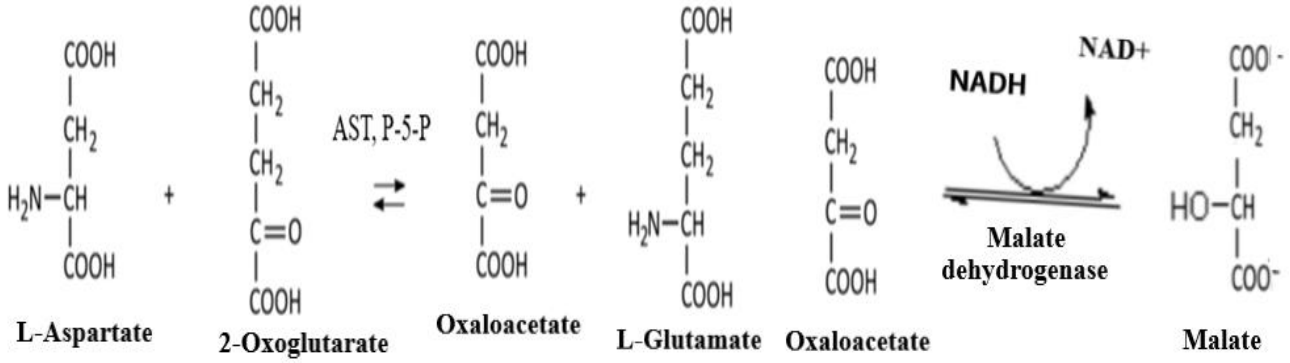


Figure 7: Reactions involved in the determination of AST activity (95).

4.16.2. Alanine aminotransferase (ALT

ALT (serum glutamic-pyruvic transaminases (SGPT)) is catalyzing the transfer of amino group from alanine to α-ketoglutarate to generate pyruvate and glutamate as shown in Figure 8. Elevated serum ALT levels frequently indicate the presence of medical issues, such as hepatic damage. The oxidation of NADH to NAD^+ in the presence of pyruvate and lactate dehydrogenase is employed in a colorimetric test to measure ALT. The rate of NADH oxidation, leads absorbance reduction at 340nm which is proportional to ALT activity (93,94).

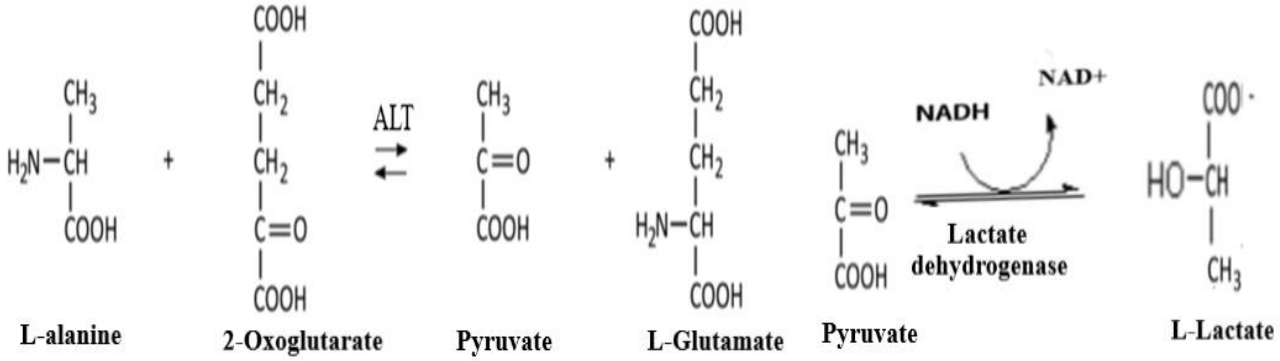


Figure 8: Reaction of ALT (93,94)

4.16.3. Alkaline phosphatase (ALP)

ALP is a membrane-bound glycoprotein that catalyzes the hydrolysis of phosphate monoesters at the basic pH levels as shown below (Figure9). Alkaline phosphatase is cytosolic and found in the canalicular membrane of hepatocytes in the liver. Its activity was shown to be higher in the serum of patients suffering from liver and bone diseases (96). ALP activity is measured using P-Nitro phenyl phosphate as the substrate. In the presence of alkali, alkaline phosphatase works on

the substrate and releases p-nitro phenol (yellow anion) which is automatically measured in a spectrophotometer 405 nm as linear function of alkaline phosphatase activity.

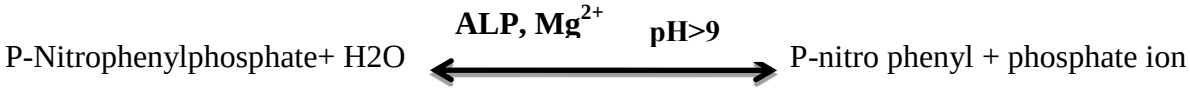


Figure 9: Reaction of ALP

4.16.4. Serum Albumin

Albumin is the most abundant protein in human plasma, accounting around 40% of total albumin in plasma and the remaining 60% in extracellular space. Albumin functions as a transport protein for a range of molecules, including bilirubin, enzymes, hormones, and medicines, and helps to regulate colloidal blood osmotic pressure. Albumin is exclusively synthesized in liver cells at a rate of about 15 g/day. The concentration of serum albumin decreases and the quality of albumin varies as a result of liver injury (97,98). Bromcresol purple (BCP) dye is used to assess the quantity of albumin in serum. A color change is detected at 600 nm when the dye binds specifically with albumin in a pH range of 5.2-6.8.

4.16.5. Total Bilirubin (TB)

Bilirubin is a metabolite of heme, which is a coordination complex that helps proteins coordinate iron. Bilirubin comes from two different sources. Breakdown of hemoglobin in senescent red blood cells and prematurely damaged erythroid cells in the bone marrow produces about 80% of bilirubin. The rest comes from the metabolism of heme-containing proteins found in other tissues, including the liver and muscles. Myoglobin, cytochromes, catalase, peroxidase, and tryptophan pyrrolase are among these proteins. Bilirubin is created at a rate of about 4 mg/kg of body weight every day(99,100). Bilirubin forms azobilirubin (violet color) when it reacts with diazotized sulphanilic acid. The synthesis of azobilirubin from free bilirubin is catalyzed by DMSO. The violet color is related to the amount of bilirubin detected at 546 nm (530-550nm).

Figure 10: metabolism of bilirubin

4.17 Histopathological Study

Histopathological examination of liver was performed to determine the effect of *C.aurea* leaf extract on hepatotoxicity induced by isoniazid and rifampicin.

4.17.1 Dissection and Tissue grossing

Liver tissue was collected from each mouse after scarification. Dissection was done from neck to pubis and the peritoneum was opened by using sterile surgical blade then the liver was taken and placed on bottle which contains 10% of buffered formalin for preservation. Then, the tissue was taken to Jimma University Pathology department tissue grossing room for tissue grossing then transferred to tissue processing room.

4.17.2 Dehydration and Infiltration

The specimen was washed by using series of ethanol (70%, 80%, 95% and 100%) for fixation and dehydration. Then ethanol was removed from tissue using xylene solution which is miscible with paraffin wax. It enhances the tissue to embed easily with the wax to form tissue blocks.

4.17.3 Embedding

Electro-thermal wax dispenser helps to embed the tissue in paraffin wax to form tissue blocks in squared metallic plates. Then blocks were labeled and placed in a refrigerator until sectioned.

4.17.4 Sectioning

Tissue blocks were sectioned at a thickness of 5μm by placing paraffin block having tissue in rotary microtome. The ribbon of sections was carefully picked from the knife by a blunt forceps to float in a water bath of 400C to remove folds in the sections. Unfolded sections were picked by clean microscope glass slides and were placed in an oven at a temperature of 560_C for 15 minutes for proper drying and better adhesion.

4.17.5 Staining

Staining is used to highlight important features of the tissue as well as to enhance the tissue contrast. The hydrated sections were immersed in hematoxylin for 3- 5 minutes with an eosin counterstained and agitated with acid alcohol to prevent over staining. Sections were immersed in a mixture of sodium bicarbonate, ethanol and distilled water to give blue color to the nucleus. Then it was immersed in 95% alcohol and eosin to give pink color to the cytoplasm. Finally, tissue sections were dehydrated in 95% alcohol, cleared in xylene and mounted by adding a drop of DPX (Dibutyl Phthalate in Xylene) mounting medium on the section to cover the microscopic glass with cover glass and to increase the refractive index of the tissue under light microscope.

4.17.6 Photomicrography

Sections of the liver were examined using light microscope at x40 magnification and microscope Camera.

4.18. Study variables

Independent variable:

Calpurnia aurea crude extract

Dependent variables

- ✓ Histopathological change
- ✓ AST,
- ✓ ALT
- ✓ ALP
- ✓ Total Bilirubin
- ✓ Albumin

4.19. Statistical Analysis

Quantitative data were expressed as mean ± SD and all statistical comparisons were made by means of one-way analysis of variance (ANOVA) test. This test was used to determine significant differences among the groups at the $p < 0.05$ level. The data was entered using Epi data 3.1 and exported to SPSS.25 statistical software for analysis. For qualitative data analysis, microscopic evaluation were carried out by a senior pathologist through preparing microscopic slides for each group and presented in the form of photomicrography.

4.20. Data quality assurance

During extraction, liver function test and histopathological examination appropriate laboratory procedures and rules were applied. And also, instruments and equipment were calibrated through known standards after being washed clearly. The expire date of chemicals and reagents was checked and histopathological examination was manipulated by professionals.

4.21. Ethical Considerations

The ethical clearance letter was obtained from Research and Ethical Review Committee of Institution of Health, Jimma University with Ref. No. IHRPGD /350/22. Support request letters were written to different responsible bodies including JUTIDRC and Pathology department. All activities in this experimental study were carried out according to recommendations from declaration of nationally and internationally conventional standards for employment of experimental animals and code of ethics of animal experiments, which comply with scientific and ethical guidelines.

4.22. Dissemination plan

The findings of the study will be submitted to Jimma University Institute of health and Graduate programs coordinating office. It will also disseminated by presenting at workshops, research meetings and by publishing on journal. Copies of the research will be submitted to the Department Biomedical Sciences.

CHAPTER FIVE

RESULT

5.1. Percentage Yield from plant material

The percentage yields of leaf extract of calpurnia aurea in different solvent system were presented in the table 2. Methanol extract has the highest yield (23.6%) and petroleum ether yielded the lowest yield (3.3 %).

$$\% \text{ Yield} = \frac{Weighted\ of\ extract\ obtained}{Weight\ of\ plant\ sample} \times 100$$

Table 1: percentage yields and appearance of leaf extracts of *C.aurea*

S/N	Solvent	Weight (g) of the crude extract	% yield	Appearance
1.	Petroleum ether	8.3	3.33	semi-solid
2.	Chloroform	18.24	7.3	semi-solid
3.	Ethanol	25.33	10.13	semi solid waxy gel
4.	Methanol	58.99	23.6	semi solid waxy gel
5.	Acetone	21.49	8.6	semi-solid

5.2 Phytochemical screening Test

Phytochemical test of leaf extract of *C.aurea* resulted from different solvent system were presented in Table 3. This table shows the presence and absence of active phytochemical constituent in petroleum ether, chloroform, ethanol, acetone and methanol extract According to the preliminary phytochemical screening test methanol crude extract contains best phyto-constituents that serve as hepatoprotective including flavonoids, alkaloids antraquinone and phenols. Therefore to conduct the main extraction on C.aurea methanol was selected as the best solvent system.

Table 2: Preliminary Phytochemical Screening result of *C.aurea* leaf extract.

Phytochemical constituent	Standard test	Solvent extract				
		Petroleum ether	Chloroform	Ethanol	Methanol	Acetone
Alkaloid	Mayer's Test	_	_	+	++	+
Flavonoid	Alkaline reagent test	+	+	+	+	+
Phenol	Gelatin test	_	+	+	+	+
Saponins	Frothing Test	_	_	+	+	_
Quinone	Conc. HCl test	_	_	_	++	+
Tannin	10% NaOH test	+	+	+	++	+
Steroid	Salkowski's test	+	+	_	+	_
Terpenoid	Liebermann test	+	_	+	+	+
Antraquinone	Borntrager's test	_	_	+	++	_
Amino acid	Xanthoproteic test	_	_	_	_	_
Fat	Stain test	_	_	+	+	_

+++ Strongly detected; + Detected; − Negative or Not Detectable

5.3. Acute Oral Toxicity Test

A single animal was taken and given 1st dosage of 2000mg/kg of *C. aurea* crude extract. But there is no physiological change like lack of appetite, trouble breathing, overall weakness, irritability, writhing, loss of motor coordination, muscular relaxation, drowsiness, and profound sleep and death. Additional four mice were taken and given sequentially 2000mg/kg of *C. aurea* crude extract in 48 hours. But there is no death recorded at dose of 2000 mg/kg within 14 days observation. Therefore oral LD50 (median lethal dose) is greater than 2000 mg/kg.

5.4 Effect of *Calpurnia aurea* on liver weight and liver index of mice

The liver weights of mice treated with RIF and INH in group II were increased significantly ($p < 0.001$) compared to that of normal control group. Whereas the mice received leaf extract of *C.aurea* with INH-RIF and Silymarin group were significantly reduced ($p < 0.01$) liver weight compared to INH-RIF only received group.

The group of mice treated with INH -RIF plus *C.aurea* extracts and Silymarin control group significantly reduced ($p<0.01$) the liver index as compared to INH-RIF only treated group as shown in Table 3.

Table 3: Effect of *C.aurea* extract on liver weight and liver index

Group	**Liver weigh (g)**	**Liver index**
Group I (Normal control group)	1.24± 0.6	3.45±0.23
Group II(INH-RIF gro up)	2.13 ± 0.24	6.90 ±1.10 α
Group III(Silymarin group)	1.44 ±1.96	4.12± 0.56
Group IV (200mg/kg Extract)	1.49 ± 0.29	4.27 ±0.31 *b*
Group V (400mg/kg Extract)	1.45± 0.10	3.43± 0.88
Group VI (600mg/kg Extract)	1.49 ± 1.45	4.8 ±0.41 *c*

Values expressed as mean ± SD, n=5. ($P < 0.001$) compare to group I.

While ($P < 0.01$), ($p < 0.001$), compared to negative control or group II

5.5 Effect of methanolic leaf extract of *C.aurea* on liver enzymes

Tables 5 show the effects of methanolic leaf extract of *C.aurea* on various liver specific enzymes and other biochemical parameters in RIF plus INH caused hepatotoxicity in mice.

5.5.1. Effect of *Calpurnia aurea* leaf extracts on the serum ALT levels

In comparison to normal control group (group I), INH - RIF treated group was significantly increased ($p<0.001$) the ALT levels from 43.8±12.95 to 94.2±12.78 IU/L. But the group of mice treated with 400mg/kg of *C.aurea* leaf extract significantly reduced ($P<0.05$) to 59.0±20.21. This reduction of ALT was comparable to Silymarin treated group.

5.5.2. Effect of *Calpurnia aurea* leaf extracts on the serum AST levels

The serum level of AST in group II (169.8±43.09) was significantly increased ($p<0.001$)) as compared to group I (35.4±7.96). The group of mice treated with 400mg/kg+ INH-RIF scored significantly reduced ($P<0.001$) (70.6016.38) ALT level compared with group II.

5.5.3. Effect of *Calpurnia aurea* leaf extracts on the serum ALP levels

The serum level of ALP in group-II (300.2±81.62) did show a significant increase ($p<0.01$) as compared to group-I (108.4±19.05). The different doses of *C.aurea* extract and Silymarin treated groups (III, IV, and V) with RIF-INH showed a significant decrement as compared with group-II mice, despite group-IV (200mg/kg *C.aurea* extract) and Silymarin control group significantly different (Table 4).

Table 4: Effect of methanolic leaf extract of C.aurea on liver enzymes (n=5)

Group	ALT (IU/L)	AST(IU/L)	ALP(IU/L)
Group I (Normal control group)	43.8 ± 12.95	35.4 ± 7.96	108.4 ± 19.05
Group II (INH-RIF group)	94.2 ± 12.78^{a}	169.8 ± 43.09^{a}	300.2 ± 81.62^{a}
Group III (Silymarin group)	46.6 ± 10.55^{b}	54.2 ± 15.19^{b}	$112.6\pm12.88^{b*}$
Group IV (200mg/kg Extract)	77.6 ± 9.48^{d}	152.2 ± 38.33^{d}	159.8 ± 60.37^{c}
Group V (400mg/kg Extract)	$59.0\pm20.21^{b*}$	70.60 ± 16.38^{b}	154.4 ± 46.77^{c}
Group VI (600mg/kg Extract)	71.8 ± 8.26^{d}	131.2 ± 11.05^{d}	$222.0\pm92.64\ d$

Significantly differ as compared to normal control group, $P<0.001$
Significantly differ as compared to group II, $P<0.001$, $P<0.01$, $P<0.05$
Insignificantly different as compared to negative control or group II, $P>0.05$

5.6. Effect of methanolic leaf extract of *C.aurea* on Serum Total bilirubin

As shown in table 6 serum level of total bilirubin were significantly increased ($P<0.01$) in group II mice treated with RIF –INH only compared to group I. Whereas INH-RIF+ *C.aurea* extract at a dose of 400 mg/kg and silymarin control group significantly decreased (p serum total bilirubin level as compared to INH-RIF treated group of mice. The effects of *C.aurea* leaf extract at doses of 200 and 600 mg/kg + in INH and RIF on serum total bilirubin were reduced but it is statistically insignificant ($P>0.05$) comparison to INH-RIF group.

5.7 Effect of *Calpurnia aurea* leaf extract on serum Albumin level

Isoniazid and rifampicin-treated group showed statistically significant decrement (p in serum albumin compared to a normal control group. Group of mice treated with INH-RIF+ 400mg/kg

of *C.aurea* extract and a silymarin control group significantly elevated (P<0.01) serum of albumin as compared to INH-RIF only treated group of mice. However insignificant elevation (P>0.05) of serum albumin was shown in INH-RIF+ plus 200mg/kg and 600mg/kg of *C.aurea* extract treated group compared to INH-RIF only treated group as shown in Table 5.

Table 5**:** Mean values of serum levels of Total bilirubin and albumin level

Group	**T-BIL (mg/dl)**	**Albumin(g/l)**
Group I (normal control group)	0.72 ± 0.42	3.86 ± 0.59
Group II (INH-RIF treated group)	1.89 ± 0.7^{a}	$0.96 \pm 0.23^{a*}$
Group III (Silymarin group)	0.83 ± 0.53^{b}	$3.34 \pm 0.47^{b*}$
Group IV(RIF-INH+200mg/kg extract)	$1.44 \pm 0.38c$	1.45 ± 0.81c
Group V(RIF-INH+400mg/kg extract)	0.70 ± 0.35^{b}	$3.12 \pm 1.44^{b*}$
Group VI(RIF-INH+600mg/kg extract)	$1.71 \pm 0.46c$	1.45 ± 0.78c

Significantly differ as compared to normal control group, P<0.05, P<0.001
Significantly differ as compared to group II, P<0.05, P<0.01
Insignificantly different as compared to group II, P>0.05,

5.8. Histopathological Investigation

Liver sections of mice observed under microscopic examination showed a visible difference in the liver architecture between control and treatment groups. The liver section of normal control showed normal hepatic architecture with normal central vein. Whereas the liver section slide of INH-RIF only treated group showed hepatic parenchymal with mild necrosis, lobular and lymphocytic inflammation, sinusoidal dilatation, cholestasis and vacuolar degeneration. However, experimental mice treated with *C,aurea* leaf extract significantly regenerated the liver architecture in isoniazid and rifampicin treated mice, and a dose-dependent difference in regenerative capacity was observed between group-IV (200 mg/kg) and group-V (400mg/kg). The silymarin treatment also significantly regenerated the liver architecture in isoniazid and rifampicin treated mice.

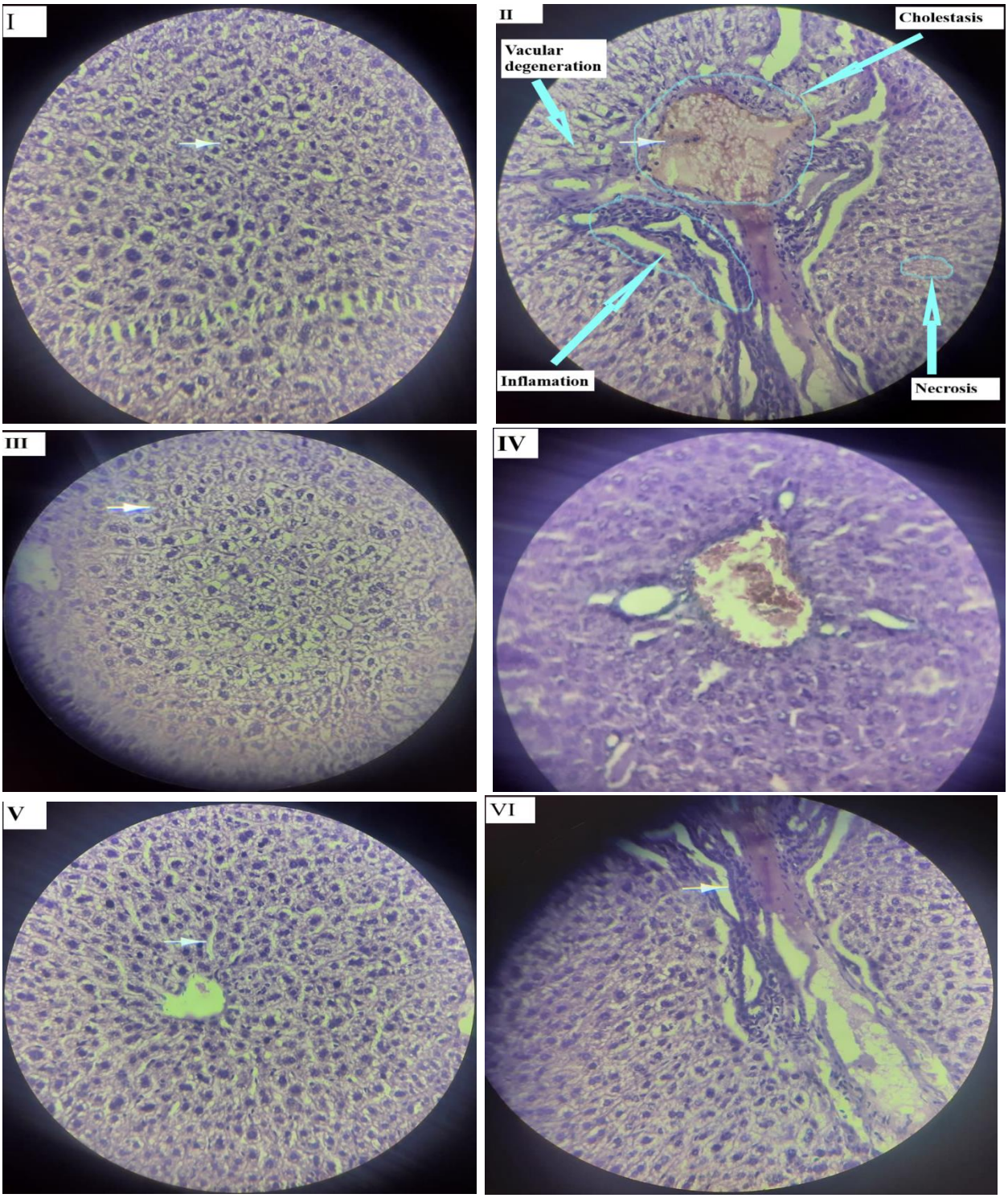


Figure 11: Photomicrograph of mice experimental liver.

I (normal control), II(received INH- RIF 100mg/kg in each),III(received 100mg/kg Silymarin),IV(received 200mg/kg extract +INH– RIF),V(received 400mg/kg extract + INH – RIF) and VI(received 600mg/kg extract + INH – RIF).

CHAPTER SIX

6. DISCUSSION

The most common reason for treatment cessation is the development of DIHD during TB chemotherapy. Hepatic damage from anti-tuberculosis chemotherapy's first-line medicines, such as isoniazid and rifampicin, is a serious concern during clinical treatment (84). In the current work to investigate the effect of methanolic leaf extract of *C. aurea* in drug-induced hepatotoxicity in mice the combination of INH and RIF were used.

The percentage yield of crude leaf extracts of *C.aurea* varied from solvent to solvent, as evidenced by the crude extract yields. This could be due to the solvents' differing polarity and extracting capacity. As a result, the percentage yields of crude leaf extracts employing petroleum ether, chloroform, ethanol, methanol, and acetone differed from solvent to solvent in the current investigation reported in Table 2. Methanol crude leaf extract had the highest yield because methanol may dissolve high phytochemical constituents in plant(17) and leaf extract may have more polar phyto constituents than non-polar compounds(71).

Preliminary phytochemical examination of plant extracts revealed the existence of many phytochemicals, according to various findings. Hydro alcoholic, 70% ethanolic and methanolic leaf extracts of *C.aurea* were screened for the presence of bioactive plant chemical constituents such as alkaloids, terpenoids, flavonoids, steroids, tannins, saponins, phenols, and cardiac glycosides (17,18,71,101). In the present study as shown in table-3, preliminary phytochemical screening of methanolic leaf extract of *C.aurea* revealed the presence of tannins, flavonoids, phenolic compounds, alkaloids, steroids, and saponins. This finding were complementary to study performed by Eyasu M *et al* (102). According to the percentage yield and phytochemical screening test methanol extract is the best one to study the hepatoprotective effect of *C.aurea* leaf extract in isoniazid and rifampicin induced hepatotoxicity.

For this study, an animal model of INH and RIF-induced liver injury was chosen to cause liver injury and test hepatoprotective effects of *C.aurea*. This is due to three reasons. The first reason, tuberculosis (TB) is still a serious health concern around the world, with 9.6 million new cases and 1.5 million deaths each year. Second, hepatotoxicity is the most prevalent major side effect of anti-TB treatment. Third, INH and RIF are still first-line anti-tuberculosis (TB) medicines, but

they have been linked to hepatotoxicity (103) and the incidence of hepatic dysfunction is more, when INH and RIF are used in combination (48).

Anti-TB drugs induced hepatotoxicity ranges from nonspecific elevation of transaminases to liver failure. The liver dysfunction is due to the synergistic effect of INH and RIF. INH is broken down into two bioactive metabolites, hydrazine and acetyl hydrazine. Hydrazine converted to toxic compound by CYP450, which leads to hepatotoxicity. RIF, aggravates hepatotoxicity by inducing CYP450, as a result more toxic metabolites are generated from hydrazine (48). Intracellular enzymes such as AST, ALT, and ALP are commonly identified in modest amounts in the blood; however, when cells are injured, these enzymes are liberated, resulting in increased serum levels (53,89). Furthermore, there is evidence that these anti -TB drugs cause cellular damage by inducing oxidative stress as a result of hepatic antioxidant defense system dysfunction, such as depletion of reserved glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase(CAT) levels within hepatocytes via CYP2E1 activation(46,50).

Several investigations show that the combination of RIF and INH causes hepatotoxicity, which manifests as hepatic steatosis and centrilobular necrosis, probably related with cholestasis, and toxic isoniazid metabolites bond covalently to cell macromolecules (66). In this study the result of liver weight and liver index of mice in the rifampicin and isoniazid induced model group (Group II) increased dramatically (p when compared to the normal control group, as shown in Table 5 and Figure 15. This result corroborated with earlier studies of X. Chen (51), Mujahid (66) and Y. Dong (88).The increased liver mass and liver index is attributed to the buildup of fat and disintegration of hepatic tissues (104). However the result of our study was inconsistent with the studies done by Y. Chen (56) and Naji (57) . Despite the exact causes for these variations are unclear, possible explanations may be due to the duration of treatment, way of administration, and different experimental animal model.

Mice treated with methanol leaf extract of *C.aurea*, on the other hand, showed a significant ($p<0.01$) reduction in liver weight and liver index as compared to the negative control group. The reduction of this liver weight and liver index was comparable to the silymarin-treated group. The finding of this study was in line with the study done by Usmani (47) and Meena (55) . Reduction

in liver weights and liver index seen in *C.aurea*-treated group may be attributed to its anti-lipidemic effect (21).

The mechanisms of hepatotoxicity caused by RIF-INH co-administration are currently unknown. (58). Rifampicin is an inducer of known microsomal enzyme (P-4502E1) which increases the concentration of toxic metabolites of isoniazid mostly hydrazine to cause hepatotoxicity(46). . During liver damage the levels of liver marker enzymes in the blood rise, acting as diagnostic indications for hepatic injury. In INH – RIF induced mice high levels of AST, ALT, LDH, ALP, TBIL and lower levels of Albumin and TP in the serum of the induced mice indicate liver injury (55,65).

In the present study the hepatotoxicity caused by co-administration of INH - RIF was confirmed by significant elevation of the serum level of liver enzymes such as ALT (P, AST (PALP (P levels in the mice treated with INH-RIF (negative control group) as compared to normal control group (G-I). This finding is in harmony with the previous studies done by Usmani (47), Nwidu and Teme (67) and Kosasih (12). Previous studies showed that co-administration of isoniazid and rifampicin can cause activation of toxic metabolites and increase the activity of CYP450 (12); that can cause cellular damage and loss of functional integrity of cell membrane resulting leakage of ALT, ALP and bilirubin enzyme levels increase in the blood (46); imbalance in endogenous oxidant-antioxidant defenses via increased lipid peroxidation and reduce glutathione homeostasis (105).

The hepatoprotective effect of methanolic leaf extract of *C.aurea* in INH and RIF induced hepatotoxicity in mice was observed through significant reduction of serum enzyme level compared to that of negative control group. In Group V mice, which received a medium dose of (400 mg/kg) methanolic leaf extract of *C.aurea* along with INH and RIF, the levels of biochemical enzymes significantly reduced ($p< 0.05$) to the normal range. The minimization of serum level enzyme with treatment of *C.aurea* extract was comparable to that of Silymarin treated group. This study was in line with the study of K. Singh (31) and Abd elhameed (106). The reduction was most likely due to active phytochemical constituent of *C. aurea* like antraquinone, flavonoids and phenols which possess anti-oxidant effect (18), highest free radical scavenging activity (75,107).

At minimum dose of *C.aurea* leaf extract in group IV there is dose dependent recovery. But at high dose of (600mg/kg) *C.aurea* methanolic leaf extract in group VI, showed insignificant ($p>0.05$) decrement in serum levels of liver biomarkers compared to negative control group (Group II). The protective effect *C.aurea* leaf extract at dose of 600mg/kg become diminished compared to that of 400mg/kg. This seems that at higher dose *C.aurea* leaf extract has negative effect on hepato-protection. It may be that when dose of *C.aurea* increases the concentration of alkaloids and tannins will be increased that leads to decrease the protective effect of the plant but it needs further study. The study done by (108) shows that tannins bound to epithelial proteins causing precipitation penetrated through the superficial cells and then caused liver damage. Alkaloids with pyrrolizidine base or pyrrolizidine alkaloids may cause liver damage due to occlusion of the hepatic sinusoids (109).

The breakdown product of HB is bilirubin, and the level of bilirubin in the blood is used to monitor liver function. Hepatobiliary disorders or liver dysfunction are indicated by an abnormal rise in bilirubin levels. A failure in bile secretion can represent an increase in serum levels in hepatic hepatotoxins insults (91, 92). From the present study the levels of TBIL in group II mice treated with RIF& INH were significantly raised ($p <0.05$) comparable to that of normal control group. This finding was in line with the study done by Meena *et al.* (55) and Dubiwak et al (13). This may because the combination of RIF-INH treatment is linked to bile salt exporter pump suppression and INH by itself cause unconjugated hyperbilirubinemia (110) and hepatocellular dysfunction and loss of cellular integrity of cell membrane (29).

Whereas the serum level of TBIL mice treated with a 400mg/kg dose of *C.aurea* leaf extract were significantly reduced ($p <0.05$) compared to that of rifampicin and isoniazid induced group approximately the same with that of Silymarin treated group. This study was in harmony with the findings of Jehangir (50), Rajan (10). The extract's ability to restore the liver's normal functioning condition was demonstrated by a decrease in serum bilirubin levels after treatment.

The levels of albumin in group II mice were significantly lowered ($P < 0.001$) as a result of RIF-INH intoxication comparable to the normal control group. The present finding supported by the previous studies Meena (55) and Darvin (111). The reason may be that RIF and INH lead to disaggregation of ribosomal profiles, a component in fatty liver disorders that contributed to

hepatocyte injury, was previously thought to be the cause of protein synthesis inhibition (13,106,112).

Furthermore, treatment with a 400mg/kg dose of *C.aurea* leaf extract and Silymarin significantly increased albumin ($P < 0.001$) serum levels to near the normal control group. This finding is consistent with the previous studies done by Nwidu and Teme (67) and Usmani ((47). This indicated that *C.aurea* leaf extract has hepato protective effect.

The histopathological observations support biochemical findings. The administration of INH and RIF produces many metabolic and morphological aberrations in the liver due to the fact that the liver is the main detoxifying site for these antitubercular drugs. The toxic metabolites of INH-RIF can induce oxidative stress in the liver of experimental animals and an evident death of hepatocytes was noted. Along with mild necrosis, inflammation, mild-moderate vacuolar change with vacuolar degeneration and fatty degeneration were observed in the liver section of mice treated with isoniazid and rifampicin. This outcome is consistent with past research Shabbir (41), Guo (109). The reason that could be hydrazine causes liver cell steatosis, vacuolation, glutathione depletion and mitochondrial swelling(12); isoniazid and rifampicin generate AcHz which is quickly converted to its active metabolites linked to the higher incidence of liver necrosis (66) and toxic INH metabolites covalently bind to macromolecules in the cell that leads to hepatocellular steatosis and centrilobular necrosis associated with cholestasis (46,55)

The hepatoprotective effect of the *C.aurea* leaf extract was further assessed by the histopathological examinations which could be only possible by the overall protective character of the extract. On phytochemical screening, methanolic leaf extract of *C.aurea* revealed the presence of flavonoids, steroidal alkaloids, saponins, terpenoids, phenols and glycosides as the major chemical constituents. Hence, it is possible that the mechanism of hepato-protection of *C.aurea* may be due to its antioxidant and higher free radical scavenging activity (107) present in these phytochemicals.

6.1. Limitations

The following were the limitations of the present study: Did not investigate the hepatoprotective effects of Solvent fractions of *C.aurea* leaf extract

CHAPTER SEVEN

7. CONCLUSION AND RECOMMENDATION

7.1 Conclusion

In conclusion, the results of the present study indicate that the co-treatment of *calpurnia aurea* leaf extract has a significant protective action against the hepatotoxicity induced by INH and RIF in mice. Treatment with the *calpurnia aurea* leaf extract restored serum level of liver enzymes level of bilirubin, liver index, total bilirubin, albumin and histopathological changes in the livers of mice near to normal. As the dosage of 400 mg/kg body weight showed maximum hepatoprotective effect in both biochemical and morphological test. This is comparable with the hepatoprotective effect of the Silymarin which is standard hepatoprotective drug. The hepatoprotective role of *calpurnia aurea* leaf extract might be due to its active phytochemical constituents that are responsible for preventing oxidative stress and the antioxidant activity.

7.2 Recommendations

As the present study focused on some aspects of hepatoprotective effects of *Calpurnia aurea*, further detailed studies including the following are recommended

- For researcher
  - Sub chronic and chronic toxicity studies
  - Protective effect of this extract on organs other than the liver
  - Hepatoprotective effects against various models of liver injury
- For environmental policymakers
  - To enact policies to protect, manage and eventually restore natural resources as they have medicinal importance

REFERENCE

1. Ozougwu J. Physiology of the liver. Int J Res Pharm Biosci. 2017;4(8):13–24.
2. Sivakrishnan S, Pharm M. Liver diseases- An overview. World J Pharm Pharm Sci. 2019;8(1):1385–95.
3. Fukaya S, Yoshioka H, Okano T, Nagatsu A, Miura N, Nonogaki T, et al. Non-toxic Level of acetaminophen potentiates Carbon Tetrachloride- induced hepatotoxicity in Mice. Biol Pharm Bull. 2017;40(9):1590–4.
4. Asrani1 SK, Devarbhavi H, Eaton J, Kamath PS. Burden of liver diseases in the world. J Hepatol. 2019;70(1):151–71.
5. Ingawale DK, Mandlik SK, Naik SR. Models of hepatotoxicity and the underlying cellular, biochemical and immunological mechanism. Environ Toxicol Pharmacol. 2013;37(1):118–33.
6. Andrade RJ, Chalasani N, Björnsson ES, Suzuki A, Ublick GAK-, Watkins PB, et al. Drug- induced liver injury. Nat Rev Dis Prim. 2019;5(58):1–22.
7. Andrade RJ, Aithal GP, Bjornsson ES, Kaplowitz N, Kullak-Ublick GA, Karlsen TH. EASL Clinical Practice Guidelines : Drug-induced liver injury. J Hepatol. 2019;70(2):1222–61.
8. Pan Y, Cao M, You D, GenggengQin, Liu Z. Research Progress on the Animal Models of Drug-Induced Liver Injury : Current Status and Further Perspectives. Hindawi BioMed Res Int. 2019;2019(4):1–13.
9. Stravitz RT, Lee WM. Seminar on Acute liver failure. Lancet. 2019;394(10):869–81.
10. Rajan T, Srinivas V, Karunakaran G. Protective effect of methanolic extract of annona squamosa linn in isoniazid-rifampicin induced hepatotoxicity in rats. Pak J Pharm Sci. 2011;24(2):129–34.
11. Ke X, Wang C, Luo W, Wang J, Li B, Lv J. Metabolomic Study to Determine the Mechanism Underlying the Effects of Sagittaria sagittifolia Hepatotoxicity in Mice. molecules. 2018;23(3087):1–11.
12. Kosasih E, Chiuman L, Lister INE, Fachrial E. Hepatoprotective Effect of Citrus Sinensis Peel Extract Against Isoniazid and Rifampicin-induced Liver Injury in Wistar Rats. Tradit Med J. 2019;24(3):197–203.
13. Dubiwak AD, Damtew TW, Senbetu MW, Yewhalaw D, Asere TG, Nemo G, et al.

Hepatoprotective Effect of Corm of Ensete ventricosum (Welw .) Cheesman Extract against Isoniazid and Rifampicin Induced Hepatotoxicity in Swiss Albino Mice. Hindawi J Toxicol. 2021;2021(5):1–8.

14. Dong D, Xu L, Yin L, Qi Y, Peng J. Naringin prevents carbon tetrachloride-induced acute liver injury in mice. J Funct Foods. 2015;12(8):179–91.
15. Rajaratnam M, Prystupa L-A, Załuska K, Wojciech P, Filip R. Herbal medicine for treatment and prevention of liver diseases. J Pre-Clinical Clin Res. 2014;8(20):55–60.
16. Umer S, Kaleab A, Veeresham C. Hepatoprotective activities of two Ethiopian medicinal plants Hepatoprotective activities of two Ethiopian medicinal plants. Pharm Biol. 2010;48(4):461–8.
17. Umer S, Tekewe A, Kebede N. Antidiarrhoeal and antimicrobial activity of Calpurnia aurea leaf extract. BMC Complement Altern Med. 2013;13(21):1–5.
18. Belay D, Kenubih A, Yesuf M, Kebede E, Yayeh M, Birhan M. Antioxidant and Antimicrobial Activity of Solvent Fractions of Calpurnia aurea (Ait .). J Exp Pharmacol. 2021;13(5):499–509.
19. Belayneh YM, Birru EM, Ambikar D. Evaluation of hypoglycemic, antihyperglycemic and antihyperlipidemic activities of 80 % methanolic seed extract of Calpurnia aurea in mice. J ofExperimental Pharmacol. 2019;11(5):73–83.
20. Melese A, Dobo B, Mikru A. Antibacterial activities of Calpurnia aurea and Ocimum lamiifolium extracts against selected gram positive and gram-negative bacteria. Ethiop J Sci Technol. 2019;12(3):203–20.
21. Mengistu W, Daniel S, Gnanasekaran N. Antilipidemic Properties of Calpurnia aurea Leaf Extract on High-Fat Diet Induced Hyperlipidemia. Pharmacognosy Res. 2019;11(4):389–95.
22. Xiao J, Wang F, Wong N-K, He J, Zhang R, Sun2 R, et al. Global liver disease burdens and research trends : Analysis from a Chinese perspective. J Hepatol. 2019;71(1):212–21.
23. Pimpin L, Cortez-Pinto H, Negro F, Corbould E, Lazarus J V, Webber L, et al. Burden of liver disease in Europe : epidemiology and analysis of risk factors to identify prevention policies. J Hepatol. 2018;9(18):1–48.
24. Ren X, Xin L, Zhang M, Zhao Q, Yue S, Chen K. Hepatoprotective effects of a traditional Chinese medicine formula against carbon tetrachloride-induced hepatotoxicity in vivo and

in vitro. Biomed Pharmacother. 2019;117(4):1–8.

25. Singh D, Cho WC, Upadhyay G. Drug-Induced Liver Toxicity and Prevention by Herbal Antioxidants : An Overview. Front Physiol. 2016;6(363):1–18.
26. M. Alshehri M, Wahab Amjad M, M. E. Mudawi M. Drugs-Inducing Hepatotoxicity. Asian J Pharm Res Heal Care. 2020;12(3):148–56.
27. Thomas AM, Lewis JH. Nonacetaminophen Drug-Induced Acute Liver Failure. Clin Liver Dis. 2018;18(1):1–24.
28. Kaplowitz N. Drug-Induced Liver Injury. Clin Infect Dis. 2011;38(2):S44-48.
29. Ramappa V, Aithal GP. Hepatotoxicity Related to Anti-tuberculosis Drugs: Mechanisms and Management. J Clin Exp Hepatol. 2013;3(1):37–49.
30. World Health Organization (WHO). 2021.
31. Singh K. Hepatoprotective Effect of Cissus quadrangularis Stem Extract Against Rifampicin - induced Hepatotoxicity in Rats. Indian J Pharm Sci 183. 2012;5(3):183–7.
32. Zeleke A, Misiker B, Yesuf TA. Drug-induced hepatotoxicity among TB/HIV co-infected patients in a referral hospital, Ethiopia. BMC Res Notes. 2020;13(1):1–5.
33. Abera W, Cheneke W, Abebe G. Incidence of antituberculosis-drug-induced hepatotoxicity and associated risk factors among tuberculosis patients in Dawro Zone, South Ethiopia: A cohort study. Int J Mycobacteriology. 2016;5(1):14–20.
34. Arage LL, Deybasso, Haji Aman Gebremichael DY, Nuramo BG, Mekuria ZN. Determinants of Drug-Induced Hepatotoxicity Among Patients with Human Immunodeficiency Virus Taking a High Dose of Rifapentine Plus Isoniazid. HIV/AIDS - Res Palliat Care. 2021;13(6):307–14.
35. Sofowora A, Ogunbodede E, Onayade A. The role and place of medicinal plants in the strategies for disease prevention. Afr J Tradit Complement Altern Med. 2013;10(5):210–29.
36. Tuasha N, Petros B, Asfaw Z. Medicinal plants used by traditional healers to treat malignancies and other human ailments in Dalle District, Sidama Zone, Ethiopia. J Ethnobiol Ethnomed. 2018;14(1):1–21.
37. Giday M, Teklehaymanot T, Animut A, Mekonnen Y. Medicinal plants of the Shinasha , Agew-awi and Amhara peoples in northwest Ethiopia. J Ethnopharmacol. 2007;110(8):516–25.

38. Kaswala D, Shah S, Patel N, Raisoni S, Swaminathan S. Hydroxycut-induced liver toxicity. Ann Med Health Sci Res. 2014;4(1):143–5.
39. Pandit A, Sachdeva T, Bafna P. Drug-induced hepatotoxicity: A review. J Appl Pharm Sci. 2012;2(5):233–43.
40. Molla Y, Wubetu M, Dessie B. Anti-Tuberculosis Drug Induced Hepatotoxicity and Associated Factors among Tuberculosis Patients at Selected Hospitals, Ethiopia. Hepatic Med Evid Res. 2021;13(10):1–8.
41. Shabbir M, Afsar T, Razak S, Almajwal A, Khan MR. Phytochemical analysis and Evaluation of hepatoprotective effect of Maytenus royleanus leaves extract against anti-tuberculosis drug induced liver injury in mice. Lipids Health Dis. 2020;19(46):1–15.
42. Daniel J. Kleina, Boukouvala S, McDonagh EM, Shuldiner SR, Laurieri N, Thorn CF, et al. PharmGKB Summary: Isoniazid Pathway, Pharmacokinetics (PK). Pharmacogenet Genomics. 2017;26(9):436–44.
43. Wang P, Pradhan K, Zhong X bo, Ma X. Isoniazid metabolism and hepatotoxicity. Acta Pharm Sin B. 2016;6(5):384–92.
44. Sarkar S, Ganguly A. Current Overview of Anti-Tuberculosis Drugs: Metabolism and Toxicities. Mycobact Dis. 2016;6(2):1–6.
45. Du Preez I, Loots DT. Novel insights into the pharmacometabonomics of first-line tuberculosis drugs relating to metabolism, mechanism of action and drug-resistance. Drug Metab Rev. 2018;50(4):466–81.
46. Yogeeta S, Rao H, Ragavender B, Yogeeta S, Rao H, Ragavender B, et al. Antihepatotoxic Effect of Punica granatum Acetone Extract Against Isoniazid- and Rifampicin- Induced Hepatotoxicity. Pharm Biol. 2007;45(8):631–7.
47. Usmani A, Mujahid M, Khushtar M, Siddiqui HH, Rahman MA. Hepatoprotective effect of Anacyclus pyrethrum Linn. against antitubercular drug-induced hepatotoxicity in SD rats. J Complement Integr Med. 2016;13(3):295–300.
48. Sankar M, Rajkumar J, Sridhar D. Hepatoprotective activity of heptoplus on isoniazid and rifampicin induced liver damage in rats. Indian J Pharm Sci. 2015;77(5):556–62.
49. Biswas A, Santra S, Bishnu D, Dhali GK, Chowdhury A, Santra A. Isoniazid and Rifampicin Produce Hepatic Fibrosis through an Oxidative Stress-Dependent Mechanism. Int J Hepatol. 2020;2020(6):1–12.

50. Jehangir A, Nagi AH, Shahzad M, Zia A. the Hepato-Protective Effect of Cassia Fistula (Amaltas) Leaves in Isoniazid and Rifampicin Induced Hepatotoxicity in Rodents. Biomedica. 2010;26(1):25–9.
51. Chen X, Xu J, Zhang C, Yu T, Wang H, Zhao M, et al. The protective effects of ursodeoxycholic acid on isoniazid plus rifampicin induced liver injury in mice. Eur J Pharmacol. 2011;659(1):53–60.
52. Boelsterli UA, Lee KK. Mechanisms of isoniazid-induced idiosyncratic liver injury: Emerging role of mitochondrial stress. J Gastroenterol Hepatol. 2014;29(4):678–87.
53. Santhosh S, Sini TK, Anandan R, Mathew PT. Hepatoprotective activity of chitosan against isoniazid and rifampicin-induced toxicity in experimental rats. Eur J Pharmacol. 2007;572(1):69–73.
54. Kalra BS, Agarwal S. Effect of cimetidine on hepatotoxicity induced by isoniazid-rifampicin combination in rabbits. Indian J Gastroenterol. 2014;1(3):3–7.
55. Meena SZ, Rahman MA, Bagga P, Mujahid M. Hepatoprotective activity of Tamarindus indica Linn stem bark ethanolic extract against hepatic damage induced by co-administration of antitubercular drugs isoniazid and rifampicin in Sprague Dawley rats. J Basic Clin Physiol Pharmacol. 2019;30(1):131–7.
56. Chen Y, Mo Q, Xie B, Ma B, Zang X. Hepatoprotective Activity of Yigan Mingmu Oral Liquid against Isoniazid / Rifampicin-Induced Liver Injuries in Rats. Chin Med. 2018;9(5):165–78.
57. Naji KM, Al-Khatib BY, Al-Haj NS, D'souza MR. Hepatoprotective activity of melittin on isoniazid- and rifampicin-induced liver injuries in male albino rats. BMC Pharmacol Toxicol. 2021;22(1).
58. Chandra AS, Shanmugapandivan P. Hepatoprotective of eclipta alba methanolic extract in isoniazid and rifampicin proved oxidative hepatic injury. Int J Pharm Res Technol. 2020;10(2):74–9.
59. Muluye , AB. Ayicheh M. Medicinal plants utilized for hepatic disorders in Ethiopian traditional medical practices : a review. Clin Phytoscience. 2020;6(52):1–11.
60. Xiong F, Guan YS. Cautiously using natural medicine to treat liver problems. World J Gastroenterol. 2017;23(19):3388–95.
61. Ali SA, Sharief NH, Mohamed YS. Hepatoprotective Activity of Some Medicinal Plants

in Sudan. Evidence-based Complement Altern Med. 2019;2019(4):1–17.

62. Asadi-Samani M, Kafash-Farkhad N, Azimi N, Fasihi A, Alinia-Ahandani E, Rafieian-Kopaei M. Medicinal plants with hepatoprotective activity in Iranian folk medicine. Asian Pac J Trop Biomed. 2015;5(2):146–57.
63. Rakotoarivelo NH, Rakotoarivony F, Ramarosandratana AV, Jeannoda VH, Kuhlman AR, Randrianasolo A, et al. Medicinal plants used to treat the most frequent diseases encountered in Ambalabe rural community, Eastern Madagascar. J Ethnobiol Ethnomed. 2015;11(68):1–16.
64. Madrigal-Santillán E, Madrigal-Bujaidar E, Álvarez-González I, Sumaya-Martínez MT, Gutiérrez-Salinas J, Bautista M, et al. Review of natural products with hepatoprotective effects. World J Gastroenterol. 2014;20(40):14787–804.
65. Mujahid M, Siddiqui HH, Hussain A, Hussain MS. Hepatoprotective effects of Adenanthera pavonina (Linn.) against anti-tubercular drugs-induced hepatotoxicity in rats. Pharmacogn J. 2013;5(6):286–90.
66. Mujahid M, Hussain T, Siddiqui HH, Hussain A. Evaluation of hepatoprotective potential of Erythrina indica leaves against antitubercular drugs induced hepatotoxicity in experimental rats. J Ayurveda Integr Med. 2017;8(1):7–12.
67. Nwidu LL, Teme RE. Hot aqueous leaf extract of Lasianthera africana (Icacinaceae) attenuates rifampicin-isoniazid-induced hepatotoxicity. J Integr Med. 2018;16(4):263–72.
68. Arabi Z, Sardari S. An investigation into the antifungal property of fabaceae using bioinformatics tools. Avicenna J Med Biotechnol. 2010;2(2):93–100.
69. Rahman AHMM, Parvin MIA. Study of Medicinal Uses on Fabaceae Family at Rajshahi , Bangladesh. Res Plant Sci. 2014;2(1):6–8.
70. Trott AR, Welch TC, Hundy GF, Trott AR, Welch TC, Qadri B. Chapter 4: Common bean (Phaseolus vulgaris). In: Refrigeration and Air Conditioning. 2016. p. 59–87.
71. Gebreslassie HB, Eyasu A. Phytochemical Screening of the Leaves Calpurnia Aurea(Ait.) Benth Extract. Int J Clin Chem Lab Med. 2019;5(4):18–24.
72. Korir E, Kiplimo JJ, Crouch NR, Moodley N, Africa S, Road B, et al. Isoflavones from calpurnia aurea subsp. Aurea and their anticancer activity. Afr J Tradit Complement Altern Med. 2014;11(5):33–7.
73. Mulata HN, Gnanasekaran N, Melaku U, Daniel S. Phytochemical Screening and

Assessment of In Vitro Antioxidant Activities of Calpurnia Aurea Seeds and Leaves. Int J Pharm Pharm Res. 2015;2(2):1–12.

74. DE D, A Z. Phytochemical Screening of Calpurnia Aurea Root Extract. Kenkyu J Pharm Pract Heal Care. 2018;4(6):61–8.
75. Daksa D. Evaluation of Free Radical Scavenging Activity of Calpurnia aurea Root Extract by Using Methanol Solvent for DPPH Selectively. Int J Sci Eng Res. 2019;10(2):177–87.
76. Festing MFW, Altman DG. Guidelines for the Design and Statistical Analysis of Experiments Using Laboratory Animals. ILAR J. 2002;43(4):244–58.
77. Charan J, Biswas T. How to calculate sample size for different study designs in medical research? Indian J Psychol Med. 2013;35(2):121–6.
78. Festing MF. On determining sample size in experiments involving laboratory animals. Lab Anim Ltd. 2018;52(4):341–50.
79. MN I, MKR A, NB S, AB A. Sample Size Calculation for Animal Studies Using Degree of Freedom (E); an Easy and Statistically Defined Approach for Metabolomics and Genetic Research. Curr Trends Biomed Eng Biosci. 2017;10(2):47–8.
80. Semakalu SC, Mtunzi F, Pillay M, Terblanche U. Screening of Variables Influencing Extraction Yield of Cotyledon orbiculata. Int J Pharmacogn Phytochem Res. 2017;9(3):303-312.
81. Shaikh JR, Patil M. Qualitative tests for preliminary phytochemical screening: An overview. Int J Chem Stud. 2020;8(2):603–8.
82. Iqbal E, Salim KA, Lim LBL. Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of Goniothalamus velutinus (Airy Shaw) from Brunei Darussalam. J King Saud Univ - Sci. 2015;27(3):224–32.
83. Fardiyah Q, Suprapto, Kurniawan F, Ersam T, Slamet A, Suyanta. Preliminary Phytochemical Screening and Fluorescence Characterization of Several Medicinal Plants Extract from East Java Indonesia. IOP Conf Ser Mater Sci Eng. 2020;833(1).
84. Abate L, Mengistu T. Phytochemical screening and peroxide value determination of methanolic extract of four traditional medicinal plants from Debre Tabor Town , Ethiopia. J Med Plants Res. 2018;12(16):203–8.
85. J. C. Garber, R. W. Barbee, L. A. Clayton, J. C. Donovan, C. F. M. Hendriksen and DF, Kohn. Guide for the Care and Use of Laboratory Animal. 8th ed. Washington DC: The

national academies press; 2011. 1–218 p.

86. OECD/OCDE. OECD guideline for testing of chemicals: Acute Oral Toxicity – Up-and-Down Procedure (UDP). In: OECD. 2001. p. 1–26.
87. Bruce RD. An up-and-down procedure for acute toxicity testing. Toxicol Sci. 1985;5(1):151–7.
88. Dong Y, Huang J, Lin X, Zhang S, Jiao Y, Liang T, et al. Hepatoprotective effects of Yulangsan polysaccharide against isoniazid and rifampicin-induced liver injury in mice. J Ethnopharmacol. 2014;152(1):201–6.
89. Wang J, Luo W, Li B, Lv J, Ke X, Ge D, et al. Sagittaria sagittifolia polysaccharide protects against isoniazid- and rifampicin-induced hepatic injury via activation of nuclear factor E2-related factor 2 signaling in mice. J Ethnopharmacol. 2018;227:237–45.
90. Parameswari SA, Chetty CM, Chandrasekhar KB. Hepatoprotective activity of ficus religiosa leaves against isoniazid+rifampicin and paracetamol induced hepatotoxicity. Pharmacognosy Res. 2013;5(4):271–6.
91. Yaschilal, Muche and Eshetie M. Antidiabetic Activities of Hydromethanolic Leaf Extract of Calpurnia aurea (Ait .) Benth . Subspecies aurea (Fabaceae) in Mice. Hindawi, Evidence-Based Complement Altern Med. 2018;2018(9):1–9.
92. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother. 2010;1(2):87–93.
93. Huang XJ, Choi YK, Im HS, Yarimaga O, Yoon E, Kim HS. Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) detection techniques. Sensors. 2006;6(7):756–82.
94. Esani MA. The physiological sources of, clinical significance of, and laboratory-testing methods for determining enzyme levels. Lab Med. 2014;45(1):e16–8.
95. Xing-Jiu Huang , Yang-Kyu Choi , Hyung-Soon Im , Oktay Yarimaga EY and H-SK. Asparate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) detection techniques. Sensors. 2006;6(7):756–82.
96. Sharma U, Pal D, Prasad R. Alkaline phosphatase: An overview. Indian J Clin Biochem. 2014;29(3):269–78.
97. Tanan MK, Mangarengi F, Mutmainnah M. Analysis of serum albumin levels in pre and post hemodialysis among Chronic Renal Failure (CRF) patients at Dr. Wahidin

Sudirohusodo Hospital, Makassar, Indonesia. Intisari Sains Medis. 2020;11(2):466–9.

98. Carvalho JR, Machado MV. New insights about albumin and liver disease. Ann Hepatol. 2018;17(4):547–60.
99. Valášková P, Muchová L. Metabolism of bilirubin and its biological properties. Klin Biochem a Metab. 2016;24(4):198–202.
100. Narwal V, Batra B, Kalra V, Jalandra R, Ahlawat J, Hooda R, et al. Bilirubin detection by different methods with special emphasis on biosensing: A review. Sens Bio-Sensing Res. 2021;33(3):100436.
101. Mulatu G. Antibacterial Activities of Calpurnia aurea against Selected Animal Pathogenic Bacterial Strains. Adv Pharmacol Pharm Sci. 2020;2020(2):1–9.
102. Eyasu M, Shibeshi W, Giday M. In vivo antimalarial activity of hydromethanolic leaf extract of Calpurnia aurea (Fabaceae) in Mice infected with chloroquine sensitive Plasmodium berghei. Int J Pharm Pharmacol. 2013;2(9):131–42.
103. Hsu YJ, Wang CY, Lee MC, Huang CC. Hepatoprotection by traditional essence of ginseng against carbon tetrachloride—induced liver damage. Nutrients. 2020;12(10):1–12.
104. Hamad Shareef S, Abdel Aziz Ibrahim I, Alzahrani AR, Al-Medhtiy MH, Ameen Abdulla M. Hepatoprotective effects of methanolic extract of green tea against Thioacetamide-Induced liver injury in Sprague Dawley rats. Saudi J Biol Sci. 2022;29(1):564–73.
105. Attri S, Rana S V., Vaiphei K, Sodhi CP, Katyal R, Goel RC, et al. Isoniazid- and rifampicin-induced oxidative hepatic injury - Protection by N-acetylcysteine. Hum Exp Toxicol. 2000;19(9):517–22.
106. Abd elhameed mohamed, Salama A, Attia T, Elbatran S, Ismaeil I, Hassan A. Protective Effects of Moringa oleifera extract on Isoniazid and Rifampicin Induced Hepatotoxicity in Rats: Involvment of Adiponectin and Tumor Necrosis Factor-α. Egypt J Vet Sci. 2018;49(1):25–34.
107. Adedapo AA, Jimoh FO, Koduru S, Afolayan AJ, Masika PJ. Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of Calpurnia aurea. BMC Complement Altern Med. 2008;8:1–8.
108. Chung KT, Wong TY, Wei CI, Huang YW, Lin Y. Tannins and human health: A review. Crit Rev Food Sci Nutr. 1998;38(6):421–64.
109. Ebmeyer J, Rasinger JD, Hengstler JG, Schaudien D, Creutzenberg O, Lampen A, et al.

Hepatotoxic pyrrolizidine alkaloids induce DNA damage response in rat liver in a 28-day feeding study. Arch Toxicol. 2020;94(5):1739–51.

110. Guo YX, Xu XF, Zhang QZ, Li C, Deng Y, Jiang P, et al. The inhibition of hepatic bile acids transporters Ntcp and Bsep is involved in the pathogenesis of isoniazid/rifampicin-induced hepatotoxicity. Toxicol Mech Methods. 2015;25(5):382–7.
111. Darvin SS, Esakkimuthu S, Toppo E, Balakrishna K, Paulraj MG, Pandikumar P, et al. Hepatoprotective effect of lawsone on rifampicin-isoniazid induced hepatotoxicity in in vitro and in vivo models. Environ Toxicol Pharmacol. 2018;61(2):87–94.
112. Guerra Ruiz AR, Crespo J, López Martínez RM, Iruzubieta P, Casals Mercadal G, Lalana Garcés M, et al. Measurement and clinical usefulness of bilirubin in liver disease. Adv Lab Med / Av en Med Lab. 2021;2(3):352–61.

ANNEXS

Annex 1: Body weight follow up checklist

Table 7: Checklist for recording body weight of mice per week

Group	No of mice	First week	Second week	Third week	Remark
Group I	1				
	2				
	3				
	4				
	5				
Group II	1				
	2				
	3				
	4				
	5				
Group III	1				
	2				
	3				
	4				
	5				
Group IV	1				
	2				
	3				
	4				
	5				
Group V	1				
	2				
	3				
	4				
	5				
Group VI	1				
	2				
	4				
	5				

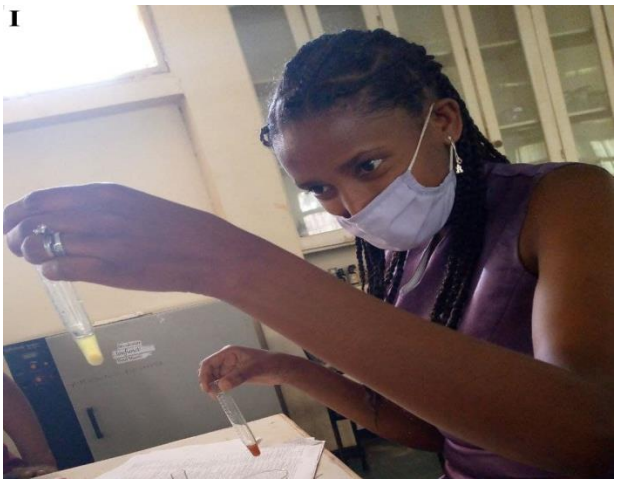
Annex 2: treatment follow up

Table: Checklists for treatment follow up

Group	Treatment component	No of mice	Day													Remark
Group I	Normal saline															
Group II	INH(100mg/kg) & RFN(100mg/kg)															
Group III	Silymarin (100mg/kg)															
Group IV	INH(100mg/kg) &RFN(100mg/kg) after 1hr. 200m/kg *C.aurea* extract															
Group V	INH(100mg/kg) &RFN(100mg/kg) after 1hr. 400m/kg *C.aurea* extract															
Group VI	INH(100mg/kg) &RFN(100mg/kg) after 1hr. 600m/kg *C.aurea* extract															

Annex 3: Photograph taken during undertaking different activities

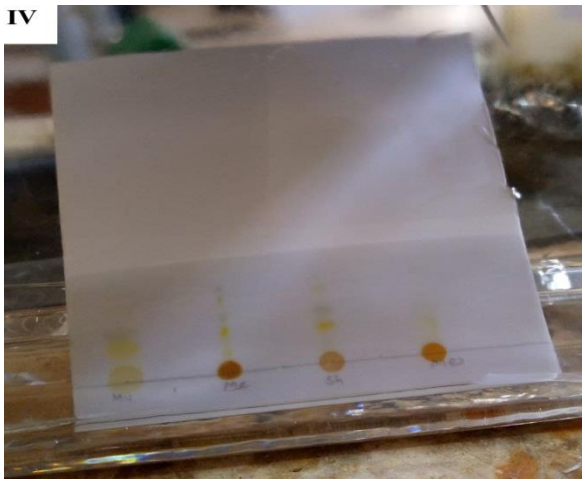
I



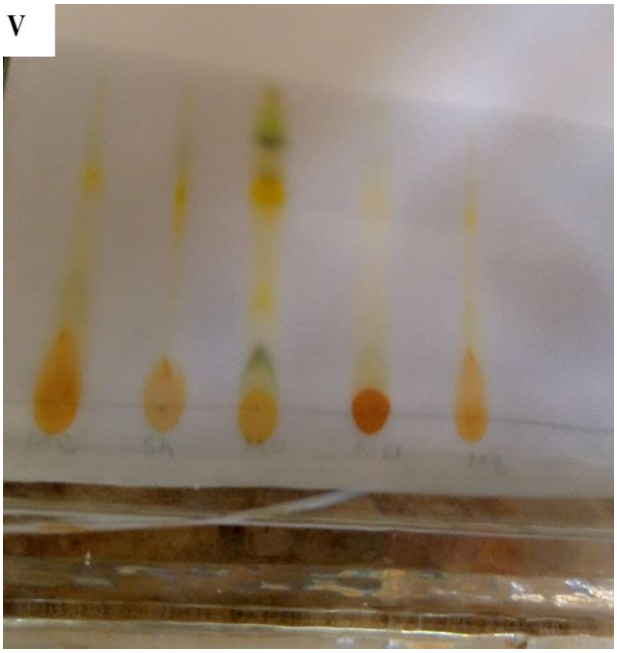
II

III

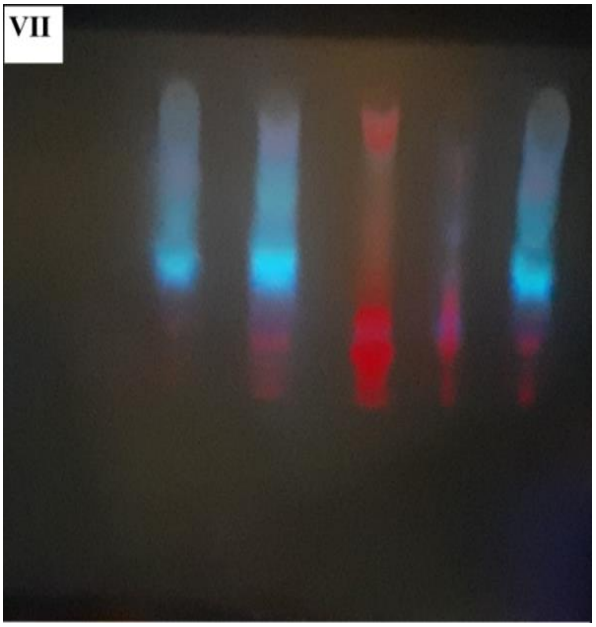
IV

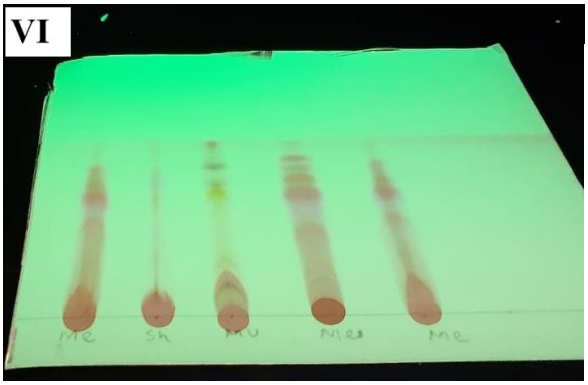


V



VII





- I- II phytochemical screening using standard procedure
- III- phytochemical screening using TLC
- IV-V movement of components with in solvent system
- VI- VII Observation of TLC under different wave length


prepared solutions for admonstration

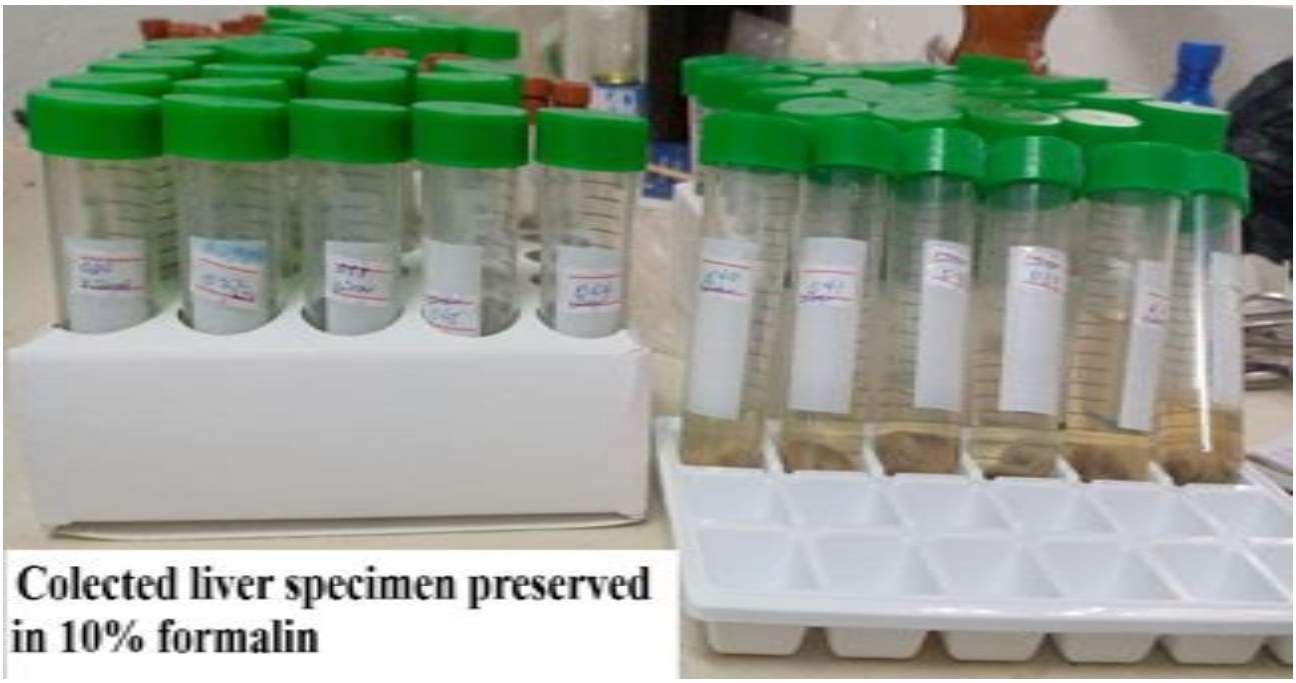
Adminstration of extract for mice per orally

Observational cheaking of mice in different group

Blood coolection via cardiac pancture

Liver collection after sacrifying of mice

serum separation using Eppendorf centrifuge



Colected liver specimen preserved in 10% formalin