HEPATOPROTECTIVE AND TOXICITY STUDIES ON *ANNONA MURICATA* LINN. LEAVES FOR THE TREATMENT OF JAUNDICE IN ANIMALS

BY:

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DECLARATION

I hereby declare that this thesis is my own work towards the PhD and that, to the best of my knowledge no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Liver diseases and jaundice have continued to be a major health problem in the world's population with most conventional drugs not being adequate for treatment. *Annona muricata* Linn. (family Annonaceae) has been listed in Ghanaian floristic studies to be effective in the treatment of jaundice. It is underutilized and grown for its ornamental purposes. Recent studies on *A. muricata* has been centred on a set of novel phytochemicals called annonaceous acetogenins. This study was designed to evaluate the acute oral and subchronic toxicity, hepatoprotective activity against carbon tetrachloride and acetaminophen (paracetamol)-induced liver damage, and bilirubin-lowering potential of aqueous leaf extract of *Annona muricata* Linn. Aqueous extract of *A. muricata* was evaluated for phytochemical constituents using standard methods. Oral acute and subchronic toxicity were assessed and hepatoprotective activity studied using carbon tetrachloride and acetaminophen-induced liver damage in rat models at doses of 50, 100, 200 and 400 mg/kg body weight administered for seven days by oral route with Silymarin (100 mg/kg b.wt) as the standard hepatoprotective drug. Hepatoprotective effect was studied by assaying the activity of serum marker enzymes like ALT, ALP, cholesterol and triglycerides. Bilirubin-lowering potential was assessed using haemolytic model using phenylhydrazine-induced hyperbilirubinaemia and anti-jaundice effect assessed by measuring serum total bilirubin and direct bilirubin concentration. The aqueous extract contained saponins, general glycosides, tannins and flavonoids. The median acute toxicity value (LD_{50}) of the aqueous extract of *A. muricata* was determined to be $\leq$ 5g/kg body weight. The aqueous extract at 100 mg/kg body weight lowered blood plasma glucose and low density lipoprotein (LDL-cholesterol) levels but raised high density lipoprotein (HDL-cholesterol) levels in both male and female rats.

Treatment had no effect on liver, kidney, heart and stomach weight while uterus weight was increased after treatment with 1000 mg/kg body weight and beyond. Haematological parameters and some biochemical parameters such as ALT, AST, ALP, urea and albumin were unaffected while creatinine levels were increased in animals treated with 2500 mg/kg body weight. In the hepatoprotective study, the activity of all the enzyme markers registered significant increases in CCl_4- and acetaminophen-treated rats. Decreases in cholesterol and triglyceride concentration and increases in total and indirect bilirubin, an indication of hepatic jaundice was observed. *A. muricata* at all doses significantly restored liver function toward normal levels which compared well against Silymarin control. Histopathological analysis of liver sections confirmed biochemical investigations. In bilirubin-lowering study, phenylhydrazine induced jaundice in animals from 1.50 µmol/L ± 0.00 total bilirubin in normal animals up to 29.25 µmol/L ± 2.21 in animals with reduced liver capacity. The hyperbilirubinaemia was reversed close to normal levels in animals treated with *A. muricata* aqueous extract at 50 and 400 mg/kg. Total bilirubin level was reduced to 6.22 µmol/L ± 0.27 at 50 mg, 5.68 µmol/L ± 0.36 at 400 mg/kg and 7.94 µmol/L ± 0.79 with Silymarin, significantly lower ($p<0.001$) compared with vehicle group (16.90 µmol/L ± 2.21) maintained on distilled water. The LD_{50} value indicated that the plant leaf extract is safe. The extract did not produce any toxic effect in the animals' tissues at low and moderate doses but could cause kidney damage at higher doses. Lowering of plasma glucose level and the positive effects of the extract on the cardiovascular risk factors were indications that the extract could have some good antidiabetic activity. The results also indicate that aqueous extract of *A. muricata* possesses hepatoprotective activity and can be used to reduce bilirubin concentration in jaundiced subjects.

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LIST OF ABBREVIATIONS

Alb	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMAE	*Annona muricata* aqueous extract
AMEE	*Annona muricata* ethyl acetate extract
AMME	*Annona muricata* methanol extract
AMPE	*Annona muricata* petroleum ether extract
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
b.wt	Body weight
CYP	Cytochrome P-450 monooxygenase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
G6PD	Glucose-6-phosphate dehydrogenase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HCT	Haematocrit
HDL	Low Density Lipoproteins
HGB	Haemoglobin concentration
HSCs	Hepatic Stellate Cells
LDH	Lactate dehydrogenase
LDL	Low Density Lipoproteins

LYM	Lymphocytes
MCH	Mean Corpscular Haemoglobin
MCHC	Mean Corpscular Haemoglobin Concentration
MCV	Mean Corpscular Volume
NADH	Reduced Nicotinamide Adinine Dinucleotide
PHZ	Phenylhydrazine
RBC	Red Blood Cell
rpm	revolutions per minute
SOD	Superoxide dismutase
TP	Total protein
UPDGT1	Uridine diphosphoglucuronate glucuronosyl transferase 1
VLDL	Very Low Density Lipoproteins
WBC	White blood cell
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Jaundice is the most common presentation of patients with liver and biliary diseases and other extrahepatobiliary causes (Beckingham and Ryder, 2001). It results from imbalance between bilirubin production and excretion. Jaundice or hyperbilirubinaemia is the appearance of yellow pigmentation in the skin, sclera and mucous membranes (Bishayi *et al.*, 2002) and is marked by elevated serum concentration of bilirubin (usually above 50 μmol/L). Serum bilirubin levels are generally less than 1-1.5 mg/dl (17-25 μmol/L) and jaundice is usually not detectable until bilirubin concentration exceed 3 mg/dl (50 μmol/L) (Lidofsky, 2006). Identified causes of jaundice include decreased hepatic bilirubin elimination (Beutler, 1994) and increased haemolysis, in response to an identifiable trigger (Valaes, 2001; Zinkham and Oski, 1996). Jaundice can be categorized as prehepatic, hepatic and posthepatic and this provides a useful framework for identifying the underlying cause (Beckingham and Ryder, 2001). In prehepatic jaundice, excess unconjugated bilirubin is produced faster than the liver is able to conjugate it for excretion. Most patients have hepatic (parenchymal) jaundice, where the liver has reduced capacity for conjugation of bilirubin for excretion or posthepatic (obstructive) jaundice due to blockages in the bile ducts (Thompson, 1970).

Visible jaundice caused by hyperbilirubinaemia occurs in over half of all healthy newborns. Even in healthy full term newborns, extreme hyperbilirubinaemia in the first few weeks of life, although very rare, can cause kernicterus, a permanent and

devastating form of brain damage (Heather and Clanton, 2003). Jaundice has been identified in glucose-6-phosphate dehydrogenase (G6PD)-deficient patients (Kaplan *et al.*, 1998), with higher rate of haemolysis and Gilbert syndrome (Muraca *et al.*, 1990), a benign condition of decreased bilirubin conjugation due to diminished activity of the conjugating enzyme, uridine diphosphoglucuronate glucuronosyl transferase 1 (EC. 2.4.1.17, UDPGT1). The most common intrahepatic causes are viral hepatitis, alcohol cirrhosis, primary biliary cirrhosis, drug-induced jaundice by analgesics (e.g. paracetamol, aspirin, non-steroidal anti-inflammatory drugs); cardiac drugs (e.g. methyldopa, aminodarone); psychotropic drugs (e.g. monoamine oxidase inhibitors, phenothiazines); and other drugs (e.g. sodium valproate and oestrogen), and alcohol hepatitis (Beckingham and Ryder, 2001). Posthepatic jaundice is most often due to biliary obstructive stone in the common bile duct or by carcinoma of the pancreas. Jaundice, and hence liver disease can be assessed in part by measurement of serum bilirubin, albumin, alanine amino transferase (ALT), aspartate amino transferase (AST), and alkaline phosphatase (ALP).

Annona muricata (Linn.) (family Annonaceae) commonly called "soursop" or "apre" in the local 'Twi' language and "aluguntugun" in the local 'Ga' language, is a small erect evergreen tree growing 5 to 6 meters in height. It is a multipurpose tree considered to be underutilized (ICUC, 2002) and is grown in Ghana for its ornamental purposes. The fruits are consumed widely and the tree is also a sources of medicinal and industrial products.

Much of the recent research on *A. muricata* has been on a novel set of phytochemicals (Annonaceous acetogenins) that are found in the leaves, seeds and stem which are cytotoxic against various cancer cells (Chang *et al.*, 2003). Root, stem and

leaves of soursop have different kinds of acetogenins. Some of them have anti-tumour activities and act preferentially against human cancer cell lines (Zeng *et al.*, 1996). Acetogenins found in the soursop leaves and stem are used to prepare extracts that have insecticidal activities (Gleye *et al.*, 1997). In soursop seeds, there are amyloids (Kooiman, 1967), acetogenins and unsaturated and saturated fatty acids.

Various plant parts are widely used in folk medicine, because of the bioactive compounds (mainly acetogenins, alkaloids and flavonoids) found in the roots, leaves bark, fruits and seeds. Acetogenins are potential for cancer treatments as they have cytotoxic effects (Chang *et al.*, 1993). Flavonoids present in the seeds, roots, bark, stem and fruits are potential chemo-protective agents, given evidence that they decrease tumour incidence (Cassady, 1990). The leaves have essential oils with parasiticidal, anti-diarrhoea, anti-rheumatological and anti-neuralgic properties (Gleye *et al.*, 1998). The boiled water infusion of the leaves have antiplasmodic, astringent, and gastric properties (Khan *et al.*, 1997). It help treat diabetes and gastric upset (Adewole and Ojewole, 2006), jaundice (Mshana *et al.*, 2000), kidney ailments (Duke, 1970) and possess antioxidant properties (Adewole and Ojewole, 2009).

Immature soursop fruits have medicinal properties against dysentery, cankers, diuretic, scorbutic, anti-thermical processes, skin diseases, rashes, fever, malaria, peptic ulcers, colic and oedema (Khan *et al.*, 1997). The peels from immature fruits have constituents that act against dyspepsia, diarrhoea and chronic dysentery; it is astringent and provokes vomiting (Calzavara and Muller, 1987). The acid pulp is used to heal foot parasites and icteric liver diseases. The fruits also have properties that act on the biliary vesicles. The seeds have anti-plasmodic and anti-parasitic properties (Philipov *et al.*, 1994). They contain amyloids, oleic acid and steroids (Asolkar *et al.*, 1992).

1.2 Problem Statement

The importance of traditional medicine as a source of primary health care was first officially recognized by the World Health Organization (WHO) in the Primary Health Declaration of Alma Ata (WHO, 1978) and has been globally addressed since 1976 by the Traditional Health Programme of WHO. That Programme defined traditional medicine as; "the sum total of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing" (Rukangira, 2001).

In Africa, traditional healers and remedies made from plants play an important role in the health of millions of people and WHO has described traditional medicine as one of the surest means to achieve total health care coverage of the world's population (Rukangira, 2001).

Among the several reasons why the use of medical plants should be studied are that herbal remedies may have recognizable therapeutic effects (Bailey and Day, 1989) as well as toxic effect (Keen *et al*., 1994).

Jaundice has been identified as a disease of the liver and a condition associated with many diseases. There is therefore the need for adequate treatment of the condition. *Annona muricata* leaves have been recognized to be used in the effective treatment of jaundice in traditional folk medicine in Ghana (Mshana *et al*., 2000). There is, therefore, the need for adequate investigation into the hepatoprotective effect of solvent extracts of *A. muricata* in treating CCl_4 – and acetaminophen – induced liver damage in Sprague-Dawley rats, toxicity of the extract *in vivo*, and the bilirubin-lowering potential of extract as an evidence of jaundice treatment.

1.3 Objectives

The general objective of the study was to elucidate the anti-jaundice hepatoprotective property of *A. muricata* and its safety.

The specific objectives of the investigations are;

1. to evaluate the hepatoprotective effect of *A. muricata* serial extracts *in vivo*;
2. to evaluate the acute and sub-chronic toxicity of *A. muricata* aqueous extract;
3. to evaluate the hepatoprotective effect of *A. muricata* against carbon tetrachloride- and acetaminophen-induced toxicity; and
4. to evaluate the bilirubin-lowering potential of *A. muricata*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Jaundice

Jaundice or hyperbilirubinaemia is defined as a bilirubin concentration above normal laboratory upper limits of 25 μmol/L. Jaundice occurs when bilirubin becomes visible within the sclera, skin and mucous membranes (Bishayi *et al.*, 2002), and blood concentrations around 50 μmol/L. It is the commonest presentation of patients with liver and biliary diseases and other extra-hepatobiliary causes (Beckingham and Ryder, 2001). Hyperbilirubinaemia may be due to the production of excess bilirubin than the normal capacity of the liver to conjugate and excrete, or it may result from the failure of a damaged liver to excrete bilirubin produced in normal amounts (Lidofsky, 2006). In the absence of hepatic damage, obstruction of the secretory ducts of the liver will also cause hyperbilirubinaemia. In all these situations, bilirubin accumulates in the blood and when it reaches a certain concentration (approximately 2 – 2.5 mg/dl), jaundice or icterus results with the concomitant yellow colouration of body tissues.

2.2 Bilirubin Metabolism

The largest repository of haem in the human body is in red blood cells, which has a life span of about 120 days (Oski, 1993). There is, thus, a turnover of about 6 g/day of haemoglobin, which presents two problems. First, the porphyrin ring is hydrophobic and must be solubilized to be excreted. Second, iron must be conserved for new haem synthesis. Other sources of haem include myoglobin and cytochrome enzymes (London *et al.*, 1950).

Normally, senescent red blood cells and haem from other sources are engulfed by cells of the reticuloendothelial system. The globin is recycled or converted into amino acids, which in turn are recycled or catabolized as required. Haem is oxidized, with the haem ring being opened by the endoplasmic reticulum enzyme, haem oxygenase (HO). The oxidation step requires haem as a substrate, and any hemin (Fe^{3+}) is reduced to haem (Fe^{2+}) prior to oxidation by HO. The oxidation occurs on a specific carbon, producing the linear tetrapyrrole, biliverdin, ferric iron (Fe^{3+}), and carbon monoxide (CO) (McKee and McKee, 1996). This is the only reaction in the body that is known to produce CO. Most of the CO is excreted through the lungs, with the result that the CO content of expired air is a direct measure of the activity of haem oxygenase in an individual (Berk *et al.*, 1974).

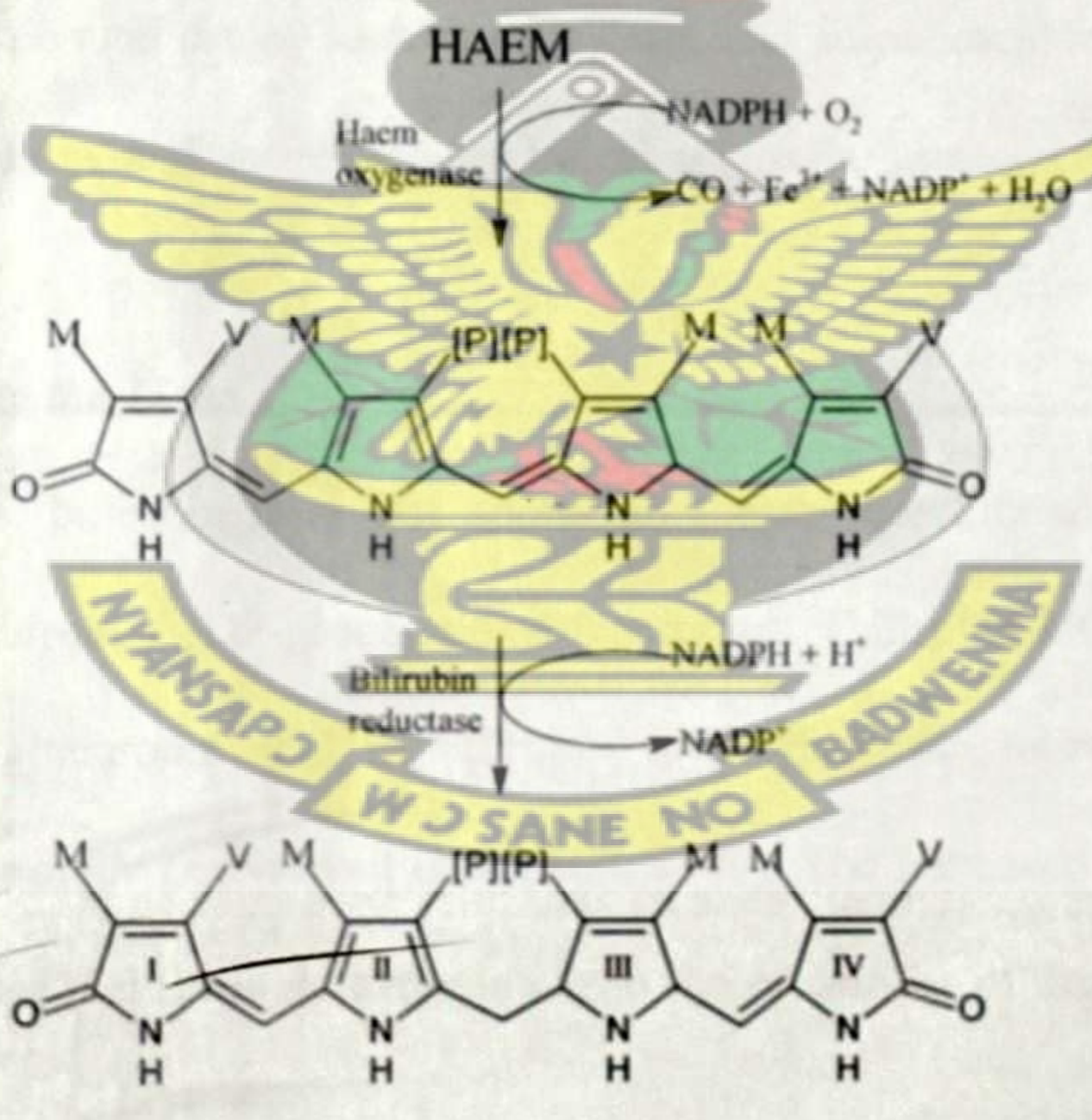


Fig. 2.1: Pathway for the degradation of haem to bilirubin

[M = methyl, V = vinyl, [P] = propionate]

In the next reaction, a second bridging methylene (between rings III and IV) is reduced by biliverdin reductase (BR), producing bilirubin. Bilirubin is

significantly less extensively conjugated than biliverdin causing a change in the colour of the molecule from blue-green (biliverdin) to yellow-red (bilirubin). The latter catabolic changes in the structure of tetrapyrroles are responsible for the progressive changes in colour of a haematoma, or bruise, in which the damaged tissue changes its colour from an initial dark blue to a red-yellow and finally to a yellow colour before all the pigment is transported out of the affected tissue (Jansen and Bittar, 2004).

In blood, bilirubin is firmly bound to albumin. Since in the adult, the albumin in 100 ml of blood can bind at least 40 mg bilirubin, it is seldom saturated (Brodersen, 1980). Nevertheless, presumably some bilirubin can become detached from albumin and when it becomes bound to proteins of the tissues gives rise to jaundice. Protein bound drugs, such as salicylates and sulphonamides, compete with bilirubin for albumin, and hence displace bilirubin into the tissues.

2.2.1 *Bilirubin in the Liver*

By itself, bilirubin does not appreciably enter the bile to any extent. In hepatocytes, bilirubin-UDP-glucuronosyltransferase (bilirubin-UGT) adds two equivalents of glucuronic acid to bilirubin to produce the more water-soluble, bilirubin diglucuronide derivative (Thompson, 1970). The increased water solubility of the tetrapyrrole facilitates its excretion with the remainder of the bile as the bile pigments.

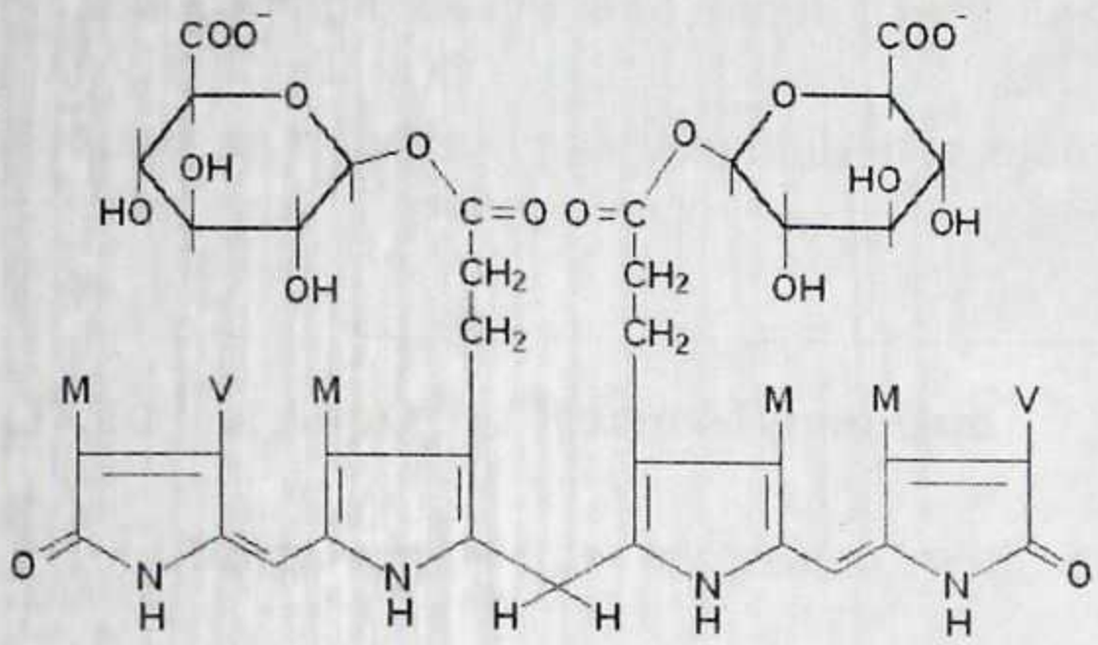


Fig. 2.2: Bilirubin diglucorunide

In humans, 15 UGT transcripts have been identified and divided into two families based upon sequence homology (Tukey and Strassburg, 2000). *UGT2* genes located on chromosome 4q13 and 4q28, consists of 6 exons, and preferentially glucuronidate endobiotic substrates including steroids and bile acids. In contrast, the *UGTlA gene* locus has been mapped to chromosome 2q37 and enables the transcription of individual gene products sharing a common carboxyl terminal portion of 280 amino acids and an exon 1 encoded divergent amino terminal portion of approximately 250 amino acids (Ritter *et al*., 1992). Utilizing a strategy of exon sharing, potentially 9 functional exon 1 sequences can be combined with the constant exons 2-5 to form individual *UGTlA* transcripts. The tissue-specific and individual regulation of the *UGTlA* first exons has recently been identified in the human hepato-gastrointestinal tract. In human liver, transcripts of *UGTlAl*, *UGTlA3*, *UGTlA4*, *UGTIA6* and *UGTlA9* have been detected and cloned, whereas the novel *UGTlA7* (oesophagus, stomach), *UGTlAX* (oesophagus, colon) and *UGTlAl0* (oesophagus, bile ducts, stomach, colon) transcripts have been identified exclusively in extrahepatic epithelial tissues (Strassburg *et al*., 1997). The genetic organization of the *UGTlA* gene locus therefore enables a tissue-specific gene expression of hepatic

and extrahepatic UGTs and most likely ensures that a broad array of differing substrates can undergo glucuronidation in man (Strassburg *et al*., 1998).

2.3 Clinical Aspect of Haem Metabolism

Clinical problems associated with haem metabolism are of two types. Disorders that arise from defects in the enzymes of haem biosynthesis termed porphyrias (Elder *et al*., 1997) with subsequent elevations in the serum and urine content of intermediates in haem synthesis and inherited disorders in bilirubin metabolism leading to hyperbilirubinaemia (Beutler, 1994). Excess circulation and accumulation of bilirubin (hyperbilirubinaemia) results in a yellow-orange discolouration of the tissues and is most easily visible as icteric (yellowish) discoloration in the sclera of the eyes. Bilirubin toxicity (bilirubin encephalopathy) can be life-threatening in neonates. Bilirubin encephalopathy is characterized by yellow discoloration of the basal ganglia in babies with intense jaundice and was first described over a century ago and the term "kernicterus" was coined to describe these physical changes (Lee and Gartner, 1983). Any increase in plasma bilirubin above 20 mg/dL is considered dangerous in neonates. However, individual differences in bilirubin sensitivity can result in kernicterus at lower bilirubin levels. Kernicterus occurs in infants with severe unconjugated hyperbilirubinaemia and in young adults with high serum levels of unconjugated bilirubin.

Bilirubin has been shown to inhibit DNA synthesis (Schiff *et al*., 1985), uncouple oxidative phosphorylation, and inhibit ATPase activity in brain mitochondria (Mustafa *et al*., 1969). Bilirubin also inhibits a variety of different classes of enzymes including dehydrogenases, electron transport proteins, hydrolyases, and enzymes of RNA synthesis, protein synthesis and carbohydrate

metabolism (Sano *et al.*, 1985). All of these toxic effects of bilirubin are reversed by binding to albumin. In fact, albumin plays a vital role in the disposition of bilirubin in the body by keeping the compound in solution and transporting it from its sites of production (primarily bone marrow and spleen) to its site of excretion which is the liver (Gourley, 1997).

2.4 Susceptibility of the Liver

The liver is the largest internal organ in the body and is often the target organ for chemically-induced injury (Dwivedi and Mishra, 2008). Several important factors are known to contribute to the liver's susceptibility. Most xenobiotics enter the liver through the gastrointestinal tracts (GIT) and, after absorption, are transported by the hepatic portal vein to the liver. Thus, the liver is the first organ perfused by chemicals that are absorbed in the gut. Also the high concentration of xenobiotic metabolizing enzymes, primarily the cytochrome P450 (CYP)-dependent monooxygenase system makes the liver a good target (Dwivedi and Mishra, 2008). Although most biotransformations are detoxification reactions, many oxidative reactions produce reactive metabolites that can induce lesions within the liver. Often, areas of damage are in the centrilobular region, and this localization has been attributed in part to the high concentration of CYP in that area of the liver (Dwivedi and Mishra, 2008).

2.5 Types of Liver Injury

Liver injury is indicated by an increase of more than twice the upper limit of the normal range in the levels of serum ALT or conjugated bilirubin or a combined increase in the levels of AST, Alkaline phosphatase (ALP) and total bilirubin,

provided that one of these was more than twice the upper limit of the normal range (Navarro and Senior, 2006). The clinical pattern of liver injury are further characterized as hepatocellular, with a predominant initial elevation of ALT level, or cholestatic, in which the serum ALT level is first elevated. There could, however be mixed if intermediate.

A Food and Drug Administration (FDA) working group stated that 'an alanine aminotransferase (ALT) level of more than three times the upper limit or normal and a total bilirubin level of more than twice the upper limit can be used as a combined test to define clinically significant abnormalities on liver, with further verification through the analysis of additional clinical data' (FDA, 1999). Elevations of serum enzyme levels (ALT, AST and ALP) are taken as indicators of liver injury, whereas increases in both total and conjugated bilirubin levels are measures of overall liver function. Liver injury is generally indicated by elevations in serum aminotransferase levels, but increases of far more than three times the upper limit of normal may not lead to clinically significant liver damage (Navarro and Senior, 2006). This is because of the great capacity of the liver to heal injury, with the subsequent development of adaptive tolerance. The regulation of serum enzyme activity is not a function of the liver, which is more accurately assessed according to the levels of total bilirubin or conjugated bilirubin – reflecting the liver's ability to move bilirubin from plasma into bile.

The types of injury to the liver depend on the type of toxic agent, the severity of intoxication, and the type of exposure, whether acute or chronic. Whereas cholestasis is specific to the liver, others such as necrosis and carcinogenesis are more general phenomena.

2.5.1 Fatty Liver

Fatty liver refers to the abnormal accumulation of fat in hepatocytes. At the same time there is a decrease in plasma lipids and lipoproteins. The onset of lipid accumulation in the liver is accompanied by changes in blood biochemistry. Although many toxicants may cause lipid accumulation in the liver, the mechanisms may be different. Basically, lipid accumulation is related to disturbances in either the synthesis or the secretion of lipoproteins. Excess lipid can result from an oversupply of free fatty acids from adipose tissues or, more commonly, from impaired release of triglycerides from the liver into the plasma. Triglycerides are secreted from the liver as lipoproteins (very low density lipoprotein, VLDL). There are a number of points at which this process can be disrupted. Some of the more important ones are interference with synthesis of the protein moiety, impaired conjugation of triglyceride with lipoprotein, interference with transfer of VLDL across cell membranes, decreased synthesis of phospholipids, impaired oxidation of lipids by mitochondria and inadequate energy (adenosine triphosphate [ATP]) for lipid and protein synthesis.

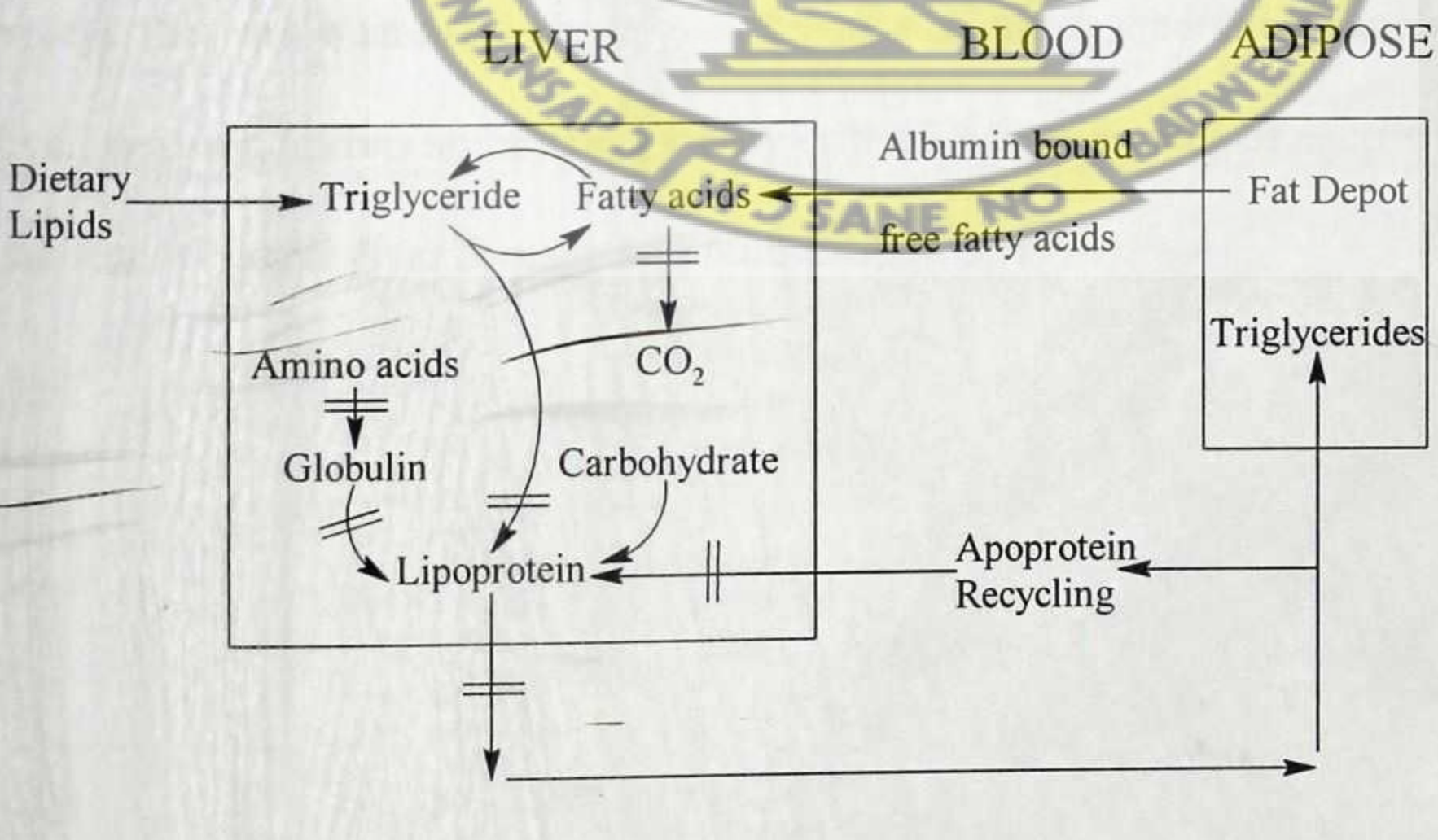


Fig. 2.3: Triglyceride cycle in the pathogenesis of fatty liver.

'=' are metabolic blocks (Courtesy Mayer *et al.*, 2005)

The role that fatty liver plays in liver injury is not clearly understood and fatty liver in itself does not necessarily mean liver dysfunction.

2.5.2 Necrosis

Cell necrosis is a degenerative process leading to cell death. Necrosis, usually an acute injury, may be localized and affect only a few hepatocytes (focal necrosis), or it may involve an entire lobe (massive necrosis). Cell death occurs along with rupture of the plasma membrane, and is preceded by a number of morphologic changes such as cytoplasmic oedema, dilation of the endoplasmic reticulum, disaggregation of polysomes, accumulation of triglycerides, swelling of mitochondria with disruption of cristae, and dissolution of organelles and nucleus. Biochemical events that may lead to these changes include binding of reactive metabolites to proteins and unsaturated lipids (inducing lipid peroxidation and subsequent membrane destruction), disturbance of cellular Ca^{+2} homeostasis, inference with metabolic pathways, shifts in Na^{+} and K^{+} balance, and inhibition of protein synthesis. Changes in blood chemistry resemble those seen with fatty liver, except they are quantitatively larger. Because of the regenerating capability of the liver, necrotic lesions are not necessarily critical. Massive areas of necrosis, however, can lead to severe liver damage and failure (Alison, 1994).

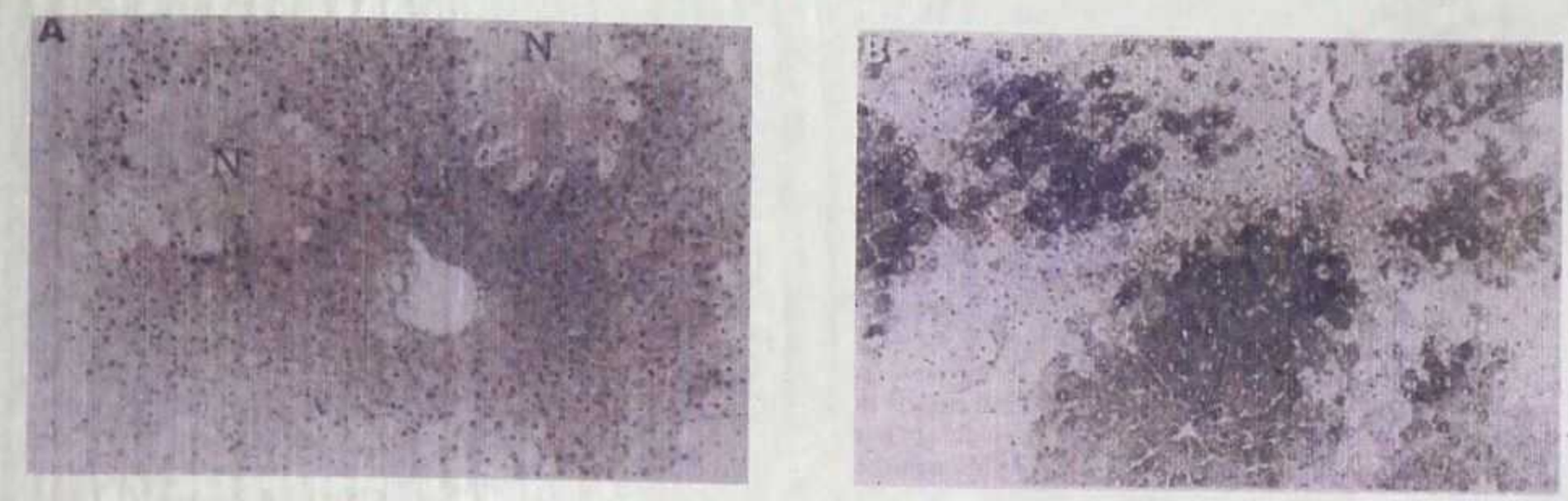


Fig.2.4: Centrilobular coagulative necrosis (N) surrounded by a cuff of ballooned hepatocytes (A) and (B) cytochrome P450 immunoreactivity located in the same area (Courtesy Alison, 1994).

2.5.3 Apoptosis

Apoptosis is a controlled form of cell death that serves as a regulation point for biologic processes and can be thought of as the counterpoint of cell division by mitosis. This selective mechanism is particularly active during development and senescence. Although apoptosis is a normal physiological process, it can also be induced by a number of exogenous factors, such as xenobiotic chemicals, oxidative stress, anoxia, and radiation. Apoptosis can be distinguished from necrosis by morphologic criteria, using either light or electron microscopy. Toxicants, however, do not always act in a clear-cut fashion, and some toxicants can induce both apoptosis and necrosis either concurrently or sequentially (Alison, 1994).

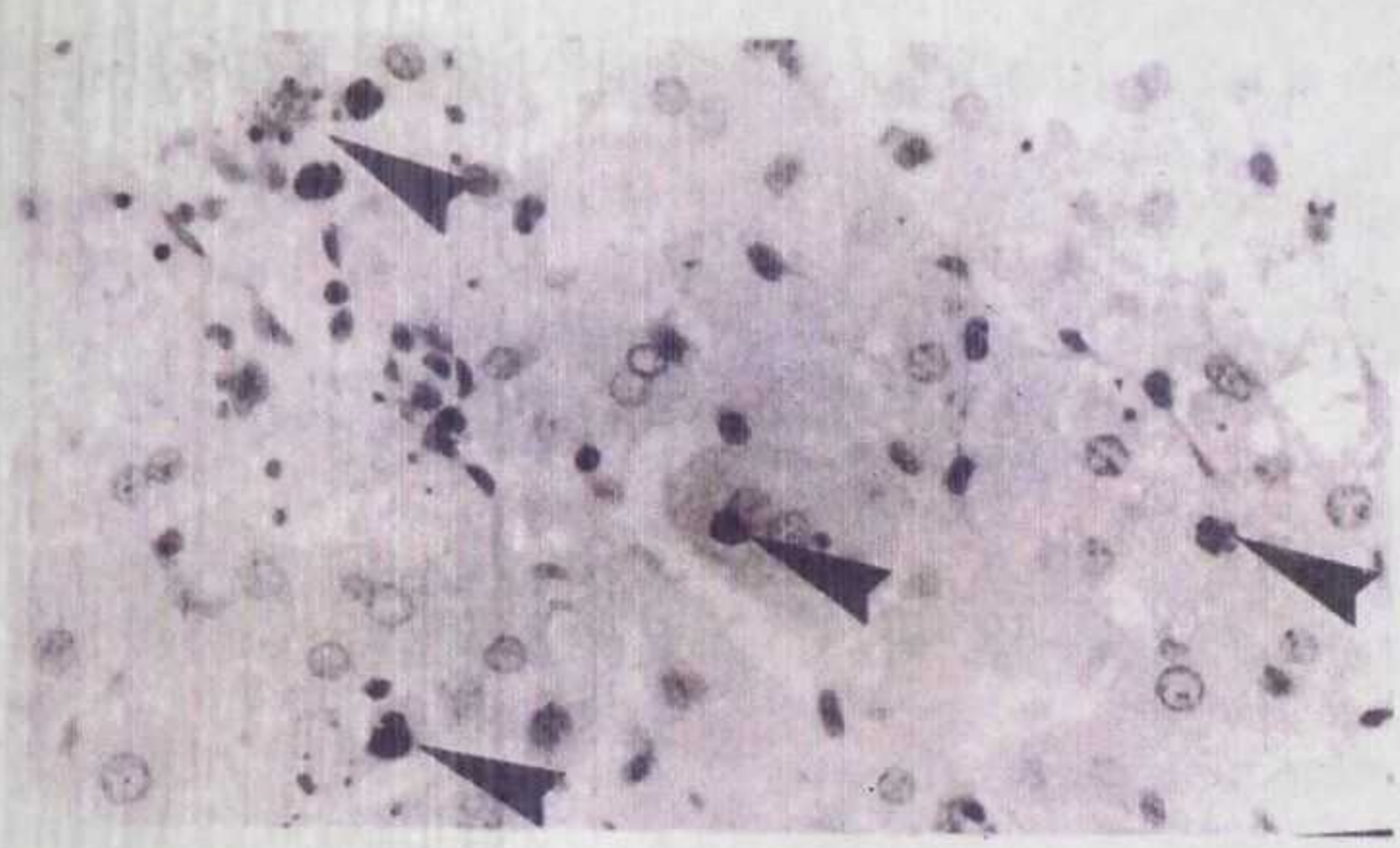


Fig. 2.5: Widespread diffuse apoptosis caused by galactosamine, which can be readily visualized by *in situ* end labelling (arrows) (Courtesy: Alison, 1994)

2.5.4 Cholestasis

Cholestasis is the suppression or stoppage of bile flow, and may have either intrahepatic or extrahepatic causes, usually drug-induced. Inflammation or blockage of the bile ducts results in retention of bile salts as well as bilirubin accumulation, an event that leads to jaundice. Other mechanisms causing cholestasis include changes in membranes permeability of either hepatocytes or biliary canaliculi.

2.5.5 Cirrhosis

Cirrhosis is a progressive disease that is characterized by the deposition of collagen throughout the liver. In most cases cirrhosis results from chronic chemical injury. The accumulation of fibrous material causes severe restriction in blood flow and in the liver's normal metabolic and detoxication processes (Bataller and Brenner, 2005). The extent of fibrosis correlates with the hydroxyproline content of the liver. This situation can, in turn cause further damage and eventually lead to liver failure. In humans, chronic use of ethanol is the single most important cause of cirrhosis,

although there is some dispute as to whether the effect is due to ethanol alone or is also related to the nutritional deficiencies that usually accompany alcoholism.

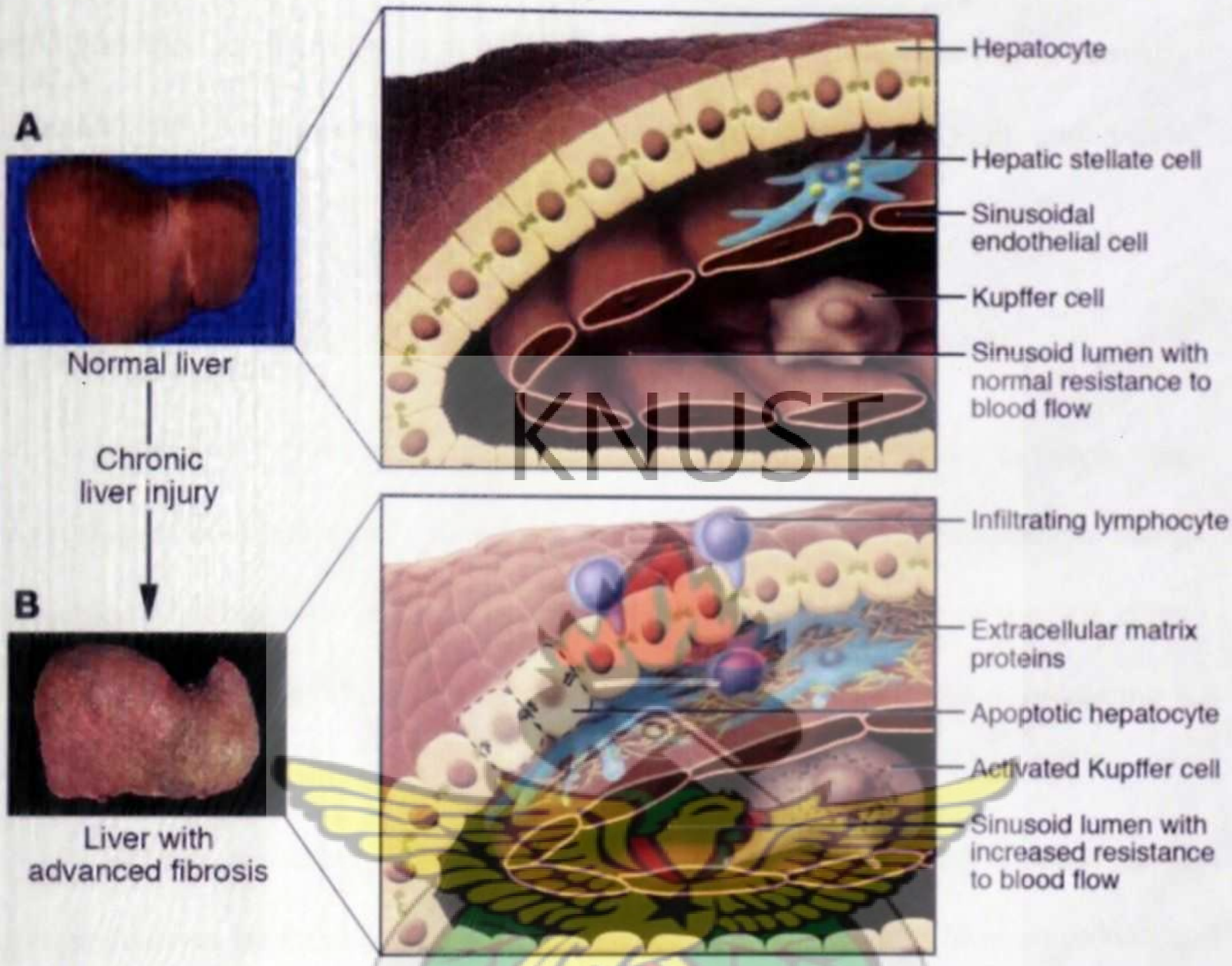


Fig. 2.6: Changes in the hepatic architecture (A) associated with advanced hepatic fibrosis (**B**) (Courtesy: Bataller and Brenner, 2005).

Following chronic liver injury, inflammatory lymphocytes infiltrate the hepatic parenchyma. Some hepatocytes undergo apoptosis, and Kupffer cells activate, releasing fibrogenic mediators. Hepatic stellate cells (HSCs) proliferate and undergo a dramatic phenotypical activation, secreting large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid (Bataller and Brenner, 2005).

2.5.6 Hepatitis

Hepatitis is an inflammation of the liver and is usually viral in origin, however, certain chemicals, usually drugs, can induce hepatitis that closely resembles that produced by viral infections. This type of liver injury is not usually demonstrable in laboratory animals and is often manifest only in susceptible individuals.

2.5.7 Oxidative Stress

Oxidative stress has been defined as an imbalance between the prooxidant/antioxidant steady state in the cell, with the excess of prooxidants being available to interact with cellular macromolecules to cause damage to the cell, often resulting in cell death. Oxidative stress can occur in almost any tissue, producing a variety of deleterious effects.

To date, a number of liver diseases, including alcoholic liver disease, metal storage diseases, and cholestatic liver disease, have been shown to have an oxidative stress component. Reactive oxygen and reactive nitrogen radicals can be formed in a number of ways, the former primarily as a by-product of mitochondrial electron transport. Superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radical can all arise from this source (Reed, 2001). Other sources include monooxygenases and peroxisomes. If not detoxified, reactive oxygen species can interact with biological macromolecules such as DNA, protein and lipids. Once lipid peroxidation of unsaturated fatty acids in phospholipids is initiated, it is propagated in such a way as to have a major damaging effect on cellular membranes. The formation, detoxication by superoxide dismutase (SOD) and by glutathione (GSH)-dependent mechanisms, and interaction at sites of toxic action are illustrated in Figure 2.7.

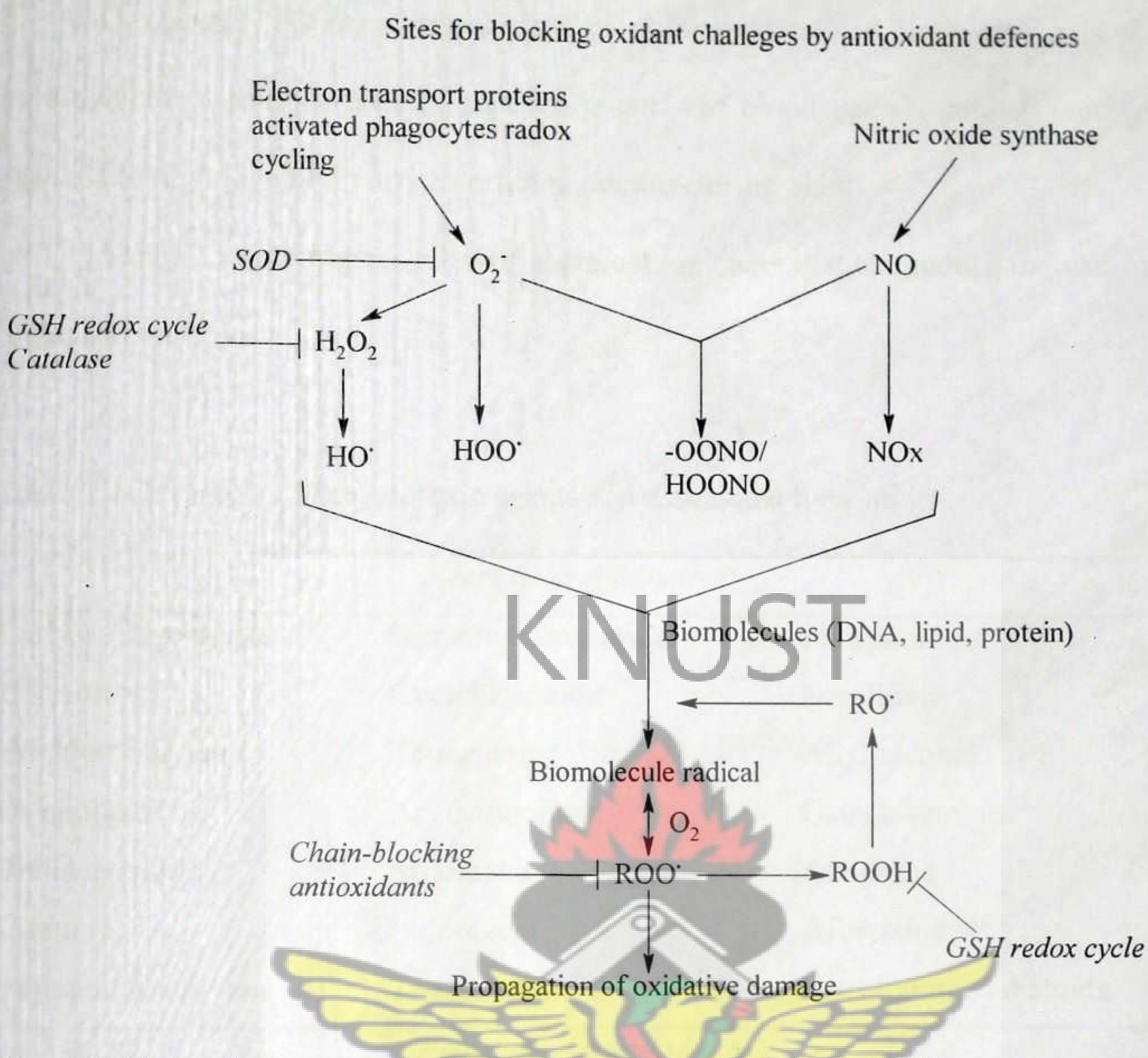


Fig. 2.7: Molecular targets of oxidative injury (Courtesy: Reed, 2001)

2.5.8 Carcinogenesis

The most common type of primary liver tumour is hepatocellular carcinoma; other types include cholangiocarcinoma, angiosarcoma, glandular carcinoma, and undifferentiated liver cell carcinoma. A wide variety of chemicals are known to induce liver cancer in laboratory animals. Some naturally-occurring liver carcinogens are aflatoxin, cycasin, and safrole. A number of synthetic chemicals have been shown to cause liver cancer in animals, including the dialkylnitrosamines, dimethylbenzanthracene, aromatic amines such as 2-naphthylamine and acetylaminofluorene, and vinyl chloride (Durr and Caselmann, 2000).

In humans, the most noted case of occupation-related liver cancer is the development of angiosarcoma, a rare malignancy of blood vessels, among workers exposed to high levels of vinyl chloride in manufacturing plants.

Table 2.1 summarizes a list of chemical toxicants that can induce the various forms of liver injury.

Table 2.1: Examples of hepatotoxic agents and associated liver injury

	Necrosis and fatty liver	
Carbon tetrachloride	Dimethylnitrosamine	Phosphorus
Chloroform	Cyclohexamide	Beryllium
Trichloroethylene	Tetracycline	Allyl alcohol
Bromobenzene	Acetaminophen	Galactosamine
Thioacetamide	Mitomycin	Azaserine
Ethionine	Puromycin	Aflatoxins
Tetrachloroethylene	Tannic acid	Pyrrolizidine alkaloids
	Cholestasis (drug-induced)	
Chlorpromazine	Imipramine	Carbarsone
Promazine	Diazepam	Chlorthiazide
Thioridazine	Methandrolone	Sulphanilamide
Mepazine	Mestranol	Methimazole
Amitriptline	Estradiol	Phenindione
	Hepatitis (drug-induced)	
Iproniazid	Methoxyflurane	Halothane
Isoniazid	Papaverine	Zoxazolamine
Imipramine	Phenyl butazone	Indomethacin
6-Mecarptopurine	Colchicine	Methyldopa
	Carcinogenesis (experimental animals)	
Aflatoxin B1	Dimethylbenzanthracene	Acetylaminofluorene
Pyrrolizidine	Dialkyl nitrosamines	Urethane
Cycasin	Polychlorinated biphenyls	
Safrole	Vinyl chloride	

Courtesy: Reed, 2001

2.6 Mechanisms of Hepatotoxicity

Chemically-induced cell injury can be thought of as involving a series of events occurring in the affected animal and often in the target organ itself:

- The chemical agent is activated to form the initiating toxic agent.
- The initiating toxic agent is either detoxified or causes molecular changes in the cell.
- The cell recovers or there are irreversible changes.
- Irreversible changes may culminate in cell death.

Cell injury can be initiated by a number of mechanisms, such as inhibition of enzymes, depletion of cofactors or metabolites, depletion of energy (ATP) stores, interaction with receptors, and alteration of cell membranes. Many compounds, including clinically useful drugs, can cause cellular damage through metabolic activation of the chemical to highly reactive compounds, such as free radicals, carbenes, and nitrenes. These reactive metabolites can bind covalently to cellular macromolecules such as nucleic acids, proteins, cofactors, lipids, and polysaccharides, thereby changing their biologic properties (Dwivedi and Mishra, 2008).

The liver is particularly vulnerable to toxicity produced by reactive metabolites because it is the major site of xenobiotic metabolism. Most activation reactions are catalysed by the cytochrome P-450 enzymes, and agents that induce these enzymes, such as phenobarbital and 3-methylcholanthrene, often increase toxicity. Conversely, inhibitors of cytochrome P450, such as SKF-525A and piperonyl butoxide, frequently decrease toxicity. Because of these interactions, cellular toxicity is a function of the balance between the rate of formation of reactive

metabolites and the rate of their removal. Examples of these interactions are presented in the following discussions of specific hepatotoxicants (Anzenbacher and Anzenbacherová, 2001).

2.6.1 Carbon tetrachloride (CCl_4)

Carbon tetrachloride is a classic example of a chemical activated by cytochrome P450 to form a highly reactive free radical (Fig. 2.8). First, CCl_4 is converted to the trichloromethyl radical ($CCl_3^{\cdot}$) and then to the trichloromethylperoxy radical ($CCl_3O_2^{\cdot}$). Such radicals are highly reactive and generally have a small radius of action. For this reason the necrosis induced by CCl_4 is most severe in the centrilobular liver cells that contain the highest concentration of the P450 isozyme responsible for CCl_4 activation. Typically, free radicals may participate in a number of events, such as covalent binding to lipids, proteins, or nucleotides as well as lipid peroxidation (Timbrell, 2009).

Lipid peroxidation (Figure 2.9) is the initiating reaction in a cascade of events, starting with the oxidation of unsaturated fatty acids to form lipid hydroperoxides, which then break down to yield a variety of end products, mainly aldehydes, which can go on to produce toxicity in distal tissues. For this reason cellular damage results not only from the breakdown of membranes such as those of the endoplasmic reticulum, mitochondria, and lysosomes but also from the production of reactive aldehydes that can travel to other tissues. It is now thought that many types of tissue injury, including inflammation, may involve lipid peroxidation (Weber *et al.*, 2003).

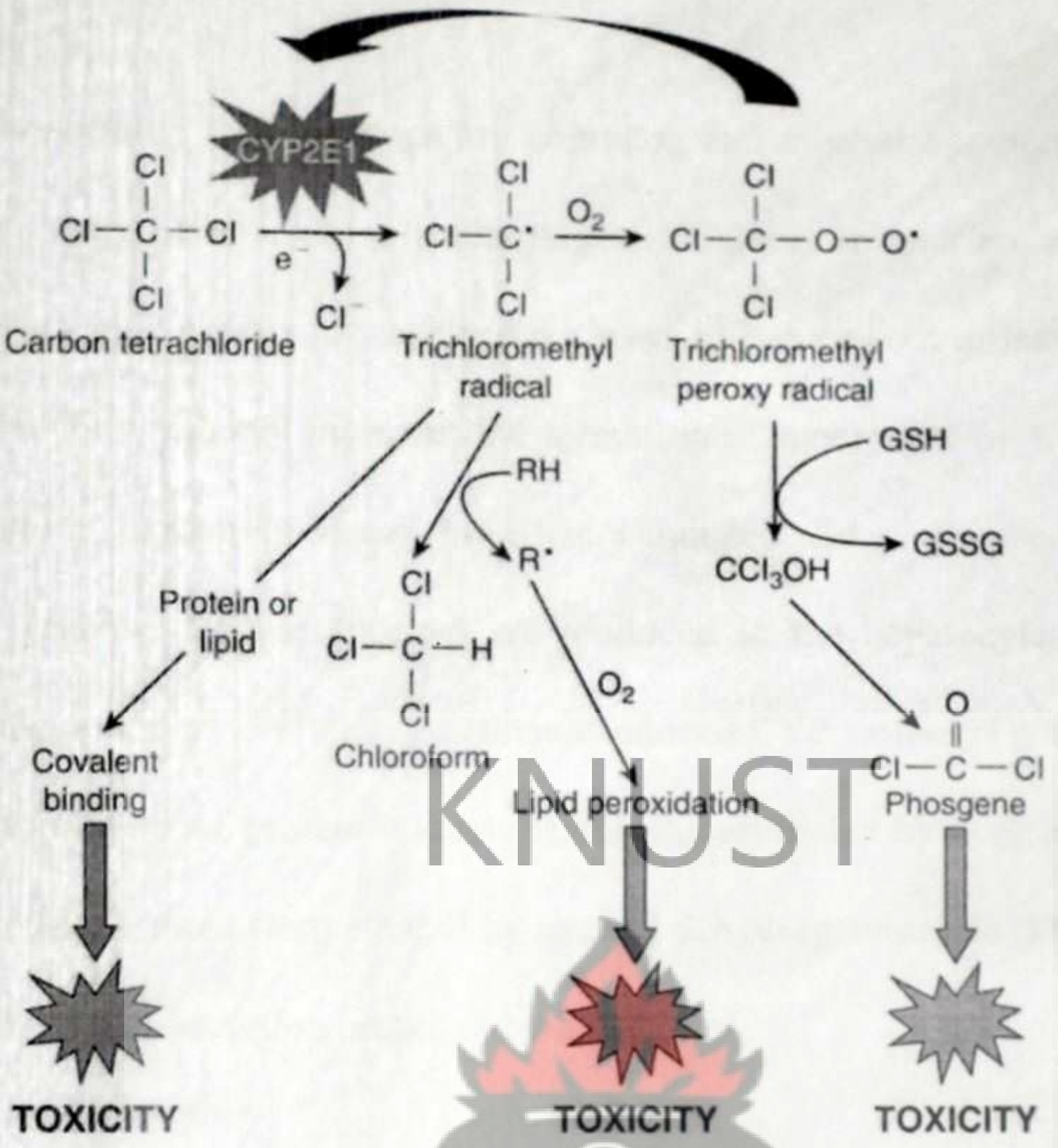


Fig. 2.8: The microsomal enzyme-mediated metabolic activation of carbon tetrachloride to the trichloromethyl radical. This radical may either react with oxygen or abstract a hydrogen atom from a suitable donor (R) to yield a secondary radical, or react covalently with lipid or protein. If R is a polyunsaturated lipid, a lipid radical (R) is formed, which can undergo peroxidation (Courtesy: Timbrell, 2009)

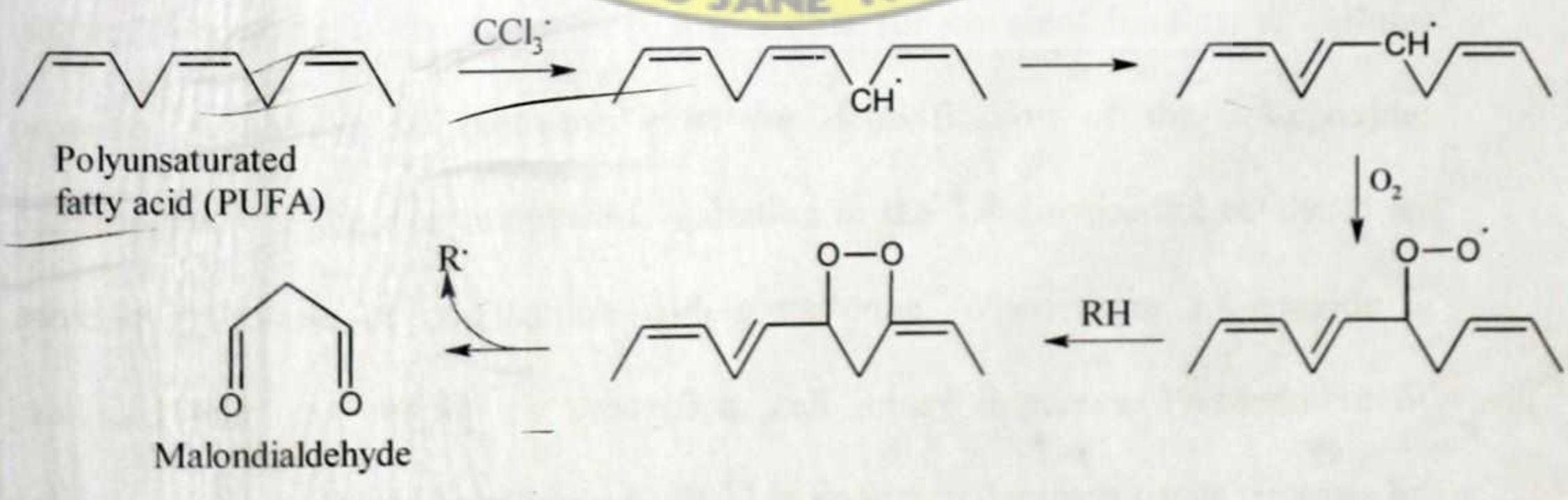


Fig. 2.9: Schematic illustration of lipid peroxidation and destruction of membranes

(Courtesy: Weber *et al.*, 2003)

2.6.2 Ethanol

Alcohol-related liver diseases are complex, and ethanol has been shown to interact with a large number of molecular targets. Ethanol can interfere with hepatic lipid metabolism in a number of ways and is known to induce both inflammation and necrosis in the liver. Ethanol increases the formation of superoxide by Kupffer cells thus implicating oxidative stress in ethanol-induced liver disease. Similarly, prooxidants (reactive oxygen species) are produced in the hepatocytes by partial reactions in the action of CYP2E1, an ethanol-induced CYP isoform (Wheeler *et al.*, 2001). The formation of protein adducts in the microtubules by acetaldehyde, the metabolic product formed from ethanol by alcohol dehydrogenase, plays a role in the impairment of VLDL secretion associated with ethanol.

2.6.3 Bromobenzene

Bromobenzene is a toxic industrial solvent that is known to produce centrilobular hepatic necrosis through the formation of reactive epoxides (Figure 2.10). Both bromobenzene-2,3-epoxide and bromobenzene-3,4-epoxide are produced by CYP oxidations. The 2,3-epoxide, however, is the less toxic of the two species, reacting readily with cellular fluid to form the nontoxic 2-bromophenol. The more stable 3,4-epoxide is the form most responsible for covalent binding to cellular proteins. A number of pathways exist for detoxification of the 3,4-epoxide: rearrangement to the 4-bromophenol, hydration to the 3,4-dihydrodiol catalysed by epoxide hydrolase, or conjugation with glutathione. When more 3,4-epoxide is produced than can readily be detoxified, cell injury increases. Pretreatment of animals with inhibitors of cytochrome P450 is known to decrease tissue necrosis by slowing down the rate of formation of the reactive metabolite, whereas pretreatment

of animals with certain P450 inducers can increase the toxicity of bromobenzene, (e.g., the P450-inducer phenobarbital increases hepatotoxicity by inducing a P450 isozyme that preferentially forms the 3,4-epoxide). However, pretreatment with another P450 inducer, 3-methylcholanthrene, decreases bromobenzene hepatotoxicity by inducing a form of P450 that produces primarily the less toxic 2,3-epoxide (Reid *et al.*, 1971).

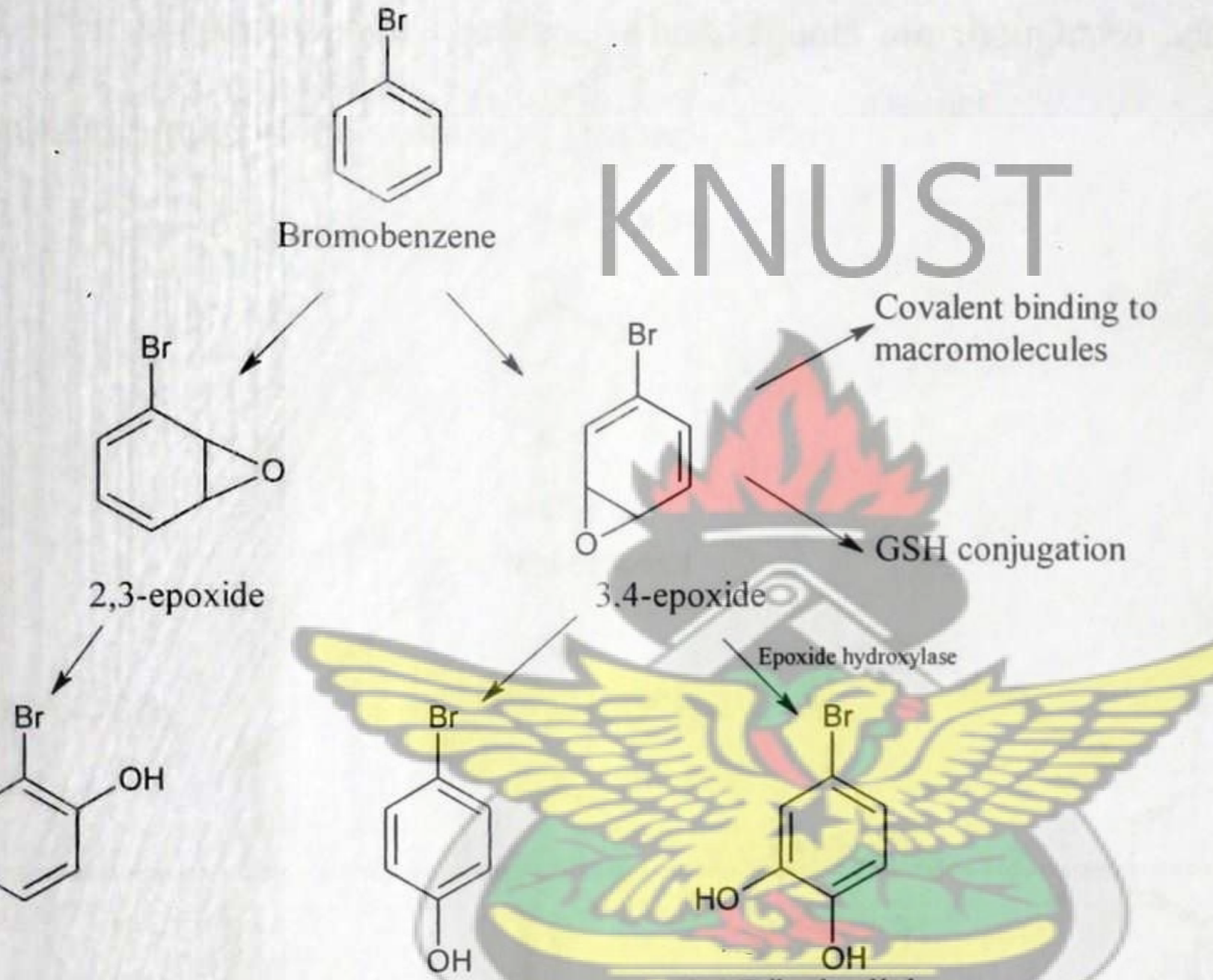


Fig. 2.10: Metabolism of bromobenzene (Courtesy: Reid *et al.*, 1971)

2.6.4 Acetaminophen

Acetaminophen (component of paracetamol) is a widely used analgesic that is normally safe when taken at therapeutic doses. Overdoses, however, may cause an acute centrilobular hepatic necrosis that can be fatal. Although acetaminophen is eliminated primarily by formation of glucuronide and sulphate conjugates, a small proportion is metabolized by cytochrome P450 to a reactive electrophilic intermediate believed to be a quinoneimine. This reactive intermediate is usually

inactivated by conjugation with reduced glutathione and excreted. Higher doses of acetaminophen will progressively deplete hepatic glutathione levels, however, resulting in extensive covalent binding of the reactive metabolite to liver macromolecules with subsequent hepatic necrosis. The early administration of sulphydryl compounds such as cysteamine, methionine, and *N*-acetylcysteine is very effective in preventing liver damage, renal failure, and death that would otherwise follow an acetaminophen overdose. These agents are thought to act primarily by stimulating glutathione synthesis (Timbrell, 2009).

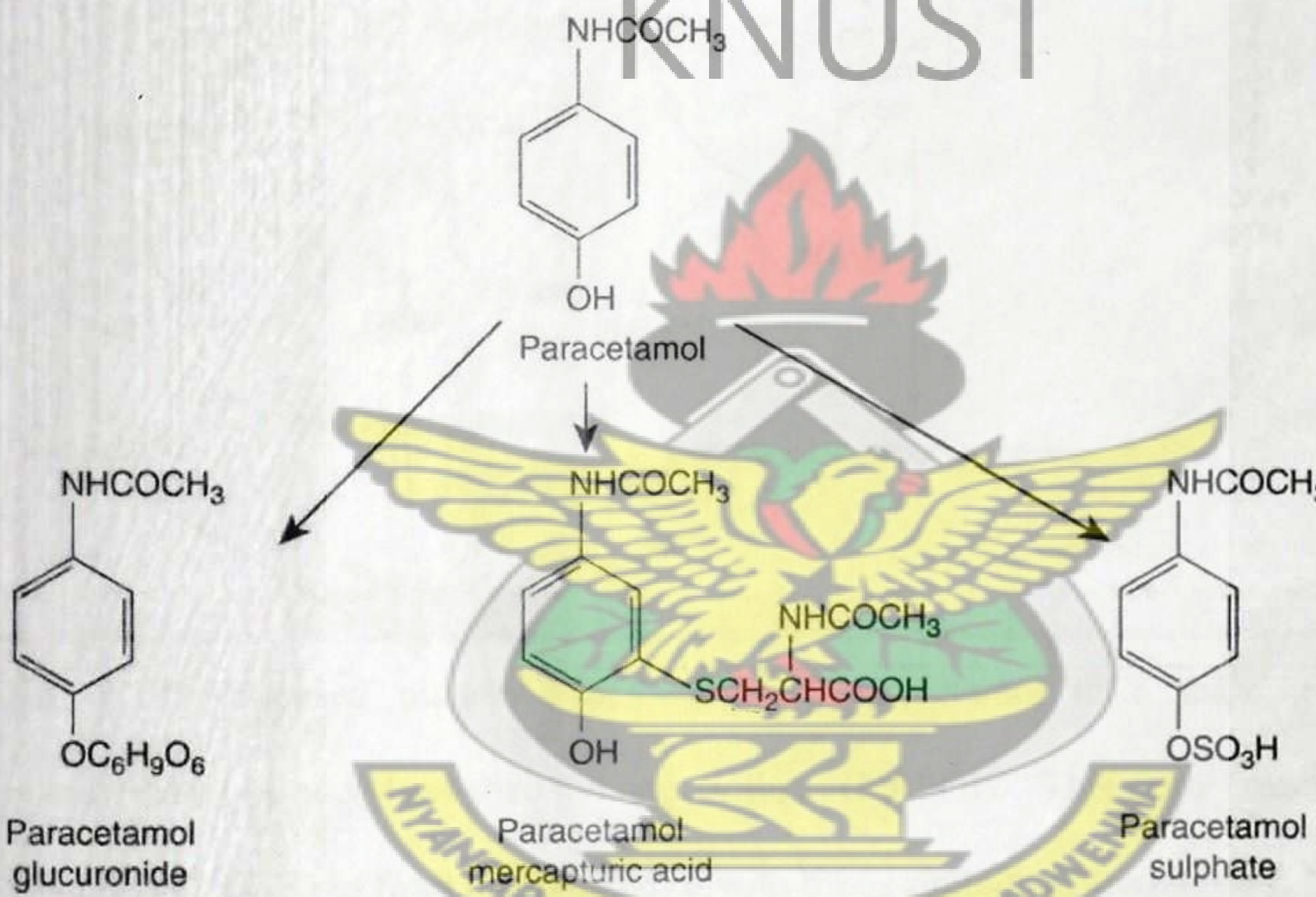


Fig. 2.11: The major metabolites of paracetamol (Courtesy: Timbrell, 2009)

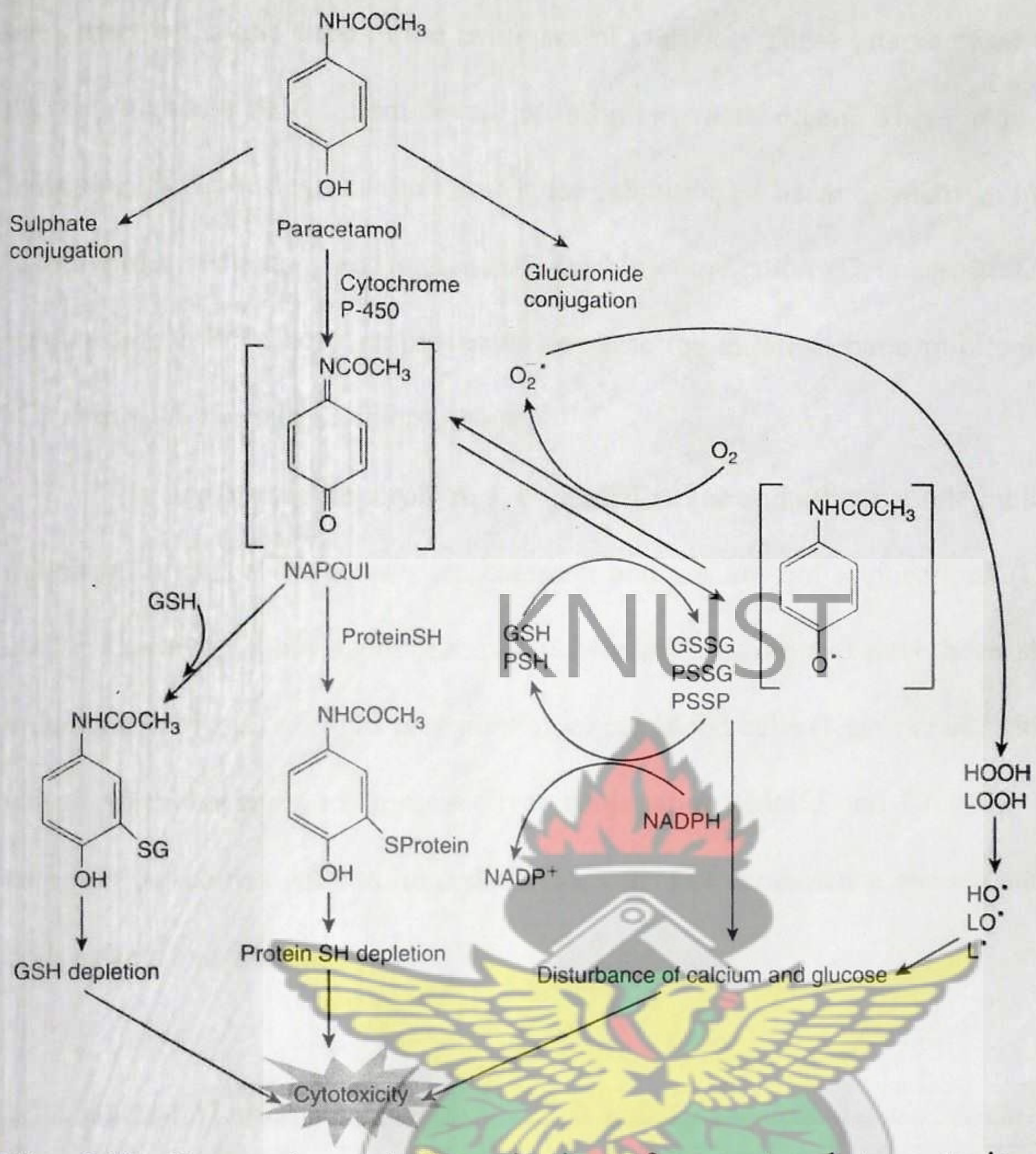


Fig. 2.12: Proposed metabolic activation of paracetamol to a toxic, reactive intermediate N-acetyl-*p*-benzoquinone imine (NAPQI). This can react with glutathione (GSH) to form a conjugate or with tissue proteins. Alternatively, NAPQI can be reduced back to paracetamol by glutathione, forming oxidized glutathione (GSSG) (Courtesy: Timbrell, 2009)

2.7 Current chemotherapy for jaundice and liver diseases

The principal method for the treatment of jaundice has been phototherapy whereby infants with hyperbilirubinaemia are exposed to white light for a period of time. However, with the elucidation of the enzymatic pathways and structure of intermediates in bilirubin metabolism, some few pharmacological interventions have

been discovered and used in the treatment of jaundice. These interventions work to prevent jaundice at different levels of bilirubin metabolism. These may include inhibition of haem degradation through the inhibition of haem oxygenase, inhibition of biliverdin reductase, and increased bilirubin conjugation (Dennery, 2002). Also, certain agents have been employed to decrease the entero-hepatic re-circulation of bilirubin and other herbal interventions.

The use of metalloporphyrins, D-penicillamine and other peptide inhibitors as inhibitors of haem oxygenase are common and has several applications (Dennery, 2002). However, these agents are photochemically active and have been shown to induce haemolysis and lipid peroxidation of red blood cells (Keino *et al.*, 1990). The use of phenobarbitals to improve liver conjugation (Marie and Cresteil, 1989) is common but comes with an immediate side effect of somnolence and even stupor in both infants and adults.

2.7.1 Herbal Medicine

The most documented herbal medicines for the clearance of bilirubin and hence jaundice are Chinese herbal remedies including 'Yin-chen' (*Artemisia scoparia*), 'Huang-qui' (scutellaria) and 'Gan-cao' (Glycyrrhiza). These have been used for centuries to promote the excretion of bilirubin in combination with Western therapies such as phototherapy in the treatment of neonatal jaundice (Chan, 1994).

In Ghana, several herbs have been documented to be used in the treatment of jaundice (Mshana *et al.*, 2000). These include *Acanthospermum hispidum*, *Alstonia boonei*, *Ananas comosus* ('Abrobe'), *Annona muricata* ('Apre'), *Bidens pilosa*, *Carica papaya* ('Borofere'), *Cassia siamea*, *Citrus aurantifolia*, *Erythrina senegalensis* ('Osurokasoro'), *Hoslundia opposita* ('Aberewa ani nsu', 'Nunum

nini'), *Jetropha curcas* ('Aboro toto', 'Akeneadua'), and several others. Though listed in floristic studies in Ghana, these have not been scientifically validated for efficacy and safety. In this study, the selected plant is *Annona muricata* because the plant is well cultivated in Ghana for its fruits and is readily available. Traditionally, *A. muricata* is boiled in water and 1 tea cup taken three times daily for the treatment of jaundice.

2.8 The Annonas

Annona is a genus of tropical fruit trees belonging to the family Annonaceae. There are approximately 119 species of which 7 and one hybrid are grown for domestic and commercial use. Five species have been selected as important under-utilised species (ICUC, 2002). Three *Annona* species are commercially important; *Annona cherimola* (cherimoya), *A. muricata* (soursop, guanabana) and *A. squamosa* (sweetsop, sugar apple), and the other two, *A. reticulata* (custard apple, bullock's heart) and *A. senegalensis* (wild soursop) are used locally. Most species are shrubs or small trees, with height varying from 5 – 7.5 m, having erect or spreading crowns and a grey-brown, rough and corrugated bark. The stems are covered with a fine layer of hair when young, later becoming smooth.

With few exceptions, Annonas are deciduous, even the tropical species, especially when cultivated in areas with a pronounced dry season and without irrigation. The fruits vary from species to species with differences in shape (round/oval/oblong/heart-shaped), size (between 2-3 cm up to 30 cm) and colour (mostly green though some cultivars are pink or red). The ripe fruit is called a syncarp. The primitive fruit form has spirally arranged carpels, resembling a raspberry, with each segment of flesh surrounding a single hard, black seed. Fruit

size is generally proportional to the number of seeds within. The flowers are pollinated by insects, although fruit production is usually very poor. Production of many good quality fruits relies largely on hand pollination (ICUC, 2002).

2.8.1 Origins of the Annonas

Of the five common species considered as underutilized, four are thought to have originated in Central and South America (cherimoya, soursop, sugar apple and custard apple) while wild soursop is thought to have originated from East Africa. The first four species are now widely distributed and can be found growing, cultivated or naturalised, throughout the tropics. Wild soursop is still restricted to Africa. All species grow well in tropical climates, at high or low altitude and on a range of soil types. They do not tolerate water logging and soils have to be well drained. Rainfall requirement ranges between species, from as low as 600 mm (for wild soursop) to 2000 mm (for the more tropical soursop) (Pinto *et al*., 2005).

Soursop (*A. muricata*) is the most tropical species and requires a moist, warm climate for good fruit production. Cherimoya has its origins on the highland plateaus of the Andes and is better adapted to cooler, drier, subtropical climates, producing a good fruit yield in the Mediterranean (Spain, Italy, Egypt, and Israel), Southern California, South Africa, Argentina and Chile. Sugar apple grows well in moist tropical climates and in drier, subtropical climates. It is the most drought-tolerant of the species and does not fruit well in high rainfall situations. It is more resistant to low temperatures than soursop and more tolerant to high temperatures than cherimoya (Pinto *et al*., 2005).

2.8.2 Uses of the Annonas

Annona is a multipurpose tree. The fruits are consumed widely and the tree is also a source of medicinal and industrial products. The fruits contain vitamin C and minerals such as calcium, phosphorous and potassium. They are also an excellent source of energy as they are high in carbohydrate. *Annona* trees can give an average fruit yield of 50-100 kg/tree and the commercial life of a tree is about 15 years. They are generally small trees or shrubs, which make maintenance and fruit harvesting easy. The trees are easy to cultivate, require comparatively little care and do not suffer from serious pests and diseases.

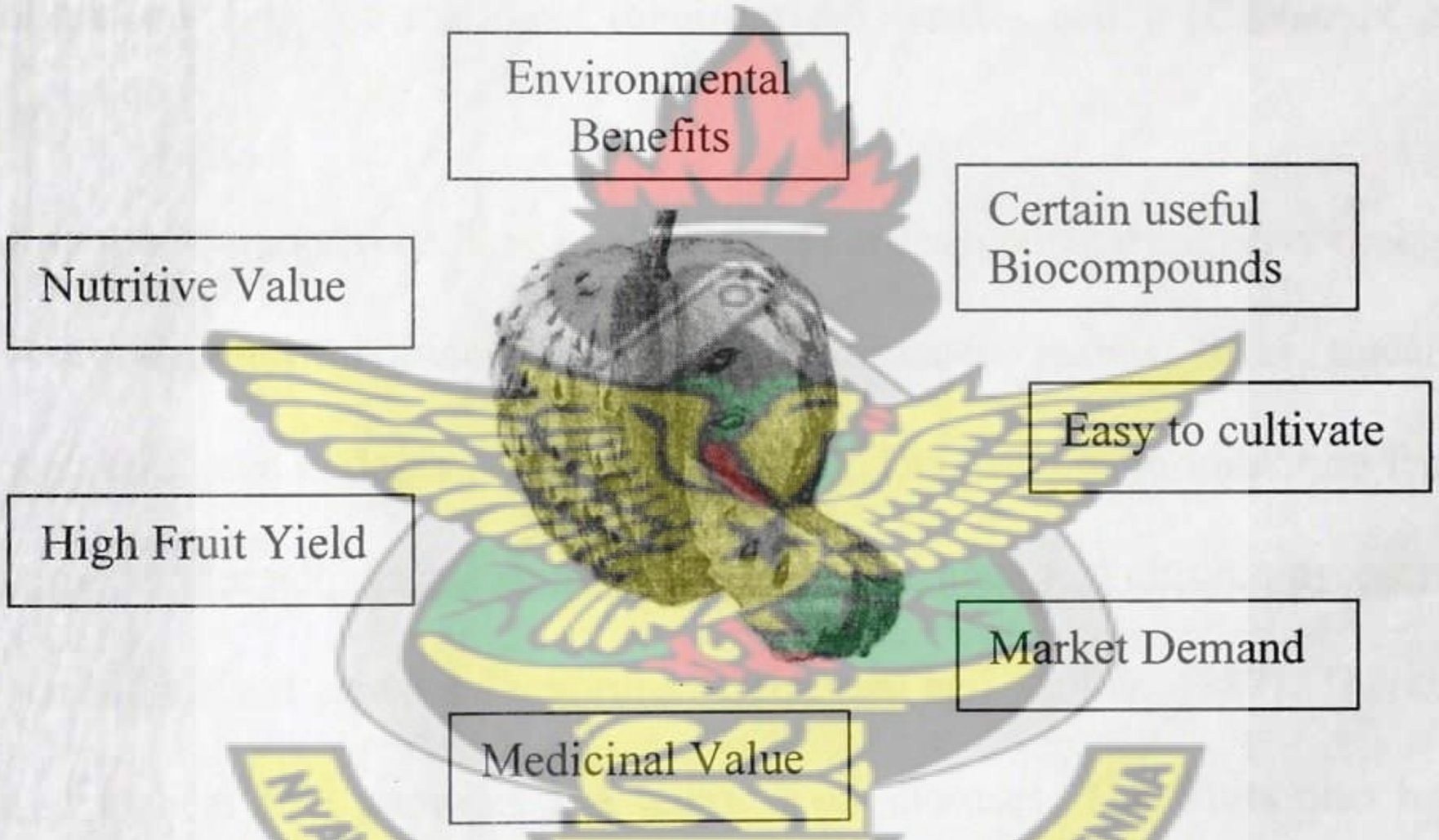


Fig. 2.13: Benefits of the Annonas (Courtesy: ICUC, 2002)

2.8.3 Medicinal value of the Annonas

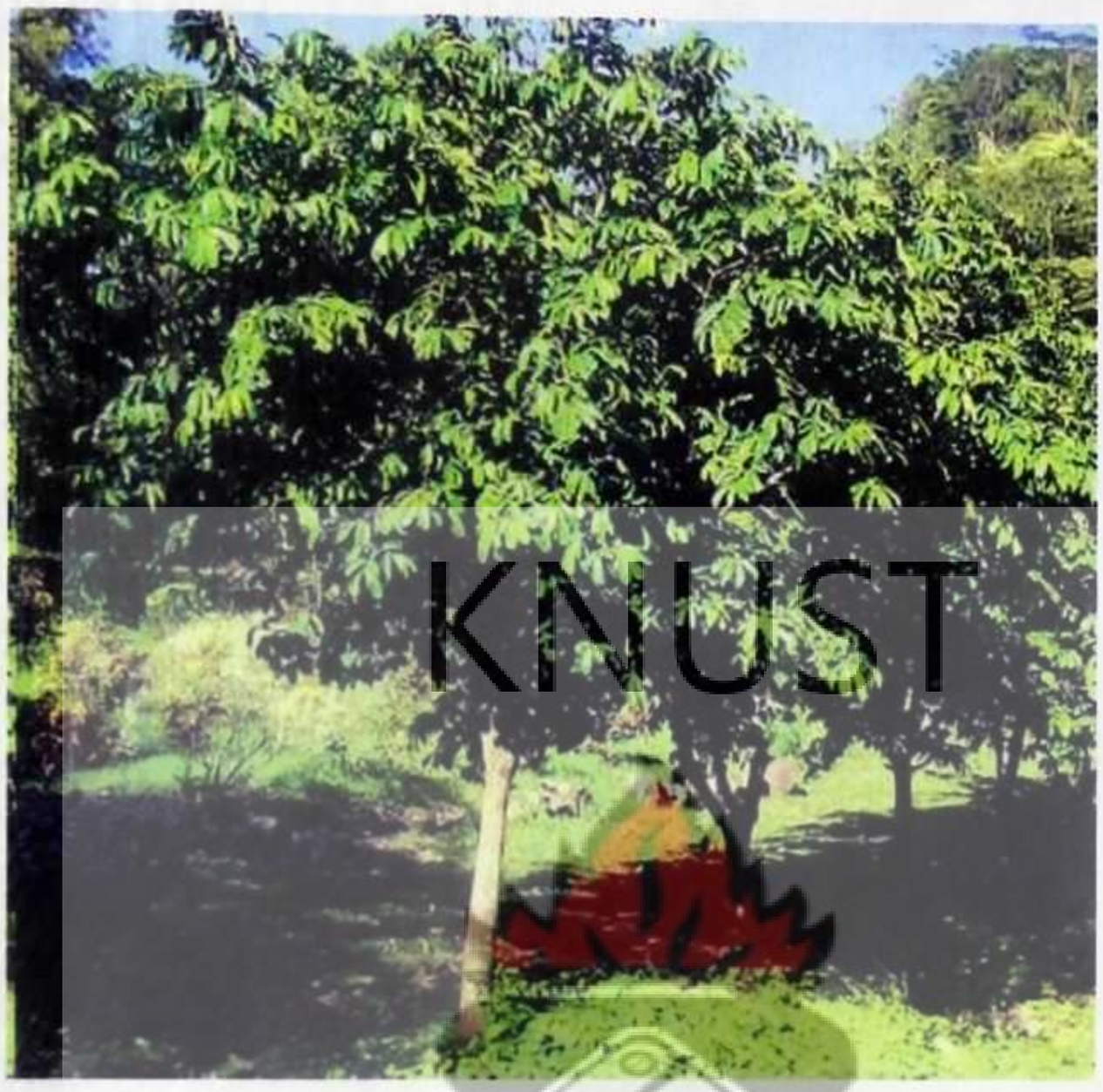
Various plant parts are widely used in folk medicine because of the bioactive compounds (mainly acetogenins, alkaloids and flavonoids) found in the roots, leaves, bark, fruits and seeds. Acetogenins are potential anti-cancer treatments, as they have cytotoxic effects (Chang *et al.*, 1993). Flavonoids present in the seeds, roots bark, stems and fruits are potential chemo-preventive agents, given evidence that they

decrease tumour incidence. Some soursop root acetogenins are known to have cytotoxic effects (Gleye *et al.*, 1998) especially panatellin, uvariamicin IV, uvariamicin I, reticulatacin, reticulatacin-10-one and solamin. The bark contains alkaloids. The leaves have essential oils with parasiticide, anti-diarrhoeal, rheumatological and antineuralgic properties (Moura, 1988). Boiled water infusion of leaves of *A. muricata* has anti-spasmodic, astringent and gastric properties (Khan *et al.*, 1997), help treat diabetes and gastric upsets (Adewole and Ojewole, 2006), as well as management of jaundice (Mshana *et al.*, 2000). They are used in kidney ailments as well (Duke, 1970). The cooked flowers and petals are used for healing eye inflammations with the treatment requiring 2-3 washes a day (Calzavara and Muller, 1987).

Immature soursop fruits have medicinal properties against dysentery, cankers, diuretic, scorbutic, anti-thermical processes, skin diseases, rashes, fever, malaria, peptic ulcers, colic and oedema (Khan *et al.*, 1997). The peels from immature fruits have constituents that act against atonic dyspepsia, diarrhoea and chronic dysentery. It is also astringent and provokes vomiting (Calzavara and Muller, 1987). The acid pulp is used to heal foot parasites and icteric liver diseases. The fruits also have properties that act on the biliary vesicles. The seeds have anti-plasmodic and anti-parasitic effect (Philipov *et al.*, 1994). They contain amyloids, oleic acid and steroids (Asolkar *et al.*, 1992).

The folk and modern medicinal uses of the Annonas are clear, but the toxic properties of most of these compounds may have undesirable side effects. Caparros-Lefebvre *et al.* (1999) showed that the alkaloids present in the leaves, bark and seeds of Annonas, when consumed for their sedative and hypnotic effects in the French West Indies, are responsible for inducing neurotoxic effects with symptoms of

Parkinsonism. Hence, any medicinal use of the Annonas should only be carried out with medical guidance (Pinto *et al*., 2005).



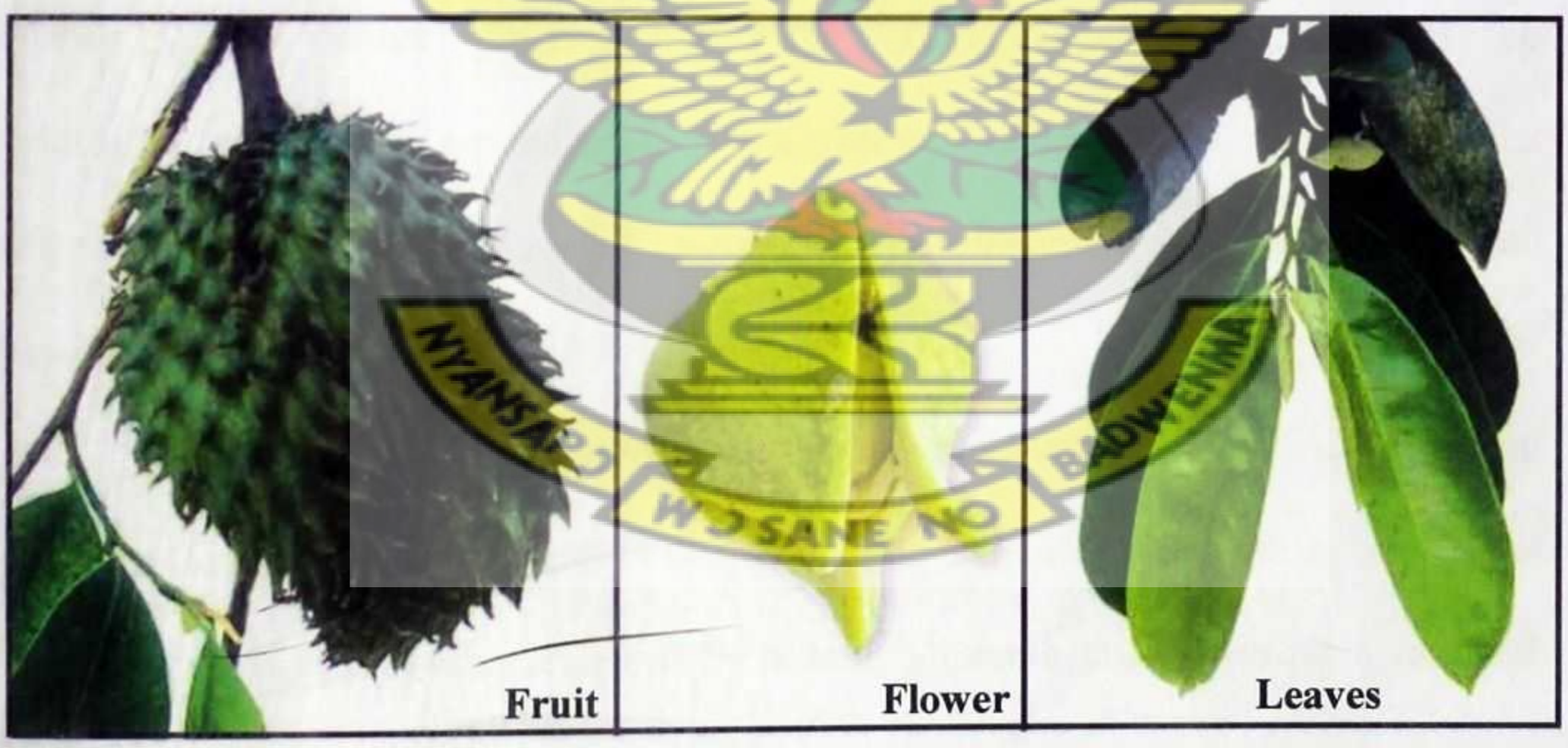


Fig. 2.14: *Annona muricata* plant and parts

2.8.4 Annonaceous acetogenins

The Annonaceae is chemically one of the least known of the tropical plant families (Leboeuf *et al.*, 1982). Phytochemical studies and, to a lesser extent, pharmacological studies on Annonaceous species have intensified in the last 15 years, this is largely due to the discovery of the Annonaceous acetogenins, a class of natural compounds with a wide variety of biological activities (Rupprecht *et al.*, 1990). Before 1982, most investigations centred upon the many isoquinoline alkaloids in this family. About 320 secondary natural products from 150 species belonging to 41 genera were summarized from 288 publications in 1982 by the group of Professor André Cavé in France (Leboeuf *et al.*, 1982). The discovery of uvaricin in 1982 (Jolad *et al.*, 1982), the first of the Annonaceous acetogenins, as an *in vivo* active antileukemic (P-388) agent, invigorated wide interest in this family.

The Annonaceous acetogenins are now one of the most rapidly growing classes of new natural products and offer exciting anti-helmintic *in vivo* and cytotoxic, antitumor, antimalarial, antimicrobial, antiprotozoal, and pesticidal activities and special promise of becoming new chemotypes for antitumor and pesticidal agents (Alali *et al.*, 1999).

Structurally, the Annonaceous acetogenins are a series of C-35/C-37 natural products derived from C-32/C-34 fatty acids that are combined with a 2-propanol unit. They are usually characterized by a long aliphatic chain bearing a terminal methyl-substituted R, â-unsaturated ç-lactone ring (sometimes rearranged to a ketolactone), with one, two, or three tetrahydrofuran (THF) rings located along the hydrocarbon chain and a number of oxygenated moieties (hydroxyls, acetoxyls, ketones, epoxides) and/or double bonds being present. To a lesser extent,

tetrahydropyran (THP) ring compounds and acyclic compounds are also found (Alali *et al.*, 1998).

The Annonaceous acetogenins are the most powerful of the known inhibitors of complex I (NADH:ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems (Hollingworth *et al.*, 1994), in addition, they are potent inhibitors of NADH oxidase of the plasma membranes of cancer cells (Morré *et al.*, 1994), with these actions decreasing oxidative, as well as, cytosolic ATP production. The consequence of such ATP deprivation is apoptosis (Wolvetang *et al.*, 1994). By 1997 scientist have shown that the acetogenins also inhibit cancer cells that are multidrug resistant (MDR) (Oberlies *et al.*, 1995), and in addition, they combat pesticide-resistant German cockroaches effectively (Alali *et al.*, 1998). Thus, they thwart biological resistance.

Several annonaceous acetogenins have been found in *Annona muricata* leaves. These vary in nature, from white wax to powder to colourless oils. Among the list are murihexocin (Kim *et al.*, 1998), annocuricin (Kim *et al.*, 1998), annopentocin A, B and C and (2,4-cis)-annomuricin-D-one and (2,4-trans)-annomuricin-D-one (Zeng *et al.*, 1996), muricatocin A, B and C (Wu *et al.*, 1995), annohexocin, murihexocin A and B, 4-acetyl gigantetrocin and cis-gigantrionin (Zeng *et al.*, 1996). The high potency, selectivity, wide chemical and biological diversity, and effectiveness of these compounds against resistance could well make them become the next class of useful natural antitumor and pesticidal agents (Alali *et al.*, 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Preparation of Serial Extract

Leaves of *Annona muricata* were collected in the month of April 2010 from the surrounding fields of Department of Biochemistry and Biotechnology Annex offices and were authenticated by Mr. G.H. Sam of the Department of Herbal Medicine, KNUST and voucher specimen (KNUST/HM1/2011/L057) deposited at the Faculty of Pharmacy and Pharmaceutical Sciences' herbarium. They were washed, air-dried under shade and milled into fine powder. A serial extraction method was employed where the residue from one solvent extraction was dried and used for the next solvent extraction. Extractions were in the order of petroleum ether, ethyl acetate, methanol and water. Cold extractions were performed in all cases and air- or freeze-dried to obtain the extracts and designated as *A. muricata* petroleum ether extract (AMPE), *A. muricata* ethyl acetate extract (AMEE), *A. muricata* methanol extract (AMME) and *A. muricata* aqueous extract (AMAE).

3.1.1 Phytochemical screening of serial extracts

Qualitative phytochemical analysis was performed on the extracts of *A. muricata* to ascertain the presence of phytochemicals and the effect of the various solvents on phytochemical extracts. Analysis was also performed on the powdered sample and the residue after the solvent extractions. Standard procedures as described by Harborne (1998), Trease and Evans (1989), and Sofowora (1993) were employed. The various phytochemicals tested for included saponins, flavonoids,

steroids and terpenoids, general glycosides, carotenoids, alkaloids, coumarins, anthraquinones and anthraquinone glycosides.

3.2 EVALUATION OF HEPATOPROTECTIVE EFFECT OF SERIAL EXTRACTS OF *ANNONA MURICATA* (LINN.) LEAVES

3.2.1 Drug formulation

Oral suspensions containing respective doses of extracts were prepared in 2% v/v aqueous Tween 80 (Sigma-Aldrich, Germany). This was used due to the variable solubilities of the extracts in water.

3.2.2 Animals

Sprague – Dawley rats of either sex weighing between 170 – 210 g were used for the studies. They were obtained from the animal facility of the Department of Biochemistry and Biotechnology, KNUST. Animals were housed in aluminium cages, suitably bedded with wood shaving. They were maintained under standard conditions of temperature and humidity and had free access to standard feed (GAFCO, Tema, Ghana) and freshly prepared distilled water except an overnight fast prior to sacrifice. In experimental grouping of the animals, their body weight and sex were taken into consideration to achieve approximately equal conditions among the groups. The animals were identified using permanent markers to mark uniquely on their tails for identification. All animal experiments were conducted in accordance with the guidelines of the committee for the purpose of control and supervision of experiment on animals (CPCSEA, New Delhi, India) and guide for care and use of laboratory animals (Washington, US).

3.2.3 Evaluation of hepatoprotective activity

Fourteen groups of 5 animals each were used for the study and treated for 7 days. The animals from Group I served as normal control and received only the vehicle, 2% v/v Tween 80, orally at a dose of 1 ml/100g body weight per day for 7 days. All the animals of Groups II – XIV received carbon tetrachloride diluted with olive oil (1:1 v/v) at a dose of 1 ml/kg b.wt orally for two successive days (2^{nd} – 3^{rd} day). In addition, Group II animals were maintained as carbon tetrachloride control without any drug treatment. The remaining groups received the following treatments: group III – V received AMAE at 100 mg/kg, 300 mg/kg and 500 mg/kg b.wt respectively; group VI – VIII received AMPE at 100 mg/kg, 300 mg/kg and 500 mg/kg b.wt respectively; group IX – XI received AMEE at 100 mg/kg, 300 mg/kg and 500 mg/kg b.wt respectively; and group XII – XIV received AMME at 100 mg/kg, 300 mg/kg and 500 mg/kg b.wt respectively. The vehicle or drug treatment was carried out from 1^{st} day to 7^{th} day with concurrent administration of CCl_4 on the 2^{nd} and 3^{rd} day orally by gastric intubation using a feeding needle connected to a syringe. During the period of drug treatment the rats were maintained on normal diet and freshly prepared distilled water *ad libitum* (Prakash *et al.*, 2008).

3.2.4 Effect of treatment on daily body weight

The body weight of each rat was taken 24 hours before the commencement of treatment as initial weight and then daily for the duration of the study. The percent change in weight was calculated by using the formula;

$$Percent\ Change\ in\ Body\ Weight = 100 \times \frac{Weight_n - Weight_{initial}}{Weight_{initial}}$$

[$Weight_{initial}$: measurement on the day 0; $Weight_n$: measurements at end of day 1, 2, 3, …. 7]

3.2.5 Assessment of hepatoprotective activity

Animals were sacrificed by cervical dislocation on the 8th day following an overnight fast. Incisions were quickly made in the sacrificed animal's cervical region with the aid of a sterile blade. Blood samples were collected from the heart and dispensed into plane tubes, allowed to clot and centrifuged at 3500 g for 10 minutes. Part of blood was dispensed into EDTA tubes for some haematological analysis using Sysmex Haematology System (USA). The sera obtained from the blood samples were used for some biochemical indices, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin (total and direct), total protein (TP) and albumin using diagnostic kits (Fortess Diagnostics, UK) according to manufacturer's instructions.

3.2.6 Effect of treatment on organ weights

Excised organs of rats were washed in buffered normal saline and weighed to obtain the absolute liver weights. Relative weights were calculated with the formula;

$$Relative\ Organ\ Weight = 100 \times \frac{Absolute\ Organ\ Weight}{Body\ Weight\ at\ Sacrifice}$$

Organs included liver, heart, lung, kidneys, spleen and stomach.

3.3 AQUEOUS DECOCTION OF *A. MURICATA* LEAVES AND TOXICITY ASSESSMENT

3.3.1 Plant preparations and extraction

The leaves of *Annona muricata* were washed, shade-dried, milled and decocted (1.41 kg with 10 L water) at 100°C for 30 minutes and allowed to cool. The aqueous extract was freeze-dried to obtain the powdered *A. muricata* aqueous extract (AMAE) weighing 211 g (14.96% w/w yield) which was used in the study.

3.3.2 Phytochemical screening of *A. muricata* aqueous extract (AMAE)

Qualitative phytochemical screening of AMAE was carried with method as described above (Section 3.1.1).

3.3.3 Acute oral toxicity study of AMAE

The toxicity study was carried out using 25 Swiss albino mice (20 – 25 g) of either sex obtained from the animal facility of the Department of Biochemistry and Biotechnology, KNUST, Kumasi-Ghana. The animals were divided into five groups of five animals per group, control group and 4 treated groups. They were maintained on standard feed (GAFCO, Tema, Ghana) and water and allowed to acclimatise for seven days to the laboratory environment before the experiment. After an overnight fast, the control group received 0.3 ml sterile distilled water while each treated group received 100 mg/kg b.wt, 1000 mg/kg b.wt, 2500 mg/kg b.wt and 5000 mg/kg b.wt administered orally with the aid of a feeding needle connected to syringe at stated doses in appropriate volume of sterile distilled water. Doses were selected based on the fixed dose method (OECD Guideline 420, 2001). The animals were observed for signs of toxicity and mortality for the first critical 4 hours and thereafter daily for 7 days. Signs of toxicity included paw-licking, stretching, respiratory distress, diarrhoea and death were observed. The oral median lethal dose (LD_{50}) was calculated as the geometric mean of dose that caused 0 % and 100 % mortality respectively. Three dose (100, 1000 and 2500 mg/kg b.wt.) were selected for the subchronic toxicity studies (Bürger *et al*, 2005).

3.3.4 Subchronic toxicity studies of AMAE

Twenty male (210 – 260 g) and 20 female (190 – 220 g) rats were separately divided into four groups of 5 animals. For each sex, group I served as the normal control and received 1 ml distilled water daily while groups II, III and IV were respectively administered 100 mg/kg b.wt, 1000 mg/kg b.wt, and 2500 mg/kg b.wt daily in appropriate volume of distilled water for 14 days. All animals were fasted 12 hours prior to first oral drug administration and had free access to food and distilled water throughout the duration of the experiment. They were observed daily for general signs of toxicity and mortality (Salawu *et al.*, 2009).

3.3.5 Effect of AMAE on body weight change

Rats in all groups were weighed on the first day (D0) and at the end of day 2 (D2), D4, D6, D8, D10, D12 and D14. The percent change in body weight was calculated using the formula;

$$Percent\ Change\ in\ Body\ Weight = 100 \times \frac{Weight_n - Weight_{initial}}{Weight_{initial}}$$

[$Weight_{initial}$: measurement on the first day (D0); $Weight_n$: measurements at end of D2, D4, …, D14]

3.3.6 Effect of AMAE on feed and water intake

The amount of feed and volume of water consumed by animals in each group were measured daily as the difference between the quantity of feed and water supplied and amount remaining after 24 hours respectively. The mean intake was calculated at the end of day 2 (D2), D4, D6, D8, D10, D12 and D14. The relative feed and water consumption was calculated as a ratio of the mean feed/water intake and the mean body weight on the respective days.

3.3.7 Effect of AMAE on some haematological parameters

On the 15th day, animals were fasted overnight and sacrificed by cervical dislocation. Incisions were quickly made in the sacrificed animal's cervical region with the aid of a sterile blade and blood samples collected from the heart and dispensed into EDTA tubes for haematological analysis using Sysmex Haematology System (USA). Determinations included haemoglobin concentration (Hb), red blood cell (RBC) count, white blood cell (WBC) count and differentials, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC).

3.3.8 Effect of AMAE on some biochemical parameters

Portions of the blood were dispensed into plain tubes, allowed to clot and centrifuged as 3500 rpm for 10 minutes. The sera were separated and used for the evaluation of biochemical parameters which included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin concentrations, total cholesterol, high density lipoproteins (HDL), total triglycerides, glucose, creatinine and urea using the Cobas Integra 400 Clinical Chemistry Analyzer (Roche, USA). Low density lipoprotein (LDL) concentration was calculated with the Friedewald's equation (Crook, 2006);

$$LDL = (TC - HDL) - \left(\frac{TG}{2.2}\right)$$

3.3.9 Effect of AMAE on absolute and relative organ weights

Different organs namely liver, heart, spleen, stomach, kidneys, testes or uterus were removed, washed with buffered normal saline, weighed to obtain

absolute organ weight (AOW) and observed macroscopically. The relative organ weights (ROW) was calculated for each rat using the formula;

$$Relative\ Organ\ Weight = \frac{Absolute\ Organ\ Weight}{Body\ Weight\ At\ Sacrifice} \times 100\%$$

3.4 HEPATOPROTECTIVE ASSESSMENT OF *A. MURICATA* AQUEOUS EXTRACT

3.4.1 Animals

Sprague-Dawley rats of either sex weighing 200 – 210 g were used in the studies. The animals were divided into seven groups of 5 animals each.

3.4.2 Hepatoprotective effect of AMAE against carbon tetrachloride – induced liver toxicity

Group I served as normal control group and received 1 ml distilled water throughout the duration of the experiment. All the animals of groups II – VII received carbon tetrachloride diluted with olive oil (1:1 v/v) at a dose of 1 ml/kg b.wt orally for two successive days (2^{nd} – 3^{rd} day). Group II animals were maintained as carbon tetrachloride control without any drug treatment. Groups III, IV, V and VI animals were pre-treated with 50 ml/kg b.wt, 100 ml/kg b.wt, 200 ml/kg b.wt and 400 mg/kg b.wt AMAE respectively by oral route. Group VII animals were treated with 100 mg/kg b.wt Silymarin standard (Legalon, Flordis, Australia) to serve as positive control. The vehicle or drug treatments were administered orally from 1^{st} to 7^{th} day with concurrent administration of carbon tetrachloride on 2^{nd} and 3^{rd} day. During the period of drug treatment the rats were maintained on normal diet and distilled water *ad libitum* (Prakash *et al.*, 2008).

3.4.3 Hepatoprotective effect of AMAE against paracetamol (acetaminophen) – induced liver toxicity

Group I served as normal vehicle control and received 1 ml freshly prepared distilled water daily. All animals in Group II – VII received 500 mg/kg b.wt acetaminophen (Trade Winds Chemists, Kumasi, Ghana) suspended in an appropriate volume of freshly prepared distilled water once daily from 2nd to 7th day. Group II were maintained as acetaminophen control without any drug treatment. Group III, IV, V and VI were pre-treated with 50 mg/kg b.wt, 100 mg/kg b.wt, 200 mg/kg b.wt and 400 mg/kg b.wt AMAE respectively while Group VII were pre-treated with 100 mg/kg b.wt Silymarin. The vehicle or drug treatments were administered orally from 1st to 7th day with concurrent administration of acetaminophen from 2nd to 7th day. During the period of drug treatment the rats were maintained under normal diet and distilled water *ad libitum* (Gupta *et al.*, 2004). All drugs were dissolved in appropriate volumes of distilled water and administered orally with the aid of a feeding needle connected to a syringe.

3.4.4 Effect of treatment on daily body weight

The body weight of each rat was taken 24 hours before the commencement of treatment as initial weight and then daily for the duration of study. The percent change in weight was calculated by using the formula;

$$Percent\ Change\ in\ Body\ Weight = 100 \times \frac{Weight_n - Weight_{initial}}{Weight_{initial}}$$

[$Weight_{initial}$: measurement on the day 0; $Weight_n$: measurements at end of day 1, 2, 3, ... 7]

3.4.5 Assessment of hepatoprotective activity

All animals were sacrificed on day 8 following an overnight fast by cervical dislocation. Incisions were quickly made in the sacrificed animal's cervical region with the aid of a sterile blade and blood samples collected from the heart and dispensed into plain tubes for biochemical tests. The blood was allowed to clot and centrifuged at 3500 rpm for 10 minutes. The plasma obtained from the blood samples were used for the liver function tests including alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin (total and direct), cholesterol, total triglycerides, total protein (TP) and albumin using the Cobas Integra 400 Clinical Chemistry Analyzer (Roche, USA)

3.4.6 Effect of treatment on liver weight and histology

Excised livers of rats were washed in buffered normal saline and weighed to obtain the absolute liver weights. Relative weights were calculated. Livers were processed for histological examination, stained with haematoxylin and eosin. Histographs were taken and the extent of CCl_4- and paracetamol-induced necrosis evaluated by a pathologist.

3.5 BILIRUBIN-LOWERING POTENTIAL OF *ANNONA MURICATA* IN TEMPORARY JAUNDICED ADULT RATS

3.5.1 Animals

Rats of either sex of Sprague-Dawley strain weighing 150 – 200 g were used in all studies. The animals were divided into 5 groups of 5 animals for each experiment.

3.5.2 Induction of hyperbilirubinaemia

In all cases, hyperbilirubinaemia was induced with aqueous solution of 40 mg/kg b.wt phenylhydrazine (PHZ) (Sigma-Aldrich Co., Germany) administered orally for two alternate days (Roque *et al.*, 2008 with modification).

3.5.3 Curative effect of *A. muricata* in temporary jaundiced rats

Group I served as normal control and received distilled water at 1ml/100 g b.wt. twice daily for the entire duration of the experiment. Groups II – V were treated orally with PHZ on day 1 and 3. In addition, Group II served as jaundice control and were sacrificed 6 hours after last PHZ treatment. Group III served as vehicle control and were maintained on distilled water from 6 hours after last PHZ treatment till day 7. Group IV and V were treated with 50 mg/kg b.wt and 400 mg/kg b.wt AMAE twice daily from day 2 till day 7. After 7 days animals in Groups I, III, IV and V were sacrificed after an overnight fast (Nag *et al.*, 2009).

3.5.4 Prophylactic effect of *A. muricata* in temporary jaundiced rats

Group I served as normal control and received distilled water at 1ml/100 g b.wt twice daily for the entire duration of the experiment. Groups II – V were treated orally with PHZ on day 2 and 4. In addition, Group II served as jaundice control and were sacrificed 6 hours after last PHZ treatment. Group III served as vehicle control and were treated with distilled water from day 1 – 7 with PHZ treatment on day 2 and 4. Group IV and V were pre-treated with 50 and 400 mg/kg AMAE on day 1 and twice daily till end of day 7 with PHZ treatment on day 2 and 4. After 7 days, animals in Groups I, III, IV and V were sacrificed after an overnight fast.

3.5.5 Prophylactic effect of *A. muricata* in temporary jaundiced rats with reduced liver capacity

Group I served as normal control and received distilled water at 1ml/100 g b.wt twice daily for the entire duration of the experiment. Groups II – VI were treated orally with PHZ on day 2 and 4 and 1 ml CCl_4/kg b.wt (1:1 v/v olive oil) on day 3. In addition, Group II served as jaundice control and were sacrificed 6 hours after last PHZ treatment. Group III served as vehicle control and were treated with distilled water twice daily from day 1 – 7 with PHZ and CCl_4 treatment on day 2 – 4. Group 4, 5 and 6 were pre-treated with 50 mg/kg b.wt AMAE, 400 mg/kg b.wt AMAE and 100 mg/kg b.wt Silymarin respectively on day 1 and twice daily till end of day 7 with PHZ and CCl_4 treatments on day 2 – 4. After 7 days animals in Groups I, III, IV, V and VI were sacrificed after an overnight fast.

3.5.6 Effect of treatment on liver, spleen and heart weight

All animals in the above grouping were sacrificed by cervical dislocation 6 hours after last treatment. Incisions were quickly made in the sacrificed animal's cervical region with the aid of a sterile blade and blood samples collected from the heart and dispensed into EDTA tubes for haematological analysis and into plain tubes for biochemical tests. Excised liver, heart and spleen of rats were washed in buffered normal saline and weighted to obtain the absolute organ weights. Relative weights were calculated.

3.5.7 Effect of treatment on some haematological parameters

After sacrifice, part of blood samples was dispensed into EDTA tubes for haematological analysis using Sysmex Haematology System (USA). Determinations

included haemoglobin concentration (HGB), red blood cell (RBC) count and haematocrit (HCT).

3.5.8 Effect of treatment on some serum biochemical parameters

Part of blood samples were collected into plain tubes for biochemical tests. The blood was allowed to clot and centrifuged at 3500 rpm for 10 minutes. The serum obtained from the blood samples were used for serum biochemistry including alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), bilirubin (total and direct), and albumin (Alb) using the Cobas Integra 400 Clinical Chemistry Analyzer (Roche, USA)

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3.6 STATISTICAL ANALYSIS

Data was analysed using GraphPad Prism 5 for Windows. The experimental results were expressed as the Mean ± standard error of mean (SEM). Data were assessed by one-way ANOVA followed by Newman-Keuls multiple comparison test. Values for which $p<0.05$ was considered as statistically significant. The percentage protection was calculated as:

$$Percent\ Protection$$

$$= 100\ x\ \frac{(Values\ of\ Toxin\ Control - Values\ of\ Test\ Sample)}{(Values\ of\ Toxin\ Control - Values\ of\ Normal\ Control)}$$

CHAPTER FOUR

4.0 RESULTS

4.1 SERIAL EXTRACT OF *ANNONA MURICATA* LEAVES

4.1.1 Phytochemical screening of serial extracts

Table 4.1 shows the phytochemical constituents of the dried powdered sample (AMFS), serial extracts (AMPE, AMEE, AMME and AMAE) and the residue after the extractions (AMR). The fresh sample contained saponins (indicated by the persistence of foam), general glycosides, flavonoids, terpenoids and steroids, coumarins, alkaloids, anthraquinone, and anthraquinone glycosides. It was however deficient in carotenoids. Petroleum ether, a non-polar solvent, extracted non-polar components of the fresh sample, saponins. The oily nature of the extract was due to the reported presence of essential oils in the leaf. The non-polar solvent, ethyl acetate, extracted general glycosides together with coumarins and alkaloids. Methanol, a slightly polar solvent, extracted saponins together with flavonoids and anthraquinones. The polar solvent, water, extracted saponins, general glycosides and flavonoids. Finally, the residue remaining after all the extractions contained terpenoids, steroids and anthraquinone glycosides.

Table 4.1 Phytochemical constituent of serial extracts of *A. muricata*

TEST	AMFS	AMPE	AMEE	AMME	AMAE	AMR
Saponins	+	+	-	+	+	-
General glycosides	+	-	+	-	+	-
Flavonoids	+	-	-	+	+	-
Terpenoids and steroids	+	-	-	-	-	+
Carotenoids	-	-	-	-	-	-
Coumarins	+	-	+	-	-	-
Alkaloids	+	+	+	-	-	-
Anthraquinone	+	-	-	+	-	-
Anthraquinone glycosides	+	-	-	-	-	+

+ present - absent

4.2 EVALUATION OF HEPATOPROTECTIVE EFFECT OF SERIAL EXTRACTS OF *A. MURICATA*

4.2.1 Effect of treatment on rat body weight

The effect of *A. muricata* serial extract on body weight of CCl_4-treated rats is as shown in Fig. 4.1. Decrease in body weight was observed following CCl_4 administration, an indication of toxicity. By day 4, increases were observed in AMAE 300 mg/kg b.wt and 500 mg/kg b.wt treated groups which improved till the end of treatment. Best increases were observed in AMAE 300 mg/kg b.wt group (161.90%) and lowest decrease in AMPE 300 mg/kg b.wt group (-188.36%). Thus AMAE had better effect on weight gain following acute liver toxicity.

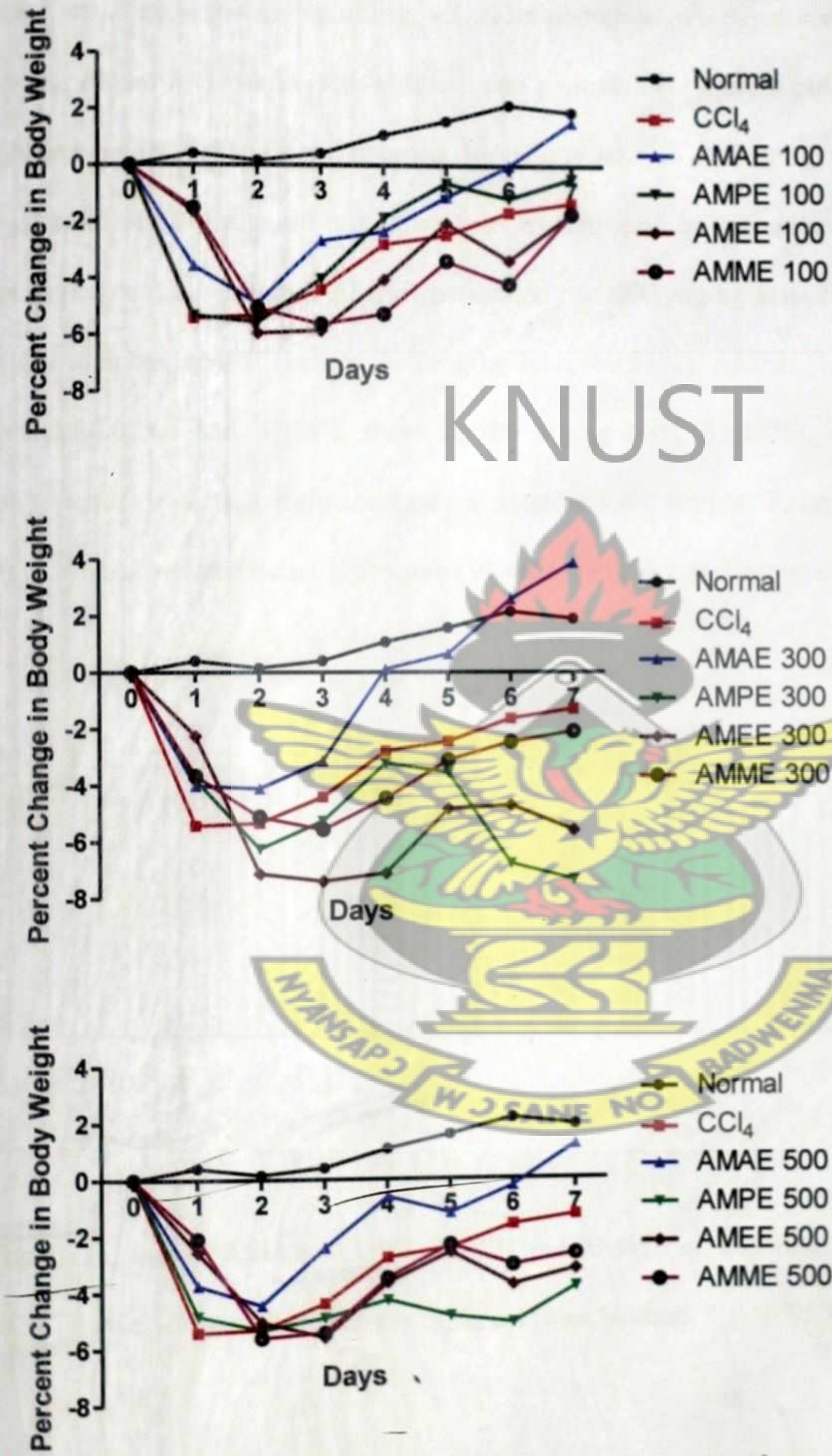


Fig. 4.1: Effect of AMAE, AMPE, AMEE and AMME on percent change in daily body weight in CCl_4-treated rats.

4.2.2 Effect of treatment on absolute and relative liver weight

The effect of *A. muricata* on absolute and relative liver weight of CCl_4-intoxicated rats is as shown in Fig. 4.2. Administration of a hepatotoxin, CCl_4 resulted in significant increase in both absolute and relative liver weight but not other organs (Appendix B, Table B1), indicating specificity of CCl_4 affecting the liver. AMAE treatment at all doses did not cause liver hypertrophy in the animals, indicating the hepatoprotective effect of AMAE particularly at 500 mg/kg b.wt (91.40%) towards CCl_4 toxicity. AMPE (best at 100 mg/kg b.wt, 60.22%), AMEE (best at 500 mg/kg b.wt, 86.02%) and AMME (best at 300 mg/kg b.wt, 88.17%), however, offered significant protection from increases in relative liver weight. Relatively, 500 mg/kg b.wt AMAE offered better protection (91.40%) (Fig. 4.2 and Appendix B, Table B1).

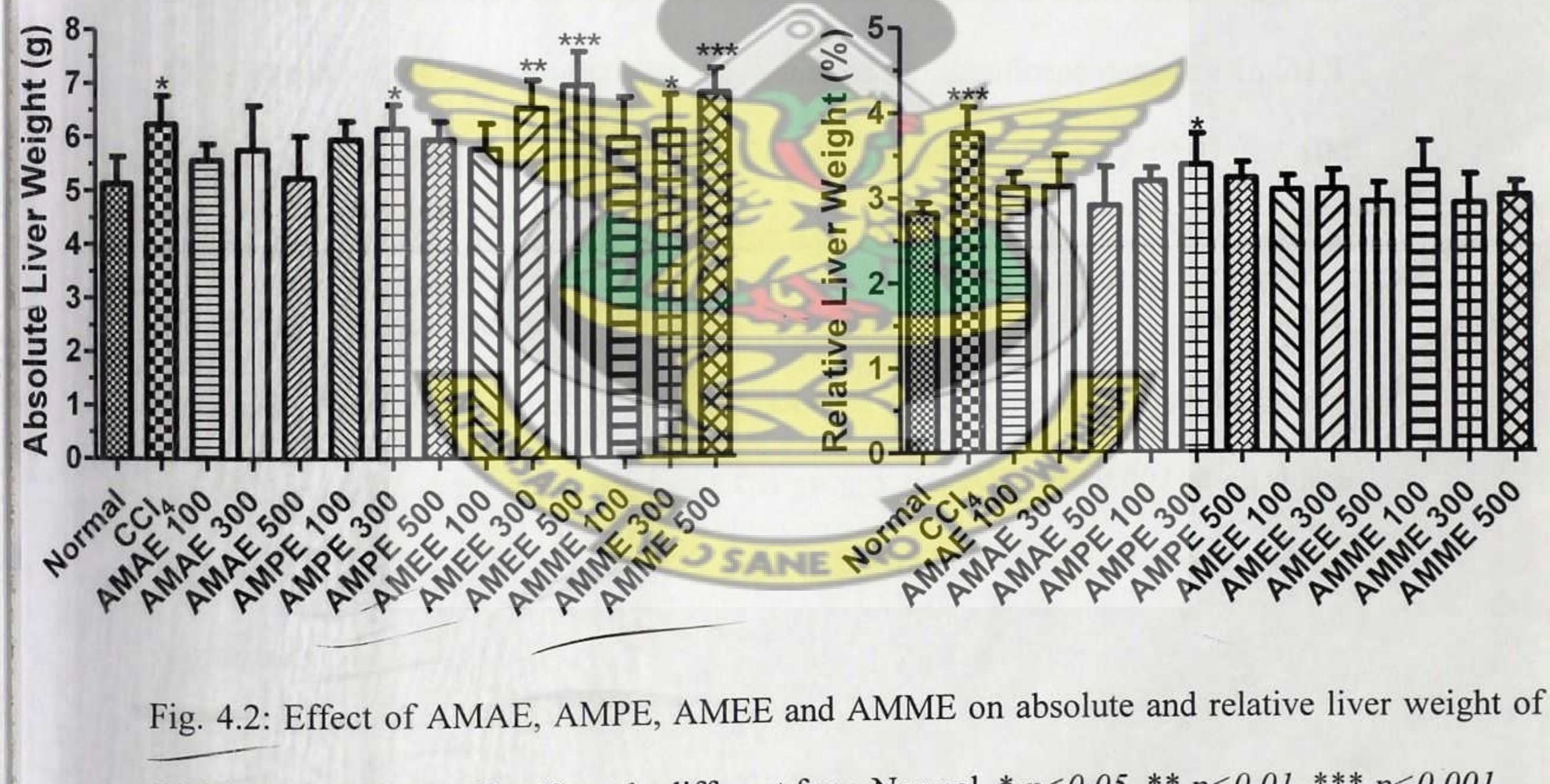
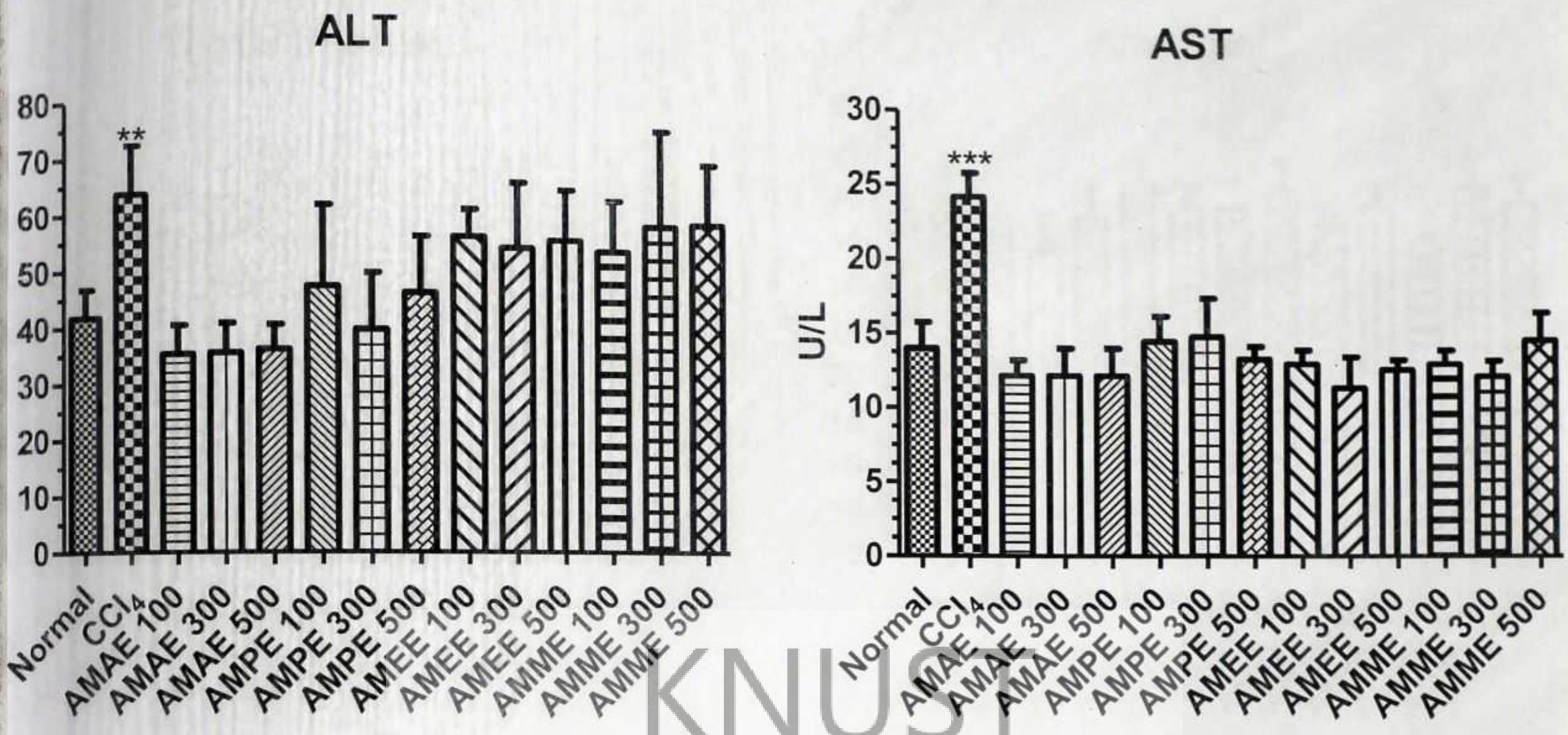


Fig. 4.2: Effect of AMAE, AMPE, AMEE and AMME on absolute and relative liver weight of CCl_4-intoxicated rats. Significantly different from Normal, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2.3 Effect of treatment on some biochemical parameters

The effect of *A. muricata* serial extracts on serum blood biochemistry of CCl_4-intoxicated rats is as shown in Fig. 4.3, 4.4 and 4.5. CCl_4 administration resulted in increases in ALT ($p<0.01$), AST ($p<0.001$), ALP ($p<0.001$) and total protein compared with normal group. Administration of AMAE at all doses resulted in significant decreases in ALT and AST ($p<0.001$ against CCl_4 group at all doses), and ALP ($p<0.001$ at 100 mg/kg b.wt and 500 mg/kg b.wt against CCl_4 group), and albumin ($p<0.001$ at 300 mg/kg b.wt against CCl_4 group), an indication of hepatoprotective activity of extracts. Significant decreases were also observed in total and indirect bilirubin concentration in AMAE 300 mg/kg b.wt ($p<0.001$) and 100 mg/kg b.wt ($p<0.05$) against CCl_4 group. However, no significant changes were observed in total protein concentration in extracts against CCl_4 group. AMPE treatment however resulted in significant decrease in ALT compared with CCl_4 group ($p<0.001$ for 300 mg/kg b.wt and $p<0.05$ for 100 mg/kg b.wt and 500 mg/kg b.wt), ALP ($p<0.01$ at all doses), and AST ($p<0.001$ at all doses) but not the other indicators of liver protection. AMEE and AMME treatment, however, resulted in increases in ALT against normal group, decreases in ALP ($p<0.01$ at all doses against CCl_4 group) and AST ($p<0.001$ at all doses against CCl_4 group). No effect was observed with the other indicators of liver protection.



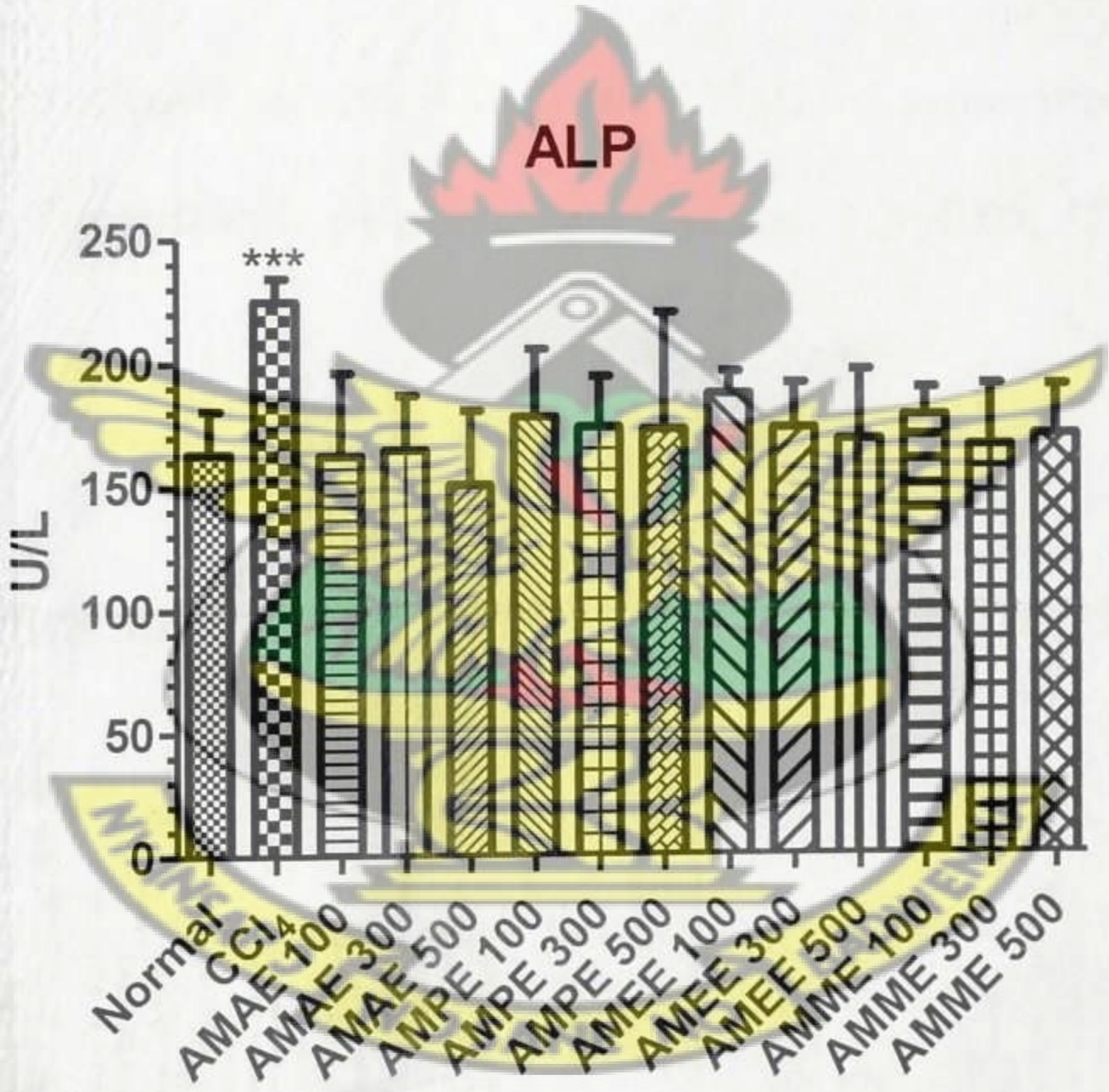


Fig. 4.3: Effect of AMAE, AMPE, AMEE and AMME on liver enzymes of CCl_4-intoxicated rats. Significantly different from Normal, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

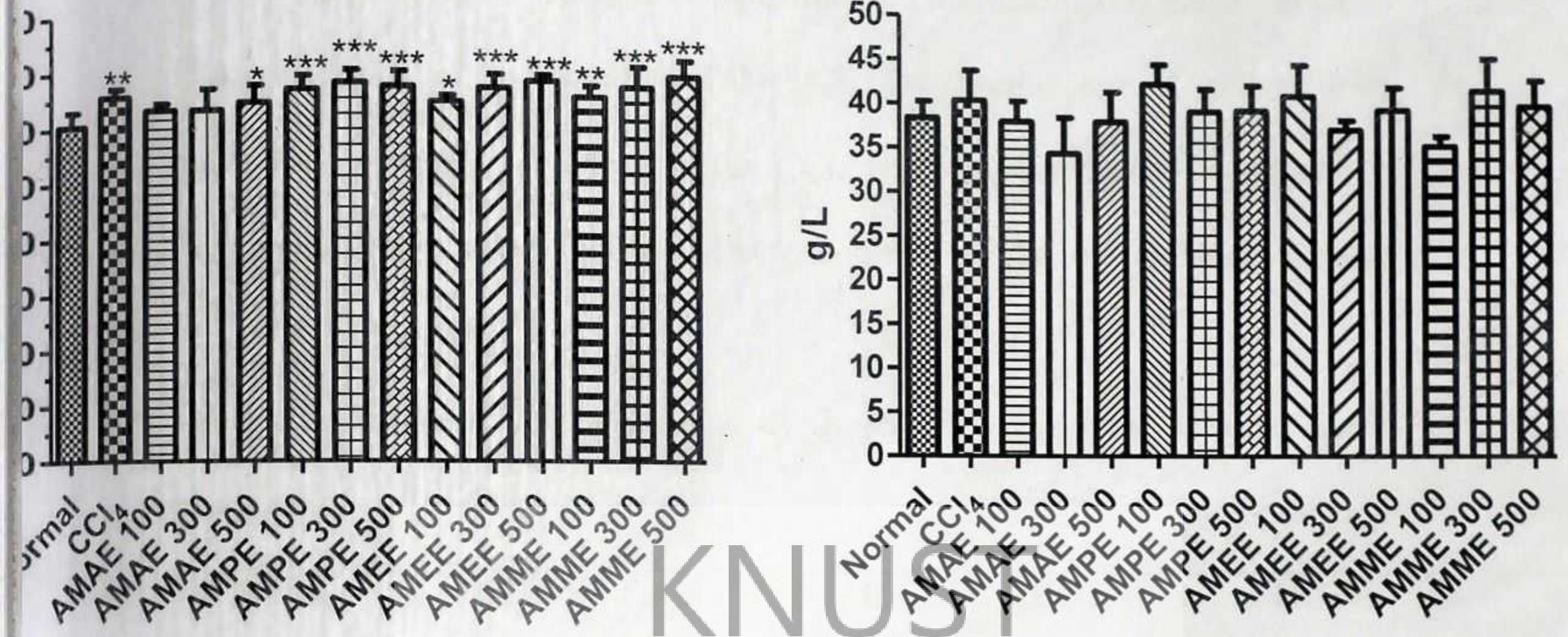


Fig. 4.4: Effect of AMAE, AMPE, AMEE and AMME on serum proteins of CCl_4-intoxicated rats. Significantly different from Normal, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

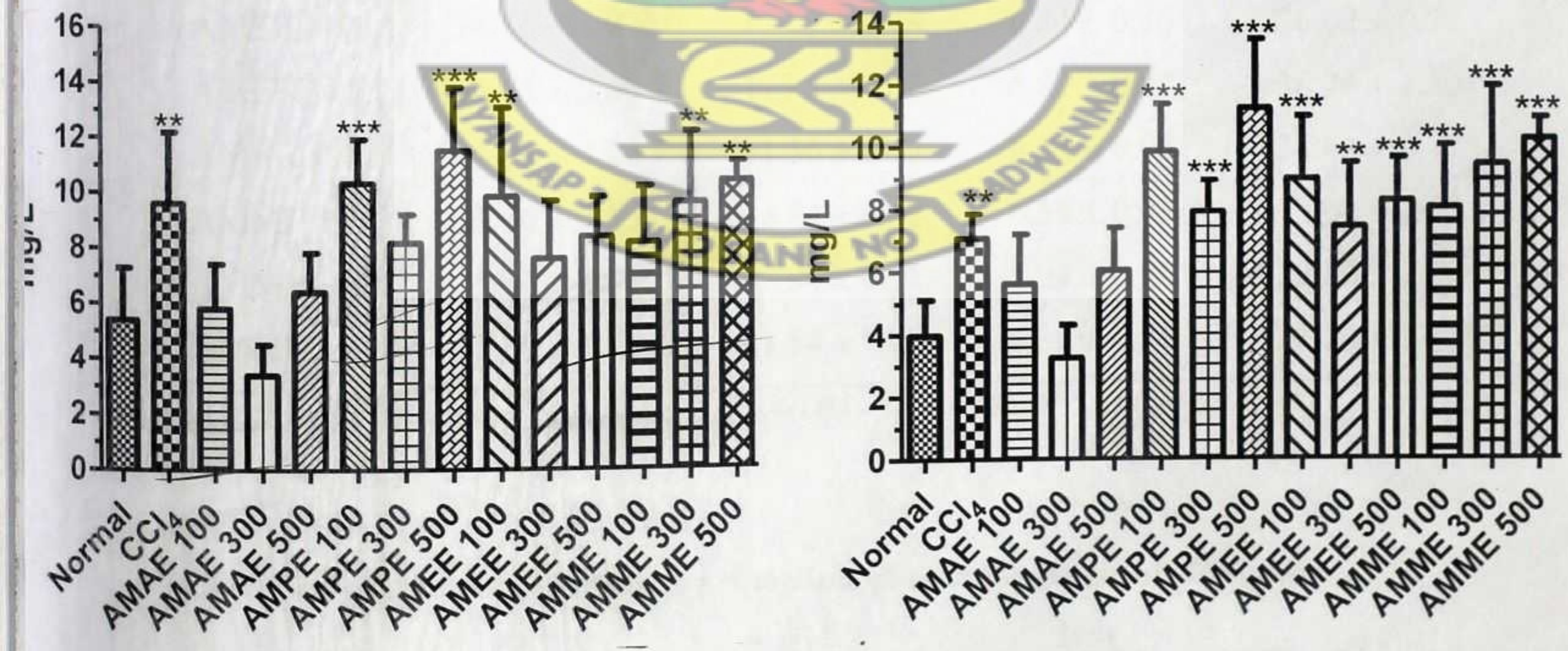


Fig. 4.5: Effect of AMAE, AMPE, AMEE and AMME on serum bilirubin concentration of CCl_4-intoxicated rats. Significantly different from Normal, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.2.4 Effect of treatment on some haematological indices

The effect of *A. muricata* serial extracts on haematological indices of CCl_4-intoxicated rats is as shown in Table 4.2. The haematological indices were measured to assess the effect of the CCl_4 and *A. muricata* on haematology. Treatments did not offer significant changes on the haematological indices of rats.

Table 4.2: Effect of serial extracts of *A. muricata* on some haematological parameters

Treatment	WBC x $10^3/\mu L$	HGB g/dL	RBC x $10^6/\mu L$	HCT%
Normal	5.66 ± 0.77	12.84 ± 0.82	6.62 ± 0.53	37.04 ± 1.16
CCl_4	6.68 ± 1.39	12.50 ± 0.48	6.73 ± 0.38	39.85 ± 1.00
AMAE 100	6.78 ± 1.73	11.25 ± 0.66	5.79 ± 0.41	33.95 ± 2.38
AMAE 300	8.45 ± 0.46	11.35 ± 0.38	5.64 ± 0.15	33.98 ± 1.35
AMAE 500	6.72 ± 0.86	10.74 ± 0.27	5.55 ± 0.21	31.82 ± 0.95*
AMPE 100	7.84 ± 0.87	11.80 ± 0.55	6.00 ± 0.16	36.08 ± 1.13
AMPE 300	7.10 ± 0.42	11.36 ± 0.28	5.85 ± 0.22	34.72 ± 1.10
AMPE 500	7.92 ± 0.61	11.16 ± 0.42	5.81 ± 0.16	34.62 ± 0.79
AMEE 100	4.76 ± 1.09	11.72 ± 0.19	6.84 ± 0.16	36.62 ± 0.71
AMEE 300	4.84 ± 0.66	11.84 ± 0.36	6.89 ± 0.27	36.24 ± 1.30
AMEE 500	3.90 ± 0.92	11.50 ± 0.12	6.54 ± 0.08	33.64 ± 0.77
AMME 100	3.86 ± 0.65	11.84 ± 0.21	6.38 ± 0.22	33.02 ± 1.00
AMME 300	5.04 ± 0.53	12.84 ± 0.21	7.04 ± 0.20	29.30 ± 6.28***
AMME 500	3.26 ± 0.81	11.54 ± 1.28	6.34 ± 0.71	31.50 ± 3.70**

Significantly different from Normal, *$p<0.05$, **$p<0.01$, ***$p<0.001$

4.2.5. Comparison of percentage protection of serial extracts of *A. muricata*

The comparison of the percentage protection of serial extracts of *A. muricata* on the liver is as shown in Fig. 4.6 and Appendix B Table B2. AMAE at all doses showed significant protective effect on the liver (88.2 – 128.6%) with

300 mg/kg b.wt showing the maximum protections. The other serial extracts showed varying levels of protection with 300 mg/kg b.wt AMPE (42.58%), 300 mg/kg b.wt AMEE (60.21%) and 100 mg/kg b.wt AMME (66.15%) showing the best protection.

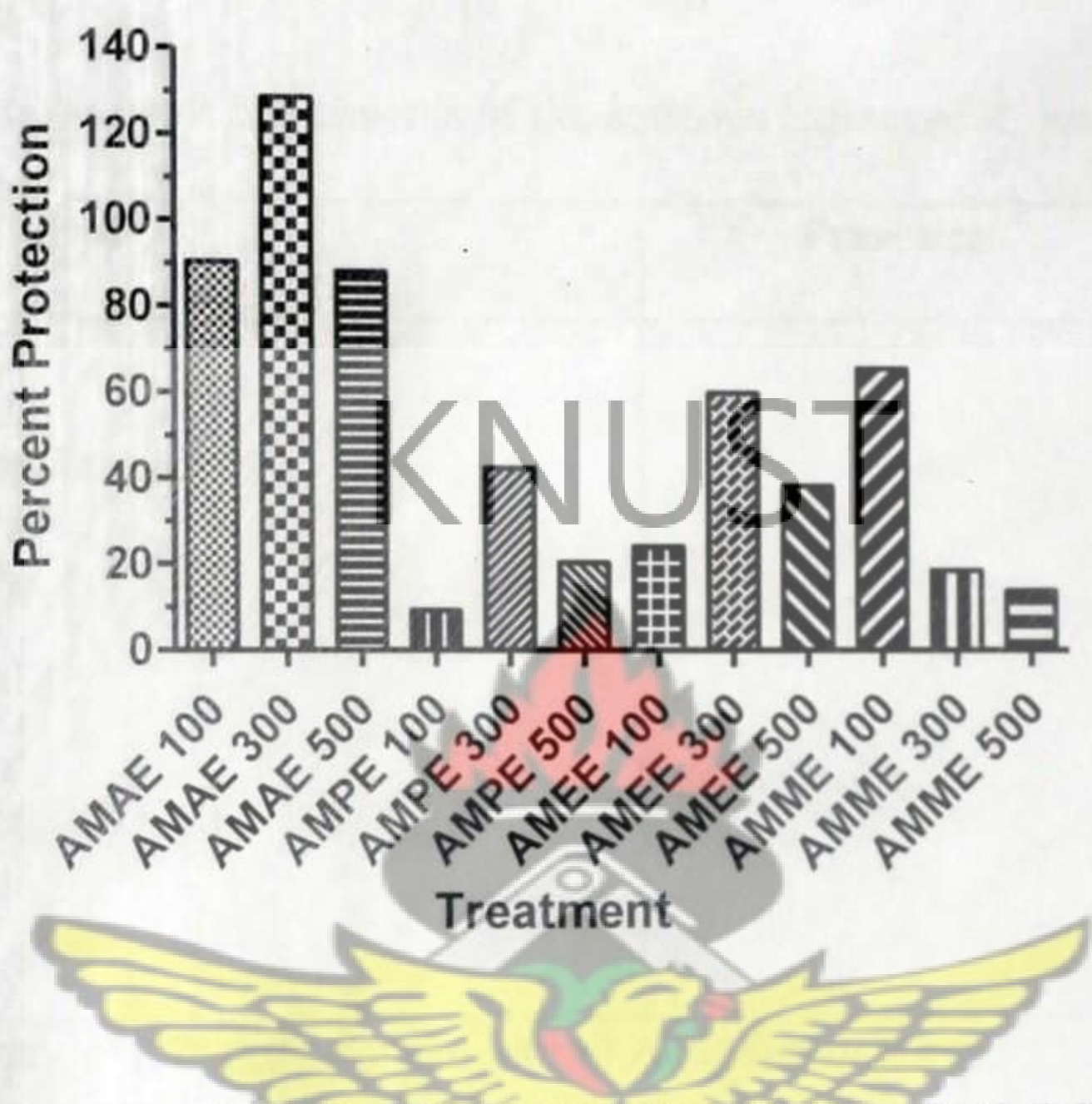


Fig. 4.6: Percent protection of AMAE, AMPE, AMEE and AMME on liver of CCl_4-intoxicated rats.

4.3 *ANNONA MURICATA* AQUEOUS DECOCTION

The aqueous decoction was performed on dried *A. muricata* leaves based on observation of hepatoprotective studies, which showed the aqueous extract (AMAE) to possess maximum liver protection effect (128.64% at 300 mg/kg b.wt).

4.3.1 Yield of *A. muricata* aqueous extract (AMAE)

The amount of plant material extracted (1.41 kg in 10 L water) yielded 211 g of freeze-dried aqueous extract representing a percentage yield of 14.96%.

4.3.2 Phytochemical constituent of *A. muricata* aqueous extract

The results of the phytochemical screening of the plant extract (Table 4.3) revealed the presence of saponins, condensed tannins, glycosides and flavonoids. Alkaloids and sterols were conspicuously absent.

Table 4.3: Phytochemical constituents of the aqueous extract of *A. muricata*

Phytochemical	**Presence**
Saponins	+++
Condensed Tannins	+++
Flavonoids	+
Alkaloids	-
Glycosides	+++
Sterols	-

(-) Absent, (+) Slightly present, (+++) Abundantly present

4.4 ACUTE ORAL TOXICITY STUDIES

At the doses studied (100 mg/kg b.wt, 1000 mg/kg b.wt, 2500 mg/kg b.wt and 5000 mg/kg b.wt), no untoward clinical signs were observed in the mice. There were no changes in the nature of stool, urine and eye colour. No mortality was observed at all dose levels from the critical 24 hours post administration to the end of the seventh day. Orally, 5000 mg/kg b.wt of AMAE was well tolerated in mice even after 7 days. Hence the LD_{50} was estimated to be $\leq$5000 mg/kg b.wt (orally).

4.5 SUBCHRONIC TOXICITY STUDIES

4.5.1 Morbidity and mortality associated with AMAE

All the rats used for the study appeared normal before, during and after treatment. Mortality was not recorded at all dose levels used for the study; 100 mg/kg b.wt, 1000 mg/kg b.wt and 2500 mg/kg b.wt of extract.

4.5.2 Effect of AMAE on percentage change in body weight

The effect of AMAE on body weight of male and female rats is as shown in Fig. 4.7 and Table 4.4. From day 2 to day 14, there were variable changes in the body weight of rats in all the groups. The normal control groups for both male and female rats gained weight throughout the duration of the treatment. Weight gains were also observed in rats administered 100 mg/kg b.wt AMAE in both males (8.53% at termination) and females (3.64% at termination) whereas decreases were observed in 1000 mg/kg b.wt (3.29% male and 1.26% female) and 2500 mg/kg b.wt (-0.39% male and -1.34% female) groups. However, male rats administered with 1000 mg/kg b.wt and 2500 mg/kg b.wt AMAE showed initial weight gains up to day 6 which declined by end of day 8 whereas female animals showed constant weight decline at the same dose. For males, no significant differences were observed between normal control and 100 mg/kg b.wt and 1000 mg/kg b.wt groups but significant decrease compared with 2500 mg/kg b.wt ($p<0.01$). For females, there were significant differences in percent change in body weight of normal and 100 mg/kg b.wt groups against 1000 mg/kg b.wt ($p<0.01$) and 2500 mg/kg b.wt ($p<0.001$) groups. Thus, higher doses of AMAE at 2500 mg/kg b.wt resulted in significant decrease in body weight in both sexes.

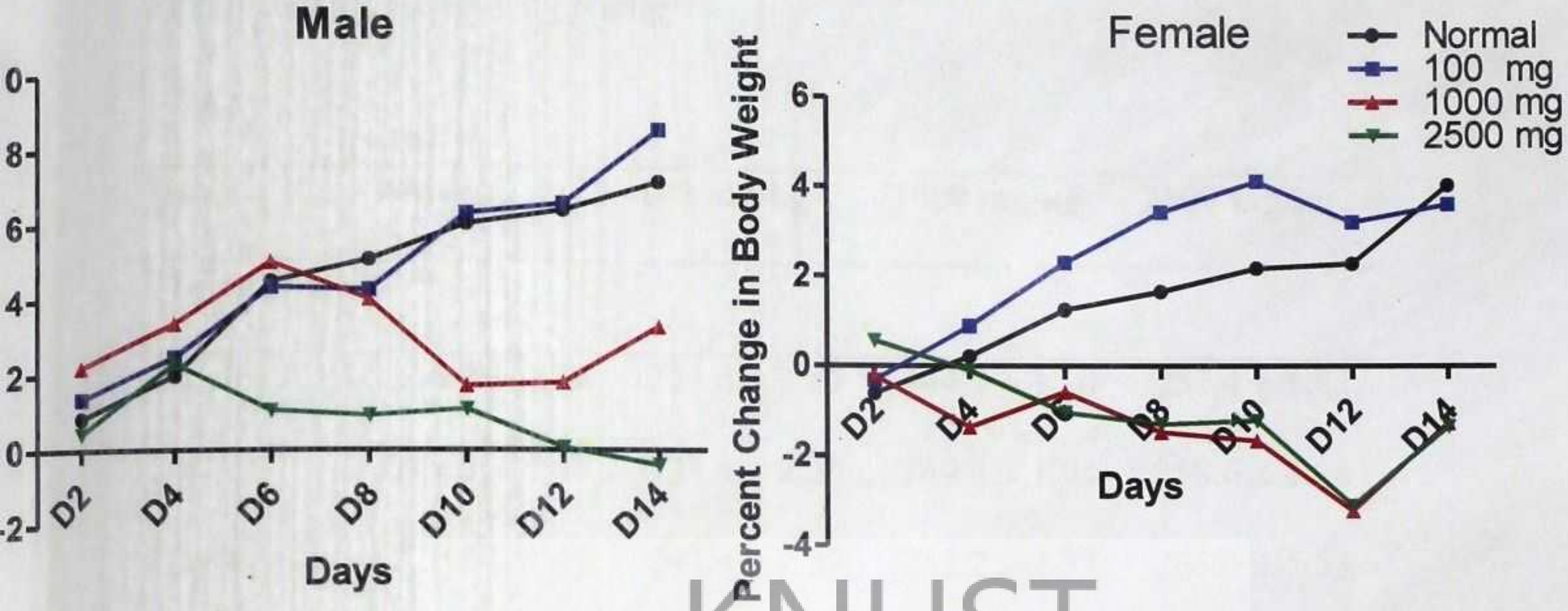


Fig. 4.7: Effect of AMAE on percentage change in body weight in rats for 14 days.

4.5.3 Effect of AMAE on feed and water consumption

The effect of AMAE on relative feed and water consumption of male and female rats is as shown in Fig. 4.8. There were varied levels of feed and water consumption in both sexes. For males, there were no significant difference in feed consumption between normal control and 100 mg/kg b.wt but significant decreases with 1000 mg/kg b.wt ($p<0.01$) and 2500 mg/kg b.wt ($p<0.001$). Significant decreases were observed with water consumption between normal control and 100 mg/kg b.wt ($p<0.01$), 1000 mg/kg b.wt ($p<0.05$) and 2500 mg/kg b.wt ($p<0.001$). For females, there were no significant differences in feed consumption but decreases ($p<0.001$) were observed in water consumption in all treated groups compared with normal group.

Table 4.4: The effect of AMAE on body weight changes in the control and treated rats in subchronic toxicity studies

Dose	**Normal**	**100 mg/kg**	**1000 mg/kg**	**2500 mg/kg**
Males				
Day 0	216.0 ± 4.59	235.6 ± 2.29	244.0 ± 1.10	257.4 ± 1.83
Day 2	217.8 ± 4.50	238.8 ± 2.20	249.4 ± 1.94	258.6 ± 4.08
Day 4	220.2 ± 4.44	241.4 ± 2.32	252.2 ± 2.33	263.4 ± 5.53
Day 6	225.6 ± 4.83	245.8 ± 2.67	256.2 ± 1.80	260.2 ± 3.96
Day 8	226.8 ± 4.09	245.6 ± 2.79	253.8 ± 2.65	259.8 ± 4.39
Day 10	228.8 ± 3.93	250.4 ± 2.73	248.2 ± 2.31	260.2 ± 2.54
Day 12	229.6 ± 4.48	251.0 ± 2.17	248.4 ± 1.86	257.6 ± 2.54
Day 14	231.2 ± 4.64	255.6 ± 1.78	252.0 ± 2.80	256.4 ± 3.67
Female				
Day 0	192.4 ± 0.93	198.2 ± 1.50	203.2 ± 1.39	211.2 ± .177
Day 2	191.2 ± 1.20	197.6 ± 3.76	202.8 ± 1.99	212.4 ± 1.47
Day 4	192.8 ± 1.39	200.0 ± 4.10	200.4 ± 1.91	211.0 ± 1.23
Day 6	194.8 ± 1.50	202.8 ± 4.62	202.0 ± 1.38	209.0 ± 3.77
Day 8	195.6 ± 3.27	205.0 ± 4.09	200.2 ± 1.91	208.4 ± 2.64
Day 10	196.6 ± 2.44	206.4 ± 2.11	199.8 ± 0.49	208.6 ± 1.99
Day 12	196.8 ± 3.11	204.6 ± 1.60	196.6 ± 0.93	204.6 ± 2.66
Day 14	200.2 ± 2.40	205.4 ± 1.25	200.6 ± 1.33	208.4 ± 3.46

Mean ± SEM, (n = 5)

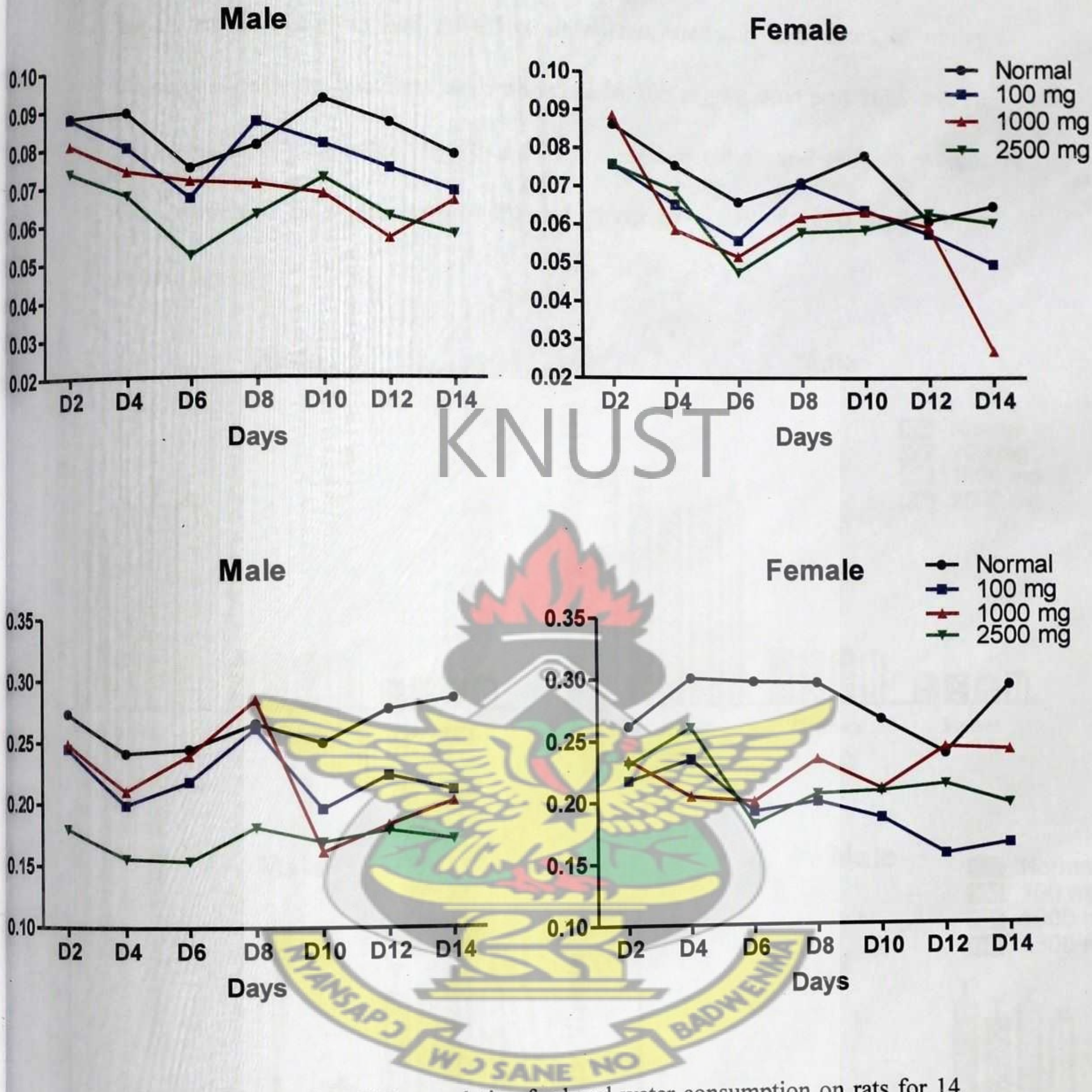


Fig. 4.8: Effect of AMAE on relative feed and water consumption on rats for 14 days. Each points represent relative consumption as a function on body weight for 5 animals.

4.5.4 Effect of AMAE on absolute and relative organ weights

The effect of AMAE on absolute and relative organ weights of male and female rats are as shown in Fig. 4.9. There were no significant changes in the

absolute and relative weights of the liver, kidney and heart in both male and female rats. Treatment had no effect on spleen, stomach and testes of males whereas significant increases were observed in 100 mg/kg b.wt and 1000 mg/kg b.wt groups. For females, significant increases in absolute and relative weight were observed in stomach ($p<0.01$) and uterus ($p<0.05$) of 1000 mg/kg b.wt treated group.

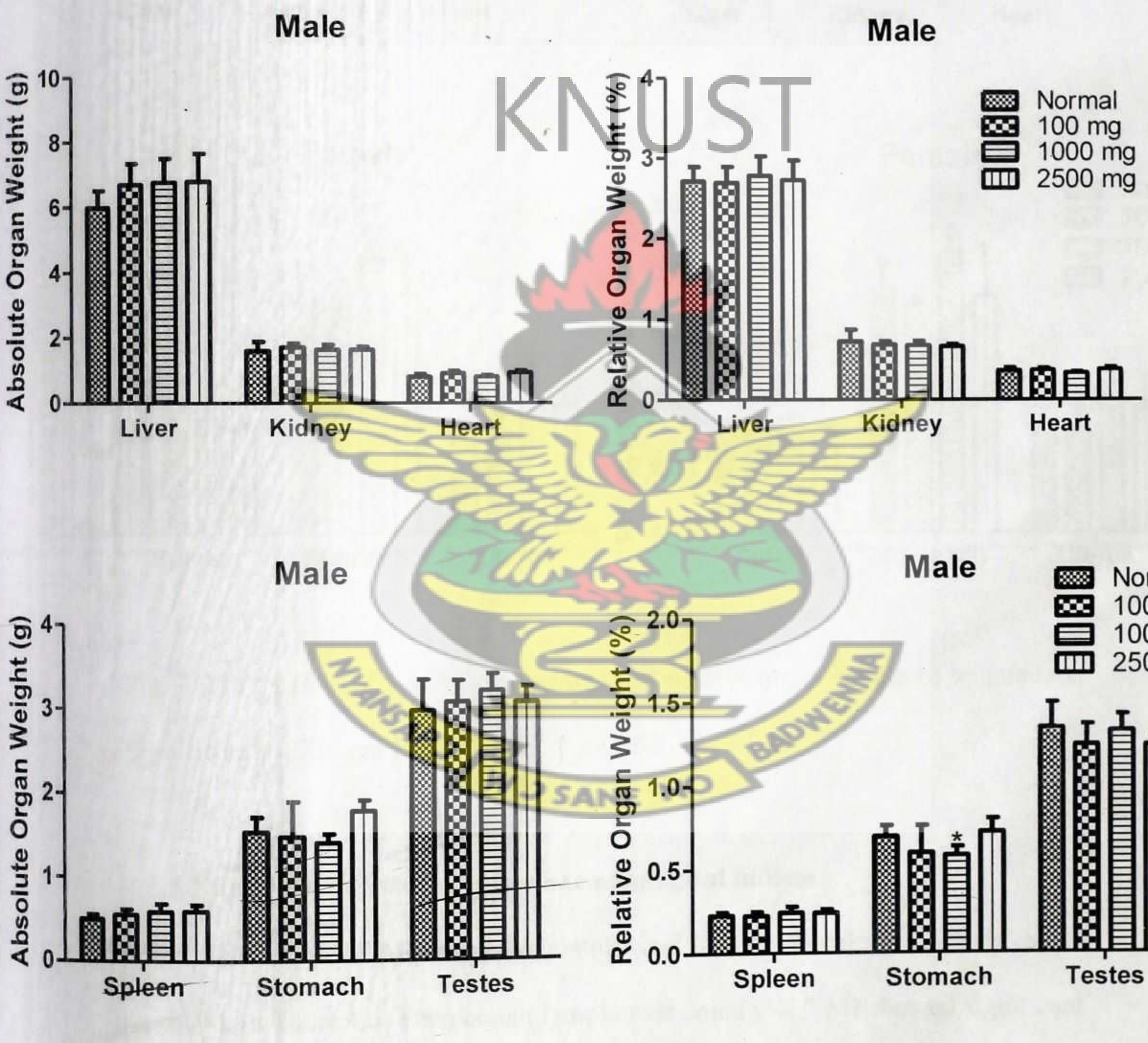
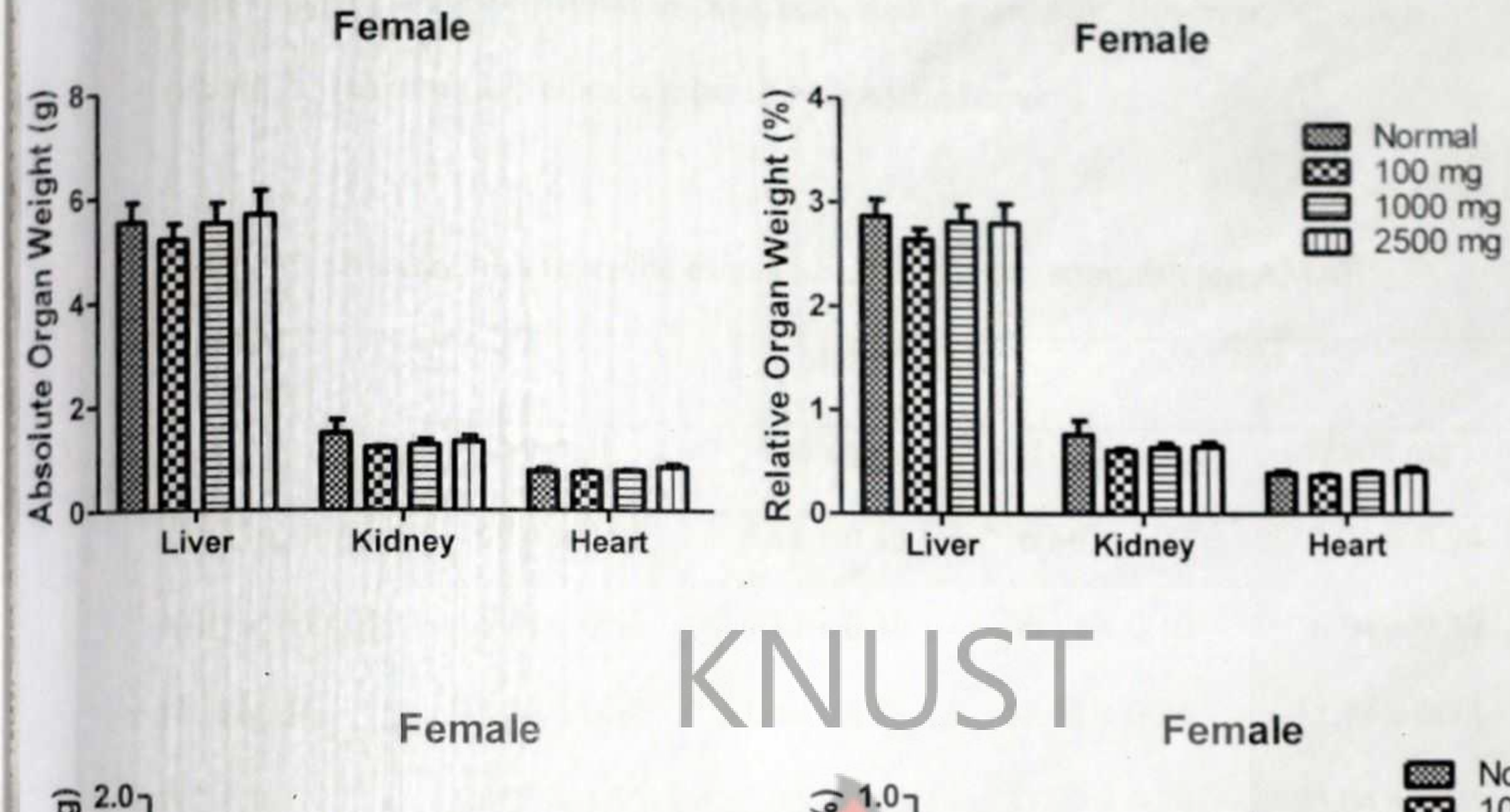


Fig. 4.9 (a): Effect of AMAE on absolute and relative organ weight of male rats.

Significantly different from Normal, * $p<0.05$



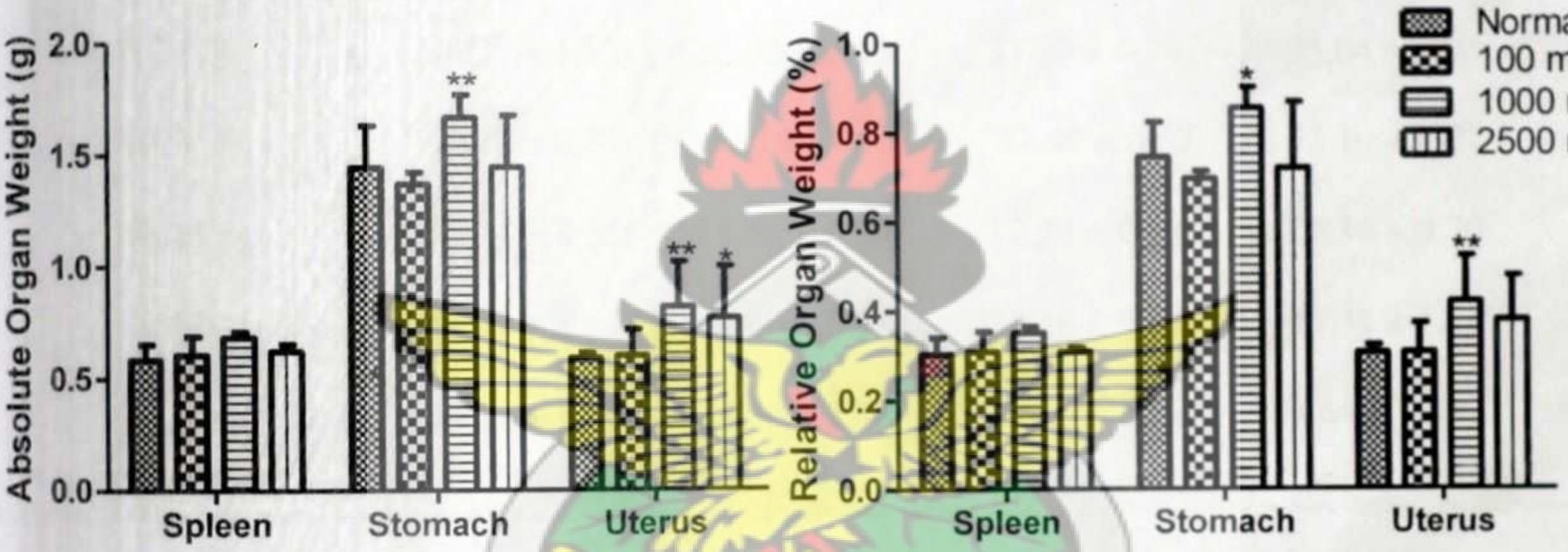


Fig. 4.9 (b): Effect of AMAE on absolute and relative organ weight of female rats.

Significantly different from Normal, * $p<0.05$, ** $p<0.01$

4.5.5 Effect of treatment on some haematological indices

The effect of AMAE on haematological indices of male and female rats is as shown in Table 4.5. The percent lymphocyte count (%LYM) showed significant increase in both male and female rats at 1000 mg/kg b.wt and 2500 mg/kg b.wt group ($p<0.05$). There were observed dose dependent non-significant increases in

white blood cell (WBC) count in both male and female rats. However, all other indices showed normal values compared with normal control.

Table 4.5: Haematological profile of male and female rats administered AMAE

	Male			
	Normal	100 mg	1000 mg	2500 mg
WBC x $10^3/\mu L$	4.64 ± 0.43	5.84 ± 0.55	6.66 ± 1.33	7.26 ± 0.94
RBC x $10^6/\mu L$	6.45 ± 0.13	6.14 ± 0.39	6.44 ± 0.10	6.04 ± 0.20
HGB g/dL	12.46 ± 0.12	11.40 ± 0.56	12.38 ± 0.26	11.74 ± 0.72
HCT %	38.90 ± 1.12	35.02 ± 2.00	37.00 ± 0.74	35.04 ± 1.06
MCV fL	60.28 ± 0.83	57.16 ± 0.91	57.42 ± 0.62	58.10 ± 0.87
MCH pg	19.34 ± 0.55	18.64 ± 0.29	19.24 ± 0.34	19.34 ± 0.70
MCHC g/dL	32.14 ± 1.11	32.60 ± 0.31	33.48 ± 0.69	33.44 ± 1.51
%LYM	79.78 ± 2.63	83.30 ± 3.16	79.56 ± 5.62	87.64 ± 3.59**
	Female			
WBC x $10^3/\mu L$	5.86 ± 0.49	5.32 ± 0.51	5.70 ± 0.76	6.26 ± 0.53
RBC x $10^6/\mu L$	5.76 ± 0.17	5.16 ± 0.45	5.54 ± 0.28	5.46 ± 0.20
HGB g/dL	11.74 ± 0.31	10.10 ± 1.34	10.90 ± 0.28	11.08 ± 0.36
HCT %	34.28 ± 1.25	29.46 ± 2.53*	30.86 ± 0.92	31.34 ± 0.89
MCV fL	59.68 ± 0.66	57.12 ± 0.53	57.20 ± 0.60	57.88 ± 0.68
MCH pg	20.40 ± 0.15	19.26 ± 1.40	20.36 ± 0.19	20.66 ± 0.14
MCHC g/dL	34.20 ± 0.43	33.68 ± 2.48	35.36 ± 0.23	35.32 ± 0.30
%LYM	80.38 ± 2.21	78.74 ± 3.16	87.46 ± 2.11**	83.74 ± 2.53

**Significantly different from Normal at $p<0.01$

4.5.6 Effect of AMAE on liver and kidney function

The effect of AMAE on liver and kidney function of male and female rats are as shown in Fig. 4.10 and 4.11. AMAE at all dose in male and female rats did not significantly alter the serum levels of liver enzymes (Fig. 4.10) except in ALP of males administered with 1000 mg/kg b.wt AMAE ($p<0.01$). Also AMAE did not alter the serum concentration of urea, albumin and creatinine (Fig. 4.11) except a significant increase in creatinine concentration in males ($p<0.05$) and females ($p<0.001$) administered 2500 mg/kg b.wt AMAE.

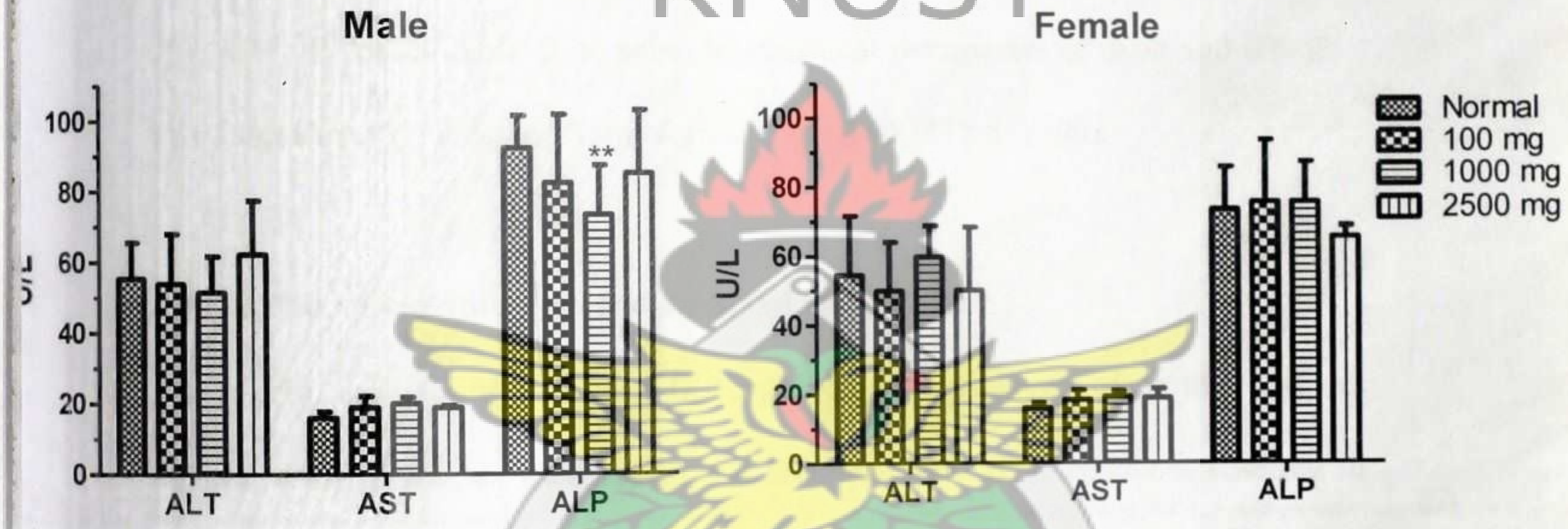


Fig. 4.10: Effect of AMAE on liver enzymes of male and female rats. Significantly different from Normal, ** $p<0.01$

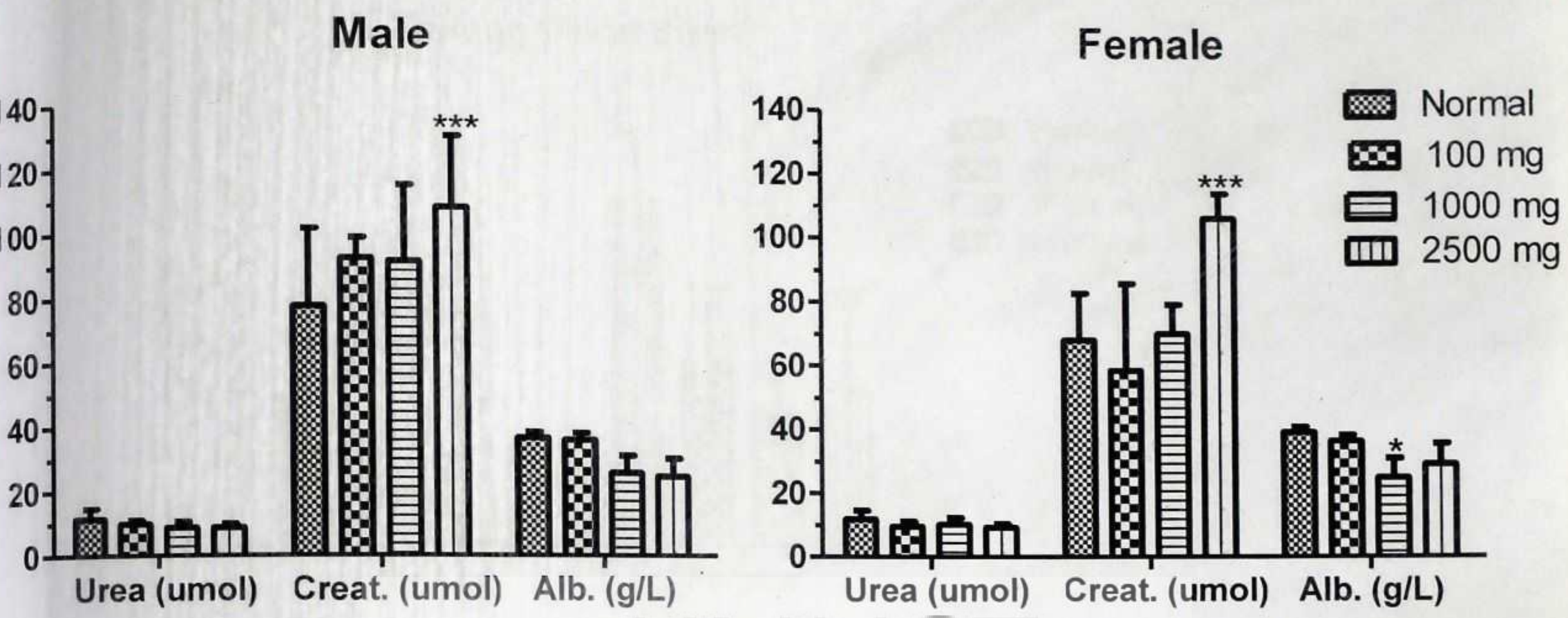


Fig. 4.11: Effect of AMAE on some biochemical parameters of male and female rats. Significantly different from Normal, * $p<0.05$, *** $p<0.001$

4.5.7 Effect of treatment on fasting blood sugar (FBS)

Fig. 4.12 shows results of fasting blood glucose levels of normal and rats treated with AMAE in both males and females. AMAE at all doses resulted in significant decreases in plasma glucose levels in both male ($p<0.01$ at 1000 mg and 2500 mg/kg b.wt) and female rats ($p<0.001$ at 100 mg/kg b.wt, 1000 mg/kg b.wt and 2500 mg/kg b.wt).

4.5.8 Effect of treatment on serum lipid profile

The effect of AMAE on serum lipid profile of male and female rats is as shown in Fig. 4.13. No significant differences were observed in total cholesterol, HDL, LDL and total triglyceride concentration in male rats at all doses of AMAE. In females, no differences were observed in HDL and total triglyceride concentrations at all doses with 100 mg AMAE inducing a decrease ($p<0.01$) in total cholesterol and LDL concentrations.

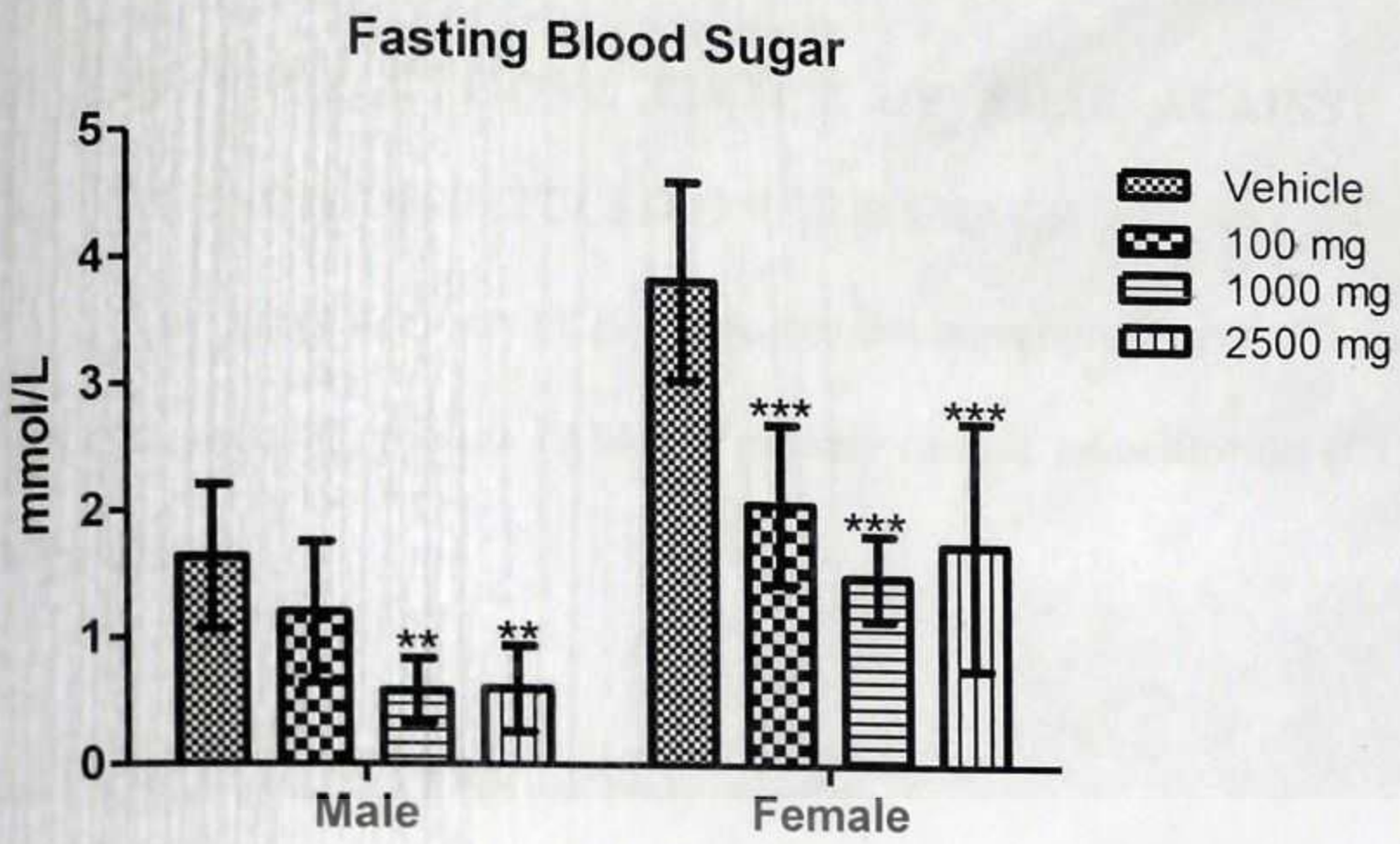


Fig 4.12: Effect of AMAE on fasting blood sugar of male and female rats.

Significantly different from Normal, ** $p<0.01$, *** $p<0.001$

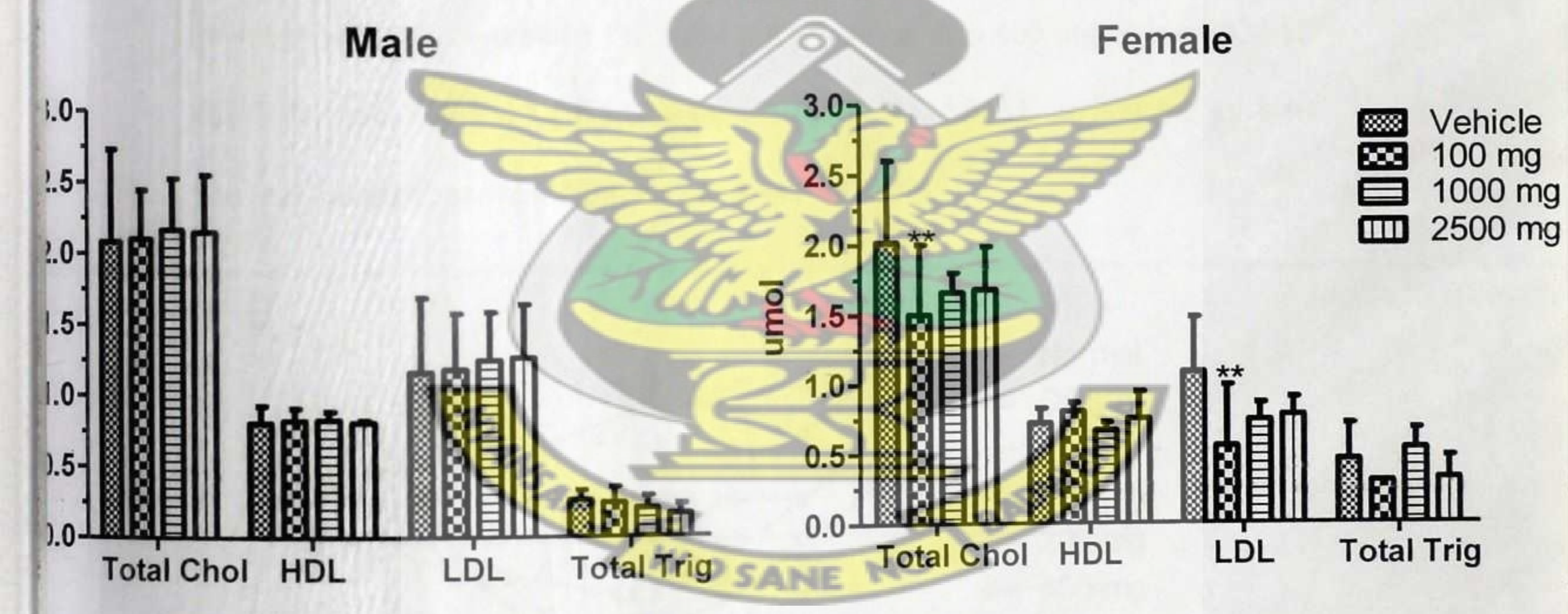


Fig. 4.13: Effect of AMAE on lipid profile of male and female rats. Significantly different from Normal, ** $p<0.01$, *** $p<0.001$

4.6 HEPATOPROTECTIVE EFFECT OF AMAE AGAINST CARBON TETRACHLORIDE-INDUCED LIVER DAMAGE

The study was carried out to assess the hepatoprotective effects of *Annona muricata* aqueous extract (AMAE) against carbon tetrachloride (CCl_4)-induced liver damage.

4.6.1 Effect of AMAE on rat body weight

The effect of AMAE on the body weight of CCl_4-intoxicated rats is as shown in Fig. 4.14. The percent daily body weight changes indicated that CCl_4-intoxication resulted in weight losses in all groups which showed recovery over time. However, AMAE administration at 100 mg/kg b.wt (59.03%) and 50 mg/kg b.wt (44.91%) showed better recovery at termination than 400 mg/kg b.wt AMAE (21.76%) and 100 mg/kg b.wt Silymarin (23.61%). AMAE at 200 mg/kg b.wt showed the least recovery from weight loss (-19.44%).

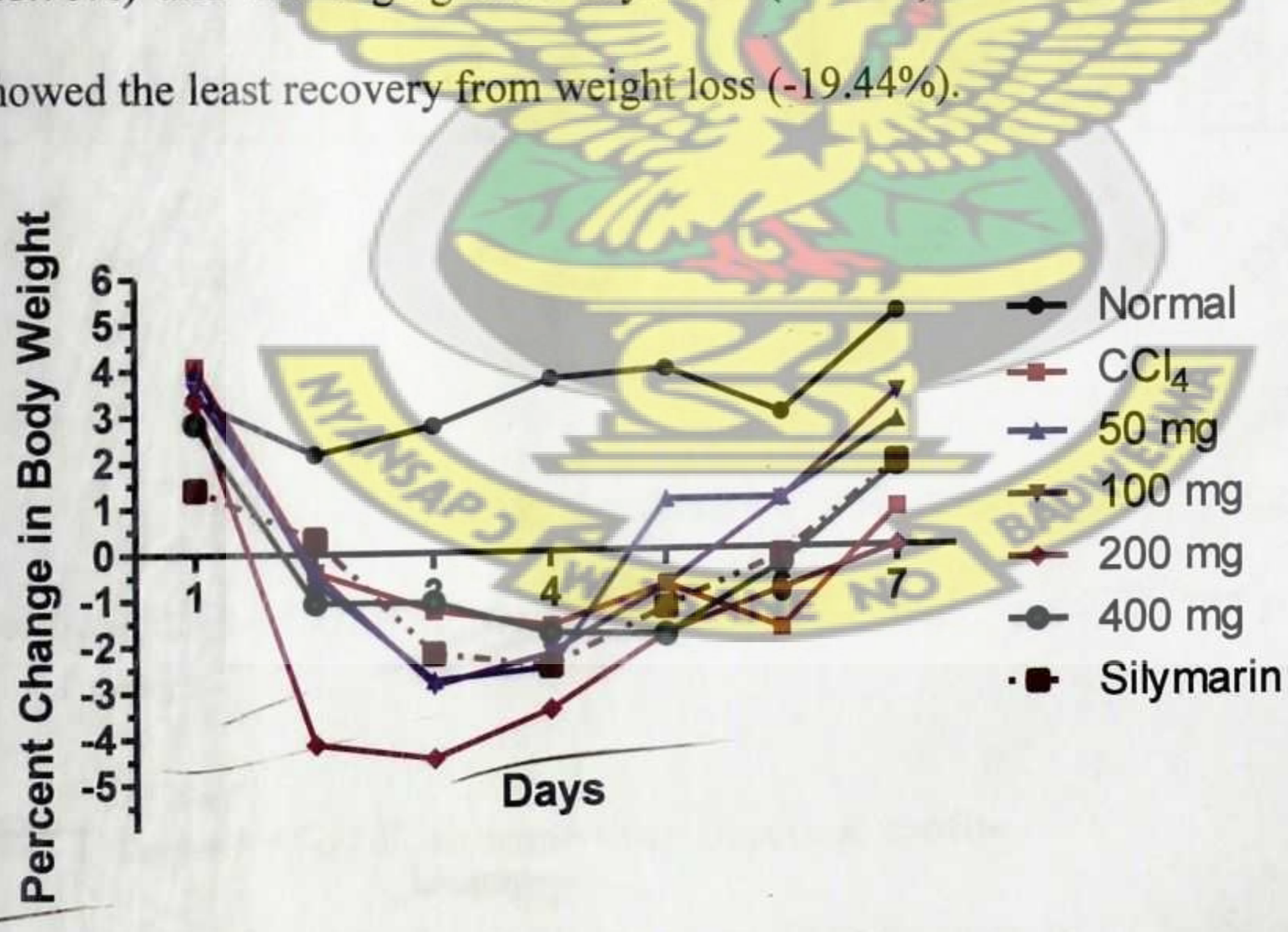


Fig. 4.14: Effect of AMAE on percent change in body weight in CCl_4-intoxicated rats.

4.6.2 Effect of AMAE on absolute and relative liver weight

The effect of AMAE on absolute and relative liver weight of CCl_4-intoxicated rats is as shown in Fig. 4.15. Significant increases in both absolute and relative liver weights were observed in animals treated with only CCl_4. However, pretreatment of animals with AMAE showed a dose dependent protection against hepatotrophy, with 50 mg/kg b.wt showing better protection (128.13%) in relative weight than 200 mg/kg b.wt (78.13%). Silymarin standard drug showed the best protection (164.06%).

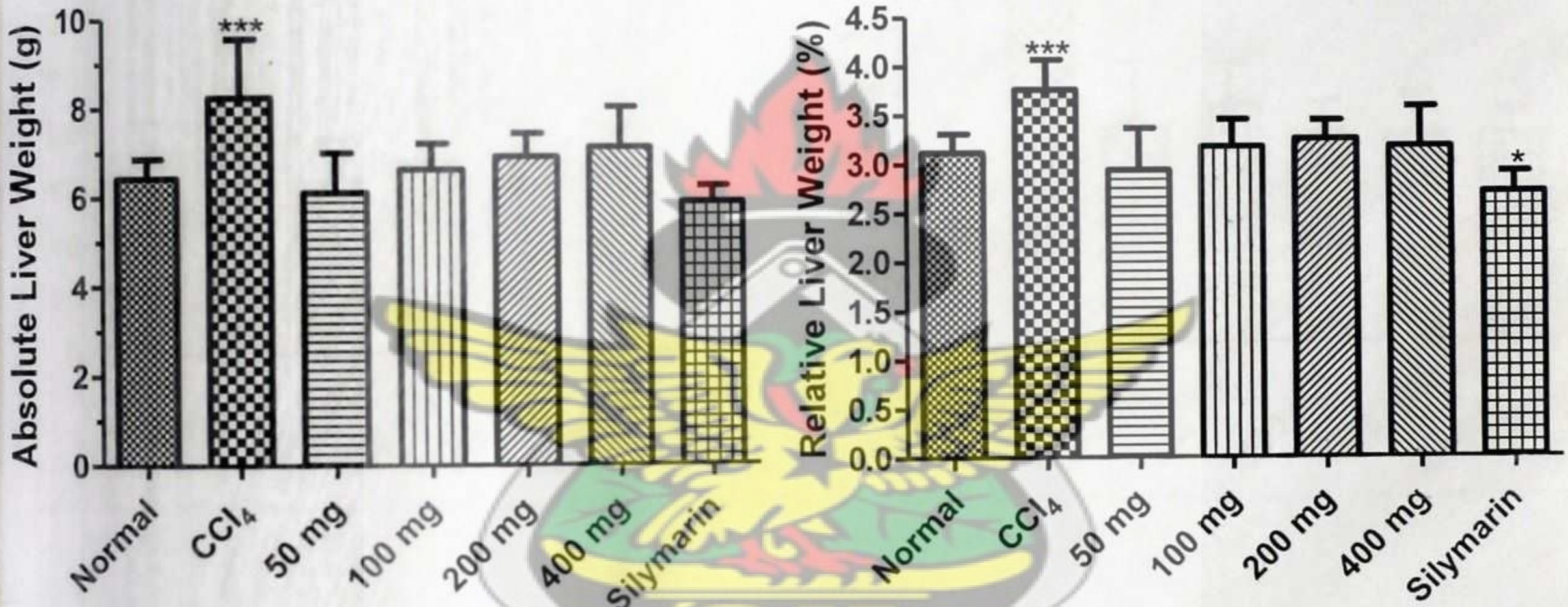


Fig. 4.15: Effect of AMAE on absolute and relative liver weight on CCl_4-intoxicated rats. Significantly different from Normal, * $p<0.05$, *** $p<0.001$

4.6.3 Effect of AMAE on some liver function profile

Effect of AMAE and Silymarin on liver function of CCl_4-intoxicated rats is as shown in Fig. 4.16, 4.17 and 4.18. In CCl_4 group, ALT, ALP and total bilirubin levels were significantly higher ($p<0.001$) than Normal control. Pretreatment with AMAE and Silymarin significantly protected the liver by reducing ALT

($p<0.001$), ALP ($p<0.001$), total bilirubin ($p<0.001$) and indirect bilirubin concentrations ($p<0.01$ for Silymarin, 200 mg/kg b.wt and 400 mg/kg b.wt AMAE and $p<0.05$ for 50 mg/kg b.wt and 100 mg/kg b.wt AMAE) in rats compared with CCl_4 group. However, no significant differences were observed in serum total protein and albumin levels of animals.

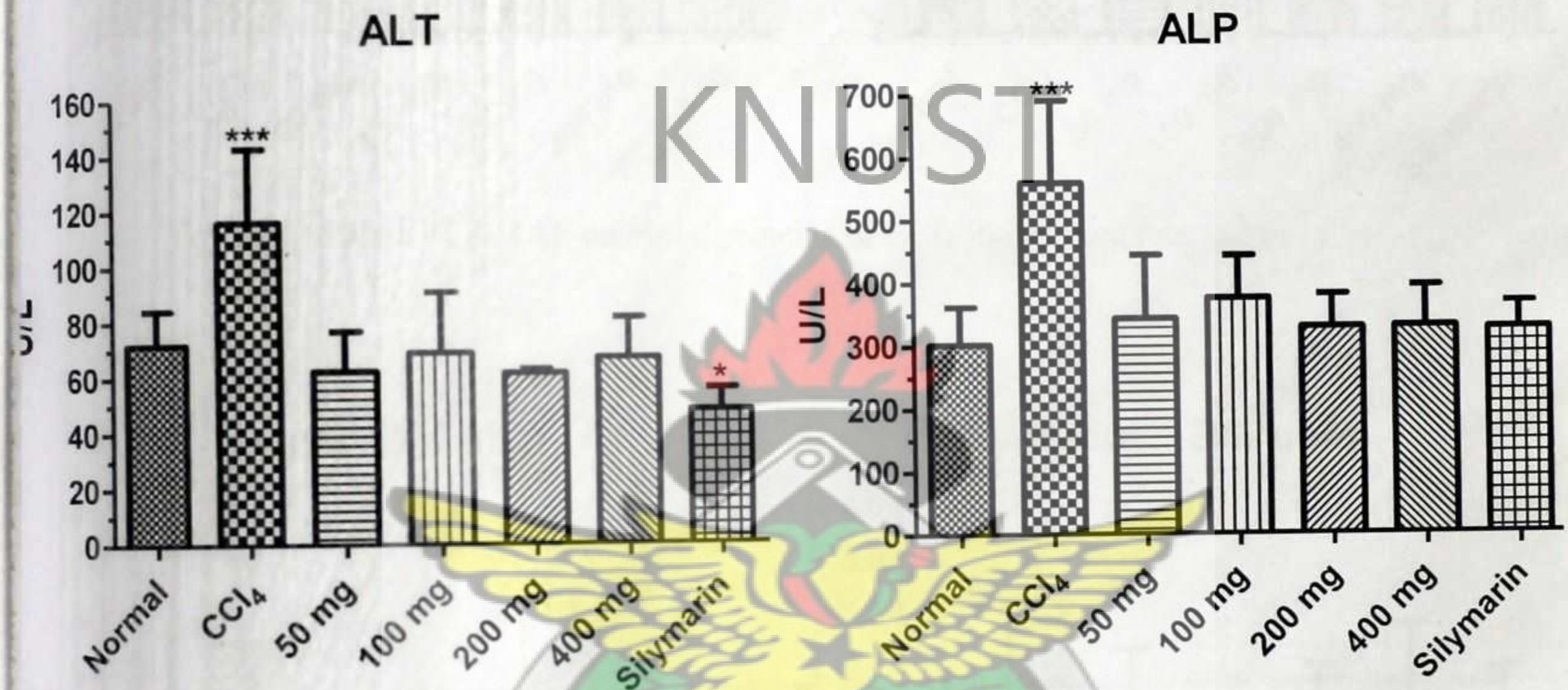


Fig. 4.16: Effect of AMAE on liver enzymes of CCl_4-intoxicated rats.

Significantly different from Normal, * $p<0.05$, *** $p<0.001$

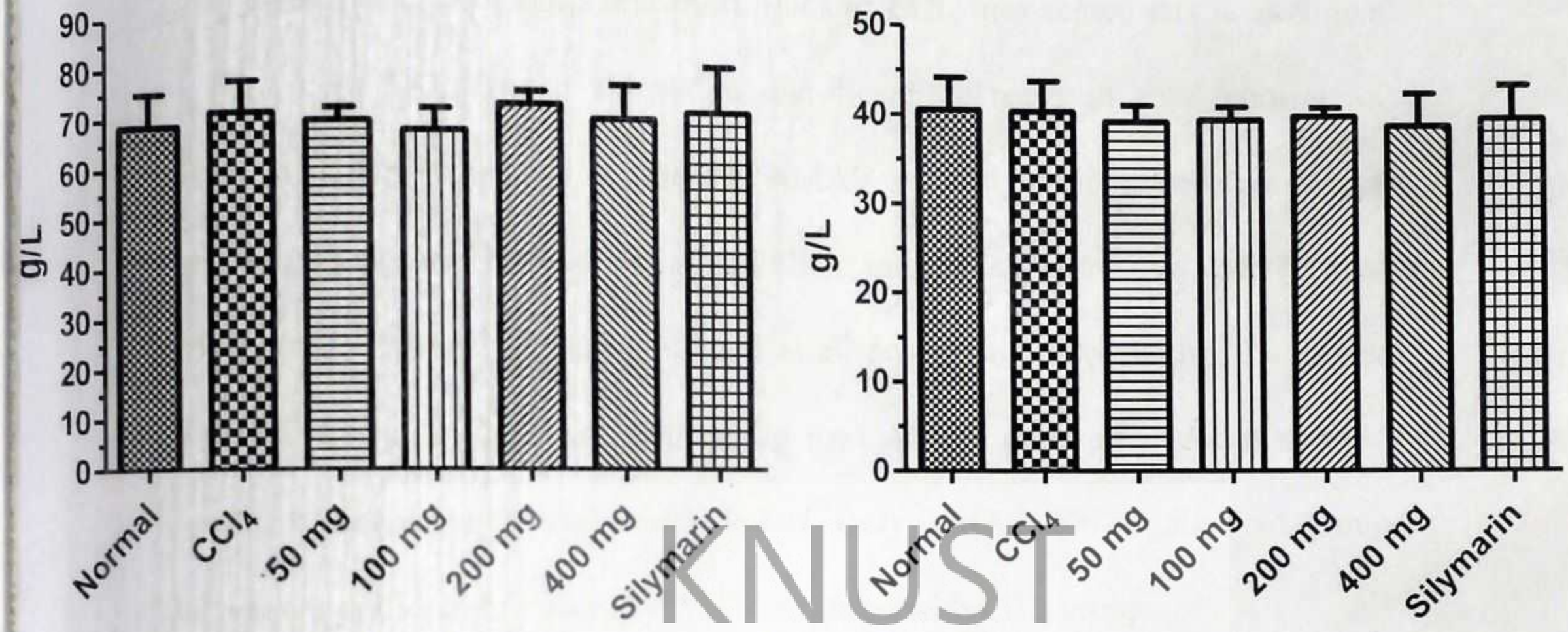


Fig. 4.17: Effect of AMAE on blood proteins of CCl_4-intoxicated rats.

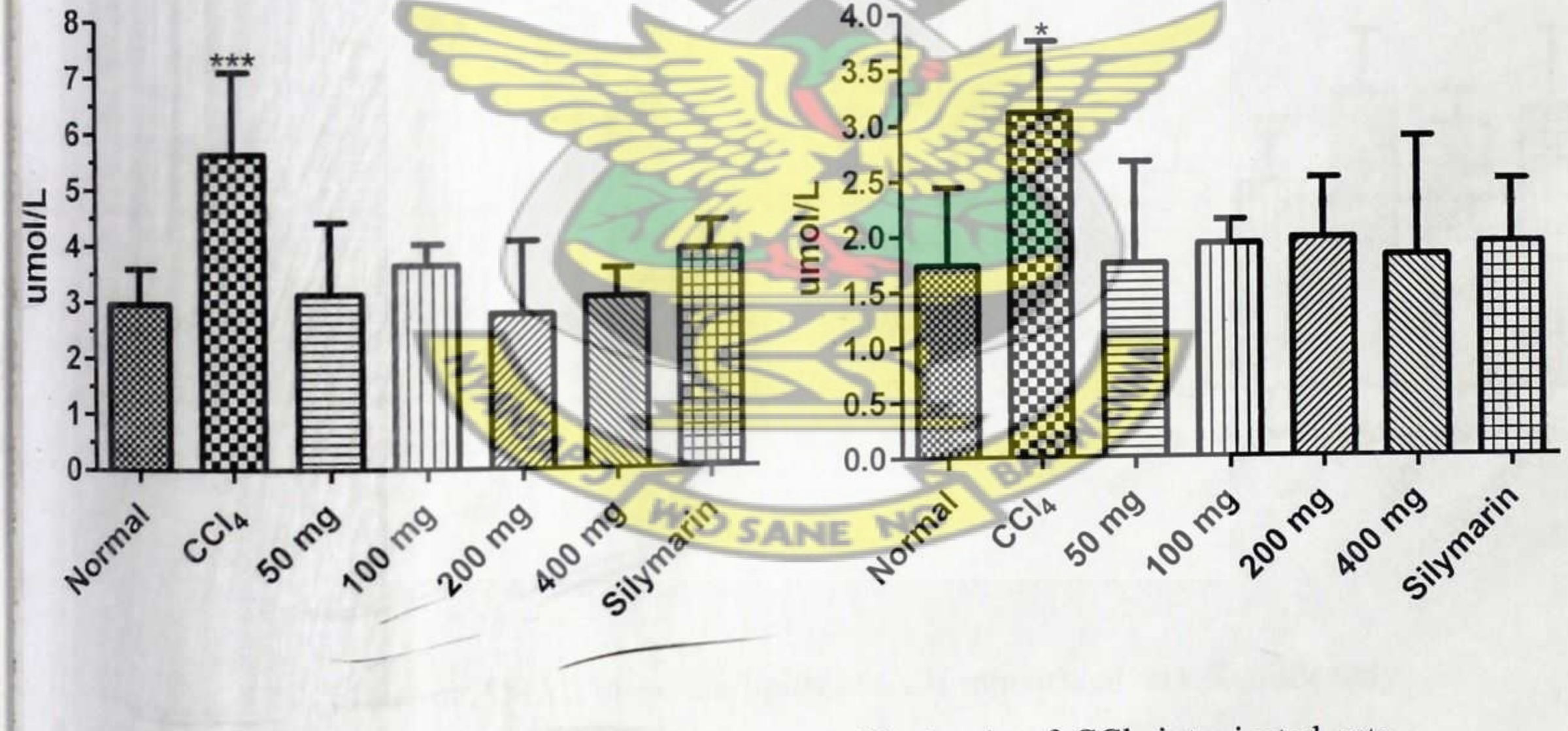


Fig. 4.18: Effect of AMAE on serum bilirubin levels of CCl_4-intoxicated rats.

Significantly different from Normal, * $p<0.05$, *** $p<0.001$

4.6.4 Effect of AMAE on serum lipid profile

The effect of AMAE on serum lipids of CCl_4-intoxicated rats is as shown in Fig. 4.19. CCl_4 group showed a significant decrease in total cholesterol ($p<0.01$) and triglycerides ($p<0.001$). AMAE and Silymarin pretreated groups showed non-significant changes in total cholesterol levels compared with Normal group but significant increase ($p<0.001$ at all doses) compared with CCl_4 group. However, 50 mg/kg b.wt and 100 mg/kg b.wt AMAE pretreated groups showed significant decreases in triglyceride levels. Only AMAE 200 mg/kg b.wt group showed a significant increase ($p<0.05$) compared with CCl_4 group.

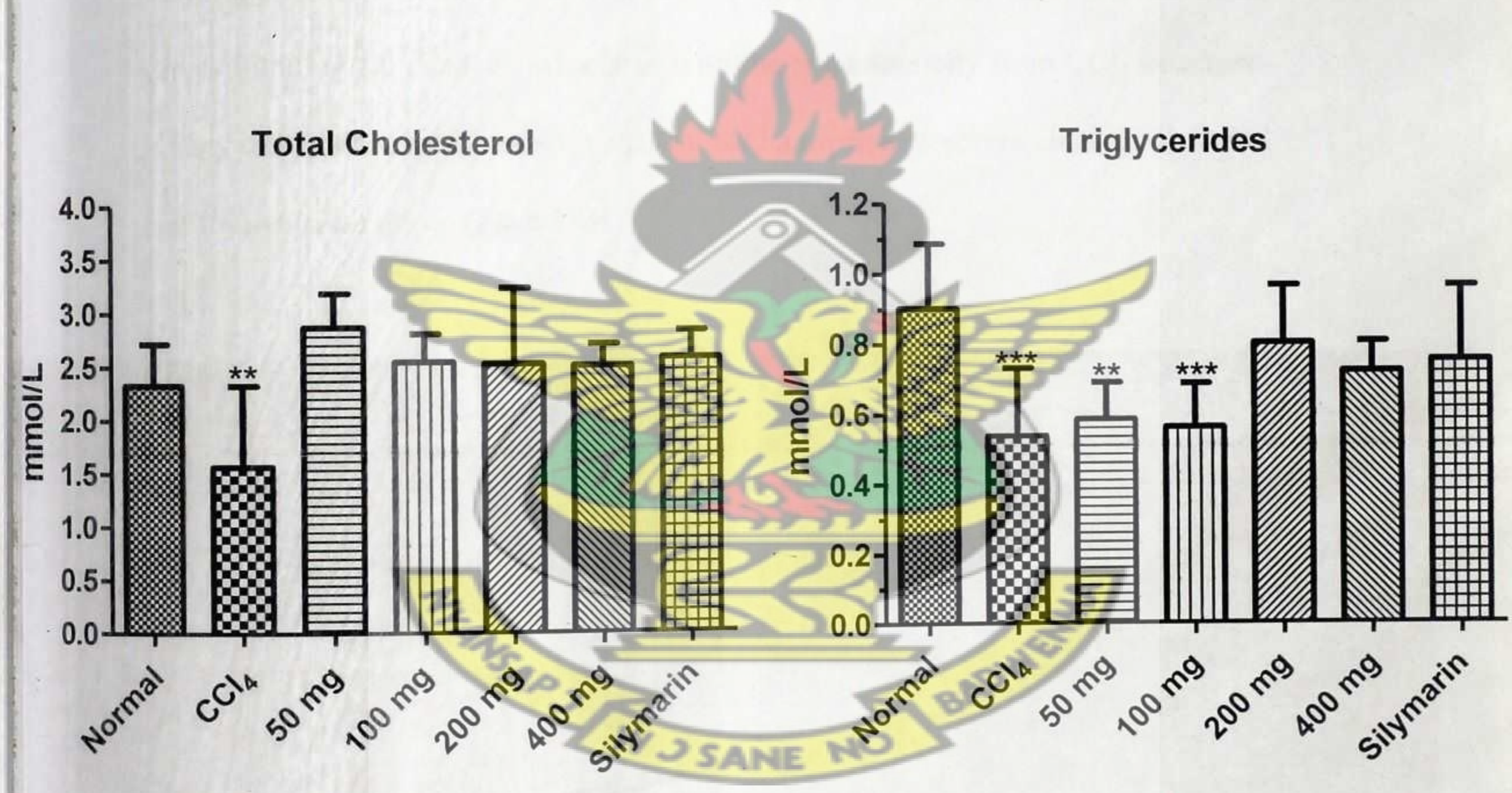


Fig. 4.19: Effect of AMAE on serum lipids of CCl_4-intoxicated rats. Significantly different from Normal, ** $p<0.01$, *** $p<0.001$

4.6.5 Effect of AMAE on liver histology

The effect of AMAE on liver histology of CCl_4-intoxicated rats is as shown in Plate 1 (A – G). Under the light microscope (100X), normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein were observed in the normal control group (Plate 1A). However, CCl_4 treatment exhibited severe histopathological changes, such as centrilobular hepatic necrosis, fatty change (arrow), ballooning degeneration, and infiltrating lymphocytes (Plate 1B). Pretreatment with 50 mg/kg b.wt, 100 mg/kg b.wt, 200 mg/kg b.wt and 400 mg/kg b.wt AMAE and 100 mg/kg b.wt Silymarin (Plate 1 C – G) prevented these histopathological changes associated with the hepatotoxicity from CCl_4 treatment. They exhibited a liver histology that showed a reverse of severe changes observed in CCl_4-treated group (Plate 1 B).

Plate 1A: Normal group (Rats received only distilled water)

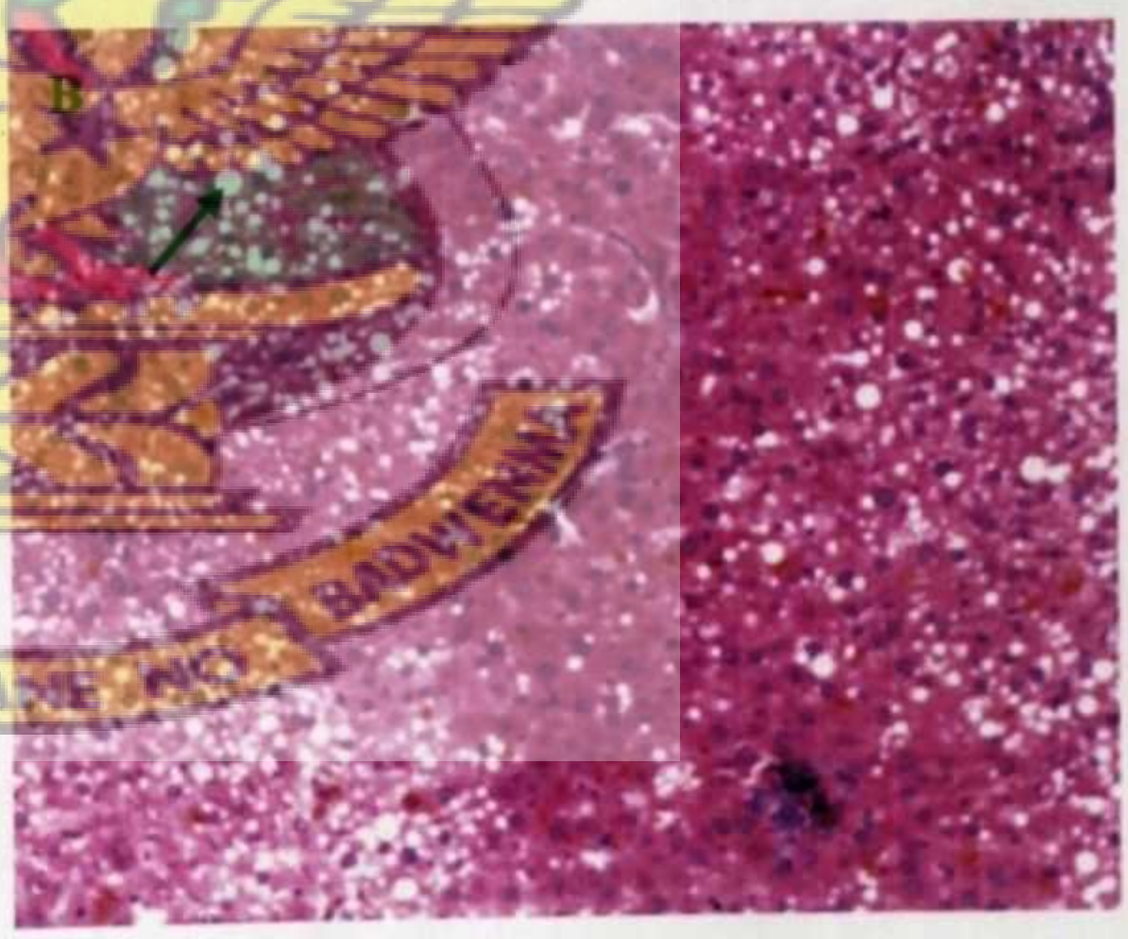


Plate 1B: CCl_4 group (Rats received 1 ml/kg CCl_4 only)

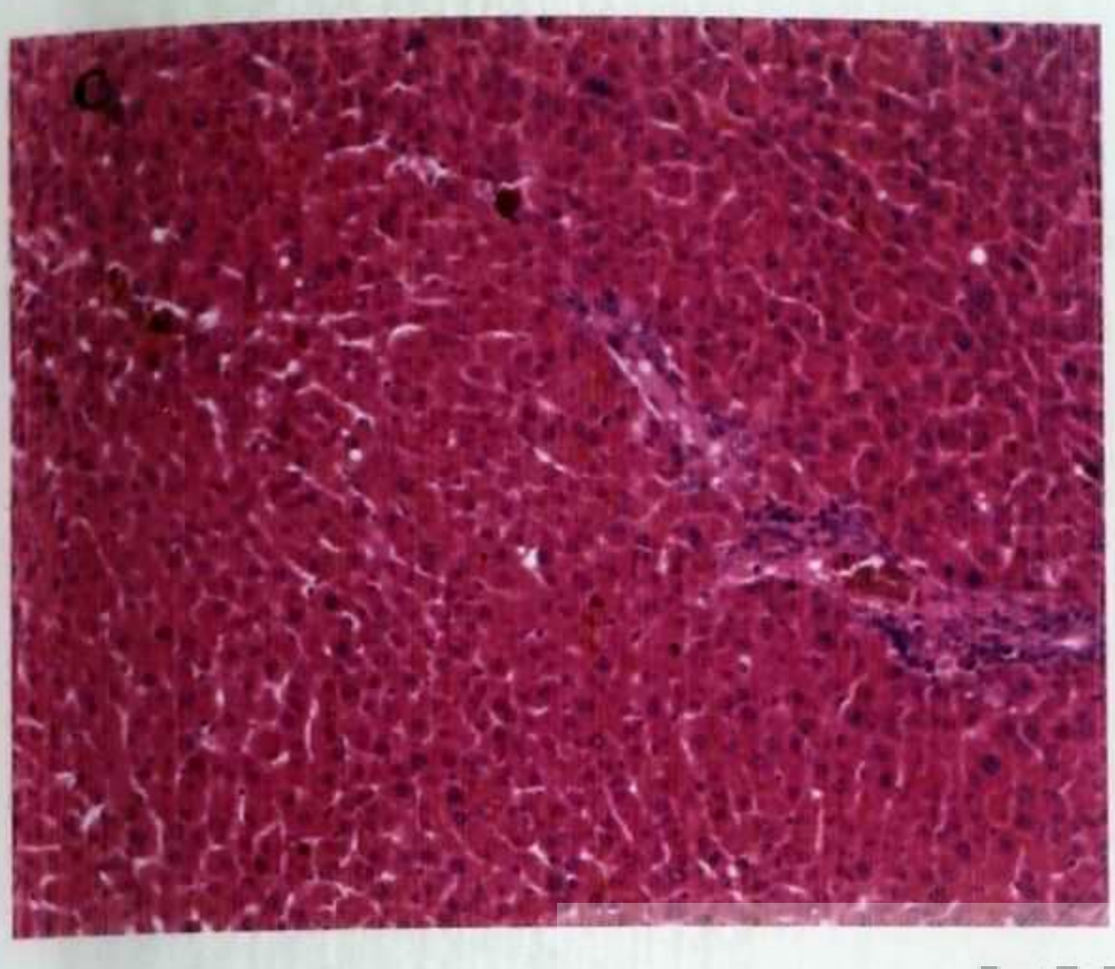


Plate 1C: AMAE 50 mg/kg b.wt group (Rats received 50 mg/kg b.wt AMAE followed by 1 ml/kg CCl_4)

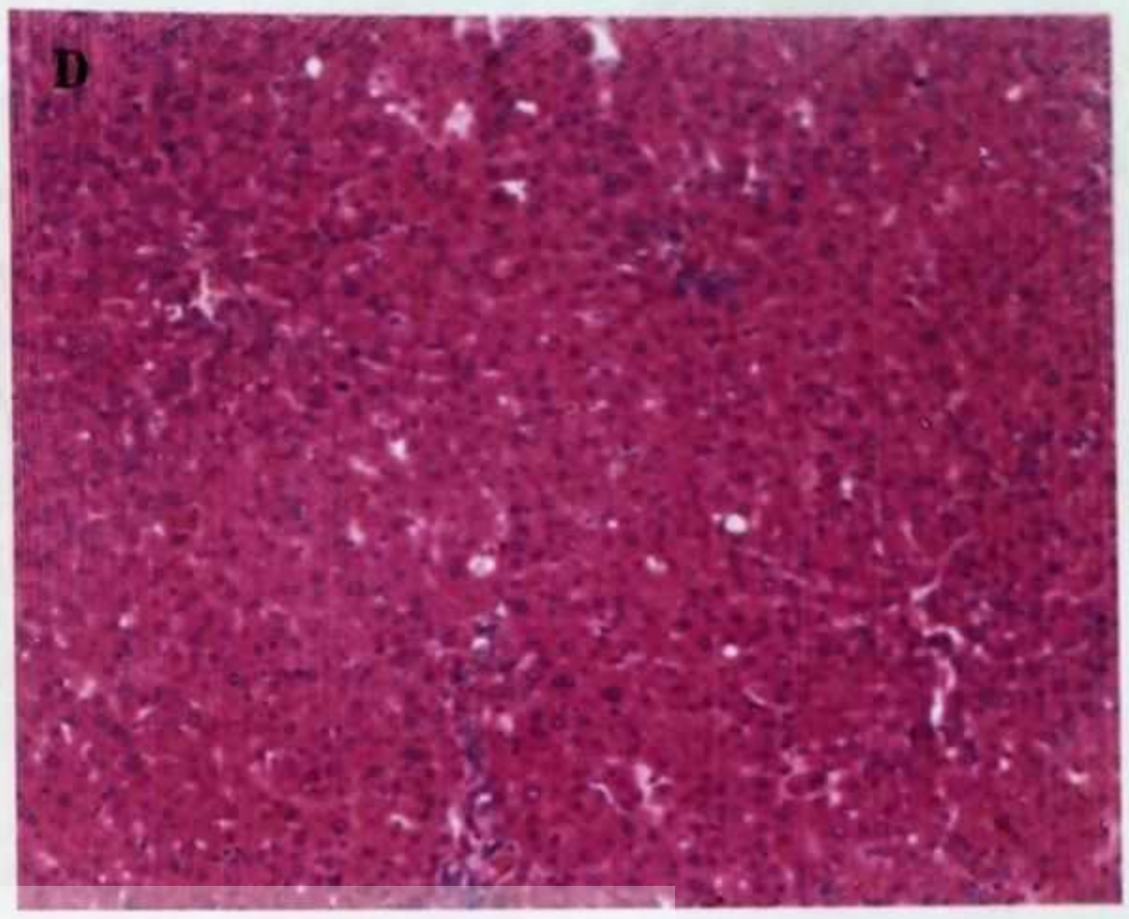


Plate 1D: AMAE 100 mg/kg b.wt group (Rats received 100 mg/kg b.wt AMAE followed by 1 ml/kg CCl_4)

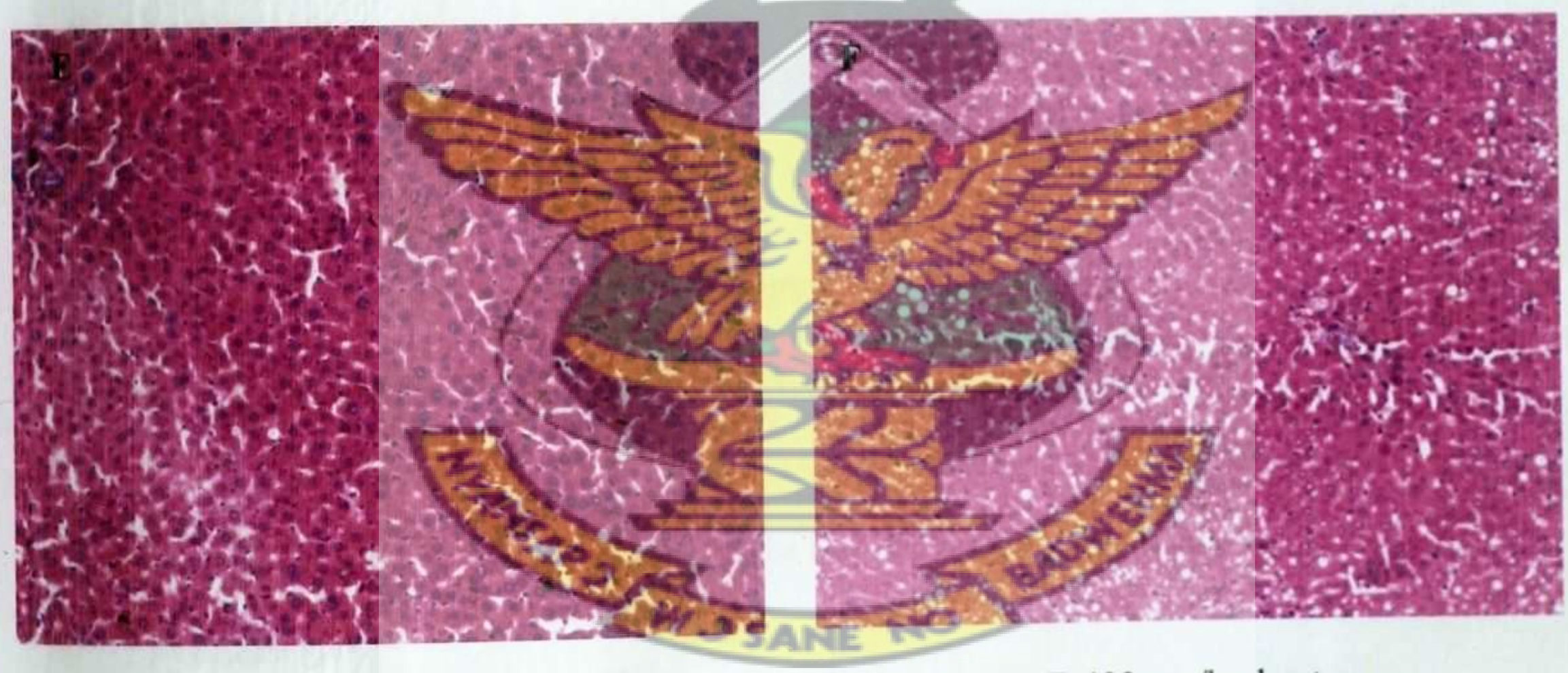


Plate 1E: AMAE 200 mg/kg b.wt group (Rats received 200 mg/kg b.wt AMAE followed by 1 ml/kg CCl_4)

Plate 1F: AMAE 400 mg/kg b.wt group (Rats received 400 mg/kg b.wt AMAE followed by 1 ml/kg CCl_4)

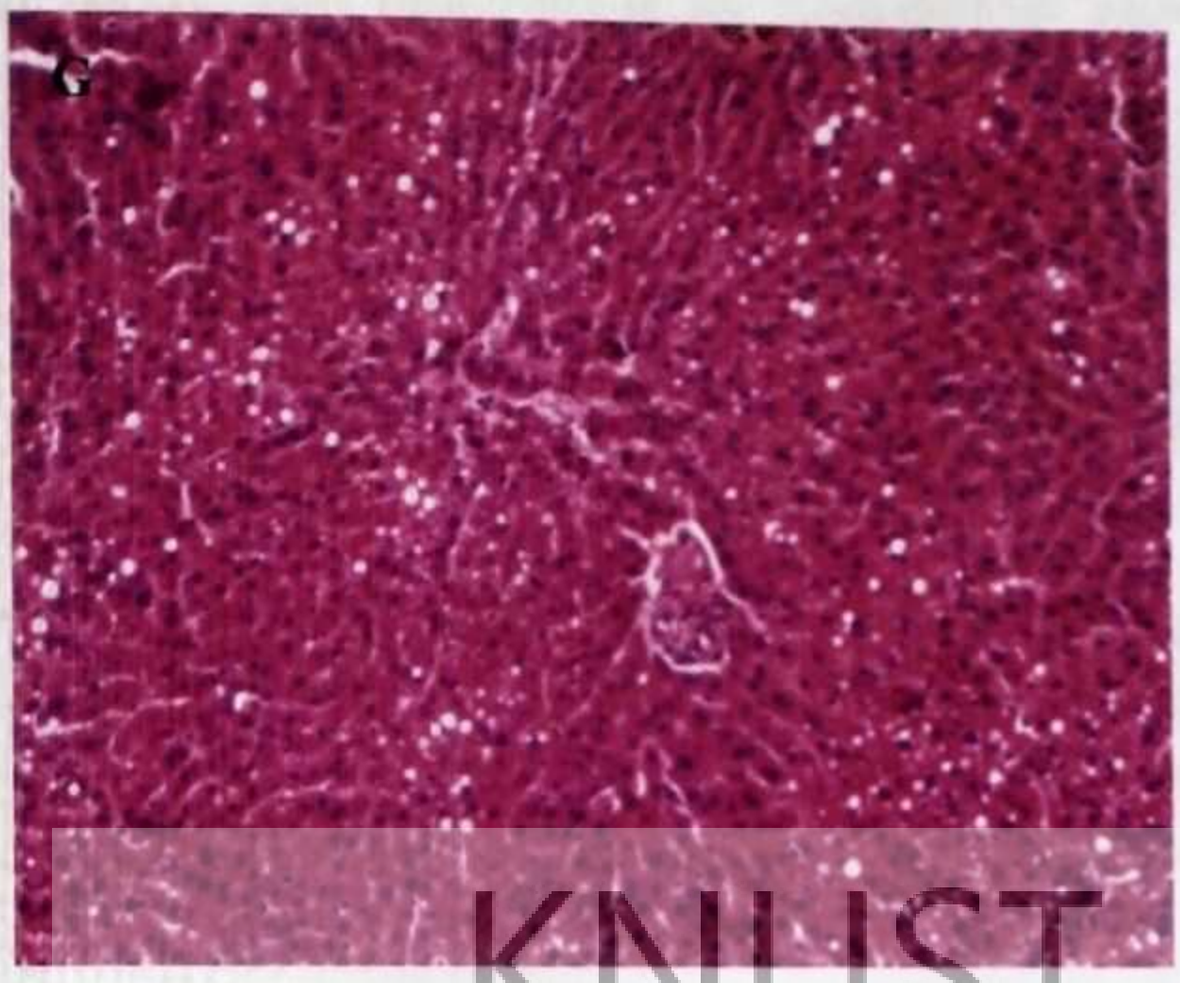


Plate 1G: Silymarin 100 mg/kg b.wt group (Rats received 100 mg/kg b.wt Silymarin followed by 1 ml/kg CCl_4)

Plate 1: Micrograph of the liver of carbon tetrachloride intoxicated rats [Magnification: X100]

4.7 HEPATOPROTECTIVE EFFECT OF AMAE AGAINST ACETAMINOPHEN-INDUCED LIVER DAMAGE

The study was carried out to assess the hepatoprotective effects of *Annona muricata* aqueous extract (AMAE) against acetaminophen (paracetamol)-induced liver damage.

4.7.1 Effect of AMAE on rat body weight

The effect of AMAE on daily percent change in body weight on paracetamol-intoxicated rats is as shown in Fig. 4.20. Varying weight changes were observed throughout the duration of the treatment. However, at termination 100 mg/kg b.wt, 200 mg/kg b.wt and 400 mg/kg b.wt AMAE showed better

protection from weight loss while 50 mg/kg b.wt AMAE showed the least protection.

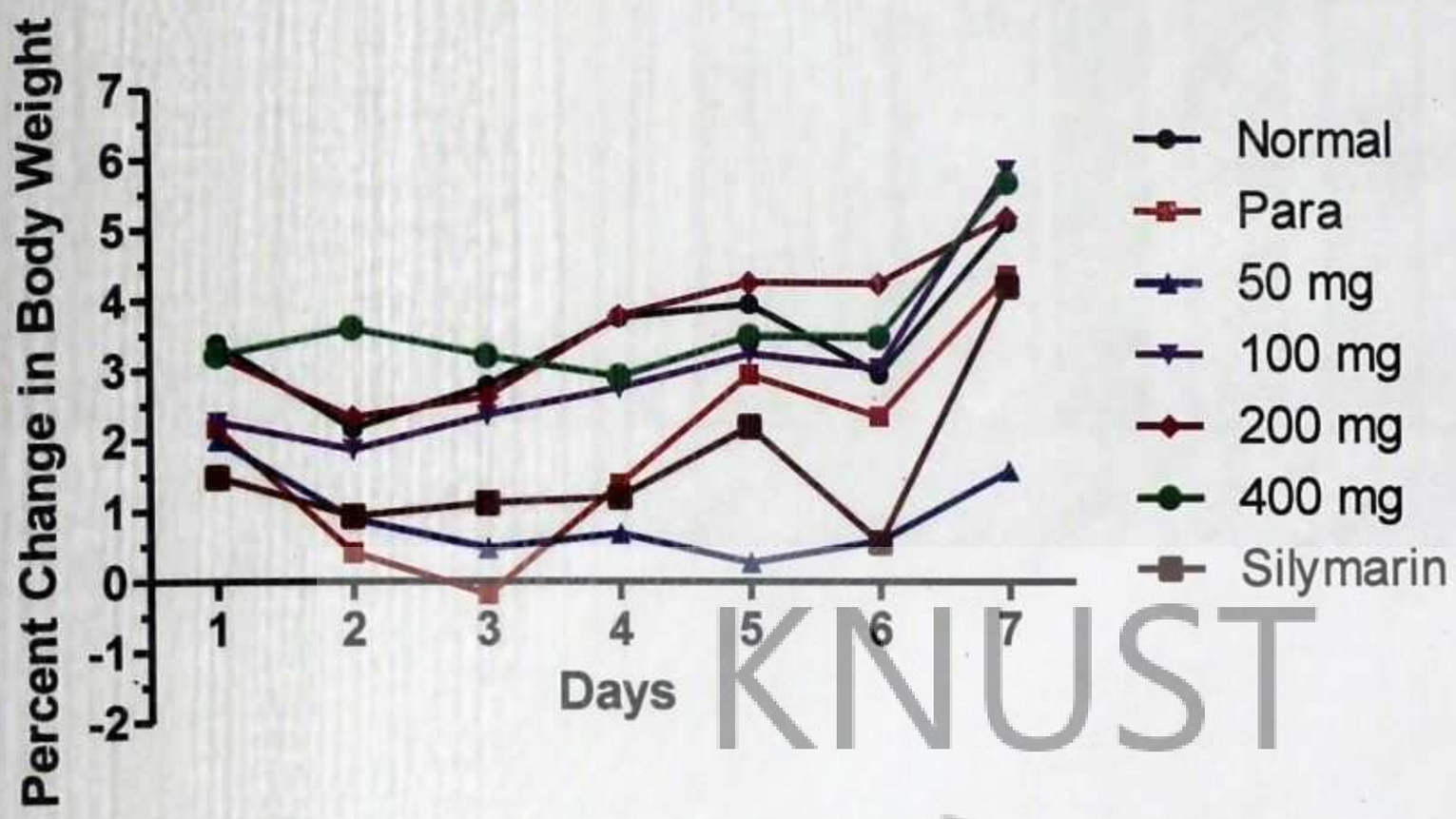


Fig. 4.20: Effect of AMAE on percentage change in body weight in paracetamol-intoxicated rats.

4.7.2 Effect of AMAE on absolute and relative liver weight

The effect of AMAE on absolute and relative liver weights of paracetamol-intoxicated rats is as shown in Fig. 4.21. Paracetamol induced a significant increase in relative liver weight of rats ($p<0.05$). AMAE at 100 mg/kg (76.92%), 200 mg/kg b.wt (56.41%) and 400 mg/kg (46.15%) and Silymarin (94.87%) protected the liver from being hypertrophied as a result of paracetamol intoxication. The 100 mg/kg b.wt AMAE showed the best protection amongst extract treatments with a gradual non-significant decrease in protection with increasing dosage.

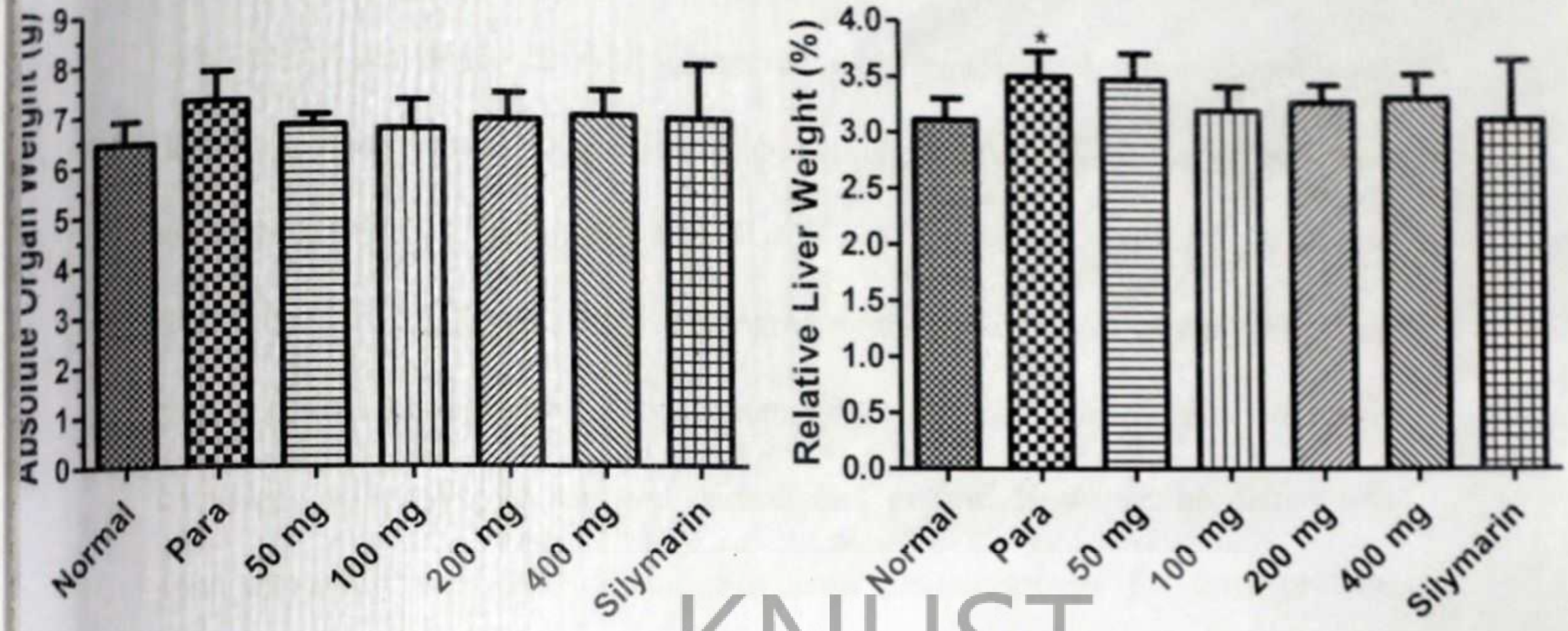


Fig. 4.21: Effect of AMAE on absolute and relative liver weights of acetaminophen-intoxicated rats. Significantly different from Normal, * $p<0.05$

4.7.3 Effect of AMAE on some liver function profile

The effect of AMAE on liver function indices of paracetamol-intoxicated rats is as shown in Fig. 4.22, 4.23 and 4.24. Paracetamol-intoxication showed no increase in serum protein and albumin levels and there were no significant differences in these determinations for both AMAE groups and Silymarin group compared with Normal group. Paracetamol-intoxication, however, resulted in significant increase in ALP and ALT levels ($p<0.001$) and total and indirect bilirubin levels ($p<0.01$) compared with normal group. AMAE at all doses and Silymarin showed significant decreases ($p<0.001$) in ALT levels compared with Paracetamol group with a non-significant dose-dependent effect in AMAE treatments. However, AMAE at 100 mg/kg b.wt and 200 mg/kg b.wt ($p<0.001$) and 400 mg/kg b.wt and Silymarin ($p<0.05$) showed significant increases in ALP levels compared with the Normal group. Also 50 mg/kg b.wt and 400 mg/kg b.wt

AMAE and Silymarin showed significant decreases ($p<0.001$) and 200 mg/kg b.wt AMAE ($p<0.01$) in ALP compared with paracetamol group. Significant decreases were observed in total bilirubin levels for AMAE 50 mg/kg b.wt and 200 mg/kg b.wt ($p<0.001$) and AMAE 100 mg/kg b.wt and 400 mg/kg b.wt and Silymarin ($p<0.01$) treatments compared with paracetamol group. All drug treatments further resulted in reductions ($p<0.001$) in Indirect bilirubin levels compared with both normal and paracetamol groups. However, no differences were observed with AMAE and Silymarin treated groups for total protein, albumin, and total and indirect bilirubin levels. Significant differences were observed in ALT levels between 50 mg/kg b.wt AMAE and 200 mg/kg b.wt AMAE, 400 mg/kg b.wt AMAE and 100 mg/kg b.wt Silymarin.

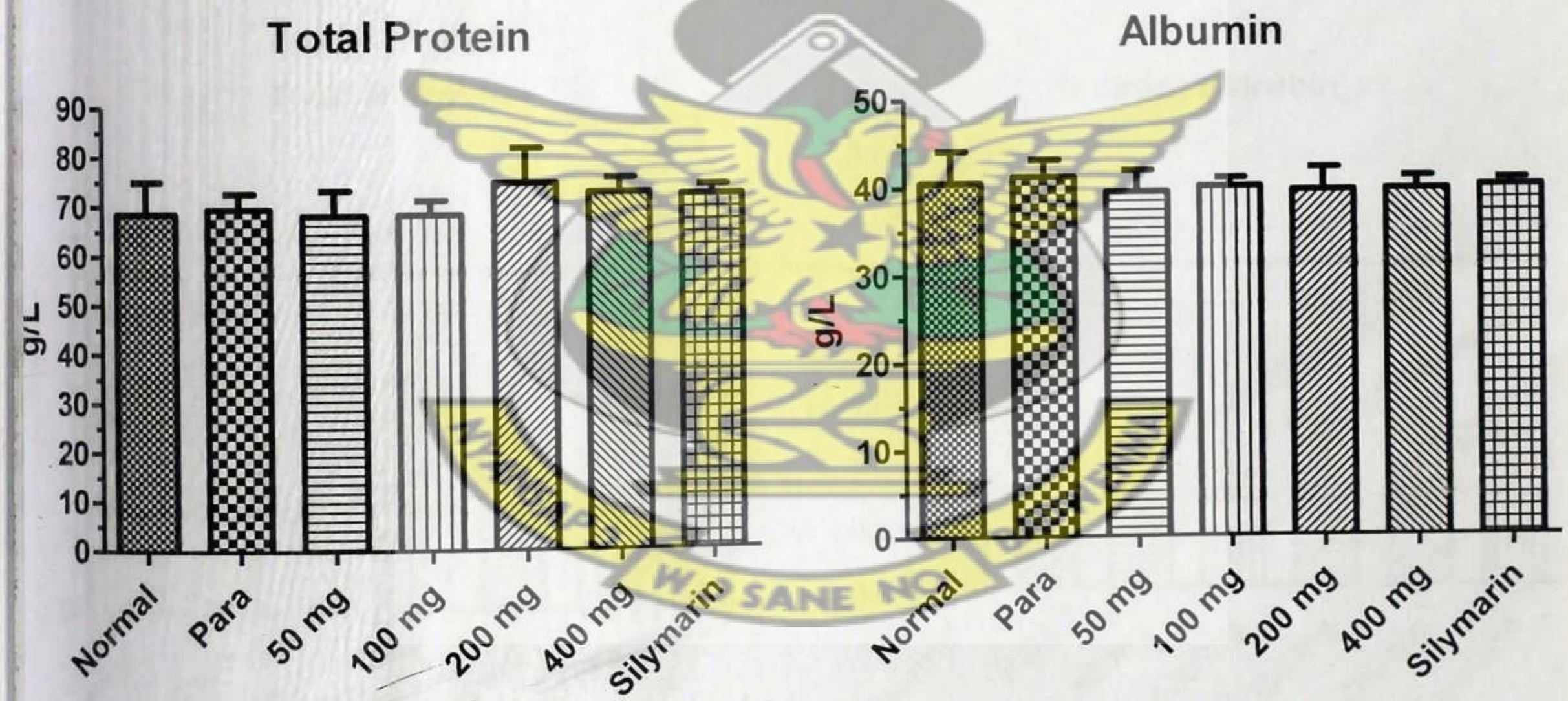


Fig. 4.22: Effect of AMAE on serum protein concentration of acetaminophen-intoxicated rats.

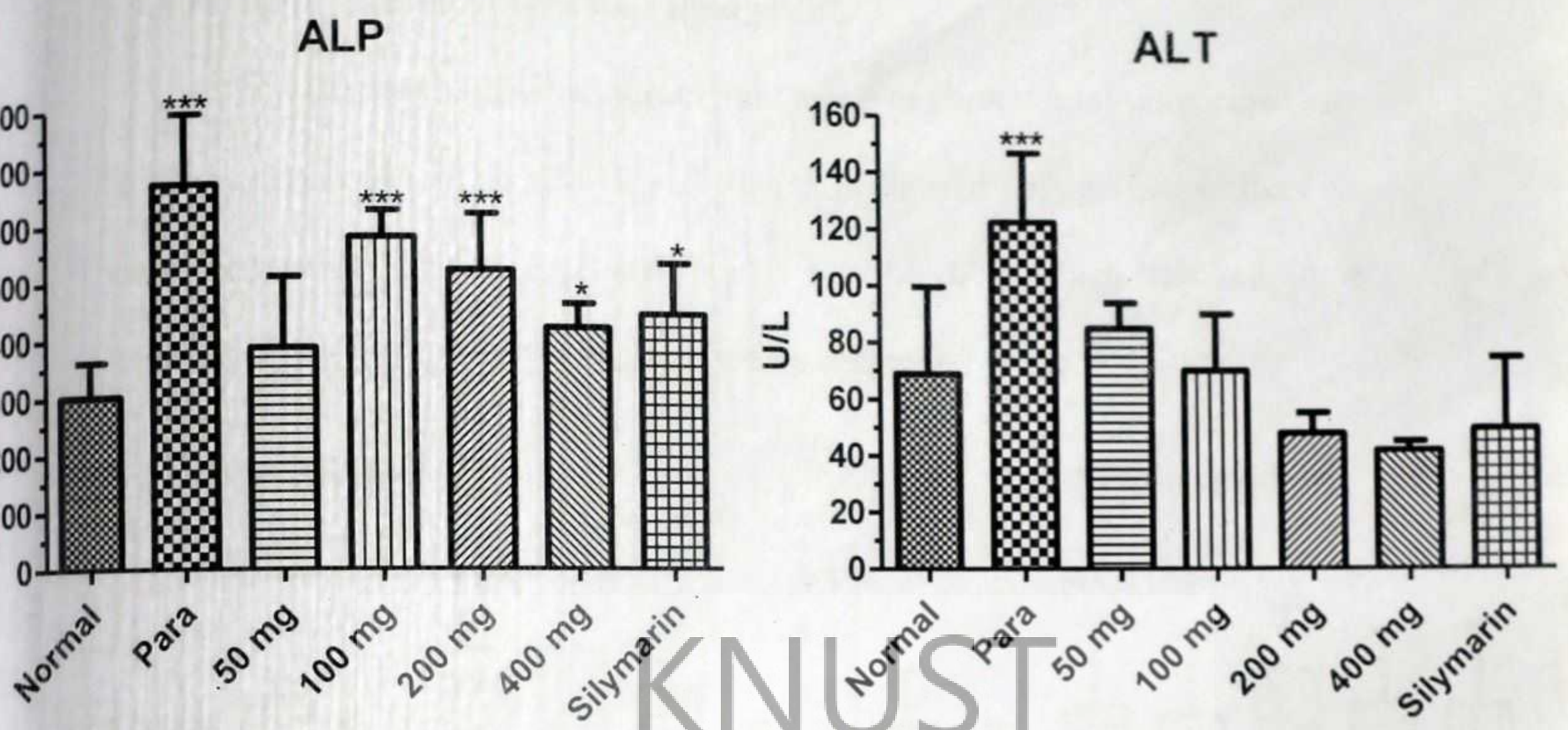


Fig. 4.23: Effect of AMAE on serum liver enzymes of acetaminophen-intoxicated rats. Significantly different from Normal, * $p<0.05$, *** $p<0.001$

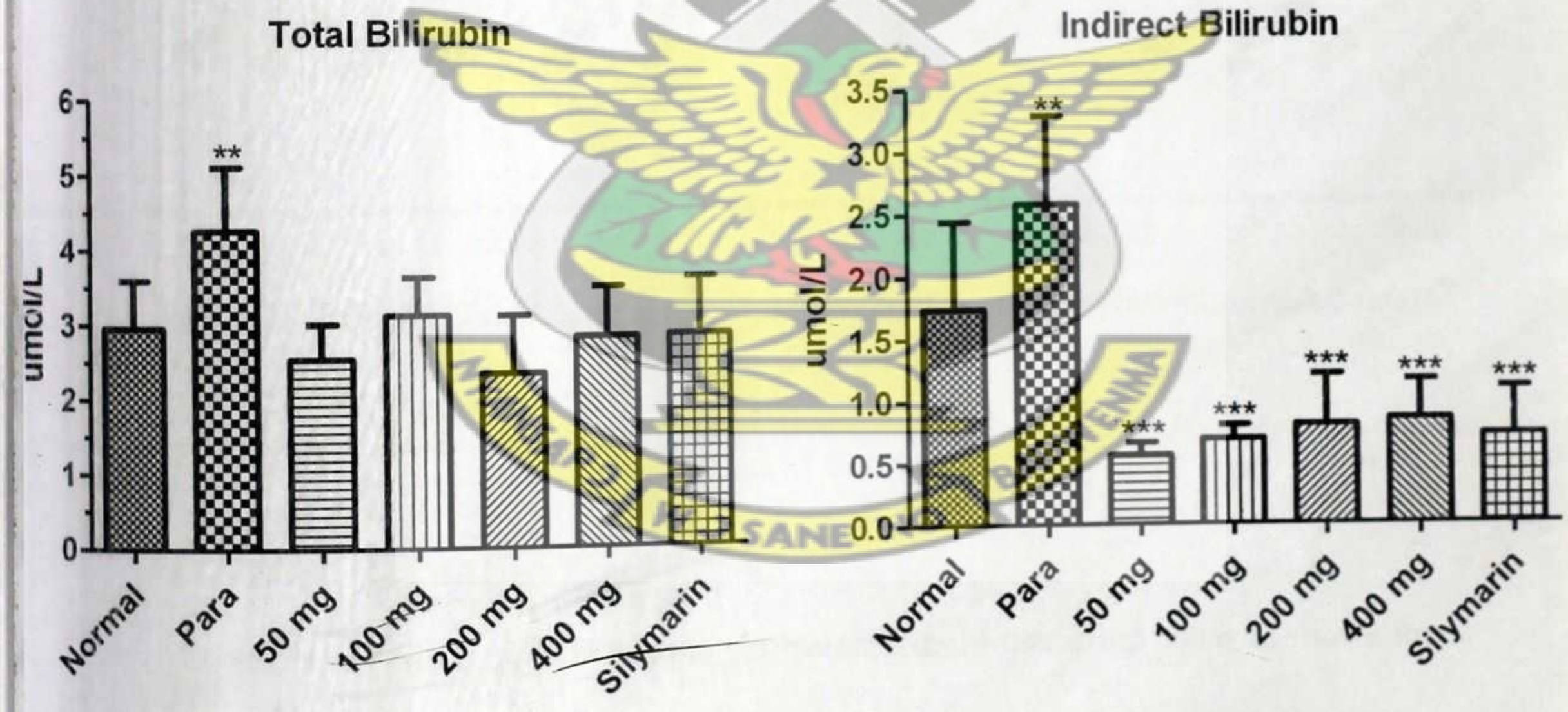


Fig. 4.24: Effect of AMAE on serum bilirubin levels of acetaminophen-intoxicated rats. Significantly different from Normal, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

4.7.4 Effect of treatment on serum lipid profile

The effects of AMAE on serum lipid levels of paracetamol-intoxicated rats are as shown in Fig. 4.25. AMAE and Silymarin showed no significant effect on total cholesterol level except 400 mg/kg b.wt AMAE which resulted in a significant increase ($p<0.05$) compared with paracetamol group.

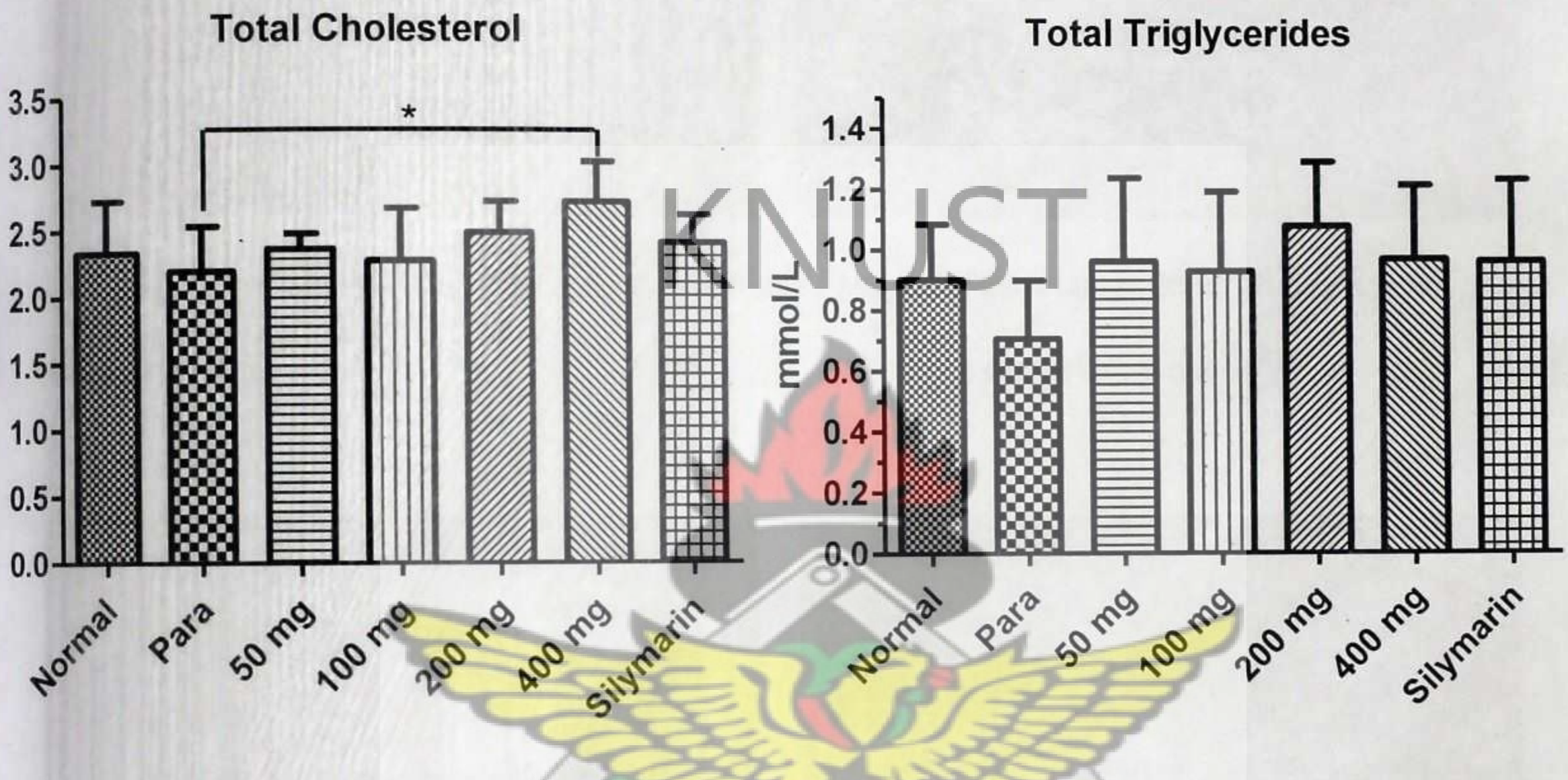


Fig. 4.25: Effect of AMAE on serum lipid levels of acetaminophen-intoxicated rats.

*$p<0.05$

4.7.5 Effect of AMAE on liver histology

The effect of AMAE on liver histology of paracetamol-intoxicated rats is as shown in Plate 2 (A – G). Paracetamol showed severe disruption in normal microarchitecture of liver cells (B). AMAE (C – F) and Silymarin (G) showed mild changes with near normal liver cells. AMAE 400 showed a better reversal of degeneration compared with paracetamol group.

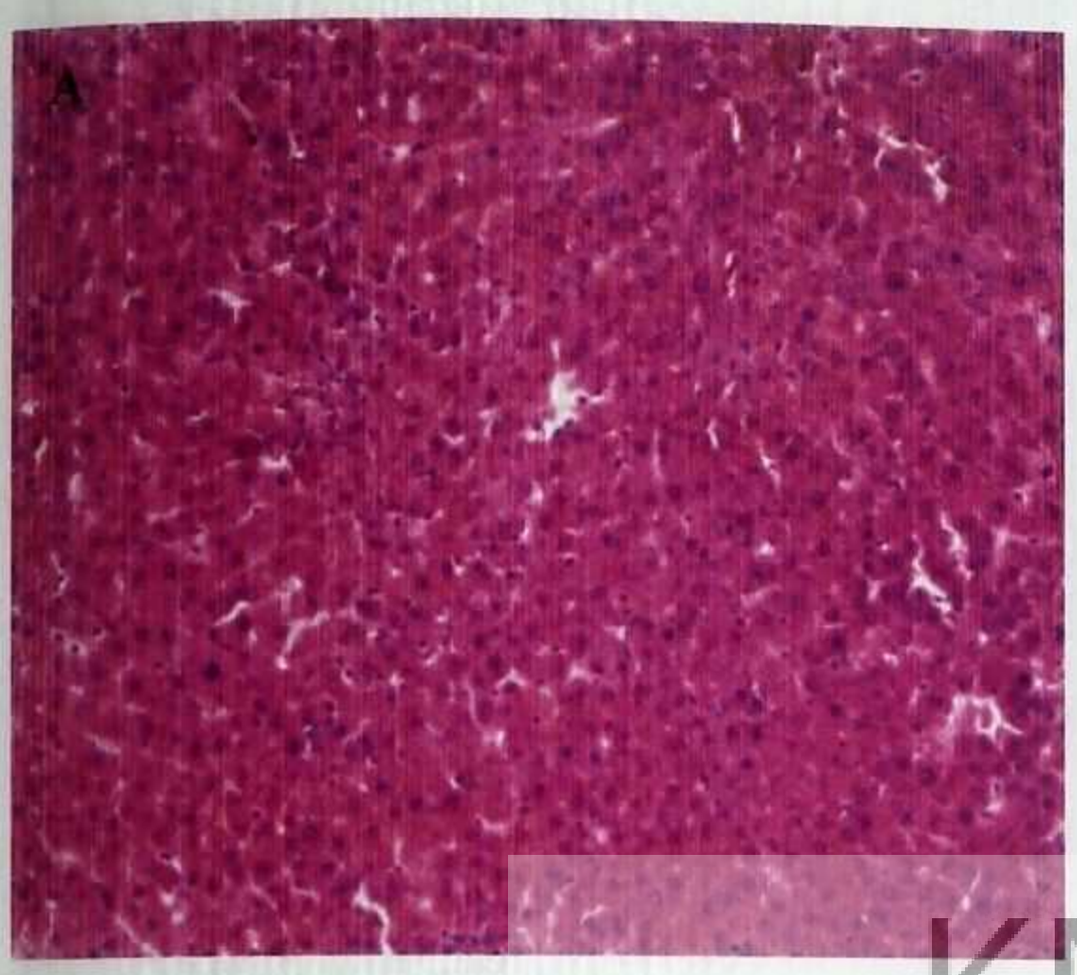


Plate 2A: Normal group (Rats received only distilled water)

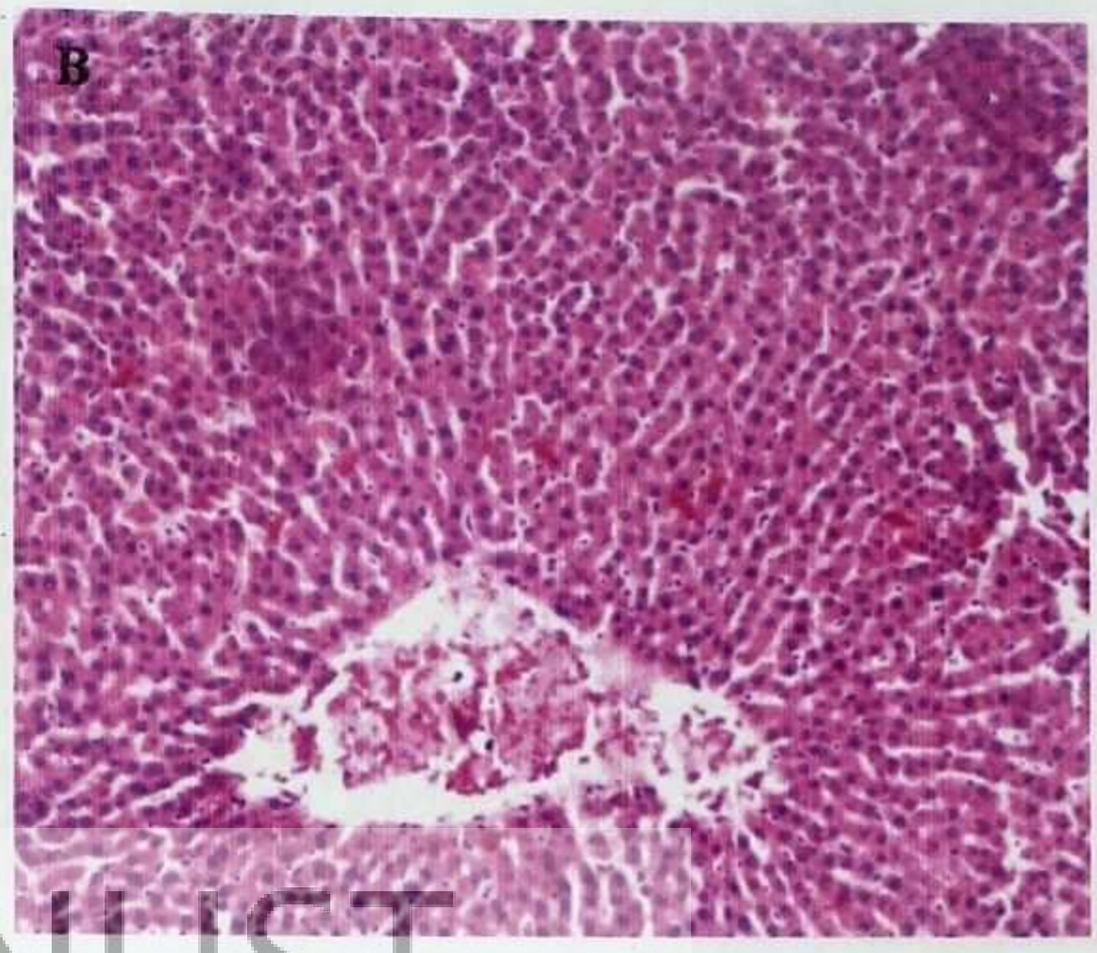


Plate 2B: Paracetamol group (Rats received 500 mg/kg paracetamol only)

Plate 2C: AMAE 50 mg/kg b.wt group (Rats received 50 mg/kg b.wt AMAE followed by 500 mg/kg paracetamol)

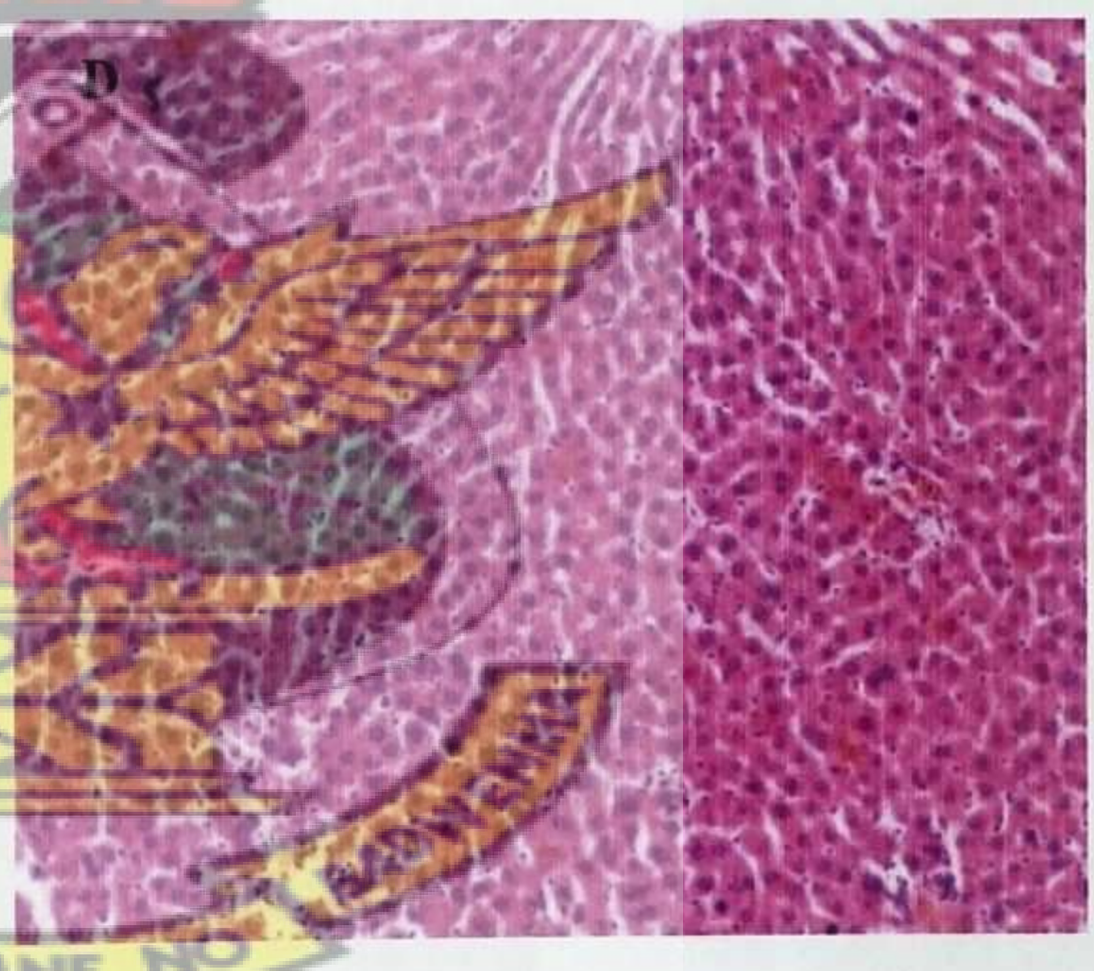


Plate 2D: AMAE 100 mg/kg b.wt group (Rats received 100 mg/kg b.wt AMAE followed by 500 mg/kg paracetamol)

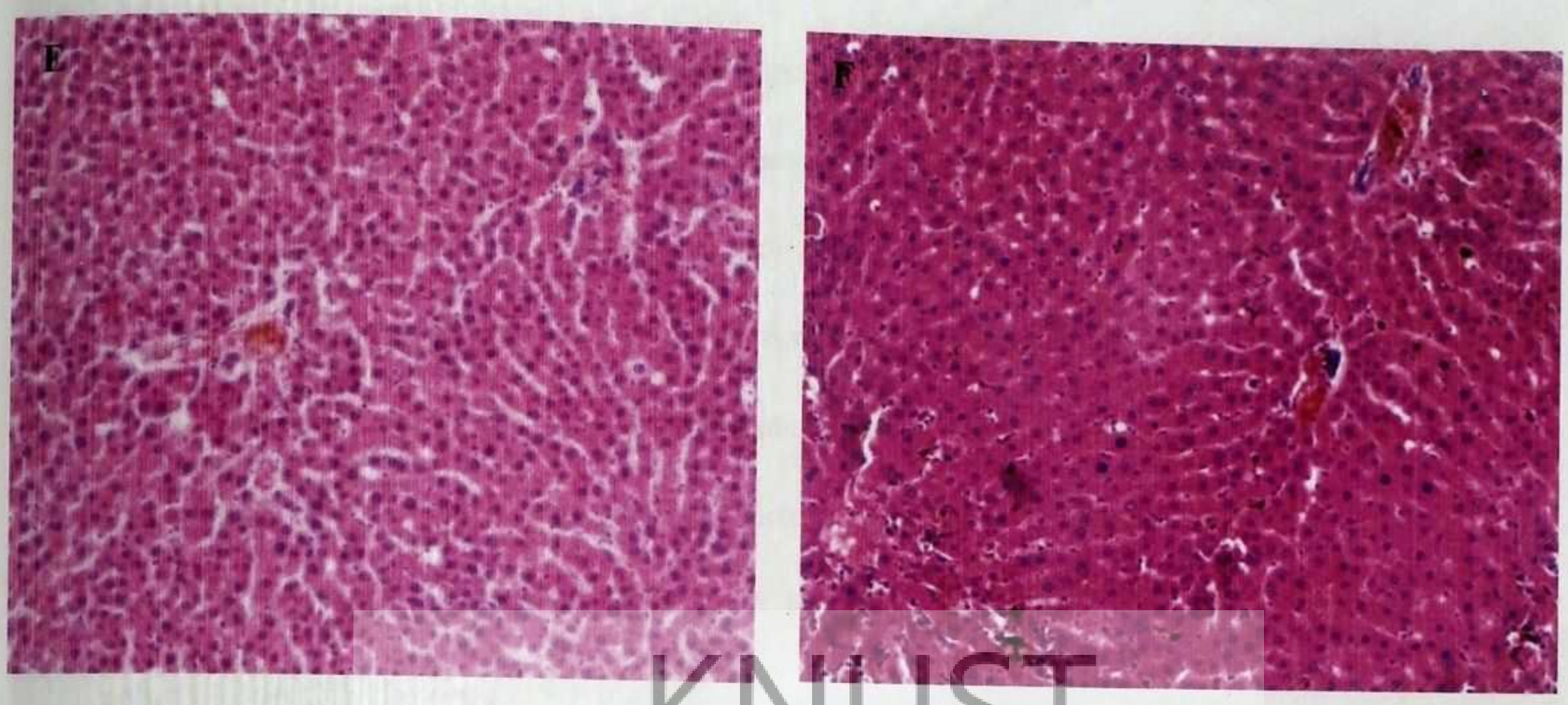


Plate 2E: AMAE 200 mg/kg b.wt group (Rats received 200 mg/kg b.wt AMAE followed by 500 mg/kg paracetamol)

Plate 2F: AMAE 400 mg/kg b.wt group (Rats received 400 mg/kg b.wt AMAE followed by 500 mg/kg paracetamol)

Plate 2G: Silymarin 100 mg/kg b.wt group (Rats received 10 mg/kg b.wt Silymarin followed by 500 mg/kg paracetamol)

Plate 2: Micrograph of the liver of paracetamol intoxicated rats [Magnification: X100]

4.8 Comparative analysis of hepatoprotective effect of AMAE against carbon tetrachloride- and paracetamol (acetaminophen)-induced liver damage

Fig. 4.26 shows the comparative analysis of hepatoprotective effect of AMAE at 50 mg/kg b.wt, 100 mg/kg b.wt, 200 mg/kg b.wt and 400 mg/kg b.wt and 100 mg/kg b.wt Silymarin against CCl_4- and paracetamol-induced liver damage. AMAE 400 mg/kg b.wt offered better protection against paracetamol while AMAE 50 mg/kg b.wt offered better protection against CCl_4 which compared well with standard hepatoprotective flavonoid, Silymarin.

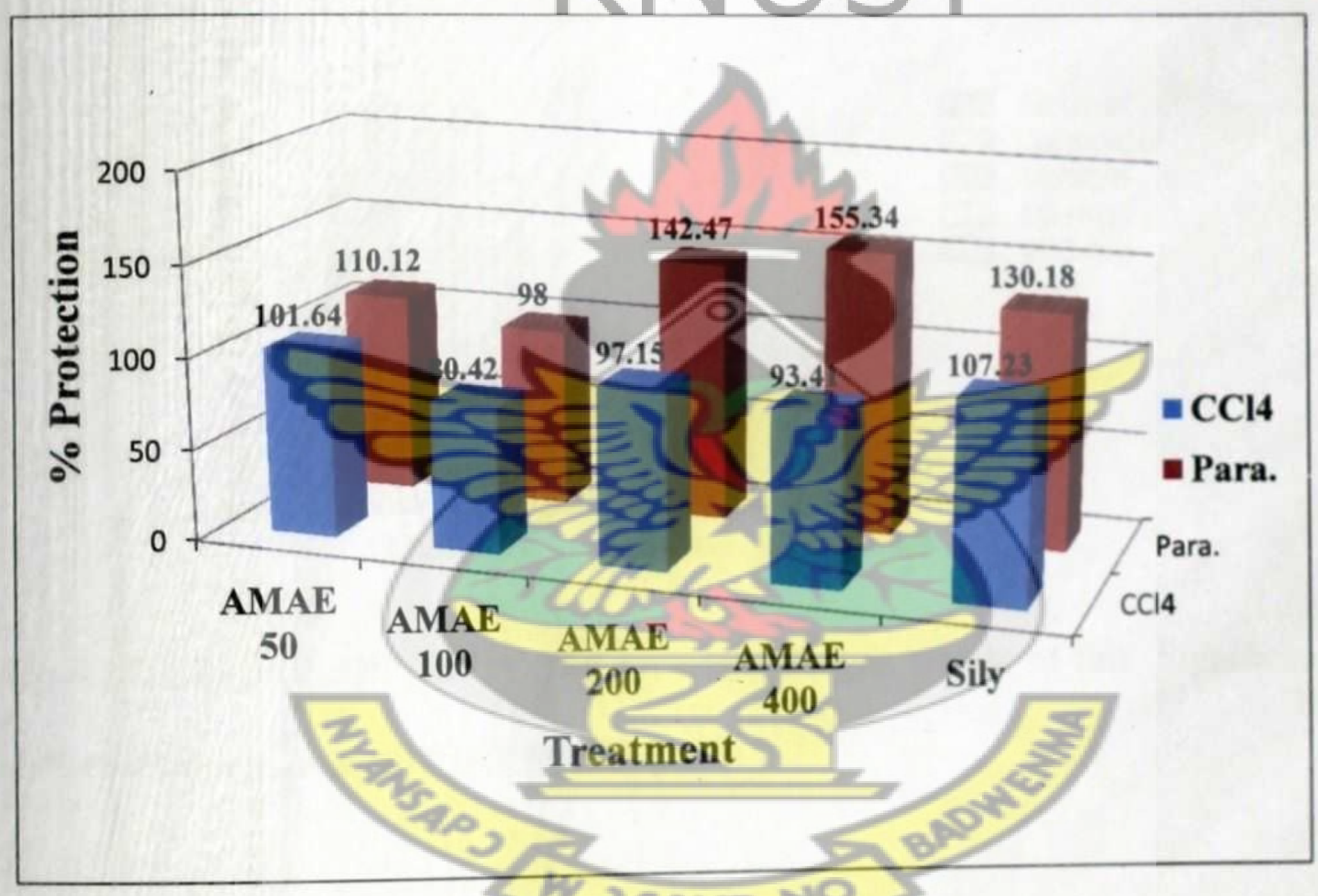


Fig. 4.26: Comparative analysis of hepatoprotective effect of AMAE and Silymarin against CCl_4- and Paracetamol-intoxicated rats

4.9 BILIRUBIN-LOWERING POTENTIAL OF *ANNONA MURICATA* IN TEMPORARY JAUNDICED ADULT RATS

4.9.1 Curative effect of *A. muricata* against PHZ-induced jaundice in rats

4.9.1.1 Effect of treatment on relative organ weights

The effect of phenylhydrazine (PHZ) and AMAE on relative organ weights of animals are as shown in Fig. 4.27. There were no significant changes in the weights of the liver and heart of animals. There were however, significant increases ($p<0.001$) in spleen weight in PHZ-treated animals.

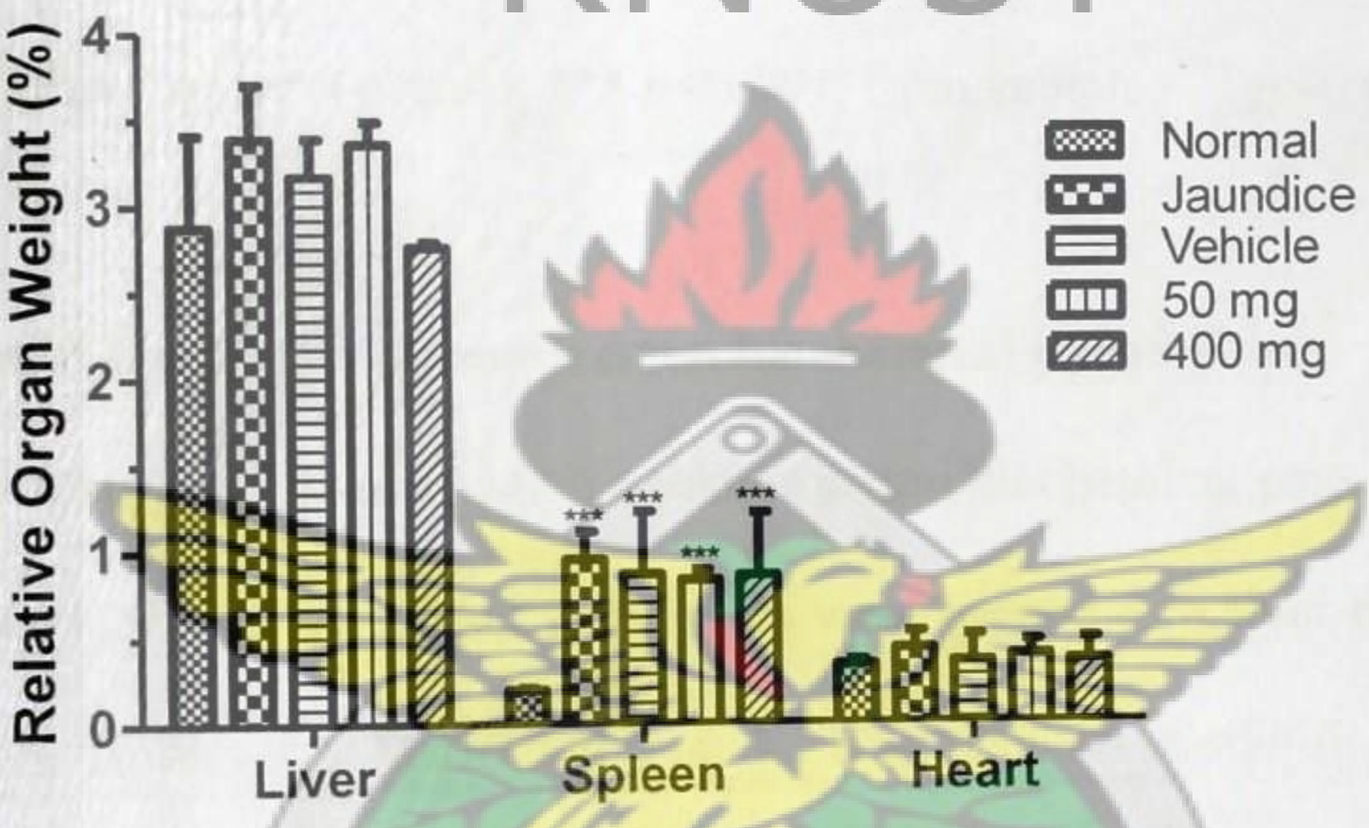


Fig.4.27: Effect of PHZ and AMAE on relative organ weight of rats. Significantly different from normal, *** $p<0.001$

4.9.1.2 Effect of treatment on some haematological parameters

The effects of PHZ and AMAE on some haematological indices of animals are as shown in Fig. 4.28. The red blood cell count and haemoglobin levels showed significant decrease in all animals treated with PHZ ($p<0.001$). Further decreases were observed in haematocrit percent following PHZ treatment. These values were, however, observed to improve with time and AMAE treatment.

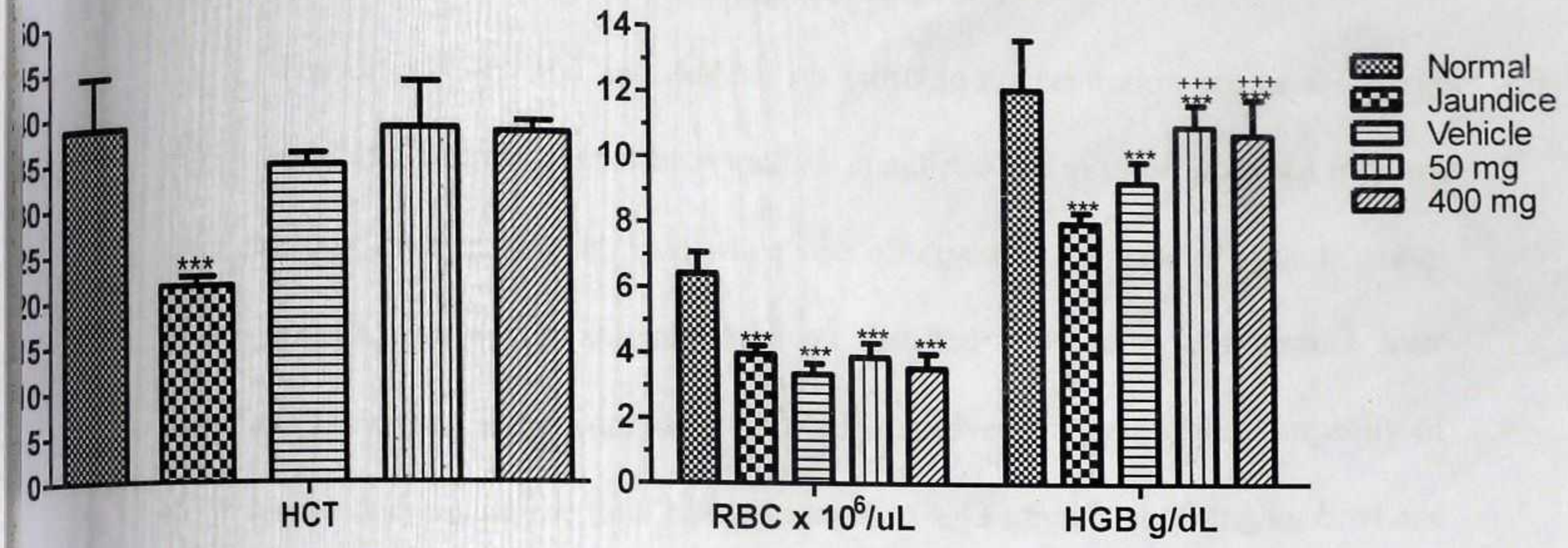


Fig.4.28: Effect of PHZ and AMAE on some haematological parameters of rats. Significantly different from normal, *** $p<0.001$; from vehicle. $^{+++}$ $p<0.001$

4.9.1.3 Effect of treatment on some serum biochemical parameters

The effects of PHZ and AMAE on some serum biochemical parameters are as shown in Fig. 4.29. No significant differences were observed in liver enzymes and albumin levels. However, AMAE at 400 mg/kg b.wt resulted in significant decrease ($p<0.001$) in ALP from both normal and vehicle groups.

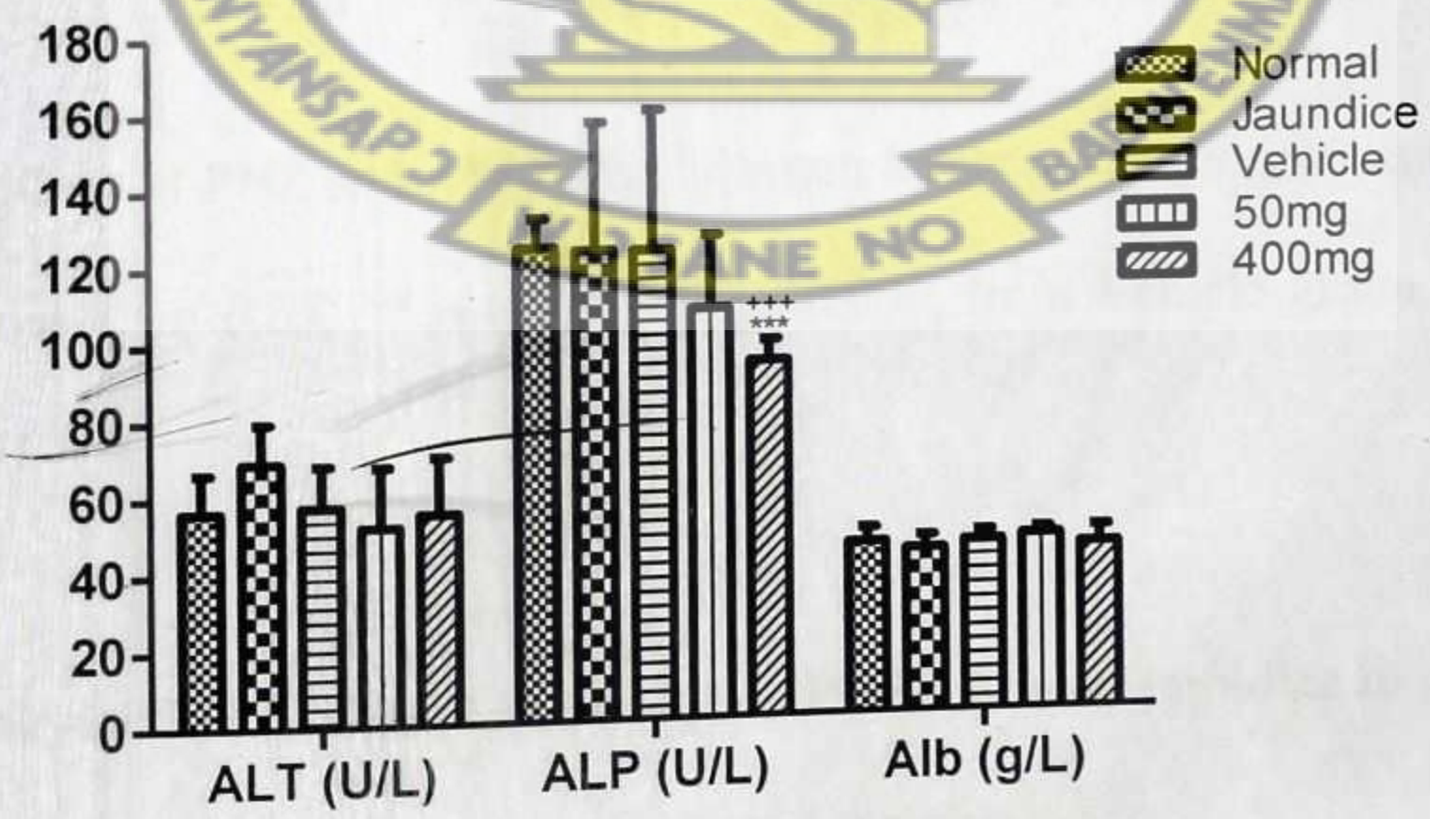


Fig.4.29: Effect of PHZ and AMAE on some serum biochemical parameters. Significantly different from normal, *** $p<0.001$; from normal. $^{+++}$ $p<0.001$

4.9.1.4 Effect of treatment on bilirubin concentration

The effects of PHZ and AMAE on bilirubin concentration are as shown in Fig. 4.30. Total bilirubin level increased in jaundiced group (9.68 μmol/L) 6 hours after the last PHZ treatment. This value was observed to decrease in vehicle group (7.43 μmol/L) in which animals received distilled water and had normal liver capacity. However, administration of AMAE improved the conjugating capacity of the liver by decreasing the total bilirubin level to 4.43 μmol/L at 50 mg/kg b.wt and 3.33 μmol/L at 400 mg/kg b.wt ($p<0.01$ from vehicle group). However, no differences were observed between the AMAE treated groups. Similar trends were observed for direct and indirect bilirubin levels.

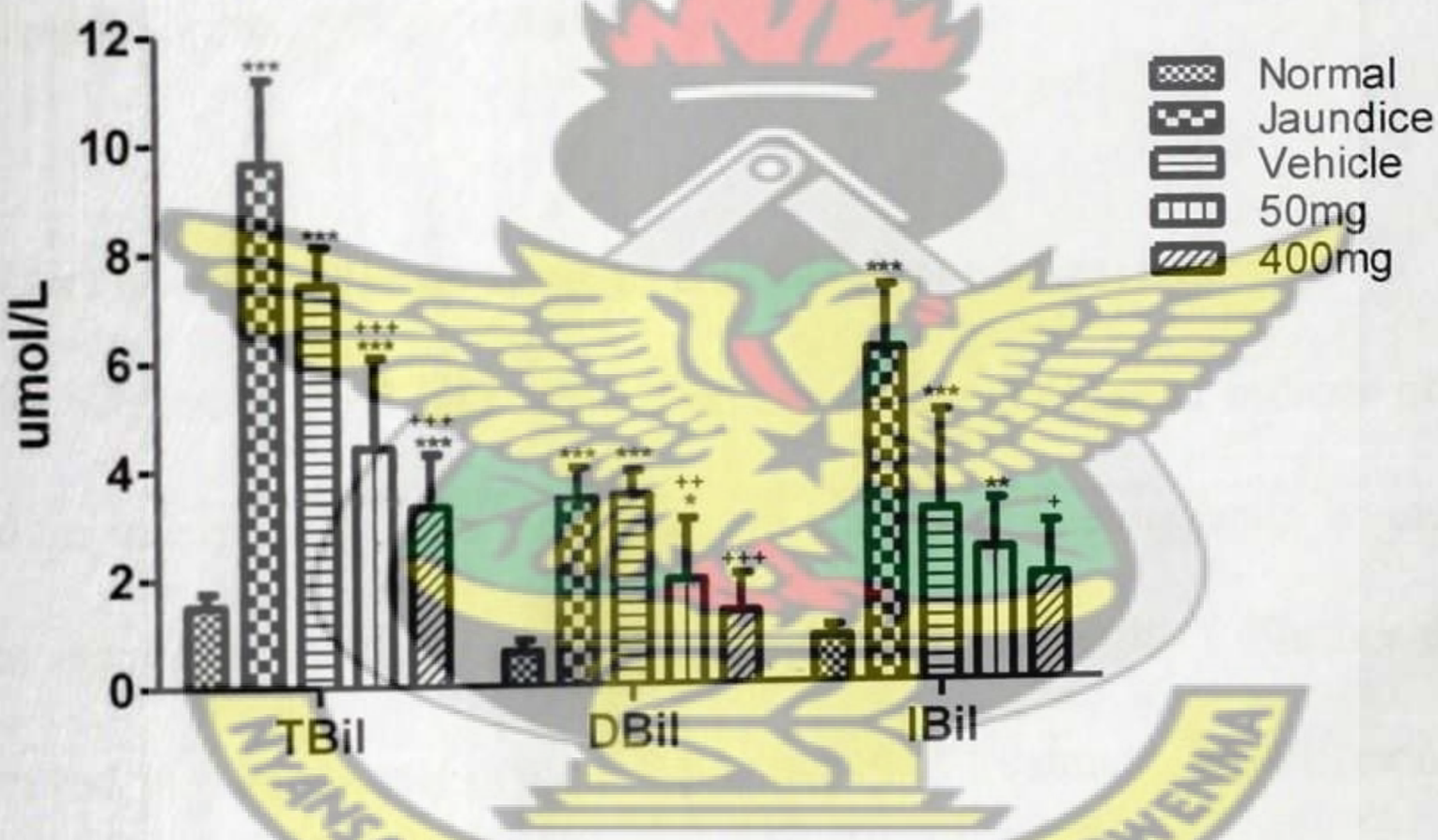


Fig.4.30: Effect of PHZ and AMAE on bilirubin levels of rats. Significantly different from Normal, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; from Vehicle group, $^{++}$ $p<0.01$, $^{+++}$ $p<0.001$

4.9.2 Prophylactic Effect of *A. muricata* on PHZ-induced jaundice in rats

4.9.2.1 Effect of treatment on relative organ weights

The effects of PHZ and AMAE on relative organ weights of rats are as shown in Fig. 4.31. There were no significant changes in the weights of the liver and heart

of animals. There were however, significant increases ($p<0.001$) in spleen weight in all PHZ treated animals.

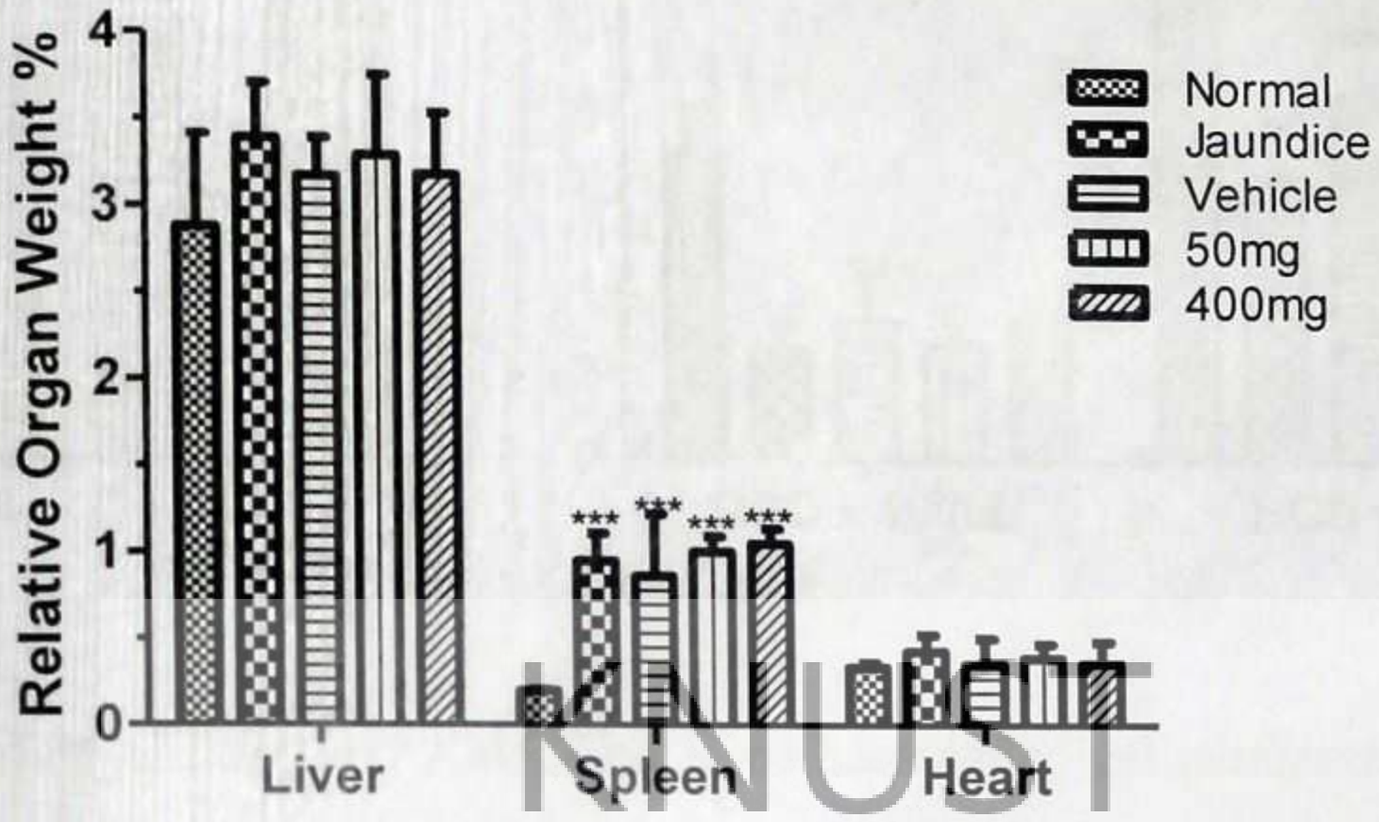


Fig.4.31: Effect of PHZ and AMAE on relative organ weight of rats. Significantly different from Normal, *** $p<0.001$

4.9.2.2 Effect of treatment on some haematological parameters

The effects of PHZ and AMAE on some haematological indices of animals are as shown in Fig. 4.32. The red blood cell count and haemoglobin levels showed significant decreases in all animals treated with PHZ ($p<0.001$). Further decreases were observed in haematocrit percent following PHZ treatment. No differences were observed among the AMAE and vehicle groups.

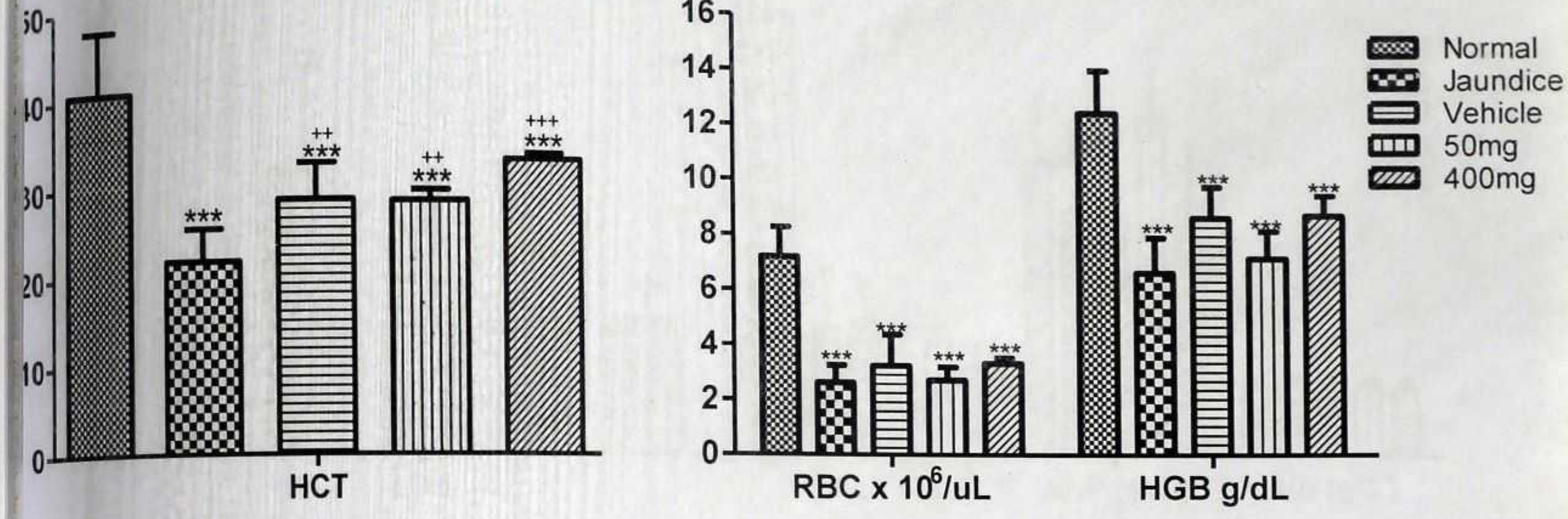


Fig.4.32: Effect of PHZ and AMAE on some haematological parameters of rats. Significantly different from normal, ** $p<0.01$, *** $p<0.001$; from Jaundice group, $^{++}p<0.01$, $^{+++}p<0.001$

4.9.2.3 Effect of treatment on some serum biochemical parameters

The effects of PHZ and AMAE on some serum biochemical parameters are as shown in Fig. 4.33. No significant differences in ALT and albumin levels were observed. However, decreases were observed in ALP levels ($p<0.05$) in jaundiced and vehicle groups. ALP level was also significantly increased in AMAE treated groups ($p<0.001$). LDH levels were significantly increased in jaundice group ($p<0.01$ from normal). However, no increases were observed for the other treatment.

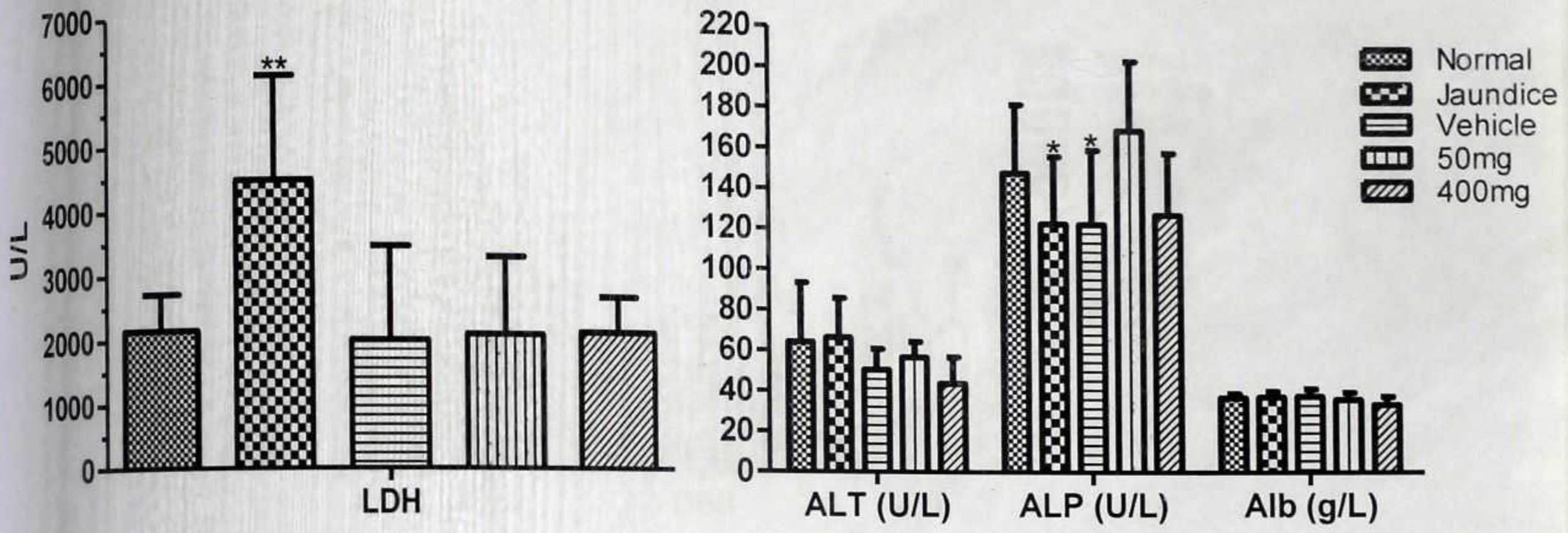


Fig.4.33: Effect of PHZ and AMAE on some serum biochemical parameters. Significantly different from Normal, $*p<0.05$, $**p<0.01$

4.9.2.4 Effect of treatment on bilirubin concentration

The effects of PHZ and AMAE on bilirubin concentration are as shown in Fig 4.34. Total bilirubin levels increased in jaundice groups (9.68 μmol/L) 6 hours after the last PHZ treatment. This value was observed to decrease in vehicle group (7.08 μmol/L) significantly different from jaundice group ($p<0.001$). However, administration of AMAE improved the conjugating capacity of the liver to reduce bilirubin concentration to 6.08 μmol/L at 50 mg/kg b.wt (no difference compared with vehicle but different from jaundice group, $p<0.001$) and 4.93 μmol/L at 400 mg/kg b.wt ($p<0.001$ from vehicle and jaundice group). No difference was observed within AMAE treated groups. Similar treads were also observed for direct and indirect bilirubin levels.

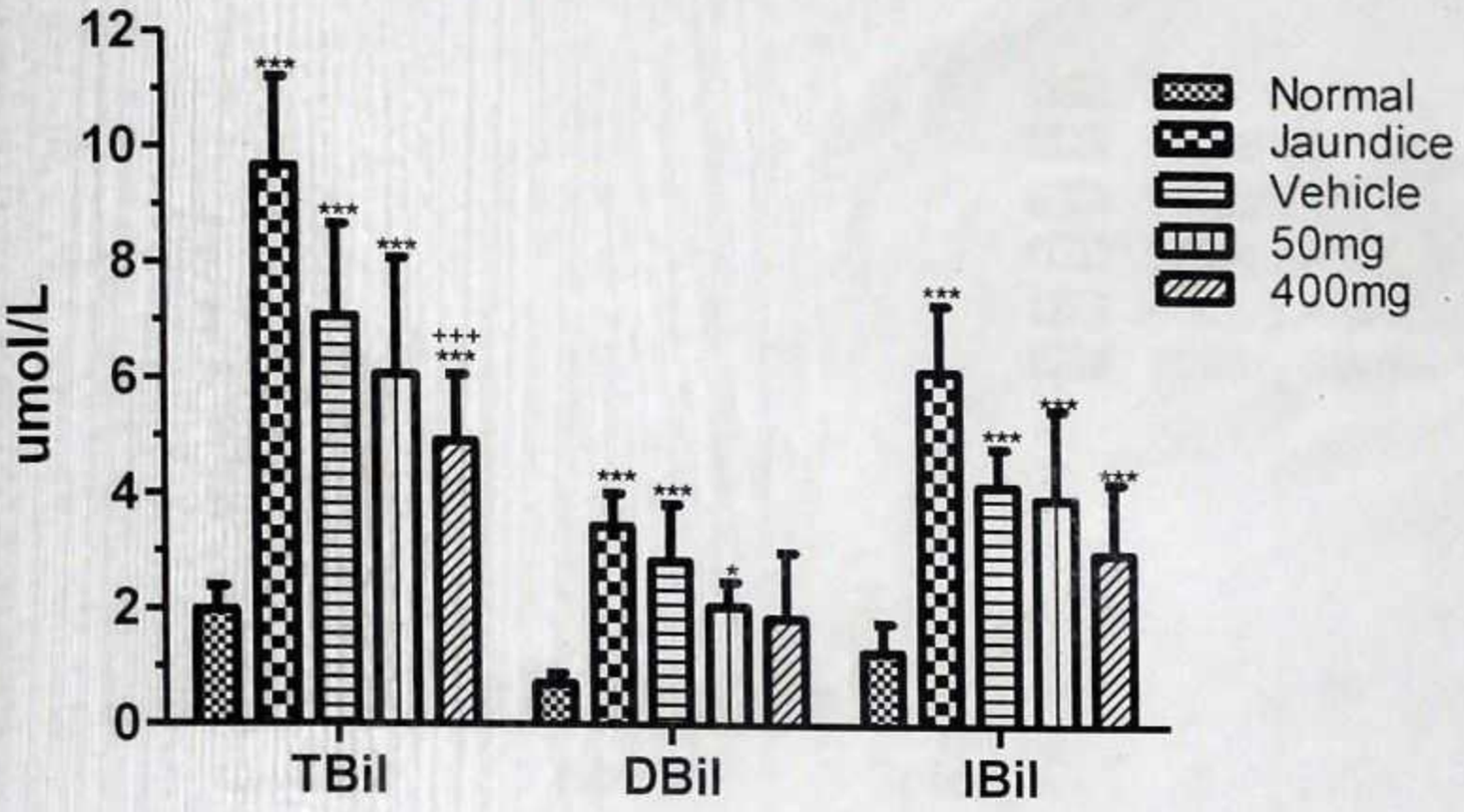


Fig.4.34: Effect of PHZ and AMAE on bilirubin levels of rats. Significantly different from Normal, * $p<0.05$, *** $p<0.001$; from Vehicle group, $^{+++}$ $p<0.001$

4.9.3 Prophylactic effect of *A. muricata* in rats with compromised liver

4.9.3.1 Effect of treatment on relative organ weights

The effects of PHZ, CCl_4 and AMAE on relative organ weights of animals are as shown in Fig. 4.35. Relative liver weights were significantly increased in jaundiced group ($p<0.001$) following the administration of CCl_4. There were also significant increases ($p<0.001$) in relative spleen weight in all PHZ-treated animals. No effect was observed on heart weight.

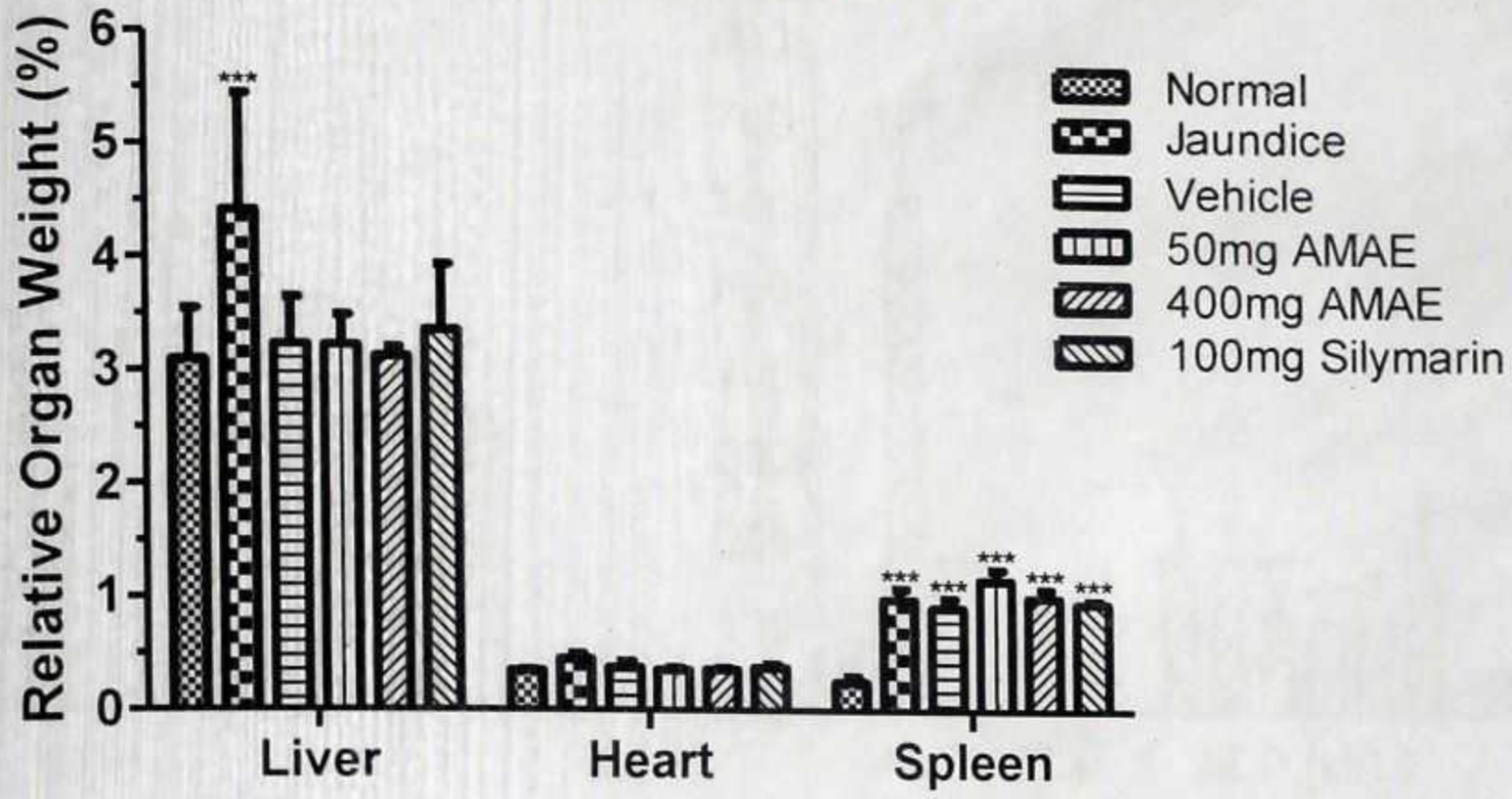


Fig. 4.35: Effect of PHZ, CCl_4 and AMAE on relative organ weight of rats. Significantly different from normal, *** $p<0.001$

4.9.3.2 Effect of treatment on liver enzymes

Hepatic effect of PHZ, CCl_4 and AMAE are as shown in Fig. 4.36. Significant increases in ALT levels were observed in jaundice vehicle groups compared with normal ($p<0.001$). Pretreatment of animals with AMAE (50 mg/kg b.wt and 400 mg/kg b.wt) and Silymarin (100 mg/kg b.wt) resulted in significant decreases in ALT ($p<0.001$) compared with jaundice and vehicle group. Nor differences were observed among AMAE and Silymarin treated groups. ALP levels were significantly decreased in vehicle and AMAE 50 mg/kg b.wt groups. Albumin levels were unaffected in all groups. LDH levels were significantly increase in jaundice group ($p<0.001$) compared with normal, vehicle and drug treatment.

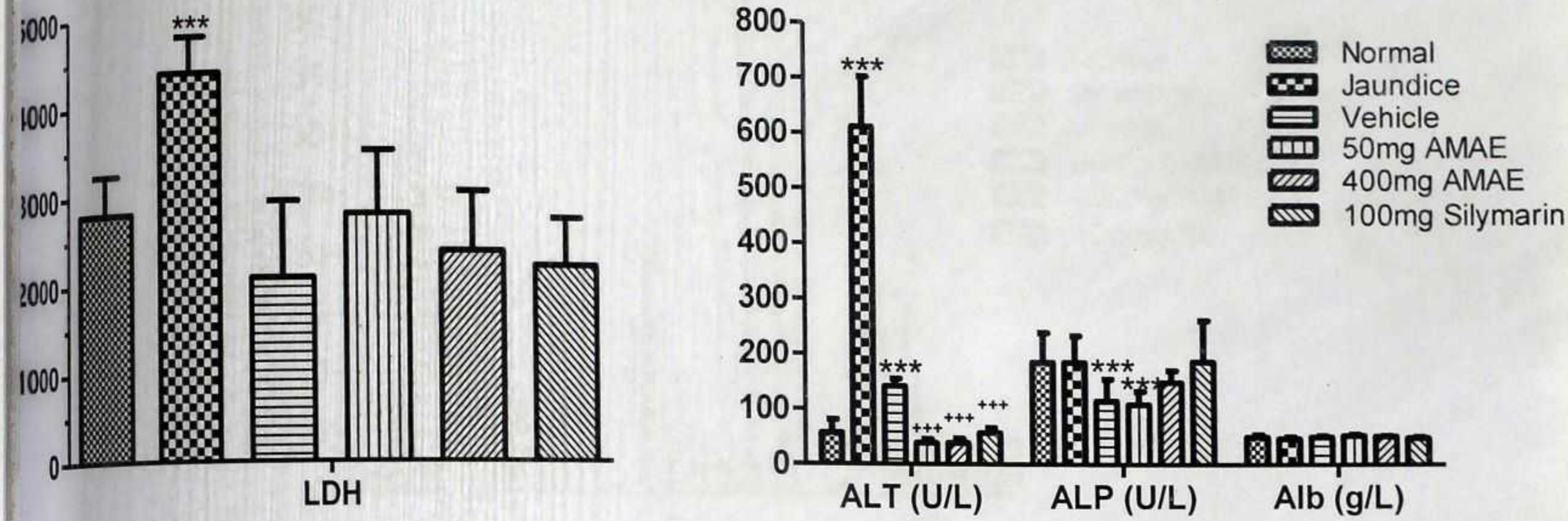


Fig. 4.36: Effect of PHZ, CCl_4 and AMAE on some serum biochemical parameters. Significantly different from normal, ***$p<0.001$; from normal, $^{+++}p<0.001$

4.9.3.3 Effect of treatment on bilirubin concentration

The effects of PHZ, CCl_4 and AMAE on bilirubin concentration are as shown in Fig. 4.37. PHZ and CCl_4 resulted in significant increases in total, direct and indirect bilirubin levels of jaundice and vehicle groups ($p<0.001$ from normal group). Pretreatment of animals with AMAE at 50 mg/kg b.wt and 400 mg/kg b.wt and 100 mg/kg b.wt Silymarin resulted in significant decrease in total bilirubin levels ($p<0.01$ – $p<0.001$) compared with jaundice and vehicle groups. No differences were observed in AMAE and Silymarin treated groups.

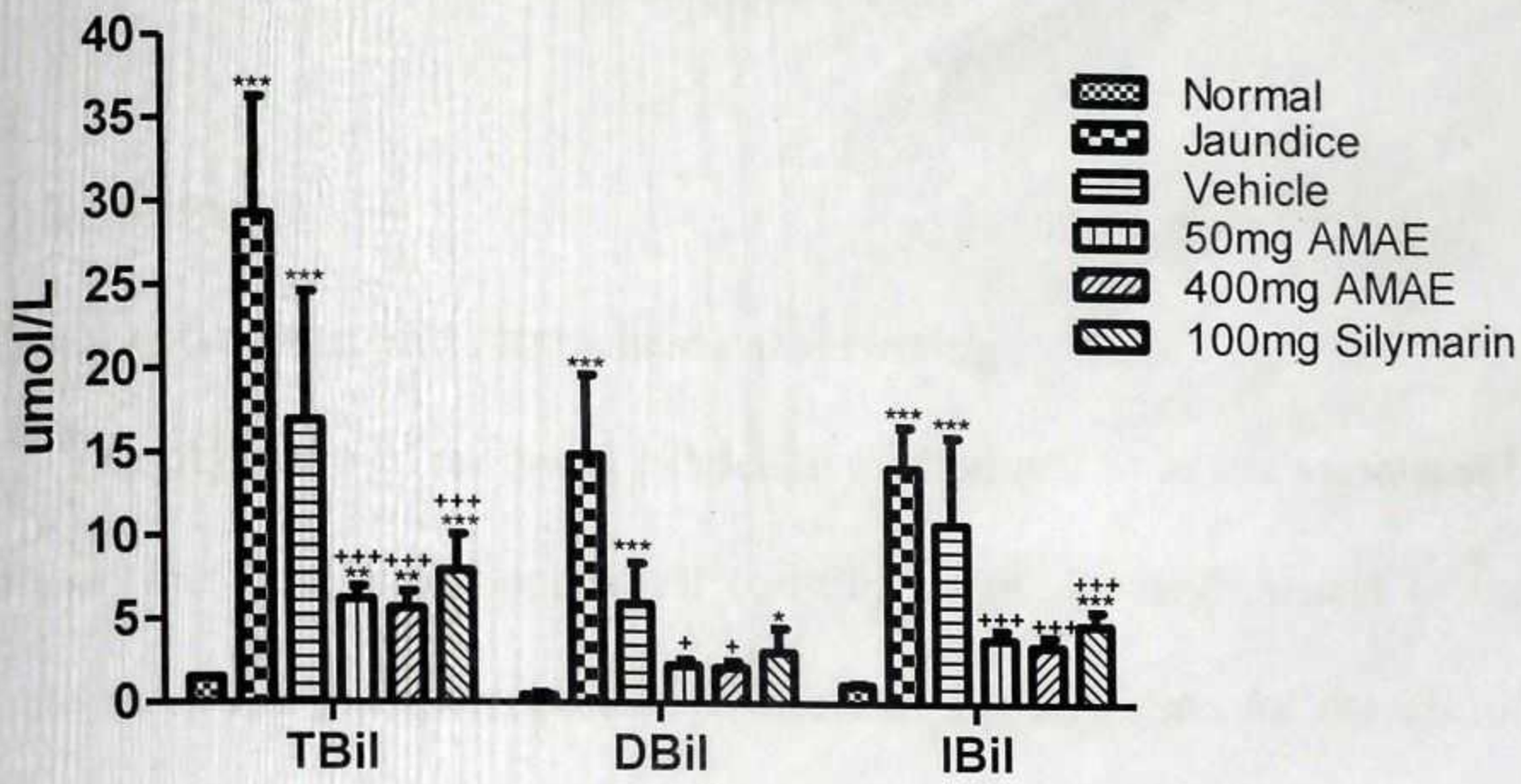


Fig. 4.37: Effect of PHZ, CCl_4 and AMAE on bilirubin levels of rats. Significantly different from Normal *** $p<0.001$; from Normal group $^{+}$ $p<0.05$, $^{++}$ $p<0.01$, $^{+++}p<0.001$

CHAPTER FIVE

5.0 DISCUSSION

5.1 Serial extraction and phytochemical screening

The objective of the serial extraction method was to assess various solvent effects on the various phytochemical constituents of *A. muricata* and to further evaluate them for the hepatoprotective effect as a justification for the use of the aqueous extract in traditional medicine. The order of solvent extraction; petroleum ether, ethyl acetate, methanol and water, was adapted based on the polarity of the selected solvent. The phytochemical screening of the leaves of *Annona muricata* showed that the leaves are rich in alkaloids, flavonoids, saponins, glycosides, anthraquinones, anthraquinone glycosides, coumarins, terpenoids and steroids. However, the various solvent extracts showed varied presence of saponins, glycosides, flavonoids, coumarins, alkaloids and anthraquinones. Flavonoids are most commonly known for their antioxidant activity. They are modifiers which modify the body's reactions to allergens, viruses, pro-oxidants and carcinogens. They show anti-allergic, anti-inflammatory, antimicrobial and anticancer activity (Ekam and Ebong, 2007), and may be useful in therapeutic roles (Jisika *et al.*, 1992). Alkaloids are organic compounds that contain nitrogen, and are physiologically active with sedative and analgesic properties. They are used in relieving pains, anxiety and depression (Jisika *et al.*, 1992). Alkaloids could be toxic due to their stimulatory effects, leading to excitation of cells and neurological dysfunction (Obochi and Malu, 2007).

Glycosides are compounds containing a carbohydrate and non-carbohydrate residue (moiety) in the same molecule and hence their extraction by both polar and non-polar solvents. In these compounds, the carbohydrate moiety is attached by an acetal linkage of carbon-1 to the non-carbohydrate residue (aglycone). They all contain steroid as aglycone component in combination with sugar molecules. They are important in medicine because of their action on the heart and are used in cardiac insufficiency (Balch and Balch, 2000). Thus, cardiac glycosides are drugs and can be used in the treatment of congestive heart failure and cardiac arrhythmia. They work by inhibiting the Na^+/K^+ pump, resulting in an increase in the levels of sodium ions in the myocytes, which then leads to a rise in the level of calcium ions. This inhibition increases the amount of Ca^{2+} ions available for contraction of the heart muscle, improves cardiac output and reduces distension of the heart (Bertorello and Apertia, 1990). These glycosides are found as secondary metabolites in several plants and animals (Beltowski *et al.*, 1998). However, some glycosides, such as ouabain (g-strophanthin, from the ripe seeds of African plant *Strophanthus gratus* and the bark of *Acokanthera ouabaio*), are toxic as it inhibits active transport of Na^+ in cardiac muscle (sodium pump inhibitor), resulting in inhibition of translocases during electron transport chain, and leading to death (Gao *et al.*, 2002). Also phlorizin (toxic glycosides, a type of flavonoid found in pear, apple and cherry), blocks the transport of sugar across the mucosal cells of small intestine and also renal tubular epithelium; it displaces Na^+ from the binding sites of carrier protein and prevents the binding of sugar molecule and produces glycosuria (Rossetti *et al.*, 1987).

Tannins (tannic acids) and saponins are responsible for the antibacterial activity of *A. muricata* leaves (Alali *et al.*, 1999). Saponins are used in veterinary

vaccines as adjuvant (e.g. foot-and-mouth disease vaccines) helping to enhance immune response. They are also mild detergents and can be used commercially as well as for research. They can also be used in intracellular histochemical staining to allow antibody access to intracellular proteins. Also, because of its ability to permeate cells without destroying cell morphology, it is used in laboratory applications to treat live cells in order to facilitate peptide or reagents such as antibodies entering cells instead of the detergents (Balch and Balch, 2000).

Coumarin is a fragrant chemical compound in the benzopyrone chemical class found in many plants including tonka bean, vanilla grass and sweet woodruff. It has a sweet odour and has been used in perfumes. Coumarin is transformed into a natural anticoagulant dicoumarol by some fungal species. Coumarins are oedema modifiers and stimulate macrophage to degrade extracellular albumin. Its presence in plants has been reported to be responsible for anti-tumour, anti-hypertensive, anti-arrhythmia, anti-inflammatory, anti-osteoporosis, antiseptic, analgesic and for treating lymphedema (Farinola and Piller, 2005).

The results in this study have shown that different solvent extraction methods affect the content of the extracts depending on the solvent used as well as the method of extraction (Table 4.1). The results of hepatoprotective effect of *A. muricata* serial extracts against carbon tetrachloride-induced liver damage are attributable mainly to the presence of flavonoids due to their known antioxidant effect. Thus, only the aqueous and methanol extracts showed significant percent liver protection due to the presence of flavonoids. The other solvent extracts, however, showed minimal hepatoprotective activity because CCl_4 is a pro-oxidant and requires an anti-oxidant to avert its effect in liver tissues.

The serial extraction method has proven useful as some solvents extracted different phytochemicals in *A. muricata* which could also be present at different concentration. Some AMPE phytochemicals were present in AMAE in addition to general glycosides. Uniquely, AMME contained flavonoids and anthraquinones while AMEE contained coumarines. In all, none of the solvents extracted terpenoids, steroids and anthraquinone glycosides which were present in the fresh sample. Other solvents need to be explored to achieve total extraction of *A. muricata* phytochemicals.

The selection of an extract for further study should have been that which contained phytochemicals not found in other extracts. Further, this will enable the study of pharmacological effect of those unique phytochemicals and the combined effect of the other phytochemicals present in all extract. The current study, however, evaluated all the extracts for hepatoprotective effects. Also, the presence of some phytochemicals in more than one solvent extract could imply that the concentration of such phytochemicals for adequate therapeutic effect will be lower and hence reduce the extent of the expected pharmacological effect.

5.2 Evaluation of hepatoprotective effect of serial extracts of *A. muricata*

Literature indicates that the hepatoprotective activity of *A. muricata* in carbon tetrachloride-induced liver damage has not been evaluated so far. An active and safe drug is needed for the treatment and management of hepatitis, jaundice and other liver diseases. In view of this, the present study was aimed at evaluating the hepatoprotective activity of serial extracts of leaves of *A. muricata* against CCl_4-induced hepatotoxicity in Sprague-Dawley rats as a justification for the use of aqueous extract in ethnopharmacology.

Decreases in body weight were observed following CCl_4 administration, an indication of toxic effect (Fig. 4.1). This was observed on day 1 which proceeded further till the end of day 3. By day 4, increases were observed in AMAE 300 mg/kg b.wt and 500 mg/kg b.wt groups which improved till end of the experiment. On termination of experiment, improved weight increases were observed in AMAE 300 mg/kg b.wt group (161.18%) and lowest decrease in AMPE 300 mg/kg b.wt group (-188.51%). Thus AMAE at all doses had better effect on body weight gain following acute liver toxicity. When animals lose appetite, weight loss is bound to ensue due to disturbances in carbohydrate, protein or fat metabolism (Klaasen, 2001). The general decrease in weight is attributable to the toxic effect of CCl_4 (Okamoto *et al.*, 2001) while the observed increase following AMAE administration could be attributed to the effect of AMAE in averting the toxic effect of CCl_4. Decreases were observed in AMPE, AMEE and AMME treatment and could be attributed to an inherent toxicity of the extracts or their inability to counter the effect of the toxin. On the other hand, it could be attributed to the lower concentration of phytochemicals in those extracts required for better pharmacological effect.

The administration of a hepatotoxin, CCl_4 resulted in significant increase in liver weight of CCl_4 group ($p<0.001$ for relative liver weight) but not other organs (Appendix B, Table B1), indicating specificity of CCl_4 affecting the liver. CCl_4 is very lipid soluble compound and is subsequently well distributed throughout the body, but despite this, its major toxic effect is on the liver, irrespective of the route of administration. This is because the toxicity of CCl_4 is dependent on the metabolic activation by CYP2E1. The liver has the greatest concentration of CYP2E1 especially in the centrilobular region where damage is

greatest (Timbrell, 2009). An attempt has been made to explain the mechanism leading to the infiltration of inflammatory cells into liver and liver necrosis resulting from CCl_4 intoxication. After CCl_4 poisoning, intracellular adhesion molecule 1 (ICAM 1) in the liver tissue increases, especially in sinusoidal endothelial cells and hepatocytes (Neubauer, 1998). Also leukocyte function antigen 1 (LFA 1) around vessel wall increases, thus, LFA 1 and ICAM 1 accumulate at necrotic areas increasing inflammation and inducing hepatotoxicity (Neubauer, 1998). No inflammation and hence hypertrophy was observed in those rats to which CCl_4 and AMAE, at all doses, were administered, indicating the hepatoprotective effect of AMAE towards CCl_4 toxicity. The observed absence of increased relative liver weights in AMPE, AMEE and AMME groups (Fig. 4.2) could also be attributed to an inherent hepatoprotection. However, prevention of liver hypertrophy is not enough proof of hepatoprotection. This has to be supported by biochemical and histological examination.

The liver can be injured by many chemicals, drugs and biological agents and carbon tetrachloride has been used as a tool to induce hepatotoxicity in experimental animals (Okuno *et al.*, 1986; Prakash *et al.*, 2008). Liver microsomal oxidizing system connected with cytochrome P-450 produces reactive metabolites of CCl_4 such as trichloromethyl radical ($CCl_3^{\cdot}$) or trichloroperoxyl radical ($CCl_3O_2^{\cdot}$) (Uličná *et al.*, 2003). This results in peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes (Mankani *et al.*, 2005). The increase in the levels of serum bilirubin reflected the level of jaundice and the increase in aminotransferases and alkaline phosphatase was the clear indication of cellular leakage and loss of functional integrity of the hepatic cell

membrane (Saraswat *et al*., 1993). The administration of hepatoprotective drugs may induce the hepatocytes to resist toxic effect of CCl_4 and other hepatotoxins.

Bilirubin is the main bile pigment that is formed from the breakdown of haem in the red blood cells. It is transported to the liver where it is secreted by the liver into the bile. Conjugation of bilirubin is a prerequisite for its secretion into the bile (Nelson and Cox, 2000). Loss of bilirubin conjugating function of the liver was evidenced by the significant increase ($p<0.01$) in the level of unconjugated or indirect bilirubin in the serum of the group treated with only CCl_4 when compared with normal group (Fig. 4.5). Increase in the level of unconjugated bilirubin in the blood may result from a defect in the function of the liver to conjugate the bilirubin being produced (Marks and Davis, 2012). The significant reduction in unconjugated bilirubin concentration in the serum of rats pretreated with each dose of AMAE before CCl_4 administration compared with CCl_4 alone indicates that the conjugating function of the liver was improved. The decrease in the unconjugated bilirubin level by the AMAE suggests that the extracts may activate the constitutive androstane receptor (CAR) which is a key regulator in bilirubin clearance in the liver (Moore *et al*., 2004). The primary function of CAR in the bilirubin clearance pathway is to direct a coordinate response to elevated levels of bilirubin by increasing the hepatic expression of each component (enzymes) of the pathway (Moore *et al*., 2004). The increased unconjugated bilirubin level in AMPE, AMEE and AMME-treated rats is an evidence of significant lack of hepatoprotection compared with AMAE. The ability of AMAE particularly at 300 mg/kg b.wt to significantly reduce the level of serum total bilirubin when compared with CCl_4-treated group ($p<0.001$) suggests the potential of the extract in clearing bilirubin from the serum when its level is elevated.

Injury to the liver, whether acute or chronic, eventually results in an increase in serum concentrations of aminotransferases. AST and ALT are enzymes that catalyze the transfer of α-amino groups from aspartate and alanine to the α-keto group of ketoglutaric acid to generate oxaloacetic and pyruvic acids respectively, which are important contributors to the citric acid cycle. Both enzymes require pyridoxal-5'-phosphate (vitamin B6) in order to carry out this reaction, although the effect of pyridoxal-5'-phosphate deficiency is greater on ALT activity than on that of AST (Dufour *et al.*, 2000). Both aminotransferases are highly concentrated in the liver. AST is also diffusely represented in the heart, skeletal muscle, kidneys, brain and red blood cells, and ALT has low concentrations in skeletal muscle and kidney (Wroblewski, 1958); an increase in serum ALT levels is, therefore, more specific for liver damage. In the liver, ALT is localized solely in the cellular cytoplasm, whereas AST is both cytosolic (20% of total activity) and mitochondrial (80% of total activity) (Rej, 1989). Zone 3 of the hepatic acinus has a higher concentration of AST, and damage to this zone, whether ischemic or toxic, may result in greater alteration to AST levels. Thus an elevated level of aminotransferases is as a result of loss of liver integrity. ALP is an enzyme that transports metabolites across cell membranes. Liver and bone diseases are the most common causes of pathological elevation of ALP levels, although ALP may originate from other tissue, such as the placenta, kidneys or intestines, or from leukocytes (Fishman, 1990). Hepatic ALP is present on the surface of bile duct epithelia. Cholestasis enhances the synthesis and release of ALP, and accumulating bile salts and precursors such as bilirubin increase its release from the cell surface (Moss, 1997).

Hepatotoxicity of CCl_4 was further evidenced by the significant increases in activities of ALT ($p<0.01$), AST ($p<0.001$) and ALP ($p<0.001$) in CCl_4 group compared with Normal (Fig. 4.3). AMAE at all doses resulted in significant decrease in ALT, AST and ALP levels ($p<0.001$). This effect could be attributed to the inherent hepatoprotection offered by the extract against carbon tetrachloride. However, the observed decreases in AST and ALP levels of the other extracts (AMPE, AMEE and AMME) could be attributed to its hepatoprotective effect but this cannot be substantiated owing to the lack of significant decrease in ALT levels, the enzyme with greater specificity to the liver.

The toxic effect of carbon tetrachloride (CCl_4) and its metabolite, chloroform ($CHCl_3$) on haematological parameters have been assessed in rats (Moritz and Pankow, 1989). CCl_4 causes a significant decrease in haemoglobin concentration in 7 days after administration whereas chloroform produces a decrease in the relative reticulocyte count at the same time though these changes were moderate and reversible. In this current study, the combined effect of CCl_4 and serial extract of *A. muricata* on haematological parameters were assessed to understand their effect. CCl_4 administration did not cause any significant difference in white blood cell count, red blood cell count, haemoglobin concentration and haematocrit (Table 4.2). The administration of serial extracts had a mixed effect on haematological parameters. AMAE at all doses caused a non-significant decrease in haemoglobin concentration, RBC and haematocrit but an increase in WBC count. This could be attributed to the inherent effect of the aqueous extract to improve immune tolerance of subjects.

The effect of AMAE to prevent increases in body weight, liver weight, and liver enzymes caused by CCl_4 is a strong justification of hepatoprotection and use

of aqueous extract of *A. muricata* in Ghanaian traditional medicine. The estimation of percentage protection (Fig. 4.6, Appendix B, Table B8) further showed AMAE 300 mg/kg b.wt to be more effective (128.64%), followed by AMAE 100 mg/kg b.wt (90.37%) and AMAE 500 mg/kg b.wt (88.20%). The toxicity of CCl_4 is due to the generation of free radicals followed by lipid peroxidation of hepatocytes and liver damage. Any active hepatoprotective drug should therefore possess significant antioxidant property to avert the toxic effects of free radicals generated by hepatotoxicants. AMAE contains flavonoids, which are known antioxidants. The antioxidant effect of *A. muricata* leaf extract has been supported by studies by Adewole and Ojewole (2009) in streptozotocin-treated diabetic rats where reduced serum levels of reactive oxygen species (ROS), oxidized glutathione (GSSG), and malondialdehyde (MDA) and an increase in hepatic catalase (CAT), glutathione peroxidise activity (GSH-Px), superoxide dismutase activity (SOD) and reduced glutathione (GSH) were observed. AMPE 100 mg/kg b.wt offered the least protection (9.30%).

This study has provided empirical proof for the use of aqueous extract of *A. muricata* in traditional medicine and thus further work was centred on the aqueous decoction of the leaves as well as assessment of acute and subchronic toxicity and a further evaluation of hepatoprotection against carbon tetrachloride and acetaminophen (paracetamol) together with bilirubin lowering potential of the extract.

5.3 Oral Acute and Subchronic Toxicity Assessment of *A. muricata* aqueous extract (AMAE)

The need to evaluate the toxicity profile of *Annona muricata* leaf aqueous extract (AMAE) was prompted by the lack of literature on toxicity and the increasing awareness and interest in medicinal plants and their preparations commonly known as herbal medicine. Consequently, herbal medicines have received greater attention as alternatives to orthodox therapy, leading to their increase in demand (Mythilypriya *et al.*, 2007).

Aqueous extract, as directed by enthomedicinal use in Ghana, contained polar extracts which are responsible for therapeutic effect. Phytochemical analysis helps detect the chemical constituents of plants extract in search of bioactive agents as basis for drug synthesis (Ogbonnia *et al.*, 2009). The presence of saponins, condensed tannins and glycosides as the major constituents and trace amounts of flavonoids contribute immensely to the bioactivity of *A. muricata* and also to its usage in treating various diseases (Table 4.3). Reported medicinal uses have included antibacterial, anthelmintic (antihelminthics), anticancerous, anticonvulsant, antidepressant, antifungal, antineoplastic, antiparasitic, antispasmodic, antitumorous, antiviral, astringent, cardiodepressant, cytotoxic, febrifuge, hypotensive, insecticidal, nervine, pectoral, piscicide, sedative, stomachic, vasodilator, and as a vermifuge (Taylor, 2002).

In the acute oral toxicity study of the extract, no changes in the behaviour of mice were observed. Also no adverse gastrointestinal effects were observed in male and female mice administered with up to 5 g/kg b.wt. The median acute toxicity value (LD_{50}) was estimated to be $\leq$ 5 g/kg b.wt, indicating safety (Ghosh, 1984). The gram equivalence of LD_{50} in an average adult man (approximate body

weight 70 kg) would translate to 350 g dose of the extract. Further, the extract is safe by oral route in relation to its folkloric therapeutic dose (1 tea cup three times a day approximately 211 mg/kg/day, Mshana *et al.*, 2000). Thus its high oral therapeutic index (≈55) might be used as a rough indication of a wide margin between the effective and toxic dose. Earlier reports have shown that if the median lethal dose of a test substance is three times more than the minimum effective dose, the substance is considered a good candidate for further studies (Salawu *et al.*, 2009). Currently the following chemical labelling and classification of acute systemic toxicity based on oral LD_{50} values are recommended by the Organisation of Economic Co-operation and Development (OECD, Paris, France): very toxic, ≤ 5 mg/kg; toxic, $> 5 \leq 50$ mg/kg; harmful, $> 50 \leq 500$ mg/kg; and no label, $> 500 \leq 2000$ mg/kg (Walum, 1998). *A. muricata* with a LD_{50} value of ≤ 5000 mg/kg b.wt is therefore safe for oral use for the management of several diseases.

But such acute oral toxicity data are of limited clinical application since cumulative toxic effects do occur even at very low doses. Hence, sub-acute and chronic toxicity studies are almost always invaluable in evaluating the safety profile of phytomedicines. This therefore explains why some authors have suggested that sub-chronic toxicity data may be needed to predict the hazard of long-term, low- and high-dose exposure to a particular compound (McNamara, 1976). A 14-day oral subchronic toxicity study was carried out in rats to determine the potential of *A. muricata* leaf extract to produce toxicity in man. Dose levels of 100 mg/kg b.wt, 1000 mg/kg b.wt and 2500 mg/kg b.wt were selected for the study to provide a better assessment of a wide range of low, medium and high dose of AMAE in rats.

The body weight changes serve as a sensitive indication of the general health status of animals. Varied responses of male and female rats to AMAE at high dose levels were observed. The 1000 mg/kg b.wt and 2500 mg/kg b.wt/day treatment resulted in decline in body weight of female rats throughout the duration of experiment whereas at the same dose level, increases were observed in male rats with a decline from day 6 (Fig. 4.7, Table 4.4). However weight gains were observed in all animals administered with 100 mg/kg/day. It can be stated that at 100 mg, AMAE did not interfere with the normal metabolism of animals as corroborated with non-significant difference with animals in normal control group. However at higher dose (1000 mg/kg b.wt and 2500 mg/kg/day), the crude extract may have been metabolised to a toxic end product which could interfere with gastric function and decreased food conversion efficiency (Chokshi, 2007). In addition, the diets were well-accepted by animals treated with 100 mg suggesting AMAE did not possibly cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. It also shows that AMAE at 100 mg did not adversely interfere with the nutritional benefits such as weight gain and stability of appetite expected of animals that are continually supplied with food and water *ad libitum*. However, the same could not be said for animals administered with 1000 and 2500 mg AMAE (Fig. 4.7 and 4.8). Thus overdose of AMAE could result in loss of appetite (as shown by decline in feed consumption) and decrease in body weight particularly in females. Such weight decreases can also be attributed the presence of tannins in the aqueous extract (Table 4.3). Tannins have been reported to be responsible for decreases in feed intake, growth rate, feed efficiency, net metabolizable energy, and protein digestibility in experimental animals. Therefore, foods rich in tannins are considered to be of low

nutritional value. Recent findings also indicate that the major effect of tannins was not due to their inhibition on food consumption or digestion but rather the decreased efficiency in converting the absorbed nutrients to new body substances hence the observed weight decrease particularly at high doses (Chung *et al.*, 1998). Also the sex difference can be attributed to the hormonal differences between the male and female system. The synthesis of the hormone oestrogen tends to make the female system more susceptible to toxic substance that hitherto will not affect the male system.

The macroscopic examination of the organs of animals treated with the various doses of AMAE did not show any changes in colour compared with normal group. Hypertrophies of organs are first hand indication of toxicity of chemical or biological substance. The significant increases in the relative weights of stomach ($p<0.05$) and uterus ($p<0.01$) in female rats administered with 1000 mg/kg b.wt AMAE is a sign of possible toxic effect of AMAE to these organs (Fig. 4.9). This is in agreement with earlier reports which demonstrated a uterine stimulant activity in rats and advised against the use of *A. muricata* during pregnancy (Taylor, 2002). The absence of organ toxicity in males at all doses gives an indication of the susceptibility of the female system to toxicity to substance that may not affect males. Thus an overdose of AMAE in females should be avoided due to the observed effect on the uterus.

The effect of AMAE on some haematological parameters of male and female rats is as shown in Table 4.5. The general lack of significant changes in blood indices is an indication of safety of AMAE. The observed non-significant increase in WBC count could emphasize the beneficial effect of AMAE in improving the immunity and general well being of the animals (Ogbonnia *et al.*,

2009). The mean corpuscular volume or mean cell volume (MCV) is a measure of the average red blood cell volume. In patients with anaemia, it is the MCV measurement that allows classification as either a microcytic (below normal), normocytic anaemia (normal MCV) or macrocytic anaemia (above normal) (Tønnesen *et al*., 1986). The observed non-significant difference in haemoglobin concentration and MCV in both male and female rats could be used to justify the fact that AMAE at all doses does not significantly induce anaemia, making it safe. Also contrary to documented use of *A. muricata* in floristic studies in Ghana as a tonic (Mshana *et al*., 2000), such was not observed in the current study as there were no significant changes in the RBC count of animals at all doses. AMAE did not confer significant increase in the production of red blood cell.

The liver releases alanine aminotransferase (ALT) and an elevation in plasma concentration is an indicator of liver damage. The liver and heart release AST and ALT, and an elevation in the levels of these enzymes are indicators of liver and heart damage (Crook, 2006). The non-significant changes in ALT and AST in both male and female rats at all doses (Fig. 4.10) indicate that the extract had no deleterious effect on liver function. ALP which has both hepatic and bone sources, showed a significant decrease ($p<0.01$) in male rats administered 1000 mg/kg b.wt AMAE. The general lack of significant changes in the aminotransferases and ALP together with normal liver weight is an indication that AMAE is safe and offers no deleterious effect on the liver.

The decrease in the plasma levels of albumin in male and female rats administered with 1000 mg/kg b.wt and 2500 mg/kg AMAE may be a sign of impaired renal function (Kāchmar and Grant, 1982). This effect was more pronounced in female rats, as higher doses showed significant decreases ($p<0.05$)

(Fig. 4.11). Further biochemical changes were observed in creatinine concentration at higher doses which suggest possible kidney damage, especially by renal infiltration mechanism (Wasan *et al.*, 2001). However, the combined decrease in albumin and increase creatinine levels occurred in animals treated with higher doses. Thus, the extract at 1000 mg/kg b.wt and beyond may likely cause kidney damage.

Effect of the extract on biochemical parameters showed a remarkable decrease in plasma glucose level especially at 1000 mg/kg b.wt in both male and female compared with normal group (Fig. 4.12). This indicates the presence of hypoglycaemic components in the extract and gives credence to the use of *A. muricata* as hypoglycaemic agent. It possesses stimulatory effect on the β-cells of the pancreas, resulting in high production of insulin for glucose absorption (Adewole and Ojewole, 2006).

The decrease in the plasma total cholesterol and triglycerides levels may be attributed to the presence of hypolipidaemic agents in the extract (Fig. 4.13). The general lack of significant changes in HDL and LDL levels indicate that the extract had no effect on lipid metabolism of animals. However, the non-significant increase in HDL-cholesterol levels and a reduction in LDL-cholesterol levels observed in female rats administered 100 mg/kg b.wt AMAE is an indication that low dose of the extract may reduce the cardiovascular risk factors which contribute to death of diabetic subjects (Barnett and O'Gara, 2003). The reduction of the cardiovascular risk factors can further give support to the traditional use of the herbal formulation of *A. muricata* as a hypoglycaemic agent and contribute to the improvement of health status of diabetic subjects. Also the general lack of

significant difference in lipid profile in male rats indicates that the extract at all doses had no effect on lipid metabolism of animals.

5.4 Hepatoprotective effect of AMAE against CCl_4 – and paracetamol – induced liver damage

The effects of aqueous extract of *Annona muricata* Linn. (AMAE) was evaluated against carbon tetrachloride and paracetamol-induced liver damage. To evaluate the hepatoprotective effect of AMAE, a well-described CCl_4 model was used (Prakash *et al*., 2008) with modification, in which the liver oxidizing systems connected with cytochrome P-450, produce reactive metabolites of CCl_4 such as trichloromethyl radical ($CCl_3^{\cdot}$) or trichloroperoxy radical ($CCl_3O_2^{\cdot}$). These free radicals cause lipid peroxidation which produces hepatocellular damage and enhance production of fibrotic tissues. Paracetamol (acetaminophen), a widely used antipyretic and analgesic, produces acute liver damage if an overdose is consumed (Savides and Oehme, 1983). Paracetamol is mainly metabolized in liver to excretable glucuronide and sulphate conjugates (Wong *et al*., 1981). However, the hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P-450 (Savides and Oehme, 1983) to a highly reactive metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI) (Vermeulen *et al*., 1992). NAPQI is initially detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid (Moore *et al*., 1985). However, when the rate of NAPQI formation exceeds the rate of detoxification by GSH, it oxidizes tissue macromolecules such as lipid or -SH group of protein and alters the homeostasis of calcium after depleting GSH.

Higher doses of paracetamol, as used in this study at 500 mg/kg body weight daily for 7 days, will progressively deplete hepatic glutathione levels with subsequent hepatic necrosis (Gupta *et al.*, 2004).

Carbon tetrachloride classically induced weight loss in animals and significant increases ($p<0.001$) in both absolute and relative liver weights in animals administered CCl_4 alone. It further resulted in elevated ALT and ALP levels ($p<0.001$), total bilirubin ($p<0.001$) and indirect bilirubin ($p<0.05$) and decline in serum cholesterol ($p<0.01$) and triglycerides ($p<0.05$) (Fig. 4.15 – 4.19). The extent of liver damage was further corroborated by histopathological observation (Plate 1 B) showing severe histopathological changes, such as centrilobular hepatic necrosis, fatty change, Kupffer cell aggregation, ballooning degeneration and infiltrating lymphocytes.

The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin, is the index of its protective effects (Prakash *et al.*, 2008). Although elevated serum liver marker enzyme levels are not a direct measure of hepatic injury, they show the status of the liver. The lowering of enzyme levels is definite indication of hepatoprotective action of the drug. The present investigation also revealed that the given dose of CCl_4 (1ml/kg, orally) produced significant elevation in ALT, ALP, bilirubin (total and indirect), and decreases in triglyceride and cholesterol indicating an impaired liver function and these parameters have been reported to be sensitive indicators of liver injury (Zimmerman and Seeff, 1970). The present study revealed that pre-treatment of animals with aqueous extract of leaves of *A. muricata* had been effective in offering protection, which is comparable to Silymarin (Fig. 4.15 – 4.19). CCl_4

treatment caused classical fatty liver as indicated by accumulation of fat in liver micro architecture and significant decrease in serum cholesterol and triglyceride levels. The serum cholesterol levels increased after Silymarin and *A. muricata* treatment following significant reduction caused by CCl_4 treatment. The disturbance in the transport function of the hepatocytes as a result of hepatic injury caused the leakage of enzymes from cells due to altered permeability of membrane (Recnagel, 1983) but was reversed by AMAE at all doses and Silymarin (Fig. 4.16). Hepatotoxic action of CCl_4 begins with changes in endoplasmic reticulum which result in loss of metabolic enzymes located in the intracellular structure (Conney and Burns, 1959). The aqueous extract of leaves of *A. muricata* when administered orally to rats showed a significant hepatoprotective activity at all doses studied. A very important observation with *A. muricata* extract was that all doses studied offered significant protection; 50 mg/kg b.wt (101.64%), 100 mg/kg b.wt (80.42%), 200 mg/kg b.wt (97.42%), and 400 mg/kg b.wt (93.41%). However, 50 mg/kg b.wt compared better with standard Silymarin (107.23%) (Fig. 4.26). The histopathological studies are the evidence of efficacy of drug as hepatoprotectant. Simultaneous treatment of AMAE and CCl_4 exhibits less damage to the hepatic cells as compared to the rats treated with CCl_4 alone. Intralobular veins though are damaged but to a lesser extent. Endothelium is disrupted at places. Hepatic cells adjoining to intralobular vein show atrophy. The sections of the liver treated with aqueous extract of leaves of *A. muricata* and CCl_4 reveals better hepatoprotective activity. Almost negligible damage to a few hepatocytes present in the close vicinity of intralobular vein was observed. Endothelium lining was almost smooth except one or two places. Hepatocytes showed normal appearance, only some cells show higher numbers of vacuoles in

the cytoplasm. The results of histopathological study also support the results of biochemical parameters.

The hepatoprotective effect of AMAE against CCl_4-induced liver damage can be attributed to the presence of flavonoids in the extract with known antioxidant effect compared with the standard flavonoid, Silymarin. AMAE possibly possesses antioxidant effect at all doses, as with Silymarin, and depleted prooxidants generated by the metabolism of CCl_4. The anti-oxidant effect of *A. muricata* leaf extract has been supported by studies by Adewole and Ojewole (2009) in streptozotocin-treated diabetic rat where *A. muricata* reduced serum levels of reactive oxygen species (ROS), oxidized glutathione (GSSG), and malondialdehyde (MDA) and an increase in hepatic catalase (CAT), glutathione peroxidise activity (GHS-Px), superoxide dismutase activity (SOD) and reduced glutathione (GSH). Silymarin has been demonstrated in several studies to possess protective effects against oxidative peroxidation (Wellington and Jarvis, 2001; Valenzuela and Garrido, 1994). In both cases, Silymarin functioned as a free radical scavenger, increasing reduced glutathione (GSH) available which functions as a detoxificant of intermediary oxygen reactive products of lipoperoxidation. Silymarin is reported to have antioxidant properties, by increasing superoxide dismutase activity in erythrocytes and lymphocytes. It is also reported to stabilize hepatocyte membrane structure, thereby preventing toxins from entering the cell through enterohepatic recirculation, and to promote liver regeneration by stimulating nucleolar polymerase A and increasing ribosomal protein synthesis (Mayer *et al*., 2005).

Increased organ weight, either absolute or relative, has been observed to be sensitive indicator of organ toxicity by known toxicant (Simmons *et al*., 1995).

However, there were no significant increases in absolute and relative liver weights in paracetamol-treated groups though increases in liver enzymes were observed, indication of hepatotoxicity. This was further corroborated by extensive fatty degeneration of cells around the portal tract. AMAE at all doses resulted in significant decrease in ALT, ALP, total and indirect bilirubin (Fig. 4.23 and 4.24) compared with increases caused by the administration of paracetamol. AMAE and Silymarin treatments also reversed decreased levels of total cholesterol and triglycerides caused by paracetamol. Histopathological observations also revealed only mild fatty changes (Plate 2 C – G). Results of the study indicated that AMAE is hepatoprotective against paracetamol-induced liver injury which corroborated well with effect of AMAE on CCl_4-induced liver injury. AMAE possibly averts the toxic effect of acetaminophen metabolism by conjugating with the metabolite NAPQI for excretion. This could be so because higher doses of AMAE (400 mg/kg b.wt) offered better protection than lower doses (50 mg/kg b.wt) and thus contained a higher concentration of the conjugating molecule. Acetaminophen undergoes detoxification mainly by glucuronidation in humans and rats which converts it to nontoxic conjugates for elimination in bile or urine (Kessler *et al*., 2002). Thus, the high levels of glycosides in aqueous extract (Table 4.3) could be metabolized to glucuronic acid for glucuronidation. Further, AMAE could modulate the synthesis of GSH for the detoxification of NAPQI attributable to the presence of flavonoids (Table 4.3). This is observable because flavonoids from vegetables have been demonstrated to increase intracellular concentration of GSH for detoxification (Myhrstad *et al*., 2002).

A comparative analysis of the protective potential of the four doses of AMAE and Silymarin against carbon tetrachloride (CCl_4) and paracetamol

indicated that AMAE had better protective effect against paracetamol toxicity than carbon tetrachloride (Fig. 4.27). A dose dependent effect was observed for paracetamol treatments with higher doses of AMAE (400 mg/kg b.wt) offering better protection attributable to the higher concentration of glycosides in the aqueous extract. However, for CCl_4 treatment, lower doses (50 mg/kg b.wt) showed better protection than higher doses attributable to the antioxidant effect of flavonoids present in the aqueous extract.

5.5 Bilirubin-lowering potential of *Annona muricata* aqueous extract (AMAE)

In vertebrates, anaemia is a common haematological disorder associated with several conditions such as drug toxicity, parasites (e.g. malaria), genetic (e.g. sickle cell diseases and G6PD deficiency) or acquired defects, and blood loss (Criswell *et al.*, 2000; Jollow and McMillan, 2001). The haemolytic activity of arylhydrazines, such as phenylhydrazine (PHZ), dapsone hydroxylamine and divicine may lead to acute haemolytic anaemia in vertebrates (Jollow and McMillan, 2001). The main action of the classical haemotoxicant, PHZ has long been associated with drug-induced oxidative stress occurring within erythrocytes (Kinuta *et al.*, 1995). This process produces an increase in the oxidation of oxyhaemoglobin, thus leading to the formation of metahaemoglobin which is subsequently converted into irreversible haemichromes which, in turn, lead to the denaturation and precipitation of haemoglobin in the form of Heinz bodies (Rifkind and Danon, 1965). Skeletal protein damage and lipid peroxidation as well as glutathione and ATP depletion, cation imbalances, and reduced membrane deformability have been proposed to be involved in the haemolytic response that is induced by oxidant drugs (McMillan *et al.*, 1998). It has also been suggested that

with high oxidative stress, lipid peroxidation and depletion of GSH, it may be expected that such changes could induce liver damage in treated animals. However, hepatotoxicity, exhibited as increases in relative liver weight and aminotransferases were not observed in the current study (Fig. 4.27, 4.28, 4.31 and 4.33). This is suggestive that PHZ only affects the red blood cells, inducing haemolysis with the release of cell content into circulation without affecting other organs such as liver and heart. This lends support to the observed decreases in red blood cell (RBC) count, haemoglobin concentration (HGB) and haematocrit (HCT) of PHZ treated groups (Fig. 4.28 and 4.32). These values were restored to normal level in vehicle and AMAE treated groups. The observed restoration of haematological parameters suggests that in a normal animal with normal liver capacity and erythroid system, anaemia is prevented mainly by compensatory erythrocytic spleen hyperplasy (Roque *et al.*, 2008) resulting in splenomegaly.

The spleen plays important roles in processing other than blood storage and immune competence, and in PHZ-induced haemolytic anaemia in rodents and rabbits it acts as the main erythrophagocytic organ (Latunde-Dada *et al.*, 2006) resulting in the observed splenomegaly in animals (Fig. 4.27, 4.31 and 4.35). This implies that damaged cells are removed intact by the spleen which accompanies intravascular lysis.

PHZ haemolysis is accompanied by hyperbilirubinaemia (Maines and Veltman, 1984). PHZ has been shown to exhibit a potent ability to increase haem oxygenase activity in a time-dependent manner in the liver and kidney and this suggest that the induction of hyperbilirubinaemia by PHZ may be related to the enhanced rate of enzymatic conversion of haemoglobin haem to bilirubin. These findings have been supported with the observation that zinc protoporphyrin, a

haem oxygenase inhibitor, markedly reduce serum total bilirubin levels (Maines and Veltman, 1984).

Four distinct steps of bilirubin metabolism have been proposed (Kamisako *et al.*, 2000). Bilirubin is first imported via the sinusoidal surface of the hepatocyte by solute carrier family 21, member 6 (SLC21A6; also known as organic anion transporter 2, OATP2) (Cui *et al.*, 2001). Ligandin, a homodimer or heterodimer of glutathione-S-transferase (GST) A1 and A2, binds bilirubin with high affinity and thus increases uptake. Bilirubin is then glucuronidated by a specific microsomal bilirubin uridine diphosphate-5′-glucuronosyltransferase (UDP-glucuronosyltransferase 1A1, UGT1A1). The resulting hydrophilic bilirubin diglucuronide is then secreted across the bile canalicular membrane of the hepatocytes by an active transporter, multidrug resistance–related protein 2 (MRP2) (cMOAT, ABC-C2) (Huang *et al.*, 2004).

In another mechanism, constitutive androstane receptor (CAR) NR113 has been shown to mediate the response of liver to phenobarbitone and other "phenobarbitone-like" compounds (Wei *et al.*, 2000). It has been demonstrated that CAR is a key regulator of the bilirubin clearance pathway and that CAR activation increases the rate of bilirubin clearance (Huang *et al.*, 2004).

The administration of PHZ at 40 mg/kg body weight significantly increased the bilirubin levels of animals 6 hours after last treatment (9.78 µmol/L ± 0.49, $p<0.001$ from normal 1.5 µmol/L ± 0.08) indicating a jaundiced state due to increased haemolysis of red cells. This could be attributed to the established modulatory effect of PHZ on haem oxygenase (Maines and Veltman, 1984), increasing the rate of haem degradation and bilirubin production. This was further evidenced by the high levels of unconjugated serum bilirubin (6.23 µmol/L ± 0.37,

$p<0.001$ from normal 0.85 μmol/L ± 0.06), an observed effect of haemolytic or prehepatic jaundice (Thompson, 1970).

The curative and prophylactic effects of AMAE were assessed. On both levels, AMAE exhibited a dose dependent effect, where 400 mg/kg b.wt showed a potent effect than 50 mg/kg b.wt. Curatively, AMAE reduced bilirubin levels to 4.43 μmol/L ± 0.53 at 50 mg/kg b.wt and 3.33 μmol/L ± 0.31 at 400 mg/kg b.wt, significantly different ($p<0.001$) from the vehicle group (7.43 μmol/L ± 0.23) maintained on distilled water (Fig. 4.30). This indicate that AMAE possesses a bilirubin-lowering potential and could help clear serum bilirubin faster than in untreated group. However, no difference existed between the AMAE treated groups.

During prophylactic studies, serum bilirubin levels were reduced to 6.08 μmol/L ± 0.64 at 50 mg/kg b.wt and 4.93 μmol/L ± 0.36 at 400 mg/kg b.wt, significantly different ($p<0.001$) from vehicle group (7.08 μmol/L ± 0.50 (Fig. 4.34). This indicates that AMAE at 400 mg protected animals against jaundice induced by a haemotoxicant.

Finally, the bilirubin lowering potential was assessed in animals with reduced liver capacity following the administration of carbon tetrachloride. The hepatotoxic agent was expected to injure the liver, reducing its liver conjugating ability and further exacerbating the jaundiced effect. This was observed when the combined effect of PHZ and CCl_4 resulted in increased serum bilirubin level (29.25 μmol/L ± 2.21, normal 1.5 μmol/L ± 0.00) (Fig. 4.37), accompanied by increased ALT and LDH levels (Fig. 4.36) characteristic of CCl_4 toxicity. The administration of AMAE, with its established hepatoprotective effect reduced the effect of PHZ and CCl_4. Bilirubin levels were reduced to 6.22 μmol/L ± 0.27 at 50

mg/kg b.wt and 5.68 µmol/L ± 0.36 at 400 mg/kg b.wt which compared well with Silymarin, 7.94 µmol/L ± 0.78. These values were significantly lower compared with animals that were maintained on distilled water (16.90 µmol/L ± 2.43 *p<0.001*). AMAE could thus help treat jaundice by offering protection on the liver, improving its bilirubin conjugating property and helping clear bilirubin for circulation. This was justified by the lack of significant difference between unconjugated or indirect bilirubin levels of normal group and AMAE treated groups.

The following mechanisms could be suggested for the bilirubin-lowering potential of *Annona muricata*. Firstly, the presence of glycosides in the extract might be converted to glucuronic acid for conjugating with bilirubin for excretion. This is evidenced by the fact that high dose of *A. muricata* (400 mg) offered significant reduction in bilirubin levels in the study than 50 mg. Glycosides are compounds consisting of a carbohydrate and non-carbohydrate (aglycone) residue in the same molecule. On hydrolysis, the carbohydrate portion, which could be glucose (glucoside), could be isomerized to glucuronic acid. Further, in some other type of glycosides (glucuronide), the carbohydrate moiety is glucuronic acid and therefore becomes available on hydrolysis for conjugation.

It could also be suggested that AMAE activated the constitutive andostane receptor (CAR), a key regulator in the bilirubin clearance pathway (Huang *et al.*, 2003), increasing the activity of glucuronyl transferases (Ostrow *et al.*, 2003), synthesis of ligandin, a transporter of bilirubin, increasing its transport to liver for conjugation (Greige-Gerges *et al.*, 2007). This has been demonstrated by administering Yin Zhi Huang, a decoction of *Arteminia capillaris* and other three herbs to humanized CAR transgenic mice for 3 days (Huang *et al.*, 2004). In that

study, CAR was observed to mediate the bilirubin clearance pathways and a potential target for new drug development. Thus *Annona muricata* aqueous extract possess a bilirubin lowering potential by the activation of constitutive androstane receptors (CAR) and providing glucuronic acid for conjugation and excretion of bilirubin.

CHAPTER SIX

6.0 CONCLUSION

The high LD_{50} value (5 g/kg body weight) obtained was a clear indication that *A. muricata* leaf extract could be safe for both internal and external uses. The study showed that the extract had some hypoglycemic activity and good reducing effects on cardiovascular factors. The study also revealed that the extract at low and moderate doses did not provoke toxic effects to the animals' tissues, but higher doses could cause kidney damage leading to renal failure during long term treatment. Kidney function should therefore be monitored regularly during the long term management of diseases with *A. muricata*. Its effect on the uterus at higher doses implies that *Annona muricata* should be avoided during pregnancy.

In line with observations of hepatoprotective studies, this study has revealed that pre-treatment of animals with AMAE protected animals against hepatic injury induced by CCl_4 and paracetamol. AMAE significantly reduced ALT, total and indirect bilirubin levels induced by CCl_4 and paracetamol. The observed decreases in bilirubin levels indicate that AMAE help treat hepatic jaundice by improving the conjugating ability of the liver. These observed decreases in biochemical parameters were confirmed by histopathological studies on the liver. The administration of CCl_4 and paracetamol alone resulted in degeneration of hepatocytes with fat depositions and ballooned cells. These pathological effects were, however, prevented by AMAE which compared well with standard drug Silymarin. Thus it can be suggested that the hepatoprotective activity of AMAE against CCl_4 and paracetamol induced hepatic damage could be due to possible antioxidant capacity.

Annona muricata aqueous extract posses bilirubin lowering potential especially at higher doses and can be used in the effective management of hyperbilirubinaemia or jaundice, possibly by improving bilirubin conjugation and excretion. Therefore, *Annona muricata* aqueous extract can be used successfully to develop a future drug for the management of prehepatic and hepatic jaundice associated with drugs, alcoholic and viral hepatitis.

6.1 Recommendations

It is recommended that other solvents and/or solvent combinations be explored in addition to achieve total extraction of phytochemicals in *Annona muricata* during serial extraction. Further, other solvents should be explored to improve the flavonoid content which is hoped to improve hepatoprotection and other pharmacological effects. Also, pure fraction of glycosides should be extracted, structurally elucidated and further studied in transgenic jaundiced rat to understand the anti-jaundice effect and also aid drug synthesis.

6.2 Contribution to Knowledge

Annona muricata has been documented for years to be used for the management of jaundice in floristic studies in Ghana. Such claims were however based on indigenous knowledge without any reported scientific evidence. Further, aqueous decoctions have been used without justification. This study has justified these assertions with scientific evidence using certified protocols and determinations. With serial extraction methods, the aqueous extract proved to be effective in hepatoprotective studies compared with other solvent extract and toxicologically safe for internal and external uses hence justifying its use in disease treatment. *A. muricata* has further been demonstrated to possess a bilirubin lowering potential in both prehepatic and hepatic jaundice. In the treatment of prehapatic jaundice as occurs in haemolytic anaemia, *A. muricata* has been demonstrated to contain the conjugating substrate, glucuronic acid, for binding with bilirubin, improving its water solubility, transport and excretion into bile. For treating hepatic jaundice, *A. muricata* has been demonstrated to possess a liver protective effect against hepatotoxicants such as carbon tetrachloride and acetaminophen, further improving the functional and structural integrity of the liver. Such protection was suggestive of the presence of flavonoids and glycosides in aqueous extract.

This study has therefore bridged the knowledge gab in enthnopharmacological use of *A. muricata* with empirical conclusions based on scientific evidence.

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APPENDICES

APPENDIX A

1.0 ANIMAL HOUSE ESTABLISHMENT

Quality and readily available animals and research laboratories are essential for pharmacological and toxicological investigations. However, the lack of such a facility in the Department of Biochemistry and Biotechnology hampers such investigations. Staff and student alike have to depend on animals and facilities available at the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Nuguchi Memorial Institute for Medical research (NMIMR), Legon, Accra, and the Centre for Scientific Research into Plant Medicine (CSRPM), Akuapem-Mampong. Also, it must be understood that such facilities offer services to a wider range of people and is under strain. It is against this background that this project first sought to establish an animal house and research laboratory for pharmacological and toxicological research to provide animals for ongoing investigations and support other students from the department and university as a whole.

1.1 Documents used

To fulfill the above, the following documents were studied and practical training sought from the Department of Pharmacology, KNUST. They were; Guide for Care and Use of Laboratory Animals (ILARCLS and NRC, 1996); Handling, restraint and techniques of laboratory rodents (Rand, 2001); Guide to the Care and Use of Experimental Animals (Olfert *et al,* 1993), and OECD Principles of Good Laboratory Practice (OECD, 1998).

1.2 Design of cages

Cages used in housing animals were locally made ['Assay', Roman Hill, Kumasi], from aluminium sheets with aluminium wire mesh opening at one side and at the top to allow ventilation and observation of animal. The cages had smooth, impervious surfaces with minimal ledges, angles, corners and overlapping surfaces to prevent accumulation of dirt, debris and moisture and also allowed for satisfactory cleaning and disinfection. The average dimension was 18 X 46 X 35 cm.

1.3 Bedding

Animal bedding is a controlled environmental factor that can influence experimental data and animal well-being. Thus, cages were lined with soft dry wood shavings, which easily soak away urine and faeces and prevented direct contact between animal and resting floor of cage. The bedding materials were changed twice weekly and cages disinfected before replacing bedding material.

1.4 Feeding vesicles

Earthen-ware bowls (average diameter, 16 cm) were provided with one each for water and feed per cage. Earthen-ware was chosen to avoid or minimize the incidence of spillage of water and feed. The inside of bowls was glazed to prevent leaching of water into the earth material. Bowls were washed daily, disinfected and allowed to dry before reuse.

1.5 Feed

Animals had free access to feed (GAFCO, Tema – Ghana). The basic nutritional composition of feed is as follows;

Protein	-	18.50%	Fat	-	4.00%
Fibre	-	4.30%	Calcium	-	3.80%
Phosphorus (available)	-	0.50%	Sodium	-	0.90%
Lysine	-	1.00%	Methionine	-	0.45%
Vitamin A	-	10,000 IU	Vitamin D	-	2500 IU
Vitamin E	-	25 mg	Vitamin C	-	220 mg
Vitamin K	-	3 mg	Vitamin B1	-	2 mg
Vitamin B2	-	5 mg	Vitamin B6	-	3 mg
Vitamin B12	-	15 mg	Choline chloride	-	200 mg
Manganese	-	45 mg	Zinc	-	35 mg
Energy	-	10.6 MJ			

1.6 Water

Animals were allowed free access to normal tap water.

1.7 Location

The animal house and research room was located at the Department of Biochemistry and Biotechnology Annex Offices, around the KNUST Commercial area. It began stocking animals in April 2009.

Fig. A1: Location of facility at the Annex of the Department of Biochemistry and Biotechnology

1.8 Stock

The facility stocks albino rats of Sprague-Dawley strain. The initial stock, comprising 10 males and 20 females were obtained from the Animal House of CSRPM, Mampong. Since November 2009, the facility has being used for this study. In January 2010, the facility supported two undergraduates in a study titled "Haematinic effect of peanut husk" under the supervision of Mr. Caleb Firempong. The facility has also provided animals to support some postgraduates from the Department of Pharmacology, KNUST.

Fig. A2: Animal facility for breeding and experimentation

1.9 Stages in Breeding

1.9.1. Gestation: Gestational period following fertilization is about 24 – 28 days.

Pregnant animals were separated from stock into separate cages and further into individual cages of two animals at about 2 – 3 days to delivery.

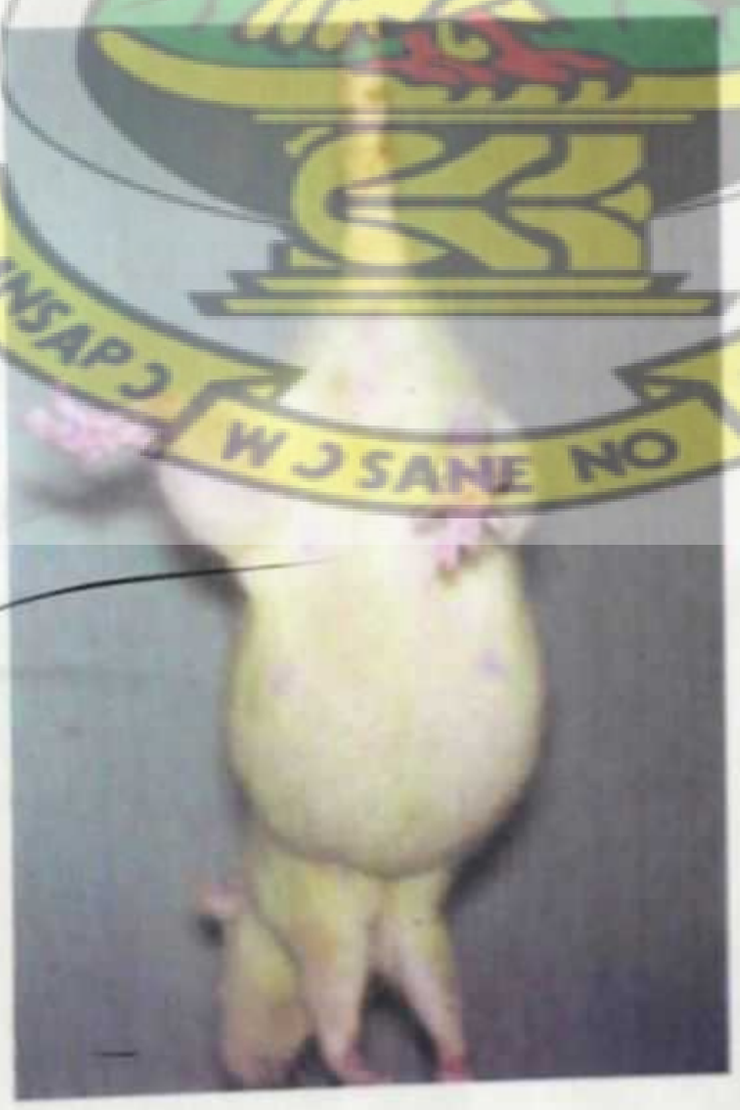

Fig. A3: A pregnant animal (gestational period about 22 days old)

Fig. A4: Day-old litters

Fig. A5: One-week old litters

Fig. A6: Three-week old litters

1.9.2. Weaning: Litters are weaned at about 28 days and kept separate on feed and water till about 8 weeks. They are then sorted into the individual sexes to avoid inbreeding.

Fig. A7: 4 week old litter ready to be weaned

Fig. A8: 6 – 8 week old rats

1.10 Funding

The facility was funded by the researcher.

APPENDIX B

TABLE OF OTHER RESULTS

Table B1: Effect of *A. muricata* serial extracts on Absolute and Relative Organ Weights of CCl_4-intoxicated rats

	Kidney	Heart	Lung	Spleen	Stomach
Normal	1.40 ± 0.10	0.72 ± 0.02	1.29 ± 0.15	0.46 ± 0.04	1.22 ± 0.08
	(0.75 ± 0.04)	(0.40 ± 0.00)	(0.71 ± 0.08)	(0.25 ± 0.01)	(0.67 ± 0.04)
CCl_4	1.45 ± 0.07	0.78 ± 0.02	1.58 ± 0.05	0.53 ± 0.01	1.40 ± 0.03
	(0.72 ± 0.03)	(0.39 ± 0.00)	(0.79 ± 0.03)	(0.26 ± 0.00)	(0.70 ± 0.02)
AMAE 100	1.34 ± 0.05	0.69 ± 0.02	1.19 ± 0.08	0.48 ± 0.04	1.35 ± 0.05
	(0.75 ± 0.04)	(0.39 ± 0.01)	(0.67 ± 0.04)	(0.27 ± 0.02)	(0.76 ± 0.03)
AMAE 300	1.18 ± 0.06	0.73 ± 0.02	1.26 ± 0.12	0.56 ± 0.04	1.42 ± 0.10
	(0.65 ± 0.03)	(0.40 ± 0.01)	(0.69 ± 0.07)	(0.30 ± 0.02)	(0.78 ± 0.05)
AMAE 500	1.33 ± 0.01	0.71 ± 0.01[b]	1.43 ± 0.16	0.53 ± 0.04	1.33 ± 0.09
	(0.74 ± 0.03)	(0.40 ± 0.01)	(0.80 ± 0.09)	(0.30 ± 0.02)	(0.74 ± 0.05)
AMPE 100	1.15 ± 0.05	0.73 ± 0.01	1.16 ± 0.03	0.53 ± 0.03	1.45 ± 0.07
	(0.62 ± 0.02)	(0.39 ± 0.01)	(0.63 ± 0.02)	(0.28 ± 0.01)	(0.78 ± 0.03)
AMPE 300	0.96 ± 0.19[b]	0.74 ± 0.02	1.38 ± 0.09	0.50 ± 0.05	1.51 ± 0.05
	(0.54 ± 0.11)	(0.41 ± 0.03)	(0.77 ± 0.06)	(0.27 ± 0.02)	(0.84 ± 0.04)
AMPE 500	1.11 ± 0.03	0.68 ± 0.01[b]	1.24 ± 0.04	0.56 ± 0.03	1.52 ± 0.00
	(0.61 ± 0.02)	(0.37 ± 0.01)	(0.68 ± 0.03)	(0.31 ± 0.01)	(0.83 ± 0.03)
AMEE 100	1.30 ± 0.06	0.73 ± 0.02	1.33 ± 0.10	0.45 ± 0.01	1.39 ± 0.05
	(0.70 ± 0.03)	(0.39 ± 0.01)	(0.72 ± 0.06)	(0.24 ± 0.01)	(0.75 ± 0.03)
AMEE 300	1.49 ± 0.05	0.85 ± 0.02[a]	1.58 ± 0.24	0.49 ± 0.02	1.67 ± 0.08[a]
	(0.71 ± 0.02)	(0.40 ± 0.01)	(0.75 ± 0.12)	(0.23± 0.01)	(0.80 ± 0.05)
AMEE 500	1.67 ± 0.08	0.87 ± 0.03[a]	1.79 ± 0.23	0.58 ± 0.07	1.84 ± 0.07[a]

	(0.71 ± 0.04)	(0.37 ± 0.01)	(0.76 ± 0.10)	(0.25 ± 0.03)	(0.78 ± 0.03)
AMME 100	1.46 ± 0.16	0.66 ± 0.02[b]	1.28 ± 0.19	0.59 ± 0.17	1.02 ± 0.23[b]
	(0.81 ± 0.08)	(0.36 ± 0.00)	(0.71 ± 0.10)	(0.33 ± 0.10)	(0.56± 0.13)
AMME 300	1.38 ± 0.07	0.83 ± 0.04[a]	1.17 ± 0.07	0.50 ± 0.04	1.54 ± 0.05[a]
	(0.66 ± 0.03)	(0.40 ± 0.02)	(0.57 ± 0.04)	(0.24 ± 0.02)	(0.74 ± 0.03)
AMME 500	1.64 ± 0.06	0.87 ± 0.02[a]	1.25 ± 0.07	0.45 ± 0.04	1.64 ± 0.07[a]
	(0.73 ± 0.03)	(0.39 ± 0.01)	(0.56 ± 0.03)	(0.20 ± 0.01)	(0.73 ± 0.03)

[a] Significant difference with respect to Vehicle [b] Significant difference with respect to Toxin

TABLE OF STATISTICS

ANOVA Tables for subchronic toxicity studies

Table B2: Table Analyzed: Percent Weight Change of Female rats

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	35.75
R squared	0.7930

Bartlett's test for equal variances	
Bartlett's statistic (corrected)	1.913
P value	0.5907
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	816.1	3	272.0
Residual (within columns)	213.0	28	7.608
Total	1029	31	

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
Vehicle vs 2500 mg	-14.15	14.51	Yes	***
Vehicle vs 100 mg	-7.450	7.640	Yes	***
Vehicle vs 1000 mg	-5.650	5.794	Yes	***
1000 mg vs 2500 mg	-8.500	8.716	Yes	***
1000 mg vs 100 mg	-1.800	1.846	No	ns
100 mg vs 2500 mg	-6.700	6.871	Yes	***

Table B3: Table Analyzed: Percent Weight Change of Male rats

One-way analysis of variance	
P value	0.0107
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	4.496
R squared	0.3251

Bartlett's test for equal variances	
Bartlett's statistic (corrected)	9.975
P value	0.0188
P value summary	*
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	62.95	3	20.98
Residual (within columns)	130.7	28	4.666
Total	193.6	31	

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
2500 mg vs 100 mg	-3.535	4.629	Yes	*
2500 mg vs Vehicle	-3.294	4.313	Yes	*
2500 mg vs 1000 mg	-1.982	2.595	No	ns
1000 mg vs 100 mg	-1.553	2.034	No	ns
1000 mg vs Vehicle	-1.312	---	No	ns
Vehicle vs 100 mg	-0.2413	---	No	ns

Table B4: Table Analyzed: Female Feed Consumption

One-way analysis of variance	
P value	0.2168
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	4
F	1.594
R squared	0.1662

Bartlett's test for equal variances	
Bartlett's statistic (corrected)	5.290
P value	0.1517
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	0.0006745	3	0.0002248
Residual (within columns)	0.003385	24	0.0001410
Total	0.004059	27	

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
1000 mg vs Vehicle	-0.01319	2.939	No	ns
1000 mg vs 100 mg	-0.004049	---	No	ns
1000 mg vs 2500 mg	-0.003151	---	No	ns
2500 mg vs Vehicle	-0.01004	---	No	ns
2500 mg vs 100 mg	-0.0008979	---	No	ns
100 mg vs Vehicle	-0.009141	---	No	ns

Table B5: Table Analyzed: Female Water Consumption

One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	16.40			
R squared	0.6722			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	0.8906			
P value	0.8277			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	0.02843	3	0.009477	
Residual (within columns)	0.01387	24	0.0005778	
Total	0.04230	27		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
100 mg vs Vehicle	-0.08620	9.488	Yes	***
100 mg vs 1000 mg	-0.03092	3.403	No	ns
100 mg vs 2500 mg	-0.02986	[illegible]	No	ns
2500 mg vs Vehicle	-0.06634	7.192	Yes	***
2500 mg vs 1000 mg	-0.01006	[illegible]	No	ns
1000 mg vs Vehicle	-0.05528	6.085	Yes	***

Table B6: Table Analyzed: Male Feed Consumption

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	4
F	9.807
R squared	0.5507

Bartlett's test for equal variances	
Bartlett's statistic (corrected)	0.3223
P value	0.9558
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	0.001664	3	0.0005546
Residual (within columns)	0.001357	24	0.00005655
Total	0.003021	27	

Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
2500 mg vs Vehicle	-0.02012	7.079	Yes	***
2500 mg vs 100 mg	-0.01392	4.898	Yes	**
2500 mg vs 1000 mg	-0.005536	1.948	No	ns
1000 mg vs Vehicle	-0.01458	5.131	Yes	**
1000 mg vs 100 mg	-0.008385	2.950	Yes	*
100 mg vs Vehicle	-0.006199	2.181	No	ns

Table B7: Table Analyzed: Male Water Consumption

One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	4			
F	14.51			
R squared	0.6447			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	9.783			
P value	0.0205			
P value summary	*			
Do the variances differ signif. (P < 0.05)	Yes			
ANOVA Table	SS	df	MS	
Treatment (between columns)	0.02978	3	0.009927	
Residual (within columns)	0.01642	24	0.0006840	
Total	0.04620	27		
Newman-Keuls Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
2500 mg vs Vehicle	-0.09200	9.307	Yes	***
2500 mg vs 100 mg	-0.05182	5.242	Yes	**
2500 mg vs 1000 mg	-0.04835	4.891	Yes	**
1000 mg vs Vehicle	-0.04365	4.415	Yes	*
1000 mg vs 100 mg	-0.003468	0.3508	No	ns
100 mg vs Vehicle	-0.04018	4.065	Yes	**

Table B8: Percentage protection of *A. muricata* serial extracts

PERCENT PROTECTION OF SERIAL EXTRACT OF *ANNONA MURICATA*

MEANS

PERCENT PROTECTION

	RLW	ALT	AST	ALP	TB	IBIL	RLW	ALT	AST	ALP	TB	IBIL	MEAN PROTECTION	%
NORMAL	2.83	41.8	14	163.4	5.4	3.96								
CCl_4	3.76	63.6	24.2	225	9.6	7.08								
AMAE 100	3.12	35.2	12.2	162.8	5.8	5.62	68.817	130.275	117.647	100.974	90.476	46.794	**92.497**	
AMAE 300	3.14	35.4	12.2	165.4	3.4	3.24	66.666	129.357	117.647	96.753	147.619	123.076	**113.520**	
AMAE 500	2.91	36	12.2	151.2	6.4	6.02	91.397	126.605	117.647	119.805	76.190	33.974	**94.270**	
AMPE 100	3.2	47.17	14.5	178.83	10.33	9.85	60.215	75.366	95.098	74.951	-17.380	-88.782	33.244	
AMPE 300	3.39	39.5	14.83	174.6	8.17	7.92	39.784	110.550	91.862	81.818	34.047	-26.923	55.190	
AMPE 500	3.24	46	13.33	173.8	11.5	11.23	55.913	80.733	106.568	83.116	-45.238	-133.012	24.680	
AMEE 100	3.1	56	13	188.5	9.83	8.97	70.967	34.862	109.803	59.253	-5.476	-60.576	34.805	
AMEE 300	3.11	54	11.4	174.4	7.6	7.44	69.892	44.036	125.490	82.142	47.619	-11.538	59.607	
AMEE 500	2.96	55.4	12.6	169.8	8.4	8.28	86.021	37.614	113.725	89.610	28.571	-38.461	52.846	
AMME 100	3.31	53.5	13	179.67	8.17	8.05	48.387	46.330	109.803	73.587	34.047	-31.089	46.844	
AMME 300	2.94	58	12.2	167.4	9.6	9.44	88.172	25.688	117.647	93.506	0	-75.641	41.562	
AMME 500	3.03	58.4	14.6	171.8	10.4	10.26	78.494	23.853	94.117	86.363	-19.047	-101.923	26.976	

Table B9: Percentage protection of AMAE against CCl_4 and paracetamol-induced liver damage

PERCENT PROTECTION OF *ANNONA MURICATA* ON CCL_4-INDUCED LIVER DAMAGE

MEANS

	RLW	ALT	ALP	TB	IBIL	TC	TG
NORMAL	3.12	71.82	301.8	2.96	1.74	2.33	0.9
CCl_4	3.76	115.78	560.5	5.63	3.12	1.57	0.54
AMAE 50	2.94	62.05	343.25	3.13	1.76	2.87	0.59
AMAE 100	3.18	68.76	375.8	3.64	1.93	2.54	0.56
AMAE 200	3.26	61.45	329.5	2.75	1.98	2.53	0.8
AMAE 400	3.18	67.04	330.4	3.08	1.82	2.51	0.72
Sily	2.71	48.04	326.8	3.92	1.94	2.59	0.75

PERCENT PROTECTION

	RLW	ALT	ALP	TB	IBIL	TC	TG	MEAN % PROTECTION
AMAE 50	128.13	122.225	83.978	93.633	98.551	171.053	13.889	101.636
AMAE 100	90.625	106.961	71.395	74.532	86.232	127.632	5.556	80.419
AMAE 200	78.125	123.590	89.293	107.865	82.609	126.316	72.222	97.146
AMAE 400	90.625	110.874	88.945	95.506	94.203	123.684	50.000	93.405
Sily	164.06	154.095	90.336	64.045	85.507	134.211	58.333	107.227

PERCENT PROTECTION OF *ANNONA MURICATA* ON PARACETAMOL-INDUCED LIVER DAMAGE

MEANS

	RLW	ALT	ALP	TB	IBIL	TC	TG
NORMAL	3.12	69.16	301.8	2.96	1.74	2.33	0.9
PARA	3.51	122.78	669.8	4.26	2.58	2.19	0.71
AMAE 50	3.49	85.32	385.8	2.54	0.56	2.35	0.96
AMAE 100	3.21	70.52	578.6	3.12	0.68	2.27	0.93
AMAE 200	3.29	48.18	521.6	2.34	0.8	2.48	1.08
AMAE 400	3.33	42.2	431.4	2.82	0.84	2.71	0.97
Sily	3.14	49.78	445.8	2.84	0.72	2.41	0.96

PERCENT PROTECTION

	RLW	ALT	ALP	TB	IBIL	TC	TG	MEAN % PROTECTION
AMAE 50	5.128	69.862	77.174	132.308	240.48	114.286	131.58	110.11
AMAE 100	76.923	97.464	24.783	87.692	226.19	57.143	115.79	97.99
AMAE 200	56.410	139.127	40.272	147.692	211.91	207.143	194.74	142.46
AMAE 400	46.154	150.280	64.783	110.769	207.14	371.429	136.84	155.34
Sily	94.872	136.143	60.870	109.231	221.43	157.143	131.58	130.18

APPENDIX C

List of Publications

1. Arthur, F. K. N., Woode, E., Terlabi, E. O. and Larbie, C., (2011). Evaluation of acute and subchronic toxicity of *Annona muricata* (Linn.) aqueous extract in animals. *Euro. J. Exp. Bio.*; **1** (4): 115 – 124.

2. Arthur, F. K. N., Woode, E., Terlabi, E. O. and Larbie, C., (2012). Evaluation of hepatoprotective effect of aqueous extract of *Annona muricata* (Linn.) leaf against carbon tetrachloride and acetaminophen-induced liver damage. *Journal of Natural Pharmaceutical*; **3** (1): 38 – 43.

3. Arthur, F. K. N., Woode, E., Terlabi, E. O. and Larbie, C., (2012). Bilirubin lowering potential of *Annona muricata* (Linn.) in temporary jaundiced rats. *American Journal of Pharmacology and Toxicology*; 7 (2): 33 – 40.