# THE AMELIORATIVE EFFECT OF *ACACIA SIEBERIANA* D.C. METHANOL ROOT BARK EXTRACT ON INDUCED LIVER INJURY AND ITS SUBCHRONIC TOXICITY PROFILE IN RATS

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**MARCH, 2018**

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**BY**

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**MARCH, 2018**

## DECLARATION

I declare that the work in this dissertation entitled **"**The Ameliorative Effect of *Acacia sieberiana* D.C. Methanol Root Bark Extract on Induced Liver Injury and its Subchronic Toxicity Profile in Rats" has been carried out by me in the Department of Pharmacology and Therapeutics. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented for another degree or diploma at this or any other institution.

Watafua, Miriam _____ _____

Name of Student Signature Date

## CERTIFICATION

This dissertation entitled "**THEAMELIORATIVE EFFECT OF *ACACIA SIEBERIANA* D.C. METHANOL ROOT BARK EXTRACT ON INDUCED LIVER INJURY AND ITS SUBCHRONIC TOXICITY PROFILE IN RATS**" by **MIRIAM WATAFUA** meets the regulations governing the award of degree of Master of Science in Pharmacology and Therapeutics of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

| | | |
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## ABBREVIATIONS, GLOSSARY AND SYMBOLS

ABU - Ahmadu Bello University

AIDS - Acquired immune deficiency syndrome

ALP - Alkaline phosphatase

ALT - Alanine aminotransferase

ANOVA - Analysis of variance

AST - Aspartate aminotransferase

BDL - Bile duct ligation

CAT - Catalase

$Cl^-$ - Chloride ion

DILI - Drug-induced liver injury

DNA - Deoxyribonucleic acid

g - Grams

g/dL- Grams per decilitre

GSH - Glutathione

GPx - Glutathione peroxidase

Hb - Haemoglobin

HB - Hepatitis B

HBV - Hepatitis B virus

HCC - Hepatocellular carcinoma

$HCO_3^-$ - Bicarbonate ion

HCV - Hepatitis C virus

H & E - Haematoxylin and Eosin

*i.p* - Intraperitoneal

IU/L - International units per Litre

$K^+$ - Potassium ion

$LD_{50}$ - Lethal dose in 50% of population

LPO - Lipid peroxidation

MDA - Malondialdehyde

mEq/L - Milliequivalents per litre

mg/dL - Milligram per decilitre

mg/kg - Milligram per kilogram

MFOs - Mixed function oxidase system

mmol - Millimoles

mmol/L - Millimoles per litre

n - Number of animals in a group

$Na^+$ - Sodium ion

NAFLD - Non-alcoholic fatty liver disease

NAPQI - N-acetyl-p-benzoquinone imine

ng/mL - Nanogram per millilitre

nmol/mL - Nanomoles per millilitre

OECD - Organisation of Economic Cooperation and Development

PCM - Paracetamol

< - Less than

≤ - Less than or equal to

*P*- Probability

PCV - Packed cell volume

*p.o* - Per Os

RBC - Red blood cell

RNA - Ribonucleic acid

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SEM - Standard error of mean

SOD - Superoxide dismutase

| | |
|---|---|
| USA - | United States of America |
| WBC - | White blood cell |
| WHO - | World Health Organisation |
| ± - | Plus or Minus |
| * - | Asterisk |
| µL - | Microlitre |
| µmol/L - | Micromole per litre |
| % - | Percentage |

## ABSTRACT

*Acacia sieberiana* D.C is traditionally used as a remedy for various ailments including hepatitis. The effect of methanol root bark extract of *Acacia sieberiana* (ASE) on paracetamol (PCM) - and bile duct ligation (BDL) –induced liver injury wereinvestigated in rats. Rats were divided into six groups (Groups I-VI) of 5 and 6 rats for PCM and BDL, respectively. For the PCM-induced liver injury study, animals were pre-treated with normal saline, silymarin (50mg/kg) and extract (250, 750, 1,500 mg/kg)for 7 days. After the last dose, liver injury was initiated by the administration of PCM (2g/kg). In BDL-induced liver injury study, 6 non-ligated but operated rats were used as control (Group I), Groups I and II received normal saline while Groups III-VI received silymarin (50mg/kg), the extract (125, 250 and 380 mg/kg), respectively, for 7 days. At the end of the experiment, blood and organs were obtainedfor biochemical and histological assay. Results showed the presence of carbohydrates, glycosides, triterpenes, cardiac glycosides, saponins, tannins, flavonoids and alkaloids. The $LD_{50}$ values for oral and intraperitoneal routes of ASE were >5,000 and 1,300 mg/kg respectively. The subchronic toxicity study revealed that ASE significantly ($P$<0.05) reduced body weight when compared to the control and significantly ($P$<0.05) elevated ALP, whereas, serum urea and blood lymphocytes were significantly ($P$<0.05) increased at the 1500mg/kg dose group. The histology of the heart revealed no effect. However, the liver produced a dose-dependent hepatocellular necrosis and vacuolations. There was also lymphocyte hyperplasia and glomerular necrosis of the kidneys and alveolar congestion of the lungs. PCM significantly ($P$<0.05) elevated ALT, AST, direct Bilirubin and significantly ($P$<0.05) decreased total protein and albumin when compared to ASE. Whereas, ASE at 250 and 750 mg/kg was seen to significantly ($P$<0.05) decrease the levels of ALT and AST, while

total protein and albumin were significantly ($P$<0.05) elevated in ASE-treated groups. However, direct bilirubin was significantly ($P$<0.05) decreased in silymarin group and 750 mg/kg ASE group. Furthermore, PCM significantly ($P$<0.05) decreased SOD and increased MDA when compared with ASE pre-treated groups. The histology revealed, PCM caused severe necrosis of the hepatocytes with vascular and sinusoid congestions but the impacts were mild with silymarin and lower doses of ASE pre-treated groups. The BDL–induced liver injury study revealed that ASE significantly ($P$<0.05) decreased AST level at lower doses and significantly decreased ALP level at higher doses. Likewise, ASE significantly ($P$<0.05) decreased direct and total bilirubin levels in silymarin and ASE treated groups. There was significant ($P$<0.05) elevation of SOD at lower ASE doses while GPx and CAT were significantly ($P$<0.05) elevated in ASE-treated groups. Furthermore, MDA was only significantly decreased at 125 mg/kg ASE group. Histology revealed that themorphology of liver tissue was preserved at 125 and 250 mg/kg ASE groups from BDL-induced moderate necrosis and vascular congestion. The study concludes that *Acacia sieberiana* is relatively non-toxic with acute administration and has hepatoprotective potentials on PCM- and BDL-induced liver injury at its lower doses but could be relatively toxic at its higher doses and/with prolonged use.

# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Hepatotoxic Injury and Disease

The liver is a vital body organ involved in several physiologic functions. It is the main organ of metabolism of xenobiotics and also the site for glycogen storage, bile formation and plasma protein synthesis (Andersen, 2012). It plays an excretory role not only as a traverse site for all ingested agents from portal artery to the general circulation, but also as bearer of bile that excretescertain substances. Due to its diverse functions, the liver is constantly exposed and vulnerable to both direct and indirect toxic injuries from foreign agents (Singh *et al*., 2011). Although it is naturally endowed with ability to regenerate its lost tissues, certain hepatic injuries or diseases sometimes tend to progress beyond this ability and may result in weakening, liver failure or death (Owais *et al*., 2006).Inflammation of the liver (hepatitis) is the most prevalent liver disease contracted from encounters with viruses, drugs or poisons. Viral hepatitis, also referred to as catarrhal jaundice is an infective or transmissible hepatitiscaused by hepatitis A, B C, D and E viruses (Mahl and O'Grady, 2006).Hepatitis of poisons or drug origin also referred to as hepatotoxin hepatitisis often due to selective toxicity of toxic substances on the liver. Liver inflammations are often part of systemic toxicity from hepatotoxins and are most predominant with highly toxic lipid soluble parent drugs with reduced metabolic rate, or that defy detoxification(eg aflatoxins) or that form metabolites of more toxicity(Thonda and Shivalinge, 2012).

Generally, undiagnosed or unmanaged hepatic injury or damage from either infective-viral or hepatotoxin origin may progress from acute hepatitis of mere inflammatory reactions to chronic fibrosis, cirrhosis or liver failure (Tag*et al*., 2015). Thus, liver damage may be acute

or chronic, but chronic liver damage is much more common, because acute effects often tend to remain latent and only appear visible in its clinical stage (McLaughlin, 2000). The early stage symptoms of liver disease are usually vague or completely absent due to the large reserve capacity of the liver and its regenerative ability.Liver damage manifests only in its overt or clinical stage and atypical sign of liver damage (jaundice or hyperbilirubinaemia)appears as sclerae or mucosae (yellow skin) (Black, 1997).

Liver damage either in its acute or chronic stage is of remarkably high morbidity and mortality as it eventually often tends to affect other body organs including the GIT and others(Younossi *et al.*, 2011).For instance, the grave consequence of accumulation of the bile fragments (jaundice) either from excessive splenic breakdown of haemoglobin of red blood cells (haemolytic jaundice) or from biliary tract obstruction due to bile stasis (cholestatic jaundice) occurs from its highly harmful effect on metabolically active tissues in the body especially of the basal nuclei of immature brain (Black, 1997; Mahl and O'Grady, 2006). Hepatotoxins can cause cytotoxic, cholestaticor a mixed pattern of injury, but thetypical morphological pattern of a diseased liver is fatty degeneration (steatosis) resulting in lipid accumulation, hypertrophy of hepatocytes and/or massive necrosis (death or large scar) often referred to as cirrhosis (Tag*et al.*, 2015).

Antiviral drugs are often used to curb the pathogenesis of the infective or viral hepatitis, and the autoimmune hepatitis had been reported to respond to corticosteroids even though its use is associated with severe morbidity and mortality (Mahl and O'Grady, 2006; Mahmood *et al.*, 2014). The hepatitis from certain hepatotoxins can only be halted by withdrawal from the causative hepatotoxin, use oflarvage, cathartics or other systemic elimination strategies or specific antidotes for the ingested toxins.These measures are not feasible in hepatitis

ofunknown causative agentand which often tend to progress uncontrollably. There has not been any therapeutic agent that stops the progression of hepatitis of unknown origin into its end cirrhotic stage. Most often, plant-derived medicines meet certain health care needs and *Acacia sieberiana* is one of such locally used plant in the management of liver derangements. The widespread use of plant-based remedies is not only due to ready accessibility, but also because of experience from long term ancestral historic use. 25% of modern medicines had been reported to have been derived from locally used plants (WHO, 2003) but still,medicinal plants remain a huge untapped indispensable source of therapeutic drugs for the continual search of agents to reverse derangements in body systems including that of liver (Suruchi *et al*., 2013). This study therefore attempts to evaluate the ameliorative effect (if any) of root bark of *Acacia sieberiana* on induced hepatotoxic injuries for scientific basis of its local use.

## 1.2 Statement of Research Problem

Insults on the liver may manifest as mere inflammations but, if unresolved or unmanaged, theyoften progress to scarring (fibrosis) or necrositic disruption of the hepatic architecture (cirrhosis) (Greshwin*et al*., 2005).

Cirrhotic liver has high risk of developing into hepatocellular carcinoma (HCC), which had been reported to be a leading cause of cancer death worldwide (WHO, 2003; Greshwin *et al*., 2005).

Hepatic injurytends to be asymptomatic at early stages because of the high reserve and/or regenerative capacity of the liver and only manifests in its overt or clinical stage. If this is not promptly managed or controlled, it usually poses serious problems from its disruption of the

liver metabolic functions that leads to severe and fatal metabolic disorders that disorganise the entire body system (Akilavalli *et al*., 2011).

Hepatitis often progresses into fibrosis and cirrhosis and no orthodox drug at present can reverse or halt this progression for the hepatitis of unidentified causeand can only be diagnosed from liver sample biopsy which is scarcely performed in many resource constrained settings (Greshwin *et al*., 2005).

Many synthetic drugs and most herbal products including those used for treating liver diseases had been reported by Rashchi and Ponti, (2015) to cause liver injury in diverse ways including undergoing metabolic activation to form toxic reactive metabolites.The increasing popularity of use of unregulated drugs for self medication and herbal products not only as supplements for medical reasons, but also for body-building, weight control etc, often results in drug-induced liver injury (DILI) of unknown cause (Rashchi and Ponti, 2015; Valdivia-Correa *et al*., 2016). Drug-induced liver damage accounts for about 5% of all hospital admissions and 50% of all acute liver failure (Singh *et al*., 2012; Mokdad *et al*., 2014).

The typical morphological pattern of a diseased liver is generally the same irrespective of its origin (viral or hepatotoxic) and cirrhosis, which is its end stage,has been associated with approximately 31,000 annual deaths in the U.S. alone, with a record of about 19,854 annual deaths in Nigeria as at 2010, thus, cirrhosis is a leading cause of death worldwide (WHO, 2003; Mokdad *et al*., 2014).About 250 million viral hepatitis infections occur annually with 1.4 million deaths, and out of about the 100 million infections in Sub-Saharan Africa, 11-13.7 million occur in Nigeria(World Hepatitis Alliance, 2010).

HCV- related cirrhotic liver has been reported in approximately 3% of the world's population,and there is also an estimated 2-3% worldwide carrier of the highly infective hepatitis B virus found mostly in tropical countries (Singh *et al*., 2012; Mokdad *et al*., 2014).

## 1.3 Justification of the Study

Readily accessible agents with liver rejuvenatingproperties for optimal liver activity may be required from time to time.Discovery of agents that halt or reversethe progression of hepatitis to the chronic stages (fibrosis, cirrhosis or liver failure) is of paramount importance in the management of liver diseases because of the high morbidity and mortality rates associated with both acute and chronic liver injuries.

Obstruction of bile flow to liver either by cannulation or ligationin animal models had been reported to induce mixed pattern liver injury morphological changes comparable to human liver inflammation, fibrosis and cirrhosis (Tag*et al*., 2015), thus, agents that can reduce these effects in experimental models may also be beneficial in related conditions of humans.

## 1.4 AimandObjectives

### 1.4.1 Aim

The aim of this study is to investigate the subchronic toxicity potential and the effect of the methanol extract of *Acacia sieberiana* root bark on paracetamol- and bile duct ligation-induced liver injuries in rats.

### 1.4.2 SpecificObjectives

i. To identify the phytochemical constituents present in the methanol root bark extract of *Acacia sieberiana*.

ii. To evaluate the acute and subchronic toxicity profile (biochemical, haematological and histological study) of the extract in rats.

iii. To evaluate theameliorative effectof the extract on some biochemical, oxidative stress and histohepatic changes of paracetamol- and bile duct ligation- induced liver injuries in rats.

## 1.5 ResearchHypothesis

The methanol root bark extract of *Acacia sieberiana* has ameliorative effect on liver injury and it is relatively non-toxic.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Toxicity Evaluation and Medicinal Plants

Herbal plants or botanical medicines have been used traditionally worldwide for the prevention and treatment of diseases including liver dysfunctions (Suruchi *et al*.,2013). Although many plant medicines have been reported to be hepatotoxic, quite a number of plants possess hepatoprotective activity and are used in the treatment of liver diseases and these includes,*Ficus hispida, Tephrosia purpure, Curcuma longa, Glychyrrhiza glabra, Cichorium intybus, Taraxacum officinale, Silybum marianum* amongst others (Mandal *et al*, 2000;Samir and Amrit, 2014). Some phytoconstituents such as phenolic compounds, terpenoids, some glycosides, saponins, flavonoids and nitrogenous compounds like alkaloids had been reported to possess hepatoprotective effects (Valan *et al*., 2010). Today's health care systems and most of the world's populations (80%) especially of developing countries rely largely on plants-derived medicines to meet daily requirements and in fact, the two most important antimalarial drugs (quinine, artemisinins) now frequently in use are derivatives of plants. The use of plant-based remedies is also widespread in Nigeria where several plants are locally used to treat various ailments. The current widespread resort to medicinal plants as components of health care particularly at grass root levels worldwide is mostly because of their readily accessibility and experience from long-term ancestral historic use. Medicinal plants, therefore, remain a huge untapped indispensable source of therapeutic drugs. Efforts are continually being made in search of agents to reverse derangements in body systems and scientific research into substances used in herbal medicine practices had led to the discovery of potential therapeutic agents (Suruchi *et al*., 2013). Substances of medicinal values from any part of medicinal plant also serve as precursors in the synthesis of useful drugs.

Many plants locally claimed to possess useful therapeutic activity are often scientifically investigated not only to authenticate the purported efficacy or identifythe phytoconstituents responsible for the activity, but also to ascertain the potential intrinsic hazards of undesirable or adverse toxic effects (David and Enegide, 2014). Frank and Ottobani, (2011)defined toxicity as 'the ability of a chemical to damage an organ system, such as the liver or kidneys, or disrupt a biochemical process, such as the blood forming mechanism, or to disturb an enzyme system at some site in the body'. The toxicity evaluations routinely performed in the investigation of safety of new agents include the acute, sub-acute and chronic toxicity studies (Bhardwaj and Deepika, 2012).Acute toxicity study investigates an agent for immediate toxic symptoms or reactions that occur within 24 hours; subchronic toxicity study investigates an agent for toxic symptoms from repeated exposures that takes days or few weeks to manifest, while chronic toxicity study investigates an agent for toxic symptoms that manifest after months or years of exposure (Frank, 2008; Bhardwaj and Deepika, 2012). In the present study, only the acute and subchronic toxicity studies were used to evaluate the toxicity profile of *Acacia sieberiana.*

## 2.2 The Plant, *Acacia sieberiana*

### 2.2.1 Taxonomic nomenclature

Plant Name: *Acacia sieberiana* var. villosa D.C

Kingdom: Plantae

Phyllum: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: Acacia

Species: Sieberiana

Variety: Villosa

This classification of *Acacia sieberiana* is according to the International Legume Database Information Service (ILDIS, 2017).

Common Names: English:white thorn, umbrella thorn, flat-topped thorn, paperback thorn or paperback;Hausa: *Farar kaya*; Yoruba: *Aluki* or *Sie*;Igbo: *siyi*; Fulani: *Daneji*; Zulu: *Umkhaya*(Owra *et al*., 2009; Paul and Nnabuk, 2014).

### 2.2.5 Description, origin and geographical distribution

*Acacia sieberiana* (Plate I) is a tree of 3-25m height and 0.6-1.8 m diameter. The bark is rough, yellowish and peels off in small, rectangular, grey-brown scales with gummy exudates.Its leaves are usually sparse and hairy and often bunched in pairs into small clusters from a common stalk, while the flowers are cream, white or pale yellow in colour. It has dehiscent shiny brownfruits of about 1.3 cm thick, 9-21 cm long and 1.7-3.5 cm wide and which slowly splits open to release about 12 seeds (Owra *et al*., 2009; Dawarung *et al*., 2012).The threerecognized varieties of the plant are *A. sieberiana var. sieberiana*, *A. sieberiana var. woodii*and *A. sieberiana var. villosa*(Owra *et al.*, 2009).

*Acacia sieberiana* grows in the savannah and woodland andseen with various botanical characteristics in the whole of the Sahel and other semi-arid regions in Africa. Itis drought and frost resistant and widely distributed in countries like Benin, Cameroon, Chad, Ethiopia, Gambia, Ghana, Kenya, Liberia, Mali, Mauritania, Mozambique, Namibia,, Nigeria, Portugal, Senegal, Sierra Leone, South Africa, Sudan, Swaziland, Tanzania, Togo, Uganda, Zambia,

Zimbabwe, India etc. In Nigeria, it is grown extensively as an economic tree in the Northern regions especially in Yobe, Jigawa and Sokoto States (Paul and Nnabuk, 2014).



Plate Ia: The whole tree



Plate Ib: leaves, flowers and thorns

**Plate I:** ***Acacia sieberiana*** **in its natural habitat**

### 2.2.3 Uses of *Acacia sieberiana*

*2.2.3.1 Non-medicinal uses*

According to Owra *et al*. (2009), *A. sieberiana* is used as/in:

Food: *A. sieberiana* produces an edible gum.

Fodder: Pods, young shoots and leaves are highly nutritious and serve as forage for livestock in the dry season. In Sudan, pods are collected for fattening sheep but are said to taint milk.

Apiculture: Flowers of the tree are good bee forage, and bee hives are often placed on the trees.

Fuel: *A. sieberiana* is a good source of firewood and charcoal.

Timber: The termite resistant, moderately hardwood has a featureless, off-white grain with little distinction between heartwood and sapwood. It is easy to work and is used in making furniture, tool handles and mortars.

Gum or resin: The tree produces gum of good quality that has been used to make ink.

Dyestuff: The bark and pods contain tannin which is used as dyestuff.

*2.2.3.2 Ethnomedicinal uses*

In most African traditional medicine practices, the tree is known as a treatment for many ailments. In Eritrea,a decoction of the root is used traditionally for the treatment of abdominal pains and stomach-aches and also for acne, tapeworms, urethral problems, oedema and dropsy (Owra *et al.*,2009). A decoction of the root is also used by the people of Plateau State, Nigeria to treat hepatitis in humans (Ohemu *et al*., 2014).The bark, leaves and gums are used to treat tapeworm, bilharzia, haemorrhage, orchitis, colds, diarrhoea, gonorrhoea, kidney problems, syphilis, ophthalmia, rheumatism and disorders of the circulatory system and the powdered bark is used to relieve fever in children. *A. sieberiana* is used by the people of plateau state,

Nigeria to treat diarrhoea in animals and humans (Offiah *et al*., 2011).The pods serve as an emollient and as astringent.

### 2.2.4 Previous Pharmacological Studies on*Acacia sieberiana*

*Anti-diarrhoeal Activity*

The acetone extract of *A. sieberiana* stem bark slowed down gastro intestinal motility and castor oil-induced diarrhoea tests in rats (Zeuko *et al*., 2015). Dawarung *et al*. (2012) also reported that its aqueous and ethanolleaf extracts had anti-diarrhoeal activity in rats.

*Anti-microbial activity*

The antimicrobial activities of *A. sieberiana* leaf and bark extracts have been reported on *Mycobacterium aurum, Bacillus subtilis, Staphyllococcus aureus, Escherichia coli, Kiebsella pneumonia,* and *Staphyllococcus epidermis* (Rabe and Van Staden, 1997; Eldeen *et al*., 2005; Eldeen and Van Staden, 2007).

## 2.3 The Liver

### 2.3.1 Location and parts of the liver

The liver is the largest organ of the human body weighing approximately 1,500g and located in the upper right corner of the abdomen beneath the diaphragm. It is a highly vascular dark reddish brown organ that performs many vital metabolic, synthetic, transport and storage functions (Arias, 1994). The liver has five (5) cell types including, hepatocytes, sinusoids, kupffer cells, bile duct epithelial cells and Ito cells (Eric, 2002).

The hepatocytes are more predominant and represent 60% of the liver cells and are responsible for most of the synthetic and metabolic activities of the liver. Each hepatocyte has an average lifespan of 150 days (British liver Trust,2011).

The liver sinusoids are the canals through which blood flows around the liver lobules into the central vein. About 75% blood supply to general circulation is often routed via the hepatic portal vein and hepatic arteries. The portal venous blood usually from parts of the body is often nutrient filled, but low in oxygen pressure, while arterial blood is directly from the aorta and is high in oxygen and pressure (Andersen, 2012).

The kupffer cells are macrophages in the sinusoids that breakdown haem-iron pigment of haemoglobins into bilirubin for removal from bile duct epithelial cells along with old red blood cells and bacterial cells (Eric, 2002). The Ito cells in presence of liver injury transform into collagen producing cells.

### 2.3.2 Functions of the liver

*2.3.2.1 Synthetic functions*

The liver synthesises:

- glucose from certain amino acids, lactate or glycerol and also converts excess glucose into glycogen for storage
- coagulation factors (thrombopoietin, fibrinogen, prothrombin etc)
- anticoagulant (heparin)
- plasma proteins such as albumin and globulin
- bile required for emulsification or breakdown of fats into smaller components for easy digestion

- foetal red blood cell production in the first trimester period is majorly from the liver
- cholesterol and its esters including triglycerides and very low density lipoproteins
- It also synthesises insulin-like growth factors, alpha1-antitrypsin, haptoglobin, ceruloplasmin, transferrin, alpha feto proteins etc. (Anderson, 2012).

*2.3.2.2 Metabolic functions*

- The hepatocyte contains enzymes including cytochrome $P_{450}$s which metabolise lipophilic substances to produce their metabolitesof either detoxified or activated types.
- The liver is the major site for metabolism and elimination of foreign agents and does not only filter out harmful substances from the bloodstream for excretion, but also synthesizes bile which excretes certain substance via its enterohepatic cycle process.
- Amino acid deamination takes place in the liver.
- The site of urea cycle which converts released ammonia into urea for excretion in urine and this represents about 90% of the total urinary nitrogen excretion (Mitra and Metcalf, 2012).

*2.3.2.3 Storage functions*

- The liver is the storage site for glycogen, fat and fat-soluble vitamins. It stores glucose in the form of glycogen with which to balance the nutrient supply on body demand.
- It also regulates the amino acid blood levels by converting excess glucose and amino acids into fatty acids for storage (Eric, 2002).
- It also stores vitamins A, D and $B_{12}$, iron and copper (Anderson, 2012).

## 2.4 Liver Diseases

### 2.4.1 Definition and consequences of liver disease

Liver injury or disease is the change in anatomy and functions of the liver. Insults to the liver may manifest as inflammations, but unresolved inflammatory cells often progress to scarring (fibrosis) or disruption of hepatic architecture (cirrhosis) (Greshwin*at al.*, 2005). Inflammation of the liver may be acute (lasting for less than six months) or chronic (persisting for longer periods). Chronic liver diseases are often more prevalent than the acute forms and also poses serious treatment or management problems. Persistent liver inflammation that leads to cirrhosis is a leading cause of death (WHO, 2003). Cirrhosis depresses the hepatocyte activity and inhibits blood flow and thus impairs liver functions and its regenerative ability (Eric, 2002). The obstruction in blood flow can cause portal hypertension and/or ascites, swollen or shunting of vein (varices), haemorrhage, hepatic encephalopathy, kidney disorders, osteoporosis and liver cancer. Worthy to note is that the onset of cirrhosis is often asymptomatic and individuals with cirrhosis can lead relatively normal lives for many years.

### 2.4.2 Causes of liver diseases

- toxins: alcohol, smokes, certain drugs and medicinal herbs, food and environmental contaminants such as vinylchloride etc
- viral infections (hepatitis A, B, C and Eviruses)
- autoimmune disorders and hereditary factors
- congenital birth defects
- nutritional deficiencies
- trauma(Tsai *et al.*, 2003; Mastrangelo *et al.*, 2004)

### 2.4.3 Types or forms of liver diseases

#### *2.4.3.1 Viral hepatitis (hepatic inflammation of viral origin)*

This is mostly caused by infections of hepatotropic viruses (hepatitis A, B, C and E viruses) and less commonly by other viruses that cause mononucleosis, yellow fever, leptospirosis and cytomegalovirus infections. Most cases of acute viral hepatitis are self-limiting and resolves spontaneously, but some tend to progress to chronic forms. Hepatitis A virus (HAV) is particularly common among children and young adults and occurs mostly in areas with poor hygiene transmitted via faecal oral route (from infected food and water)(Ryder and Beckingham, 2001).

Hepatitis B virus (HBV) is usually transmitted parenterally from transfusion of donor bloods with hepatitis B surface antigen (HBsAg). Serum HBsAg has a high potential for infectivity, but HBV can also be contacted from body fluids such as saliva, sneeze specimens or semen and it is usually of severe or fatal consequences. An estimated 2-3% of the world's populations, mostly of the tropical countries, are carriers of hepatitis B (Mahl and O'Grady, 2006).

Hepatitis C virus (HCV) infection is also transmitted via piercing of the body with infected or contaminated used sharp objects and can also very uncommonly be transmitted sexually or from mother to infants. Hepatitis D virus (HDV) is a defective RNA virus that can replicate only in the presence of HBV and this often occurs as a co- or super- infection with HBV. Hepatitis E virus (HEV) is an enteric RNA virus often transmitted sporadically or waterborne (Ryder and Beckingham, 2001).

About 250 million viral hepatitis infections occur annually with 1.4 million deaths, and of about the 100 million infections in Sub-Saharan Africa, 11-13.7 million occur in Nigeria

(World Hepatitis Alliance, 2010). Generally, chronic viral hepatitis is commonly caused by hepatitis B and C viruses. Worldwide, approximately 170 million people show chronic infection of hepatitis C virus and 16,000 die annually; and of the 2.7 – 3.9 million annual infections in U.S, 31,000 million annual deaths occurs from HBV and 1.2 million deaths from HCV infections (Younossi *et al.*, 2011). Thus, HCV infections have been reported as the most prevalent viral hepatitis and the leading cause of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (Davis *et al*., 2003).

*2.4.3.2 Alcoholic and non-alcoholic fatty liver disease:*

Alcoholic liver disease is damage to the liver caused by excessive intake of alcohol and which present in variant features either as fatty liver, hepatitis or cirrhosis. Hepatocellular carcinoma may also develop especially in association with iron accumulation (haemochromatosis) (Eldon, 2009a).

Non-alcoholic fatty liver disease (NAFLD) is majorly common in children and young adults. In a study conducted in the U.S., the leading causes of NAFLD were obesity and AIDs amongst others like HCV and HBV, dyslipidaemia and glucose intolerance (Younossi *et al.*, 2011).

Fatty liver disease (hepatic steatosis) typical of excessive alcohol consumption is characterised by hepatomegally, which is potentially reversible and steatohepatitis or alcoholic hepatitis which is a fatty liver with necrositic inflammations that results in cirrhotic shrinkage of the liver (Eldon, 2009a). Fatty liver disease is the excessive accumulation of lipid (fatty infiltration) in the hepatocytes in response to injury (Herrine, 2009).

*2.4.3.3 Liver cancer (hepatocellular carcinoma or hepatoma)*

This is a primary liver cancer amongst others like fibrolamellar carcinoma, cholangiocarcinoma, hepatoblastoma and angiosarcoma. Hepatocellular carcinoma usually occurs in patients with cirrhosis and in areas where infections with HBV and HCV are prevalent. Other causes of hepatocellular carcinoma are haemochromatosis (Herrine, 2009).

## 2.5 Symptoms of liver diseases

There are usually no obvious symptoms at the early stages of liver disease except vague signs of weakness and loss of energy. In the acute liver disease, symptoms may include jaundice, dark urine, loss of appetite, nausea, vomiting and diarrhoea. Chronic liver disease presents typically with abdominal pain and swelling due to ascites, weight loss or gain (Mastrangelo *et al.*, 2004). The most frequently reported common symptoms of liver disease as reported by Ryder and Beckingham, (2001) include:

i. Jaundice: this usually is the first sign and sometimes the only sign that manifests as yellow sclera due to abnormally high levels of bilirubin (bile pigments) in the blood. Bilirubin makes the urine dark and the increase in its level may be due to blockade of the bile duct or excessive breakdown of red blood cells.
ii. Cholestasis (reduction in bile flow): intrahepatic or extrahepatic reduction in bile flow may result in jaundice,enlarged spleen, enlarged gallbladder etc.
iii. Hepatomegally: this is usually a positive indicator of liver disease
iv. Portal hypertension (abnormally high blood pressure in the portal vein): due to resistance to blood flow in the liver and this may result in bleeding of the varicose veins at the lower end of the esophagus and in stomach lining.

v. Ascites: this is fluid build-up in the abdominal cavity.

vi. Liver encephalopathy (portal systemic encephalopathy, hepatic encephalopathy or hepatic coma): this is the deterioration of brain function caused from build-up of removed toxic substances normally removed by the liver and this often impairs consciousness.

vii. Liver failure: this occurs in severe deterioration of large portion of the liver and/or liver functions.

viii. Wilson's disease: this is a hepatic degenerative disease due to disturbances in copper metabolism manifested by irregular and spasmodic uncontrollable jerky movements.

## 2.6 Diagnosis of Liver Disease

Damage to liver could occur in the form of biochemical or histologic changes resulting in its physiological dysfunction, and which can be detected by quantification of certain liver biomarkers in laboratory tests. Tests can also be used to detect the prognostic course, severity or drug response of liver disease. Some laboratory tests used for hepatic dysfunction include determination of serum levels of liver marker enzymes, bilirubin (total, indirect and direct fractions), serum levels of albumin and total protein and other liver function tests (Alan, 2007).Liver function testassesses the biochemistry and excretory performances of the liver and the other specific diagnostic laboratory tests according to Eldon (2009b) that could be measured include:

- Immunoglobulin M (IgM) antibody to hepatitis A (antiHAV), for acute hepatitis A
- Hepatitis B surface antigen (HBsAg), for hepatitis B
- Antibody to hepatitis C virus (anti-HCV) and HCV.RNA, for hepatitis C

- Antimitochondrial antibody, for primary biliary cirrhosis

### 2.6.1 Diagnostic markers of liver injury and disease

#### *2.6.1.1 Liver enzymes*

In hepatic injury or loss of liver integrity, the transport function of the liver cells is impaired and thus liver enzymes leak into the blood stream or plasma (Usha *et al*., 2008). An augmented serum level of liver marker enzymes is an indication of hepatocellular leakage and/or loss of functional integrity from liver damage. Liver enzymes are sensitive indicators of liver injury and include:

i. alanine aminotransferase (ALT) predominantly found in the liver
ii. aspartate aminotransferase (AST) found also in the heart, skeletal muscle, kidney and pancreas

Usually, marked increase in aminotransferases coincides with hepatocellular necrosis, thus, high values of aminotransferases indicate acute hepatocellular injury (acute hepatitis). A moderate elevation persists in chronic hepatitis or biliary obstructions as in duct stones, but normal or only slightly elevated values can be seen in certain chronic injuries or liver diseases when few hepatocytes are still left to be damaged as in haemochromatosis, drug-induced liver injury and chronic hepatitis C (Giannini *et al*., 2005; bpac, 2007; Eldon, 2009b). A transaminase enzyme catalyses the amino acids catabolic transfer of an α-amino group from an α-amino acid to an α-keto acid inmaintenance of the citric acid cycle.

iii. Alkaline phosphatase: is of a widespread extrahepatic distribution predominating the liver and bone and also found in the intestine and placenta of the 3$^{rd}$ trimester pregnancy. Thus, it is a non-specific marker of cholestasis, hepatitis or

cirrhosis.However, the liver specific alkaline phosphatase has presence of γ-glutamyltransferase or 5'-nucleotidase and these are used to differentiate the hepatic from the extrahepatic sources of the enzyme. Thus, jaundice with concurrent elevation in alkaline phosphatase of γ-glutamyltranspeptidase signifies cholestasis (liver injury from reduced bile flow or obstruction) and this always occurs with pain, whilethe elevation of ALP with normal bilirubin level and without pain or jaundice signifies primary sclerosing cholangitis (PSC)(Alan, 2007). The enzyme level is also increased in bone diseases associated with increased activity of the osteoblasts (rickets, osteomalacia). Children have higher values than adults due to bony tissue development process (Black, 1997; Hall and Cash, 2012). In liver, ALP is found histochemically in the microvilli of bile canaliculi and on the sinusoids surface of the hepatocytes andit is produced in the membranes of cells lining bile duct and canaliculi **(**Hall and Cash, 2012)**.** For liver function tests, moderate increase in ALP with slight increase in AST and ALT depicts cholestasis; moderate increase in ALP with slight or no increase in AST and ALT signifies primary sclerosing cholangitis (Hall and Cash, 2012), while intense increase in ALP with slight or no increase in AST and ALT is an indication of primary biliary cirrhosis.

iv. Lactate dehydrogenase (LDH): is present in liver and in other tissues and thus not specific for hepatocellular injury, but its level is elevated in ischaemic hepatitis and cancers (Alan, 2007).

*2.6.1.2 Bilirubin*

Increased level of serum bilirubin occurs in liver damage. The first physical sign of liver disease is usually an increased serum bilirubin level and thus tests to assess the hepatobiliary

excretion of bilirubin can also be undertaken.Bilirubin is produced from haem proteins like haemoglobins of old or damaged RBCs. Elevated blood levels of bilirubin results in jaundice and dark urination. Total bilirubin is a measure of all the unconjugated (indirect) bilirubin in the body and which increases during defective uptake in liver for conjugation (Eldon, 2009a). However, increase in serum bilirubin could also be due to excess production, obstruction of biliary excretion and haemolysis. Bilirubinalso accumulates in reduced albumin level because bilirubin normally binds to albumin on a ratio of 1:1 (Hansen, 2007).The unconjugated bilirubin is water insoluble and highly albumin-bound, thus the blood transport of bilirubin is usually as attachment to albumin and which reduces its toxic likelihood to enter and damage brain cells. Thus, reduced serum albumin often tends to worsen bilirubin accumulation.The conjugated (direct) bilirubin in the blood is elevated only in conditions of reduced bile formation or excretion (cholestasis), and in markedly elevated blood levels (bilirubinaemia), conjugated bilirubin spills into urine (bilirubinuria) (Hall and Cash, 2012).

#### *2.6.1.3 Serum proteins (albumin and globulin)*

Decreased total protein and albumin levels occur in extensive liver damage, but these are usuallynon-specific markers for liver disorders. Decreased albumin level also occurs in dehydration, malnutrition etc., while high serum globulin also occurs in rheumatoid arthritis, leukaemia etc(Kaslow, 2011). Usually, the level of globulin is inversely related to that of albumin such that the concentration of the serum albumin is reduced in severe liver diseases, that of globulin is usually increased such that the total concentration is rarely low. Increased level of total protein shows hepatoprotective activity as stimulation of protein synthesis accelerates the regeneration process and production of liver cells (Van-leeuwen *et al*., 2011).

*2.6.1.4 Liver synthetic ability*

This couldbe assessed using prothrombintime -PT for fibrinogen synthesis and international normalised ratio – INR for synthesis of vitamin K- dependent clotting factors (II,V, VII and X). An increased PT or INR indicates severe hepatocellular dysfunction (Thapa and Anuj, 2007).

*2.6.1.5 Elevated levels of ammonia*

Most blood ammonia is formed from the breakdown of protein by intestinal bacteria. Ammonia is then converted to urea in the liver which is eliminated in the urine via the kidney. Elevated ammonia level in the blood indicates a liver disorder which is seen in hepatic encephalopathy of advanced liver disorders(Albrecht, 2012).

*2.6.1.6 Marked elevation of serum immunoglobulins (Ig)*

This is seen in autoimmune hepatitis (IgG), biliary cirrhosis (IgM) and alcoholic liver disease (IgA) (Thapa and Anuj, 2007).

*2.6.1.7 Positive antimitochondrial antibodies*

This is seen in primary biliary cirrhosis, autoimmune hepatitis and drug-induced hepatitis (Thapa and Anuj, 2007).

*2.6.1.8 Slight increase in alpha-fetoprotein (AFP)*

This suggests primary hepatocellular carcinoma (HCC).

*2.6.1.9 Leucopenia, thrombocytopenia or pancytopenia:* are implicated in cirrhosis.

*2.6.1.10 Iron accumulation (hemochromatosis):* occurs in liver damage.

#### *2.6.1.1 Liver sample biopsy*

The structural integrity of liver cells can be microscopically evaluated in absence of a known cause. Medical imaging tests are examinations that detect focal liver lesions, abscesses and tumours and include, ultrasonography – a sensitive imaging technique that can differentiate between intrahepatic and extrahepatic causes; computed tomography (CT) scan – a highly accurate imaging technique that identifies small hepatic metastases; magnetic resonance imaging (MRI) technique – that gives more detailed images than CT scan (Califonia Pacific Medical Center, 2010)

### 2.6.2 Oxidative stress markers of liver injury and disease

Oxidative stress tends to worsen liver injury and disease and it occurs when there is an imbalance in the production and elimination of reactive oxygen and nitrogen species and/or a decrease in production of antioxidants that scavenge or mop up these radicals. The reactive free radicals often modulates the pathways that control normal biological functions and/or cause irreversible cellular alterations of lipids, proteins and DNAs resulting in tissue necrosis or death (Mittler, 2002; Yoshikawa and Naito, 2002). Oxidative stress from free radicals is a pathologic mechanism that can either initiate or worsen the progression of liver diseases and other chronic degenerative disorders (Sha *et al,* 2015). The level of the antioxidant system of the body is often assessed in serum to evaluate the effect of oxidative stress on both the enzymatic and non-enzymatic types of antioxidants (Sha *et al,* 2015).

Superoxide dismutase, catalase and glutathione peroxidase (GPx) are the enzyme antioxidants, while glutathione and malondialdehyde (MDA) are typical examples of the non-enzymatic antioxidants. Superoxide dismutase scavenges for cellular superoxide radicals and catalyses

the conversion of any two radicals into hydrogen peroxide and molecular oxygen.Catalase is a haem protein that catalyses the conversion of hydrogen peroxide into water and oxygen.

Glutathione peroxidase is selenium containing oxidative enzyme of glutathione that catalyses the conversion of hydroperoxides as well as hydrogen peroxides into water. Hydroperoxides are non-radical intermediates of unsaturated fatty acids (phospholipids, glycolipids, cholesterol and its esters) (Weydert and Cullen, 2010).Glutathione (GSH) is a tripeptide of glycine, glutamic acid and cysteine that acts as a non-enzymatic reducing agent. It maintains the cysteine thiol sulfydryl group side chains of proteins in reduced states and thus maintains low levels of lipid peroxides and/or peroxidation (oxidative deteriorative reactions of lipids) (Yoshikawa and Naito, 2002).

## 2.7 Treatment of Liver Diseases

### 2.7.1 Symptomatic management

Liver disease treatment is mostly a symptomatic relief of complications and requires bed rest, rehydration, adequate nutrition and use of anti-nausea medications. Specific pharmacological intervention depends on the type and severity of the disease and includes use of:

i. Penicillamine or zinc acetate (for Wilson's disease)

ii. Cholestyramine (for itching related to cholestatic hepatitis)

iii. Corticosteroid (prednisone) with or without azathioprine (for autoimmune hepatitis and maintaining aminotransferases level); prednisolone (for severe alcoholic hepatitis (Ryder and Beckingham, 2001)

iv. Antiviral treatment (for HBV-related elevated aminotransferases), the antiviral drugs used include oral nucleoside analogue (entecavir, adefovir, lamuvudine, telbivudine), interferon alpha (IFN-α), pegylated IFN-α2a (pegiinterferon α-

2a);Combination therapy of pegylated IFN-α2b or 2a plus ribavirin (for HCV-chronic hepatitis related elevated aminotransferases). Viral hepatitis are generally, vaccine preventable and treatable (NTA news transmission, 26th June, 2017)

v. Baclofen and disulfiram (for reducing symptoms of abstinence from alcohol); baclofen reduces symptoms of cravings and withdrawal, while disulfiram produces unpleasant alcoholside effects for restraint.

vi. Benzodiazepines (as calmative and for sedation in withdrawal symptoms)

vii. Thiazolidinediones (has preliminary evidence for correcting biochemical and histologic abnormalities)

viii. Weekly removal of a pint of blood as to reduce excess iron (for hemochromatosis)

ix. Kasai surgery (placing part of the intestine as bile duct for biliary atresia)

x. Liver transplantation (for liver cancer or cirrhosis) (Ryder and Beckingkam, 2001)

### 2.7.2 Protectants of the liverhepatotoxic injury or disease (silymarin)

Viral hepatitis is infectious and can be prevented by adhering to practicing good hygiene and healthcare safety precautions. However, silymarin is the only standardised drug that had shown and had been reported to exhibit potent hepatoprotective activity in various experimental and clinical studies (Wills and Asha, 2006; Salam *et al.*, 2007). Silymarin is a flavonoid extract of *Silybum marianum* (a milk thistle) plant whose seeds had been used for more than 2000 years to treat liver and gall bladder disorders, including hepatitis, cirrhosis and jaundice and to protect the liver against poisoning from chemicals, environmental toxins, snake bites, insect stings, mushroom poisoning and alcohol (Kren and Walterova, 2005). The hepatoprotective properties of silymarin and its structurally related compound, silibinin are well documented and their preparations are now clinically available for use in liver

diseaseconditions. However, the mechanism of action of silymarin and silibinin are still poorly understood, but literature data relatedthis tofour different activities as:

(i) antioxidants or free radical scavengerswith ability to regulate the intracellular content of glutathione.

(ii) stabilisers of cell membrane and permeability regulators that prevent hepatotoxic agents from entering hepatocytes.

(iii) promoters of ribosomal RNA synthesis and stimulators of liver regeneration.

(iv) inhibitors of the transformation of stellate hepatocytes into myofibroblasts a process that is responsible for the deposition of collagen fibres that leads to cirrhosis.

The flavonoids also have documentations of anti-inflammatory and anticarcinogenic properties (Fraschini *et al*., 2002). Silymarin had been found to be able to neutralise the hepatotoxicity of several agents, including *Amanita phalloides*, ethanol, paracetamol (acetaminophen) and carbon tetrachloride in animal models. It protects against the toxic principle of *Amanita phalloides* (a-amanitin) by preventing its uptake through hepatocyte membranes and inhibiting its effects of tumour necrositic factor, which exacerbates lipid peroxidation (Singh *et al*., 2012).Clinical trials have shown that silymarin exerts hepatoprotective effects in acute viral hepatitis, poisoning by *A. phalloides*, toxic hepatitis produced by psychotropic agents and alcohol-related liver disease, including cirrhosis (Fraschini *et al*., 2002).Due to its proven hepatoprotective activity, silymarin is being used as one of the standard agents for comparison in the evaluation of hepatoprotective effects of plants.

## 2.8 Experimental models of hepatotoxicity

Animalmodels of induced liver toxicity such as rats or mice are often used to evaluate drugs for potentials of hepatoprotective effects. These hepatotoxic models include:

I. Hepatotoxin-induced
II. Common bile duct ligation-induced
III. Immune-mediated induced
IV. Targeted introduction of gene defect-induced e.g., there are defects for over-expression of transgenes that affect critical signaling pathways involved in the pathogenesis of liver fibrosis (Tag*et al*., 2015).

The most commonly used experimental approach for liver injury isdrug (hepatotoxin)-induced and common bile duct ligation-induced liver injury.

### 2.8.1 Drug (hepatotoxin)-induced liver injury

The various chemical agents used to induce hepatotoxicity in experimental animals act by disrupting both the liver parenchymal and non-parenchymal cells to produce a mixed pattern of injuriousconditions including acute and chronic hepatocellular hepatitis, fibrosis/cirrhosis, cholestasis, steatosis, as well as sinusoidal and hepatic artery/vein damage (Thonda and Shivalinge, 2012). These hepatotoxic drugs or agents include: acrlamide, adriamycin, alcohol, alphanaphthyl-isothiocyanate, anti-tubercular agents (isoniazid - INH, rifampicin, pyrazinamide),erythromycin, galactosamine, lead, cadmium, tamoxifen, tert-butylhydroperoxide - t-BHP, thioacetamide, paracetamol, phallodin (Thonda and Shivalinge, 2012). There are also some natural products hepatotoxins such as fungi or moulds of *Aspergillus flavus* or *A. Parasiticus* (Adedara *et al*., 2010), as well as natural chemicals like

carbontetrachloride ($CCl_4$) and microcystin. The advanced stage of $CCl_4$-induced liver injury is usually associated with risks of tumour growth because it is also a carcinogenic agent (Marques *et al*., 2012).

The degree of toxicity of these agents is dependent on the rate of metabolic detoxification that converts them into water-soluble forms in the smooth endoplasmic reticulum of hepatocytes. Thus, usually, toxicity is less in reduced activity of mixed function oxidase system (MFOs) due to reduced production of toxic metabolites and also with agents of no or little enzyme inducibility(Thonda and Shivalinge, 2012).

Hepatotoxins are of two broad categories, intrinsic and idiosyncratic hepatotoxins and each has both direct and indirect mechanisms of injuries. Intrinsic (or true) hepatotoxins are dose dependent toxic agents that cause predictable and reproducible hepatic disruptions in a relatively short period of time and which are often used in animal experimentsexample paracetamol (William and Lee, 2003). Idiosyncratic hepatotoxins are agents of highly unpredictable toxicity that cause time and host related hepatotoxicity example isoniazid (William and Lee, 2003). Their effects usually take a long latent period (days to weeks) and lack relationship between dose and extent of injury and/or reproducibility (Fisher *et al*., 2015). Usually, drugs of predictable dose-response curves have well characterised mechanisms of pharmacological heptotoxicity effect and act either by directly damaging the liver tissue or blocking its metabolic processes especially when taken in overdosage (Aashish *et al.,* 2012; Fisher *et al*, 2015) as typified in the over dose of paracetamol.

#### *2.8.1.1 Mechanism of paracetamol-induced liver injury*

Paracetamol is an analgesic-antipyretic drug that produces acute liver damage at high doses. It causes nuclear pyknosis and eosinophilic cytoplasm that result in centrilobular necrositic hepatocytes and excessive hepatic lesion (Thonda and Shivalinge, 2012). About 90% of each dose of paracetamol is normally completely metabolised by glucuronidation and sulfation, but small amount of it is often oxidised into hepatotoxic intermediate (N-acetyl-p-benzoquinone imine - NAPQI) by cytochrome $P_{450}$ E1 and other $P_{450}$ isoenzymes. Cellular glutathione often converts this toxic intermediate to its inactive mercapturic acid form, but in acute dosage of paracetamol, more of the NAPQI is produced beyond glutathione inactivation and this result in toxicity (Jaeschke, 2011).

### 2.8.3 Bile duct ligation-induced liver injury

Bileduct ligation induces an extrahepatic cholestatic liver injury in rodents resembling obstruction of the common bile duct as with gallstone impaction or extrinsic compression by tumours or enlarged lymph nodes(Teixeira *et al*.,2013). Duct ligation results in cholangiohepatitis (inflammation of the liver and bile ducts) that progress to fibrosis and cirrhosis.Irrespective of the source of damage, the anatomical and functional alterations of the liver tissue integrity are generally similar (Aller *et al*., 2008). The interaction of inflammatory signals with the liver cell macrophages (kupffer cells) triggers a response against the insult, but which on persistence results in chronic inflammation characterised by:

i. accumulated deposition of extracellular matrix (ECM) and formation of scar tissues that subsequently substitute the normal liver parenchymal cells (Tacke and Weiskirchen, 2012).

ii. transformation of stellate hepatocytes into myofibroblasts that causes functional alterations of liver tissue integrity. Generally, myofibrosis or excessive development of connective tissues in muscles often leads to inadequate functioning (Aller *et al*., 2008;Abshagen *et al*., 2015).

# CHAPTER THREE

# 3.0 MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Collection and identification of plant material

The plant *Acacia sieberiana* was collected from Samaru, Zaria, Kaduna State in March, 2016. It was identified and authenticated by Mr. Namadi Sanusi of the Department of Biological Sciences, Ahmadu Bello University, Zaria. A voucher number; 16,136 was obtained.

### 3.1.2 Experimental animals

Adult Wistar rats of either sexes weighing between 120-200g obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University (ABU), Zaria were used. They were acclimatised for one week to the laboratory conditions before commencement of the experiment with food and water provided *ad libitum*. All experiments were carried out in compliance with the institution's Ethics on Animal Use and Care (ABUCAUC) with an approval number: ABUCAUC/2016/049, and in accordance with the regulation for the Care and Use of Laboratory animals as accepted internationally **(**NIH, rev 1996).

### 3.1.3 Drugs and chemical reagents

All drugs and chemical reagents used included:

Silybon-140 (Micro LabsLimited, India)

Paracetamol 500 mg Tabs (Evans Medical Plc, Nigeria)

Thiopental Sodium (Rotex medica, Germany)

Reagent Kits (Vital Scientific, Netherlands)

The chemical reagents used for the study were from either Sigma-Aldrich, USA or BDH, UK and included: Methanol, Chloroform, Hydrochloric Acid (HCl), Sodium Hydroxide (NaOH), Molisch Reagent, Ferric Chloride Solution, 10% Ammonia Solution, Acetic Acid Anhydride, Dragendoff Reagent, Dettol (Disinfectant), Formalin, Normal saline (0.9% normal saline isotonic solution

### 3.1.4 Equipment and instruments

Electronic weighing machine (Precision Electronic Instrument Company, India)

Water bath (Rajat Scientific Instrument, India)

Automated haematology analyser (Sysmex model 2X-12N, USA)

Colorimeter (Uptima digital colorimeter AC-114, Indonesia)

The other materials include centrifugedissecting set, orogastric cannula, syringes and needles, spatula, mortar and pestle, desiccators and refrigerator, glass and ceramic wares, cotton wool, hand gloves, nose masks, Whatman filter paper (No. 1), cages and feeders.

## 3.2 Methods

### 3.2.1 Plant preparation and extraction

The collected fresh root bark of *A. sieberiana* were washed clean with water to remove dirt and contaminants and then air-dried to a constant weight and crushed into a coarse powder using mortar and pestle. The powdered sample (2,500g) was soaked in 10 litres of 70%$^{v}/_{v}$ methanol in a conical flask for 72 hours with occasional shaking to stir. The mixture was filtered using muslin cloth followed by Whatman filter paper (No. 1) and the resultant filtrate was concentrated to a constant weight over water bath maintained at 50°C. Percent (%) yield of the extract was calculated The obtained extract was stored in a labelled sample bottle and

was thenceforth referred to as ASE (*Acacia sieberiana* extract). Fresh concentrations of the extract were prepared on each day of experiment by reconstituting in normal saline.

### 3.2.2 Phytochemical screening

Preliminary phytochemical screening of ASE was carried out to identify the constituents, using standard procedures(Sofowora, 1993; Trease and Evans, 2002; Ayoola *et al.*, 2008).

#### *3.2.2.1 Test for carbohydrates (Molisch Test)*

Few drops of Molisch reagent was added to a small portion of the extract in a test tube, and concentrated sulphuric acid was added down the side of the test tube to form a lower layer, a reddish-coloured ring at the interphase indicates the presence of carbohydrates.

#### *3.2.2.2 Test for anthraquinone (Bontrager's Test)*

About 0.5 g of the extract was taken in a dry test tube and 5ml of chloroform was added and shaken for at least 5 minutes. This was filtered and equal volume of 10% ammonia solution was added to the filtrate and shaken. The presence of a bright pink colour in the aqueous (upper) layer indicates free anthraquinones

#### *3.2.2.3 Test for glycosides (Fehling's test)*

To a portion of the extract, 5ml of dilute sulphuric acid was added and boiled on water bath for 10-15 minutes. The cooled mixture was neutralized with 20% KOH and then divided into two portions for reactions with fehling's solution and ferric chloride solution.

- To the first portion, 5ml of a mixture of fehling's solutions A and B was added and boiled and the presence of a brick red precipitate indicates release of reducing sugar from hydrolysis of glycosides.
- To the second portion, about 3ml of ferric chloride solution was added; a green to blue colour change indicates release of phenolic aglycones from hydrolysis glycosides.

*3.2.2.4 Test for steroids and triterpenes (Lieberman Bucchard Test)*

About 0.5 g of the extract is dissolved in 2 ml of acetic anhydride and 1ml concentrated sulphuric acid was added down the side of the test tube to form a lower layer. Change of colour from violet to bluish-green indicates the presence of steroidal ring of the glycosides, while a reddish, pink or purple colour indicates the presence of triterpene.

*3.2.2.5 Test for cardiac glycosides (Keller-Kiliani Test)*

About 0.5 g of the extract was dissolved in 1ml of glacial acetic acid and with traces of ferric chloride solution and 1ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer at the bottom. Presence of a purple-brown ring at the interphase indicates the presence of deoxy-sugars and a pale green colour in the upper acetic acid layer indicates the presence of cardiac glycosides.

*3.2.2.6 Test for saponins (Frothing Test)*

About 10 ml of distilled water was added to 0.5 g of the extract and was shaken vigorously for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30 minutes. A honeycomb froth that persists for 10-15 minutes indicates presence of saponins.

*3.2.2.7 Test for tannins (Ferric chloride Test)*

Ferric chloride was added in drops of 3-5 to 0.5 g of the extract and the presence of a greenish-black precipitate indicates condensed tannins while a blue or brownish-blue precipitate indicates hydrolysable tannins.

*3.2.2.8 Test of flavonoids (Sodium Hydroxide Test)*

Few drops of 10% sodium hydroxide were added to the 0.5 g of the extract yellowish coloration indicates the presence of flavonoids.

*3.2.2.9 Test for alkaloids (Dragendoff's Test)*

Few drops of Dragendoff's reagent were added to 0.5 g of the extract and a reddish brown precipitate indicates the presence of alkaloids.

### 3.2.3 Acute toxicity study

The acute toxicity test of the methanol root bark extract of *Acacia sieberiana* were evaluated in rats using both oral and intraperitoneal routes of administration. Lorke's method (1983) was adopted. The acute toxicity testfor each route of administration was conducted with 12 rats in which 9 were used for phase I in 3 groups of 3 rats per group and administered with 10, 100 and 1000 mg/kg ASE per body weight with 24 h observation for signs of toxicity or death. With the absence of death in the 1st phase, the 2nd phase of the test was conducted, with 3 rats divided into 3 groups of 1 rat each and administeredhigher doses of 1600, 2900 and 5000 mg/kg respectively in another 24 h. The animals were observed and the median lethal dose ($LD_{50}$) calculated by the geometric mean of the highest non-lethal and the lowest lethal doses as:$LD_{50} = \sqrt{}$(maximum nonlethal dose × minimum lethal dose)

### 3.2.4 Subchronic toxicity study

The guideline of OECD 407 (2008) was used for the study. Forty rats of both sexes were randomly divided into 4 groups of 10 animals each (5 males and 5 females). The rats were administered orally per body weight with normal saline and extract doses of 250, 750 and 1,500 mg/kg respectively for 28 consecutive days using orogastric cannula. Rats were maintained under standard conditions with food and water *ad libitum* for the entire period with close observation for signs and symptoms of toxicity. The body weights of the rats were subsequently obtained on weekly intervals for 4 weeks. On day 29, animals were anaesthetised with chloroform and then euthanised. Blood samples were collected via cardiac

puncture into plain bottles for serum biochemical analysis and into coagulated (EDTA) bottles for plasma haematological analysis. Some vital organs were placedinto 10% formalin fixative for histological examinations.

#### *3.2.4.1 Biochemical analysis*

Biochemical tests for liver function such as levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (Alb), total and direct bilirubin (TBil and DBil); as well as that for kidney function – urea, creatinine and electrolytes (chloride, sodium, potassium and bicarbonate ions) were evaluated using standard kit.

#### *3.2.4.2 Haematological analysis*

Blood indices including red blood cells (RBC), haemoglobin (Hb), platelet, packed cell volume (PCV), white blood cells (WBC) and differentials [(neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E), basophils (B)] were analysed using an automated haematology analyser.

#### *3.2.4.3 Histological studies*

The excised organs including liver, kidneys, lungs and heart were fixed in 10% formalin fixative solution for histological analysis. The samples were grossed to isolate the particular area to be sectioned. The tissues were processed with reagents (70% alcohol, 90-95% alcohol) to make them more hydrophobic to enable embedding with paraffin wax. Embedding secures a specimen and then enables it to be cut and stored.Sectioning was done by using a microtome to cut very fine sections of the embedded tissues which were then floated-out on a water bath then picked and placed on microscope slides. The slides weredried on a hot plate to remove

moisture and help the tissue adhere to the slide.De-waxingwas done by using a solvent to remove the wax from the slide prior to staining.The tissue on the slide was then stained with haematoxylin and eosin (H&E) stain and covered with a cover glass to make the preparation permanent.Tissue slides were then viewed at a magnification of ×250 and photomicrographs of the tissues were obtained.

### 3.2.5 Effect of ASE on paracetamol-induced liver injury in rats

Animals were pre-treated for 7 days in the following order; 250, 750, 1500 mg/kg of ASE in 3 respective groups and standard drug silymarin in the standard control andthe normal control group had normal saline. After the last administration, liver injury was induced using PCM (2g/kg)in accordance to Mahmood *et al*. (2014). 24 h after liver injury induction, the rats were anaesthetisedwith chloroform soaked in cotton and put into an inhalation chamber and euthanised. Livers were excised and placed in 10% formalin for histology examination. Blood samples were also collected from the various groups in plain bottles for analysis of both biochemical and oxidative stress biomarkers. Following centrifugation, the obtained sera was assayed for ALT, AST, ALP, total protein (TP), albumin (Alb), total bilirubin (TB) and direct bilirubin (DB).The antioxidant markers; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA) were also assayed from the sera using standard kits.

### 3.2.6 Effect of ASE on bile duct ligation-induced liver injury

The method described by Tag*et al.,* 2015was used.A total of fifty (50) male rats were anaesthetised with thiopental sodium (40 mg/kg,*i.p*).The abdominal furs of the rats were shaved off and a short incision of about 2cm made below the xiphoid process of the abdomen

to expose the bile duct region. Double ligation was placed at the common bile duct of 44 of the rats such that the bile does not flow. The incisions were then sutured and the animals kept to recover (fully awake and active). Thirty (30) out of the 44 double ligated rats that were fully awake and active within 24 h were selected and divided into 5 groups of 6 rats each, while the 6 non-ligated rats served as negative control ($6^{th}$ group) for the study. The various groups were then treated *i.p*. per for 7 days with normal saline (1 ml/kg) for groups 1 and 2, ASE extract doses (125, 250 and 380 mg/kg) for groups 4, 5 and 6, while group 3 was treated orally with 50 mg/kg silymarin (standard drug). The rats were anaesthetized and euthanized to isolate the liver into formalin fixative for histology examination. Blood samples were collected from the various groups in plain bottles for the analysis of both biochemical and oxidative stress biomarkers. Samples were centrifuged to obtainthe sera for the following analysis; ALT, AST, ALP, total protein (TP), albumin (Alb), total bilirubin (TB) and direct bilirubin (DB). Oxidative stress markers; superoxide dismutase (SOD), catalase (CAT), gluthatione peroxidase (GPx), malondialdehyde (MDA) were also assayed from the obtained sera using standard laboratory kit and procedures.

### 3.2.7 Data analysis

The data obtained were expressed as mean ± SEM.One way Analysis of Variance (ANOVA), Split Plot ANOVA (for weekly body weights) followed by Bonferonni multiple comparison *post hoc* test (for level of significance between means) were used for the analysis. $P \leq 0.05$ was considered as level of statistical significance.

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Extraction Yield of *A. sieberiana* Root Bark Extract

A sticky dark brown solid residue of 115.78g (4.63%$^{w}/_{w}$) with a mild sweet smell was obtained from 2,500gcrude root bark of *A. sieberiana* powdered sample.

### 4.2 Phytochemical Constituents

Preliminary phytochemical screening of *A. sieberiana* root bark extract showed the presence of carbohydrates, glycosides(cardiac glycosides), triterpenes, saponins, tannins, flavonoids and alkaloids, while anthraquinones and steroids were absent (Table 4.1).

**Table 4.1: Phytochemical constituents of methanol extract of *A. sieberiana* root bark**

| **Phytoconstituents** | **Result** |
|---|---|
| Alkaloids | + |
| Carbohydrates | + |
| Flavonoids | + |
| Glycosides | + |
| - Cardiac glycosides | + |
| - Anthraquinones | - |
| Saponins | + |
| Steroids | - |
| Triterpenes | + |
| Tannins | + |

Key: (-) = Absent, (+) = Present

## 4.3 Estimation of the Median Lethal Dose($LD_{50}$) of ASE

Acute oral administration of methanol root bark extract of *A. sieberiana* showed no death at doses up to 5,000mg/kg and thus, the oral $LD_{50}$ of the extract was estimated to be >5,000 mg/kg body weight and there were no signs of abnormal behaviours. Death was not recorded in the first phase of*i.p.* acute ASE treatments, the rats at all the increased doses (1,600, 2,900 and 5,000 mg/kg) of the second phase died and the*i.p.* $LD_{50}$ was thus estimated to be about 1300 mg/kg.

## 4.4 Subchronic Toxicity Study of 28 days ASE Administrations in Rats

### 4.4.1 Effect of ASE on body weights of rats

The result of the weekly body weight changes for the subchronic toxicity study revealed that in all the groups, there was a progressive increase in weight. However the animals that were treated with the extract showed lesser weight gain when compared to the control group. In week 1, the mean weight of all the extract-treated groups was almost similar, however, the extract groups showed lesser weight gain in the subsequent weeks (2, 3 and 4)that was significantly ($P$<0.05) decreased when compared to the control group (Figure I).

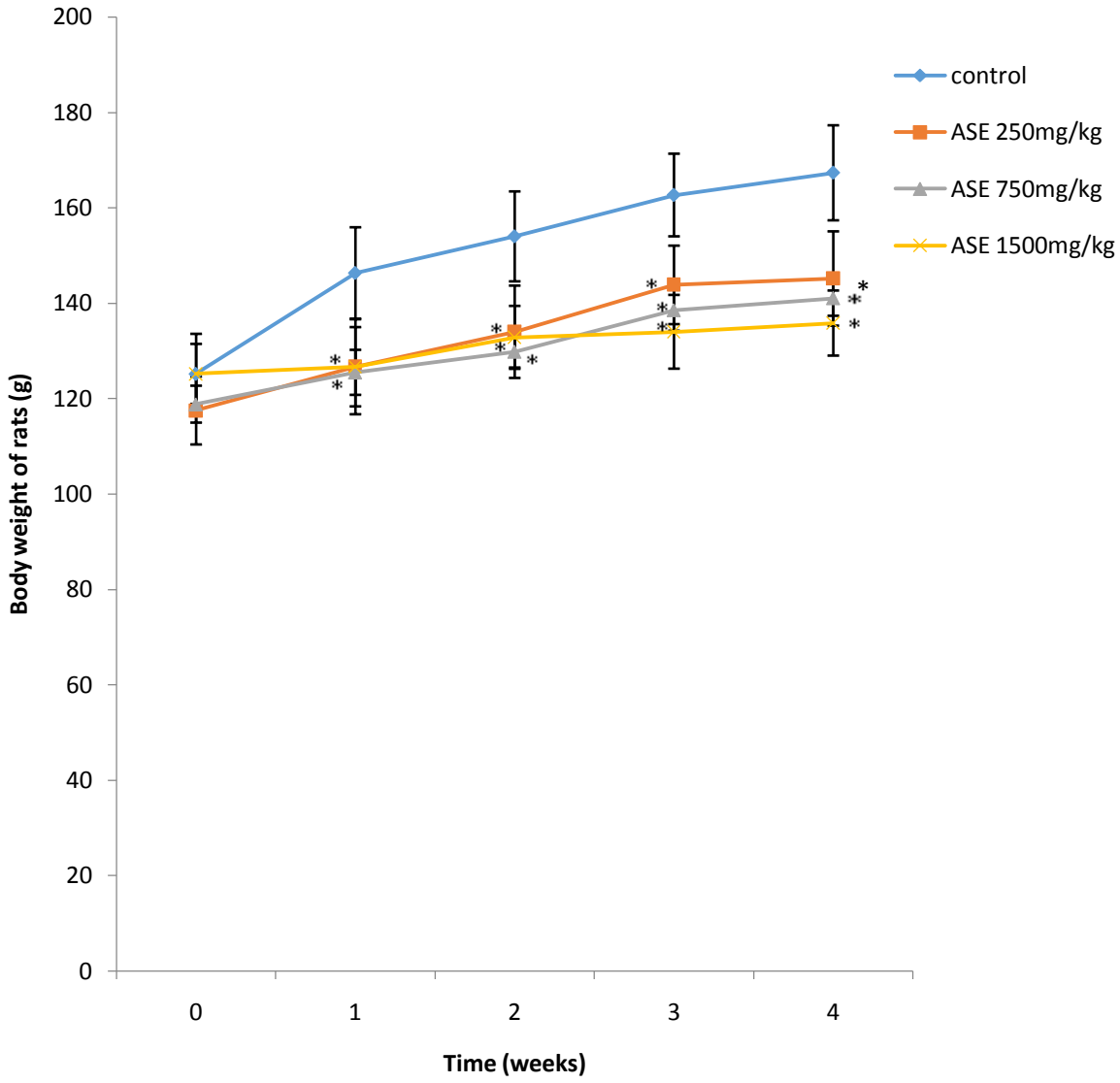



**Figure 4.1: Weekly body weight changes of rats after 28 days daily oral *A. sieberiana* extractadministration**

n = 10; statistics: Split plot ANOVA and Bonferonni multiple comparison *post hoc* test; * = significance at $P<0.05$ compared to the control group.

### 4.4.2 Effect of ASE on serum biochemical parameters of liver function

In this study, a non-significant increase in ALT and decrease in AST were observed in animals treated for 28 days with the extract, however a significant ($P$<0.05) dose-dependent increase in serum ALP level occurred. The ALP increase did not differ significantly amongst the extract dose groups. The total protein, albumin and bilirubin were not significantly altered (Table 4.2).

**Table 4.2: Liver function biomarkers in rats treated for 28 days with ASE**

| Liver biomarkers | Treatment groups(per kg) | | | |
|---|---|---|---|---|
| | **N/S (1 ml)** | **ASE 250 mg** | **ASE 750mg** | **ASE 1,500 mg** |
| **ALT (IU/L)** | 6.00 ± 0.68 | 7.17 ± 0.80 | 8.00 ± 1.24 | 7.33 ± 0.67 |
| **AST (IU/L)** | 12.67± 0.92 | 12.00± 0.58 | 11.00± 1.67 | 10.33± 1.28 |
| **ALP (IU/L)** | 26.76± 3.38 | 43.78±4.32* | 45.40±3.24* | 50.21±4.03* |
| **Total Protein (g/dL)** | 6.54±0.24 | 6.69±0.32 | 6.44±0.30 | 6.93±0.34 |
| **Albumin (g/dL)** | 3.14±0.07 | 3.17±0.08 | 3.08±0.06 | 3.03±0.09 |
| **Direct Bilirubin (mmol/L)** | 0.34±0.06 | 0.26±0.03 | 0.24±0.04 | 0.30±0.02 |
| **Total Bilirubin (mmol/L)** | 0.56±0.08 | 0.52±0.06 | 0.42±0.07 | 0.50±0.06 |

n = 10; data expressed asmean ±SEM; statistics: one way ANOVA and Bonferonni*post hoc* test; * indicates significance at $P$<0.05 compared to the N/S group; N/S = normal saline; ALT= alanine aminotransferase; AST; aspartate aminotransferase; ALP; alkaline phosphatase.

### 4.4.3 Effect of ASE on serum biochemical parameters of kidney excretory functions and electrolytes

This result showed a dose-dependent increase in serum urea level that wasstatistically significant ($P$<0.05) at 1,500 mg/kg extract group compared to the normal control. Creatinine and all the assessed electrolytes showed slight and inconsistent changes that were not statistically significantat $P \leq 0.05$ (Table 4.3).

**Table 4.3: Kidney excretory function biomarkers and electrolyte concentrations in rats treated for 28 days with ASE**

| Kidney biomarkers | Treatment groups (per kg) | | | |
|---|---|---|---|---|
| | **N/S (1 ml)** | **ASE 250 mg** | **ASE 750 mg** | **ASE 1,500 mg** |
| **Urea (mg/dL)** | 29.38± 1.22 | 29.80 ± 0.89 | 31.50 ± 2.70 | 34.23 ± 1.82* |
| **Creatinine (mEq/L)** | 0.97±0.05 | 1.03±0.11 | 0.97±0.12 | 1.24±0.10 |
| **$K^+$ (mmol/L)** | 23.49± 2.10 | 20.66± 1.83 | 22.78± 1.36 | 24.87± 2.27 |
| **$Na^+$ (mmol/L)** | 225.32± 7.44 | 225.26±2.99 | 223.08±6.91 | 224.83±8.24 |
| **$Cl^-$ (mg/dL)** | 96.67±5.39 | 98.67±5.26 | 87.83±3.02 | 89.40±1.50 |
| **$HCO_3^-$ (mg/dL)** | 22.17±1.33 | 23.00±1.77 | 22.17±1.35 | 21.00±1.92 |

n = 10; data expressed asmean ±SEM; statistics: one way ANOVA and Bonferonni*post hoc* test; * indicates significance at $P$<0.05 compared to the N/S group. N/S=normal saline; $K^+$=potassium ion; $Na^+$=sodium ion; $Cl^-$=chloride ion; $HCO_3^-$=bicarbonate ion.

### 4.4.4 Effect of ASE on haematological parameters

PCV and Hb concentrations were dose dependently reduced, but not significantly ($P \leq 0.05$) compared to control. There was an insignificant increase ($P > 0.05$) in WBC at the lower doses and a decrease ($P > 0.05$). Of the WBC-differentials that were assessed, significant increase in lymphocytes was observed at the dose of 1,500mg/kg, while the monocytes and eosinophils reduced slightly and consistently, but not significantly. The neutrophil as with the WBC showed similar slight insignificant increase at the lower extract doses (250 mg/kg, 750 mg/kg), but was reduced when compared to the normal control at the dose of 1,500mg/kg (Table 4.4).

**Table 4.4: Haematological parameters in rats treated for 28 days with ASE**

| Haematological indices | Treatment groups(per kg) | | | |
|---|---|---|---|---|
| | **N/S (1 ml)** | **ASE 250 mg** | **ASE 750 mg** | **ASE 1,500 mg** |
| **PCV (%)** | 37.16±1.86 | 36.67 ±3.18 | 35.00±1.97 | 35.17±1.30 |
| **Hb (g/dL)** | 12.72±0.33 | 12.62±0.97 | 11.15±0.69 | 11.12±0.51 |
| **WBC ($\times 10^9$L)** | 3.85±0.27 | 4.07±0.22 | 4.27±0.10 | 3.85±0.15 |
| **RBC ($\times 10^6$L)** | 5.85±0.17 | 5.98±0.15 | 5.70±0.14 | 5.93±0.19 |
| **Platelet ($\times 10^5$L)** | 7.20±0.10 | 7.18±0.10 | 7.18±0.11 | 7.08±0.06 |
| **Neutrophils (%)** | 17.33±2.08 | 19.00±0.97 | 18.00±1.15 | 15.00±0.97 |
| **Lymphocytes (%)** | 78.17±1.17 | 77.67±0.72 | 78.50±0.99 | 81.33±1.38* |
| **Monocytes (%)** | 2.33±0.56 | 1.67±0.21 | 1.50±0.34 | 2.00±0.37 |
| **Eosinophils (%)** | 2.17±0.30 | 1.66±0.33 | 2.00±0.36 | 1.83±0.31 |
| **Basophils (%)** | 0 | 0 | 0 | 0 |

n = 10; data expressed asmean ±SEM; statistics: one way ANOVA and Bonferonni *post hoc* test; * indicates significance at $P<0.05$ compared to the N/S group; N/S=normal saline; PCV=packed cell volume; Hb=haemoglobin; WBC=white blood cell; RBC=red blood cell.

### 4.4.5 Effect of ASE on organ histology

No structural changes were identified by histological studies in the liver, kidney, heart and lungs of the control group. Also, there were no pathological changes in the heart muscles of rats in all treated groups. However, there were dose-dependent pathological changes (slight to moderate hepatocellular necrosis and slight vacuolation) in the liver tissues of the treated groups. The kidneys showed slight glomerular necrosis, lymphocyte hyperplasia and slight tubular distortion. The lungs showed a dose dependent pathological change (slight to moderate alveoli congestion and slight lymphocyte hyperplasia) in all the treated groups. (Plate III-VI)

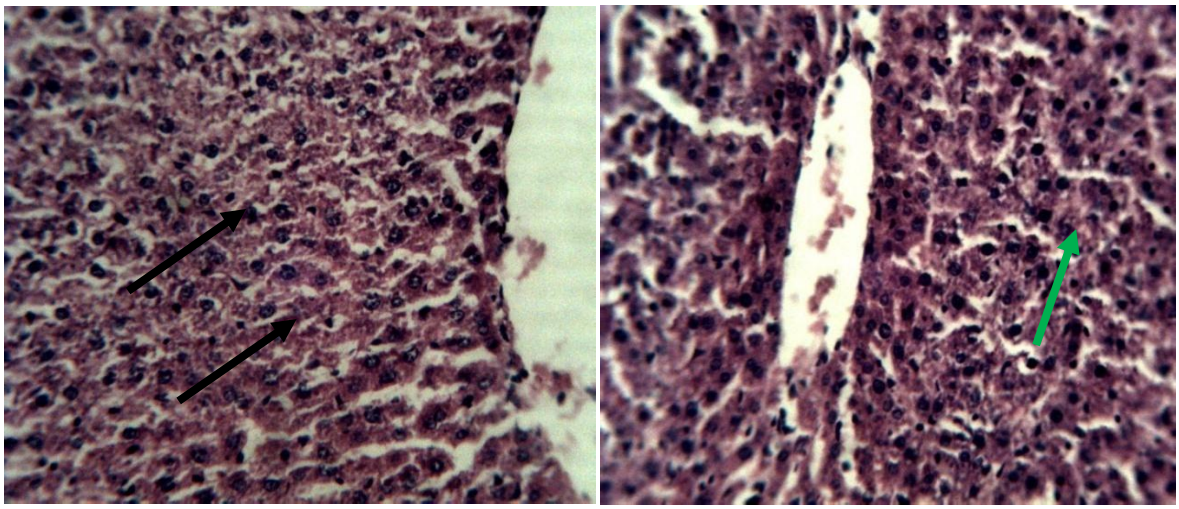


Plate IIa**:** Control Plate IIb:250mg/kgASE

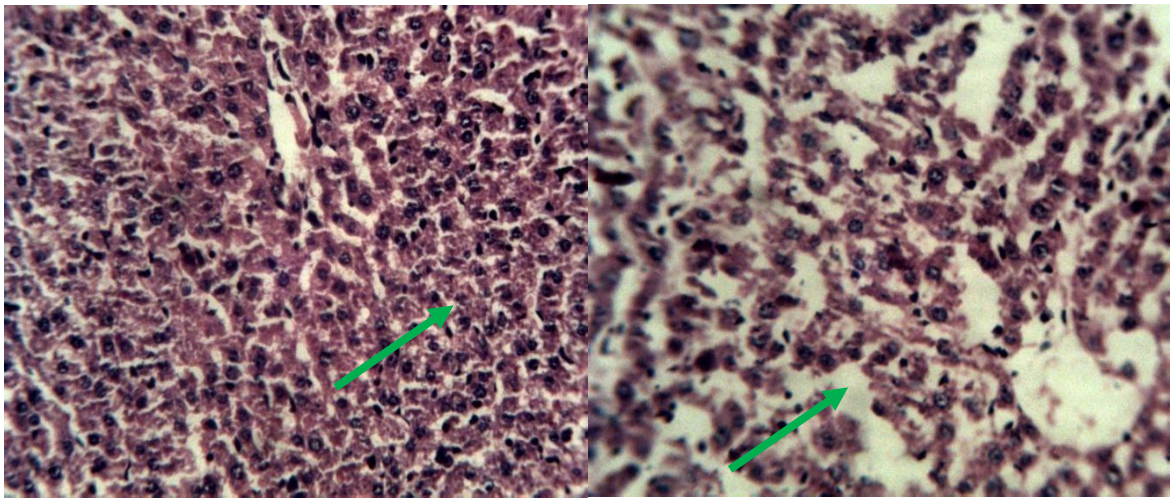


Plate IIc:750mg/kgASEPlate IId:1,500mg/kgASE

**Plate II: Photomicrographs of liver sections (H & E stained at ×250 magnification) of ratsfollowing 28 days daily oral administrations of ASE**

IIa: Section showing normal hepatocellular features (black arrows)

IIb: Section showing slight vacuolation and hepatocellular necrosis (green arrow)

IIc: Section showing slight vacuolation and hepatocellular necrosis (green arrow)

IId: Section showing moderate vacuolation and hepatocellular necrosis (green arrow)

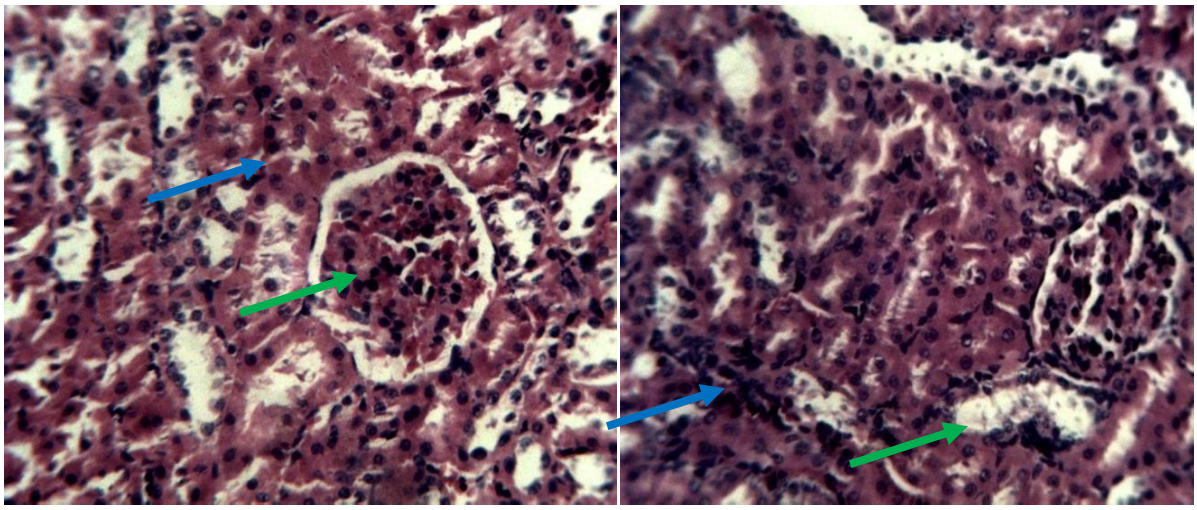


Plate IIIa: Control Plate IIIb: 250mg/kg ASE

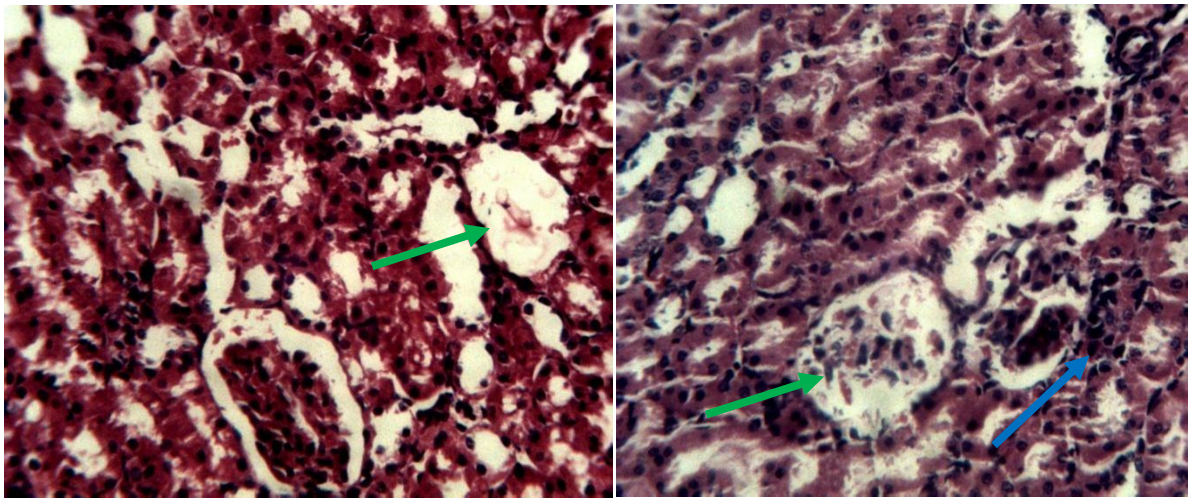


Plate IIIc: 750mg/kg ASE Plate IIId: 1,500mg/kg ASE

**Plate II: Photomicrographs of kidney sections (H & E stained at ×250 magnification) of ratsfollowing 28 days daily oral administrations of ASE**

IIIa: Section showing normal kidney tubules (blue arrow) and glomerulus (green arrow)

IIIb: Section showing slight tubular distortion and necrosis (green arrow) and lymphocyte hyperplasia (blue arrow)

IIIc: Section showing slight glomerular necrosis (green arrow)

IIId: Section showing slight glomerular necrosis (green arrow) and lymphocyte hyperplasia (blue arrow).

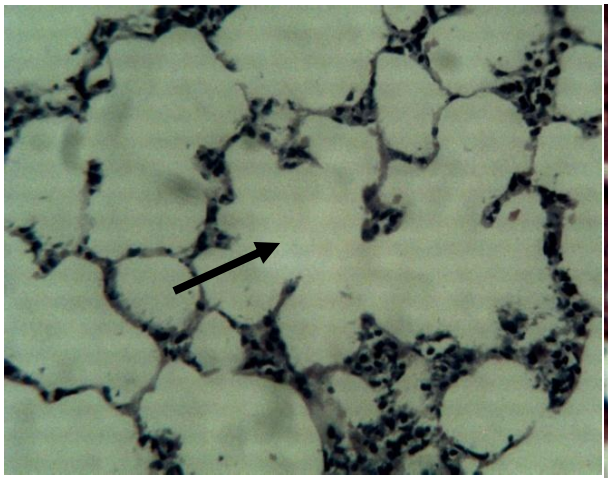


Plate IVa: Control

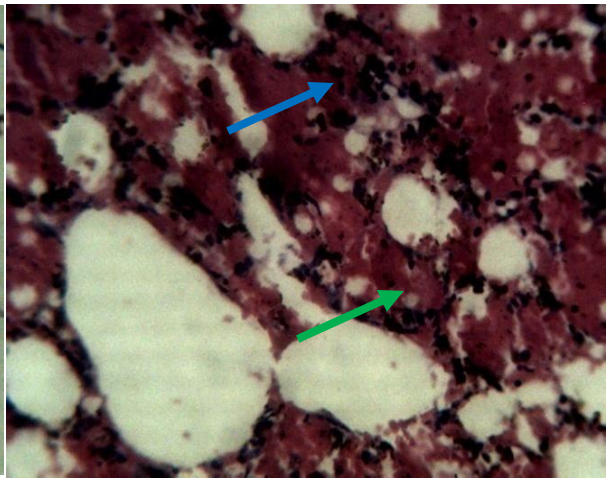


Plate IVb: 250mg/kg ASE

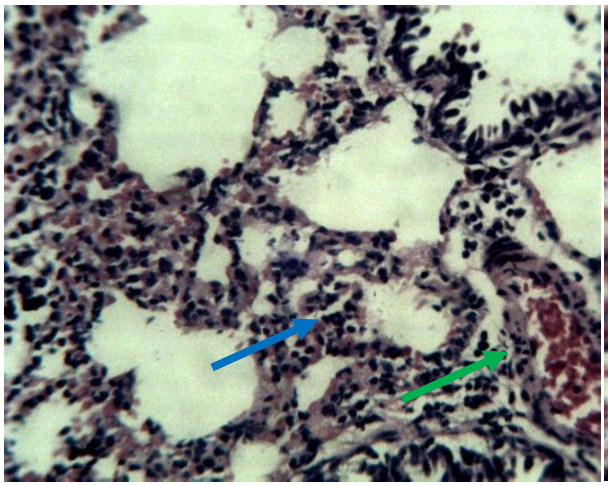


Plate IVc: 750mg/kg ASE

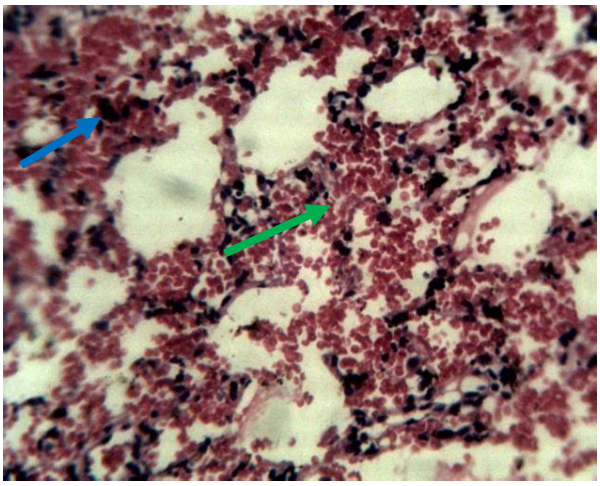


Plate IVd: 1,500mg/kg ASE

**Plate IV: Photomicrographs of lung sections (H & E stained at ×250 magnification) of ratsfollowing 28 days daily oral administrations of ASE**

IVa: Section showing normal lung alveoli (black arrow)

IVb: Section showing alveoli congestion (green arrow) and slight lymphocyte hyperplasia (blue arrow)

IVc: Section showing slight alveoli congestion (green arrow) and slight lymphocyte hyperplasia (blue arrow)

IVd: Section showing moderate alveoli congestion (green arrow) and slight lymphocyte hyperplasia(blue arrow)

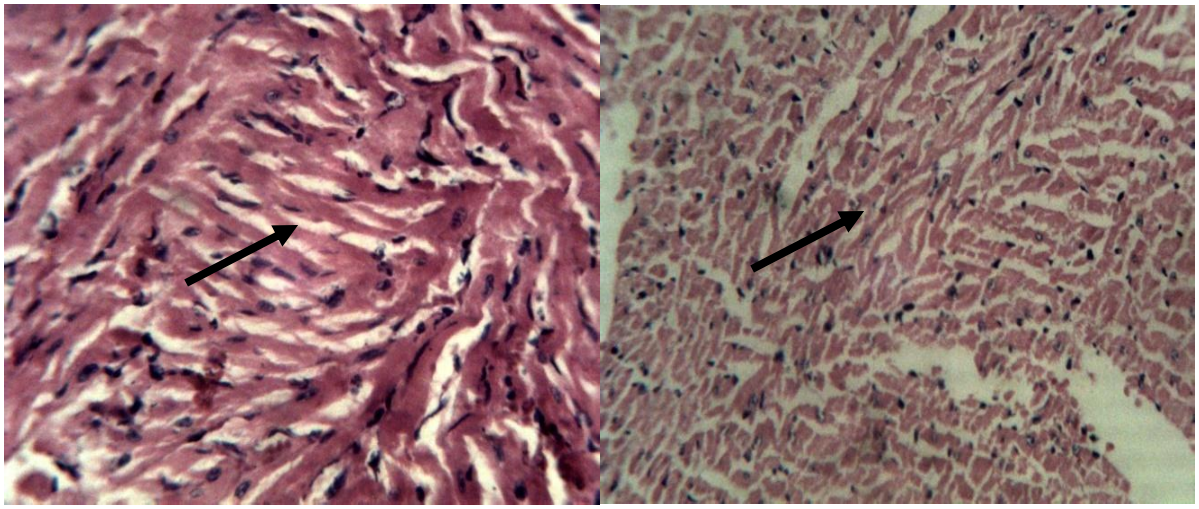


Plate Va: Control Plate Vb: 250mg/kg ASE

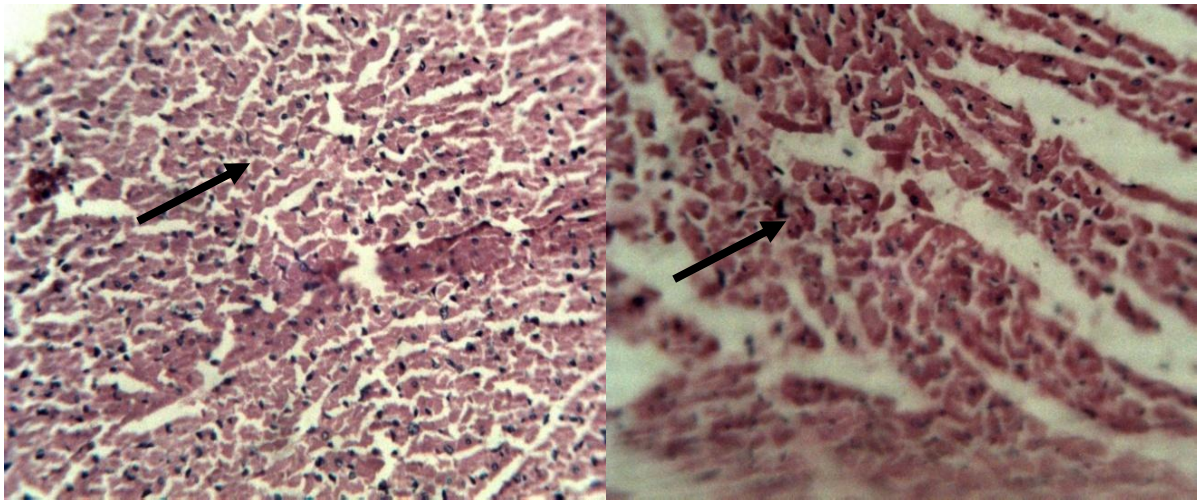


Plate Vc: 750mg/kg ASE Plate Vd: 1,500mg/kg ASE

**Plate V: Photomicrographs of heart sections (H & E stained at ×250 magnification) of ratsfollowing 28 days daily oral administrations of ASE**

V (a, b, c, d): Sections showing normal cardiac muscles (black arrows)

## 4.5 Effect of ASE on PCM-Induced Liver Injury in Rats

### 4.5.1 Effect of 7 day ASE pre-treatment on liver biomarkers of rats induced withliver injury using PCM

The PCM toxic injury on the liver reflected significant ($P$<0.05) increase in ALT, AST and direct bilirubin and a significant decrease ($P$<0.05) in total protein and albumin. Silymarin group andthe extract doses of 250 mg/kg and 750 mg/kgsignificantly decreased the PCM-induced increase in ALT and AST, while the significant decrease in total protein by PCM was significantlyreversed ($P$<0.05)by silymarin and all the extract dose groups. The extract in all its dose groups significantly increased ($P$<0.05) the albumin level while silymarin treated group showed a non-significant increase. The PCM-induced increase in direct bilirubin was significantly ($P$<0.05) reduced in the silymarin treated group and 750 mg/kg ASE dosegroup(Table 4.5).

**Table 4.5: Liver biomarkers in ASE pre-treated rats following paracetamol-induced liver injury**

| Liver biomarkers | Treatment groups (Per kg) | | | | | |
|---|---|---|---|---|---|---|
| | **N/S (1 ml)** | **NS, 1ml + PCM** | **Sily 50mg + PCM** | **ASE 250mg + PCM** | **ASE 750mg + PCM** | **ASE 1,500mg + PCM** |
| **ALT (IU/L)** | 11.33±0.88 | 30.33±2.03[a] | 17.00±1.15[a,b] | 16.33±1.45[a,b] | 17.00±2.65[a,b] | 26.33±3.76[a] |
| **AST (IU/L** | 38.67±3.48 | 45.33±0.40[a] | 41.00±3.79[b] | 37.33±1.45[b] | 38.33±4.63[b] | 41.35±4.61 |
| **ALP (IU/L)** | 12.49±1.18 | 13.58±0.53 | 14.55±1.05 | 14.05±1.16 | 11.70±1.03[a] | 12.64±1.05 |
| **Total Protein (g/dL)** | 9.37±0.53 | 8.05±0.09[a] | 9.14±0.19[b] | 8.86±0.17[b] | 10.16±0.91[b] | 10.14±0.95[b] |
| **Albumin (g/dL)** | 3.85±0.81 | 2.64±0.27[a] | 3.03±0.56 | 3.25±0.18[b] | 3.46±0.19[b] | 3.00±0.12[b] |
| **Direct bilirubin (mmol/L)** | 3.03±0.44 | 4.24±0.68[a] | 3.23±0.21[b] | 3.56±0.43 | 3.18±0.26[b] | 4.07±1.19 |
| **Total bilirubin (mmol/L)** | 5.30±0.35 | 5.78±0.41 | 5.33±0.43 | 5.45±0.30 | 5.16±0.97 | 5.69±0.46 |

n = 5; data expressed asmean ±SEM; statistics: one way ANOVA and Bonferonni *post hoc* test; [a] - statistically significant to NS at $P<0.05$; [b] – statistically significant to NS + PCM at $P<0.05$.Sily = silymarin; NS=normal saline; PCM=paracetamol; ALT=alanine aminotransferase; AST=aspartate aminotransferase; ALP=alkaline phosphatise.

### 4.5.2 Effect of 7 day ASE pre-treatment on oxidative stress markers in rats induced with liver injury using PCM

This result showed that PCM-induced liver injury caused a significantreduction ($P$<0.05) in superoxide dismutase (SOD). However,silymarin treatment group and ASE at all its doses significantly ($P$<0.05) and dose dependently increased the serum level of the SOD when compared with the normal control. Catalase (CAT) concentration was not affected by PCM-intoxication, but was significantly increased ($P$<0.05) when compared with the normal control at the extract doses of 250 and 750 mg/kg and with the standard drug - silymarin. PCM caused a significant increase ($P$<0.05) in the oxidative stress biomarker (malondialdehyde-MDA) but, the extract at all tested doses significantly reversed ($P$<0.05) this and was slightly better than the reduction by silymarin. Glutathione peroxidase (GPx) concentration was slightly and insignificantly reduced by paracetamol-induced injury. However, its serum concentration was increased when compared with the normal control in all the extract treated groups, as with the silymarin treated group, but only the silymarin treated group was statistically significantat $P$<0.05 (Table 4.6).

**Table 4.6: Serum concentrations of oxidative stress markers in ASE pre-treated rats following paracetamol-induced liver injury**

| Treatment groups(mg/kg) | Oxidative stress markers | | | |
|---|---|---|---|---|
| | SOD (ng/mL) | CAT (ng/mL) | MDA(nmol/mL) | GPx (ng/mL) |
| **NS** | 9.47 ±1.64 | 40.94 ±1.45 | 13.71 ±0.65 | 92.39 ±4.42 |
| **NS + PCM** | 6.58 ±0.74[a] | 41.31 ±2.39 | 27.00 ±3.60[a] | 89.62 ±5.80 |
| **Sily (50) + PCM** | 11.45 ±1.61[b] | 48.90 ±6.64[a] | 14.17 ±1.19[b] | 99.92 ±1.61[a,b] |
| **ASE(250)+PCM** | 10.71 ±0.81[b] | 45.58 ±1.26[a,b] | 12.28 ±0.50[b] | 99.88 ±9.81 |
| **ASE(750) +PCM** | 12.55 ±0.81[a,b] | 53.91 ±3.17[a,b] | 11.93 ±0.96[b] | 97.06 ±4.62 |
| **ASE(1500) +PCM** | 13.22 ±0.97[a,b] | 40.82 ±3.07 | 12.11 ±0.65[b] | 94.59 ±9.77 |

n = 5; data expressed asmean ±SEM; statistics: one way ANOVA followed by Bonferonni *post hoc* test at $P<0.05$; [a] - statistically significant to NS at $P<0.05$; [b] – statistically significant to NS + PCM at $P<0.05$.Sily = silymarin; NS=normal saline; PCM=paracetamol; SOD=superoxide dismutase; CAT=catalase; MDA=malondialdehyde; GPx=glutathione peroxidise.

### 4.5.3 Effect of 7 day ASE pre-treatment on liver histology in rats induced with liver injury using PCM

The result of thehistology of tissue sections of livers of rats after paracetamol-induced liver injuries showed that PCM caused an intense hepatocellular necrosis with sinusoid and vascular congestion while the group pre-treated with standard drug silymarin showed slight focal necrosis, lymphocyte hyperplasia and vascular congestion. There was slight kupffer cell hyperplasia and slight sinusoidal congestion in the group pre-treated with 250mg/kg of ASE while the group pre-treated with 750mg/kg of ASE showed vascular congestion and slight vacuolation and necrosis. However the group pre-treated with 1,500mg/kg of ASE showed intense hepatocellular necrosis and lymphocyte hyperplasia (Plate VI).

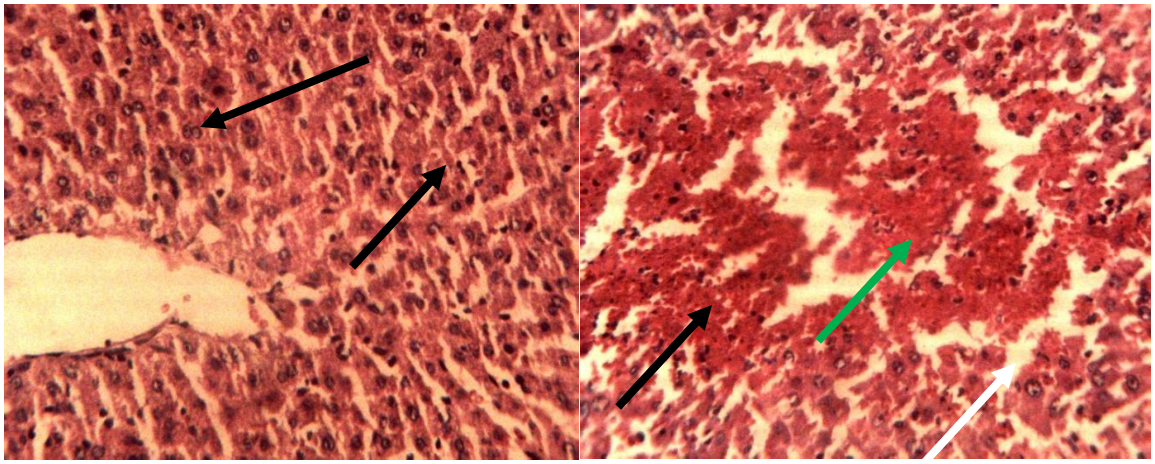


Plate VIa: Normal saline

Plate VIb : NS + PCM

**Plate VI: Photomicrographs of liver sections (H & E stained at ×250 magnification) of 7 days ASE pre-treated rats following paracetamol-induced liver injury I**

VIa: Section showing normal histological appearance of liver tissue with hepatocytes (Black arrows)

VIb: Section showing intense necrosis (white arrow) with sinusoid congestion (black arrow) and vascularcongestion (green arrow)

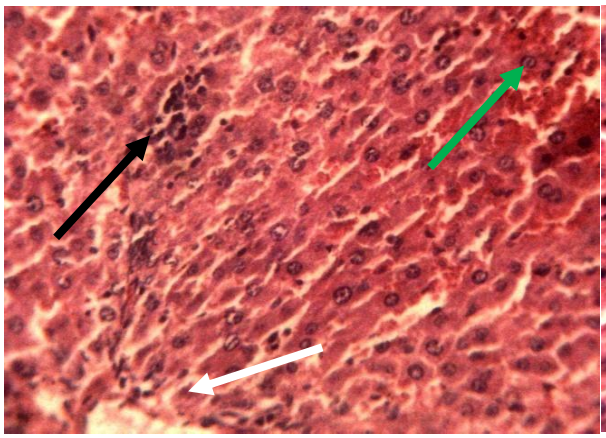


Plate VIc: Sily + PCM

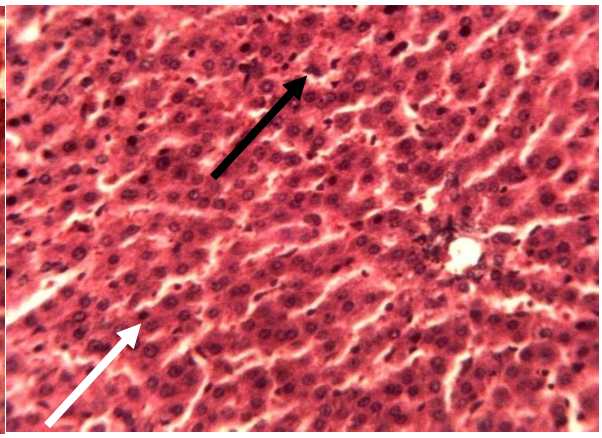


Plate VId: ASE 250mg/kg + PCM

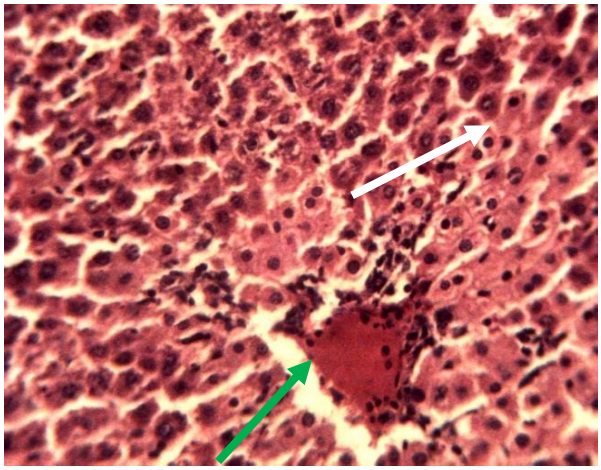


Plate VIe: ASE 750mg/kg + PCM

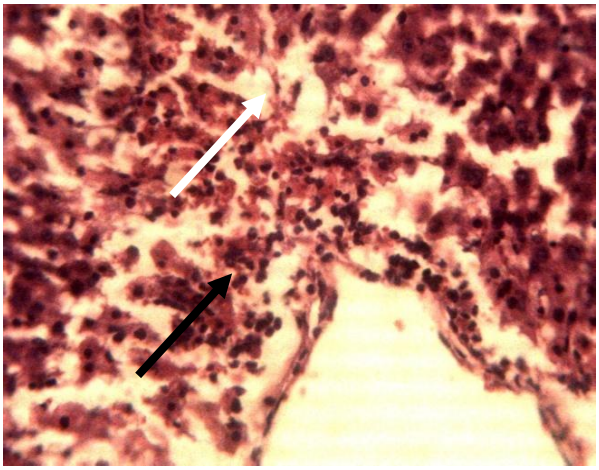


Plate VIf: ASE 1,500mg/kg + PCM

**Plate VI: Photomicrographs of liver sections (H & E stained at ×250 magnification) of 7 days ASE pre-treated rats following paracetamol-induced liver injury II**

VIIc: Section showing slight focal necrosis (white arrow) and lymphocyte hyperplasia (black) with vascular congestion (green arrow)

VIId: Section showing slight kupffer cell hyperplasia (black arrow) with slight sinusoidal congestion (white arrow)

VIIe: Section showing vascular congestion (green arrow) with moderate necrosis (white arrow)

VIIf: Section showing intense hepatocellular necrosis (white arrow) and lymphocyte hyperplasia (black arrow)

## 4.6 Effect of ASE on Bile Duct Ligation-Induced Liver Injury in Rats

### 4.6.1 Effect of 7 day ASE administration on liver biomarkers followingbile duct ligation-induced liver injury in rats

In this study, bile duct ligation (BDL) -induced injury increased all liver biomarkers, but significantly ($P$<0.05) only for ALP, AST and bilirubin (direct and total) when compared with the operated but non-ligated control. However, BDL caused an insignificant decrease in the serum level of albumin when compared with the operated but non-ligated control.There was a non significant decrease in the level of ALT by the lower extract doses (125 mg/kg and 250 mg/kg) when compared to the increase seen with BDL, however, silymarin increased ALT although insignificantly.The BDL-increased AST was decreased by all extract dose groups but significantly at 125 and 250 mg/kg ASE doses. ALP was also decreased by all ASE dose groups but was significant for 250 and 380 mg/kg dose groups. Total protein concentration was decreased at all the extract doses although non-significantly, but silymarin was seen to insignificantly increase the level of total protein when compared to the increase with BDL.Silymarinand the higher extract doses (250 mg/kg, 380 mg/kg) increased the serum level ofalbumin but this increase wassignificant ($P$<0.05) only for the silymarin group. Both Direct and total bilirubin were significantly reduced ($P$<0.05)at all extract dose groups and silymarin group when compared to the BDL control (Table 4.7).

**Table 4.7: Liver biomarkers in rats treated for 7 days with ASE following bile duct ligation-induced liver injury**

| Liver biomarkers | Treatment groups (per kg) | | | | | |
|---|---|---|---|---|---|---|
| | **Non-ligated + NS, 1ml** | **BDL + NS, 1ml** | **BDL+ Sily 50mg** | **BDL+ASE 125mg** | **BDL+ASE 250mg** | **BDL+ASE 380mg** |
| **ALT (IU/L)** | 7.67±0.88 | 8.33±1.45 | 9.02±1.00 | 6.67±0.33[c] | 7.00 ±1.0 2 | 7.67±1.45 |
| **AST (IU/L)** | 9.00 ±1.15 | 24.67±4.17[a] | 20.00±3.58[a] | 12.05±2.08[b,c] | 10.50±0.50[b,c] | 19.67±1.76[a] |
| **ALP (IU/L)** | 12.87± 2.04 | 16.23±0.84[a] | 13.32±2.18 | 15.93±0.38[a] | 12.57±0.26[b] | 13.93±1.21[b] |
| **Total Protein (g/dL)** | 5.98±0.09 | 6.35±0.90 | 6.78±1.16 | 5.39±0.60 | 5.59±0.50 | 5.62±0.64 |
| **Albumin (g/dL)** | 1.88±0.37 | 1.43±0.38 | 2.20±0.16[b] | 1.42±0.31 | 1.64±0.30 | 1.91±0.13 |
| **Direct bilirubin (mmol/L)** | 0.33±0.13 | 2.83±0.08[a] | 0.39±0.08[b] | 0.27±0.04[b] | 0.23±0.10[b] | 0.27±0.04[b] |
| **Total bilirubin (mmol/L)** | 0.56 ±0.17 | 2.86±0.08[a] | 1.18±0.23[a,b] | 0.82±0.27[b] | 0.58±0.10[b] | 1.53±0.10[a,b] |

n = 5; data expressed asmean ±SEM; statistics: one way ANOVA and Bonferonni post hoc test; [a] - statistically significant to Non-ligated + NS at $P<0.05$; [b] – statistically significant to BDL + NS at $P<0.05$, [c] – statistically significant to BDL + Sily at $P<0.05$.Sily = silymarin; NS=normal saline; BDL=bile duct ligation; ALT=alanine aminotransferase; AST=aspartate aminotransferase; ALP=alkaline phosphatise.

### 4.6.2 Effect of 7 day ASE administration on serum oxidative stress markers on bile duct ligation-induced liver injury in rats

The result of this study revealed that BDL-injury showed significant reduction ($P$<0.05) in the concentrations of both SOD and CAT. MDA level was increased significantly ($P$<0.05) by BDL. However, GPx level was slightly elevated by BDL. The standard drug - silymarin and lower extract doses (125 and 250 mg/kg) significantly increased ($P$<0.05) the BDL-reduced level of SOD. The BDL-reduced level of CAT was significantly increased ($P$<0.05) in all the treatment groups and dose dependently in the extract groups, with silymarin showing more increase. The BDL-increase in MDA remained at its elevated level at the 250 and 380 mg/kg ASE doses, but was significantly reduced ($P$<0.05) at the lowest extract dose (125mg/kg) as with silymarin standard drug. BDL-injury caused a slight increase in GPx level, but this enzyme level was increased at all the extract doses and the silymarin treated group but was found significant($P$<0.05) atonly the extract treated groups (Table 4.8).

**Table 4.8: Serum concentrations of oxidative stress markers following 7 day ASE administration on bile duct ligation-induced liver injury in rats**

| Treatment groups(mg/kg) | Oxidative stress markers | | | |
|---|---|---|---|---|
| | **SOD (ng/mL)** | **CAT (ng/mL)** | **MDA(nmol/mL)** | **GPx (ng/mL)** |
| **Non-ligated+NS** | 5.47 ±0.65 | 40.94 ±1.45 | 36.71 ±1.54 | 95.06 ±4.05 |
| **BDL + NS** | 1.27 ±0.18[a] | 32.48 ±8.27[a] | 68.61 ±6.09[a] | 99.61 ±1.02 |
| **BDL + Sily 50** | 2.01 ±0.13[a,b] | 61.38 ±5.30[a,b] | 47.30 ±9.44[b] | 105.28 ±7.40 |
| **BDL + ASE 125** | 2.35 ±0.40[a,b] | 45.06 ±3.24[b,c] | 52.45 ±3.91[a,b] | 112.62 ±8.31[a,b] |
| **BDL + ASE250** | 2.61 ±0.31[a,b] | 49.11 ±4.95[a,b,c] | 67.45 ±7.45[a] | 116.50 ±7.50[a,b] |
| **BDL + ASE 380** | 1.65 ±0.37[a] | 53.70 ±3.75[a,b] | 68.23 ±6.23[a] | 113.54 ±7.54[a,b] |

n = 5; data expressed asmean ±SEM; statistics: one way ANOVA and Bonferonni *post hoc* test; [a] - statistically significant to Non-ligated + NS at $P<0.05$; [b] – statistically significant to BDL + NS at $P<0.05$, [c] – statistically significant to BDL + Sily at $P<0.05$. Sily =silymarin; NS=normal saline; BDL=bile duct ligation; SOD=superoxide dismutase; CAT=catalase; MDA=malondialdehyde; GPx=glutathione peroxidise.

### 4.6.3 Effect of 7 day ASE administration on liver histology on bile duct ligation induced liver injury in rats

The hepatocytes of the operated, non-ligated normal saline (control) group of rats were observed to be intact and normal unlike the BDL-injury group which showed moderate necrosis of the hepatocytes and congestion of the central vein (vascular congestion). This effect was seen to be reversed at the 125mg/kg treated group, while the 250mg/kg treated group had lymphocyte hyperplasia. The silymarin group showed moderate necrosis similar to that in the BDL-injury group, but with no vascular congestion, while the 380mg/kg extract group had Kupffer cell hyperplasia and vascular congestion (Plate VII).

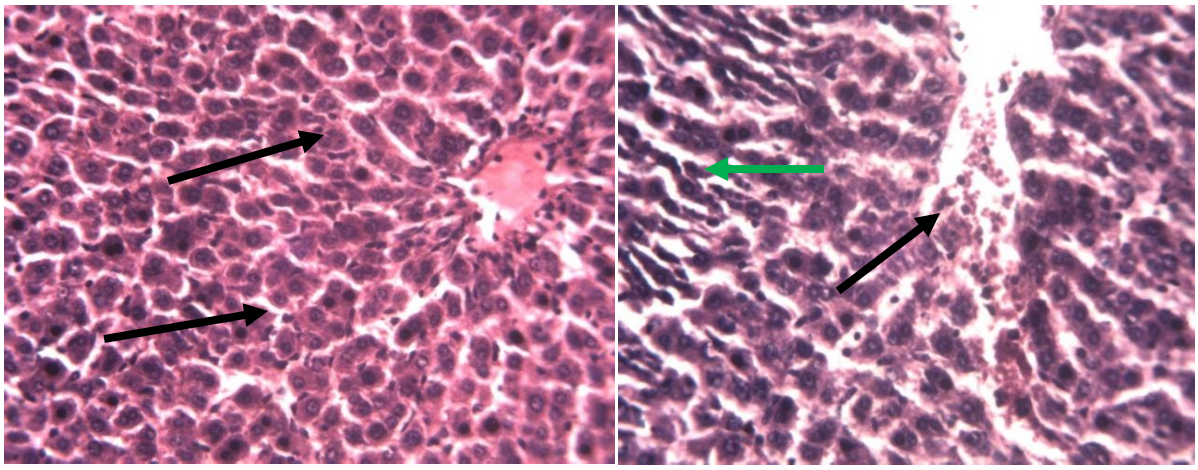


Plate VIIa: Operated, non-ligated + NS  Plate VIIb: BDL + NS

**Plate VII: Photomicrographs of liver sections (H & E stained at ×250 magnification) of rats on bile duct ligation induced liver injury following 7 days ASE administration I**

VIIa: Section showing normal histological appearance of the liver tissue and normal hepatocytes (black arrows)

VIIb: Section showing vascular congestion (black arrow) with moderate necrosis (green arrow)

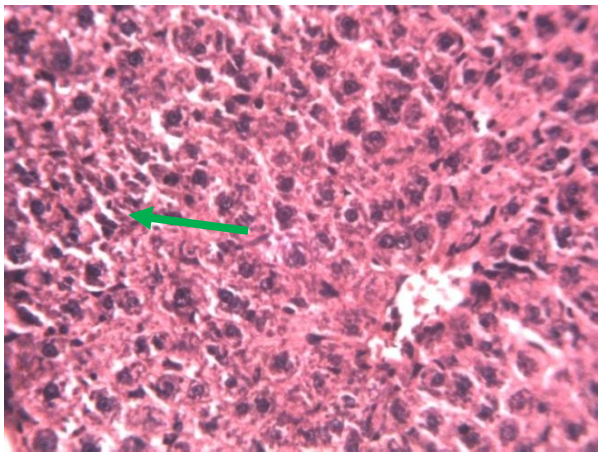


Plate VIIc: BDL + Sily 50mg/kg

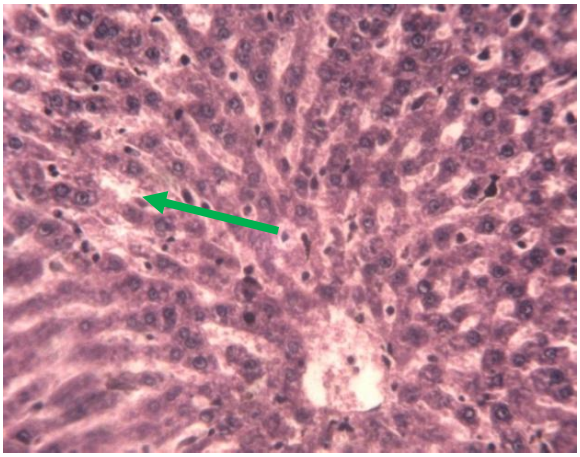


Plate VIId: BDL + ASE 125mg/kg

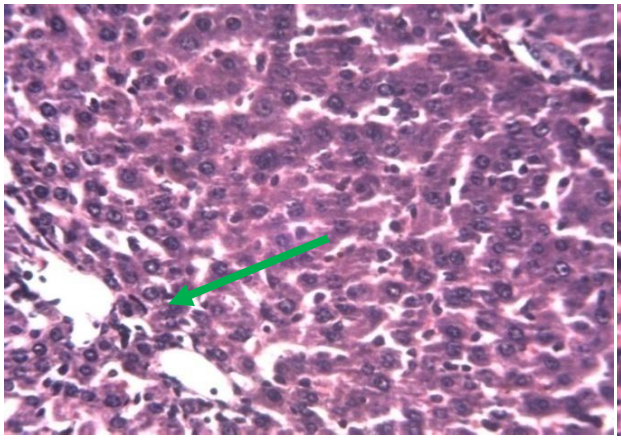


Plate VIIe: BDL + ASE 250mg/kg

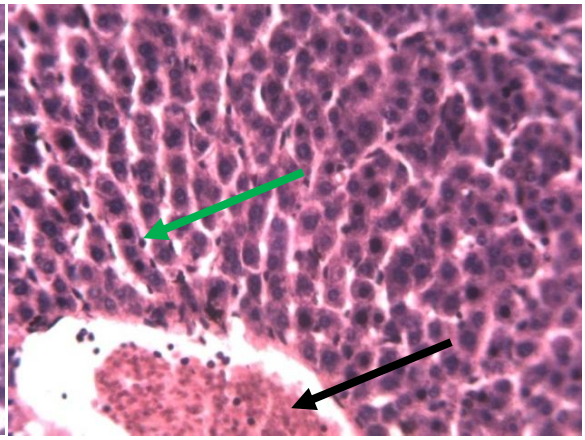


Plate VIIf: BDL + ASE 380mg/kg

**Plate VII: Photomicrographs of liver sections (H & E stained at ×250 magnification) of rats on bile duct ligation induced liver injury following 7 days ASE administration II**

VIIc: Section showing moderate necrosis (green arrow)

VIId: Section showing slight necrosis (green arrow)

VIIe: Section showingslight lymphocyte hyperplasia (green arrow)

VIIf:Section showing kupffer cell hyperplasia (green arrow) and vascular congestion(black arrow)

# CHAPTER FIVE

## 5.0 DISCUSSION

The liver is often exposed to diverse endogenous and exogenous toxic xenobiotics, drugs, waste materials, viral or bacterial agentswhich traverse it for detoxification or excretion. The early stageliver inflammatory insult or injury from harmful agents is usually latent and asymptomatic and even on detection has no drugs with which to halt its deteriorative progression and this tends to resultin chronic end stage liver diseaseassociated with numerous metabolic disorders.Of the several herbs and herbal formulationsof claimed hepatoprotective efficacy,the flavonoid extract of *Silybum marianum* plant is currently standardised as silymarinfor use(Sheikh *et al*., 2012).This studyevaluated methanol extract of the root bark of *Acacia sieberiana*on paracetamol and bile duct ligation induced rat models of liver injury in attempt to validate its folkloric and safe use in liver diseases.

This study found many of the phytochemicals that had been reported to possess hepatoprotectiveproperties to be present in the methanol root bark of ASE, including triterpenoids, glycosides, saponins, flavonoids and alkaloids (Valan *et al.*, 2010; Samir and Amrit, 2014). The extract,however, did not contain anthraquinones and steroids.In a previous study by Zeuko *et al*. (2015), an acetone extract of the stem bark of the plant showed the presence of steroids which was not found in the methanol root bark extract in the present study, and which on the contrary showed presence of flavonoids and alkaloids. The therapeutic or biological activities of any medicinal plant are usually a direct function of the chemical constituents present in the plant and phytochemical constituents often vary with

plant parts, method of extraction and solvent for the extraction (Ayinde and Agbakwuru, 2010).

The acute toxicity effect in 24 hour period of single exposure to graded doses of the ASEshowed no death at 5,000mg/kg body weight oral treatments in rats and thus its oral median lethal dose ($LD_{50}$) was estimated as >5,000mg/kg suggesting it to be non-toxic with oral acute administration.Death was howeverrecorded with intraperitoneal treatment at the extract dose of 1,600 mg/kg body weight of rats resulting in a calculated $LD_{50i.p}$ of 1,300mg/kg suggesting the extract to be relatively non-toxic with acute *i.p.* administration.

Body weight changes can be an adverse effect of certain substances(Tucci, 2010). The obtained result suggested that ASE treatment slowed the progressive increase in body weight of the rats in all its doses used in this study compared to the control group. The decrease in weightin groups treated with ASE could be attributed to the effect of the extract on the appetite or food intake of the rats or the extract may have interferredwith nutrientuptake from the gut or the absorption processes.

Liver enzymes are sensitive indicators of liver injury and a moderate elevation of aminotransferases (ALT and AST) in the blood stream or plasma usually suggests chronic hepatitis or biliary obstructions (Karthikeyan *et al*., 2006; Usha *et al*., 2008). In the 28 days subchronic toxicity study,the activity of ALTincreased, while that of ASTdecreased, but none was significant with respect to the control. These slight changes therefore suggested containment within the limits of the normal biochemical or metabolic adjustments of the body system. Although the presence of γ-glutamyltransferase or 5'-nucleotidase which are used to differentiate the hepatic from the extrahepatic sources of alkaline phosphatase (ALP) were not

investigated in this study, there was a significant dose-dependent increase in the serum level of ALP in rats treated with ASE for 28 days. According to Hall and Cash (2012), moderate increase in ALP, with little or no increase in ALT and AST suggests primary biliary cirrhosis or primary sclerosing cholangitis.In as much as the total protein and albumin were not altered in a consistent pattern, the levels of direct and indirect (total) bilirubin were also insignificantly altered.

Urea is a non-protein nitrogen compound produced in the liver from ammonia as an end product of protein metabolism and which is often carried in blood to the kidneys for excretion. A decrease in urea level is an indication of liver disorders and not only is urea a biomarker for liver function, it can also, like creatinine, be used to measure renal function (Fischbach and Dunning, 2005). Thus, the observed significant increase in serum urea at the 1,500 mg/kg extract dose in this study could be related to decreased kidney function and subsequent inability to excrete the urea. Normally, hepatic abnormalities do not have effects on serum creatinine. High serum creatinine level indicates kidney problems, while reduced level of it signifies reduced muscle activity as in health problems and normal aging. The effect of the extract on creatinine in this study was neither significant nor in a consistent pattern. Generally,increase in serum creatinine level had been reported to start only when about half of the nephrons are impaired (Van-leeuwen *et al*., 2011)**.**The insignificant changes observed in the electrolyte levels were not in a consistent manner, but slight decrease in the chloride level occurred at the higher doses of the extract. The liver and kidneys are considered highly useful in toxicity studies because of their involvement in several essential detoxification functions and which exposes them to harmful compounds.

Assessment of the haematological indices showed that *Acacia sieberiana* did not cause any significant change in the level of Hb, PCV, RBC, WBC, platelets, neutrophils, eosinophils, and monocytes but there was a significant increase in the level of lymphocytes at the 1,500 mg/kg dose group.This increase could suggest that the extract may contain biologically active compound(s) that may have activated the immune system (WHO, 2004) or may suggest toxicity of the extract at that dose. The above increase could also be due to an imbalance in the rate of the lymphocyte synthesis and catabolism (Cooper, 2004).

The histological examination of body organs is one of the golden standards for evaluating treatment related pathological changes (OECD, 2005) and four vital organs were used to assess ASE for toxicity.The heart was not affected, while the morphology of liver, kidney and lungs were altered. The hepatocellular necrosis and vacuolations that occurred in the liver may have resulted in the increase in ALP that was seen in the liver function test. It had been reported that liver ALP is located histochemically in the microvilli of bile canaliculi and on the sinusoids surface of the hepatocytes (Hall and Cash, 2012), thus agents that affect the liver as with ASE may also cause alterations in the concentration of this enzyme. The necrosis that was seen in the glomerulus of the kidney may also cause the increase in serum urea concentration, observed in this study, and which probably reduced its filtration ability. The alveolar congestion of the lungs found in this study may have caused a delay in the absorption of oxygen and other gaseous substances required by the body and may contribute to the reduction in weight of the rats since the extremely rapid absorption of the alveolar epithelium is desirable for diffusion of gaseous substances into the pulmonary capillaries for normal body functions.

The hepatoprotective activity of ASE was also evaluated on someinduced liver injury alteredbiochemical and oxidative stress biomarkers as well as liver histologic changes. The liver injury induction with PCM caused significant elevation of ALT and AST. AST and ALT had been reported to be particularly elevated in conditions that cause extensive cell necrosis as in toxic injuries of the liver (Giannini *et al*., 2005)alsoseen with PCM in the present study. ALT is sorely produced in the hepatocytes and, thus, is a liver specific enzyme localised in the cytosol of the hepatocytes, while AST is produced in both the heart and skeletal muscle tissue, but often localised in the mitochondria and cytosol of hepatocytes (Hall and Cash, 2012). In cell necrosis of the heart and liver, the increase in serum AST is usually of its mitochondrial isoenzyme (Gaskill *et al*., 2005). Thus, the hepatocellular membrane damage causes leakage of the enzymes into the blood. Significant reduction in ALT and AST of PCM toxic injury was seen at the lower doses of 250 and 750 mg/kg ASE and this was similar to that of silymarin standard drug used for the study. Usually, in hepatocellular injury, AST increases in conjunction with ALT which is a specific hepatic marker. The 1,500 mg/kg extract group showed a little and non-significant reduction in ALT and AST of the PCM injury when compared to the significant reductions seen with the lower doses (250, 750 mg/kg) of the extract. This may suggest that the extract may be toxic at the1,500 mg/kg dose. Toxic injury had been reported to notoften affect ALP concentration (Hall and Cash, 2012), and this seemed to be confirmed in this study in that the PCM toxic injury did not significantly increase the ALP activity unlike ALT and AST.

Decrease in total protein and albumin levels usually occurs in extensive liver damage (Kaslow, 2011)and both of which were found reduced in PCM-induced toxic injury in this study. ASE at all its doses increased the concentrations of the total protein and albumin

significantly. However, the increase caused by silymarin was not significant for albumin as with the total protein. Moreover, the extract at the higher doses increased the level of total protein more than silymarin. Increased levels of total protein and albumin show hepatoprotective activity as albumin is mainly produced in the liver and stimulation of protein synthesis accelerates the regenerative process and production of liver cells (Van-leeuwen *et al*., 2011).

In the current study, PCM liver injury was also reflected in both the direct and total bilirubin which were increased, but this was significant only for the direct bilirubin. Bilirubin is synthesised in the liver and elevation of the direct bilirubin usually occurs in conditions of liver dysfunction or bile obstruction (Hansen, 2007; Eldon, 2009a). ASE reduced the PCM elevated bilirubin level, which was significant only with silymarin and 750 mg/kg ASE treated group for the direct bilirubin, but none of the reductions was significant for total bilirubin. These reductions were also seen to be more with the lower extract doses (250, 750 mg/kg) than the highest dose (1,500mg/kg).

The antioxidant enzymes (SOD, CAT and GPx) form a team of defence mechanism against reactive oxygen and nitrogen species (ROS and RNS). The significant increase in SOD concentration in all ASE-treated groups suggested protection against PCM toxic injury that caused its decrease and the higher ASE doses showed more of the increase than the standard drug. SOD converts superoxide radical into $H_2O_2$ and $O_2$ to maintain the steady state of oxygen (Palanivel *et al*., 2008). Thus, decrease in SOD as seen with PCM toxic injury in this study suggests hepatocellular damage reported to be often associated with it (Sha *et al*., 2015). CAT and GPx often convert $H_2O_2$ to $H_2O$ and $O_2$ and thus, tend to complete the SOD's scavenging activity (Sha *et al*., 2015), and although the PCM toxic injury did not affect

catalase concentration, ASE as with silymarin at 250 and 750 mg/kg doses significantly increased its level, with slightly higher increase in the750mg/kg group than silymarin. The 1,500 mg/kg dose may have caused a dose-related toxic effect to mask the ASE increase in CAT as with the lower doses. The injury from PCM-induced reduction in GPx was also ameliorated by ASE, which increased the level of this enzyme beyond normal control and almost to the same extent with the standard drug, but this was not significant for all the ASE groups. Only the silymarin group showed significant increase in GPx from PCM-induced injury, which was also significant when compared with the normal control. The PCM-induced toxic injury caused enhanced lipid peroxidation as seen with the increase in malondialdehyde level beyond the normal control in this study. Oxidised products of lipid peroxidation, such as MDA tend to contribute to or amplify cellular damage and, thus, are often used as biomarkers of oxidative injury(Weydert and Cullen, 2010). Both silymarin and ASE dose groups decreased the MDA level to almost that of normal control, and the ASE groups were slightly better than silymarin.

The reduction in the PCM-induced intense necrosis and congestion in the treatment groups of both silymarin and ASE doses suggested hepatoprotective activity resulting probably by increasing the activity of antioxidant system in the body thereby preventing liver damage. This protection seemed better with the lower ASE doses than with the 1,500 mg/kg dose which may be associated with dose-related toxic effect.

The result obtained for the BDL-liver injury in this study seemed to suggest milder toxicity than the paracetamol-induced model. The extract, as in the paracetamol-induced model, also demonstrated amelioration of induced injury. All the extract groups reduced the BDL-increase in ALT, but the standard drug increased it more than that of the BDL-injury. Although the

reduced ALT level at the lowest extract dose which showed more of the reduction was not significant to the BDL-injury, it was significantly reduced compared to the increase seen with silymarin. The BDL-injury elevated AST was also reduced in all the extract groups more than the standard drug, but only the lower extract doses had significant reduction in AST. However, ALP which was also reduced in all treatment groups, was only significant for the higher extract doses, but not for silymarin and lowest extract dose. The better result exhibited by the lower extract doses for ALT and AST seemed to suggest a dose related toxic effect at the highest dose of the extract.

The effect of ligation was slight for both total protein (which increased) and albumin (which decreased slightly). Silymarin increased the albumin level significantly, but the increase in the total protein was not significant. ASE higher doses increased albumin level, but total protein decreased in all the ASE doses, but these were slight and insignificant.

Both the direct and total bilirubin (DBil and TBil) that were significantly elevated with the BDL injury were significantly reduced in all the treatment groups and this was better for extract groups than the standard drug.Since the increase in total bilirubin level occurs in defective liver uptake for conjugation(Alan, 2007), it seemed likely that the extract increased bilirubin uptake or its conjugation. The conjugated (direct) bilirubin in the blood is elevated only in conditions of reduced bile formation or excretion (cholestasis) (Alan, 2007), thus the ASE extract might have increased the bile formation.

The decrease in SOD from BDL-injury was reversed/prevented significantly in the lower ASE doses (125 mg/kg 250 mg/kg) as with silymarin standard and this was dose-dependent for the extract groups andthe results were better than silymarin. The increase at the highest ASEdose

(380 mg/kg) was not significant when compared to the BDL injury group and this seemed to suggest a dose-related toxic effect.Although SOD level was increased in all the treatment groups, the level was still significantly below that of the normal control unlike the result obtained in the PCM-induced injury study above, in which SOD in all the treatment groups increased beyond the normal control. A dose-dependent increase in CAT also occurred with all the ASE-treated groups and in this case, the increase at the380 mg/kg extract dose was more and was not significantly different from the silymarin group, unlike the lower extract doses. In both the PCM- and BDL- induced injury, the GPx concentration were not significantly reduced, but it was increased in all the treatment groups of both the standard drug and ASE dose groups beyond the normal control. GPx is an enzyme that protects haemoglobin from oxidative degradation in the red blood cells. This enzyme has been reported to be over-expressed when in attempt to protect cells against oxidative damage and apoptosis induced by $H_2O_2$ (Yoshikawa and Naito, 2002). Although the PCM-induced increase in MDA was significantly reduced in the treatment groups of both the standard and all the ASE dose groups, only the standard drug and the lowest extract dose reduced its BDL-increase significantly.

The moderate necrosis and vascular congestion observed with the BDL were reversed/preventedwith 125mg/kg extract group. Milder effectswas observed at the higher dosesASE dose groups (250 and 380 mg/kg) and the silymarin group. These effects could be suggestive of some level of protection by the ASE extract or liver regenerative potentials of the extract on the liver.

The hepatoprotective effect of ASE in the BDL-induced liver injury was more at the extract dose of 125 mg/kg as also with the dose of 250 mg/kg dose in the PCM-induced injury study

and showed better protection than the standard drug (silymarin). The better protection seen with the two lower extract doses suggested that the highest ASE dose used in this study may be relatively toxic.

# CHAPTER SIX

## 6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

### 6.1 Summary and Conclusion

This study found that many of the phytochemicals present in the methanol root bark of ASE had been reported to possess hepatoprotective properties.

The acute toxicity study revealed that the extract is relatively safe orally (Lorke, 1983) since the $LD_{50}$ was greater than 5,000mg/kg and relatively toxic intraperitoneally with $LD_{50}$ of 1,300mg/kg.

The continuous oral daily dosing (subchronic toxicity study) of ASE for 28 days showed a decrease in the body weight gain that was significant when compared to the control.A significant dose-dependent increase in ALP with insignificant ALT increase and AST decrease was observed in ASE-treated groups. There was a dose dependent increase in urea that was significant only at 1,500 mg/kg extract dose when compared to the control. The total protein, albumin, bilirubin, creatinine and all the assessed electrolytes were not significantly altered by ASE treatments. The lower extract doses showed slight increase in WBC though not significantly, but there was a significant increase in lymphocytes at 1,500 mg/kg.

The organs histology following the 28 days subchronic toxicity study showed no changes in heart, a dose-dependent hepatocellular necrosis and vacuolations in liver; lymphocyte hyperplasia with slight tubular distortion and glomerular necrosis in kidney and lymphocyte hyperplasia with alveolar congestion in lungs.

ASE ameliorated the PCM and BDL hepatotoxic changes in the morphology and biomarkers of liver dose-dependently and was relatively toxic at increasing doses and its effect showed that:

For the significant PCM hepatotoxic increase in ALT, AST, direct bilirubin, and malondialdehyde (MDA), all extract doses reduced ALT and AST levels, but significantly at lower doses and silymarin; direct bilirubin reduced significantly only at 750 mg/kg ASE dose and silymarin, and MDA level reduced significantly in all treatment groups.

For the significant PCM hepatotoxic decrease in total protein, albumin and superoxide dismutase (SOD); significant increase in total protein at all treatment groups; in albumin only at all extract doses and in SOD even beyond normal control at all treatment groups and dose-dependently for the extract doses was seen.

The insignificant PCM hepatotoxic glutathione peroxidase (GPx) decrease was at all treatment groups increased beyond normal control, but significant only for silymarin.

PCM had no hepatotoxic effect on catalase (CAT) but, showed significant increase at silymarin and lower extract (250, 750 mg/kg) treatments that occurred dose-dependently, with no effect at highest dose.

The PCM hepatotoxic intense necrosis and congestion on the liver histology, was considerably reduced in all treatment groups.

For the BDL hepatotoxic significant increase in ALP, AST, bilirubin (direct and total) and MDA; AST reduced significantly at lower extract doses and ALP at higher doses; MDA reduced significantly in the lowest extract and silymarin groups, while bilirubin (direct and

total) reduced significantly at all treatment groups and below normal for the direct bilirubin of the extract dose groups.

For the BDL significant decrease in SOD and CAT, CAT at all treatment groups increased significantly beyond normal control; SOD also increased significantly only at lower extract and silymarin groups, though still significantly below normal; while the significantly reduced albumin of BDL was increased slightly above normal at highest extract dose and significantly in silymarin group, while the GPx at all the extract doses increased significantly.

ASE extract at the lower doses (125 and 250 mg/kg) protected the liver cells from the BDL-injury more than silymarin and the highest extract dose of 380mg/kg.

In conclusion, this study has shown that the methanol root bark extract of *Acacia sieberiana* was found relatively non-toxic especially for the oral route than for *i.p*. in the acute toxicity study. This study however showed that the extract has ameliorative effect on liver injury at its lower doses (125mg/kg, 250mg/kg) which may justify its local use for hepatitis; but, at higher doses (750mg/kg, 1500mg/kg) the extract was found relatively toxic particularly with prolonged use.

## 6.2 Recommendations

i. The extract treated rats showed decrease in weight gaincompared to the control rats and thus there may be need to evaluate food and water intake and/or lipid profile that assesses excessive breakdown of fat.

ii. Hepatotoxins of highly lipid soluble toxic parent drugs that either defy detoxification or are of reduced metabolic rate are the predominant cause of liver inflammations as with toxic metabolites, thus sleeping experiment to determine the rate of ASE metabolism may be carried out to ascertain whether the observed toxic effects are due to reduced ASE metabolism.

iii. Since the lowest extract dose used for this study showed reasonable efficacy and reduced toxicity, extract doses below this dose may be assessed for both efficacy and toxicity.

iv. Since the methanol root bark extract of *Acacia sieberiana* showed potential ameliorative effect on both PCM and BDL induced liver injury in the present study, there may be need for characterisation and standardisation of the ASE extract.

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## APPENDICES

### Appendix I: Weekly body weight changes of rats in 28 days daily oral ASE administration

| Treatment groups (mg/kg) | Mean ± SEM weekly body weights of rats | | | | |
|---|---|---|---|---|---|
| | **0** | **1** | **2** | **3** | **4** |
| **N/S** | 125.50 ±8.37 | 146.33 ±9.58 | 154.00 ±8.43 | 162.67 ±8.66 | 167.33 ±9.96 |
| **ASE 250** | 117.50 ±7.12 | 126.67 ±9.96 | 134.00 ±8.68 | 143.83 ±8.23 | 145.17 ±9.88 |
| **ASE 750** | 118.83 ±3.87 | 125.50 ±4.72 | 129.83 ±3.32 | 138.50 ±4.28 | 141.00 ±3.65 |
| **ASE1500** | 125.17 ±6.29 | 126.67 ±8.31 | 132.83 ±6.60 | 134.00 ±7.73 | 135.83 ±6.82 |

n = 10; statistics: split-plot ANOVA followed by Bonferonni multiple comparison *post hoc* test at $P<0.05$. N/S=normal saline