MODULATION OF CARBON TETRACHLORIDE (CCL_4) AND ACETAMINOPHEN INDUCED LIVER DAMAGE IN RATS BY *MORINDA LUCIDA* BENTH. (RUBIACEAE).

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by

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

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DEDICATION

To my mother, Victoria Danquah, for the gift of life, love and the support.

ABSTRACT

Morinda lucida is a tropical West Africa rainforest tree also called Brimstone tree. The plant is employed in the traditional settings for the treatment of malaria and severe jaundice. The alcoholic leaf extract of the plant was investigated for its possible toxicity in experimental rats and its hepatoprotective activity against carbon tetrachloride and acetaminophen-induced liver damage, also in rats.

Administration of *Morinda lucida* extract (MLE) (250, 500, 1000, 2000, and 4000 mg/kg) to rats daily for two weeks did not cause significant changes in most of the haematological parameters assessed between the MLE treated groups and the control except for a significant increase in the mean corpuscular volume (MCV) at the 250 mg/kg. Also, there were no significant changes in the biochemical parameters assessed as well as the body weight and the selected organ weights (heart, spleen, liver and kidney).

In the pentobarbitone-induced sleeping time assessment, pentobarbitone at 50 mg/kg, administered one hour after the oral administration of MLE (250, 1000 and 4000 mg/kg) caused a dose dependent decrease ($P < 0.05$) in the rat sleeping time.

The hepatoprotective effect of the extract was assessed using two widely used models, CCl_4 and acetaminophen. Intraperitoneal administration of CCl_4 to rats caused severe liver damage as was indicated by the elevation of serum enzymes, bilirubin levels as well as the reduction in the protein levels. These effects were attenuated by the pre and post treatments with MLE and the effects were comparable to that of silymarin, used as the positive standard drug. A

histopathology study of the liver samples from the MLE treated rats showed recovery from the injury caused by CCl_4 to the toxin control group of rats whereas samples from the CCl_4 did not.

In the acetaminophen-induced liver damage, the effect of a single dose and multiple doses of the acetaminophen caused extensive liver damage, resulting in the elevation of the serum enzymes, bilirubin and a decrease in protein levels as well as damage to the hepatocytes as seen in the histopathology of the liver. Treatment with MLE assuaged this injurious effect caused by pre- and post-administration of acetaminophen by reducing the levels of the serum enzymes and bilirubin levels as well as increasing the protein levels. This effect was comparable to silymarin the standard positive drug used in the study.

Overall, this study has shown that leaves of Morinda lucida is safe up to a dose of 4000 mg/kg in rats, does not interfere with the normal functioning of the hepatic microsomal drug metabolizing enzymes (MDME) and also protects the liver from injury caused by CCl_4 and acetaminophen.

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ABBREVIATIONS

WHO – World Health Organizaton

ALT – Alanine transaminase

AST – Aspartate Transaminase

ALP – Alkaline phosphatase

GGT - Gamma glutamyl transpeptidase

ATP – Adenosine Triphosphate

NASH – Non Alcoholic Steatohepatitis

NAFLD – Non Alcoholc Fatty Liver Diseases

VLDL – Very Low Density Lipoprotein

HMGB1 – High Mobility Group Box-1

DAMPs – Damage-Associated Molecular Patterns

NAD – Nicotinamide Adenine Dinucleotide

NADP – Nicotinamide Adenine Dinucleotide Phosphate

DNA – Deoxyribonucleic Acid

CAD – Caspase-Activated DNase

APAP – Acetaminophen

CFCs – Chlorofluoro carbons

CCl_4 – Carbon Tetrachloride

CYP – Cytochrome P450

NAPQI – N-acetyl-p-benzoquinone

AIF – Apoptosis-inducing factor

H and E – Haematoxylin and eosin

PUFAs – Polyunsaturated Fatty Acids

TNF – Tumour Necrosis Factor

Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Plants have been used for medicinal purposes for as long as history has been recorded. Plants have been used in traditional medicine for several years. Either the whole plant, or a specific part of it (root, leaves, fruit, flowers, seeds), is formulated into suitable preparations – compressed as tablets or made into pills, used to make infusions (teas), extracts, tinctures, etc, or mixed with excipients to make lotions, ointments, creams etc. Few herbal drugs are subjected to legislative control. In Cherokee medicine, three categories of herbs are distinguished, namely; food herbs, medicinal herbs and poison herbs (Winston, 1992). The food herbs are gentle in action, have very low toxicity, and are unlikely to cause an adverse response. Examples include lemon balm, ginger, garlic, dandelion root and leaf, and fresh oat extract. These herbs can be utilized in substantial quantities over long periods of time without any acute or chronic toxicity. The second category is the medicine herbs. These herbs are stronger acting – they need to be used with greater knowledge (dosage and rationale for use) for specific conditions (with a medical diagnosis) and usually for a limited period of time. These herbs are not daily tonics and they should not be taken just because they are good. These herbs have a greater potential for adverse reaction and in some cases, drug interactions. The medicine herbs include andrographis, blue cohosh, *Cascara sagrada.* The last category is the poison herbs. These herbs have strong potential for either acute or chronic toxicity and should

only be utilized by clinicians who are trained to use them and clearly understand their toxicology and appropriate use (Winston, 1992).

The World Health Organization (WHO) estimates that 4 billion people, eighty percent (80%) of the world population presently use herbal medicine for some aspect of primary health care (WHO, 1993). Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental, and Native American Indian medicine. WHO notes that 119 plant-derived pharmaceutical medicines, about seventy four percent (74%) are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value (Kumar *et al.*, 2011).

The use of herbal medicines has certain advantages. Herbal medicines are thought to be more effective for long- standing health complaints that do not respond well to traditional medicine. Herbs typically have fewer side effects, and may be safer to use over time (Grunert, 2011). Another advantage is cost. Herbs cost much less than prescription medications. Research, testing and marketing add considerably to the cost of prescription medicines and their availability. Herbs are available without a prescription, and some simple herbs, such as peppermint and chamomile, can be grown at home. In some remote parts of the world, herbs may be the only treatment available to the majority of people. Many find the easy availability of herbs appealing.

Herbs are not without some disadvantages. Modern medicine treats sudden illness and accidents much more effectively than herbal or alternative treatments (Grunert, 2011). Another disadvantage of herbal medicine is the very risks of doing oneself harm through self-dosing with herbs.

1.2 MORINDA LUCIDA BENTH.

1.2.1 DESCRIPTION

Figure 1: Leaves of *Morinda lucida*

Morinda lucida Benth. belongs to the coffee family Rubiaceae. It is a tropical West Africa rainforest tree also called Brimstone tree. In Ghana, it is called *Konkroma* in Twi and *Amake* in Ewe. It is an evergreen shrub or small to medium-sized tree up to 18-25 m high. It has a characteristic yellow wood from which it derived its name ‘‘brimstone tree’’. It has bole and branches often crooked or gnarled, slender branchlets and a dense crown. The bark is smooth to roughly scaly, grey to brown, often with some distinct purple layers (Abbiw, 1990). The leaves

are broadly elliptical to broadly ovate, acuminate, and entire, about 20 x 15 cm. Also the leaves are opposite, simple and entire; stipulate ovate or triangular, 1-7 mm long, falling early; petiole up to 1.5 cm long; blade elliptical, 6-8 cm x 2.9 cm, base rounded to cuneate, apex acute to acuminate, shiny above, sometimes finely pubescent when young, later only tufts of hairs in vein axils beneath and some hairs on the midrib. Inflorescence is a stalked head, 4-7 mm in diameter, 1-3 at the nodes opposite a single leaf. The peduncle is up to 8 cm long at base a stalked cup-shaped gland. Flowers are bisexual, regular, 5-merous, heterostylous and fragrant. The calyx is cup-shaped, c. 2 mm long, persistent while the corolla is salver-shaped, c.2.5 mm. The ovary is inferior and 2-celled. The style is 8-11 mm long with 2 stigma lobes 4-7 mm long. The stamens are 5, inserted in corolla throat, with short filaments. The fruit is a drupe, several together arranged into an almost globose succulent syncarp 1-2.5 cm in diameter which is soft and black when mature, pyrene compressed ovoid, up to 6.5 mm x 4 mm, dark red-brown, very hard, 1-seeded. Seed ellipsoid, c. 3.5 mm x 2 mm x 0.5 mm, yellow, soft (Burkhill, 1997).

Morinda lucida produces white fragrant flowers from January – July and September – October. It fruits in March – April. (Irvin, 1961).

Morinda lucida grows in grassland, exposed hillsides, thickets, forests, and often on termite mounds, sometimes in areas which are regularly flooded, from sea-level up to 1300 m altitude. The species also occur in fringe forests and sometimes it takes over secondary clearings in rain forests.

1.2.2 TRADITIONAL USES OF MORINDA LUCIDA BENTH.

1.2.2.1 Non-medicinal uses

The wood of *Morinda lucida yields* yellow to red dyes. In Nigeria and Gabon the root bark is used to dye textiles into scarlet red. On occasions of national grief or the death of a chief, the Ashanti people of Ghana dye cotton red with the root bark of *Morinda lucida*. These cloths, called 'Kobene', are worn as mourning dress by official people and by the family of the deceased. The root is the most important traditional source of yellow dye for textiles in the Kasai province of Democratic Republic of Congo (DR Congo). It can be used without a mordant. The root is also added to indigo vats in Cote d' Ivoire, to contribute both to the fermentation and reduction process necessary for dyeing with indigo and to get darker blues. In the process, it is often combined with leafy twigs of *Saba comorensis* (Bojer) Pichon (synonym; *Saba florida* (Benth.) Bullock). In the region of Kasongo in northern DR Congo, young leaves of *Morinda lucida* are combined with leaves of *Philenoptera species* (a source of indigo) to obtain a pale green dye used in baskets weaving. The bitter-tasting roots are used as flavouring for food and alcoholic beverages in Nigeria and they are also popular as chewing sticks. The wood is excellent for making charcoal but is also used for cleaning and scouring, e.g. of calabashes (Burkhill, 1997).

1.2.2.2 Traditional medicinal uses

In West Africa *Morinda lucida* is an important plant in traditional medicine. The leaves are used in the preparation of fever teas, which are used not only for the treatment of malaria but as a general febrifuge and analgesic. All parts of the plant are used as laxative. A weak decoction

of the stem bark is administered for the treatment of severe jaundice, often characterized by haemoglobinura and haematura (Oliver-Bever, 1986). The treatment induces vomiting, diarrhoea, and dieresis, and cure is determined from the clearance of yellow colouration of the urine. The extract of the leaves and stem bark has been recommended for the prevention and treatment of hypertension and its cerebral complication (Van Ho, 1955). It is widely used in the treatment of malaria (Watt and Breyer-Brandwijk, 1962) and diabetes (Kamenyi *et al.,* 1994) in sub-Saharan Africa. In Nigeria, the plant is used for the treatment of malaria, typhoid fever, jaundice and dressing of wounds to prevent infections (Akinmoladun *et al.,* 2010). The leaves are used as "oral teas", which are usually taken orally for the traditional treatment of malaria, and as a general febrifuge, analgesic, laxative and as anti-infections (Makinde and Obih, 1985, Raji *et al.,* 2005). The leaves have been reported to possess strong trypanocidal and aortic vasorelaxant activities (Asuzu and Chineme, 1990, Ettar and Emeka, 2004). Decoctions and infusions or plasters of root, bark and leaves are recognized remedies against different types of fever, including yellow fever, malaria, trypanosomiasis and feverish condition during childbirth. The plant is also employed in cases of diabetes, hypertension, cerebral congestion, dysentery, stomach-ache, ulcers, leprosy and gonorrhoea. In Cote d'Ivoire a bark or leaf decoction is applied against jaundice and in DR Congo it is combined with a dressing of powdered root bark against itch and ringworm (Irvin, 1961).

1.2.3 CHEMICAL CONSTITUENTS OF MORINDA LUCIDA

From the wood and bark of *morinda lucida,* eighteen (18) anthraquinones have been isolated, including the red colourants 1-methylether-alizarin, rubiadin and derivatives, lucidin, soranjidiol, damnacanthal, nordamnacanthal, morindin, munjistin and purpuroxanthin. Two anthraquinols, oruwal and oruwalol, have also been found (Adewumi and Adesogan, 1984), these give a yellow colour and possibly are intermediates in the formation of anthraquinones. In addition to anthraquinones, tannins, flavonoids, and saponosides have also been isolated (Adesogan, 1973).

1.2.4 PHARMACOLOGICAL EFFECTS OF MORINDA LUCIDA

Tests with animals confirm the attributed activity of several traditional medicinal applications of *Morinda lucida.* The extract of the leaf and stem bark have been shown to posses strong but short acting antihypertensive activity. This therapeutic activity may be due to its pronounced diuretic and tranquilizing properties (Jansen and Cardon, 2005). Due to its apparent lack of acute toxicity, the drug is recommended for chronic treatment of hypertension and cases requiring high doses or frequent medication to arrest elevated blood pressure (La Barre and Weitheimer, 1962). The anthraquinone fraction has been shown to posses' molluscкcidal (against *fasciola sp*. and *schistosoma sp.)* properties and the activity may be due to oruwacin (Adewumi and Adesogan, 1984). Acqueous alcohol extracts of the leaves showed significant activity against *Trypanosoma brucei* infection in mice at a dose of 1000 mg/kg given intraperitoneally (Asuzu and Chineme, 1990). The extract was also found to posses' purgative activity and the LD_{50} was calculated to be 2000 mg/kg (Olajide *et al.*, 1998). The leaf extract

demonstrated significant schizonticidal activity against *Plasmodium bergei* in mice (Makinde and Obih, 1985; Obih *et al.,* 1985). Extracts showed anti-inflammatory, anti-fever and pain reducing activity in tests with rats (Awe *et al.,* 1998) and promoted gastric emptying and intestinal motility (Olajide *et al*., 1998). Leaf extracts showed *in vitro* anti-malarial activity against *Plasmodium falciparum* while in several other tests anti-diabetic properties were confirmed (Adeneye and Agbaje, 2008). Inhibiting effects on cancer tumours in mice have also been reported. A leaf extract gave 100% mortality in the freshwater snail *Bulinus globulus* at a concentration of 1000 ppm (Jansen and Cardon, 2005).

1.3 HEPATOTOXICITY

1.3.1 The Liver

1.3.1.1 General Description

A large, reddish-brown, glandular vertebrate organ located in the upper right portion of the abdominal cavity, resting just below the diaphragm. The liver lies to the right of the stomach and overlies the gall bladder. The liver accounts for approximately 2.5% of the total body weight. The liver is the largest gland in the human body that secretes bile and is active in the formation of certain blood proteins and in the metabolism of carbohydrates, fats and proteins. The liver is a unique organ anatomically located to serve its dual role as a metabolic and biochemical transformation 'factory'. The liver receives blood containing substances absorbed or secreted by the gastrointestinal organs including the spleen, intestines, stomach and pancreas. It uses these substances as raw materials and modifies them or synthesizes new

chemicals. These are then returned to the blood stream or to the bile for excretion. The liver is divided into four lobes. The right and left lobes are separated by the falciform ligament.

1.3.1.2 Structural Organization of the Liver

Two concepts exist for organization of the liver into operational units, namely, the lobule and the acinus (McCuskey, 2005b). The liver is divided into hexagonal lobules oriented around terminal hepatic venules (also known as central veins). At the corners of the lobule are the portal triads, containing a branch of the portal vein, a hepatic arteriole, and a bile duct. Blood entering the portal tract via the portal vein and hepatic artery is mixed in the penetrating vessels, enters the sinusoids, and percolates along the cords of parenchymal cells (hepatocytes), eventually flows into terminal hepatic venules, and exits the liver via the hepatic vein. The lobule is divided into three regions known as centrolobular, midzonal, and periportal.

The acinus is the preferred concept for a functional hepatic unit. The terminal branches of the portal vein and hepatic artery, which extends out from the portal tracts, from the base of the acinus. The acinus has three zones: zone 1 is closest to the entry of blood, zone 3 abuts the terminal hepatic vein, and zone 2 is intermediate.

Despite the utility of the concept, lobular terminology is still used to describe regions of pathologic lesions of hepatic parenchyma. Fortunately, the three zones of the acinus roughly coincide with the three regions of the lobule (Harmut, 2008).

1.3.1.3 Functions of the Liver

The liver has three main functions: storage, metabolism, and biosynthesis. Glucose is converted to glycogen and stored; when needed for energy, it is converted back to glucose. Cholesterol uptake also occurs in the liver. Fat, fat-soluble vitamins and other nutrients are also stored in the liver. Fatty acids are metabolized and converted to lipids, which are then conjugated with proteins synthesized in the liver and released into blood stream as lipoproteins. Numerous functional proteins such as, enzymes and blood-coagulating factors are also synthesized by the liver. In addition, the liver, which contains numerous xenobiotic metabolizing enzymes, is the main site of xenobiotic metabolism (Hogson and Levi, 2004)

1.3.1.4 Susceptibility of the Liver to Toxicants

The liver, the largest organ in the body, is often the target organ for chemically induced injuries. Several important factors are known to contribute to the liver's susceptibility. First, most xenobiotics enter the body through the gastrointestinal tract and, after absorption, are transported by the hepatic portal vein to the liver. The liver has a high concentration of binding sites. It also has a high concentration of xenobiotic-metabolizing enzymes (mainly cytochrome P-450) (Hogson and Levi, 2004), which render most toxicants less toxic and more water-soluble, and thus more readily excretable. But in some cases the toxicants are activated to reactive metabolites to be capable of inducing lesions. The fact that hepatic lesions are often centrolobular has been attributed to the higher concentration of cytochrome P-450 there. In addition, the relatively lower concentration of glutathione there, compared to that in other parts of the liver, may also play a role (Smith *et al.*, 1979).

1.3.2 Mechanisms and types of Toxin – Induced Liver Injury

Several factors contribute to a high incidence of toxicity in the liver. These include remarkable exposure to high drug concentrations, enormous metabolic activity and the presence of several enzymes held responsible for generation of reactive metabolites, most notably reactive oxygen species (ROS). Possible targets of toxic substances are

- macro molecular structures or individual molecules such as the bile acid transporters.
- members of the nuclear receptor family
- intracellular lipids
- proteins
- nucleic acids.

These targeted molecules become dysfunctional units and activate secondary pathways to result in programmed events, such as apoptosis, necrosis and autophagy, mitochondrial failure and immunological reactions (Kaplowitz, 2002; Lee and Ferguson, 2003). In addition, the functional integrity of a cell can also be disturbed by direct cytolytic reactions, membrane disruption and distortion of trans-membrane transport mechanisms. Even though several different mechanisms might be involved in the onset and progression of hepatotoxicity of a single substance, there are likely to be only a few central mechanisms that are activated within the general toxic response of the liver.

The development of toxic liver injury follows a two-staged course; the first phase is characterized by initiation of the injury and may involve direct interaction with a toxicant. This

phase may exhibit dose-dependency (Mehendale and Limaye, 2005). In contrast, the second phase is characterized by progress of the injury in a toxicant-independent fashion that is dominated by secondary events. Among the mechanisms contributing to phase two of toxic liver injury, the three leading mechanisms proposed are contribution of

- inflammatory cells (Czaja *et al.*, 1994; Laskin and Pendino, 1995; Piguet *et al.*, 1990)
- oxidative stress and lipid peroxidation (Poli, 1993; Slater, 1984)
- leakage of degrading enzymes (Mehendale and Limaye, 2005; Poli *et al.*, 1987)

Furthermore, tissue repair has been determined to be one of the major factors that influence the fate of the liver after toxic injury, which may either be directed towards regeneration or loss of function and necrosis. Therefore, the toxicological response to different toxicants to a significant part obeys similar principles and may be initiated by mechanisms, although the acute and first phase of liver injury may be initiated by mechanisms specific to the toxicant. This may explain why the morphology of toxic or drug-related liver injury usually differentiates into one of the following four phenotypes:

- hepatocellular injury, often associated with elevated liver enzymes (e.g. isoniazid)
- cholestatic injury, which is due to damage of the bile duct epithelia displays predominantly elevated alkaline phosphatase (ALP) (e.g., amoxicillin-clavunic acid)
- mitochondrial injury involving microvesicular steatosis (e.g. valproic acid)
- the often delayed immunologic injury that is associated with fever, rash or eosinophilia (e.g. phenytoin) (Navarro and Senior, 2006).

Some drugs, as indicated by the examples given in parenthesis, typically cause certain morphologies, but do not have a specific correlation to them. Thus, they can induce a mixed injury that is characterized by more than one feature of the different morphologic phenotypes. It is not known why certain drugs induce certain morphologies of the liver injury but it is likely that these drugs share similar mechanisms of initiating toxicity in the liver.

In the following, mechanisms recognized in different types of liver injury will be discussed, and general features of toxicity will be portrayed in detail.

1.3.2.1 Cholestatic Liver Injury

This form of liver dysfunction is defined physiologically as a decrease in the volume of bile formed or an obstruction of bile flow or an impaired secretion of specific solutes into bile. Cholestasis is characterized biochemically by elevated serum levels of compounds normally concentrated in bile, particularly bile salts and bilirubin. When biliary excretion of the yellowish bilirubin pigment is impaired, this pigment accumulates in skin and eyes, producing jaundice, and spills into urine, which becomes bright yellow or dark brown. This is because drug-induced jaundice reflects a more generalized liver dysfunction, it is considered a more serious warning sign in clinical trials than mild elevation of liver enzymes (Zimmerman, 1999).

The mechanism underlying cholestatic liver injury has been linked to an impairment of bile salt transport by inhibition or downregulation of ATP-dependent bile salt transporters and also to alterations of actin resulting in disruption of the cytoskeleton and an impaired transport of bile along the canalicular system and into the bile ducts (Cullen, 2005). Interaction with bile acid

transporters is a mechanism that has been observed under administration of several drugs and toxicants leading to toxic liver injury and is believed to be a central event in the development of cholestatic liver injury (Lewis, 2000)

1.3.2.2 Fatty Liver (Steatosis)

Fatty liver (steatosis) is defined biochemically as an appreciable increase in the hepatic lipid (mainly triglyceride) content in the hepatocytes, which is less than five percent (5%) by weight in normal human liver. At the same time there is a decrease in plasma lipids and lipoproteins. The term hepatic steatosis also refers to an intracellular accumulation of lipid droplets in the cytoplasm.

Steatosis is of two types. Primary steatosis is often observed in patients displaying symptoms of the metabolic syndrome including obesity, diabetes, hypertriglycerinaemia and insulin resistance. Secondary hepatic steatosis is extrinsically induced by alcohol, several drugs, copper accumulation in Wilson's disease and other factors (Pessayre *et al.,* 2001).

Histologically, in standard paraffin-embedded and solvent-extracted sections, hepatocytes containing excess fat appear to have multiple round, empty vacuoles that displace the nucleus to the periphery of the cell. Hepatic steatosis may also be accompanied by hepatocellular necrosis, inflammation and fibrosis, in which case it is termed as 'non-alcoholic steatohepatitis' (NASH). The morphology of steatosis has been linked to primary mitochondrial failure and has been implicated for microvesicular steatosis, non-alcoholic steatohepatitis (NASH) and cytolytic hepatitis (Fromenty and Passayre, 1995).

Currently, the most common cause of hepatic steatosis is insulin resistance due to central obesity and sedentary life style (Jaeschke, 2008). However, acute exposure to many hepatotoxicants, e.g., carbon tetrachloride, and drugs can induce steatosis (Zimmerman, 1999). Compounds that produce prominent steatosis associated with lethality include the antiepileptic drug valproic acid (Scheffner *et al.,* 1988) and the antiviral drug fialuridine (Honkoop *et al.,* 1997). Ethanol is by far the most relevant drug or chemical leading to steatosis in humans and experimental animals. Although steatosis alone may be benign, it can develop into steatohepatitis (alcoholic or non-alcoholic), which is associated with significant liver injury (Farrel, 2002; Passayre *et al.,* 2002; Stravitz and Sanyal, 2003; Neuschwander-Tetri, 2006; Saito *et al.,* 2007). Steatohepatitis can progress to fibrosis and even hepatocellular carcinoma. Livers with steatosis are more susceptible to additional insults such as hepatotoxins (Donthamsetty *et al.*, 2007) or hepatic ischemia (Selzner and Clavien, 2001).

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 over supply 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). As might be expected, there are a number of points at which this process can be disrupted. Some of the more important ones are as follows:

- interference with synthesis of the protein moiety
- impaired conjugation of triglycerides with lipoprotein

- interference with transfer of VLDL across cell membranes
- decreased synthesis of phospholipids
- impaired oxidation of lipids by mitochondria
- inadequate energy (adenosine triphosphate [ATP] for protein and lipid synthesis.

1.3.2.3 Cell Death

Based on morphology, liver can be destroyed or damaged by two different modes, oncotic necrosis (necrosis) or apoptosis.

1.3.2.3.1 Liver 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 (including 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 (Hogson and Levi, 2004). Necrosis is characterized by cell swelling, leakage of cellular contents, nuclear disintegration, and an influx of inflammatory cells (Jaeschke, 2008). Because necrosis is generally the result of an exposure to toxic chemicals or other traumatic conditions, e.g., ischemia, large numbers of contiguous

hepatocytes and nonparenchymal cells may be affected. Cell contents released during oncotic necrosis includes proteins such as a high mobility group box-1 (HMGB1) and other alarmins, which are a subset of the larger class of damage-associated molecular patterns (DAMPs) (Bianchi, 2007). These molecules are recognized by cells of the innate immune system including Kupffer cells through their toll-like receptors and trigger cytokine formation, which orchestrate the inflammatory response after tissue injury (Scaffidi *et al.,* 2002). Thus, an ongoing oncotic necrotic process can be identified by the release of liver-specific enzymes such as alanine (ALT) or aspartate (AST) aminotransferases into the plasma and by histology, where areas of necrosis with loss of nuclei and inflammatory infiltrates are easily detectable in haematoxylin and eosin (H&E) sections. Disturbance of Ca^{2+} homeostasis may also play an important role through an activation of molecular oxygen resulting in an oxidative stress (Thomas and Reed, 1989). Other biochemical changes include depletion of adenosine triphosphate (ATP), shifts of the Na^{+} and K^{+} balance between hepatocytes and blood, depletion of glutathione, damage to cytochrome P-450, and loss of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Kulkarni and Hogson, 1980). As a result 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.

1.3.2.3.2 Apoptosis

Apoptosis is a controlled form of cell death that serves as a regulation point for biological 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 is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, and generally a lack of inflammation. The characteristic morphological features of apoptosis are caused by the activation of caspases, which trigger the activation of enzymes such as caspase-activated DNase (CAD) responsible for internucleosomal DNA fragmentation (Nagata *et al.*, 2003). In addition, caspases can directly cleave cellular and nuclear structural proteins (Fischer *et al.*, 2003). Apoptosis is always a single cell event with the main purpose of removing cells no longer needed during development or eliminating aging cells during regular tissue turnover. Under these conditions, apoptotic bodies are phagocytosed by Kupffer cells or taken up by neighbouring hepatocytes. In the absence of cell contents release, the remnants of apoptotic cells disappear without causing an inflammatory response. Because of effective regeneration, apoptotic cell death during normal tissue turnover or even a moderately elevated rate of apoptosis is of limited pathophysiological relevance in the liver. However, if the rate of apoptosis is substantially increased, the apoptotic process cannot be completed. In this case, cells undergo secondary necrosis with breakdown of membrane potential, cell swelling, and cell contents release (Ogasawara *et al.*, 1993).

1.3.2.4 Cirrhosis / Fibrosis

Fibrosis is a progressive disease that occurs in response to chronic injury and is characterized by the accumulation of excessive amounts of fibrous tissue, specifically fibril forming collagens type I and III, and a decrease in normal plasma membrane collagen type IV.

Formation of so-called pseudo-septa and regeneration nodules leads to a diversion of the blood flow and impairs supply with nutrients and oxygen. Furthermore, toxic liver fibrosis is accompanied by both bile ductular proliferation and inflammation under various conditions (Muller *et al.,* 1996). Fibrosis can develop around central veins and portal tracts or within the space of Disse. The excessive extracellular matrix protein deposition and the loss of sinusoidal endothelial cell fenestrae and of hepatocyte microvilli limit exchange of nutrients and waste material between hepatocyte and sinusoidal blood. With continuing collagen deposition, the architecture of the liver is disrupted by interconnecting fibrous scars. When the fibrous scars subdivide the remaining liver mass into nodules of regenerating hepatocytes, fibrosis has progressed to cirrhosis and the liver has limited residual capacity to perform its essential functions. The primary cause of hepatic fibrosis/cirrhosis in humans worldwide is viral hepatitis. However, biliary obstruction and in particular alcoholic and non-alcoholic steatohepatitis are of growing importance for the development of hepatic fibrosis (Bataller and Brenner, 2005). In addition, fibrosis can be induced by heavy metal overload (Gutierrez-Ruiz and Gomez-Quiroz, 2007).

1.3.2.5 Tumours

Hepatocellular carcinoma and cholangiocarcinoma are the most common types of primary malignant neoplasms of the liver. Other types include angiosarcoma, glandular carcinoma, and undifferentiated liver cell carcinoma. Hepatocellular cancer has been linked to chronic abuse of androgens, alcohol, and a high prevalence of aflatoxin-contaminated diets. In addition, viral hepatitis, metabolic diseases such as hemochromatosis and α 1-antitrypsin deficiency, and non-

alcoholic steatohepatitis are major risk factors for hepatocellular carcinoma (Zimmerman, 1999; McKillop *et al.,* 2006; Wands and Moradpour, 2006). The

synergistic effect of co-exposure to aflatoxin and hepatitis virus B is well recognized (Henry *et al*., 2002).The prevalence of hepatitis B and C viruses and environmental factors make hepatocellular carcinoma one of the most common malignant tumours worldwide (Bosch *et al.,* 2005).

The molecular pathogenesis of the hepatocellular carcinoma is complex and poorly understood. The malignant transformation of hepatocytes occurs as a result of increased cell turnover due to chronic liver injury, persistent inflammation, regeneration, and cirrhosis (Wands and Moradpour, 2006). Direct DNA binding of carcinogens or their metabolites (e.g. aflatoxin metabolites) or indirect DNA modifications by reactive oxygen species generated during inflammation and cell injury can lead to genetic alterations in hepatocytes resulting in impaired DNA repair, the activation of cellular oncogens, and inactivation of tumour suppressor genes. An overall imbalance between stimulation of proliferation and inhibition of apoptosis in the liver leads to the survival and expansion of these preneoplastic cells (Fabregat *et al.,* 2007).

1.3.3 Hepatotoxicants

1.3.3.1 Carbon Tetrachloride (CCl_4)

Carbon tetrachloride is an industrial chemical that does not occur naturally. Most of the carbon tetrachloride produced is used in the production of chlorofluorocarbons (CFCs) and other chlorinated hydrocarbons. It was once used widely as a solvent, cleaner and degreaser, both for industrial and home use. Today, the scientific database on the effects of haloalkanes is so vast

that it is no longer employed for such purposes although it is used as a model of experimental liver injury (Weber *et al.,* 2003).

CCl_4 is a well known hepato-and nephrotoxicant (Cassilas and Ames, 1986; Kotsanis and Metcalfe, 1991; Thrall *et al.,* 2000; Ogeturk *et al.,* 2005), and proves highly useful as an experimental model for the study of certain hepatotoxic effects (Muriel *et al.,* 2003; Moreno and Muriel, 2006). CCl_4-induced toxicity, depending on dose and duration of exposure, covers a variety of effects. At low doses, transient effects prevail, such as loss of Ca^{2+} homeostasis (Muriel and Mourelle, 1990), lipid peroxidation (Muriel, 1997), release of noxious or beneficial cytokines (Kyung-Hyun *et al.,* 2006; Muriel, 2007) and apoptotic events followed by regeneration. Other effects, with higher doses or longer exposure, are more serious and develop over a long period of time, such as fatty degeneration, fibrosis, cirrhosis and even cancer (Weber *et al.,* 2003). In addition, acute intoxication with CCl_4 at high doses, when the hepatocellular necrosis exceeds the regenerative capacity of the liver, fatal liver failure will ensue. Extreme doses of CCl_4 result in nonspecific solvent toxicity, including central nervous system depression and respiratory failure and death (Berger *et al.,* 1986).

CCl_4 metabolism begins with the formation of the trichloromethyl free radical, $CCl^{\cdot}_3$ (McCay *et al.,* 1984) through the action of the mixed function cytochrome P-450 oxygenase system of the endoplasmic reticulum (Recknagel *et al.,* 1989). This process involves reductive cleavage of a carbon-chlorine bond. Free radical activation of CCl_4 in mitochondria has also been observed (Tomasi *et al.,* 1987) and may contribute significantly to its toxicity. The major cytochrome isoenzyme to execute biotransformation of CCl_4 is cytochrome P450 isoenzyme 2E1

(CYP2E1). This is evidenced by the absence of toxicity in CYP2E1 knockout mice (Wong *et al.*, 1998). In humans, CYP2E1 dominates CCl_4 metabolism at environmentally relevant concentrations, but at higher concentrations other cytochromes, particularly CYP3A, also contribute importantly (Zanger *et al.*, 2000). The $CCl^{\cdot}_3$ radical reacts with several important biological substances, like fatty acids, proteins, lipids, nucleic acids and amino acids (Weber *et al.*, 2003). $CCl^{\cdot}_3$ also acts by abstracting hydrogen from unsaturated fatty acids to form chloroform. DNA adducts is a mechanism for CCl_4-induced carcinogenesis (DiRenzo *et al.*, 1982).

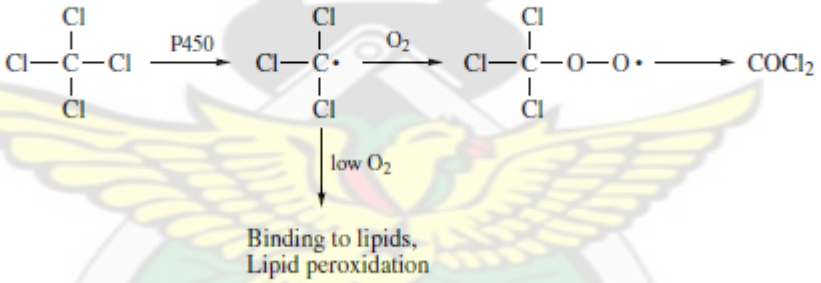

Figure 2: Metabolism of carbon tetrachloride and formation of reactive metabolites. From P.E. Levi, *A Textbook of Modern Toxicology*, 2nd ed., Appleton and Lange, 1997.)

1.3.3.1.1 Lipid Peroxidation

The $CCl^{\cdot}_3$ radical in the presence of oxygen, is transformed into trichloromethyl peroxy radical CCl_3OO. This radical is more reactive and thus shorter lived than the $CCl^{\cdot}_3$ radical (Mico and Pohl, 1983). The lifetime of the CCl_3OO radical is in the millisecond range and disappears from the tissue by reacting with suitable substrates to complete its electron pair. CCl_3OO is more likely than $CCl^{\cdot}_3$ to abstract hydrogen from polyunsaturated fatty acids (PUFAs) (Forni *et*

al., 1983) leading to lipid peroxidation (Comporti, 1985; Trible *et al.,* 1987). The abstraction of hydrogen of PUFAs starts sequential reactions that finish in the complete disintegration of the fatty acid molecule with the consequent formation of aldehydes, other carbonyls and alkanes in a process called lipid peroxidation. CCl_4-induced lipid peroxidation increases the permeability of the plasma membrane to Ca^{2+}, leading to severe disturbances of the calcium homeostasis and necrotic cell death (Weber *et al.,* 2003). In addition, the $CCl^{\cdot}_3$ radical can directly bind to tissue macromolecules and some of the lipid peroxidation products are reactive aldehydes, e.g. 4-hydroxynonenal, which can form adducts with proteins (Weber *et al.,* 2003).

Figure 3: Schematic illustrating lipid peroxidation and destruction of membrane. (From P.E. Levi, *A Textbook of Modern Toxicology*, 2nd ed., Appleton and Lange, 1997.)

1.3.3.1.2 Aldehyde Toxicity

The products of lipid peroxidation may produce further liver damage (Esterbauer *et al.,* 1982, 1991). They may inhibit enzymes (Hruszkewycz *et al.,* 1978), damage DNA (Ueda *et al.,* 1985), and block lipoprotein secretion. Reactive aldehydes first appear in liver about six hours after CCl_4 intoxication, show a maximum at twenty four hours and disappear by thirty six to seventy two hours later (Hartley *et al.,* 1999). Aldehydes are less reactive than $CCl^{\cdot}_3$ or

CCl_3OO, and thus they may reach other organs. However, they do not usually reach toxic concentrations. This is because of their unspecific action because they react typically with amino or sulfhydryl groups, and so a large number of groups need to be inactivated before measurable damage occurs (Sundari and Ramakrishna, 1997). The activities of aldehydes displaying a 4-hydroxy-2, 3-*trans*-unsaturated configuration and especially of 4-OH-2, 3-*trans*-nonenal, is favoured, and they are formed in high concentrations after exposure of cells to CCl_4 (Benedetti *et al.,* 1980, 1984). These compounds inhibit several enzymes, including adenylate cyclase, at pathophysiological concentrations (Poli *et al.,* 1989). There is also evidence that 4-hydroxynonenal decreases cytochrome P450 (Poli *et al.,* 1989) and inhibits the activities of Ca^{2+} (Parola *et al.,* 1990) and Na^+, K^+ (Morel *et al.,* 1998), ATPases, and phosphatase and protein kinase activities (Omura *et al.,* 1999). The effect of CCl_4-derived haloalkanes on these enzyme activities may contribute to CCl_4-induced toxicity.

1.3.3.2 Acetaminophen

One of the most widely used analgesics; acetaminophen (APAP) is a safe drug when used at therapeutically recommended doses. However, an overdose can cause severe liver injury and even liver failure in experimental animals and in humans (Lee, 2004). Acetaminophen at therapeutic doses is rapidly metabolized in the liver principally through glucuronidation and sulfation, and only a small portion is oxidised by cytochrome P450 2E1 to generate a highly reactive and cyrotoxic intermediate, *N*-acetyl-p-benzoquinone (NAPQI) (Lee *et al.,* 1996, Vermuulen *et al.,* 1992) which is quickly conjugated by hepatic glutathione to yield a harmless water-soluble product, mecapturic acid.

When an overdose occurs, a fraction of APAP is activated metabolically to the reactive metabolite *N*-acetyl-p-benzoquinone imine (NAPQI) that first consumes cellular glutathione and then binds covalently to proteins. This leads to increases in the intracellular Ca^{2+} concentration, 'Bax' and 'Bid' translocation to the mitochondria and oxidant stress and peroxynitrite formation in the mitochondria. Reactive oxygen species and peroxynitrite induce the membrane permeability transition that causes the collapse of the mitochondrial membrane potential and abolishes ATP synthesis. In fact, some free radical scavengers prevent experimental APAP induced liver injury (Muriel *et al.*, 1992; Muriel, 1997). The decreasing of ATP levels appears to prevent caspase activation by the release of cytochrome c and Smac. The apoptosis-inducing factor, (AIF) (Susin *et al.*, 2000) and endonuclease G (van Loo *et al.*, 2001) induce chromatin condensation and nuclear DNA fragmentation, respectively. The extensive DNA damage and the rapid elimination of mitochondria, together with activation of intracellular proteases (calpains), conduce to plasmatic membrane breakdown and oncotic necrosis of the hepatic cells. The massive cell death and liver failure after APAP intoxication can be explained by these intracellular events. Laboratory studies may show evidence of hepatic necrosis with transaminases, bilirubin, and prolonged coagulation times, particularly elevated prothrombin time (Bartlett, 2004). The hepatotoxicity may precipitate jaundice and coagulation disorders and progress to encephalopathy, coma and death.

1.4 MANAGEMENT OF LIVER DISEASES

1.4.1 Epidemiology of Liver Diseases

The World Health Organization has estimated 170 million worldwide are infected with hepatitis C (WHO, 1997). An estimated 8-10 thousand death occurs annually in the United States as a result of hepatitis C-related liver disease compared to 16,685 AIDS death in 1997 (Birth and Death, 1997). Non alcoholic fatty liver diseases (NAFLD) affects 10 – 24% of the general population from different countries. The prevalence of NAFLD, however, increases significantly to 57.5–74% (Ballentani *et al.,* 2000, Luyckx *et al.,* 1998) in obese individuals. NAFLD affects 2.6% of children (Tominaga *et al.,* 1995) and this figure increases to 22.5 – 52.8% (Franzese *et al.,* 1997) in the obese child population.

1.4.2 Treatment

No effective treatment has been demonstrated to alter the natural history of NAFLD. In the absence of therapeutic modalities of proven efficacy, therapy is directed towards correction of the risk factors for non alcoholic steatohepatitis (NASH). A multifaceted approach is taken for the management of patients with NAFLD. The aims of treatment in NAFLD are to reduce liver-related morbidity/mortality and cardiovascular morbidity/mortality. As the knowledge of the biochemical mechanisms leading to both the development and progression of NAFLD has improved, the goal of treatment has shifted from simply trying to clear fat from the liver to address and treat the metabolic risk factors for fatty liver.

Potential therapeutic targets for NAFLD are insulin sensitizers as insulin resistance is central to the pathogenesis of NAFLD, weight loss as therapy targeting obesity, lipid-lowering agents for

dislipidaemia, antioxidants and pro-biotics for oxidative stress, cytoprotective agents for cellular apoptosis and anti-tumour necrosis factor (TNF) agents for pro-inflammatory cytokines.

1.4.2.1 Weight Loss

The therapeutic measure with the best potential for treating NAFLD is sustained weight loss by dietary changes with or without exercise, or bariatric surgery or by pharmacological measures.

1.4.2.1.1 Weight Loss through Lifestyle Changes

Overweight or obese patients with NAFLD should be encouraged to adopt a program of dietary self-management and moderate daily physical activity. Adherence to combined dietary restriction and increased physical activity has been shown to result in larger and progressive weight loss that can be maintained over time (Saris *et al.,* 2003). Moderate weight loss and exercise improve insulin sensitivity and are ideal treatments for overweight patients with diabetes mellitus and NAFLD. Weight loss can occur through caloric restriction, physical exercise, pharmacotherapeutic agents or bariatric surgery. A 6.5–10.0% weight loss through lifestyle modification improves liver biochemistry and reduces hepatic steatosis (Johnson and George, 2000, Larson-Meyer *et al.,* 2006). However, because lifestyle changes associated with dietary restriction and exercise are so difficult to sustain for most patients, attention has turned to other means of achieving sustainable weight loss.

1.4.2.1.2 Weight Loss by Pharmacological Measures

Pharmacological treatment for obesity may be offered to patients who have failed to lose body weight by lifestyle interventions alone.

Orlistat, an enteric lipase inhibitor which reduces dietary fat absorption, has been evaluated in NAFLD in several studies. In a pilot study of 10 obese patients with non-alcoholic steatohepatitis (NASH) treated with orlistat for 6 months, Harrison and colleagues demonstrated a reduction in aminotransferase levels with improved liver histology in 9 out of the 10 patients (Harrison *et al.*, 2004). They noted that improvements in steatosis and fibrosis were generally associated with a weight loss of 10% or more.

Sibutramine is a serotonin and norepinephrine reuptake inhibitor that acts by enhancing satiety, thus helping to reduce food intake.

Despite these encouraging findings, questions have been raised about the long-term safety profile of both these agents and whether sustained weight loss can be achieved. Orlistat causes gastrointestinal side effects and malabsorption of fat soluble vitamins in up to 30% of patients, and sibutramine can elevate heart rate and blood pressure (Padwal *et al*., 2004; Kim *et al.* 2003). In fact, sibutramine has recently been withdrawn from use by the European Medicines Agency (EMA) after an interim analysis of the sibutramine Cardiovascular Outcome (SCOUT) Study found the drug increased morbidity from cardio-vascular disease (Williams, 2010).

1.4.2.1.3 Weight Loss through Bariatric Surgery

In patients with NAFLD who are severely obese (BMI >40 kg/m^2) or have a BMI >35 kg/m^2 with obesity-associated co-morbidities, lifestyle modification alone may not be enough to achieve sustained weight loss, and so bariatric surgery can be considered. Gastric restrictive procedures (such as vertical banded gastroplasty, adjustable gastric banding and Roux-en-Y gastric bypass) reduce gastric volume and thereby create a mechanical barrier to the ingestion

of food. They promote gradual weight reduction and are currently the bariatric techniques of choice. At least five small studies have examined the effect of Roux-en-Y gastric bypass on patients with NASH (Furuya *et al.*, 2007; Liu *et al.*, 2007; de Almeida *et al.*, 2006; Barker *et al.*, 2006; Clark *et al.*, 2005).

There is evidence to suggest that rapid weight loss can lead to progression of hepatic inflammation and fibrosis (Andersen *et al.*, 1991), and some authors remain concerned that the risk of liver disease progression due to rapid weight loss during the first few months post-surgery makes the role of bariatric surgery in the treatment of NAFLD unclear.

1.4.2.2 Insulin-Sensitizing Agents

Insulin resistance plays a crucial role in the pathogenesis of NAFLD and NASH. This forms the basis for the use of insulin sensitizers in the treatment of NASH (Stein *et al.*, 2009). Metformin is an insulin-sensitizing agent that is associated with reduced cardiovascular mortality in patients with type II diabetes mellitus (T2DM) (Selvin, 2008). The biguanide metformin is widely used worldwide either alone or in combination with sulfo-nylureas, thiazolidinediones (TZDs) or insulin for the treatment of T2DM. The primary anti-hyperglycaemic action of metformin results from improved insulin sensitivity, primarily in the liver and secondarily in skeletal muscle (Scarpello and Howlett, 2008). Within the liver, the principle of action of metformin is to reduce hepatic glucose production, largely by inhibiting gluconeogenesis but also by inhibiting glycogenolysis (Scarpello and Howlett, 2008; Natali and Ferrannini, 2006). The increase in peripheral glucose ulitization (between 10% and 30%)

arises largely through non-oxidative glucose ulitization in skeletal muscle (Natali and Ferrannini, 2006).

Several studies have examined the effect of metformin in patients with NAFLD, showing it to consistently improve insulin resistance and serum liver enzymes without weight gain, but with more variable improvements in liver histology (Bugianesi *et al.*, 2005; Schwimmer *et al.*, 2005; Duseja *et al.*, 2004; Nair *et al.*, 2004; Uygun *et al.*, 2004; Marchesini *et al.*, 2001b). However, only two of these have been randomized controlled trials (Bugianesi *et al.*, 2005; Uygun *et al.*, 2004).

TZDs are a novel class of drugs that directly reduce insulin resistance by enhancing insulin action in adipose tissue, skeletal muscle and liver (Yki-Jarvinen, 2004). Currently, two TZDs, rosiglitazone and pioglitazone, are approved for the treatment of hyperglycaemia in T2DM, as mono therapy or in combination with other oral hypoglycaemic agents. TZDs act as agonists of peroxisome proliferators activator receptor gamma (PPAR-γ) receptor, a nuclear ligand-activated transcription factor that regulates multiple target genes. PPAR- γ is most abundantly expressed in adipose tissue, but also in pancreatic b -cells, vascular endothelium, macrophages and skeletal muscle cells. TZDs improve hepatic and peripheral insulin sensitivity by promoting fatty acid uptake into adipose tissue, decreasing serum FFA concentrations, and increasing hepatic fatty acid oxidation. They also increase the production of the insulin sensitizing cytokine adiponectin and reduce circulating levels of several pro-inflammatory mediators.

Several pilot studies examining the effect of TZDs on NAFLD and NASH have reported favourable results, with improvement in both liver function tests and liver histology (Belfort *et al.,* 2006; Promrat *et al.,* 2004; Sanyal *et al.,* 2004; Neuschwander-Tetri *et al.,* 2003). Also, the effect of TZDs on inflammation, fibrosis, and other histological features of NASH are less uniform (Argo *et al.,* 2009; Chalasani *et al.,* 2009; Juurlink *et al.,* 2009; Aithal *et al.,* 2008; Lutchman *et al.,* 2007; Belfort *et al.,* 2006; Ratziu *et al.,* 2006, 2008; Promrat *et al.,* 2004; Sanyal *et al.,* 2004; Neuschwander-Tetri *et al.,* 2003; Shadid and Jensen, 2003; Caldwell *et al.,* 2001).

On a comparative basis, TZDs seem to be more effective than metformin (Tiikkainen *et al.,* 2004).

Despite these favourable results, further, adequately powered, randomized, placebo- controlled trials of longer duration with histological data from paired biopsies are required to confirm the histological benefits of TZDs and metformin in NAFLD. In addition, the side-effect profile of both classes of drug should be borne in mind. TZDs can cause weight gain and congestive cardiac failure (Aithal *et al.,* 2008; Lago *et al.,* 2007). In addition, rosiglitazone has deleterious effects on bone mineral density and may increase cardio-vascular morbidity and mortality (Grey *et al.,* 2007; Nissen and Wolski, 2007). Metformin is generally well tolerated with the most commonest adverse effects being gastrointestinal (Scarpello and Howlett, 2008). Neither metformin nor TZDs have so far been approved by United States or European agencies for the treatment of NAFLD (Targher *et al.,* 2010).

1.4.2.3 Lipid Lowering Agents

Interest in the use of lipid lowering agents for the treatment of NAFLD has stemmed from the close association between NAFLD and dyslipidaemia (Kotronen and Yki-Jarvinen, 2008; Assy *et al.,* 2000).

Statins reduce cholesterol production and hence serum cholesterol by competitively inhibiting hepatic 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase.

Only a handful of studies have examined the efficacy of statins for NAFLD treatment. Rallidis and colleagues performed a very small pilot study in which they examined pravastatin use in four patients with NASH for 6 months (Rallidis *et al.,* 2004). They reported improvement in inflammation in three patients and improvement in steatosis in one patient.

Fibrates, such as clofibrate, fenofibrate and gemfibrozil may be of benefit in the treatment of NAFLD.

1.4.2.4 Antioxidants

The number of antioxidants with potential beneficial hepatic effects is increasing. Antioxidants have therapeutic potential because fatty acid oxidation produces reactive oxygen species, which cause direct cellular damage and activate pro-inflammatory cytokines. These agents include vitamins E and C, Nacetylcysteine (NAC), mitoquinone (MitoQ) and polyenylphosphatidylcholine (PPC). Of the antioxidants, vitamin E has been studied the most, with a few studies showing some positive preliminary results (Argo *et al.,* 2009; Lutchman *et al.,* 2007; Erso¨z *et al.,* 2005; Kawanaka *et al.,* 2004; Vajro *et al.,* 2004; Harrison *et al.,* 2003; Kugelmas *et al.,* 2003; Hasegawa *et al.,* 2001; Lavine, 2000).

NAC acts by increasing hepatic GSH levels and serving as a free thiol itself. It is widely used for the treatment of acetaminophen overdose. N-acetylcysteine has been studied in animal models of hepatic steatosis (Nakano *et al.*, 1997). In a small human study of 11 patients with NASH managed with diet regulation followed by N-acetylcysteine therapy, Gulbahar and colleagues reported improvements in aminotranferases, although no liver biopsies were performed (Chang *et al.*, 2006).

Oral administration of NAC is usually well tolerated. The most common side effects are nausea, vomiting, and abdominal pain (Mullins *et al.*, 2004). The distasteful odor of NAC (eg. akin to rotten eggs) may contribute to intolerance and vomiting of the administered dose. Serious adverse effects related to oral NAC use are rare. There is one report in the literature of a patient that developed an anaphylactoid reaction (tongue swelling and rash) after administration of the 8th dose of NAC in a treatment regiment (Mroz *et al.*, 1997).

Intravenous use of NAC is associated with a higher incidence of adverse reactions. Nausea and vomiting are the most common adverse effects reported (Whyte *et al.*, 2007, Mullins *et al.*, 2004) The most serious adverse effects are anaphylactoid reactions. Most commonly these reactions are characterized by the development of rash, urticaria, and pruritis. However, more serious and potentially fatal reactions can occur and manifest with bronchospasm and hypotension. Patients with asthma appear to be at higher risk for developing serious anaphylactoid reactions (Schmidt and Dalhoff, 2001, Appelboam *et al.*, 2002, Ho and Beilen, 1983, Pizon and LoVecchio, 2006).

1.4.3 HERBAL TREATMENT IN LIVER DISEASE

The single most commonly used modality in most Western surveys is herbal therapy (Eisenberg *et al.,* 1998, Angell and Kassirer, 1998, Vickers and Zollman, 1999). Indeed, annual spending on herbal products by the general population is said to exceed £40 million per year in the UK and $5 billion per year in the USA (Eisenberg *et al.,* 1998, Vickers and Zollman, 1999).

The most researched herbal treatment for liver diseases is Silybum or milk thistle. Its active constituents are collectively known as silymarin. Silymarin has been reported to protect liver cells from a wide variety of toxins, including acetaminophen, ethanol, carbon tetrachloride, and D-galactosamine. (Muriel and Mourelle, 1990, Bosisio *et al.,* 1992, Muriel *et al.,* 1992, Halim *et al.,* 1997, Chrungoo and Singh1997, Campos *et al.,* 1989). Silymarin has also been found to protect liver cells from ischemic injury (Wu *et al.,* 1993), radiation (Kropacova *et al.,* 1998), iron toxicity (Pietrangelo *et al.,* 1995), and viral hepatitis (McPartland, 1996). Silymarin's hepatoprotective effects are accomplished via several mechanisms including antioxidation (Wagner, 1981), inhibition of lipid peroxidation (Bosisio *et al.,* 1992), enhanced liver detoxification via inhibition of Phase I detoxification and enhanced glucuronidation (Baer-Dubowska *et al.,* 1998, Halim *et al.,* 1997) and protection of glutathione depletion (Campos *et al.,* 1989). Studies have also shown silymarin exhibits several anti-inflammatory effects, including inhibition of leukotriene, and prostaglandin synthesis, Kupffer cell cell inhibition, mast cell stabilization and inhibition of neutrophil migration (Fiebrich and Koch, 1979, Dehmlow *et al.,* 1996, Fantozzi., 1986, De La Puerta *et al.,* 1996). In addition, silymarin has

been shown to increase hepatocyte protein synthesis, thereby promoting hepatic tissue regeneration (Sonnenbichler and Zetl, 1986).

Animal studies have demonstrated that silybin reduces the conversion of hepatic stellate cells into myofibroblasts, slowing or even reversing fibrosis (Fuchs *et al.*, 1997). Clinical studies conducted in Hungary also demonstrated silymarin to have immunomodulatory effects on the diseased liver (Deak *et al.*, 1990, Lang *et al.*, 1990)

While much progress has been made in the epidemiology, natural history, and pathogenesis of NAFLD, as yet no single treatment modality has established efficacy in the treatment for NASH. To date, no pharmacological agent has been approved for treatment of NASH or could be recommended for routine use in clinical practice. While a number of agents targeting insulin resistance and pro-inflammatory factors demonstrate some potential, additional research is warranted to establish their safety and efficacy.

1.5 JUSTIFICATION

Liver diseases remain one of the major threat to public health and a worldwide problem (Asha and Pushpangdan, 1998). Hepatotoxicity from drugs and chemicals is the commonest form of iatrogenic diseases.

In spite of the tremendous advances in modern medicine, there is no effective drug available that stimulates liver functions, offer protection to the liver from damage or help regenerate hepatic cells (Chattopadhyay, 2003). Treatment options for common liver diseases such as cirrhosis, fatty liver and chronic hepatitis are problematic. The effectiveness of treatments such as interferon, colchicines, penicillamine, and corticosteroids are inconsistent at best and the incidence of side-effects profound. The treatment worsens the disease condition.

It is therefore necessary to search for alternative drugs for the treatment of liver diseases to replace the currently used drugs of doubtful efficacy and safety. In recent years, many researchers have examined the effects of plants used traditionally by indigenous healers and herbalists to support liver function and treat diseases of the liver. In most cases, research has confirmed traditional experience and wisdom by discovering the mechanism and modes of action of these plants as well as reaffirming the therapeutic effectiveness of certain plant extracts in clinical studies (Luper, 1998).

Paracetamol over dosage is of global concern especially in Africa since N-acetylcysteine, the antidote is not readily available at an affordable price to most people as well as its associated side effects. Traditionally, *Morinda lucida* is employed in the treatment of jaundice and malaria. *Morinda lucida* has been shown to be hepatoprotective against paracetamol-induced

liver damage in mice (Udem *et al.*, 1997). Though the mechanism of hepatic damage caused by hepatotoxic agents may differ considerably, it is not known presently if *Morinda lucida* has an effect on hepatic damage induced by other hepatotoxic agents. Literature survey reveals that, this plant, *Morinda lucida* has not been subjected to pharmacological screening for its hepatoprotective activity against carbon tetrachloride and paracetamol-induced liver damage in rats. This project therefore aims at investigating the possible modulation of paracetamol and carbon tetrachloride-induced liver damage in rats by *Morinda lucida.*

1.6 AIM OF THE RESEARCH

Morinda lucida Benth. is employed traditionally in the treatment of malaria, liver diseases (jaundice) and other disorders. Herbal medicines are gaining popularity in the treatment of various liver diseases because the pharmacological approach to the treatment of these liver diseases have made little or no impact (. This work was therefore carried out to evaluate the hepatoprotective potential of the ethanolic leaf extract of *Morinda lucida*.

1.6.1 Specific Objectives

- To evaluate the toxicity of the extract in acute and subacute toxicity studies.
- To evaluate the effect of the extract on the normal functioning of the hepatic microsomal drug metabolizing enzymes (MDME) using the pentobarbitone-induced sleeping time model.
- To evaluate the hepatoprotective activity (both prophylactic and curative studies) of the alcoholic leaf extract of *Morinda lucida* against Carbon tetrachloride (CCl_4).

- To evaluate the hepatoprotective activity (both prophylactic and curative studies) of the alcoholic leaf extract of *Morinda lucida* against acetaminophen.

Chapter 2

MATERIALS AND METHODS

2.1 PLANT COLLECTION AND EXTRACTION

The leaves of *Morinda lucida* Benth. were collected from around the School of Business, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi in October 2009.

The leaves were sun dried for five (5) consecutive days at the Department of Pharmacology after which it was ground with a mechanical mill.

The powdered leaves (1400 g) was cold macerated in seventy percent (70%) alcohol for three days (72 hours) in a bell jar and the alcohol was drained. The drained ethanol was concentrated using a rotary evaporator attached to a thermo chiller (ROTAVAPOR® R-210/R-215, BUCHI SWITZERLAND). The concentrated extract was dried in an oven to get the dried extract. The extract is subsequently refered to as *Morinda lucida* extract (MLE). The dried extract was dissolved in distilled water to prepare various concentrations and administered by means of oral gavage to the experimental animals.

2.2 ANIMALS

Sprague-Dawley (SD) rats were obtained from the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The animals were housed in stainless steel cages with soft wood shavings as their bedding. The animals were fed with normal commercial pellet purchased from the Ghana Agro Company Limited, Tema, Ghana and given water *ad libitum* throughout the experimental period. The

animals were treated humanely in accordance with internationally accepted principles for the treatment of laboratory animals.

2.3 DRUGS AND CHEMICALS

Paracetamol (Acetaminophen)	– SIGMA® ST LOUIS, M.O U.S.A
Bio-Silymarin	-ALOHA Medicinals Incl, U.S.A
Carbon Tetrachloride	– BDH Chemicals Ltd Poole, England
Olive oil	– Bell, Sons & CO. England.
Pentobarbitone	- SIGMA® ST LOUIS, M.O U.S.A
Formaldehyde	- Fisher Scientific, U.S.A
Sodium Dihydrogen Orthophosphate	– Hopkins & Williams Ltd, Essex, England.
di-Sodium Hydrogen Orthophosphate	– BDH Chemical Ltd, Poole, England.

2.4 QUANTITATIVE PHYTOCHEMICAL SCREENING (CRUDE EXTRACT)

Test for alkaloids

The plant extract (0.5g) was added to 5ml of dilute sulphuric acid H_2SO_4 (1%) on a steam bath. The solution was filtered, and the filtrate was treated with a few drops of Dragendorff's reagent. Reddish brown turbidity or precipitate indicated the presence of alkaloids.

Test for saponins

To about (0.5g) of the plant extract was shaken vigorously with 5ml of water in a test tube and warmed. Frothing formed persisted for 15 minutes indicated the presence of saponins.

Test for tannins

About 0.5 g of extract was dissolved in10ml of distilled water. The mixture was filtered, and the filtrate was treated with1% ferric chloride. A black-green precipitate formed indicated the presence of condensed tannins.

Test for steroids

The plant extract of about (0.5g) was dissolved in 2ml chloroform and filtrated. To about 2ml of the filtrate, acetic acid anhydride (2 ml) was added. Two milliliters of concentrated H_2SO_4 was then added. A blue-green colour showed the presence of steroids.

Test for triterpenoids

The extract was mixed with 2ml of chloroform and filtered. To the filtrate three drops of concentrated H_2SO_4 was then carefully added to form a thin layer. A reddish brown coloration at the interface was not observed indicated a negative result for terpenoids

Test for flavonoids

Dilute ammonia solution was added to (0.5g) of the extract followed by addition of concentrated H_2SO_4. A yellow coloration that disappeared on standing indicated the presence of flavonoids.

2.5 TOXICITY STUDIES

Thirty male Sprague-Dawley (SD) rats were grouped into six (6) groups of five (5) animals in each group (groups a, b, c, d, e, and f). Doses of *Morinda lucida* Benth. were prepared (250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg, and 4000 mg/kg). Animals in group *a* served as the control and received water throughout the experimental period. The animals in the other groups received the MLE as follows:

- group *B* received 250 mg/kg,
- *group C* received 500 mg/kg,
- group *D* received 1000 mg/kg,
- group *E* received 2000 mg/kg
- group *F* received 4000 mg/kg;

all by means of oral gavage.

2.5.1 Acute Toxicity Studies

Acute toxicity for *Morinda lucida* was determined in rats consisting of five (5) rats per group. The *Morinda lucida* extract (MLE) was dissolved in distilled water and a single dose was administered by gavage at doses 0, 250, 500, 1000, 2000 and 4000 mg/kg to each animal per group. The animals were observed closely for any toxic or abnormal behaviour in the first two (2) hours after dosing and were kept under further observation for twenty four (24) hours. Close observation of treated animals throughout the study period included: general appearance (compared to control animals), onset, intensity, and duration of toxic effects, changes in

behaviour, activity, respiration, appetite, fluid intake, or food retention and monitoring of body weight, food intake, water consumption, and skin or fur turgor.

2.5.2 Subacute Toxicity Studies

After the 24 hour observation, the extract (0, 250, 500, 1000, 2000 and 4000 mg/kg) was given to the animals for thirteen (13) days. During this period, the animals were observed daily for signs of toxicity. At the end of the two week study period, the animals were sacrificed by cervical dislocation, the jugular vein was cut and blood allowed to flow freely and collected for analysis.

2.5.2.1 Assessment of Haematological Parameters

Blood was taken into tubes containing ethylenediaminetetraacetic acid (EDTA), which served as an anticoagulant. Haematological parameters including red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and haemoglobin (HGB) were measured by an automatic analyzer (CELL-DYN 1700, Abbot Diagnostics Division, Abbot Laboratories, Abbot Park, IL, USA).

2.5.2.2 Assessment of Biochemical Parameters

Blood was taken into tubes without the anticoagulant and allowed to clot. After the clotting of the blood, it was centrifuged at 6000 rpm (revolutions per minute) for ten (10) minutes with Hettich Mikro 220R centrifuge (Hettich ZENTRIFUGGEN), DJB lab care, UK). Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatise (ALP), gamma-glutamyl transpeptidase (GGT), total proteins, albumin, globulin, total bilirubin, direct

(unconjugated) bilirubin, and indirect (conjugated) bilirubin were determined using an automatic analyzer (Random Access Chemistry System ATAC 8000, elan diagnostics laboratories, Brea, CA, USA).

2.5.2.3 Assessment of Body and Selected Organ Weights.

Weights of animals in grams (g) were taken at the start of the experiment (Day 1), half way through the experiment (Day 7) and on the final day of the experiment (Day 14). Mean body weight gains were calculated for each group at each interval and for the overall (Days 1–14) testing interval.

For the selected organ weights, after the animals have been sacrificed by the cervical dislocation, the selected organs including the liver, kidney, spleen and the heart were excised, trimmed of fat and other connective tissues, blotted dry and weighed on a balance. The relative weights of the organs were then calculated.

Histopathology of the liver and the kidney was done. Sections of liver and kidney from the autopsy samples were dewaxed, washed with phosphate buffered saline (PBS) and then stained with haematoxylin and eosin (H&E). Slides were then prepared and inspected under a light microscope.

2.6 PENTOBARBITONE–INDUCED SLEEPING TIME IN RATS/FUNCTIONAL TEST.

In this experiment, five (5) groups of five SD rats in a group were used. Animals in group A served as the normal control and received water and pellets *ad libitum* throughout the experimental period, that is, for all the five days of the experiment. Animals in group B served

as the toxin control and received carbon tetrachloride (CCl_4) (1 ml/kg) intraperitoneally (i.p) on the first, third and the fifth days of the experiment to induce liver damage. The animals in groups C, D, and E received doses MLE; 250 mg/kg, 500 mg/kg and 750 mg/kg for five days. On the fifth day, all the animals were challenged with 50 mg/kg body weight of pentobarbitone (i.p) thirty minutes after extract and CCl_4 administration. Sleeping time in the different groups of animals was recorded as the time between the loss and gain of the righting reflex. (Lim, 1964; Dandiya and Collumbine, 1959).

2.7 EFFECT OF MLE ON CARBON TETRACHLORIDE-INDUCED LIVER DAMAGE.

2.7.1 Prophylactic Studies

Thirty SD rats were grouped into six with five animals in each group. Group A served as the normal control and received only water and pellet *ad libitum* throughout the experimental period. Group B served as the toxin control and received a single injection of CCl_4 (1 ml/kg) intraperitoneally (i.p) on the sixth (6^{th}) day of the experiment. Group C, D, and E received 250 mg/kg, 1000 mg/kg and 4000 mg/kg of MLE respectively while animals in group F received silymarin for six consecutive days. Thirty minutes after the last administration of the extract or silymarin, the animals in these groups were also injected with CCl_4 (1 ml/kg). Twenty four hours later, the animals were weighed and later sacrificed by cervical dislocation. Blood was collected into tubes for biochemical analysis.

2.7.1.1 Assessment of Biochemical Parameters

Blood was collected into tubes without the anticoagulant and allowed to clot. After the clotting of the blood, it was centrifuged at 6000 rpm (revolutions per minute) for ten (10) minutes with Hettich Mikro 220R centrifuge (Hettich ZENTRIFUGGEN), DJB lab care, UK). Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatise (ALP), gamma-glutamyl transpeptidase (GGT), total proteins, albumin, globulin, total bilirubin, direct (unconjugated) bilirubin, and indirect (conjugated) bilirubin were determined using an automatic analyzer (COBAS INTEGRA® 400 Plus, COBAS INTEGRA® SYSTEMS, Canada).

2.7.1.2 Histopathology

After the animals have been sacrificed by cervical dislocation, the liver was excised, trimmed of fat and other connective tissues, blotted dry and weighed on a balance. Sections of the liver from the autopsy samples were stored in 10% formalin and taken to the Department of Pathology, Komfo Anokye Teaching Hospital (KATH) for the histopathology. Slides were prepared, observed under the light microscope and photomicrographs were taken.

2.7.2 Curative Studies

Thirty SD rats were placed into six groups of five rats in each group. Group A served as the normal control and received only water and pellet throughout the experiment. Group B served as the toxin control and received CCl_4 injection (i.p) (1 ml/kg) on day 1, day 3, day 5 and day 7 of the experimental period. Groups C, D, E and F received same injection on days 1, 3, and 5 of the experimental period. Thirty minutes after the last injection, the animals in these groups

received 250 mg/kg, 500 mg/kg, 750 mg/kg and silymarin respectively and also for days 6 and 7. Twenty four hours after the last administration of the extract and silymarin, the animals were weighed and later sacrificed by cervical dislocation. Blood was collected into tubes for biochemical analysis.

2.7.2.1 Assessment of Biochemical Parameters

Blood was collected into tubes without the anticoagulant and allowed to clot. After the clotting of the blood, it was centrifuged at 6000 rpm (revolutions per minute) for ten (10) minutes with Hettich Mikro 220R centrifuge (Hettich ZENTRIFUGGEN), DJB lab care, UK). Serum levels of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatise (ALP), gamma-glutamyl transpeptidase (GGT), total proteins, albumin, globulin, total bilirubin, direct (unconjugated) bilirubin, and indirect (conjugated) bilirubin were determined using an automatic analyzer (COBAS INTEGRA® 400 Plus, COBAS INTEGRA® SYSTEMS, Canada).

2.7.2.2 Histopathology

After the animals have been sacrificed by cervical dislocation, the liver was excised, trimmed of fat and other connective tissues, blotted dry and weighed on a balance. Sections of the liver from the autopsy samples were stored in 10% formalin and taken to the Department of Pathology, Komfo Anokye Teaching Hospital (KATH) for the histopathology. Slides were prepared, observed under the light microscope and photomicrographs were taken.

2.8 EFFECT OF MLE ON ACETAMINOPHEN-INDUCED LIVER DAMAGE.

2.8.1 Prophylactic Studies

Thirty SD rats were grouped into six with five animals in each group. Group A served as the normal control and received only water and pellet *ad libitum* throughout the experimental period. Group B served as the toxin control and received a single oral dose of acetaminophen (2500 mg/kg) on the sixth (6^{th}) day of the experiment. Group C, D, and E received 250 mg/kg, 500 mg/kg and 750 mg/kg of MLE respectively while animals in group F received silymarin for six consecutive days. Thirty minutes after the last administration of the extract and silymarin, the animals in these groups were also given acetaminophen orally. Twenty four hours later, the animals were weighed and later sacrificed by cervical dislocation. Blood was collected into tubes for biochemical analysis.

2.8.1.1 Assessment of Biochemical Parameters

Blood was collected into tubes without the anticoagulant and allowed to clot. After the clotting of the blood, it was centrifuged at 6000 rpm (revolutions per minute) for ten (10) minutes with Hettich Mikro 220R centrifuge (Hettich ZENTRIFUGGEN), DJB lab care, UK). Serum levels of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatise (ALP), gamma-glutamyl transpeptidase (GGT), total proteins, albumin, globulin, total bilirubin, direct (unconjugated) bilirubin, and indirect (conjugated) bilirubin were determined using an automatic analyzer (COBAS INTEGRA® 400 Plus, COBAS INTEGRA® SYSTEMS, Canada).

2.8.1.2 Histopathology

After the animals have been sacrificed by cervical dislocation, the liver was excised, trimmed of fat and other connective tissues, blotted dry and weighed on a balance. Sections of the liver from the autopsy samples were stored in 10% formalin and taken to the Department of Pathology, Komfo Anokye Teaching Hospital (KATH) for the histopathology. Slides were prepared, observed under the light microscope and photomicrographs were taken.

2.8.2 Curative Studies

Thirty SD rats were placed into six groups of five rats in each group. Group A served as the normal control and received only water and pellet throughout the experiment. Group B served as the toxin control and received oral dose of acetaminophen (2500 mg/kg) throughout the experimental period. Groups C, D, E and F received the oral dose of acetaminophen for the first five days of the experimental period. Thirty minutes after the last oral administration of the acetaminophen, the animals in these groups received 250 mg/kg, 500 mg/kg, 750 mg/kg and silymarin respectively and also for days 6 and 7. Twenty four hours after the last administration of the extract and silymarin, the animals were weighed and later sacrificed by cervical dislocation. Blood was collected into tubes for biochemical analysis.

2.8.2.1 Assessment of Biochemical Parameters

Blood was collected into tubes without the anticoagulant and allowed to clot. After the clotting of the blood, it was centrifuged at 6000 rpm (revolutions per minute) for ten (10) minutes with Hettich Mikro 220R centrifuge (Hettich ZENTRIFUGGEN), DJB lab care, UK). Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatise (ALP),

gamma-glutamyl transpeptidase (GGT), total proteins, albumin, globulin, total bilirubin, direct (unconjugated) bilirubin, and indirect (conjugated) bilirubin were determined using an automatic analyzer (COBAS INTEGRA® 400 Plus, COBAS INTEGRA® SYSTEMS, Canada).

2.8.2.2 Histopathology

After the animals have been sacrificed by cervical dislocation, the liver was excised, trimmed of fat and other connective tissues, blotted dry and weighed on a balance. Sections of the liver from the autopsy samples were stored in 10% formalin and taken to the Department of Pathology, Komfo Anokye Teaching Hospital (KATH) for the histopathology. Slides were prepared, observed under the light microscope and photomicrographs were taken.

2.9 STATISTICS

Results presented as mean ± SD or mean ± SEM were computed and analysed with Graph Pad Prism version 5.0 (Graph Pad Prism Software, San Diego, CA, USA). Significant difference among means of the groups were determined by one way ANOVA using Neuman-Keuls multiple comparism test as a posthoc test with $p < 0.05$ set as the level of significance.

Chapter 3

RESULTS

3.1 PRELIMINARY PHYTOCHEMICAL SCREENING

Table 1: Phytochemical constituents of the alcoholic leaf extract of *Morinda lucida*

CONSTITUENTS	*Morinda lucida*
Alkaloids	+
Triterpenoids	+
Steroids	-
Saponins	+
Condensed Tannins	+
Reducing sugars	+
Flavonoids	+

Key (+) = Present (-) = Absent

3.2 ACUTE TOXICITY STUDIES

The single oral administration of MLE to rats was non toxic up to a dose of 4000 mg/kg) as no mortality was detected at the end of experiment (24 h later). No abnormalities of condition or behaviour, significant changes in behaviour, breathing, skin effects, defecation, yellowing or loss of hair, postural abnormalities, impairment in food intake and body weight in experimental

animals were detected. In all cases the faeces of the experimental animals were darkly coloured for the control and treated groups.

3.3 SUBACUTE TOXICITY STUDIES

3.3.1 General Observations

Over the study duration of 14 days no death was recorded during treatment with MLE in doses up to 4000 mg/kg by the oral route. The animals did not show any alteration in general behaviour or clinical signs of toxicity. Consumption of food and water did not change over the course of the experiment.

The only significant observation made in this 14 day study was the watery stool that the *Morinda lucida* extract treated rats passed throughout the experimental period as compared to the control animals.

3.3.2 Effect of MLE on Body Weight

Average overall (test days 1–14) body-weight data indicated that the *Morinda lucida* extract treated rats, regardless of dose level, were comparable to the controls (Fig 2). Comparison of mean body weight between the extract administered and the control groups revealed no significant changes throughout the experiment.

Table 2: Effect of *Morinda lucida* on body weights of rats treated for two weeks (14 days). Animals were weighed on day one, day seven and day fourteen. Values are expressed as means ± SEM (n = 5).

Day	Doses (mg/kg)					
	control	250	500	1000	2000	4000
One	131.0±11.22	132.0±6.04	131.0±4.30	131.0±5.79	131.0±8.28	131.0±3.67
Seven	170.0±10.84	173.0±6.44	185.0±5.24	185.0±8.06	173.0±11.14	182.0±6.63
Fourteen	193.0±8.60	193.0±6.63	205.0±4.18	205.0±5.00	198.0±10.56	206.0±5.34

3.3.3 Effect of MLE on Selected Organ Weights in rats

There were increases in the weight of the liver at two levels of treatment (250 and 500 mg/kg), but the increases were not statistically significant as compared to the control ($P < 0.05$). There were decreases in the weight of the liver at three dose levels (1000, 2000, and 4000 mg/kg). The decrease was only significant ($P < 0.05$) at a dose of 4000 mg/kg which was 6.22 ± 0.53 compared to the control value of 8.24 ± 0.52.

Also, there were increases in the weight of the heart in all treated animals but the increase was only significant ($P < 0.05$) at the 4000 mg/kg which was 0.584± 0.087 compared to the control

value of 0.46± 0.0296. There were no significant changes in the kidney and the spleen (Table 1).

Table 3: Effect of *Morinda lucida* on organ weights in rats treated for two weeks (14 days).

Organ	Doses (mg/kg)					
	control	250	500	1000	2000	4000
Liver	8.24±0.52	9.02±0.23	8.99±0.79	7.72±0.37	7.90±0.28	6.22±0.53
Spleen	0.56±0.04	0.50±0.02	0.492±0.01	0.57±0.05	0.59±0.03	0.45±0.03
Kidney	0.59±0.02	0.57±0.02	0.58±0.04	0.61±0.02	0.61±0.02	0.54±0.03
Kidney	0.61±0.01	0.57±0.03	0.57±0.03	0.58±0.02	0.60±0.01	0.54±0.04
Heart	0.46±0.03	0.55±0.04	0.60±0.04	0.60±0.04	0.58±0.01	0.58±0.09

Values are expressed as means ± SEM (n = 5).

3.3.4 EFFECT OF MLE ON SERUM BIOCHEMICAL PARAMETERS

The biochemical parameters of treated and control groups are presented in Fig. 3 and 4. Sub-acute treatment did not cause significant change in the serum levels of bilirubin (total, direct and indirect), proteins (total, albumin and globulin), ALT, AST, ALP and GGT in treated groups as compared to the control group.

3.3.5 EFFECT OF MLE ON HAEMATOLOGICAL PARAMETERS

The analyzed haematological parameters revealed no significant changes in the levels of red blood cells (RBC), white blood cells (WBC), haemoglobin (HGB), haematocrit (HCT), mean

corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelet (PLT) among the control and *Morinda lucida* extract treated groups. However, for MCV, there were increases at all levels of treatment except at 500 mg/kg. The increase at the 250 mg/kg was significant ($P < 0.05$), which was 76.98 ± 11.72 compared to the control value of 56.00 ± 1.420 (Table 2). In contrast, the levels of HCT reduced over the dosage range used from the control value of 24.56 ± 2.42 to a level of 21.16 ± 0.68. These decreases were not statistically significant ($P < 0.05$).

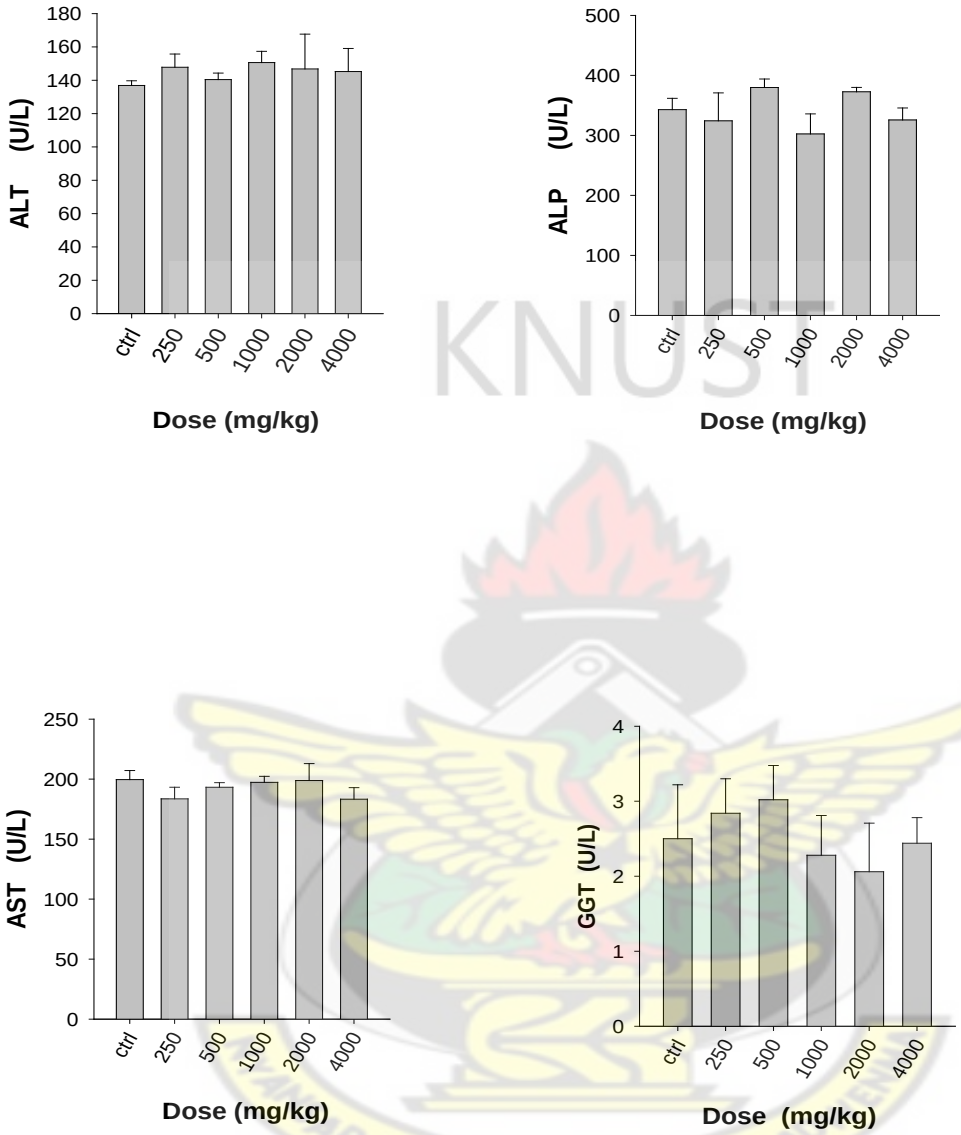

Figure 4: Effect of MLE on serum enzymes in rats treated for 14 days.The extract did not cause significant changes in these serum enzymes (a) ALT, (b) AST, (c) ALP and (d) GGT in the treated groups as compared to the control. Values are presented as the mean ± SEM (n = 5).

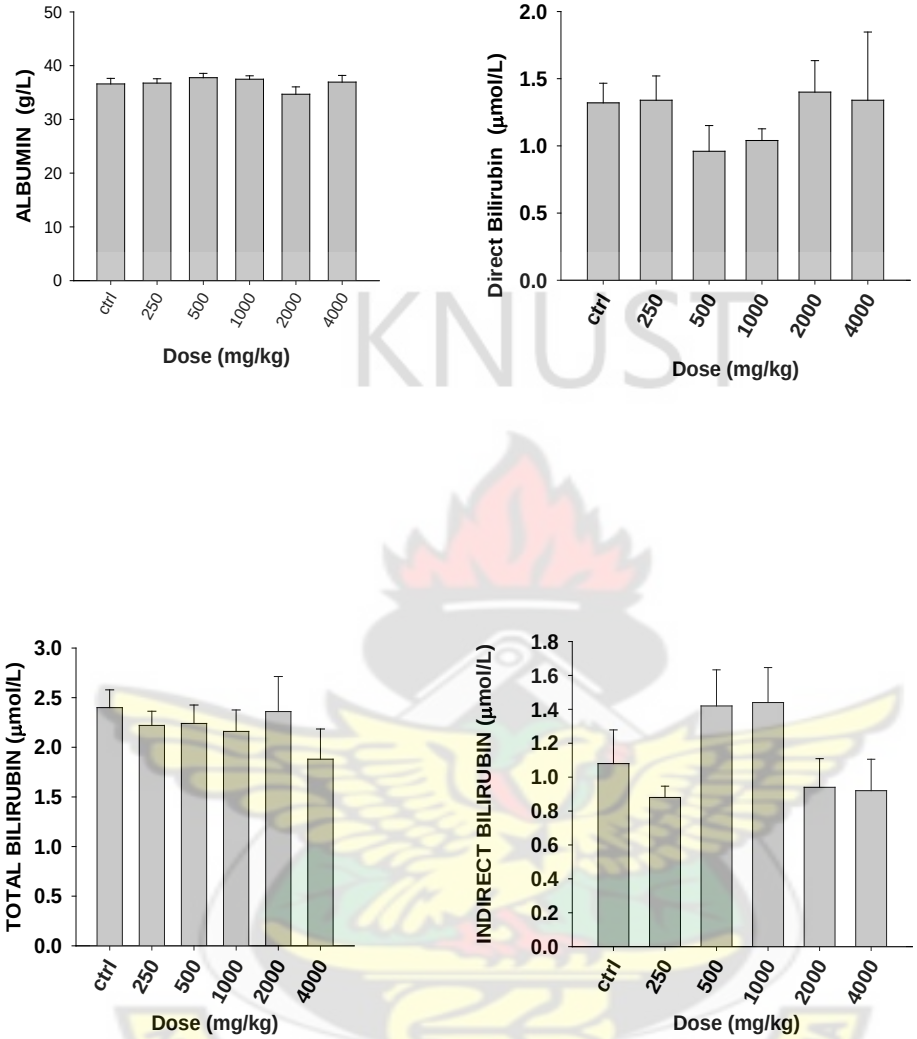

Figure 5: Effect of MLE on (a) albumin and (b) direct bilirubin (c) total bilirubin and (d) indirect bilirubin levels in rats treated for 14 days. The extract did not cause any significant change in the levels of albumin and bilirubin levels in the treated groups as compared to the control group. Values are presented as the mean ± SEM (n = 5).

Table 4: Effect of *Morinda lucida* on haematological indices in rats treated for two weeks (14 days).

parameter	Doses (mg/kg)					
	control	**250**	**500**	**1000**	**2000**	**4000**
RBC	4.36±0.36	3.32±0.45	4.28±0.38	3.85±0.76	3.86±0.21	4.07±0.48
WBC	4.18±0.68	4.56±1.44	5.26±1.43	4.14±0.32	3.26±0.21	4.00±0.65
HGB	10.64±0.78	11.18±1.14	10.48±0.84	10.46±0.52	9.76±0.65	9.74±0.61
HCT	24.56±2.42	23.46±0.64	23.26±2.02	22.78±4.80	21.08±1.18	21.16±0.68
MCV	56.00±1.42	76.98±11.72*****	54.40±1.42	59.12±2.39	56.30±0.99	59.32±1.27
MCH	24.52±0.99	37.24±7.81	24.56±1.02	32.02±6.57	24.44±1.69	27.02±1.52
MCHC	43.98±2.18	47.36±3.71	45.18±1.04	54.26±10.35	45.14±3.04	52.30±3.79
PLT	904.6±178.8	1123±217.2	931.6±152.1	1288±64.66	1045±180.2	1239±116.7

Values are expressed as means ± SEM (n = 5). * indicates significance ($P < 0.005$) compared to the control group by Newman-Keuls's test.

3.4 EFFECT OF MLE ON SLEEPING TIME IN RATS

The CCl_4-induced liver injury was delaying the metabolism of barbiturates, thereby, slowing their excretion rate and leading to an increase in the duration of barbiturate induced hypnosis, from 194.5 ± 4.17 min) in normal control to 273.5 ± 2.53 min in CCl_4 induced hepatotoxicity group; ($P < 0.05$) (Fig 5). Treatment of animals with MLE (250, 1000, and 4000 mg/kg) inhibited the effect of CCl_4 and reduced the sleeping time close to the normal control.

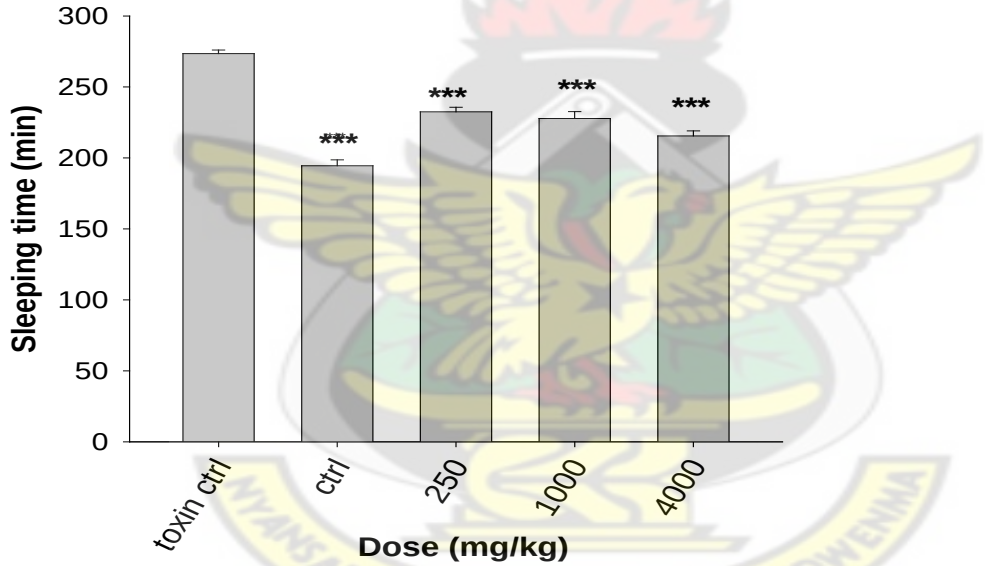

Figure 6: Effect of MLE on pentobarbitone-induced sleeping time in rats. Animals received the MLE for three days and one hour after the last administration; the animals were challenged with pentobarbitone (50 mg/kg). The sleeping times in the different groups were recorded as the time between the loss and gain of the righting reflex. Values are presented as the mean ± SEM (n = 4). *** indicates significance ($P < 0.005$) compared to the toxin control (CCl_4) group by Newman-Keuls's test.

3.5 HEPATOPROTECTIVE STUDIES (CARBON TETRACHLORIDE MODEL, PROPHYLACTIC STUDIES)

The hepatoprotective efficacy of the ethanolic leaf extract of *Morinda lucida* against CCl_4-induced hepatotoxicity is shown in Figs. 6 and 7. A single intraperitoneal administration of CCl_4 caused significant liver damage as evidenced by altered biochemical parameters. CCl_4 at 1 mg/kg *i.p.* significantly ($P < 0.05$) raised serum levels of ALT, AST, GGT, ALP and total, direct and indirect bilirubin levels. Pre-treatment with the ethanolic leaf extract of MLE attenuated the elevated enzyme levels to levels comparable to silymarin, which was the standard hepatoprotective drug. The hepatoprotective efficacy of the test drug was comparable with that of standard drug silymarin.

The hepatoprotective efficacy of the extract against CCl_4 intoxication was further confirmed by histopathological examinations (Fig 8). The liver samples of CCl_4 treated rats showed cell vacuolation, necrosis and degeneration of nuclei and bile capillaries. However, in the test drug treated groups, mild degenerative changes and marked recovery from necrosis and degeneration of bile capillaries in the liver samples indicate the effectiveness of the plant extract in comparison with the standard drug silymarin.

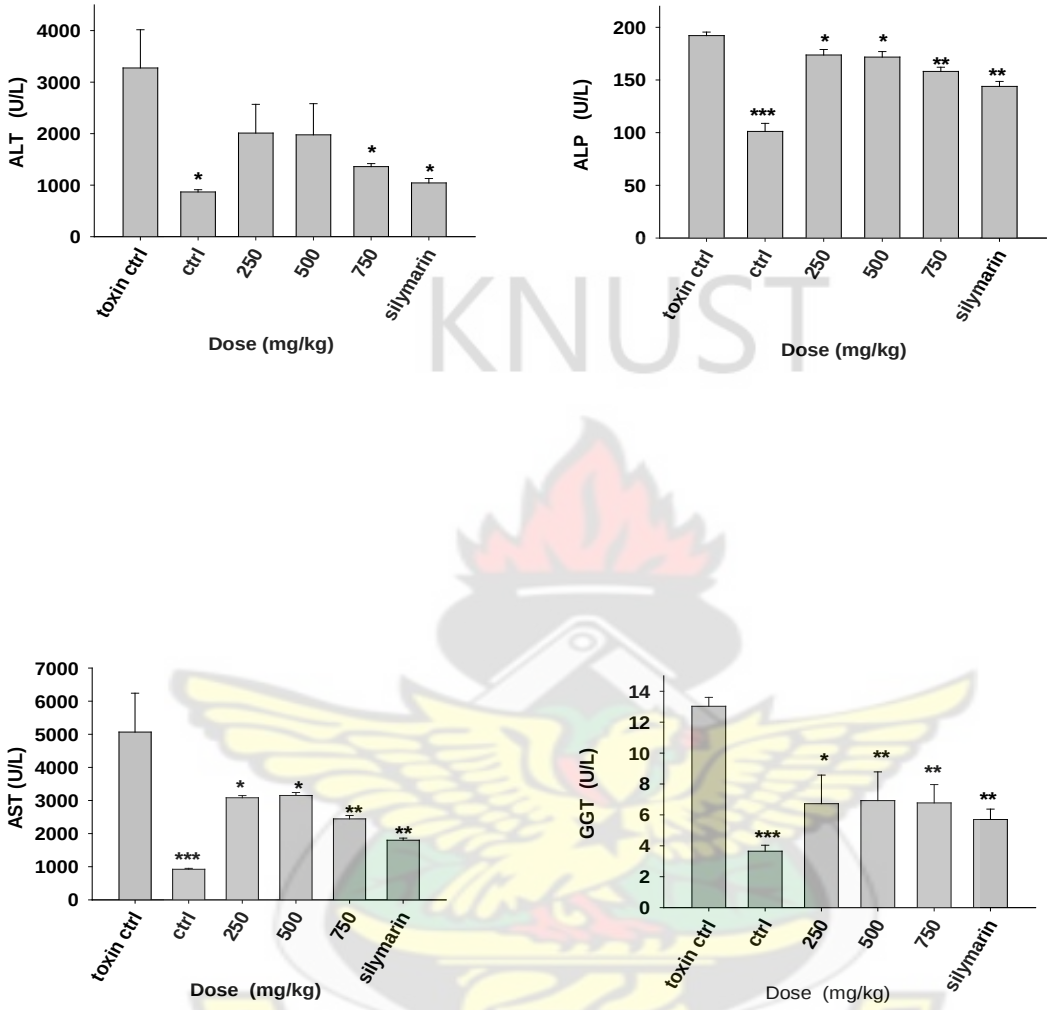

Figure 7: Effect of pre-treatment of MLE on serum enzymes; ALT, AST, ALP, and GGT levels in CCl_4 intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (CCl_4) group.

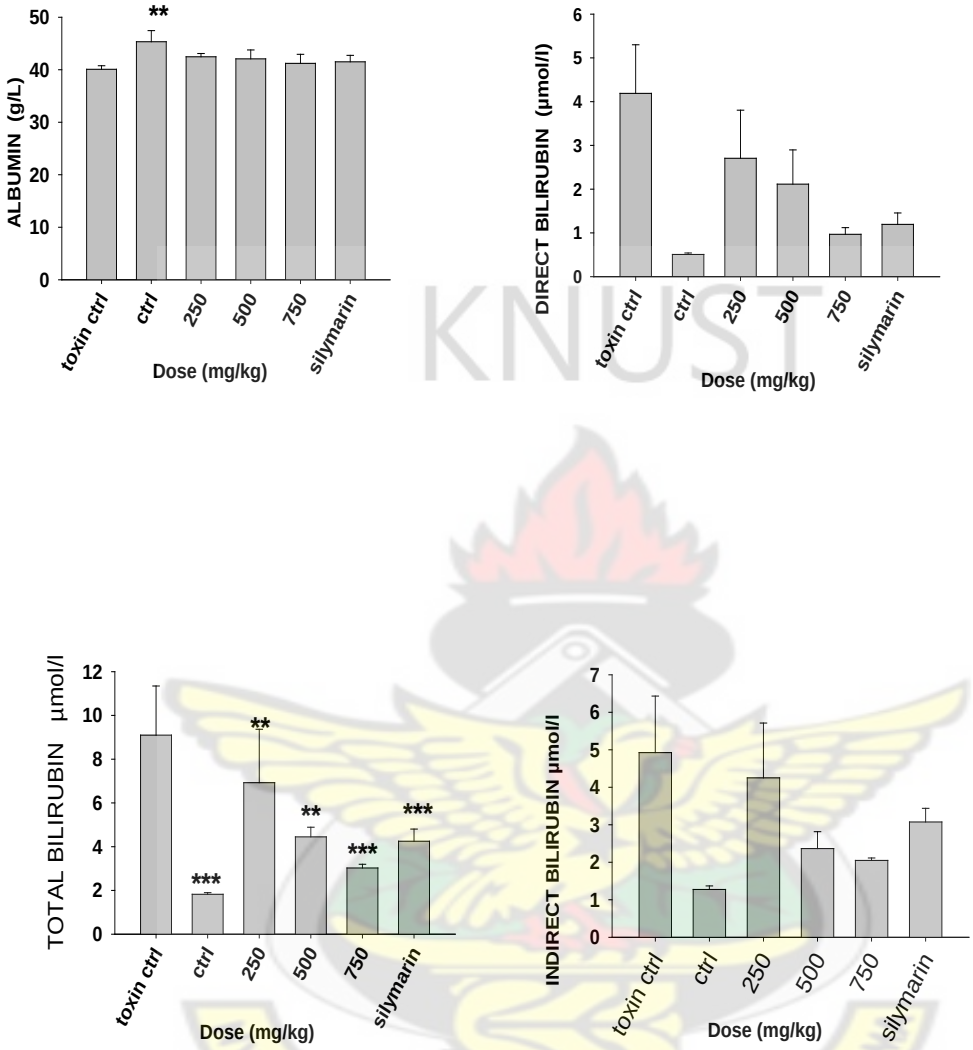

Figure 8: Effect of MLE pre-treatment on protein and bilirubin levels in CCl_4 intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (CCl_4) group.

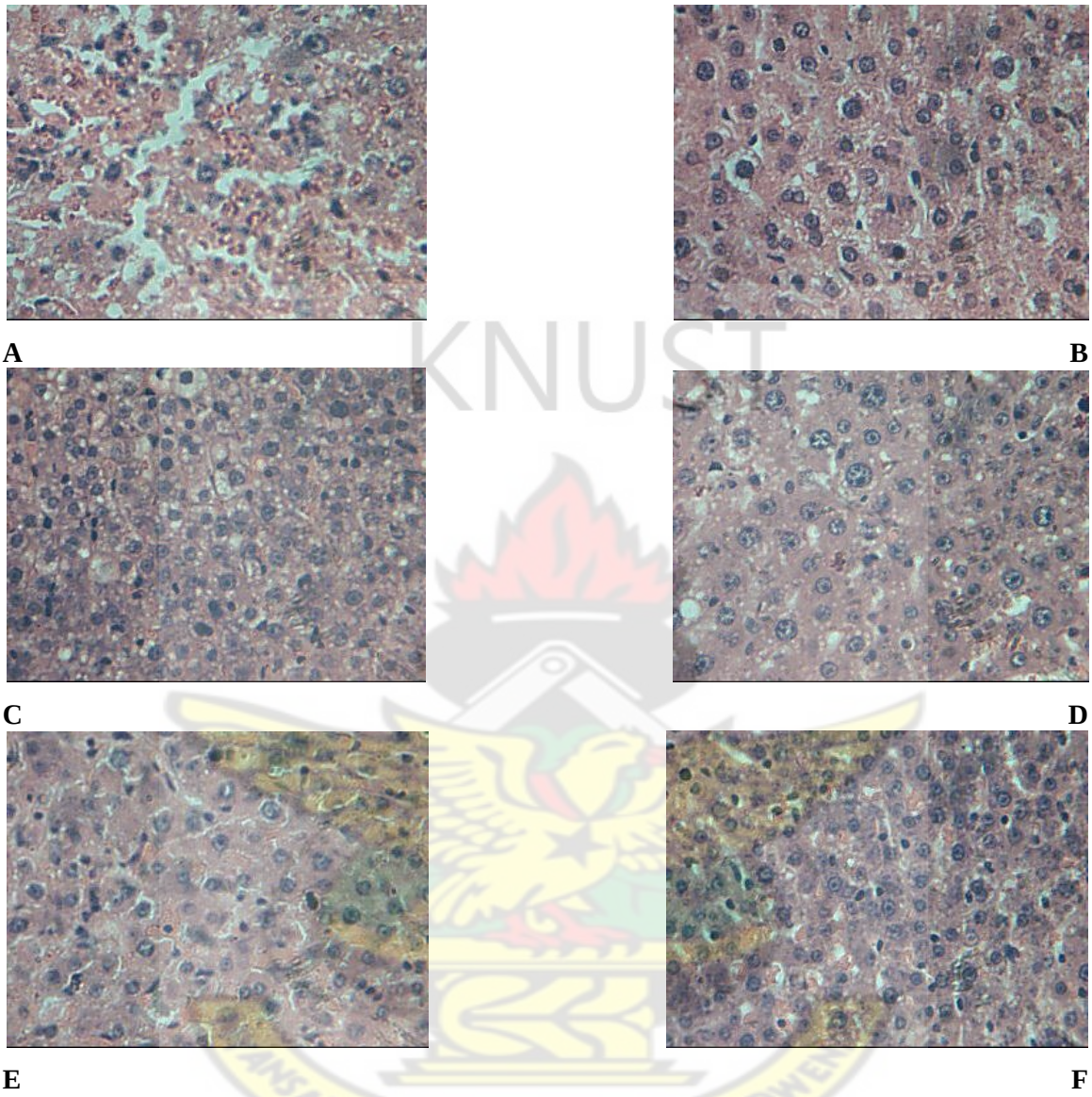

Figure 9: The photomicrographs of liver sections from rats treated with CCl_4, the pre-treatment of MLE at 250, 500 and 750 mg/kg, and vehicle. Liver tissues were stained with H&E (100×). (A) Liver section of CCl_4 treated rat; (B) liver section of control rat; (C) liver section of the CCl_4-treated rat pre-dosed by MLE at 250 mg/kg; (D) liver section of the CCl_4-treated rat pre-dosed by MLE at 500 mg/kg; (E) liver section of the CCl_4-treated rat pre-dosed by MLE at 750 mg/kg; (F) liver section of the CCl_4-treated rat pre-dosed by silymarin.

3.6 HEPATOPROTECTIVE STUDIES (CARBON TETRACHLORIDE MODEL, CURATIVE STUDIES)

The hepatoprotective efficacy of the ethanolic leaf extract of *Morinda lucida* against CCl_4 induced hepatotoxicity is shown in Figs. 11 and 12. Intraperitoneal administration of CCl_4 caused significant liver damage as evidenced by altered biochemical parameters. CCl_4 at 1 mg/kg *i.p.* significantly ($P < 0.05$) increased serum levels of ALT, AST, GGT, ALP and total, direct and indirect bilirubin levels. Treatment with the ethanolic extract attenuated the elevated enzyme levels to normal level. The hepatoprotective efficacy of the test drug was comparable with that of standard drug silymarin.

The hepatoprotective efficacy of the extract against CCl_4 intoxication was further confirmed by histopathological examinations (Fig 13). The liver samples of CCl_4 treated rats showed cell vacuolation, necrosis and degeneration of nuclei and bile capillaries. However, in the test drug treated groups, mild degenerative changes and marked recovery from necrosis and degeneration of bile capillaries in the liver samples indicate the effectiveness of the plant extract in comparison with the standard drug silymarin.

The relative weight of the liver increased significantly in the CCl_4 treated group (Fig. 9) and post treatment with MLE reduced the weight which was comparable to silymarin. Also, CCl_4 caused a reduction in the body weight in the CCl_4 treated group (Fig. 10) as well as the MLE treated groups but not in the silymarin treated group.

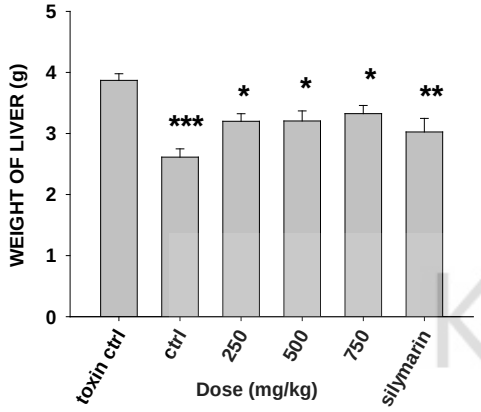

Figure 10: Changes in relative liver weight of rats during CCl_4 induced hepatotoxicity and treated with *Morinda lucida* leaf extract or silymarin. Values are presented as the mean ± SEM (n = 5).). (*) indicates significance compared to the toxin control (CCl_4) group.

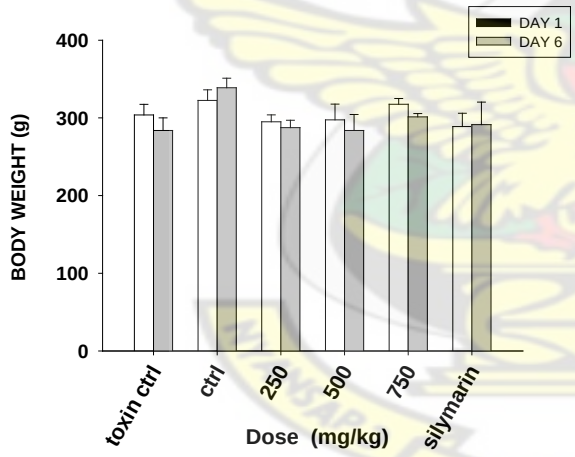

Figure 11: Changes in body weight of rats during CCl_4 induced hepatotoxicity and treated with *Morinda lucida* leaf extract or silymarin. Values are presented as the mean ± SEM (n = 5).

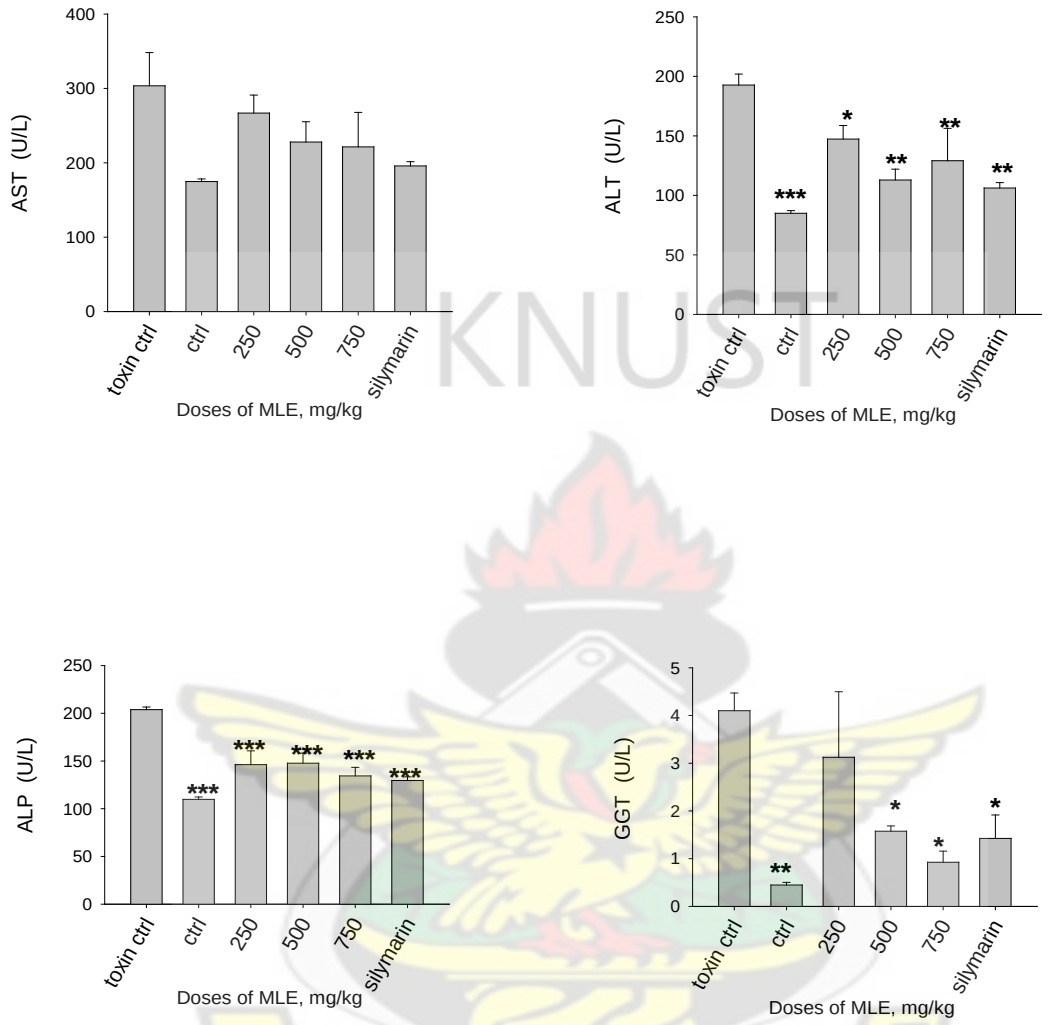

Figure 12: Effect of MLE post-treatment on serum enzymes; ALT, AST, ALP, and GGT levels in CCl_4 intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (CCl_4) group.

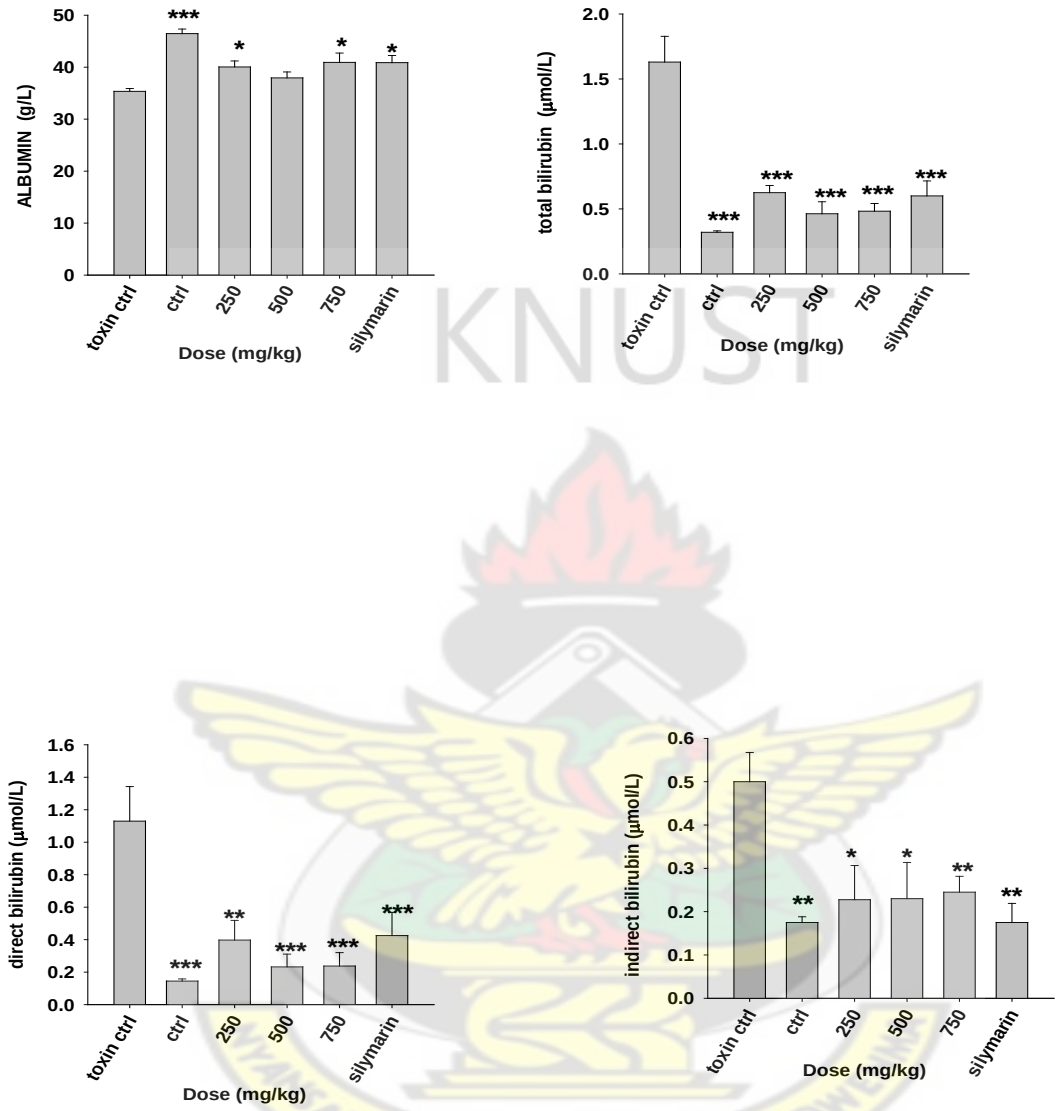

Figure 13: Effect of MLE post-treatment on protein and bilirubin levels in CCl_4 intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (CCl_4) group.

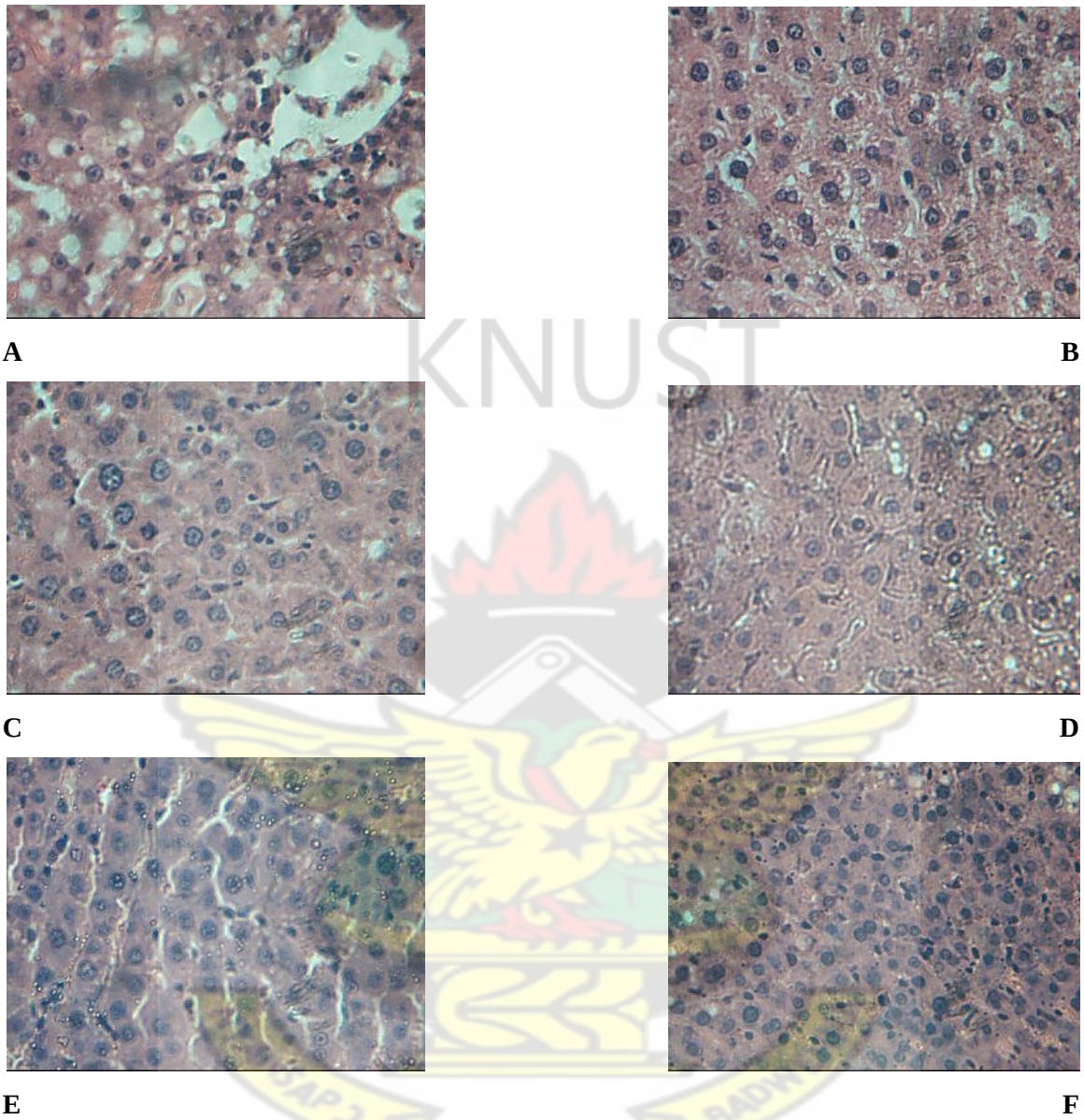

Figure 14: The photomicrographs of liver sections from rats treated with CCl_4, the post-treatment of MLE at 250, 500 and 750 mg/kg, and vehicle. Liver tissues were stained with H&E (100×). (A) Liver section of CCl_4 treated rat; (B) liver section of control rat; (C) liver section of the CCl_4-treated rat post-dosed by MLE at 250 mg/kg; (D) liver section of the
CCl_4-treated rat post-dosed by MLE at 500 mg/kg; (E) liver section of the CCl_4-treated rat post-dosed by MLE at 750 mg/kg; (F) liver section of the CCl_4-treated rat post-dosed by silymarin.

3.7 HEPATOPROTECTIVE STUDIES (ACETAMINOPHEN MODEL, PROPHYLACTIC STUDIES)

The liver function markers in normal control, toxin control, and MLE treated groups are shown in Figs 14 and 15. Acetaminophen significantly ($P < 0.05$) produced severe liver damage as indicated by a marked increase in the serum levels of ALT, AST, ALP, GGT, and bilirubin (total, direct and indirect) of the toxin control group after a single oral administration. Also, there was a decrease in the serum protein levels. This indicates hepatocellular damage as well as biliary obstruction. Pre-treatment with MLE produced a significant reduction of the levels of serum marker enzymes; ALT, AST, ALP, GGT, bilirubin (total, direct, and indirect) and an increase in the levels of protein (total protein and albumin) similar to the silymarin treated group which seems to offer the protection and maintain the functional integrity of hepatic cells.

Histopathological studies, showed acetaminophen produced extensive vascular degenerative changes and centrilobular necrosis in hepatocytes. Pre-treatment with silymarin and MLE produced mild degenerative changes and absence of centrilobular necrosis when compared with control (Fig. 16). All these results indicate a hepatoprotective potential of the extract.

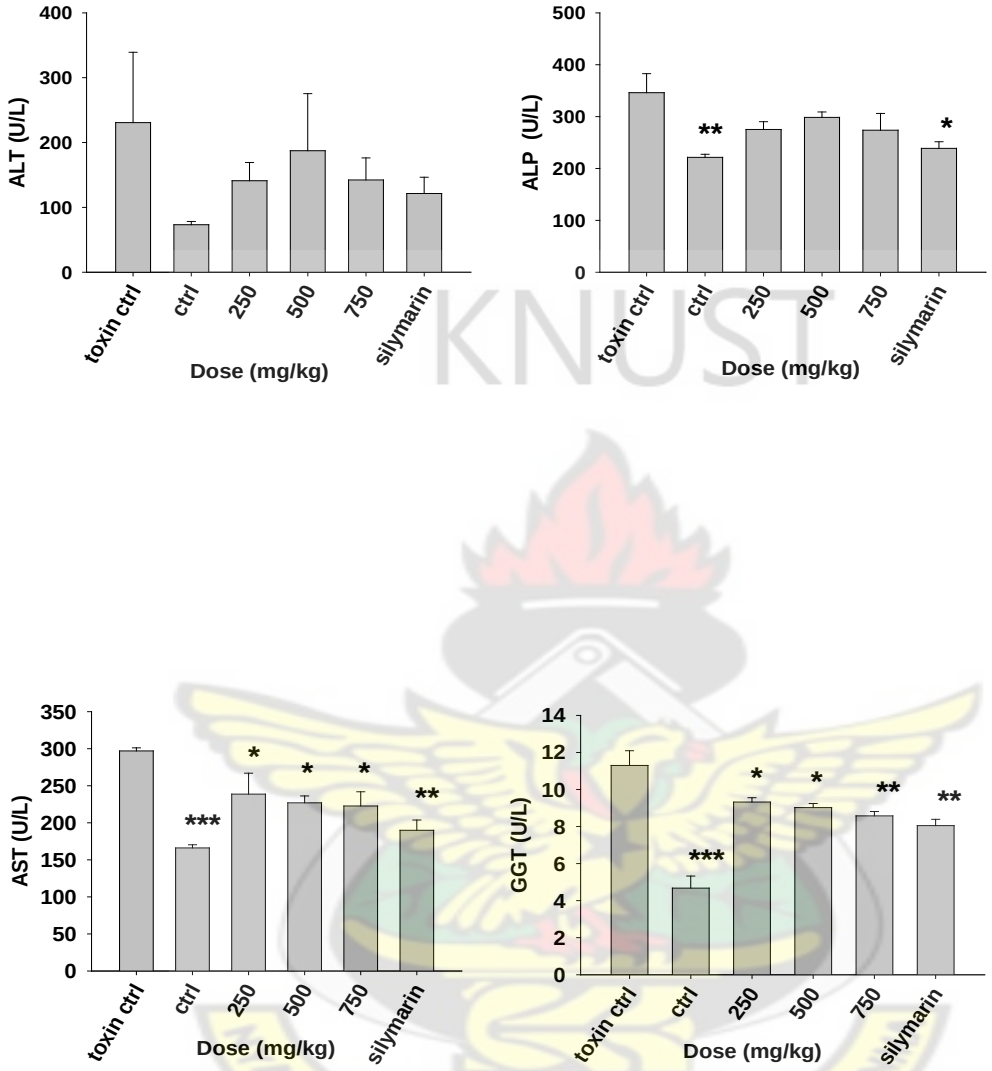

Figure 15: Effect of MLE pre-treatment on serum enzymes; ALT, AST, ALP, and GGT levels in acetaminophen intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (acetaminophen) group.

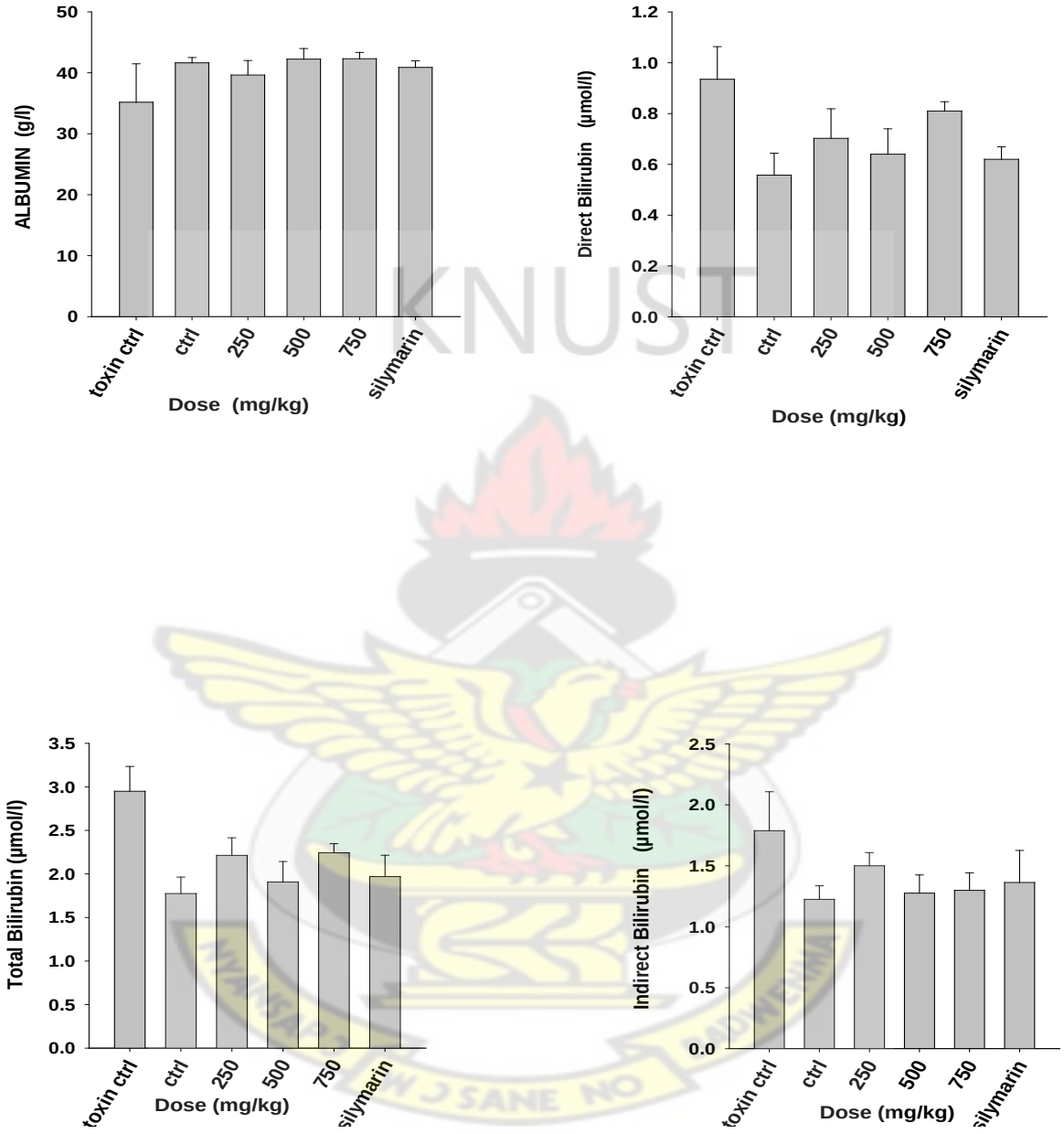

Figure 16: Effect of MLE pre-treatment on protein and bilirubin levels in acetaminophen intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (acetaminophen) group.

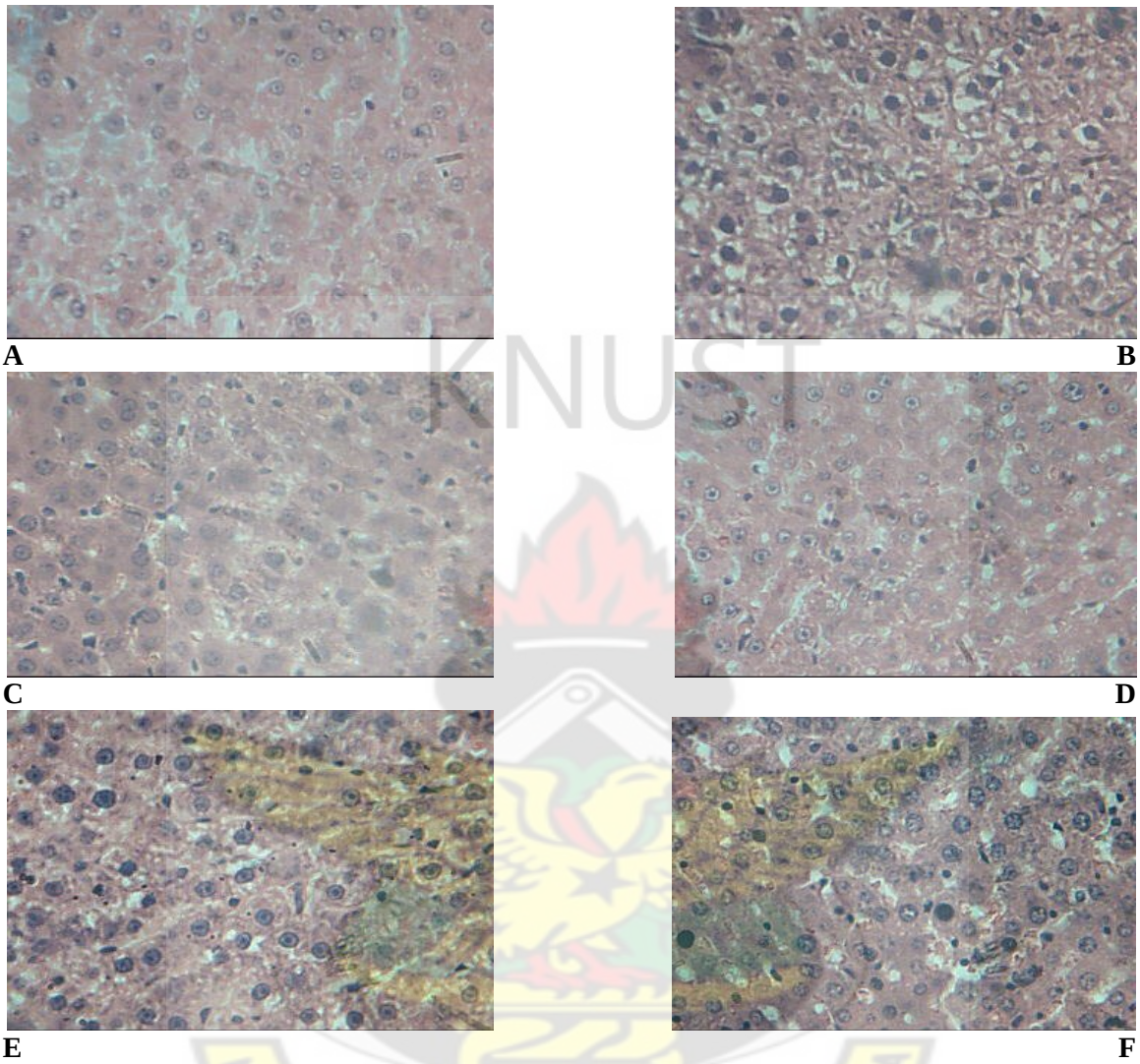

Figure 17: The photomicrographs of liver sections from rats treated with acetaminophen, the post-treatment of MLE at 250, 500 and 750 mg/kg, and vehicle. Liver tissues were stained with H&E (100×). (A) Liver section of APAP treated rat; (B) liver section of control rat; (C) liver section of the APAP-treated rat pre-dosed by MLE at 250 mg/kg; (D) liver section of the APAP-treated rat pre-dosed by MLE at 500 mg/kg; (E) liver section of the APAP-treated rat pre-dosed by MLE at 750 mg/kg; (F) liver section of the APAP-treated rat pre-dosed by silymarin.

3.8 HEPATOPROTECTIVE STUDIES (ACETAMINOPHEN MODEL, CURATIVE STUDIES)

The liver function markers in normal control, toxin control, and MLE treated groups are shown in Figs. 19 and 20. Acetaminophen significantly ($P < 0.05$) produced severe liver damage as indicated by a marked increase in the serum levels of ALT, AST, ALP, GGT, and bilirubin (total, direct and indirect) of the toxin control group. Also, there was a decrease in the serum protein levels. This indicates hepatocellular damage as well as biliary obstruction. Treatment with MLE produced a significant reduction in the levels of serum marker enzymes; ALT, AST, ALP, GGT, bilirubin (total, direct, and indirect) and an increase in the levels protein (total protein and albumin) similar to the silymarin treated group which seems to offer the protection and maintain the functional integrity of hepatic cells.

Fig. 18 shows that when APAP was administered orally to rats at a dose of 2500 mg/kg without further treatments, a rapid drop in body weight was observed. When rats were treated with MLE after APAP administration, an improvement in the body weight variation (Fig. 14) was observed.

Histopathological studies showed acetaminophen produced extensive vascular degenerative changes and centrilobular necrosis in hepatocytes. Treatment with silymarin and MLE produced mild degenerative changes and absence of centrilobular necrosis when compared with control (Fig. 21). All these results indicate a hepatoprotective potential of the extract.

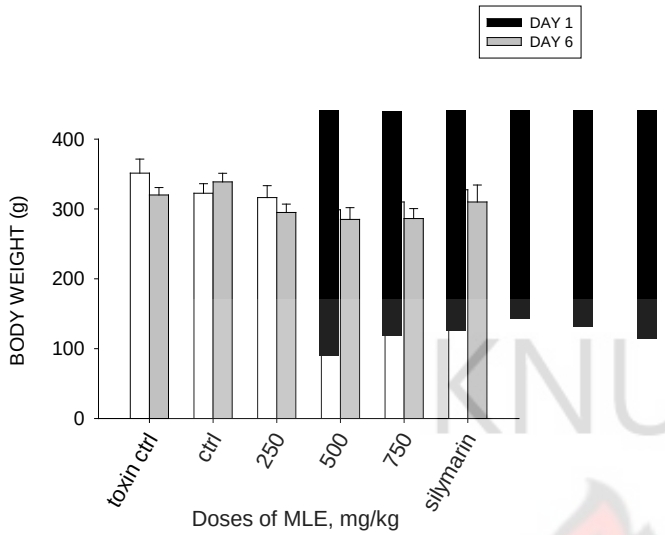

Figure 18: Changes in body weight of rats during acetaminophen induced hepatotoxicity and treated with *Morinda lucida* leaf extract or silymarin. Values are presented as the mean ± SEM (n = 5).

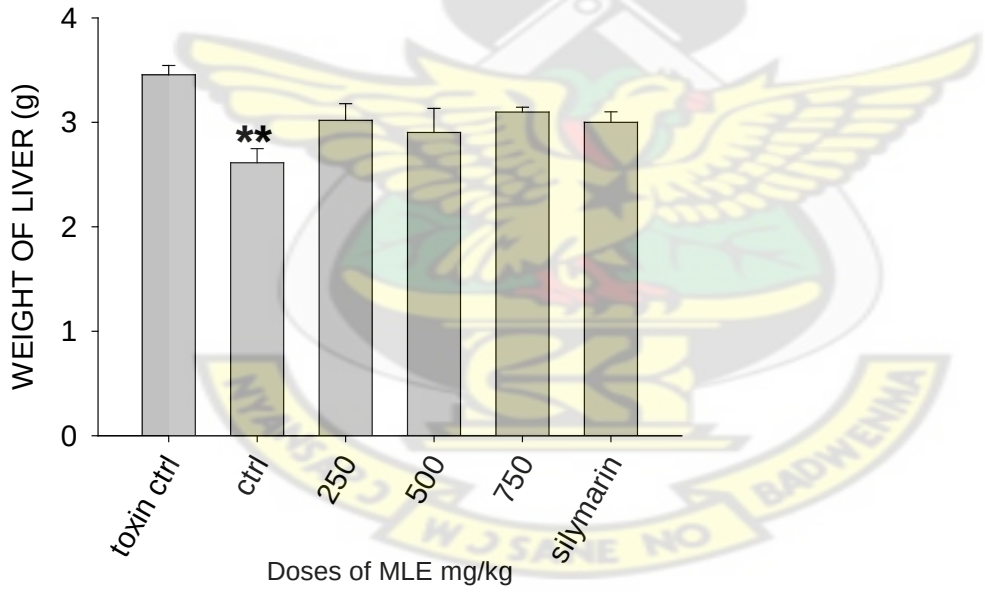

Figure 19: Changes in relative liver weight of rats during acetaminophen-induced hepatotoxicity and treated with *Morinda lucida* leaf extract or silymarin. Values are presented as the mean ± SEM (n = 5).). (*) indicates significance compared to the toxin control (CCl_4) group.

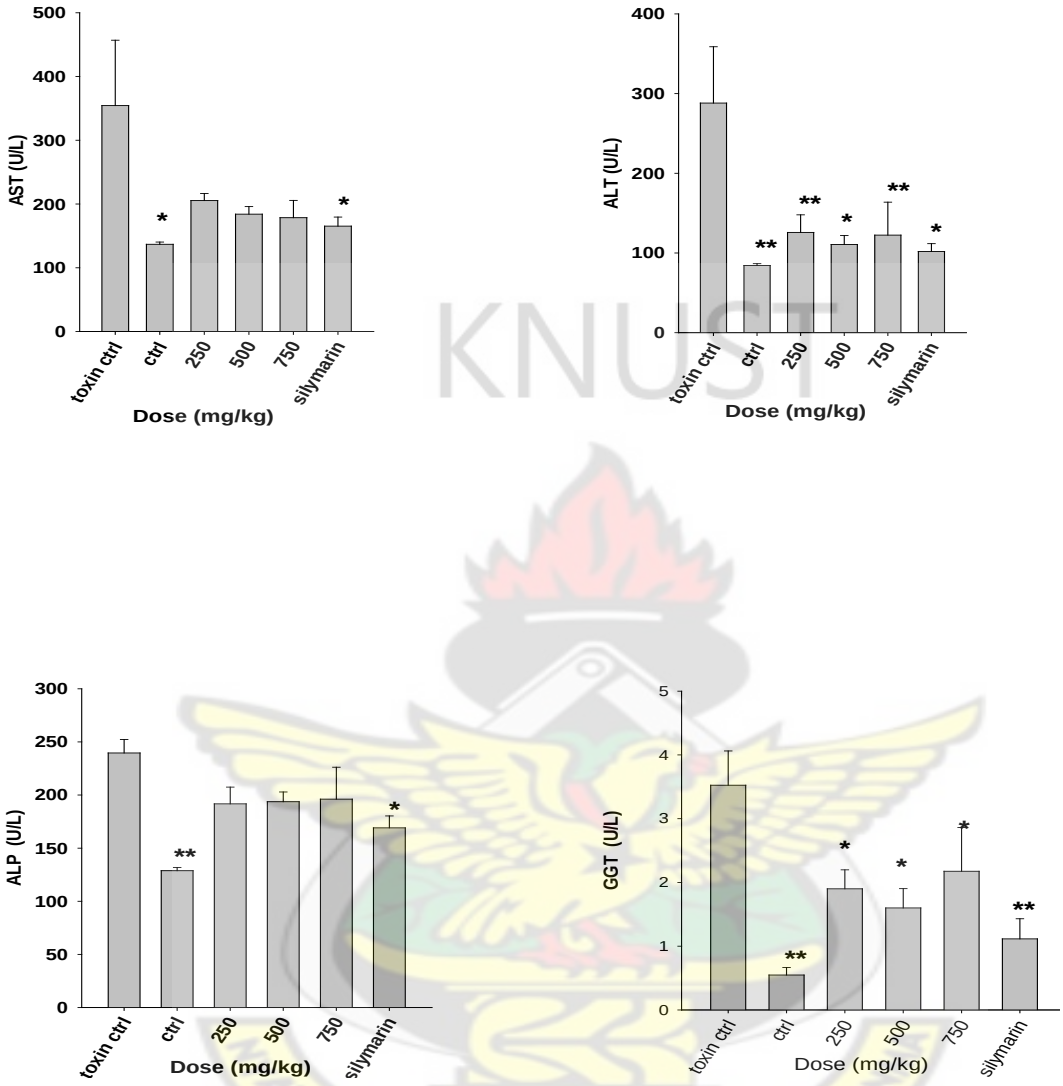

Figure 20: Effect of MLE post-treatment on serum enzymes; ALT, AST, ALP, and GGT levels in acetaminophen intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (acetaminophen) group.

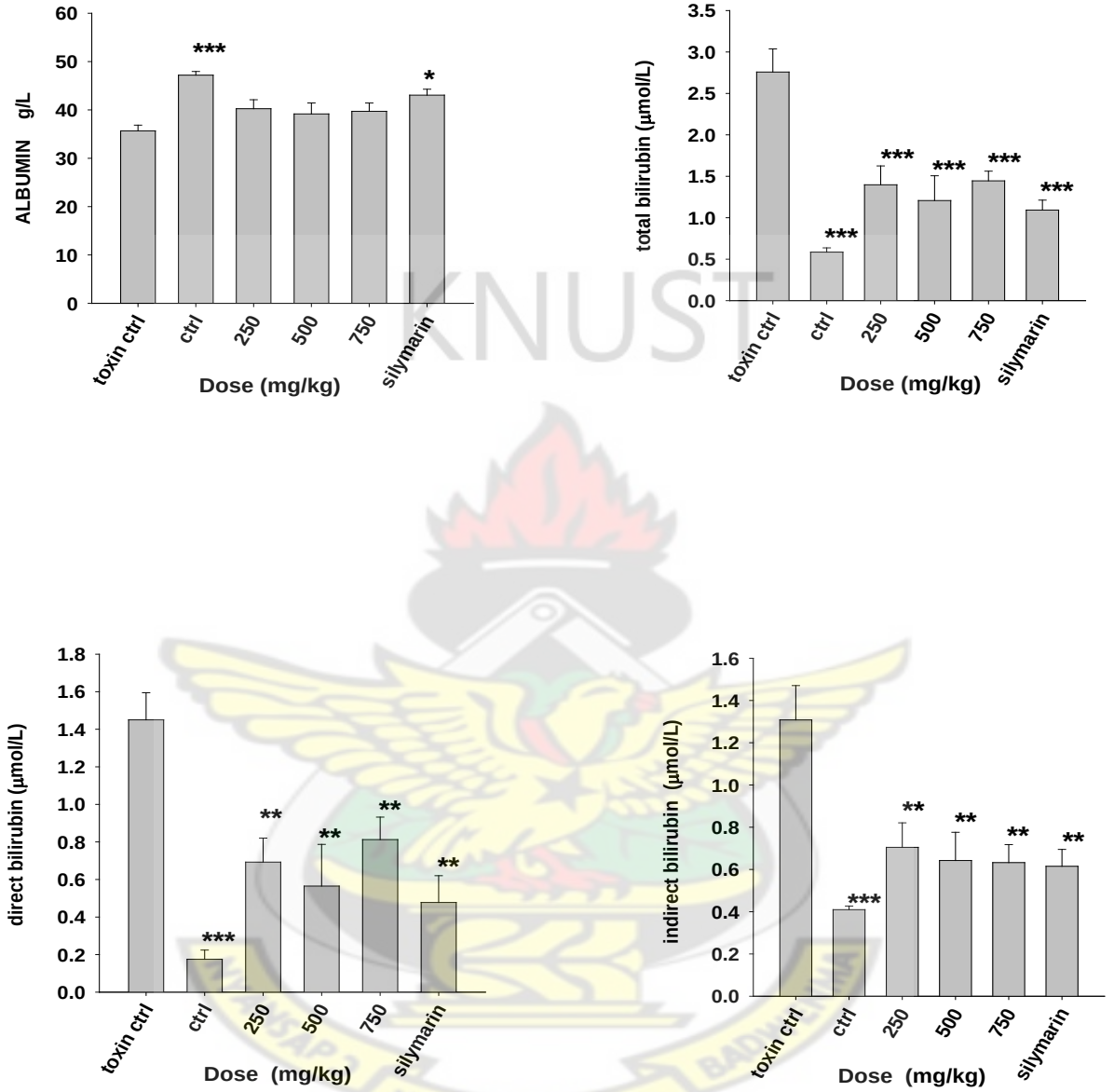

Figure 21: Effect of MLE post-treatment on protein and bilirubin levels in acetaminophen intoxicated rats treated with MLE or silymarin. Bar represents means ± SD (n = 5). (*) indicates significance compared to the toxin control (acetaminophen) group.

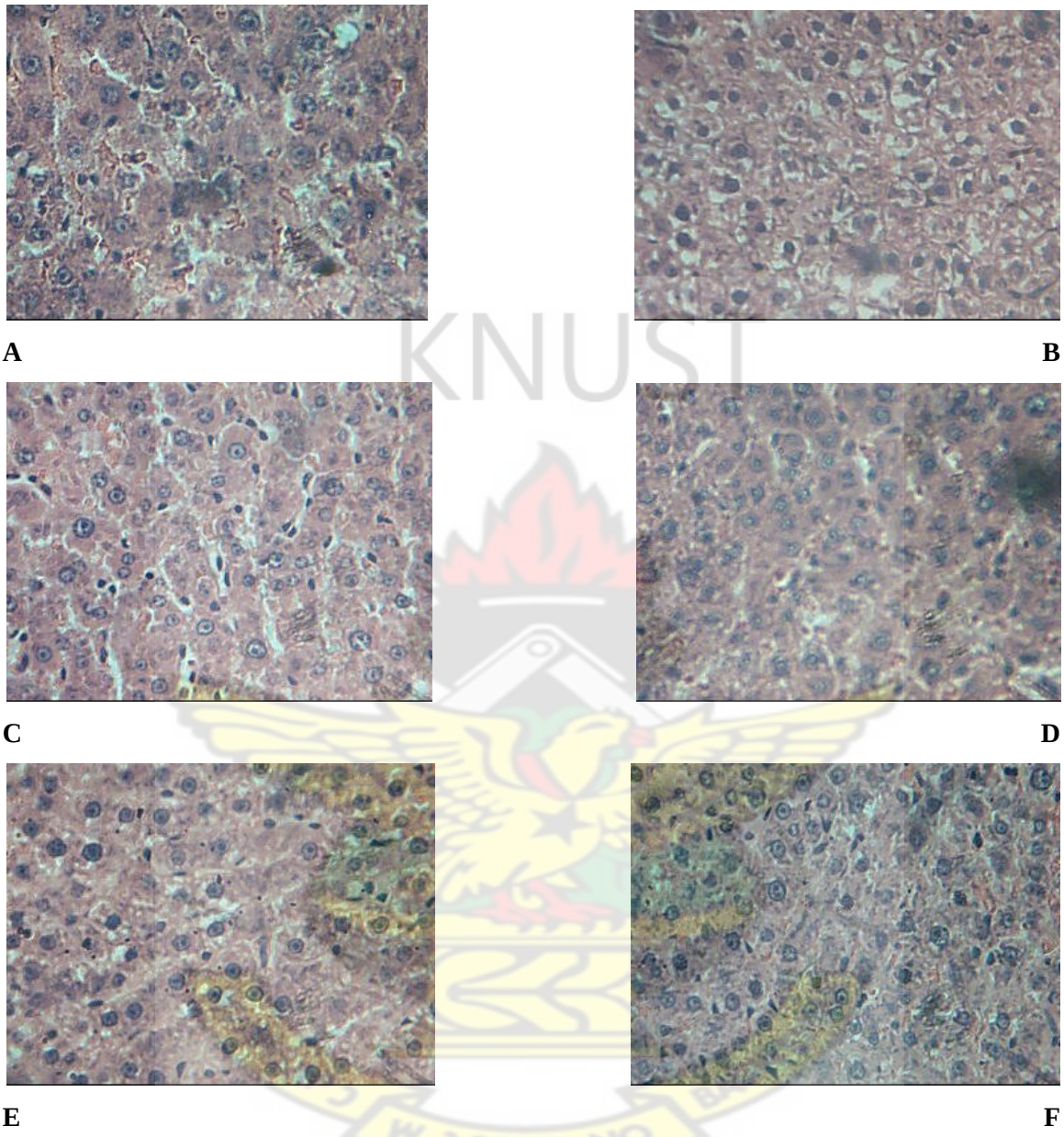

Figure 22: The photomicrographs of liver sections from rats treated with acetaminophen, the post-treatment of MLE at 250, 500 and 750 mg/kg, and vehicle. Liver tissues were stained with H&E (100×). (A) Liver section of APAP treated rat; (B) liver section of control rat; (C) liver section of the APAP-treated rat post-dosed by MLE at 250 mg/kg; (D) liver section of the APAP-treated rat post-dosed by MLE at 500 mg/kg; (E) liver section of the APAP-treated rat post-dosed by MLE at 750 mg/kg; (F) liver section of the APAP-treated rat post-dosed by silymarin.

Chapter 4

GENERAL DISCUSSION

Use of herbal medicine remains and will remain for a long time the main source and method to maintain health for most peole in developing countries (Letouzey, 1972). The use of herbal medicines continues to expand rapidly across the world. Many people now take herbal medicines or herbal products for their health care. However, mass media reports of adverse effects tend to be sensational and give a negative impression regarding the use of herbal medicines in general rather than identifying the causes of these effects, which may be due to a lot of factors. The safety of herbal medicines has become a major concern to both national health authorities and the general public. Safety is a fundamental principle in the provision of herbal medicines and herbal products for health care, and a critical component of quality control.

Morinda lucida has been used in traditional medicine to treat severe malaria (Watt and Breyer-Brandwijk, 1962), jaundice and diabetes (Kamanyi *et al.,* 1994). In the present study, the effect of the extract of *Morinda lucida* was determined on the general toxicity, pentobarbitone induced-sleeping time, prophylactic and curative hepatoprotective studies using carbon tetrachloride (CCl_4) and acetaminophen (paracetamol) models or as the hepatotoxicants.

The haematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status for both animals and humans (Adeneye *et al.,* 2006).

Hematotoxicology is the study of adverse effects of drugs, non-therapeutic chemicals and other agents in our environment on blood and blood-forming tissues (Bloom, 1997). This subspecialty draws on the discipline of hematology and the principles of toxicology.

The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, makes the hematopoietic system unique as a target organ. Accordingly, it ranks with liver and kidney as one of the most important considerations in the risk assessment of individual patient populations exposed to potential toxicants in the environment, workplace, and medicine cabinet

The delivery of oxygen to tissues throughout the body, maintenance of vascular integrity, and provision of the many affector and effector immune functions necessary for host defense, requires a prodigious proliferative and regenerative capacity. The various blood cells (erythrocytes, granulocytes, and platelets) are each produced at a rate of approximately 1–3 million per second in a healthy adult and up to several times that rate in conditions where demand for these cells is high, as in hemolytic anemia or suppurative inflammation (Kaushansky, 2006). This characteristic makes the hematopoietic tissue a particularly sensitive target for cytoreductive or antimitotic agents, such as those used to treat cancer, infection, and immune-mediated disorders. This tissue is also susceptible to secondary effects of toxic agents that affect the supply of nutrients, such as iron; the clearance of toxins and metabolites, such as urea; or the production of vital growth factors, such as erythropoietin and granulocyte colony stimulating factor (G-CSF).

Blood is an important index of physiological and pathological status in man and animals, and the parameters usually measured include red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and haemoglobin (HGB) (Schlam *et al.*, 1975).

The normal range of these parameters can be altered by the ingestion of some toxic plants (Abatan and Arowolo, 1989; Ajagbonna *et al.*, 1999).

The results of the haematological studies showed that *Morinda lucida* leaf extract has little or no effect on RBC, WBC, Hb concentration, platelet number, HCT, MCH, and the MCHC. However, there was an increase in the MCV, which measures the average volume of the cell.

Xenobiotics may affect the production, function and/or survival of erythrocytes. These effects are most frequently manifest as a change in the circulating red cell mass, usually resulting in a decrease (anaemia). Occasionally, agents that increase oxygen affinity lead to an increase in red cell mass (erythrocytosis), but this is distinctly less common. There are two general mechanisms that lead to true anaemia-either decreased production or increased erythrocyte destruction.

Evaluation of a peripheral blood sample can provide evidence for the underlying mechanism of anaemia (Prchal, 2006). The usual parameters of a complete blood count (CBC)-including the red blood cell (RBC) count, hemoglobin concentration (Hbg) and hematocrit (also referred to as packed cell volume, or PCV)-can establish the presence of anaemia. Two additional parameters helpful in classifying an anaemia are the mean corpuscular volume (MCV) and the

reticulocyte count. Abnormalities that lead to decreased haemoglobin synthesis are relatively common (e.g., iron deficiency) and are often associated with a decrease in the MCV and hypochromasia (increased central pallor of RBCs on stained blood films due to the low hemoglobin concentration).

Since anaemia is in a part as a result of a decrease in MCV, the increase in MCV in this toxicity studies means the MLE at the dose of 250 mg/kg will perhaps boost the RBC and haemoglobin level in an individual. The platelet number and the lymphocyte number were not affected by the oral administration of the MLE in the fourteen day study.

In addition to these parameters, body weight changes are the indication of adverse effects of drugs and chemicals and will be significant if the body weight losses were more than ten percent (10%) from the initial body weight (Tofovic and Jackson, 1999; Raza *et al.,* 2002; Teo *et al.,* 2002). When animals lose appetite (anorexia), weight loss is bound to ensue due to disturbances in carbohydrate, protein or fat metabolism (Klaassen *et al.,* 2001). There were no significant differences among groups in weight gain or final weight indicating that the MLE did not alter protein, carbohydrate or fat utilisation. It is then clear that the experimental diets were well accepted by rats.

Also, the most widely used criteria for the toxic action of a drug in animals are reduction in body weight gain, changes in organ weights/body weight ratio and detection of histological abnormalities in the vital organs. In the present study the comparable body weight gain of the experimental animals with controls suggests no gross toxicity of the extract.

The determination of food- and water-consumption parameters is important in the study of the safety of a product with therapeutic purpose, as proper intake of nutrients and water are essential to the physiological status of the animals and to the accomplishment of the proper response to the drug tested, instead of a "false" response due to improper nutritional conditions (Stevens and Mylecraine, 1994; Iversen and Nicolaysen, 2003). The experimental animals consumed food and water very well throughout the period; this indicates that the MLE did not interfere with the dieting patterns of the animals.

Biochemical parameters are an important marker to evaluate the organs and cellular functions. Among the evaluated parameters, such as AST, ALT, and ALP, total bilirubin, unconjugated bilirubin and conjugated bilirubin, total protein, albumin, globulin, and A/G ratio are considered as liver function markers (Palmeiro *et al.*, 2003; Hilaly *et al.*, 2004). The analysis of these parameters is important because several reports of liver toxicity are related to the use of phytotherapeutic products (Corns, 2003; Pittler and Ernst, 2003). It is known that many toxic plant compounds accumulate in the liver, where they are detoxified (Clarke and Clarke, 1977).

These tests help to characterize injury patterns and provide a crude measure of the synthetic capacity of the liver or measure the ability of the liver, either to uptake and clear substances from the circulation or to metabolize and alter test reagents. Albumin is the most commonly used indicator of synthetic function, meaning, low albumin levels indicate poor synthetic function of the liver.

The two major ways where the liver gets injured are damage, or destruction, of liver cells, which is classified as hepatocellular, and impaired transport of bile, which is classified as

cholestatic. Hepatocellular injury is most often due to viral hepatitis, autoimmune hepatitis, and various toxins or toxicants and drugs.

The most specific test for hepatocellular damage is the ALT level. The AST may also be elevated but is not as specific as the ALT. The ALT is the gold test in hepatocellular damage. AST and ALT are enzymes that are present in all the cells, however, they are found in highest concentration in the hepatocytes: ALT in the cytoplasm and AST in the cytoplasm as well as the mitochondria (Al-Habori *et al.,* 2002). Cholestatic injury is best diagnosed by an elevated ALP level. Bile acids stimulate ALP production, but duct obstruction or damage prevents bile acid secretion into the duodenum. Therefore, the ALP level in serum rises dramatically. Because ALP can be derived from other body tissues (e.g. bone, intestine), a concurrent elevation of GGT helps to support a cholestatic mechanism.

Bilirubin, a breakdown product of RBCs, exist in two forms: conjugated and unconjugated. Unconjugated bilirubin appears in the serum when blood is broken down at a rate that overwhelms the processing ability of the liver. A major function of the liver is to convert the bilirubin to a water-soluble form by attaching sugar derivatives onto two of the pyrrole rings, a process called conjugation. The sugar derivative, glucuronic acid; is added in its activated form, UDP-glucuronic acid; this is derived in the liver by the action of a soluble dehydrogenase upon UDP-glucose. A specific enzyme in the endoplasmic reticulum, UDP-glucuronyltransferase, catalyses the addition of glucuronic acid to the two propionnyl side chains of bilirubin in ester linkages (Dutton, 1996, Colleran and O'carra, 1977). The resulting conjugated form of the bile pigment, bilirubin diglucuronide (BDG), has the hydrophilic sugar

residues to offset the hydrophobic property of the rest of the molecule. The conjugated and unconjugated bilirubin molecules may then be passed from the hepatic cells into the bile canaliculi, utilising the ATP-dependent canalicular multidrug resisitant protein. They then percolate down the biliary tree to the gallbladder before being released into the intestinal tract. In the intestine, BDG is metabolised to urobilinogen, some of which is reabsorbed and is in turn excreted in urine, i.e., an enterohepatic circulation of bile pigment metabolites. Urobilinogen that is not reabsorbed is excreted in faeces.

When bile pigments accumulate in the blood and other body fluids, either because of excessive formation or because of inadequate removal in the bile, they may produce intense yellow colouration of the skin. This condition, termed icterus or jaundice, may result from:

- Massive breakdown of erythrocytes (haemolysis) leading to overproduction of bilirubin from haemoglobin catabolism
- A defect in the mechanisms by which the liver disposes of the normal flux of bilirubin.

It is possible to determine if the bilirubin has already been conjugated, and this helps in searching for the causes of the hyperbilirubinaemia. Unconjugated (or indirect) hyperbilirubinaemia is the result of excess production of bilirubin (eg. Hemolysis) or decreased hepatic uptake. Conjugated hyperbilirubinemia results when intrinsic parenchymal injury or biliary obstruction exists. Therefore, acute changes of the conjugated bilirubin levels are related to acute hepatocyte injury in situations such as viral hepatitis or ischemic hepatitis and will be related to the increase in the transaminases.

The extract did not alter the levels of these serum markers of toxicity, suggesting that the MLE is safe in rats up to a dose of 4000 mg/kg.

To determine whether the plant extract has a direct inhibitory effect on hepatic microsomal drug metabolizing enzymes (MDME), it was administered with pentobarbital, a barbiturate, to rats and possible change in the duration of sleep was recorded. The duration of pentobarbital-induced sleep in intact animals is considered as a reliable index for the activity of hepatic MDME (Conney, 1967). Barbiturates are a class of xenobiotics that are extensively metabolized in the liver. Pentobarbital is a short acting barbiturate metabolized by microsomal drug enzymes primarily in the liver. Barbiturate-induced anaesthesia is a popular model of pharmacological or toxicological response, because it is a non-destructive measure of liver function (Lovell, 1986 a, b). Deranged liver function leads to delay in the clearance of barbiturates, resulting in a longer duration of the hypnotic effect (Kulkarni, 1999).

Pre-treatment with the drugs that stimulate liver drug metabolizing enzymes considerably shortens the duration of pentobarbitone-induced narcosis (Singh *et al.,* 1998). Thus, in the present study, pretreatment of animals with plant extract prevented the $CC1_4$-induced prolongation of pentobarbital sleeping time confirming its protective effect against $CC1_4$-induced damage to hepatocytes including MDME. The damage conferred by CCl_4 to hepatocytes as well as hepatic microsomal drug metabolizing enzymes (MDME) in the animals treated with CCl_4 caused loss of drug metabolizing capacity of the liver, resulting in prolongation of pentobarbital induced sleeping time (Javatilaka *et al.,* 1990).

Pentobarbital is metabolized by the hepatic MDME to inactive metabolites and any drug with inhibitory effect on MDME is expected to prolong pentobarbital sleeping time. The fact that the plant extract did not prolong pentobarbital sleeping time suggests that it is devoid of inhibitory effect on hepatic MDME such as cytochrome P450 and its hepatoprotective effect is mediated perhaps through other mechanism(s).

The liver is the major organ responsible for the metabolism of drugs and toxic chemicals, and therefore is the primary target organ for nearly all toxic chemicals (Bissel *et al.,* 2001; Kaplowitz, 2000). CCl_4 is a well known hepato-and nephrotoxicant (Cassilas and Ames, 1986; Kotsanis and Metcalfe, 1991; Thrall *et al.,* 2000; Ogeturk *et al.,* 2005), and proves highly useful as an experimental model for the study of certain hepatotoxic effects (Muriel *et al.,* 2003; Moreno and Muriel, 2006). Liver injury induced by CCl_4 is the best-characterized system of the xenobiotic-induced hepatotoxicity and is a commonly used model for the screening the anti-hepatotoxic and hepatoprotective activity of drugs (Recknagel et al., 1989; Williams and Burk, 1990; Brautbar and Williams, 2002).

CCl_4 metabolism begins with the formation of the trichloromethyl free radical, $CCl^{\cdot}_3$ (McCay *et al.,* 1984) through the action of the mixed function cytochrome P-450 oxygenase system of the endoplasmic reticulum (Recknagel *et al.,* 1989). The major cytochrome isoenzyme to execute biotransformation of CCl_4 is CYP2E1. The $CCl^{\cdot}_3$ radical reacts with several important biological substances, like fatty acids, proteins, lipids, nucleic acids and amino acids (Weber *et al.,* 2003).

The results of the present study show that CCl_4 administration causes severe acute liver damage in rats, demonstrated by the remarkable elevation of serum AST and ALT levels. The increased serum levels of AST and ALT have been attributed to the damaged structural integrity of the liver. This is because these serum enzymes are intracellular enzymes, released into circulation after hepatocyte damage or necrosis (Sallie *et al*., 1991). Serum aminotransferase activities have long been considered as sensitive indicators of hepatic injury (Molander *et al.,* 1955). Injury to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells (Zimmerman and Seeff, 1970). These findings were also confirmed by histological observation. The changes mostly included hepatocellular necrosis or apoptosis, fatty accumulation, inflammatory cells infiltration and other histological manifestations, which were also consistent with the findings of other authors (Brattin *et al.,* 1985; Sun *et al.,* 2001).

Pre-treatment with MLE ameliorated the CCl_4-induced hepatotoxicity in rats, as demonstrated by the lower serum aminotransferase activities. This effect is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and regeneration of hepatocytes (Thabrew *et al,* 1987). A microscopic examination showed that the severe hepatic lesions and necrosis induced by CCl_4 were ameliorated by the administration of MLE, with the most significant at the 250 mg/kg dose level.

Hypoalbuminaemia is most frequent in the presence of advanced chronic liver diseases. Hence decline in total protein content can be deemed as a useful index of the severity of cellular

dysfunction in chronic liver diseases. The lowered level of total proteins recorded in the serum of CCl_4 treated rats revealed the severity of hepatopathy. The attainment of near normalcy in total protein content of serum of MLE-treated rats further support the hepatoprotective effect of the extract. The ability of a hepatoprotective drug to reduce the injurious effects or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effects (Yadav and Dixit, 2003).

Administration of MLE at different dose levels attenuated the increased levels of the serum enzymes, produced by CCl_4 and caused a subsequent recovery towards normalization comparable to the control group's animals.

CCl_4-induced liver damage, a free radical damage model, results from oxidative stress that could directly injure hepatocellular membrane by lipid peroxidation, followed by a series of cascades of cellular events such as the massive release of inflammatory mediators or cytokines, which eventually lead to liver injuries (Pessayre, 1995; Dizdaroglu *et al.,* 2002; Higuchi and Gores, 2003). It has been hypothesized that one of the principal causes of CCl_4 induced liver injury is formation of lipid peroxides by free radical derivatives of CCl_4 (CCl_3). Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 induced hepatopathy (Castro *et al.*, 1974). The body has an effective defense mechanism to prevent and neutralize the free radical induced damage. This is proficient by a set of endogenous antioxidants such as glutathione (GSH), superoxide dismutase (SOD), and catalase. These enzymes constitute a mutually supportive team of defense against reactive oxygen species (ROS) (Amresh *et al.,* 2007c). In CCl_4 induced

hepatotoxicity, the balance between ROS production and these antioxidant defenses may be lost, resulting in an oxidative stress, which through a series of events deregulates the cellular functions leading to hepatic necrosis. The MLE treated groups recovering from the damage caused by CCl_4 might be as a result of it ability to stimulate these anitoxidants to fight the ROS generated by the CCl_4. Regarding non enzymic antioxidants, GSH is a critical determinant of tissue susceptibility to oxidative damage and the depletion of hepatic GSH has been shown to be associated with an enhanced toxicity to chemicals, including CCl_4 (Hewawasam *et al.*, 2003).

These findings, therefore, suggest that the hepatoprotective activity of MLE might be due to its ability to stabilize the cell membrane in hepatocytes, thereby preventing the loss of its functional integrity as well as cellular leakage (Dvorak *et al.*, 2003; Najmi *et al.*, 2005).

In this study, using the SD rats, it was demonstrated that both prophylactic and curative treatments with MLE restored all the enzymes studied showing its potential to maintain the normal functional status of the liver. A possible explanation for the effect is that the constituents detected in MLE posses prominent antihepatoxic activity. This claim can be supported by scientific findings on phenolic acids mainly present in plants, like chlorogenic, caffeic and ferulic acids, as potent hepatoprotective agents (Kapil *et al.*, 1995; Janbaz *et al.*, 2004; Srinivasan *et al.*, 2005). Furthermore, comprehensive investigation of Kinjo *et al.*, 2006, described the *in vitro* hepatoprotective activity of 40 phenolic compounds mostly present in plants, like caffeic acid, ferulic acid and quercetin, and they were also proved to be effective in experimental animal studies.

Acetaminophen (APAP) or paracetamol (PCM) is a common antipyretic agent, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man, rats and mice with toxic doses (Mitchell *et al.,* 1973, Kuma and Rex, 1991, Eriksson *et al.,* 1992). Protection against paracetamol-induced toxicity has been used as a test for potential hepatoprotective activity by several investigations (Ahmed and Khater, 2001, Asha *et al.,* 2004, Kumar *et al.*, 2004, Singh and Handa, 1995, Visen *et al.,* 1993).

It is established that, a fraction of acetaminophen is converted via the cytochrome P450 pathway to a highly toxic metabolite; N–acetyl–p–benzoquinamine (NAPQI) (Dahlin *et al.,* 1984) which is normally conjugated with glutathione and excreted in urine. Overdose of acetaminophen depletes glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction (Parmar and Kandakar, 1995) and the development of acute hepatic necrosis. This NAPQI can further interact with the glutathione (GSH) in the liver (Dahlin *et al*, 1984). If an overdose of APAP is administrated, it reduces hepatic GSH, and NAPQI thus binds covalently to cysteine residues on proteins, causing the production of 3-(cysteine-S-yl) APAP adducts (Mitchell *et al*., 1973). A large number of the metabolites produced by APAP are found to generate superoxide anion and other free radicals in the biological systems (Vries, 1984)

APAP induced hepatotoxicity is pathologically characterized by its centrilobular hepatic necrotic features. Several P450 enzymes are known to play an important role in acetaminophen bioactivation to NAPQI. P450 2E1 have been suggested to be primary enzyme for acetaminophen bioactivation in liver microsomes (Raucy *et al*., 1989). Since cytochrome 450 CYP2E1 plays a crucial role in biotransformation of toxic chemicals, it appears that any

reagent which can down-regulate the cytochrome 450 CYP2E1 activity would be a potential hepatoprotective regimen candidate to protect the hepatocytes from APAP induced toxicity. Studies demonstrated that acetaminophen induced hepatotoxicity can be modulated by substances that influence P450 activity (Mitchell *et al*., 1973).

The present study attempted to develop an experimental design that mimics the actual clinical situation of patients accidentally consuming lethal doses of APAP. Under such circumstances, acute liver toxicity was observed with extensive pathological necrotic features, leading to the animals becoming weak in the general appearance and also with a loss in body weight. A seventy two hour post-treatment or pre-treatment with MLE, however, improved the general appearance of the animals as compared to the toxin control group. The relative changes in body weight of rats indicated that MLE may gradually decrease the toxic effects of APAP. The relative liver weight, which was increased after PCM administration may be due to haemorrhages, hydropic degeneration and fatty changes associated with necrosis. Water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells, resulting in increased total liver mass and volume (Blancho and Gentil, 1990) as observed in the present study. This hepatotoxic agent-induced increase in total wet-liver weight and volume were prevented by pre-treatment with MLE, thus indicating a hepatoprotective effect.

Necrosis or membrane damage releases the enzyme into circulation and hence it can be measured in the serum. Assessment of liver function can be made by estimating the activities of serum levels of ALT, AST, ALP, bilirubin and total protein which are enzymes originally present in higher concentration in the cytoplasm (Manokaran *et al.*, 2008).

Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman and Lawhan, 1978). The abnormally high level of serum ALT, AST, ALP, total bilirubin and decrease in total protein content observed in the toxin control group in the study are the consequence of paracetamol-induced liver dysfunction and denote the damage to the hepatic cells. The elevated levels of serum marker enzyme activities such as ALT, AST enzyme levels are a direct measure of hepatic injury and they show the status of liver. The elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in the liver. Thus, the lowering of the enzyme content in serum is a definite indication of the hepatoprotective action of a drug.

MLE decreased the levels of both AST and ALT as compared to the standard drug silymarin.

Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure (Muriel and Garcipiana, 1992). Elevated ALP level may indicate cholestasis (partial or full blockade of the bile ducts). Since bile ducts bring bile from the liver into gall bladder and intestine, inflammation/damage of the liver can cause spillage of ALP into the blood stream. ALP and gamma-glutamyl transpeptidase (GGT) levels typically rise to several times the normal level following bile obstruction or intrahepatic cholestasis. Causes of elevated ALP may also include biliary cirrhosis, fatty liver and liver tumour. Significant reduction in ALP levels in the extract treated groups indicated that MLE was able to offer protection to the liver against acetaminophen induced hepatotoxicity.

Also, the reversal of increased serum enzymes in acetaminophen-induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew *et al*, 1987). Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretary mechanism of the hepatic cells.

A reduction in total serum protein observed in the paracetamol treated rats may be associated with the decrease in the number of hepatocytes which in turn may result into decreased hepatic capacity to synthesize protein. Hepatotoxicity impairs the synthetic function of the liver. In particular, it reduces albumin production by the liver, and by extension, its serum quantity (David, 1999). The raised in protein and albumin levels suggests the stabilization of endoplasmic reticulum leading to protein synthesis.

Oxidative stress associated with increase in the formation of thiobarbituric acid reactive substances (TBARS) in the paracetamol intoxicated group which is indicative of lipid peroxidation has been adduced to be a crucial step in acetaminophen toxicity (Younes *et al.,* 1986). The antioxidant constituents present in the extracts might have been responsible for their ability to reduce the acetaminophen induced lipid peroxidation (Patrizia *et al.,* 2005; Olaleye and Rocha, 2008). The reactive species mediated hepatotoxicity can be effectively managed upon administration of agents possessing anti-oxidant (Attri *et al.,* 2000), free radical scavenger (Sadanobu *et al.,* 1999) and anti-lipid peroxidant (Lim *et al.,* 2000) activities. MLE treatment was able to ameliorate paracetamol induced hepatocellular damage as evidenced by prevention

of increase in serum transaminase (AST and ALT) levels subsequent to its exposure. Reactive oxygen species are normally detoxified by antioxidation system consisted with non enzyme antioxidation system and antioxidase. SOD, a special antioxidase, converts superoxide anion into hydrogen peroxide, which is detoxified by CAT and GSH-Px. Also, GSH-Px, making GSH as its substrate, removes redundant free radicals and peroxidate in coordination with SOD. The extract might have increased the levels of these antioxidants enzymes.

Bilirubin, a yellow pigment is produced when heme is catabolized. Hepatocytes render bilirubin water-soluble and therefore easily excretable by conjugating it with glucuronic acid prior to secreting it into bile by active transport. Hyperbilirubinaemia may result from the production of more bilirubin than the liver can process, damage to the liver impairing its ability to excrete normal amounts of bilirubin or obstruction of excretory ducts of the liver. Serum bilirubin is considered as one of the true test of liver functions since it reflects the ability of the liver to take up and process bilirubin into bile. High levels of total bilirubin in the acetaminophen induced treated rats may be due to acetaminophen toxicity. This may have resulted in hyperbilirubinemia (Rang *et al.*, 2003). The reduction in the serum bilirubin levels in the MLE treated groups is indicative of the hepatoprotective activity of the extract.

Possible mechanism that may be responsible for the protection against paracetamol induced liver damage by MLE include the following- (a) MLE could act as a free radical scavenger intercepting those radicals involved in paracetamol metabolism by microsomal enzymes. (b) A significantly higher content GSH in blood and liver would afford the tissue a better protection

against antioxidative stress, thus contributing to the abolishment of paracetamol induced hepatotoxicity.

The protection from hepatic diseases in experimental animals had been achieved by administering the chemopreventive agents that modulate the metabolic processing of xenobiotics include phenolic antioxidants, indoles, isothiocyanates, coumarins, flavanones, allyl sulfides, etc. (Kensler, 1997). Preliminary phytochemical studies reveal the presence of flavonoids in the ethanolic leaf extract of *Morinda lucida*. Flavonoids are hepatoprotectives (Seevola *et al*., 1984; Wegner and Fintelmann, 1999). Flavonoids constituents also possess free radical scavenging properties (Hesham *et al.,* 2007). Treatment with MLE showed a protection against the injurious effects of PCM that may result from interference with cytochrome P450, resulting in the hindrance of the formation of hepatotoxic free radicals. The presence of antioxidant compounds in the MLE might possibly be related to the exhibited hepatoprotective activity.

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been disturbed by a hepatotoxin. The plant extract decreased acetaminophen induced elevated enzyme levels in test group, indicating the protection of structural integrity of hepatocytic cell membrane of damaged liver cells.

Extensive vascular degenerative changes and centrilobular necrosis in hepatocytes was produced by acetaminophen. Treatment with alcoholic leaf extract of *Morinda lucida* produced

only mild degenerative changes and absence of centrilobular necrosis, indicating its hepatoprotective efficiency.

In the study, the normal liver tissue showed the typical architecture with a central vein and hepatocytes radiating from it. The portal triad consisted of hepatic artery, portal vein and bile duct which constituted various zones (1, 2 & 3) surrounding these areas. PCM treatment produced centrizonal necrosis (zone 3), fatty and hydropic changes with congestion of sinusoids (Grawford, 2005).

Treatment with MLE restored the hepatic architecture and protected the liver tissue from fatty and degenerative changes, by preventing the toxic chemical reaction, oxidative stress, lipid peroxidation, molecular changes in the liver tissues, micro and macro vesicular fatty changes ultimately leading to necrosis (Koul and Kapil, 1994, Handa and Sharma, 1990). The hepatoprotective effect of MLE may have a role in the process of regeneration, prevention of fibrosis, or formation of nodules which may be expressed in the long term use of the drug (Srivastava *et al.,* 1994, Thresiamma and Kuttan, 1996).

Chapter 5

CONCLUSIONS

This study has shown that the alcoholic leaf extract of *Morinda lucida*

1. Is safe in experimental Sprague-Dawley (SD) rats up to a dose of 4000 mg/kg.
2. Reduces the pentobarbitone induced sleeping time, meaning that it does not affect the normal functioning of the hepatic microsomal drug metabolizing enzymes (MDME).
3. Has hepatoprotective activity against
    - carbon tetrachloride-induced liver damage
    - acetaminophen-induced liver damage

This activity of the extract could be due to its antioxidant activity by the presence of flavonoids and other phytochemical constituents present in the ethanolic leaf extract.

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APPENDIX