THE HISTOMORPHOLOGICAL AND BIOCHEMICAL HEPATORESTORATIVE EFFECTS OF SILYMARIN MILK THISTLE ON PARACETAMOL INDUCED HEPATOTOXICITY IN ADULT ALBINO RATS

(*Rattus norvegicus*)

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University. I wish to declare that works from other authors used in the development of this research thesis has been duly acknowledged in the reference section.

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DEDICATION

I wish to dedicate this thesis to my family members; Samwel Bor, Jackline Bor and Emmauel Ngetich

ABSTRACT

Liver toxicity has been on a steady rise worldwide and it is attributed to various causes including paracetamol-induced when taken singly in high dose or prolonged use. Paracetamol is a common, most preferred and widely tolerated first line analgesia for mild and moderate acute pain for all age groups. Since its cheap and easily accessible as an over-the-counter drug, it can be easily misused especially with increasing causes of pain in the society. Its long-term use causes liver toxicity and eventually liver failure. Most plant medicinal extracts have been found to either prevent or ameliorate the hepatotoxic effects either alcohol or drug induced. Silymarin milk thistle is a medicinal herb that grows widely in various climatic conditions, available locally and affordable to all. It has been used over time to prevent or treat liver diseases though there is inadequate data on its histo-morphologic restorative effects that occur on the liver following drug induced hepatotoxicity. This study aimed at evaluating the restorative histo-morphological changes of silymarin milk thistle on paracetamol induced hepatotoxicity. Specifically, the study determined; the histo-morphological injurious effects that occur on the liver following toxicity, the restorative histo-morphological effects of various doses of silymarin milk thistle on paracetamol hepatotoxicity and assessed the changes in liver biochemical parameters of Aspartate transaminase (AST), Alkaline phosphatase (ALP) and Alanine transaminase (ALT) following administration of silymarin milk thistle and paracetamol toxicity. A post-test only true experimental study design was used with a total of 24 adult albino rats randomly sampled into intervention and non-intervention groups. The non-intervention further into control of 3 rats received no drug interventions and 3 rats that received high dose of paracetamol (750 mg/kbwt) for 5 days. A total of 3 intervention groups each having 6 rats received same dose of paracetamol (750 mg/kbwt) for 5 days to induce hepatotoxicity and varying doses of silymarin (low dose- 200mg/kbwt, medium dose- 400 mg/kbwt and high dose- 600 mg/kbwt) to each group respectively for the remaining 16 days of the experimental process. Humane sacrificial was done on day 21 and liver tissues harvested and processed for both gross and histological examinations and stained using Hematoxylin & Eosin (H&E). Morphological data were entered into excel sheet, analyzed through Statistical Package for the Social Sciences (SPSS) version 26, and One-way Analysis of Variance (ANOVA) was used to test the mean groups and a post hoc was used to test the difference between the mean groups. The results showed that there was a significant reduction (P=0.0001) in length, width and volume in the control group as compared with the paracetamol only group. It also had areas of hemorrhagic necrosis, vacuolated hepatocytes and dilated sinusoids. Unlike the low dose and medium dose silymarin groups, there was a significant increase in all the parameters (volume, weight, length and width) in the high dose silymarin when compared with the control. In comparison with the control group the medium dose and low dose groups registered a significant difference in all the parameters while the high dose group showed no significant difference to signify some level of restoration. Histologically, the paracetamol only group liver had areas of hemorrhagic necrosis, pocket foci of hemorrhage and dilated sinusoids and compared with those of the low dose and medium dose silymarin groups. The high dose group had even distribution of normal hepatocytes and sinusoids and was similar with the control liver. The liver biochemical parameters were significantly (P=0.0001) elevated in the paracetamol only group**,** low dose and medium dose silymarin groups when compared with the control whose parameters presented no significant difference with those of the high dose silymarin group. In conclusion, high dose silymarin had hepato-restorative effects, therefore more studies should be done on the safe human dose and its pharmacokinetics so that it can be taken alongside those drugs causing hepatotoxicity and to treat liver related conditions, ultimately reducing liver related mortalities.

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LIST OF ABBREVIATION

COX : Cyclo-oxygenase

ATP : Adenosine Triphosphate

NSAIDs : Non-Steroidal Anti-Inflammatory Drugs

WHO : World Health Organization.

SM/ SMT /SIL: Silymarin Milk Thistle.

AST : Aspartate transaminase

ALT : Alanine transaminase

ALP : Alkaline Phosphatase

RNA : Ribose Nucleic Acid

NAPQI : N-acetyl-para-benzoquinoneimine

SPSS : Statistical Package for the Social Sciences

SEM :Standard Error of Mean

Mg/kbwt : Miligrams per kilo body weight

H&E : Hematoxylin & Eosin

ANOVA : Analysis of variance

IL : Interleukins

CYP : Cytochrome

RNA : Ribose Nucleic Acid

UEAB : University of Eastern Africa, Baraton

NACOSTI : National Commission for Science, Technology and Innovation

DEFINITION OF TERMS

Dosage : Quantity and frequency of paracetamol and silymarin milk thistle used.

Antioxidant : A substance that can down grade oxidation.

Histo-morphological : Utilization of knowledge of microscopy to learn about the structure and shape of the liver and its cellular structure

Morphometric : Study of the liver in terms of their variations in weight, volume, length and width.

Restorative : To bring back the liver to its original self after damage.

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

There has been a steady increase in liver toxicities worldwide from various causes such as alcohol and drugs. This has led to increased morbidity and mortality in the general population with approximately 30% from drug induced hepatotoxicty (Reddy 2017). These drugs are either prescribed or found over the counter.

With increasing effects of hepatotoxic effects from either alcohol or drug induced, liver damage presents with histological and functional changes. These include changes in the hepatic architecture disorganization that present with vacualation of hepatocytes, congestion of the central vein, congestions of the portal area, dilatations of the sinusoids or even obliteration of the sinusoids and increasing number of kuppfer cells that line the blood sinusoids Nabil, (2015). These changes due to the injuries to the hepatocytes and bile duct cells causes accumulation of bile acid within the liver causing liver damage (Seif, 2016).

Milk thistle (*Silybum marianum)* is a plant used locally for medicinal purposes and contains an active chemical called silymarin, which is a mixture of flavonolignans like silybin, isosilybin, silydianin and silychristin. This edible plant can be eaten raw or cooked, and it has leaf-spines with all parts of the plant being edible or roasted to substitute coffee as a beverage. It grows in a wide variety of climatic conditions and it is widely available. Its powdered seeds and leaves are used as an herb locally in the management of liver conditions, oral thrush and flu, stomach upsets and heartburns. This extract has been used as an antioxidant and is believed to contain hepatoprotective effects (Soleimani *et al.,* 2019). They have anti-inflammatory, anti-oxidative, anti-lipid, membrane

stabilizers and liver cell regenerating mechanisms and properties that are believed to have protective effects against drug-induced hepatotoxicity (Pradhan & Girish, 2013).

The anti-oxidant properties that have been found in silymarin milk thistle reduces free radicals that are produced secondary to the metabolism of toxic substances such as paracetamol and alcohol. This would therefore improve the integrity of the mitochondria and maintain redox balance, thus maintaining liver function (Vargas-Mendoza *et al.,* 2014; Surai, 2015). Milk thistle has been found to normalize the levels of liver biomarkers of bilirubin, aspartate transaminase (AST) as well as alanine transaminase (ALT), with a significant improvement in liver histology for patients with chronic alcohol disease and those with chronic active hepatitis (Rainone, 2015).

Silymarin milk thistle has been used over time and is believed to have medicinal components. It has been found to anticancer activities where it has antiproliferation action against the cancer cell, hepatoprotective effects by having anti-inflammatory and immunomodulatory effects in the liver and the bronchial epithelial cells of the lung thus reducing congestion from cigarette smoking(Valková *et al.,* 2020).

Paracetamol is the most commonly used analgesic and antipyretic around the world, readily available for use among the population. It is regarded as the drug of choice for people who have sensitivities to non-steroidal anti-inflammatory drugs, the underage, and pregnant and lactating women (Jozwiak-Bebenista & Nowak, 2014). It has been found that paracetamol is the most common drug that causes liver toxicity since it's metabolized in the liver and its overdose may cause injury to the liver. Paracetamol has been readily found across the world with or without prescription, thus steadily increasing the number of paracetamols-induced liver intoxications (Jozwiak-Bebenista & Nowak, 2014).

Prolonged use of paracetamol or at high doses causes the oxidative metabolite N-aacetyl-para-benzoquinoneimine (NAPQI) to cause the liver cells to be under oxidative stress. This will therefore cause the hepatocellular mitochondria to burst, leading to free oxygen radicals and nitrogen ions that cause necrosis of hepatocellular cells, leading to liver damage. Paracetamol is metabolized in the liver, with its metabolites being sulphate and glucuronide, with a small amount of the drug being converted into a very reactive alkalytic metabolite that is inactivated with a reduction in glutathione levels (Majee *et al.,* 2013).

Paracetamol has been for a long time considered non-toxic when given in therapeutic doses, though it might result in hepatotoxicity when taken singly, repeated in high doses or after it has been chronically ingested (Tittarelli *et al.,* 2017). Toxicity may also arise when there is poor dietary intake and nutritional status of an individual or in alcohol intake even when administered at a therapeutic dose (Offor *et al.,* 2022).

With the overuse of paracetamol and the known side effects it causes in the liver, there is a need for research to understand the hepatorestorative dosage of silymarin, which has proven to have hepatoprotective properties, so as to counteract the side effects of paracetamol. This will go a long way toward reducing the rates of hepatotoxicity and liver failure related deaths.

1.2 Problem statement

There are several incidences of liver toxicity and accounts to almost 50% of all cases of acute liver failure (Offor *et al.,* 2022). This has led to high costs of treatment and hospitalization worldwide, with almost 2 million deaths annually (Asrani *et al.,* 2019). With growing sedentary lifestyle contributing to obesity and diabetes, this contributes to non-alcoholic fatty liver and hepatocellular carcinoma. There is an increasing global burden of chronic liver disease arising from drug induced,

increased alcohol misuse and even metabolic syndromes. It is estimated that approximately 1.5 billion people worldwide have chronic liver disease (Moon *et al.,* 2020). Mortality rates have also increased due to liver related problems with sub-Sahara Africa leading in the mortalities (32% mortality rate) with high clinical burden in terms of cost of treatment and management of liver conditions also witnessed (Sepanlou 2020).

Most commonly used drugs, including analgesics, anticancer, antibiotics, antituberculous, antiretrovirals, and anesthetics either regulated or non-regulated, have been the major cause of drug-induced liver toxicity (David & Hamilton, 2010). Paracetamol is the most commonly used analgesic and is widely accepted by most communities to manage different forms of pain (Freo *et al.,* 2021). It is easily accessible as an over-the-counter drug and cheap; therefore, it is easily misused. With increasing causes of pain within the society, paracetamol becomes the drug of choice by many and its long-term use causes liver toxicity and eventually acute liver failure diagnosis (Przybyła *et al.,* 2021).

Several traditional methods and herbal formulations have been used over time to manage liver conditions, though these herbal formulations have a paucity of data on pharmacokinetics and pharmacodynamics. One example is the silymarin milk thistle that is commonly used in the treatment of liver conditions, oral thrush and flu, stomach upsets and heartburn and has been proven to have beneficial therapeutic effects. However, there is a paucity of information on the histomorphological and biochemical restorative effects of silymarin milk thistle and the accurate dosage required to produce histological and morphological effects on the liver cells following paracetamol-induced hepatotoxicity.

1.3 Justification and significance of the study

With the increasing level of liver failures arising from toxicities from various agents such as drugs or alcohol (David & Hamilton 2010), a restorative remedy should be sought to avoid the progression of the disease and to help in improvement of quality of life and health in the population. Scientific data on dosage and administration is of great importance in the application of various types of medicinal herbs to counter liver toxicities. Silymarin milk thistle is a medicinal herb that grows in most of the climatic conditions worldwide and has been used to manage liver conditions.

The choice of this plant was due to the fact that its formulation is readily available, affordable and accessible to the general population. These therefore enable it to be utilized for medicinal purposes and for the management of different conditions, including liver failures, arising from different causes, such as drug-induced or alcohol-induced. The effects of silymarin on the hepatocytes of alcohol-induced liver cirrhosis were found to have a significant reduction in tumor cell proliferation, angiogenesis and resistance to insulin, liver fibrogenesis and promotion of hepatocyte regeneration (Feher & Lengyel 2012). However, there is paucity of data on the amount of Silymarin required to provide the restorative histo-morphological changes associated with paracetamol toxicity, which this study sought to find out. Breaching this gap will help in countering liver complications at an early stage, as well as preventing progression to hepatotoxicity and eventually mortality.

Findings from this study will be helpful to the healthcare workers in administration of the silymarin milk thistle in liver toxicities to counter its effects. This will also help in the close monitoring of the restoration of the liver by using the biochemical parameters of ALT, AST and ALP while administering silymarin milk thistle in liver problems.

1.4 Objectives of the Study

1.4.1 Broad objective

To evaluate the hepato-restorative effects of silymarin milk thistle in paracetamol induced hepatotoxicity in adult albino rats.

1.4.2 Specific objectives

1. To determine histo-morphological injurious effects of paracetamol on the liver cells of adult albino rats.
2. To determine the restorative histo-morphological effects of silymarin milk thistle on paracetamol induced liver toxicity among albino rats when administered in varied doses.
3. To evaluate the biochemical restorative changes of silymarin milk thistle on liver biochemical parameters (AST, ALT and ALP) following administration of silymarin milk thistle and paracetamol among adult albino rats.

1.5 Research Questions

a) What are the histo-morphological changes that occur on the liver following paracetamol induced liver toxicity?
b) What are the histo-morphological restorative effects of silymarin milk thistle on paracetamol induced hepatotoxicity?
c) What are the changes in liver biochemical markers (AST, ALT and ALP) with administration of silymarin milk thistle?

1.6 Study model assumption

The albino rats have been found to replicate the actual histo-architectural effects on liver as it can occur in human beings due to their close known similarities biologically, structurally and

functionally between these albino rats and human beings hence these animals were used in this study.

1.7 Possible Limitations and Delimitations

The study limitations that were likely to be anticipated included sickness or death of animals that could occur during the experimental process while administering paracetamol and silymarin milk thistle using the gastric lavage needle and the variant doses which could not be tolerated by animals.

In order to overcome the limitations, the animals that would not have gained the desired weight by the time of the experimental process were to be isolated into different cages and be fed so that they can attain the weight and then the experiment be conducted separately while those that became sick or died during the experiment were replaced from the initial population.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

This chapter focuses on various pieces of literature from other authors that have been written concerning the anatomy of the liver, effects of silymarin on the liver and biochemical markers to illustrate the similarities in their findings, the differences they found and even the gaps identified.

2.3 The comparative microstructure of the liver of the albino rat and human

2.3.1 Microscopic and structural pattern of the Human Liver

The human liver is the largest organ in the body proportionate to 2%- 3% of the human body weight. It has two lobes; the right and left lobes lying on the right upper quadrant of the abdominal cavity. It is held in position by various ligamentous attachments including the falciform ligament which divides the liver into the two lobes, the ligamentum teres which is a remnant of the obliterated ductus venosus, the ligamentum venosum lying between the caudate lobe and left lobe (Abdel-Misih & Bloomston, 2010).

The superior aspect of the liver is convex along the inferior surface of the diaphragm without any ligamentous attachment to form the bare area of the liver and posterior to it is the coronary ligament The innervation of the liver is via sympathetic and parasympathetic neural innervation with its lymphatic drainage through the deep and superficial lymphatic network (Vernon *et al.,* 2018).

Histologically, the human liver has large hepatocytes with majority being binucleated that are hexagonal in shape, less and scanty connective tissue septa with a central vein close to the hepatic lobule and a thick capsule (Al-Hamdany, 2019), (Madhan & Raju, 2014).

2.3.2 Microscopic and structural arrangement of the Rat Liver

The liver of a rat has four lobes; left, middle, right and caudate lobes with a single lobe made up of the left and middle lobe with the middle lobe having a deep notch for the attachment of the round ligament with the entire liver covering the entire sub diaphragmatic space. It represents approximately 5% of the total body weight and weighs 250g -350g. The liver provides both the exocrine and endocrine functions in the body with its chief functions being production of bile for metabolism, blood glucose level regulation by storing glycogen and regulating the blood stability by secretion of factors involved in blood clotting and proteins in the blood (Stan, 2018).

2.3.3 Histo-morphological comparative characteristics of the human liver and rats when induced with paracetamol hepatotoxicity

In comparative studies on the liver cells of both rats and humans, paracetamol overdose has been found to cause necrosis of hepatocytes and apoptosis secondary to activation of the Kupffer cells and increase in production of cytokine leading to general loss of liver mass and significant reduction in liver parenchyma (Islam *et al.,* 2021). In human liver, paracetamol toxicity leads to centrilobular hepatic necrosis, necrotic hepatocytes and significant loss in mitochondrial membrane and general change in hepatic structure (Hinson *et al.,* 2010).

2.2 Silymarin Milk thistle components and postulated protective mechanism on liver cells

Milk thistle is from the *Asteraceae* family of plants and has medicinal effects. The major components of the milk thistle extracts are silymarin and other flavonolignans mixtures such as silybin, silydianin, and silychristin, which are found in the whole plant but have a higher concentration of silymarin in the fruits and seeds. This medicinal plant has been found to have hepatoprotective, anti-cancer, anti-alzheimer, anti-Parkinson and anti-diabetic effects in the body. It has been considered to be tolerated in the human system at therapeutic doses or high doses of

2100 mg daily with slight gastrointestinal discomfort (Soleimani *et al.,* 2019; Gillessen & Schmidt 2020).

Silymarin, an edible herb, has been found to have hepato-protective effects on the liver because it has antioxidant, scavenging, and regulation of glutathione contents within the cell, thus causing cell membrane stabilization and regulation of permeability into the cell, thereby preventing hepatotoxic agents from getting into the hepatocytes (Gillessen & Schmidt 2020). These anti-oxidant properties of silymarin have been found to greatly reduce free radicals that are produced secondary to the metabolism of toxic substances such as paracetamol and alcohol, thereby improving the integrity of the mitochondria and maintaining redox balance, thus maintaining liver function. Silymarin also increases hepatic glutathione which will promote the antioxidant defense of the liver (Vargas-Mendoza *et al.,* 2014; Surai 2015).

Silymarin, an edible herb, has been found to have effective properties on the liver, such as being an antioxidant, radical scavenging and regulating glutathione contents within the cell, thus causing cell membrane stabilization and regulation of permeability into the cell, as well as anti-fibrotic, anti-inflammatory and regeneration of liver mechanisms with it being absorbed orally and excreted as conjugates and sulphates through bile (Aghemo *et al.,* 2022).

Silymarin and silybin, which are the main components of the milk thistle plant, have been used for their hepatoprotective properties, with emerging issues in the treatment and management of other conditions such as liver issues, kidney and gastrointestinal problems, as well as skin problems and cancers, since they lead to the production of interleukins IL-1 and IL-6, Tumor Necrosis Factor, granulocyte macrophages and interferon gamma (Khazaei *et al.,* 2022).

2.2 Paracetamol structure and action mechanism in induction of hepatotoxicity

2.2.1 Paracetamol structure

Paracetamol is an analgesic and antipyretic that works by inhibiting the synthesis of prostaglandins and its effects are central secondary to the serotonergic pathways being activated (Graham & Scott, 2005). Its structure is made of acetanilide, paracetamol and aniline, with a molecular formula of C8H9NO2.

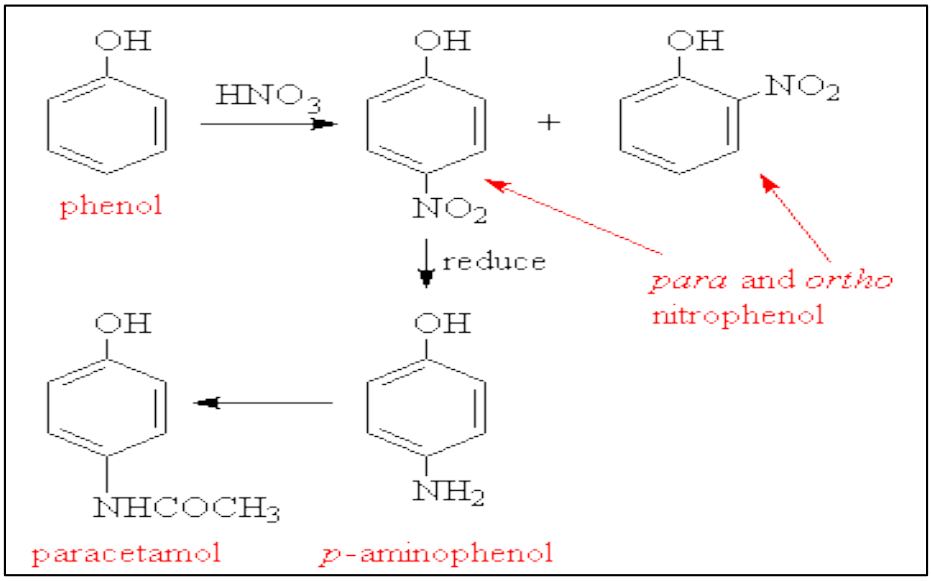


Figure 2.2:Molecular structure of paracetamol drug. (Adapted from www.ch.ic.ac.uk)

2.2.2 Mode of action of paracetamol in inducing hepatotoxicity

Paracetamol is one of the ancient analgesics that has been used over the years in the management of various types of pains and fevers as it has no anti-inflammatory activity and has better gastro-intestinal tolerance. It is a selective cyclooxygenase (COX) inhibitor, though it is less strong than NSAIDs or COX-2 selective inhibiting drugs (Freo *et al.*, 2021).

It has been found to have a lesser strength in analgesic activity than NSAIDS, though its action mode is uncertain, but it inhibits both COX-1 and COX-2 via the metabolism of isoenzymes, leading to a reduction in the formation of phenoxyl radicals from critical tyrosine residues from

the activity of cyclo-oxygenases 1 and 2 and the synthesis of prostaglandin. Its effect is selective in inhibiting synthesis of prostaglandins, depending on the arachidonic acids and peroxides available. Its general mode of action has both central and peripheral effects (Davies *et al.,* 2013).

Paracetamol is rapidly absorbed in the small intestines and it is greatly affected by the rate of gastric emptying with the plasma half-life of 1.5-2 hours and 55% of it being excreted via urine as glucuronide and 30% as sulphate conjugates (Arana *et al.,* 2001). Its metabolism is also dependent on age and dose administered and its clearance depends on urine flow rate (McCrae *et al.,* 2018).

2.2.3 Pattern of hepatotoxicity pathways associated with paracetamol

Paracetamol overdose causes damage to the liver by forming a highly reactive metabolite that is inactivated by glutathione. Any overdose leads to glutathione being depleted, causing the accumulation of metabolites that are toxic, covalently binding with liver cells to cause necrosis (Alchin *et al.,* 2022). According to Athersuch *et al.,* 2018, liver toxicity arises when the metabolite N-acetyl-p-benzoquinonemine (NAPQI/NABQI) is produced thus depleting glutathione and causes reaction with cellular macromolecules to cause liver cell death.

An overdose of the drug causes hepatic centrilobular necrosis through a complex cascade of events in the liver where the Cytochrome P450 (CYP) metabolism into a reactive metabolite causes depletion of glutathione to covalently bind with proteins. This depletion of glutathione causes an increase in production of reactive oxygen and nitrogen species in the liver cells thus undergoing necrosis and causes a surge in oxidative stress due to alteration of calcium homeostasis thereby initiating signal transduction and mitochondrial permeability transition causing the mitochondria to lose its ability to synthesize ATP leading to hepato-necrosis (Massart *et al.,* 2021).

With paracetamol toxicity, there is hepatic congestion, dilatations of the sinusoid and the central vein, necrosis of the hepatocytes and their cells undergoing signs of pyknosis and karyorrhexis. There is also vacuolation of the hepatocytes and lysis coupled up with leakage. This is due to the depletion of glutathione in the cells. The liver weight also decreases within the first 24 hours due to hepatocyte lysis. There is also an increase in the levels of ALT and AST (McGill & Hinson 2020).

This hepatotoxicity leads to an initial state of nausea and vomiting which progresses rapidly to metabolic acidosis or even coma. Between 24-72 hours after toxicity, there is right upper quadrant abdominal pain with worsening liver biochemical parameters. After few days there is massive liver encephalopathy, multiple organ failure and death that arises from liver necrosis due to coagulation defects (Adio *et al.,* 2022).

2.4 Restorative mechanism of silymarin milk thistle

According to Vargas-Mendoza *et al.,* 2014, silymarin milk thistle was found to have significant improvement in liver histology and liver biochemical markers for patients that had chronic liver disease with it also playing a critical role in liver defense by increasing liver glutathione and hepatocyte protein synthesis by stimulating RNA polymerase I activity. The effects of silymarin on the hepatocytes of alcohol induced liver cirrhosis were found to have significant reduction in tumor cell proliferation, angiogenesis and resistance to insulin, liver fibro-genesis and promotion of hepatocyte regeneration (Feher & Lengyel 2012).

From literature reviewed, there is still deficiency of information on the restorative microstructural changes associated with paracetamol toxicity.

2.5 The changes in liver bio-chemical parameters following administration of silymarin milk thistle and paracetamol among adult albino rats

Silymarin has been postulated to reduce the elevated levels of ALT and AST biochemical serum levels amongst patients who had non-alcoholic fatty liver though there is still inadequate data on it (Camilla *et al.,* 2017). Similar studies conducted on Non-alcoholic fatty liver disease showed that there was significant reduction in liver enzymes post administration of silymarin milk thistle extracts (Kalopitas *et al.,* 2021).

A study on the effects of silymarin milk thistle on liver cirrhosis found out that there was great reduction in the levels of ALT, AST, gamma-glutamyl transpeptidase and total bilirubin. This potrayed that the silymarin phytochemical had that ability in normalization of liver biochemical parameters (Gillessen *et al.,* 2022).

According to Ibrahim *et al.,* 2022 in a study on the role of silymarin milk thistle oil in the improvement of carbon tetrachloride-induced liver fibrosis and cirrhosis in albino mice, there was improvement in histological changes and representation of α-fetoprotein in the livers of the mice. On further evaluation of the liver biochemical markers of ALT, ALP and AST, there was a decrease in all those parameters signifying that silymarin milk thistle had ameliorating effects on liver fibrosis and cirrhosis.

A double -blind randomized control trial to evaluate the effects of silymarin milk thistle on liver enzymes and serum lipid profile, it showed that silymarin greatly reduced the levels of AST and ALT levels. It also led to silymarin having a recuing effect on the levels of triglycerides, total cholesterol and LDL-cholesterol implying that silymarin has a restorative effect (Dabiri *et al.,* 2023).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

This chapter focused on the methodology used in this current study. It gives descriptions of the study locations, design and study subjects and their eligibility criteria used in this study. It also spells out the study procedures of drug administration, handling and feeding of the study subjects, harvesting and processing of tissues, data analysis and presentation.

3.2 Study Location

The study was conducted in Maseno University, Western Kenya, Kisumu County, and the former Nyanza Province along Kisumu- Busia Highway. The university is accredited by the Commission of University Education to offer both Science and Arts courses in various fields in undergraduate and postgraduate programs. All experiments that involved handling, weighing, administration of paracetamol and silymarin milk thistle formulation and harvesting of liver tissues was done at the school of biomedical sciences at the animal house. The Department of Zoology has a well-established animal house for rearing and keeping rats. They also have a capability to keep the animals for experimental purposes and the expertise for handling of the animals. Tissue preparation, processing, histological study and analysis was done at the School of Medicine, Department of Human Anatomy. This department have the correct and necessary laboratory equipment for histological processing. Ethical Approval was sought at University of Eastern Africa- Baraton, Kenya and liver biochemical markers done at University of Nairobi, Kenya.

3.3 Study design

A posttest only true experimental study design was used. Intervention was implemented to determine the restorative effects of silymarin milk thistle on hepatocellular toxicity against the control.

3.4 Study subjects

This study was conducted on a pure breed of albino rats. They were of the species of *Rattus norvegicus* obtained from a pure colony sourced from the Department of Zoology, Maseno University. At every research period at the Department of Zoology, there is a population of 250 albino rats that can be held at the animal house and these numbers of albino rats are used for experimental purposes by different groups within and outside the Maseno University. Albino rats of both genders were recruited into the study with provision of only single gender within the same cage to prevent breeding during the experimental process.

This study used albino rats as study subjects due to various facts; that they are readily available, have relatively low cost of maintenance, they are small in size and easy to handle during the experimental study and are able to withstand a wide range of medicine and they share similar biological characteristics with humans therefore they replicate same results (Pritchett & Corning, 2016). The data obtained will be helpful to the healthcare workers in countering liver toxicity induced by paracetamol and any other agents.

Phenotypically, both gender of this species of albino rats which are a progeny of the same genetic ancestor appear to be red eyed and their fur are white and uniformly distributed throughout their bodies (Pritchett & Corning, 2016).

These albino rats have an average lifespan of 3 years and rapidly develop during infancy to reach their sexual maturity between 30-35 days in females while males at 45-48 days. Male and female rats are different in relation to size and weight. Male rats are usually longer than female rats with a length of about 23-28 cm. adult female and male rats weigh 350-450 grams and 450 – 650 grams respectively.

3.4 Sample size determination

The study had two major groupings; non-intervention group and intervention group which was further split into three subgroups for experimental purposes.

The sampled size was arrived at using Modified Resource equation method" of which there was no previous research done to determine the standard deviation, (Arifin & Zahiruddin, 2017).

n*=DF/K+1*

N*= nXk*

n*- number of animals per group*

DF*-Error of degree of freedom*

K*-Number of groups*

N*=Total number of subjects*

DF *range from 10 to 20 to obtain minimum and maximum number of each group*

Sample size in each group will be calculated as follows;

K=4

=20/4+1

=5+1 =6

=6

n=6 rats in each group

=6x4

N=24

3.5 Sampling Technique

The sampling technique utilized was a simple random sampling method to obtain the 24 study subjects from the 250 albino rats in the animal house and they were assigned to the groups with replacement done for those that did not meet the inclusion criteria.

3.6 Sample Selection criteria

3.6.1 Inclusion criteria.

All pure breed albino rats of both sexes within the cages which had attained the desired weight, age and were found to be healthy. Only adult albino rats were recruited and those that had attained the minimum required weight.

3.6.2 Exclusion criteria

Any animal that had not achieved the desired average weight and had not attained the desired age. The rats that died during the experimental process were replaced from the initial population and the experimental process done separately.

3.7 Grouping of animals

The 24 albino rats were assigned to two major groups; Non-intervention and intervention groups using simple random sampling method was used. The non-intervention group had 6 rats while the intervention group had 18 rats under three sub-groups of 6 animals each.

3.7.1 Non-intervention group

Non-intervention group; 6 albino rats received no silymarin intervention. Only water and rodent pellets (formulated feeds made from wheat germ, corn meal and sodium chloride) *ad libitum* for a period of 21 days afterwards they were humanely sacrificed at the end of the experiment but under two subgroups (three albino rats each); One subgroup was given rat diet and water only- control group; while the other subgroup was given high dose paracetamol (750 mg) for 5 days and normal diet plus water- paracetamol only group.

3.7.2 Intervention group

The remaining 18 animals were randomly assigned into three groups and they received 750 mg of paracetamol for 5 days for induction of hepatotoxicity and varying doses of silymarin milk thistle for the remaining 16 days and then they were humanely sacrificed at day 21 of the experiment for histo-morphological studies. This intervention group was divided into three sub groups of different dosages of silymarin milk thistle of 200mg/kgbwt (low dose), 400mg/kgbwt (Medium dose) and 600mg/kgbwt (high dose) respectively.

3.8 Procedure for each objective

3.8.1 Procedure for determining the gross morphology and histo-architectural injurious effects due to paracetamol toxicity on the liver cells in paracetamol induced hepatotoxicity.

At the end of the 21 days of the study, all the animals were humanely sacrificed. To meet this objective, the animals that had been randomly sampled into the paracetamol only group (those given high doses of paracetamol for 5 consecutive days, rodent pellets, and water only) and the control (those fed a normal diet and water *ad* libitum) were recruited. The liver tissues of rats in the two subgroups were harvested and the morphological features such as length, width, volume

and weight were studied. Later, the histo-architectural study was done, where histological processing and staining using H&E was done and studied under a microscope (Olympus BP). The length and width measurements were obtained using digital Vanier calipers, while volume was obtained using Archimedes' principle. The results found between the two subgroups were analyzed and compared.

3.8.2 Procedure for determining the histo-morphological restorative effects of silymarin milk thistle on paracetamol induced liver toxicity among adult albino rats

After the 21 days of administration of silymarin and paracetamol to the rats, they were humanely sacrificed and liver tissues extracted for the study. All 24 animals in both the intervention group, control group and paracetamol only group were recruited. The liver tissues of rats in all the groups were harvested and the morphological features such as length, width, volume and weight were studied. Later, the histo-architectural studies were done, where histological processing and staining using the H&E technique were done and studied under a microscope (Olympus BP). The length and width measurements were obtained using digital Vanier calipers and ruler while volume was achieved using Archimedes' principle. The results found on the liver tissues of the rats in the intervention group were compared against the results from the liver tissues of the control group to find out if there were any restorative effects both morphologically and histologically.

3.8.3 Procedure for assessing the changes in liver biochemical parameters following administration of silymarin milk thistle and paracetamol among adult albino rats

After the 21 days of administration of silymarin milk thistle and paracetamol to the rats, they were humanely sacrificed for the extraction of liver tissues. A blood sample was drawn from the heart of all the animals in the intervention group and the animals in the control group and paracetamol

only group. Analysis for liver biochemical parameters of AST, ALP and ALT was obtained and entered in an Excel sheet. Results obtained from the liver biochemical parameters (AST, ALP and ALT levels) of intervention group were compared with those obtained from the control group and paracetamol only group. Blood sample analysis was only done at the end of the experiment where the liver biochemical parameters of the intervention group was compared with control group and the paracetamol only group to check in the difference in the levels. The non- intervention group was used as the baseline hence done at the end of the experiment.

3.9 Experimental design and grouping of animals

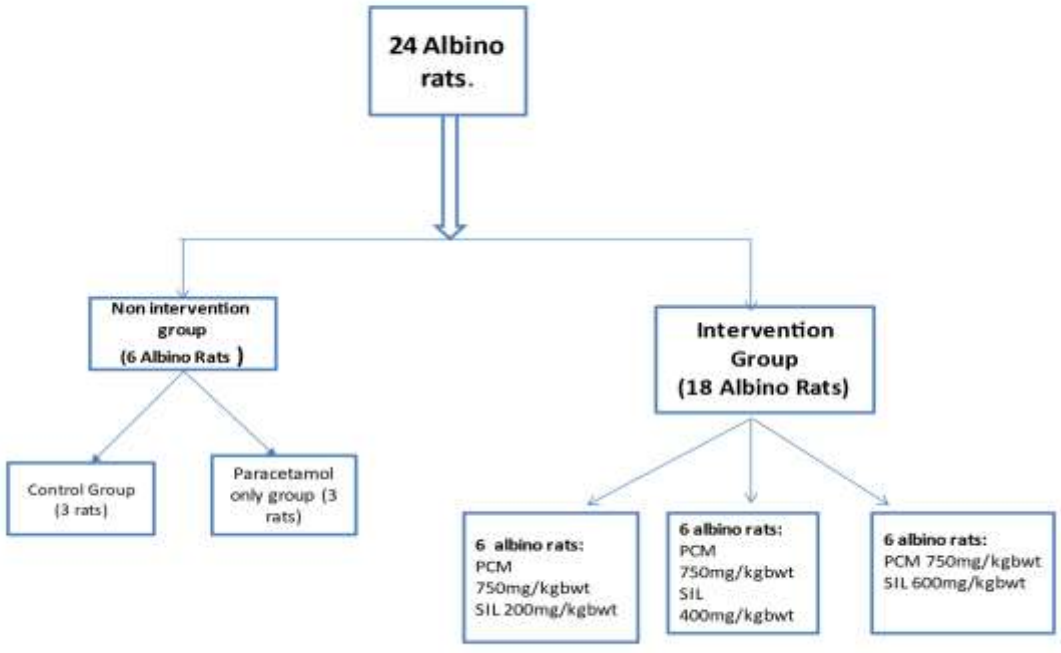


Figure 3.1. Group Distribution Chart- Experimental design

3.10 Feeding of rats

The albino rats were left to acclimatize for a period of one week and thereafter they were fed in according to the groups of study; the control group, paracetamol only group and the intervention group. Their feeds comprised of standard rodent pellets that were obtained from Unga Feeds Limited Company in Kisumu City, Kenya, and water *ad libitum (in plenty).* They were given clean water that was changed daily using the lixit chew proof flat sided water bottles (drinkers). The animals were fed each morning at 0900 hours within the polycarbonate cages.

Figure 3.2. Animal feeding within the polycarbonate cages

3.11 Occupational safety and Handling of rats

The albino rats were solely handled by the lead researcher with the assistance of a trained and certified animal attendant for the purposes of collecting daily data such as weights plus feeding and administration of the drugs between 0900 hours and 1200 hours. The procedures and steps in handling of laboratory animals were followed to the latter as adopted from Guide to the Care and use of laboratory Animals (Albus 2012). Occupational safety and health protocols and guidelines in animal handling were strictly followed to avoid injuries and cross infections. This included the

lead investigator wearing personal protective equipment such as examination gloves, mask, apron and face shield. Any eventuality that would have arisen such as animal bites would necessitate prompt medication attention.

3.12 Acquisition and determination of drugs doses

Administration of paracetamol and silymarin milk thistle was administered between 1000hrs and 1200hrs daily. The paracetamol tablets were obtained from Litein Central Chemist, Kericho with batch numbers of BN:2H103 while the silymarin milk thistle tablets (BN: D2231H) were obtained from Dynapharm Kenya Limited-Nakuru, Kenya.

3.12.1 Materials

Paracetamol tablets, Silymarin milk thistle tablet formulations, 24 albino rats, gastric gavages needles, Syringes 5 ml each, one 20 ml dilution beaker, Syringes, Deionized water (500ml), Table towel.

3.12.2 Method of administration; feeding time and drug administration

To induce hepatotoxicity from paracetamol, the paracetamol dosage was administered to the animals between 1000 hrs. -1200 hours daily for five consecutive days using a gastric gavage needle.

3.12.3 Procedure followed in administration of paracetamol and silymarin milk thistle formulation using gastric gavage needle

i. Each albino rat was held and wrapped using a table towel. This was to prevent the animal from soiling the primary investigator’s garments.
ii. The animal was held behind the neck region with one hand.

iii. After that, the rat was put to resting position to lie on the researcher with its mouth put to face forward.

iv. The gastric gavage needle was gently directed into the albino rat's oral cavity while gently to maneuvering it through the esophageal constrictors and finally through the cardiac sphincter.

v. Eventually the Paracetamol dose was eventually deposited into the rat's stomach

vi. The gastric gavage needle was removed slowly and gently to avoid injuring the animal.

3.13 Determination of paracetamol doses for the experiment

To induce liver toxicity in the albino rat, Paracetamol dosage of 750 mg/kbwt was used for 5 days.

Animal equivalent dose=750 mg× rat weight =dose of the rat

Example if an albino rat weighs example 160grams

750mg×160/1000=120 mg per day

Or 200 grams albino rat; 750mg x 200/1000= 150 mg per day

Or 150 grams albino rat; 750 mg x 150/1000= 112.5 mg per day

3.14 Preparation of silymarin dosages

Human adult dose of Silymarin is 200mg taken thrice a day (after every 8 hours), with the dose being changed to proportionately match the animal dose, (Shin *et al.,* 2010)

Animal equivalent dose =human equivalent dose (Mg/kg) ×converting factor

Animal equivalent dose=100mg×rat weight in kg

For silymarin 200mg 600mg/60×6.2= 62 mg/kgbwt

For silymarin 400mg 1200/60×6.2=124mg/kgbwt

For silymarin 600mg 1800mg/60×6.2=186 mg/kgbwt

3.14 Administration of silymarin milk thistle tablets

3.14.1 Materials needed

Silymarin milk thistle tablets, gastric gavages needle, syringes 5ml each, 20 ml beaker for mixing, water for injection, table towel

3.14.2 Procedure of administration of silymarin milk thistle dose

i. Each albino rat was held and wrapped using a table towel. to avoid the animal from soiling the primary researcher's garments.

ii. The animal was held behind the neck region with one hand.

iii. After that, the rat was put to resting position by lying on the researcher with its mouth put to face forward

iv. The gastric gavage needle was gently introduced into the albino rat's oral cavity while gently to maneuvering it through the esophageal constrictors and finally through the cardiac sphincter.

v. Eventually the silymarin milk thistle solution was deposited right to the stomach of the rat.

vi. The gastric gavage needle was removed slowly and to avoid injuring the animal.

3.15 Dissolution and preparation of Paracetamol and Silymarin milk thistle dosages

Paracetamol tablets and Silymarin milk thistle formulation tablets were diluted with water for injection, and their concentration administered in residual volumes not exceeding 3 mls per administration.

3.16 Humane sacrificing of animals and harvesting of liver tissues for study

Supplies Required

24 Albino rats, electronic weighing machines, 10 mls of chloroform, mounting board and pins, 1 roll of cotton wool, dissector jar, Pair of scissors, normal saline 0.85% concentration, toothed forceps, Surgical razorblade, 5% formalin, 2 Drip set, 24 Hypodermic needle gauge 20, 1 box latex Gloves, Magnifying glass, digital calipers, Specimen collection bottles.

Figure 3.3. Humane Sacrificing of the albino rats

3.17 Procedure for anaesthetizing of albino rats

i. Concentrated chloroform was opened into a heavy tight fitting bell jar.

ii. The albino rats will be euthanized by being put into the bell jar for approximately 3-5 minutes.

iii. After being euthanized they were removed from the tight fitted lid jar and put on the dissecting board and well mounted using mounting pins to lie of the rear side facing supine.

iv. With the use of the toothed forceps and pair of scissors, the researcher made an incision from the point of the rat's sternal angle of Louis up to symphysis pubis and opened for the liver to be identified.

v. The entire liver tissue with all the lobes intact were removed and fully dipped into formaldehyde solution for 24 hours to fix it.

Figure 3.4. Anaesthetizing of albino rats Concentrated chloroform in tight fitting jar

3.18 Evaluating gross morphometric of liver weight, width, length and volume

After the liver tissues had been extracted, liver specimens were cleaned using 5% normal saline, their gross morphometric including lengths, width and thickness measured using digital calipers (SEAHAVEN Digital caliper) and a ruler. Liver weights were measured using digital weighing scale (Equipment details- KERN EMB 200-2).

Figure 3.5. Weighing of liver tissue using digital weighing scale

3.19 Blood collection and biochemical analysis

Blood samples for biochemical analysis were drawn directly from the heart at the end of the experiment during the human sacrificial of the rats. The samples were then centrifuged at 3000rpm for 10 minutes, sera collected and analyzed using ELISA kits.

3.20 Estimation of liver volumes using Archimedes' Principle

To estimate liver volume, Archimedes' principle was used to obtain the independent volumes of all rats' livers. This principle works by estimating the volume of the liver by inserting the fixed liver organ that was extracted into the 100 ml graduated beaker half-filled with normal saline with measurements of displaced volumes taken. The amount of normal saline that was displaced in the graduated beaker by the liver represented the actual volume of the liver (Sherle, 1970).

Figure 3.6. Estimation of liver volume using Archimedes' principle

3.20 Processing for light microscopy

i. The liver tissue excised underwent fixation using formaldehyde solution for a period of 24 hours

ii. They were immersed in ascending strengths of alcohol from 50%, 60%, 70%, 80%, 90% up to 100% for a period of one hour in each concentration to be dehydrated.

iii. After dehydration it was cleared with xylene and infiltrated using paraffin wax for 12 hours at 56^0c

iv. It was then positioned longitudinally and paraffin wax used to embed it onto the wooden blocs

v. The surplus wax was removed till there was full exposure of the whole length of the liver tissue.

vi. Thin longitudinal sections of 5µm thickness were obtained by cutting using a Leitz sledge rotary microtome

vii. The thin longitudinal sections that were cut were left to float on water at 37^0 to stretch the liver sections

viii. These cut longitudinal sections were sticked onto the glass slide and applied using a micro-dropper as a thin film onto the glass slide.

ix. The glass slides prepared were left to dry in an oven of temperature 37^0 for 24 hours

x. Thereafter staining using Hematoxylin and eosin (H&E) was done.

Figure 3.7.Liver tissue fixation and blocking

3.21 Materials for staining

Liver tissues, Specimen bottles, distilled water, Paraffin wax, Microtome blades, Rotary microtome, Slide holders, Distilled water, water heating container, Acetic acid, DPX mount ant, Glass slides and cover slips, Glass staining square jar, Hematoxylin and Eosin, 40% concentrated Formaldehyde, Xylene, ethanol, embedding wood blocks, 50 ml dilution Beakers, Dropper,

3.22 Staining of liver slides

The sections of the liver tissues that were prepared and staining was done using hematoxylin and eosin solution using with the steps used in staining as outlined by (Ghosh *et al*., 2014) in preparation for microscopy.

3.23 Equipment and procedures for photography

3.23.1 Equipment

LABOMED IV 3200 fitted with digital camera and USB drive

3.23.2 Steps followed in obtaining photomicrograph

i. The slides that were prepared for histological study were secured on the microscope' stage.

ii. The fine and coarse adjustment knobs of the microscope were adjusted to obtain a clear focus of the image to be photographed and magnified appropriately.

iii. Photograph images of the regions under focus were taken

iv. Photographs obtained were stored in a computer and a flash disc

v. The photograph images taken were then uploaded and carefully labelled using the Adobe fireworks Program me

3.24 Data management and analysis

The data was then entered into an Excel sheet, analyzed using SPSS for Windows version 26, (Chicago Illinois). To test for statistical significance, a one-way analysis of variance (ANOVA) was used to analyze the group means. A P value of $\leq$ than 0.05 was considered statistically significant at a 95% confidence interval. A post hoc test for independence was used to determine the histo-morphological effects of silymarin milk thistle on paracetamol-induced hepatotoxicity

and to determine the dosage of silymarin milk thistle that provided hepato-restorative effects following paracetamol-induced hepatotoxicity.

The gross morphological parameters of volume were determined using Archimedes' principle, while weights, length, weights of the liver samples and biochemical parameters were analyzed descriptively using the mean and the standard error of the mean and presented in tables.

The data acquired through the photomicrograph at different magnifications from the histological images were collected, stored on a flash disc and analyzed qualitatively.

3.25 Ethical approval

The proposal was first forwarded to the School of Graduate Studies for approval to undertake research at Maseno University. The ethical approval to carry out the study was from University of Eastern Africa, Baraton, Kenya number UEAB/ISERC/11/01/2023. License to do the research was granted by National Commission for Science, Technology and Innovation (NACOSTI), license No: NACOSTI/P/23/23588. All protocols for handling and humane sacrificing of animals were adhered to throughout the entire study period.

CHAPTER FOUR

RESULTS

4.1 Introduction

This chapter outlines the findings of this current study. These results have been arranged chronologically as per the objectives and presented in tables. Objective 1 presents the histo-morphological changes on the liver following administration of high dose of paracetamol; objective 2 examines the histo-morphological effects of different doses of silymarin milk thistle on paracetamol-induced hepatotoxicity and objective 3 examines the effects of various doses of silymarin milk thistle on the liver biochemical parameters of ALT, ALP and AST on paracetamol-induced hepatotoxicity.

4.2: Results for Objective 1

4.2.1 Morphological changes on the liver on administration of high dose paracetamol

The liver weight, width, length and volume were measured immediately after humane sacrificial of the adult albino rats. The measurement for the variables in the paracetamol only group was compared to the control group (water + feed) to demonstrate hepatotoxicity.

Table 4.1: Morphological changes in paracetamol induced hepatotoxicity

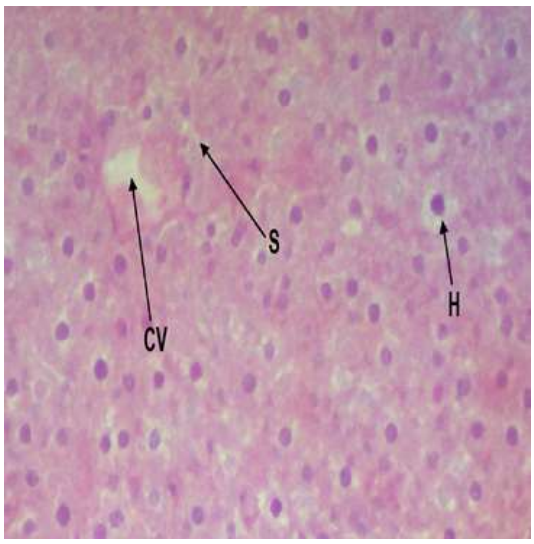
Variable per groups	Volume of the liver (ml) Mean ±SEM	Width of the liver (mm) Mean ±SEM	Length of the liver (mm) Mean ±SEM	Weight of the liver (g) Mean ±SEM
Control group (water + feeds)	11.76 ± 0.04	41.14 ± 0.23	64.61 ± 0.19	11.04 ± 0.11
Paracetamol only group (*750mg/kgbwt of paracetamol only)*	8.88 ± 0.11	35.50 ± 0.19	50.97 ± 0.10	8.41 ± 0.09
	P = 0.0001	*P* = 0. 0001	*P* = 0.0001	*P* = 0.0001

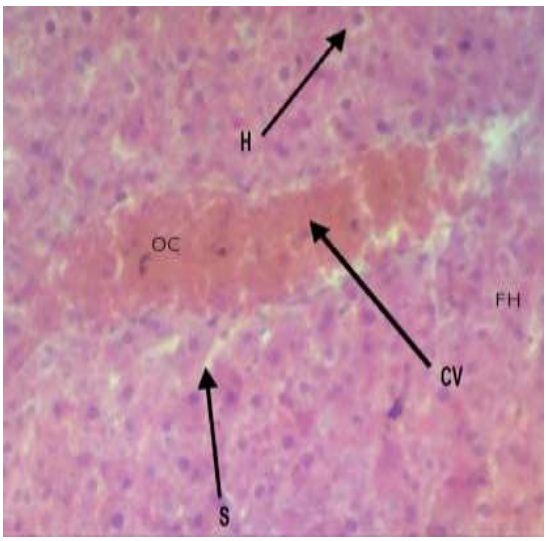
Key*: SEM- standard error of mean, mg/kgbwt -milligrams per kilo body weight*

There was significant (p = 0.0001) reduction in the mean length, weight, width and volume of the liver in the paracetamol only group as compared to the control group. One way ANOVA was used to test the significance and the $p \leq 0.05$ at 95% confidence interval was found significant. The results were expressed as a standard error of mean and a t test used to analyze the difference between the two groups.

4.2.2 Histological changes on the liver when administered with hepatotoxic dose of paracetamol

The liver histological slides of the paracetamol only group were compared with the control group. The sinusoids, hepatocytes, central part and the central vein were observed.

A 

B 

A- Control group **B- Paracetamol only group**

***Key**: CV-Central vein, S- sinusoid, H- hepatocyte, OC- Occlusions, FH- Focal Hemorrhage*

Figure 4.1. Photomicrographs of the control group and the paracetamol only group after staining with Hematoxylin and Eosin (H & E)

The control group (Fig 4.1-A) had evenly distributed hepatocytes, normal sinusoidal dilatation and visible central vein. The paracetamol only group (Fig 4.1-B) group had dilated central vein, dilated sinusoids, focal areas of hemorrhage and atrophied hepatocytes. It also presented with occlusions in central veins and numerous Kupffer cells.

4.3 Results for Objective 2

4.3.1: Morphological effects of different doses of silymarin milk thistle on paracetamol induced hepatotoxicity

The mean volume, width, length and weight of the liver of the paracetamol only group was compared with the high, medium and low dose of silymarin milk thistle groups. Histological slides of the silymarin milk thistle groups were also compared to demonstrate histo-structural restoration.

Table 4.2: Comparison of the morphological changes in paracetamol induced hepatotoxicity following administration of different doses of silymarin milk thistle and paracetamol only group

Variable per groups	Volume of the liver Mean ±SEM	Width of the liver Mean ±SEM	Length of the liver Mean ±SEM	Weight of the liver Mean ±SEM
Paracetamol only group (750mg/kbwt of paracetamol)	8.88 ± 0.11	35.50 ± 0.19	50.97 ± 0.10	8.41 ± 0.09
High dose (600 mg/kbwt of silymarin)	11.23 ± 0.07 *P*= 0.0001	40.35 ± 0.10 *P*= 0.0001	62.48 ± 0.20 *P*= 0.0001	10.87 ± 0.10 *P*= 0.0001
Medium dose (400 mg/kbwt of silymarin)	8.62 ± 0.18 *P*= 1.000	35.67 ± 0.22 *P*= 1.000	50.95± 0.13 *P*= 1.000	8.61 ± 0.25 *P*= 1.000
Low dose (200 mg/kbwt of silymarin)	8.76 ± 0.19 *P*= 1.000	35.59 ± 0.19 *P*= 1.000	50.59 ± 0.27 *P*= 1.000	8.35 ± 0.04 *P*= 1.000

Key: *SEM- standard error of mean. Mg/kbwt -milligrams kilo body weight.*

There was significant (p= 0.0001) increase in mean of all liver parameters (length, width, weight and volume) in the High dose SIL group as compared to the paracetamol only group. There was no statistical significance (p= 1.000) in the liver morphologic parameters (length, width, weight and volume) of Medium and low dose group respectively as comparable to the paracetamol only group, (**Table 4.2**). One way ANOVA was used to test the significance and the $p \leq 0.05$ at 95% confidence interval was found significant.

The mean volume, width, length and weight of the liver of the control group was compared to the high, medium and low dose of silymarin milk thistle groups.

Table 4.3: Comparison of the morphological changes in paracetamol induced hepatotoxicity following administration of different doses of silymarin milk thistle and control group

Variable per groups	Volume of the liver Mean ±SEM	Width of the liver Mean ±SEM	Length of the liver Mean ±SEM	Weight of the liver Mean ±SEM
Control group (water + feeds)	11.76 ± 0.04	41.14 ± 0.23	64.61 ± 0.19	11.04 ± 0.11
High dose (600 mg/kbwt of silymarin)	11.23 ± 0.07 *P*= 1.000	40.35 ± 0.10 *P*= 1.000	62.48 ± 0.20 *P*= 1.000	10.87 ± 0.10 *P*= 1.000
Medium dose (400 mg/kbwt of silymarin)	8.62 ± 0.18 *P*= 0.0001	35.67 ± 0.22 *P*= 0.0001	50.95± 0.13 *P*= 0.0001	8.61 ± 0.25 *P*= 0.0001
Low dose (200 mg/kbwt of silymarin)	8.76 ± 0.19 *P*= 0.0001	35.59 ± 0.19 *P*= 0.0001	50.59 ± 0.27 *P*= 0.0001	8.35 ± 0.04 *P*= 0.0001

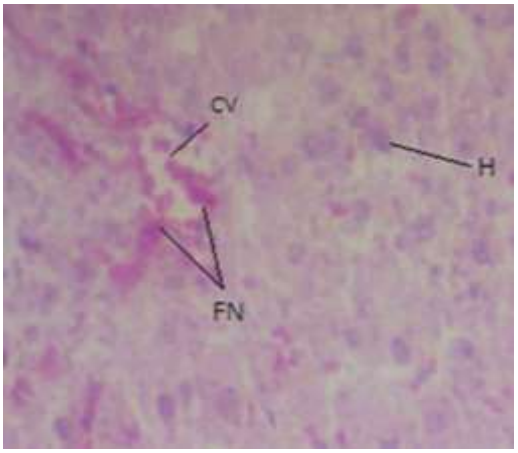
Key: *SEM- standard error of mean. Mg/kbwt -milligrams kilo body weight.*

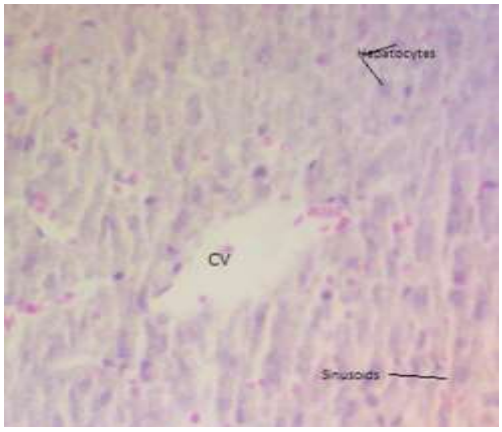
There was significant (p= 0.0001) reduction in mean of all liver parameters (length, width, weight and volume) in the low dose SIL and medium dose SIL groups as compared to the control group. There was no significant difference (p= 1.000) in the liver morphologic parameters of high SIL

dose group as compared to the control group (**Table 4.3**). One way ANOVA was used to test the significance and the $p \leq 0.05$ at 95% confidence interval was found significant.

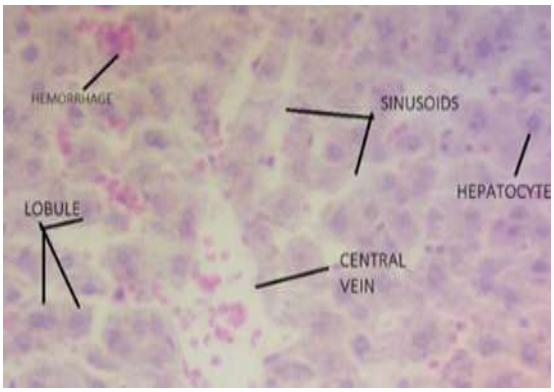
4.3.2 Histo-morphological changes on the liver on paracetamol induced hepatotoxicity following administration of different doses of silymarin

The histological slides of the silymarin intervention groups were examined. The central vein, sinusoids and the hepatocytes were compared among the silymarin intervention groups with those of the control group.

A 

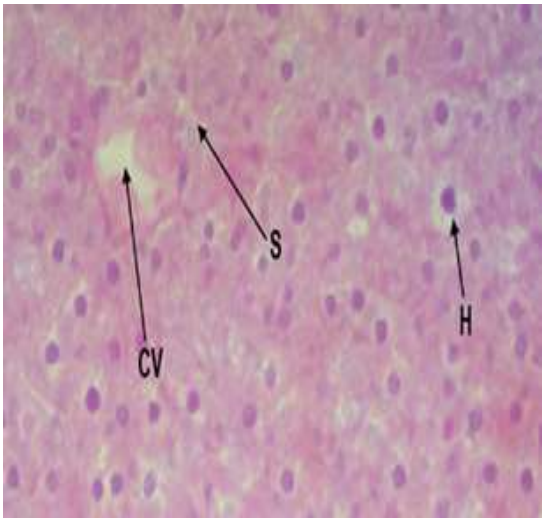
B 

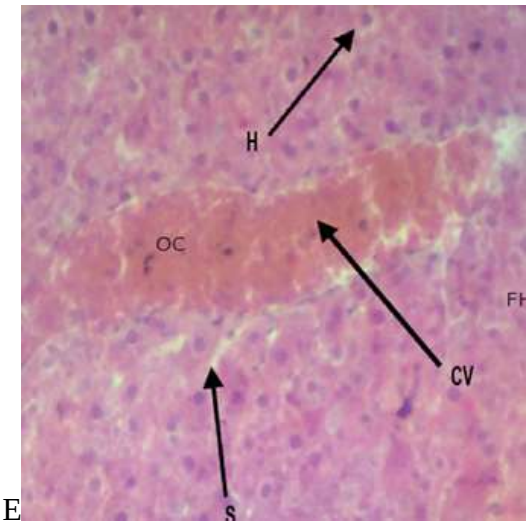
A. LOW SIL GROUP **B. MEDIUM SIL GROUPC**



C. HIGH SIL GROUP

Key: *H& E – hematoxylin and eosin. CV-Central vein, S- sinusoid, H- hepatocyte, DS- Dilated Sinusoids, MV- Mild Vacuolation, N- Nucleus, FN- Focal Necrosis, NH- Normal Hepatocytes, OC- Occlusions, FH- Focal Hemorrhage*

D 

E 

D. CONTROL GROUP **E. PARACETAMOL ONLY GROUP**

Key: *H& E – hematoxylin and eosin. CV-Central vein, S- sinusoid, H- hepatocyte, DS- Dilated Sinusoids, MV- Mild Vacuolation, N- Nucleus, FN- Focal Necrosis, NH- Normal Hepatocytes, OC- Occlusions, FH- Focal Hemorrhage*

Figure 3.2. Photomicrographs of the Control group compared to paracetamol only group and Silymarin Intervention groups that were treated with different doses of silymarin milk thistle and stained with H & E

The central veins and sinusoids were dilated in the paracetamol only group, medium dose and low dose group as compared to the High dose SIL group. There was also reduction in size of the hepatocytes in the paracetamol only group, medium dose SIL and low dose SIL group as compared to the High dose SIL group and control group that had normal hepatocytes. The low dose SIL had dilated hepatocytes, vocal areas of hemorrhage. The central veins of the paracetamol only group, medium dose SIL and low dose SIL group had also been occluded while High dose SIL had evenly distributed hepatocytes and no dilated sinusoids (**Figure 4.2**). This could be due to the hepatotoxic effects of paracetamol on the liver cells that causes these changes in the central vein. The occlusion in these groups could also be due to the hemorrhage brought about by the reactive oxygen and protein radicals within the liver affecting the normal liver functions. The focal areas of necrosis

and hemorrhages is possibly due to the increase in the toxic levels of NAPQI triggering the necrotic changes.

The control group and the high dose group had normal central vein, evenly distributed normal hepatocytes, normal arrangements and dilations of sinusoids as compared to the low dose, medium dose SIL groups and the paracetamol only group. The liver histo-architecture of the high dose SIL group and the control group had similar histological presentations of the sinusoids, hepatocytes and central vein illustrating restoration on high dose silymarin (**Figure 4.2**). These changes observed in these two groups could be due to the restorative effects of high dose of silymarin milk thistle on the liver that restores the normal liver histo-architecture and restores normal functioning.

4.4: Results for Objective 3

4.4.1: Biochemical parameters following administration of paracetamol followed by silymarin milk thistle

The normal adopted ranges of the biochemical parameters were as follows; Alanine transaminase (14- 30 Units/Litre), Alkaline phosphatase (56.8 – 128 Units/ Litre) and Aspartate transaminase (45.7- 80.0 Units/Litre).

Table 4.4: Mean of the biochemical parameters of ALT, ALP and AST between the control group and paracetamol only group

Groups	ALT		ALP	AST
Control group	22.00 0.95	±	77.00 ± 0.70	94.62 ± 0.71
paracetamol only group	35.84 0.51	±	108.30 ± 1.39	113.27± 0.21
	P= 0.0001		*P*= 0.0001	*P*= 0.0001

***Key**: ALT- Alanine transaminase, ALP- Alkaline phosphatase, AST- Aspartate transaminase*

There was statistical (*P*= 0.0001) increase in the levels of the ALP, AST and ALT in the paracetamol only group as compared to the control group. The AST and ALT biomarkers tested were all above the normal ranges for the paracetamol only group (**Table 4.4**). The results were expressed as a standard error of mean and a t test used to analyze the difference between the two groups.

Table 4.5: Mean of the biochemical parameters between the paracetamol only group and the silymarin intervention groups

Groups	ALT	ALP	AST
paracetamol only group	35.84 ± 0.51	108.30 ± 1.39	113.27± 0.21
High dose SIL group	25.72 ± 1.33 P= 0.0001	78.33 ± 0.89 P= 0.0001	95.66 ± 0.25 P= 0.0001
Medium dose SIL group	34.28 ± 0.10 *P*= 1.000	106.81 ± 1.86 *P*=1.000	112.35 ± 0.76 *P*=1.000
Low dose SIL group	35.22 ± 0.70 *P*=1.000	106.58 ± 1.71 *P*=1.000	113.60 ± 0. 22 *P*=1.000

Key: *ALT- Alanine transaminase, ALP- Alkaline phosphatase, AST- Aspartate transaminase, SIL- Silymarin milk thistle*

There was significant (p= 0.0001) reduction in the levels of the AST and ALT in the high SIL group as compared to the paracetamol only group. There was no significant difference (*P*=1.000) in the medium dose and low dose group as compared to the paracetamol only group (**Table 4.5**). One way ANOVA was used to test the significance and the $p \leq 0.05$ at 95% confidence interval was found significant.

Table 4.6: Mean of the biochemical parameters between the control and the silymarin intervention groups

Groups	ALT	ALP	AST
Control group	22.00 ± 0.95	77.00 ± 0.70	94.62 ± 0.71
High dose SIL group	25.72 ± 1.33 *P*=1.000	78.33 ± 0.89 *P*=1.000	95.66 ± 0.25 *P*=1.000
Medium dose SIL group	34.28 ± 0.10 P=0.0001	106.81 ± 1.86 *P*=0.0001	112.35 ± 0.76 *P*=0.0001
Low dose SIL group	35.22 ± 0.70 P=0.0001	106.58 ± 1.71 *P*=0.0001	113.60 ± 0. 22 *P*=0.0001

Key: *ALT- Alanine transaminase, ALP- Alkaline phosphatase, AST- Aspartate transaminase, SIL- Silymarin milk thistle*

There was a significant (*P*=0.0001) increase in ALT, AST and ALP in low dose SIL and Medium dose SIL groups as compared to the control group respectively**.** There was no significant difference (*P*=1.000) in high dose SIL in all the biomarkers tested as compared to the control group. There were no significant changes between the biochemical parameters of high dose SIL group and the control group implying that restoration had occurred. However, in both the control and silymarin intervention groups, ALP were within the normal adopted ranges. (**Table 4.6**). One way ANOVA was used to test the significance and the $p \leq 0.05$ at 95% confidence interval was found significant.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

This chapter focuses mainly on the correlation between findings from this current study and those from other authors on the effects of silymarin milk thistle on paracetamol hepatotoxicity. It has been done with accordance to the objectives. Objective 1 was to determine the histo-morphological injurious effects of paracetamol that occur on the liver following paracetamol toxicity; objective 2 was to determine the restorative histo-morphological effects of various doses of silymarin milk thistle on paracetamol hepatotoxicity and objective 3 was to assess the changes in liver biochemical parameters of AST, ALP and ALT following administration of silymarin milk thistle on paracetamol toxicity.

5.2 Discussion for objective 1

Paracetamol is one of the most commonly accessible over-the-counter drugs used to manage pain and its overdose and abuse have been associated with liver damage (Offor *et al.,* 2022). The present study found out that there was significant change in liver morphometric parameters. There were reductions in all parameters of liver length, width, volume and liver weight among the group that received high doses of paracetamol as compared to the group that never had any paracetamol intake (Table 4.1).

These changes may be due to the depletion of glutathione and release the of reactive proteins, causing necrotic changes in the liver tissues. With high levels of paracetamol intake, there will be toxic production of N-acetyl-p-benzoquinonemine (NAPQI) from the liver, which will react with cellular membrane molecules, leading to hepatocyte damage and necrosis of liver tissues. The current findings are in tandem with studies done by Hudaa & Laila 2015, Mahmood *et al.*, 2014,

Rono 2017 and Sato *et al.,* 2023, who recorded acetaminophen-induced reductions in liver size, liver length and width. A study by Vakiloddin *et al.,* 2015 on paracetamol-induced hepatotoxicity in wistar rats found that there was an increase in wet liver volume and wet liver weight in the wistar rats treated with high-dose paracetamol, which has contrary findings to this study on liver volume on assessment using the Archimedes' principle. The current study noted that the liver volume of the high dose paracetamol only group was lower as compared to that of the control group; however, in the current study, wet liver volume was not recorded.

According to the histo-morphologic findings in this study, the paracetamol only group was found to have areas of hemorrhagic necrosis with the presentation of vacuolated hepatocytes, deranged and dilated sinusoids, pockets of foci areas of hemorrhage, and abnormally high numbers of Kupfer cells, while those of the control group had a normal arrangement of sinusoids, normal hepatocytes and no areas of necrosis (Figure 4.1). These morphological changes could have been due to an increase in NAPQI in the liver tissue, causing damage and disruption in cytoplasmic organelles. This could have a negative effect in the normal liver physiology and cause deranged liver biochemical markers causing further liver damage. These changes also in the liver parenchyma could lead to mitochondrial dysfunction thus affecting the ATP generation in the cells causing necrotic cell death (Jaeschke *et al.,* 2021). A study by Manal & Emal 2014, on the effects of paracetamol on liver histology of adult rabbits also observed necrosis, vacuolization of hepatocytes with irregular nuclei and the presence of abnormally high Kupfer cells on administration of high-dose paracetamol. In addition, Fazil *et al.,* 2019, in a study on histopathological changes in acetaminophen-induced liver injury in wistar albino mice, reported focal points of hemorrhagic necrosis on the liver, which could be due to inflammatory responses at these hepatotoxic levels, as was observed in the current study.

5.3 Discussion for objective 2

Silymarin milk thistle an edible herb, is believed to have antioxidant, radical scavenging, and regulation of glutathione levels properties. It has been used over time in treating various conditions, including stomach upset, liver and kidney problems; however, its pharmacokinetics and pharmacodynamics have not been well documented. In this current study, different doses of silymarin milk thistle (high dose of SIL: 600 mg/kbwt, medium dose of SIL: 400 mg/kbwt, low dose of SIL: 200 mg/kbwt) were administered to the silymarin interventional group and there was an improvement in liver morphological data of weight, volume, width, and length as compared to the paracetamol only group, with a significant increase in the high dose SIL group (Tables 4.2 and 4.3). The high-dose SIL group showed that there was marked normalization of these parameters as compared to the other two groups. This might have been due to silymarin improving the glutathione levels in the blood and liver, counteracting the effects on paracetamol toxicity, hence lowering the effects of NAPQI. These changes may also be due to the reduction in superoxide anion levels in the bloodstream, causing improvement and normalization of these parameters compared to those of the control group. According to studies by TajMohammadi *et al*., 2018 and Abenavoli *et al*., 2011, silymarin milk thistle was found to potentiate the normalization and restoration of relative liver weights, glutathione levels, and plasma lipid levels in metabolic syndromes and nonalcoholic fatty liver disease. Khazaei *et al.*, 2022 also recorded that silymarin milk thistle led to a reduction in the weights of the spleen in laboratory animals, hence concurring with the current study.

From the literature reviewed, Tsai *et al.,* 2008, found out that post-administration of silymarin milk thistle on carbon tetrachloride-induced liver fibrosis, liver morphological parameters were greatly

improved, leading to resolution of liver fibrosis, hence concurring with this present study that demonstrates restorative effects of silymarin milk thistle on paracetamol-induced hepatotoxicity.

On the histo-architectural findings of the silymarin intervention groups, the low-dose SIL group was found to have some aspects of sinusoidal dilatations, vacuolated hepatocytes and areas of focal necrosis, which could be due to the minimal impact of silymarin on the liver cell. The medium-dose SIL group had relatively normal hepatocytes with mild dilation of sinusoids and minimal focal areas of necrosis. The high-dose SIL group had minimal cell damage, possibly due to the changes brought about by the high dose of silymarin that might have possibly counteracted the hepatotoxicity (Figure 4). These changes in the liver parenchyma in the three groups could possibly be due to the varying doses of silymarin, thus affecting the levels of glutathione and reducing the levels of reactive superoxides and anions, which eventually affect the liver histology. These findings could also be due to the neutralization of free oxygen radicals and nitrosative stress from the liver bringing about these changes.

From different literature reviewed, a study by Mohammed *et al.,* 2016, on streptozotocin-induced diabetic rats found that silymarin and silybinin caused repair on the histoarchitectural damaged pancreatic islets of Langerhans and normalization of arrangements of acinar cells, and improvements on beta cells. Zuzana *et al.,* 2018 recorded that during concurrent administration of silymarin and acetaminophen in wistar rats, silymarin protected the liver tissue from damage and necrosis, noting that it had antioxidant properties. Saheed *et al.,* 2015 observed that administration of silymarin and vitamin C led to a reduction of reactive oxygen species, bringing about normalization of the liver histo-architecture on acetaminophen-induced liver damage in wistar rats, concurring with this study that there are restorative effects brought about by administration of silymarin milk thistle.

5.4 Discussion for objective 3

Paracetamol toxicity has been found to cause significant changes by increasing the total bilirubin, total protein, ALT, ALP and AST levels in the body. The current study found a significant increase (P=0.0001) in the levels of ALP, AST and ALT in the medium-dose SIL group, the low-dose SIL group, and the paracetamol only group as compared to the control group and high-dose SIL groups (Table 4.4, Table 4.5 and Table 4.6). The persistent elevation of these markers even after giving silymarin milk thistle at low and medium doses is an indication that the restorative effects on the liver tissue did not occur using these doses. However, at higher doses of 600 mg/kbwt there was normalization of all liver biochemical markers (Table 4.6).

In agreement with the current results, Tsai *et al.,* 2008 in a study on the effects of silymarin on the resolution of carbon tetrachloride-induced liver fibrosis, observed that low doses of silymarin were ineffective and could not cause any restoration or normalization of the elevated liver biochemical markers. In addition, Felipe *et al.,* 2015, also reported that lower doses of less than 100 mg/kgbwt of silymarin milk thistle had no restorative effects on the liver histoarchitecture. He further observed no normalization of the elevated liver biomarkers that had increased due to acetaminophen toxicity on liver tissues.

The levels of AST, ALP and ALT in the high-dose SIL group and the control group were within the normal ranges, indicating that restoration of the liver tissue was achieved through high-dose silymarin (Table 4.6). This confirms that the phytochemical silymarin in the milk thistle may facilitate reconstruction of the hepatic histo-architecture of the liver tissue and counteract the hepatotoxin effects of paracetamol, thereby improving the levels of glutathione and thus improving the liver's functional biochemistry. Authors from the literature reviewed (Tsai *et al.,* 2008; Saeed *et al.,* 2017; Amir *et al.,* 2022) recorded that administration of silymarin milk thistle was found to

normalize elevated AST, ALP and ALT since silymarin at a high dose of 500 mg/kbwt was found to potentiate normalization of liver tissues, causing improvements in liver biochemical parameters. Moreover, Rahul *et al.,* 2014, also reported restoration of liver histoarchitecture after administering *Rhodiola imbricata* and silymarin milk thistle to injured livers in rats; therefore, these studies concluded that silymarin milk thistle may have a restorative effect on damaged liver tissues.

The outcomes of this study indicate that the restorative effect of silymarin milk thistle is dose-dependent so only higher doses may give the desired results. These findings mirror observations from Hamza *et al.,* 2015 and Kidd & Head 2005, who showed that the normalization of liver biomarkers was directly dependent on the dose of silymarin and *Nigella sativa,* where a high dose of silymarin portrayed normalization of liver functions and improvement of its histoarchitecture. However, more studies may be needed to assess the effects of high doses of silymarin milk thistle and its pharmacodynamics.

CHAPTER SIX

CONCLUSSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

In conclusion;

1. High doses of paracetamol cause damage to liver tissue and also interfere with the normal anatomy. High dose affects the synthesis of paracetamol in the liver by causing depletion of glutathione and the release of NAPQI causing histo-morphological damage to the liver.
2. Silymarin milk thistle has been found to have restorative effects on liver tissue at higher doses., high doses were likely to bring the histo-morphological changes in the liver parenchyma due to high concentration of the restorative phytochemical. These eventually leads to changes in the anatomy of the liver itself
3. A high dose of silymarin milk thistle caused normalization of the liver biomarkers ALP, AST, and ALT. the phytochemical silymarin in the milk thistle brings about normalization of liver biochemical parameters at high doses and low doses have no restorative effects.

6.2 RECOMMENDATIONS

According to the study findings, this study recommends that:

1. Since paracetamol was found to cause hepatotoxicity, its recommended dosages should be strictly adhered to, with education given to the general population on the hazardous effects of overdosing on the liver.
2. Studies should be done to identify the safe human dose range for silymarin milk thistle to bring about gross morphological and histological restoration of the damaged liver.

3. Studies should be done to identify the safe human dose range for silymarin milk thistle to restore the biochemical markers of the damaged liver.

6.3 Recommendation for further studies

Studies on further tests and trials on silymarin milk thistle should be done on the rats' species to check on replication of the results before the regulatory bodies conduct tests that may lead to the registration of this traditional herb as a drug.

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APPENDICES

APPENDIX I: TIMEFRAME

TASK	Jan 2022	Feb 2022	March 2022	April 2022	May 2022	June 2022
Proposal writing						
Proposal presentation						
Data collection						
Data analysis and interpretation						
Theses development						
Theses presentation						

APPENDIX II: BUDGET

ITEM	**QUANTITY**	**AMOUNT (Ksh)**
CAGES	4	120,000/=
Unga rat pellets (feeds)	75 kgs	6,000/=
Experimental drugs	20 bottles	20,000/=
Tissue processing	20000/=	50,000/=
Travel expenses & stationery	50000/=	50,000/=
Reagent &jik/detergents	65000/=	65,000/=
Labour	50000/=	50000/=
Total		**361,000**

APPENDIX III: DATA COLLECTION TOOLS

SHEET 1.

DATA CAPTURE SHEET- GROSS MORPHOLOGY ALBINO RAT LIVER

DATE……..

GROUP CODE……..

ID CODE…….

GENDER……..

DATE OF HARVESTING…….

FIXATIVE USED……...

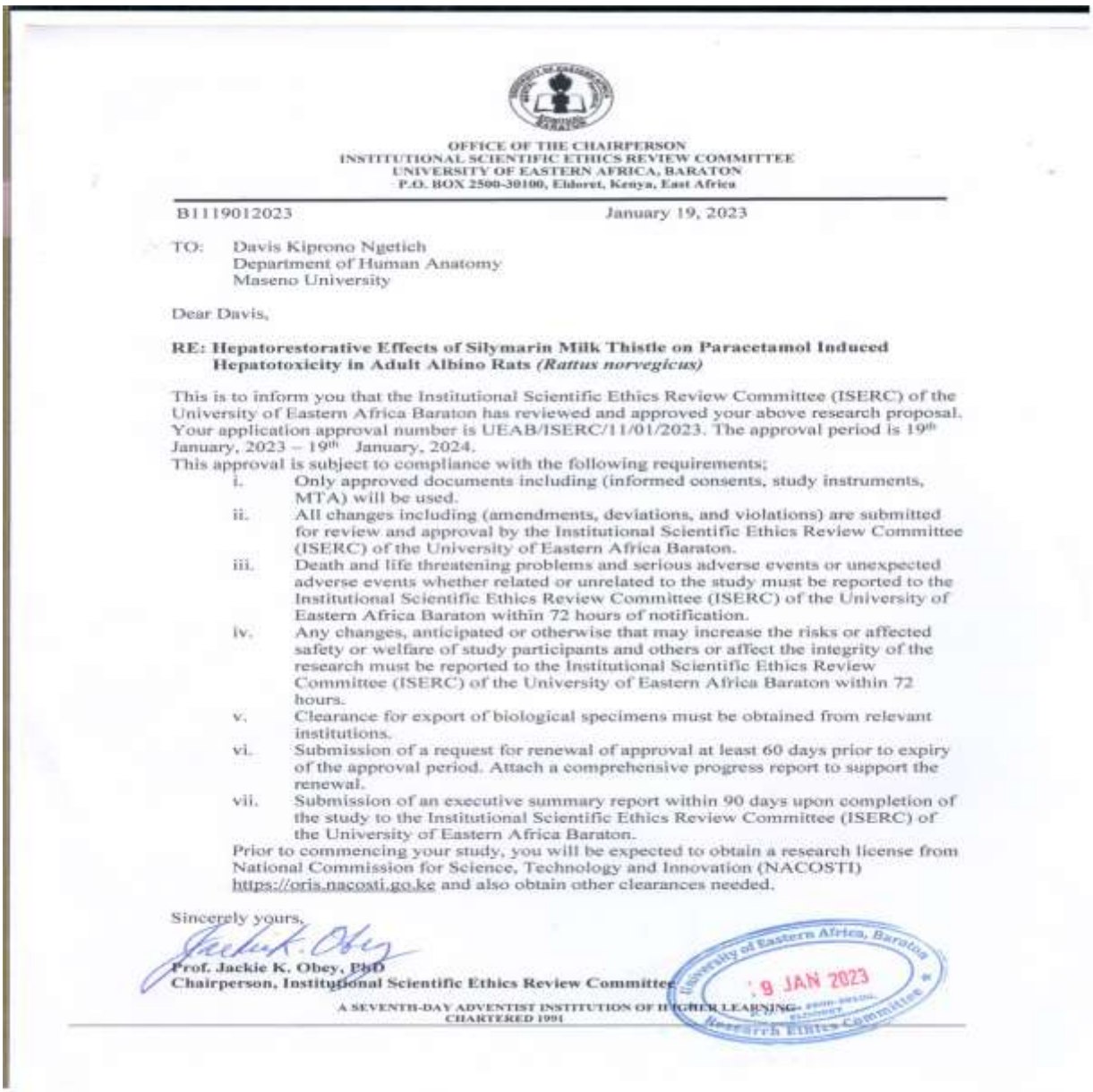
No.	Gross appearance	thickness (mm)	Length (mm)	Width (mm)	Total volume

DAILY WEIGHT.

RAT UNIQUE NUMBER/ GROUP **DAILY WEIGHT.**

GROUP 1	**BEFORE**	**DURING**	**POST**
Rat Unique No	**ADMINISTRATION OF SIL**	**EXPERIMENT**	**EXPERIMENT.**
Rat 1			
Rat 2			
Rat 3			
Rat 4			
Rat 5			
Rat 6			

APPENDIX IV: APPROVAL LETTER FROM ETHICS COMMITTEE



OFFICE OF THE CHAIRPERSON
INSTITUTIONAL SCIENTIFIC ETHICS REVIEW COMMITTEE
UNIVERSITY OF EASTERN AFRICA, BARATON
P.O. BOX 2500-30100, Eldoret, Kenya, East Africa

B1119012023 January 19, 2023

TO: Davis Kiprono Ngetich
Department of Human Anatomy
Maseno University

Dear Davis,

RE: Hepatorestorative Effects of Silymarin Milk Thistle on Paracetamol Induced Hepatotoxicity in Adult Albino Rats *(Rattus norvegicus)*

This is to inform you that the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton has reviewed and approved your above research proposal. Your application approval number is UEAB/ISERC/11/01/2023. The approval period is 19th January, 2023 – 19th January, 2024.
This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Sincerely yours,

Prof. Jackie K. Obey, PhD
Chairperson, Institutional Scientific Ethics Review Committee

University of Eastern Africa, Baraton
19 JAN 2023
Research Ethics Committee

A SEVENTH-DAY ADVENTIST INSTITUTION OF HIGHER LEARNING
CHARTERED 1991

APPENDIX V: RESEARCH LICENSE FROM NACOSTI

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 410333

Date of Issue: 01/March/2023

RESEARCH LICENSE

This is to Certify that Mr.. DAVIS KIPRONO NGETICH of Maseno University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Kisumu on the topic: HEPATORESTORATIVE EFFECTS OF SILYMARIN MILK THISTLE ON PARACETAMOL INDUCED HEPATOTOXICITY IN ADULT ALBINO RATS (RATTUS NORVEGICUS) for the period ending : 01/March/2024.

License No: NACOSTI/P/23/23588

410333

Applicant Identification Number

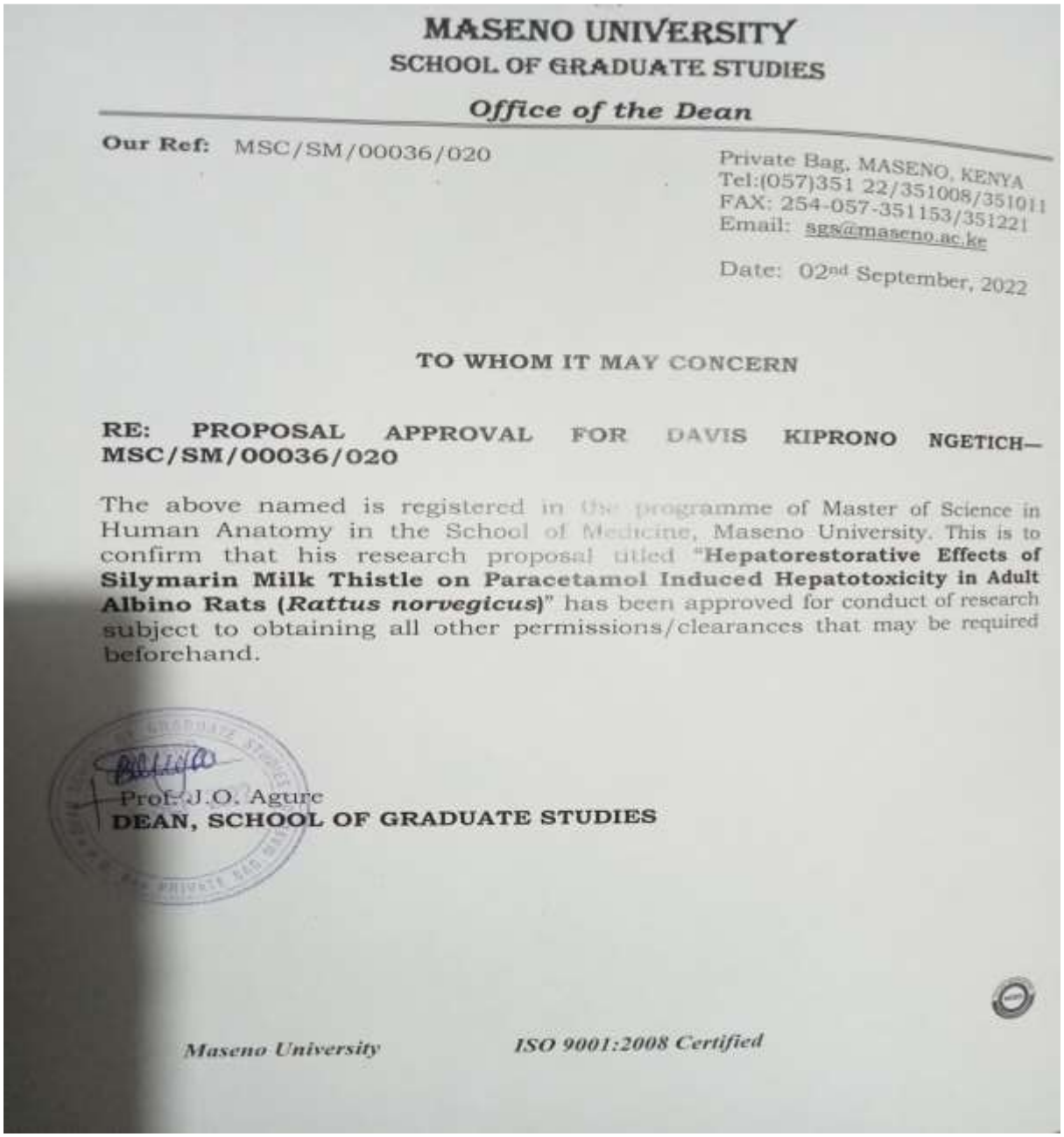
Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

APPENDIX VI: PROPASAL APPROVAL FROM MASENO UNIVERSITY



MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES
Office of the Dean

Our Ref: MSC/SM/00036/020

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FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 02nd September, 2022

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR DAVIS KIPRONO NGETICH– MSC/SM/00036/020

The above named is registered in the programme of Master of Science in Human Anatomy in the School of Medicine, Maseno University. This is to confirm that his research proposal titled "**Hepatorestorative Effects of Silymarin Milk Thistle on Paracetamol Induced Hepatotoxicity in Adult Albino Rats (*Rattus norvegicus*)**" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*