SAFETY EVALUATION AND HEPATOPROTECTIVE ACTIVITY OF THE AQUEOUS STEM BARK EXTRACT OF *Spathodea campanulata*

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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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ABSTRACT

Spathodea campanulata, a widely used traditional African medicinal plant for skin diseases and stomach aches has not been scientifically assessed for toxicity and hepatoprotection. The aqueous extract of the stem bark of the plant *Spathodea campanulata* (SCE) was therefore evaluated for safety and potential hepatoprotective activity in rodents. The extract (1250, 2500, 5000 mg/kg, p.o) administered to rats and mice over a 24-hour period did not show any signs of toxicity or mortality, suggesting that the oral LD_{50} of the aqueous extract (in rats and mice) was beyond 5000 mg/kg. Daily administration (1250, 2500, 5000 mg/kg; p.o) of extract for 14 days did not cause any changes in behavior or alterations in haematological parameters of the animals. Serum biochemical analysis in rats showed a rise in alkaline phosphatase (ALP) (339.50±90.50 in the control to 582.5±28.50 U/L in the highest dose of 5000 mg/kg) and a decrease in Na^+ concentration (147.3±1.856 mmol/L in the control to 135.0±1.00 in the 5000mg/kg dose), but no change in other parameters. These observations did not reflect in the histopathology of the liver and kidneys of treated animals. However, the diuretic test on the extract was positive. Urine output, Na^+ and K^+ concentration in urine were increased significantly by the administration of extract for seven days. In hepatoprotective studies, rats were pre-treated with 625 mg/kg , 1250 mg/kg, 2500 mg/kg p.o for 4 days before intoxication with carbon tetrachloride (CCl_4) (1 ml/kg, 20 % in liquid paraffin, p.o) or mice and rats with 100 mg/kg, 300 mg/kg, 625 mg/kg for 4 days before intoxication with paracetamol or aflatoxin B1 (AFB1) respectively. Generally, the administration of these hepatotoxic agents caused an increase in liver weights which were prevented or restored by pre-treatment with extract in both rats and mice. The aspartate transaminase (AST), alanine transaminase (ALT), gamma glutamyl transpeptidase (GGT), bilirubin-induced by these hepatotoxic agents (CCl_4, parcetamol and AFB1) were significantly reduced with SCE treatment. Additionally, administration of CCl_4 daily for 5 days, followed by treatment with 100, 300, and 625 mg/kg p.o daily for 3days (curative) decreased profoundly the AST, ALT and GGT levels induced by CCl_4. These results

correlated well with the histopathological studies observed in the photomicrographs of liver for treated and control groups. Treatment with the extract decreased the extent of fatty liver and necrosis caused by the hepatotoxic agents. Lipid peroxidation measured as TBARS was elevated by CCl_4 but this was reversed by treatment with the extract at all dose levels. Again, pretreatment with SCE (625 mg/kg) before hepatotoxicity by CCl_4 or paracetamol restored the decrease in super oxide dismutase (SOD) and glutathione peroxidase (GPx) activity caused by the hepatotoxin. This decrease was more profound after paracetamol intoxication than it was after CCl_4 intoxication. The extract (625 mg/kg) also showed strong enzyme inhibition when it reduced the total cytochrome P450 in both mice and rats when given for seven consecutive days. This decrease was also reflected in the presence of CCl_4 and paracetamol. The preliminary phytochemistry which showed the presence of tannins, sterols and reducing sugars as well as the *in vitro* testing which gave positive results for reducing power and total phenolic content also support the activity of the plant extract in interference with hepatotoxicity. Collectively, the results indicate that the aqueous extract of *Spathodea campanulata* is fairly nontoxic and may exhibit hepatoprotective activity at lower doses by enhancing antioxidant protection in the cell and inhibiting total cytochrome P450 hence interfering with bioactivation of hepatotoxic agents.

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ABBREVIATIONS

SCE- *Spathodea campanulata* stem bark extract

WHO- World health organisation

STZ- Streptozotocin

GTT- Glucose tolerance test

DPPH- 2, 2-diphenyl-1-picrylhydrazyl

MIC- Minimum inhibitory concentration

CML- Chronic myeloid leukemia

MDR- Multidrug resistance

ICF- Intracellular fluid

ECF- Extracellular fluid

GFR- Glomerular filtration

CYP- Cytochrom P450

TCA- Trichloro acetic acid

AUC- Area under curve

DILI- Drug induced liver injury

CCl_4- Carbon tetrachloride

NAC- n-acetyl cysteine

NSAIDS- Non steroidal anti-inflammatory drugs

NAPQI- N-acetyl-p-benzo quinone imine

GSH- Reduced glutathione

GSSG- Oxidized glutathione

AFB1- Aflatoxin B1

NAFLD- Non-alcoholic fatty liver disease

HCC- Hepatocellular carcinoma

SOD- Superoxide dismutase

LPO- Lipid peroxidation

MDA- Malondialdehyde

SD- Spraque-Dawley

ICR- Imprinting Control Region

EDTA- Ethylene diamine tetra acetic acid

WBC- White blood cell

HCT- Haematocrit

PLT- Platelet

MCV- Mean corpuscular volume

MCH- Mean corpuscular haemoglobin

MCHC- Mean corpuscular haemoglobin concentration

AST- Aspartate aminotransferase

ALT- Alanine aminotransferase

ALP- Alkaline phosphatase

GGT- Gamma glutamyl transpeptidase

T-BIL- Total bilirubin

D-BIL- Direct bilirubin

I-BIL- Indirect bilirubin

BUN- Blood urea nitrogen

SEM- Standard error of maen

ALB- Albumin

T Pro- Total protein

CCl_3- Trichloro methyl radical

ROS- Reactive oxygen species

PMSF- Phenyl methane sulphonyl fluoride

NADPH- Nicotinamide Adenine dinucleotide phosphate-reduced

NBT- Nitroblue tetrazolium chloride

GPx- Glutathione peroxidase

TBARS- Thiobarbituric acid reactive substance

TBA- Thiobarbituric acid

CO- Carbon monoxide

CHAPTER 1

GENERAL INTRODUCTION

1.1 OVERVIEW

Herbal medicines have recently attracted much attention as alternative medicines useful for treating or preventing life-style related disorders (Agyare *et al.*, 2009). However, relatively very little knowledge is available about their mode of action and safety. The aqueous stem bark extract of *Spathodea campanulata* (SCE) is known in traditional medicine in Ghana and other parts of Africa for treating dysentery, stomachache, gonorrhoea, backache and skin diseases. It also has laxative and antiseptic properties while the dried seeds are used to prepare arrow poison to kill game (Abbiw, 1990) but there is no scientific work on safety or toxicity of other parts of the plant. In this study, SCE, which is considered a panacea in Ghanaian traditional medicine, is assessed *in vivo* for potential toxicity and possible hepatoprotection.

1.2 GLOBAL USE OF HERBAL REMEDIES

The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into the African continent. Herbal medicine has been widely used and formed an integral part of primary health care in China, Ethiopia, Argentina and Papau New Guinea (Akinyemi *et al.*, 2005).

The earliest recorded use of herbal remedies (Greek) comes from Hippocrates, who advocated using a few simple plants, such as garlic (Rivlin, 2001). Other cultures, which do not have a well recorded history such as the native peoples of Africa, South America, North America and the indigenous tribes of Australia, have also used plants for medicinal purposes (Amusan *et al.*, 2002; Desmarchelier *et al.*, 1996; Hart, 1981).

Herbal remedies have a therapeutic effect and are acceptable interventions for diseases and symptoms. Interestingly, demand for medicinal plants is progressively rising in industrialized nations as it is in developing countries (Abere *et al.*, 2010). The World Health

Organisation (WHO) estimates that about 80% of the developing world's population meets their primary healthcare needs through traditional medicine (Abere *et al.*, 2010; Calixto, 2000; Green, 2000; Jadeja *et al.*, 2011). About 25% of drugs prescribed and dispensed in the United States contain at least one active component derived from plant matter.

While some are synthesized to mimic a natural plant compound, others are made from plant extracts. It has been estimated that one out of every three people in the United States had tried at least one form of alternative medicine (Eisenberg *et al.*, 1993). A follow up study reported that the number of respondents using alternative therapies increased from 33.8% in 1990 to 42.7% in 1997 (Eisenberg, 1997) most of which are from plant sources.

In Ghana however, though speculations abound, there is no accurate data on the true extent of the use of alternative therapies. It is anticipated that there is one (1) traditional doctor to approximately two hundred (200) people as opposed to one (1) orthodox doctor to twenty thousand (20000) people (Briggs, 2007).

Within the last few decades, many plants have been screened for their biological and pharmacological properties by researchers. These steps are continually being taken to study the intrinsic worth of traditional medicine in the light of modern science with the scrutiny aimed at adopting effectively beneficial medical practice and discouraging harmful ones (Abere *et al.*, 2010). Note worthily, components of herbs serve as starting materials for a number of old and new pharmaceutical products. About 25% of modern pharmaceutical drugs have botanical origins. For example, the herb foxglove is the source for digitalis and the herb salicin is the source for aspirin. The breast-cancer-fighting drug taxol (tamoxifen) comes from the pacific yew tree. However, the lack of standards in manufacturing and oversight by regulatory agencies has exposed patients to possible hazards from these herbal medications (Eisenberg *et al.*, 1998).

There has been a growing interest in the analysis of plant products which has stimulated intense research on their potential health benefits. Testing identifies the purity and

concentration of the active ingredient that delivers the therapeutic effect. It could also identify the amount of the herb that can provoke serious and potentially lethal toxic effects.

In this project, the aqueous stem bark extract of *Spathodea campanulata* is evaluated for safety and hepatoprotective activity.

1.3 THE PLANT SPATHODEA CAMPANULATA

Also referred to as African Tulip tree (Blundell *et al.*, 1987) or Flame tree, *Spathodea campanulata* is the botanical name. It is a single species of the monotypic genus *Spathodea* in the flowering plant family Bignoniaceae which is composed of around 800 species distributed in 112 genera (Spangler and Olmstead, 1999).

In Ghana, it is locally known in Twi as Kokonisu/ Kokonsu, meaning red tears and in Ewe as Adatigo, meaning tear pod.

Spathodea campanulata, is a large upright tree with a stout, tapering often somewhat buttressed trunk, thickish, marked with small white lenticels, subglabrous to thinly puberulent; leaves are usually opposite (rarely 3 at a node), very widely diverging, up to 50 cm long, leaflets broadly elliptic or ovate glossy deep green pinnate leaves and glorious orange scarlet flowers.

It may grow to eighty (80) ft. but most species are much smaller (Corner, 1997).

The flower bud is ampule-shaped and contains water. The sap sometimes stains yellow on fingers and clothes. The open flowers are cup-shaped and hold rain and dew, making them attractive to many species of birds. The wood of the tree is soft and is used for nesting by many hole-building birds such as barbets. In neotropical gardens and parks, their nectar is popular with many hummingbirds, such as the Black-throated Mango (*Anthracothorax nigricollis*), the Black Jacobin (*Florisuga fusca*), or the Gilded Hummingbird (*Hylocharis chrysura)* (Joly, 1985).

Figure 1.1 *Spathodea campanulata* showing stem, branches, leaves and flowers. Adopted from www.floridata.com

Spathodea campanulata is widely distributed throughout Africa, specifically in the rainforest of equatorial Africa and found abundantly in Cameroon. It is distributed through Guinea to Angola, Sudan and Uganda (Joly, 1985). It is native to Ghana, Zambia, Uganda, Tanzania, Sudan, Angola, Ethiopia and Kenya (Orwa *et al.*, 2009) and also widely planted throughout the tropical and subtropical areas including South America as well as the pacific. It is favoured by moist habitats below 3,000 ft (914m)

1.3.1 Medicinal Uses

Traditional uses

In traditional medicine, the tree is known for the treatment of various ailments. Although most of the traditional medical practice is in Africa, it is also found in Indo-China where the flowers are used to heal ulcers (Perry and Metzger, 1980).

In Ghana, it is a locally known medicinal plant used for the treatment of various disorders almost as a universal remedy. The bark has laxative and antiseptic properties, and the seeds, flowers and roots are used as medicines for several conditions (Abbiw, 1990; Joly, 1985).

An infusion of the bark is known in Ivory Coast as an enema for back ache. Also, the leaf decoction has been used as a poison antidote, for treatment of gonorrhoea and women's pelvic disorders. In Ghana the bark infusion is used for the treatment of dysentery and stomach ache (Mendes *et al.*, 1986). The bark pulp is used in wound healing in Ghana while decoctions of the stem bark are used as an enema in Rwanda, to treat insulin and non-insulin dependent diabetes (Niyonzima *et al.*, 1990). The bark pulp is also used for rubbings and plasters in treating oedemas, skin diseases like herpes and sores. In Gabon, the crushed bark and flowers have been applied to ulcers. The cold leaf infusion is used to treat urethral inflammation much as the bark decoction has been reported to be used to treat kidney disorders (Abbiw, 1990). In Senegal, the bruised leaves and flowers are used in wound treatment and ulcers. The flowers are employed as diuretic and anti-inflammatory, while the leaves are used against kidney disease, urethra inflammation and as an antidote against animal poisons (Mendes *et al.*, 1986).

1.3.2 Biological Activities of Spathodea campanulata

Molluscicidal Activity

Flowers and stem bark extracts have been shown to have molluscicidal activity when hexanic and ethylic extracts of *Spathodea campanulata* was evaluated against adults and egg masses of *Biomphalaria glabrata* (Mendes *et al.*, 1986).

Hypoglycaemic Activity

Spathodea campanulata stem bark decoction has shown a hypoglycaemic activity in streptozotocin (STZ) diabetic mice. The decoction, washed with chloroform, was divided in water and butanol fractions. Both had a hypoglycaemic activity but were devoid of any influence on insulin levels in STZ diabetic mice (Niyonzima *et al.*, 1999). The decoction also decreased blood glucose levels in a glucose tolerance test (GTT) in normal mice with the most polar fraction, when separated by column chromatography, exerting by far the most prominent effect in different biological models (Niyonzima *et al.*, 1999).

Following four insulin-dependent diabetes patients as they started to use *Spathodea campanulata* decoctions, as enema, daily for several weeks and later once a week, the four male patients who were 42 to 48 years old and had been known diabetics for 4**,** 17, 22 and 24 months showed remarkable improvement. All four had tried glibenclamide without success and they used respectively 40, 28, 20 and 40U insulin daily. Insulin treatment was abandoned without side effects with two of them having completely normal glycaemia and the other two presenting slight hyperglycaemia (Laekeman *et al.*, 1991).

Antimalarial Activity

In antimalarial research, *Spathodea campanulata* stem bark extract demonstrated activity on *Plasmodium berghei* in mice (Makinde *et al.*, 1988).

Anti-inflammatory and antioxidant properties

Studies on extracts from the bark and leaves have shown that the tree (leaves and bark) has anti-inflammatory and antioxidant properties due to flavonoids, triterpenoids, diterpenoids and caffeic acid derivatives. The antioxidant property of the stem bark extract was demonstrated using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) test, with the extract showing a strong reactive oxygen species scavenging effects (Houghton *et al.*, 2005).

Antibacterial Activity

The antibacterial activity of the aqueous, ethanol, methanol and petroleum ether soxhlet extracts of sundried stem bark of *Spathodea campanulata* P. Beauv. (Bignoniaceae) was investigated by testing the extracts against *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus*. The minimum inhibitory concentration (MIC) of the methanol extract was determined against the four bacteria strains and *C. albicans* using the broth dilution method (Mensah *et al.*, 2006; Ofori-Kwakye *et al.*, 2009). *S. Campanulata* showed significant inhibition against these microbacteria.

Wound healing Activity

The evolution over recent years of tests for wound healing, from *in vivo* tests to cell-based systems and chemical reactions and on to investigations into effects on secondary messengers and protein expression, has been described (Houghton *et al.*, 2005).

1.3.3 Some Identified Chemical Constituents of Spathodea campanulata

The widespread use of *Spathodea campanulata* in traditional medicine has stimulated more pharmacological studies over recent years. Phytochemical investigation of *S. campanulata* bark has shown the presence of triterpenes and sterols (Ngouela *et al.*, 1988).

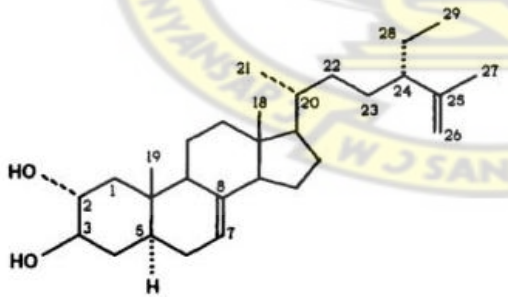
The predominant component of *S. campanulata* is n-alcohols (35%), with octacosanol and triacontanol as the most abundant ones (Gormann *et al.*, 2004). Spathoside, a new

cerebroside was isolated from the stem bark of *Spathodea campanulata*, besides known compounds (n-alkanes, linear aliphatic alcohols, sitosterol and their esters, beta-sitosterol-3-O-beta-D-glucopyranoside, oleanolic acid, pomolic acid, p-hydroxybenzoic acid and phenylethanol esters (Mbosso *et al.*, 2008)). Seven (three new and four known) iridoids have been isolated from the leaves of *Spathodea campanulata*. Provisionally, the new three have been named as spathosides A, B and C respectively. The known iridoids have been identified as verminoside, 6'-O-trans-caffeoyl-loganic acid, catalpol and ajugol (Gouda, 2009). The structures of the isolated compounds were established by spectroscopic studies and when the antibacterial activity of the isolated compounds was examined against a wide range of microorganisms, they inhibited significantly the growth of some gram-positive and -negative bacteria (Mbosso *et al.*, 2008).

A few of the compounds known to have been isolated from *Spathodea campanulata* are mentioned below.

Spathodol (1) was isolated as well as caffeic acid, phenolic acid and flavonoids from the leaves of *Spathodea campanulata* (El-Hela, 2001). In the fruits are known polyphenols, tannins, saponins and glucosides (Amusan *et al.*, 2002).

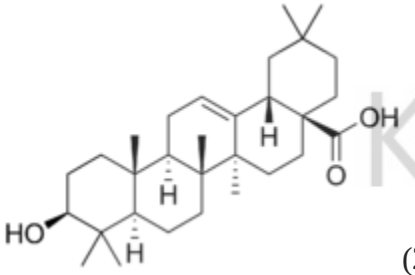
Acid-base extraction of fresh *Spathodea campanulata* root peels and exhaustive purification yielded two phenolic compounds, identified as methyl *p*-hydroxy- benzoate and *p*-hydroxy-benzoic acid. Both phenolic constituents exhibited an evident aromatic 1,4 substitution pattern in 1 H-NMR spectra (El-Hela, 2001).



(1)

Spathodol is a new dihydroxylated sterol isolated from the leaves of *Spathodea campanulata*.

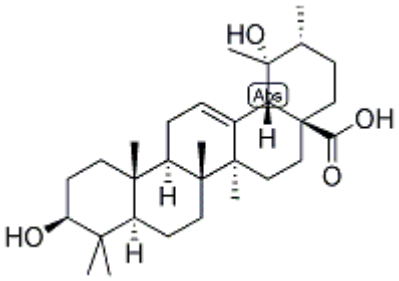
Another important compound which has successfully been isolated from *Spathodea campanulata* is oleanolic acid (2).



(2)

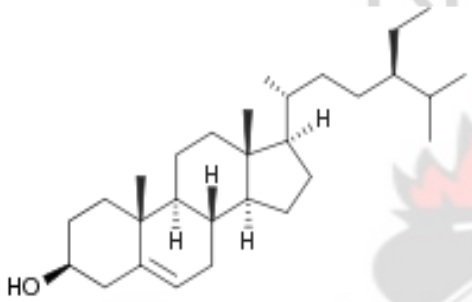
Oleanolic acid is a pentacyclic triterpenoid carboxylic acid compound that exists widely in food, medicinal herbs and other plants. It is an isomer of ursolic acid. Oleanolic acid is effective in protecting against chemically induced liver injury in laboratory animals (Liu, 1995). Oleanolic acid has been marketed in China as an oral drug for human liver disorders. The mechanism of hepatoprotection by this compound may involve the inhibition of toxicant activation and the enhancement of the body defense systems. Oleanolic acid has also been long-recognized to have anti-inflammatory and anti-hyperlipidemic properties in laboratory animals (Liu, 1995).

Pomolic acid (PA) (3) also a pentacyclic triterpene has been isolated. It has been previously described as active in inhibiting the growth of K562 cell line-originated from chronic myeloid leukemia (CML) in blast crisis as well as its vincristine-resistant derivative K562-Lucena1, that displays multidrug resistance (MDR) phenotype (Fernandes *et al.*, 2005).



(3)

β-sitosterol (4), also isolated from *Spathodea,* is one of several phytosterols with chemical structures similar to that of cholesterol. Beta-sitosterol differs from cholesterol by the presence of an extra ethyl group. It is white in colour and waxy in nature.



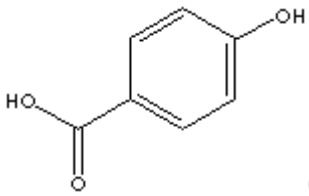
(4)

Alone and in combination with similar phytosterols (one study shows a positive effect on male hair loss in combination with saw palmetto), β-sitosterol reduces blood levels of cholesterol and is sometimes used in treating hypercholesterolemia (Prager *et al.*, 2002).

It is also used for the treatment of prostatic carcinoma and breast cancer, although the benefits are still being evaluated in the United States (Awad *et al.*, 2008). Beta-sitosterol is an antioxidant able to reduce DNA damage, reduce the level of free radical in cells and to increase the level of typical antioxidant enzymes.

The use of sitosterol as a chemical intermediate was for many years limited due to the lack of a chemical point of attachment on the side chain that would permit its removal. Extensive efforts on the part of the many laboratories eventually led to the discovery of a pseudomonas

microbe that efficiently affected that transformation. Fermentation digests the entire aliphatic side chain at carbon seventeen (17) to afford a mixture of 17-keto products including dehydroepiandrosterone (Lenz, 1983).



(5)

P-Hydroxybenzoic Acid (5), one of three crystalline derivatives of benzoic acid (isolated from *Spathodea campanulata)*, is a white crystalline powder; slightly soluble in water, soluble in hot alcohol, ether; melts at 215^0C. It contains both hydroxyl and carboxyl group at para-position, which react with either acid or alcohol. p-Hydroxybenzoic acid is used for the preparation of biocides, antiseptics and bacteriostatic agents. It is used as a chemical intermediate for synthetic pharmaceutical drugs, dyes and plasticizers.

1.3.4 Non-medicinal uses

The seeds of *Spathodea campanulata* are edible and used in many parts of Africa.

Timber: In its original habitat, the soft, light brownish-white wood is used for carving and making drums. It is also used in making paper (Nathan and Chee, 1987). In West Africa, their homeland, the wood is used to make drums and blacksmith's bellows.

Poison: The hard central portion of the fruit is used to kill animals. The bark is chewed and sprayed over swollen cheeks. The bark may also be boiled in water used for bathing newly born babies to heal body rashes. Other services for which *Spathodea campanulata* is used include:

Shade or shelter: Recommended as a shade tree for parks and yards, *Spathodea campanulata* has been used for coffee shade.

S. campanulata also helps rehabilitate disturbed lands through its quick invasion and rapid growth (Corner, 1997).

Ornamental: *S. campanulata* has been planted as an ornamental throughout the tropics. The flowers bloom with great profusion, and the trees can be seen from great distances. It is not browsed by domestic animals and is popular as a decorative tree for avenues. The species, either planted or growing naturally, is frequently used for living fence posts.

It has shallow roots and a tendency for branches to break off in a storm and therefore considered unsuitable as a roadside tree.

1.4 SAFETY EVALUATION OF MEDICINAL PLANTS

Drugs in general need to be safe at the dose recommended for use. For this reason, the safety of drugs needs to be ascertained before approval for use by regulatory authorities in most countries. When found to be toxic, drugs are usually withdrawn from circulation; for example, troglitazone (Rezulin), a new antidiabetic drug, was removed from the market after close to 100 of the 1.9 million patients treated with the drug suffered liver failure (Chojkier, 2005). _Adequate investigation is therefore necessary to ascertain the safety dose and toxic dose of a drug.

Plants in general are rich in antioxidants and other helpful constituents; therefore use of their various parts for treatments focuses on these properties to the neglect of possible toxicity. Herbal medicines have therefore been considered to be safe and are being used for the treatment of various problems by the general public as well as traditional doctors worldwide (Oduola *et al.*, 2007).

Although toxicity resulting from the use of herbs have been documented in literature on many occasions, the potential toxicity of herbs has not been acknowledged by the general

public or by some professional groups of traditional medicine practitioners (O'Hara *et al.*, 1998).

The extent or mode of toxicity of a substance can be affected by many different factors, such as the pathway of administration, the time of exposure, the number of exposures, the physical form of the toxin (solid, liquid, and gas), the genetic makeup of an individual, an individual's overall health, and many others. Many substances regarded as poisons are toxic only indirectly. An example is alcohol, or methanol, which is chemically converted to formaldehyde and formic acid in the liver. It is the formaldehyde and formic acid that cause the toxic effects of methanol exposure.

On the other hand, idiosyncratic toxicity is a leading cause for failure of drugs in clinical testing and it is the most frequent reason for posting warnings, restricting use, or even withdrawal of the drug from the market.

1.4.1 Factors influencing Toxicity

Human fatalities caused by poisonous plants - especially resulting from accidental ingestion - are rare in the USA (Krenzelok and Mrvos, 2011). Unfortunately, there is little or no documentation available in Ghana and most parts of Africa on incidents of fatalities caused by poisonous plants. This is because, almost every plant material ingested is considered medicine or food.

Generally, the toxicity of plants is caused by the presence of certain compounds such as cyanogenic glycosides and lectins like phytohaemaglutinin.

Many food plants possess toxic parts, are toxic unless processed, or are toxic at certain stages of their life. Notable examples include: Apple (*Malus domestica*) whose seeds contain cyanogenic glycosides; in most species, the amount found in a single fruit won't kill a person; but it is possible to ingest enough seeds to provide a fatal dose.

Other plants with toxic effects include: *Cerbera odollam* (colloquially known as the suicide tree). The nut contains cerberin, which is known to stop the heart (Gaillard *et al.*, 2004). *Helleborus niger* (also known as Christmas rose) contains protoanemonin (Olson, 2003), or ranunculin (Spoerke and Smolinske, 1990), which has an acrid taste and can cause burning of the eyes, mouth and throat, oral ulceration, gastroenteritis and hematemesis (Olson, 2003).

Castor bean, *Ricinus communis*, often used as an additive to the product mix of sweets and food in Africa has been documented amongst the world's five most poisonous plants (www.prlog.org). Seeds are very toxic, if ingested can lead to nausea, stomach cramps, vomiting (after effect of nausea), bleeding, and finally failure in the metabolic system.

1.4.2 Assessment of Toxicity

Toxicity is the degree to which a substance is able to damage an exposed organism. The study of the adverse effects of chemicals on living organisms; the study of symptoms, mechanisms and treatments is called toxicology. Toxicity can refer to the effect on a whole organism, such as an animal, bacterium or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or organ (organotoxicity), such as the liver (hepatotoxicity).

Toxicity may be acute, sub-acute/sub-chronic or chronic.

Acute Toxicity

Acute toxicity looks at lethal effects following oral, dermal or inhalation exposure to a toxin. In acute toxicity, there is a single exposure to a toxicant which may result in severe biological harm or death; acute exposures are usually characterised as lasting no longer than twenty four (24) hours.

The information on acute systemic toxicity generated by the acute toxicity testing is used in hazard identification and risk management in the context of production, management, and utilization of chemicals.

The LD_{50} value, (the statistically derived dose that, when administered in an acute toxicity test, is expected to cause death in 50% of the treated animals in a given period), is currently the starting point for toxicological classification of chemicals. Laboratory mice and rats are the species typically selected for a classic LD_{50} study. Often both sexes must be used for regulatory purposes. When oral administration is combined with parenteral, information on the bioavailability of the tested compound is obtained (Walum, 1998).

Efforts have also been made to develop *in vitro* systems; e.g., it has been suggested that acute systemic toxicity can be broken down into a number of biokinetic, cellular, and molecular elements, each of which can be identified and quantified in appropriate models. The various elements may then be used in different combinations to model large numbers of toxic trial to predict hazard and classify compounds.

Chronic Toxicity

Chronic exposure to a toxin is over an extended period of time, often measured in months or years, and can cause irreversible side effects. Two distinct situations need to be considered:
Prolonged exposure to a substance, for example ingestion of too much alcohol on a regular basis; Alcohol has a short half-life but if ingested on a regular basis, results in health hazards); Prolonged internal exposure because a substance remains in the body for a long time; for example if a person were to ingest radium, much of it would be absorbed into the bones where it would exert a harmful effect in the blood cell-forming bone marrow.
Periods of exposure between acute and chronic could be referred to as sub-acute or sub-chronic.
A central concept of toxicology is that effects are dose dependent; even water can lead to water intoxication when taken in large enough doses, whereas for even a very toxic

substance such as snake venom, there is a dose below which there is no detectable toxic effect.

Drugs that exert their toxic effects on the liver may do so through the hepatic production of a toxic metabolite. Drug toxicity may occur in all individuals exposed to a sufficient concentration of a particular drug. A drug may even be toxic in some individuals at concentrations normally tolerated by most patients prescribed the drug. This phenomenon is known as idiosyncratic drug toxicity, and may be due to a genetic or immunologic cause.

1.4.3 Target organ Toxicity

The extent to which an organ is susceptible to toxicity varies from organ to organ. For example the kidneys and liver are more highly vascularised making them more susceptible to toxicity than the bone tissues.

Blood Toxicity

Blood which forms the main medium of transport in the body is a very important tissue. It serves to transport many drugs and xenobiotic. Since all foreign compounds are distributed via the bloodstream, the various components, cellular and non-cellular, are initially exposed to significant concentrations of toxic compounds (Timbrel, 2009).

Some plant materials when ingested either in the raw state or their extract, have been reported to cause anaemia which may result from sequestration of red blood cell in the spleen, impaired red cell production or primary bone marrow dysfunction (Cheeke, 1998).

Damage to and destruction of the blood cells results in a variety of consequences such as a reduction in the oxygen-carrying capacity of the blood if the cells affected are the red blood cells. The assessment of blood is relevant to the evaluation of risks since the haematological system carries a higher predictive value for toxicity in humans (90%) (Olson *et al.*, 2000).

Kidney Toxicity

The kidneys have several functions and serve important regulatory roles in most animals. They are essential in the urinary system in serving homeostatic functions such as the regulation of electrolytes, maintenance of acid-base balance, and regulation of blood pressure (via maintaining salt and water balance) and serving the body as a natural filter of the blood, removing wastes which are diverted to the urinary bladder. The kidneys excrete wastes such as urea and ammonium in urine; they are also responsible for the re-absorption of water, glucose, and amino acids and produce hormones including calcitriol, renin, and erythropoietin.

Exposure to chemical substances can cause adverse effects on the kidney, ureter, or bladder (Kidney Toxicity). The kidney is unusually susceptible because of its role in filtering harmful substances from the blood. Toxic injury to the kidney is known to occur as a result of exposures to halogenated hydrocarbons, such as carbon tetrachloride and trichloroethylene, and the heavy metals cadmium and lead. Some of these toxicants cause acute injury to the kidney, while others produce chronic changes that can lead to end-stage renal failure or cancer (Toback, 1992).

Chloroform, for example, is nephrotoxic following metabolic activation via the microsomal enzyme system. Chloroform is also hepatotoxic, and again this involves cytochromes P-450-mediated activation, although in male mice the kidney is susceptible at doses that are not hepatotoxic. It is clear that the tissues of the kidney are often exposed to higher concentrations of potentially toxic compounds than most other tissues (Timbrel, 2009). Common disorders that affect the kidney include nephropathy, congenital hydronephrosis, and obstruction of urinary tract among others. Several mechanisms and drugs are employed to heal these disorders.

Diuretics

Diuretics are generally necessary in Chronic Kidney Disease for control of extracellular fluid (ECF), volume expansion and for their associated effects on blood pressure.

Body water is distributed between the intracellular fluid (ICF) and the extracellular fluid (ECF) compartments (interstitial tissue, vascular space), the volume of which depends on the osmotic pressure exerted by their electrolyte composition. Owing to the selective permeability of biological membranes, sodium (Na+) and accompanying anions are mostly restricted to the ECF compartment, while potassium (K+) is confined to the ICF compartment (Nadeau *et al.*, 2010).

Diuresis may refer to urine production as an aspect of fluid balance. A diuretic is any drug that elevates the rate of urination and thus provides a means of forced diuresis. There are several categories of diuretics. All diuretics increase the excretion of water from bodies, although each class does so in a distinct way.

Depending on the mode by which they excrete water from the bodies, there are about seven types (though some may overlap with some similarities) of diuretics namely: High ceiling loop diuretics, Thiazides, Carbonic Anhydrase Inhibitors, Potassium-sparing diuretics, Calcium-sparing diuretics, Osmotic diuretics, Low ceiling diuretics.

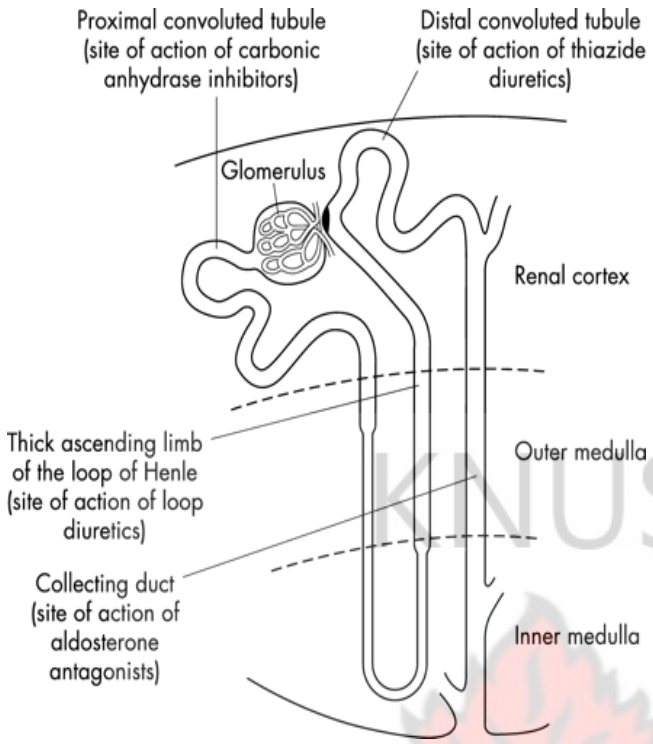


Figure 1.2 Portion of the renal physiology showing site of action of some classes of diuretics. (Adopted from www.pmj.bmj.com)

High ceiling loop diuretics may cause diuresis up to 20% of the filtered load of NaCl and water as compared to the normal renal sodium reabsorption which leaves only about 0.4% of filtered sodium in the urine. Loop diuretics hinder the body's ability to reabsorb sodium at the ascending loop in the nephron leading to retention of water in the urine (Myrvang, 2011). Examples include; furosemide, ethacrynic acid, torsemide and bumetanide.

Thiazide-type diuretics act on the distal convoluted tubule and inhibit the sodium-chloride symporter leading to retention of water in the urine, as water normally follows penetrating solutes. Frequent urination is due to the increased loss of water concomitant with sodium loss from the convoluted tubule. Thiazides decrease preload, decreasing blood pressure which gives them a short-time antihypertensive action. On the other hand, a decreased resistance caused by an unknown vasodilator effect (long-term effect) also decreases blood pressure, example hydroflumethiazide.

Carbonic anhydrase inhibitors inhibit the enzyme carbonic anhydrase which is found in the proximal convoluted tubule. This results in bicarbonate retention, potassium retention and decreased sodium absorption. Examples of carbonic anhydrase inhibitors include acetazolamide and methazolamide.

Potassium-sparing diuretics do not promote the secretion of potassium into the urine thus sparing them as opposed to other diuretics (Ovaert *et al.*, 2010). The term potassium-sparing refers to an effect rather than a mechanism or location. However, the term refers to two specific classes that have their effect at similar locations: Aldosterone antagonists (spironolactone, a competitive antagonist of aldosterone) and Epithelial sodium channel blockers (amiloride and triamterene).

Calcium-sparing diuretics result in relatively low calcium excretion rate (Shankaran *et al.*, 1995). The thiazides and potassium-sparing diuretics are considered to be calcium-sparing diuretics (Bakhireva *et al.*, 2004). The thiazides cause a net decrease in calcium lost in urine , whereas the potassium-sparing diuretics cause a net increase in calcium lost in urine although the increase is much smaller than the increase associated with other diuretic classes (Champe *et al.*, 2006).

Osmotic diuretics cause water retention in the urine thus maintaining osmotic balance. Mannitol as well as glucose can cause osmotic diuresis.

Multiple diuretic classes are available for use in Chronic Kidney Disease including thiazides, loop diuretics, and potassium-sparing diuretics. Thiazide diuretics may lower blood pressure and reduce cardiovascular disease (CVD) risk in addition to reduction in extra cellular fluid (ECF) volume. Selection of diuretic agents depends on the level of Glomerular Filtration Rate (GFR) and need for reduction in ECF volume (Chobanian *et al.*, 2003). Diuretic therapy enhances the antihypertensive effect of most antihypertensive agents. The mechanism for this effect is that most antihypertensive agents stimulate renal tubular sodium reabsorption, thereby increasing ECF volume and blunting the antihypertensive effect. Diuretics interfere with sodium reabsorption, lower ECF volume,

and potentiate the antihypertensive effect of the antihypertensive agent (Sica, 2002). At the same time, reducing ECF volume activates neurohumoral pathways, especially the renin-angiotensin system, leading to vasoconstriction and increased systemic vascular resistance, which blunts the antihypertensive effect of diuretics.

Liver Toxicity

The liver is the main organ of metabolism and energy production. Its other main functions include: bile production, storage of iron, vitamins and trace elements, detoxification, conversion of waste products for excretion by the kidneys.

The liver is functionally divided into two lobes, right and left. The external division is marked on the front of the liver by the falciform ligament, which joins the coronary ligament at the superior margin of the liver. It receives 30% of the resting cardiac output and acts as a giant chemical processing plant in the body. These chemical reactions, called metabolism, are central in the regulation of body homeostasis. It also stores nutrients and other useful substances, as well as detoxifying or breaking down harmful compounds. These can then be excreted from the body in bile via the liver; in urine via the kidney, or by other means. The liver metabolises both beneficial and harmful substances. It has an immense task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents. Thus, the liver is subjected to a variety of diseases and disorders (which may be acute or chronic) caused by these agents or their metabolites. Acute and chronic liver diseases constitute a global concern, but medical treatments for these diseases are often difficult to handle and have limited efficacy (Kim *et al.*, 2009).

The liver performs a variety of important host defense and metabolic functions that include synthesis of acute-phase proteins, gluconeogenesis, detoxification, and clearance of endogenous mediators, as well as secretion of pro-inflammatory cytokines (Pastor *et al.*, 1995). It is a unique organ in the sense that a significant loss of liver cells due to drug toxicity or other insults can be overcome by regeneration (Mehendale, 2005).

Several plant extracts have been examined for use in a wide variety of liver disorders, *Glycosmis pentaphylla* protects membrane integrity in mice hepatocytes (Nayak *et al.*, 2011), *Silybum marianum* (source of silymarin) is a chemo-preventive agent that exhibits antitumor activity against human tumors in rodents (Agarwal *et al.*, 2006) just to mention a few; while others have been reported to cause injury to the liver. For example, *Artemisia macivera* Linn which is a traditional antimalarial in Nigeria is reported to be hepatotoxic in rats at the onset of treatment, especially at higher doses (Atawodi *et al.*, 2011) and several cases of hepatotoxic side effects of green tea have been reported (Rohde *et al.*, 2011).

1.4.4 Drug Metabolism in liver

The ability of humans to metabolize and clear drugs is a natural process that involves the same enzymatic pathways and transport systems that are utilized for normal metabolism of dietary constituents. The human body identifies almost all drugs as foreign substances (xenobiotic) and subjects them to various chemical processes to make them suitable for elimination. This involves chemical transformations to reduce fat solubility and to change biological activity. Although almost all tissues in the body have some ability to metabolise chemicals, smooth endoplasmic reticulum in the liver is the principal metabolic clearing house for both endogenous chemicals (example, fatty acids, and steroid hormones) and exogenous substances like drugs (Blumenthal *et al.*, 2006).

Drug metabolism is usually divided into two phases: phase 1 and phase 2. Phase 1 reaction, in which enzymes carry out oxidation, reduction, or hydrolytic reactions, is thought to prepare a drug for phase 2 in which enzymes form a conjugate of the substrate (the phase 1 product). These processes tend to increase water solubility of the drug and can generate metabolites which are more chemically active and potentially toxic. Most of phase 2 reactions take place in the cytosol and involve conjugation with endogenous compounds via transferase enzymes. Chemically active products from phase 1 are made relatively inert and suitable for elimination by the phase 2 step (Liston *et al.*, 2001).

Interactions are not only experienced with drugs: constituents of food may also take part in drug interactions. An example of this effect is the well-known inhibitory action of grapefruit juice on some cytochrome P450 isoenzymes, which was discovered rather by chance (Anzenbacher and Anzenbacherová, 2001). In this case, the *in vivo* effect can be quite dramatic with a single glass of grapefruit juice resulting in fivefold increase in the values of the main pharmacokinetic parameters such as the Cmax and Area under pharmacokinetic curve (AUC) for dihydropyridine beta-blocking agents (e.g. nifedipine) (Anzenbacher and Anzenbacherová, 2001).

A group of enzymes located in the endoplasmic reticulum, known as cytochrome P-450 is the most important family of metabolizing enzymes in the liver.

Role of cytochrome P450 enzymes

Cytochromes P450 constitute a superfamily of heme enzymes found from bacteria to humans (Nelson *et al.*, 1996). It is reasonable to suppose that a P450 enzyme is present in every living species on Earth, as this enzyme has been found in archaebacteria, in plants, and in various animal species.

Cytochrome P450 (CYP) is responsible for the oxidative metabolism of many clinically available drugs from a diverse number of drug classes (e.g., thiazolidinediones, meglitinides, NSAIDs, antimalarial and chemotherapeutic taxanes). This system, while not the only group of enzymes that catalyze oxidative metabolism, has been studied more extensively than others (Daily and Aquilante, 2009).

P450 isoenzymes catalyze oxidative reactions in the liver, intestine, kidney, lung, and brain (Jefferson, 1998a). They evolved to enable organisms to metabolize xenobiotics (foreign plants or chemicals), a category that includes exogenous medications as well as prostaglandins and endogenous hormones that resemble foreign substances (Jefferson, 1998a; Nemeroff *et al.*, 1996). Because of substrate overlap, many medications are metabolized by more than one P450 isoenzyme and many inhibit or activate more than one

isoenzyme. Three important characteristics of the CYP system have roles in drug-induced toxicity:

Genetic diversity: Each CYP 450 protein is unique and accounts to some extent for the variation in drug metabolism between individuals.

Change in enzyme activity: Drugs interact with the enzyme family in several ways (Michalets, 1998). They may inhibit or induce their activity. This effect usually occurs immediately. Depending on the inducing drug's half-life, there is usually a delay before enzyme activity increases (Lynch and Price, 2007).

Competitive inhibition: some drugs may share the same P-450 specificity and thus competitively block their bio-transformation. This may also reduce the rate of generation of toxic substrate. While enzyme induction is gradual, inhibition is rapid (Jefferson, 1998a). Inhibition of an enzyme that plays a major role in the metabolism of a particular drug can, for any given dose, increase elimination half-life, plasma concentration, and area under the concentration-time curve and therefore enhance both therapeutic and adverse effects.

Because the P450 family metabolizes numerous exogenous materials, the consequences of enzyme inhibition are not restricted to alterations in medication levels or to adverse effects. For example, because activation of CYP 1A isoenzymes by cigarette smoke and charred meat may increase carcinogenesis, (Jefferson, 1998a), inhibition of this enzyme by substances such as disulfiram might decrease carcinogenesis. P450 interactions are further complicated by the fact that metabolites of psychotropic medications may be active or inactive, so that inhibition or induction of metabolism of the parent drug may result in a net increase or decrease of therapeutic effects or adverse effects. Amino acid sequences within the same family (e.g., CYP2) have about 40% similarity. There is at least a 55% similarity within the same subfamily (e.g., 2D), although sequences may differ minimally between enzymes within a subfamily (e.g., between 3A3 and 3A4).

Fifty to sixty percent of the P450 class of enzymes consists of the 3A subfamily. CYP3A4 is apparently the most important P450 enzyme for drug metabolism in humans. (Nemeroff *et al.*, 1996). This is not only because of its amount in the liver (which may be increased by induction to more than 60%) but mainly because it participates in the metabolism of the majority of drugs with known metabolic pathways. Unfortunately, it is not only a rather unstable enzyme with a complicated mechanism of action, which makes *in vitro* studies difficult, but it is also a P450 enzyme for which a suitable probe drug (*in vivo* 'marker' activity) has not yet been found (Schmider *et al.*, 2000).

1A2 metabolizes caffeine, phenacetin, paracetamol, tacrine, and clozapine. It also metabolizes 90% of pro-carcinogens into active carcinogens. It is induced by cigarette smoke, charcoal-broiled foods, and cabbage; it is inhibited by fluvoxamine and disulfiram (Shen, 1995). Induction of 1A2 by alcohol leads to accumulation of a hepatotoxic metabolite of paracetamol, which is why paracetamol with alcohol can lead to fatal hepatotoxicity. Inhibition of 1A2 by fluvoxamine can result in dangerously elevated levels of clozapine (Jefferson, 1998b).

The 2C family metabolizes diazepam, citalopram, moclobemide, phenytoin, tolbutamide, warfarin, and some non-steroidal anti-inflammatory drugs (Nemeroff *et al.*, 1996) but various members of this subfamily are inhibited by fluvoxamine, fluoxetine, and sertraline. Inhibition of the 2Cs becomes particularly important when selective serotonin reuptake inhibitors (SSRIs) are coprescribed for patients in treatment for clotting disorders or diabetes.

2D6 substrates include fluoxetine, many neuroleptics, risperidone, and other antidepressants such as venlafaxine and trazodone. Common 2D6 inhibitors include neuroleptics such as fluphenazine, haloperidol, and thioridazine, as well as clomipramine, desipramine, quinidine, and all selective serotonin reuptake inhibitors (SSRIs) except fluvoxamine (Nemeroff *et al.*, 1996). Inhibition of 2D6 and other P450 isoenzymes by citalopram was not considered clinically significant until it was reported that a 50% increase in the imipramine AUC when a single 100 mg dose was given to normal subjects who had taken 40 mg/day of citalopram for

one week (Preskorn, 1998). Potent 2D6 inhibition by fluoxetine and paroxetine, in particular, is reason for caution in co-prescribing these medications and antipsychotics (Preskorn, 1998).

1.4.5 Drug Induced Liver Injury

This central role played by liver in the clearance and transformation of chemicals makes it susceptible to drug-induced injury.

Drug-induced liver injury (DILI) can be broadly classified into two categories, based on incidence, animal model predictability and dose-dependency.

Drug-induced liver disease can be predictable (high incidence and dose-related), type A or unpredictable (low incidence and may or may not be dose-related), type B. Unpredictable reactions, also referred to as idiosyncratic, can be viewed as either immune-mediated hypersensitivity or non-immune reactions (Davies, 1985). Most potent predictable hepatotoxins are recognized in the animal testing or clinical phase of drug development.

Drugs or toxins that have a pharmacological (type A) hepatotoxicity are those that have predictable dose-response curves (higher concentrations cause more liver damage) and well characterized mechanisms of toxicity, such as directly damaging liver tissue or blocking a metabolic process. As in the case of paracetamol overdose, this type of injury occurs shortly after some threshold for toxicity is reached. Type A drug reaction accounts for 80% of all toxicities (Pirmohamed *et al.*, 1998).

1.4.6 Mechanism of liver Damage

Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria, an intracellular organelle that produces energy. Its dysfunction releases excessive amount of oxidants which, in turn, injure hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP 2E1 also lead to oxidative stress (Jaeschke *et al.*, 2002). Injury to hepatocyte and bile duct cells

lead to accumulation of bile acid in the liver. This promotes further liver damage (Patel *et al.*, 1998). Non-parenchymal cells such as Kupffer cells, fat-storing stellate cells, and leukocytes (neutrophil and monocyte) also have roles in the mechanism.

Many hepatotoxicants including CCl_4, nitrosamines, and polycyclic aromatic hydrocarbons require metabolic activation, particularly by liver CYP enzymes to form reactive toxic metabolites, which in turn cause liver injury in experimental animals and humans.

The presence of a lesion in the cellular parenchyma is common to a large number of chronic liver diseases, such as viral hepatitis, alcoholic hepatitis, chronic cholestasis and steato hepatitis. Although the pathogenesis may vary, Kupffer cell activation and inflammatory cell recruitment, free oxygen radical formation and the development of oxidative stress, cytokine production, mainly TNFα and TGFβ, and inflammatory mediator release due to arachidonic acid oxidation through the COX-2 and 5-LO pathways are the usual (Claria *et al.*, 2008).

1.4.7 Patterns of Injury

Inflammatory disease of the liver is termed hepatitis, and may be of short (acute) or long (chronic) duration. Viral infections, particularly hepatitis A, B, and C, are common infectious causes of acute hepatitis, whereas alcohol and paracetamol are the most common toxicological causes. Hepatic dysfunction after sepsis is a frequent event that is characterized by loss of synthetic function, hepatocellular necrosis, and release of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, prostaglandins, and nitric oxide (NO) (Harbrecht *et al.*, 1994; Kumins *et al.*, 1996; Nadler *et al.*, 2001).

Hepatitis B and C virus (HBV, HCV) infection can lead to the development of chronic liver disease that can lead to liver fibrosis and eventually to liver cirrhosis (Gutierrez-Reyes, 2007).

Fibrosis occurs as a result of initial liver injury, including hepatocyte damage, Kupffer cell activation, hepatic stellate cell (HSC) activation and proliferation. In hepatic fibrosis, the hepatocyte, Kupffer cell, and HSC communicate by way of oxygen stress, intracellular free

calcium [Ca^{2+}] increasing and cytokines imbalance. Macrophages stimulate the progression of fibrogenic process, while stellate cells synthesize most of the matrix protein (Jodynis-Liebert *et al.*, 2009; Poli and Parola, 1997). In theory, it should be possible to halt or prevent fibrosis if cell communication was blocked (Hu *et al.*, 1997). Prevention as the most effective approach toward cirrhosis often fails; therefore, antifibrotic treatment that halts the progression of cirrhosis is important. Several plant-derived substances such as colchicine, silymarin, trans-resveratrol, Gingkobiloba-composita and Sho-saiko-to (extract of seven herbs in Chinese folk medicine) have been proposed as antifibrotic agents (Stickel *et al.*, 2002).

The Chinese herbal medicines have been used for many years by Chinese investigators treating liver cirrhosis in the advantage of their low toxicity.

Herbal medicines have marked effects against liver injury and fibrosis. Blocking cell communication is associated with their action of antioxidant or/and anti-inflammatory activity (Hu *et al.*, 1997).

It has been shown that some flavonoids interfere with fibrogenic functions of hepatic stellate cells (HSC) and Kupffer cells *in vitro* and are inhibitors of different protein kinases involved in signal transduction. Since protein kinases also play a role in the activation of HSC, such an inhibitory effect of flavonoids may be of significance for interrupting the pathogenic process (Gebhardt, 2002).

In Taiwan, it has been shown that long-standing necro-inflammatory liver disease resulting from viral super-infection strongly influences the rate of progression to cirrhosis (Chu, 2006). Cirrhosis is characterized by widespread nodules in the liver combined with fibrosis and by increased deposition and altered composition of extracellular matrix (Friedman, 1993).

Liver architecture is distorted by dense bands of collagen that link vascular structures and surround islands of regenerating parenchymal cells.

Generally, cirrhosis is considered to be irreversible even after removal of the injurious agent. Thus, the disease is characterized by the incapacity of the injured liver to remodel the fibrotic matrix (Arias, 2001).

By contrast, other studies in animal experimental models of liver cirrhosis have shown spontaneous resolution of fibrosis once the toxic agent or cell insult is removed (Abdel-Aziz, 1990; Varga *et al.*, 1966). Therefore, experimental models of cirrhosis have been useful only in testing the efficacy of some drugs in reversing fibrosis.

The term 'hepatic failure' denotes a clinical condition in which the biochemical function of the liver is severely, and potentially fatally, compromised.

Cholestasis may occur in the small bile ducts, in the liver itself, or in the larger extrahepatic ducts. Biochemical tests cannot distinguish between these two possibilities, which generally have radically different causes; imaging techniques such as ultrasound are required.

Jaundice is clinically obvious when plasma bilirubin concentrations exceed 50μmol/L (3 mg/dL) in humans. It may be hepatic, intra-hepatic or post-hepatic.

1.4.8 Experimental Models of Hepatotoxicity

Some drugs and chemicals with which is associated hepatotoxicity are employed as inducers of hepatotoxicity in testing for various hepatoprotective agents.

Although individual analgesics rarely induce liver damage due to their widespread use, NSAIDs have emerged as a major group of drugs exhibiting hepatotoxicity. Both dose-dependent and idiosyncratic reactions have been documented (Manov *et al.*, 2006). Aspirin and phenylbutazone are associated with intrinsic hepatotoxicity; idiosyncratic reaction has been associated with ibuprofen, sulindac, phenylbutazone, piroxicam, diclofenac and indomethacin.

Glucocorticoids, so named due to their effect on the carbohydrate mechanism, promote glycogen storage in the liver. An enlarged liver is a rare side effect of long-term steroid use in children. The classical effect of prolonged use both in adult and paediatric population is steatosis.

Isoniazid is one of the most commonly used drugs for tuberculosis; it is associated with mild elevation of liver enzymes in up to 20% of patients and severe hepatotoxicity in 1-2% of patients (Sarich *et al.*, 1999).

Natural products, example fungi or moulds like *Aspergillus flavus* and *Aspergillus parasiticus* produce toxins that have a number of toxic effects, particularly hepatotoxicity (Adedara *et al.*, 2010a).

Industrial toxins with negative effect on the liver include arsenic, carbon tetrachloride, and vinyl chloride.

Carbon Tetrachloride-Induced Hepatotoxicity

Carbon tetrachloride, once used extensively in dry cleaning and even as an anesthetic, is primarily hepatotoxic causing two different types of pathological effect.

It is said to be present in low quantities in foods in Britain. For example, milk contains about 0.2ug/kg; cheese, 5.0ug/kg; butter, 14.0ug/kg; pork liver, 9.0ug/kg; tomatoes, 4.5ug/kg; fresh bread, 5.0ug/kg (Klaassen, 2008).

The hepatotoxicity of carbon tetrachloride has probably been more extensively studied than that of any other hepatotoxic agent, and there is now a wealth of data available. Its toxicity has been studied both from the biochemical and pathological viewpoints, and therefore the data available provide particular insight into mechanisms of toxicity.

Carbon tetrachloride is a simple molecule which, when administered to a variety of species, causes centrilobular hepatic necrosis (zone 3) and fatty liver. It is a very lipid-soluble compound and is consequently well distributed throughout the body, but despite this, its major toxic effect is on the liver, irrespective of the route of administration. It should be noted that it does have other toxic effects, and there are species and sex differences in toxicity.

Chronic administration or exposure causes liver cirrhosis, liver tumors, and also kidney damage. The reason for the liver being the major target is that the toxicity of carbon tetrachloride is dependent on metabolic activation by CYP2E1 (Figure 1.3) (Timbrel, 2009).

The liver is a major inflammatory organ and inflammatory processes contribute to a number of pathological events after exposure to various hepatotoxins. Kupffer cells release pro-inflammatory mediators either in response to necrosis or in direct response to an activated hepatotoxin; these pro-inflammatory mediators are believed to aggravate CCl_4-induced hepatic injury (Badger *et al.*, 1996). Although carbon tetrachloride was originally thought to be resistant to metabolic attack, it is now clear that it is metabolized by cytochromes P-450.

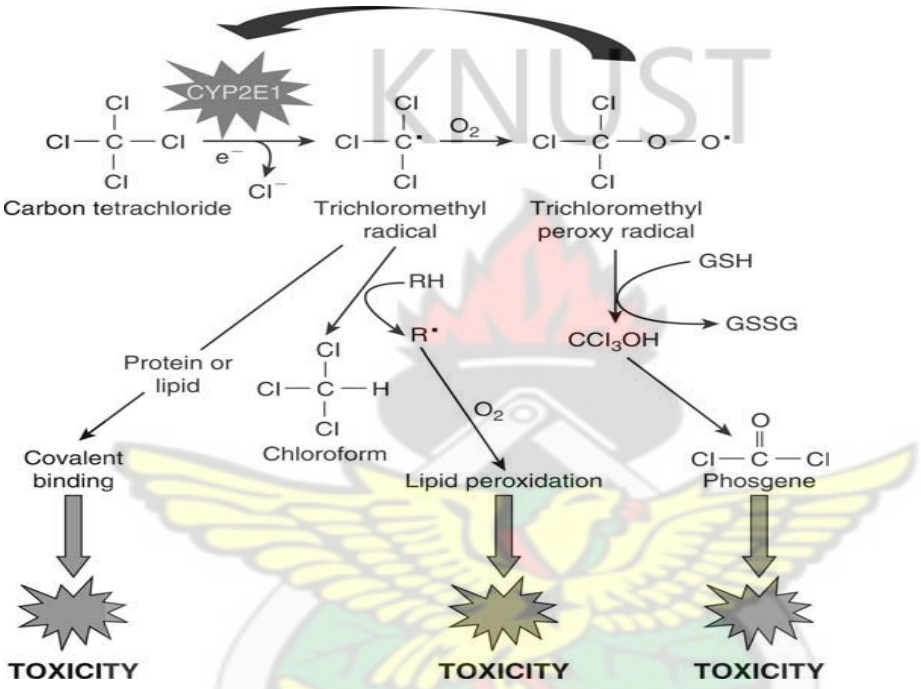


Figure 1.3: Mechanism of hepatotoxicity of CCl_4
Adopted from Timbrel (2009)

The metabolic activation of CCl_4 involves primarily CYP2E1 *in vivo* as indicated by the absence of toxicity in CYP2E1 knockout mice Reactive oxygen species are the main causes of carbon tetrachloride-induced acute liver injury (Campo *et al.*, 2008). The hepatic necrosis caused by CCl_4 is thought to be bioactivated by cytochrome P450 2E1 (CYP2E1), resulting in the formation of trichloromethyl free radicals and reactive oxygen species (ROS), which

initiate lipid peroxidation and protein oxidation and damage the hepatocellular membranes (McCay *et al.*, 1984). This process is followed by the release of inflammatory mediators from activated hepatic macrophages, which are believed to potentiate the CCl_4-induced hepatic injury (Kim *et al.*, 2009; Raabe *et al.*, 1998).

Alterations in the activity of CYP2E1 can affect the susceptibility to hepatic injury from CCl_4. The trichloromethyl radical formed during the metabolism of CCl_4 is capable of binding to lipids, and this binding initiates lipid peroxidation and liver damage (Ahmed *et al.*, 2000).

Paracetamol-Induced Hepatotoxicity

Paracetamol is a safe and effective analgesic/antipyretic drug when used at therapeutic levels (Rumack, 2004).

Paracetamol induced liver injury is a classic case of DILI-1 and accounts for nearly half of acute liver failure in the United States (Larson *et al.*, 2005). It can be modeled in more than one strain of rodents (Mehendale, 2005) and has a clear dose-dependency in both animals and humans.

Paracetamol hepatotoxicity has been related with several cases of cirrhosis, hepatitis and suicide attempts. Notably, oxidative stress plays a central role in the hepatic damage caused by paracetamol and antioxidants have been tested as alternative treatment against paracetamol toxicity (Avila *et al.*, 2011) An acute or cumulative overdose of the analgesic drug paracetamol can lead to severe liver injury in humans and in experimental animals. In fact, paracetamol overdose is the most frequent cause of drug-induced liver failure in the United States and in Great Britain (Lee, 2004). Although intensely studied for more than 25 years, the mechanism of this injury is still not entirely clear. Based on the mechanistic insight gained from early preclinical studies (Jollow, 1973; Mitchell *et al.*, 1973a; Mitchell *et al.*, 1973b) N-acetylcysteine was introduced in the 1970s and still is the only clinical antidote

against paracetamol-induced liver injury (Polson and Lee, 2005a). Seventy percent of the covalent binding of paracetamol in the liver is to cysteine residues in hepatic proteins through the 3-position on the benzene ring. In addition, this binding does not seem to be accidental and is to specific proteins in both mouse and human liver *in vivo*, and in microsomes and isolated hepatocytes *in vitro*. Some of the binding seems to be similar in both mice and humans. Thus, both species show binding of a paracetamol metabolite to various proteins, but especially to a 58-kDa protein, which has been termed "paracetamol-binding protein" and is also a target for other reactive metabolites.

Paracetamol is directly conjugated with glucuronic acid or sulphate through glucuronyl transferases or sulfonyltransferases. The conjugated compound is either excreted into the bile by multidrug resistance-associated protein (Mrp2) or into the blood by Mrp3. The remaining unconjugated paracetamol is then metabolized by P450 enzymes into N-acetyle P-benzoquinone imine (NAPQI), the most relevant isoenzyme being CYP2E1, especially in humans (Gonzalez, 2005; Raucy *et al.*, 1989).

It is undisputed that the metabolism of a fraction of the paracetamol dose by the P450 system is the initial step of the injury process (Figure 1.4). The product of this reaction is a reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI) which is detoxified by glutathione. NAPQI reacts with glutathione (GSH) spontaneously or is catalyzed by glutathione-S-transferases to form a GSH-adduct, which is mainly excreted into bile through Mrp2 (Chen *et al.*, 2003). One of these secondary effects of reactive metabolite formation and protein binding is mitochondrial dysfunction which results in ATP depletion and oxidant stress (Jaeschke, 2011; Jaeschke *et al.*, 2002).

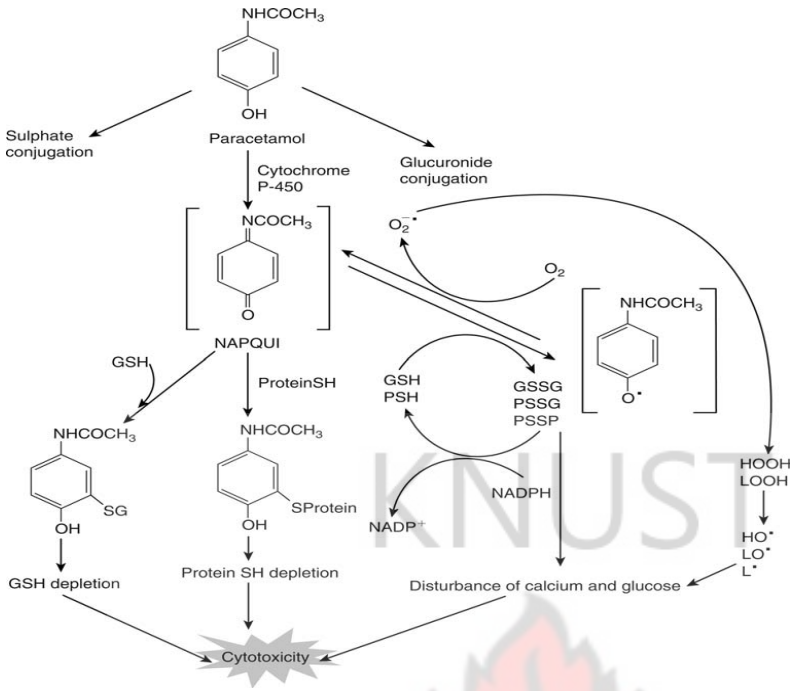


Figure 1.4: Metabolic activation of paracetamol. (Adopted from Timbrel, 2009)

Aflatoxin B1 induced liver injury

Aflatoxins are naturally-occurring mycotoxins that are produced by many species of *Aspergillus*, a fungus, most notably *Aspergillus flavus* and *Aspergillus parasiticus*. The aflatoxins including aflatoxin B1 (AFB1) are secondary metabolites of some strains of the molds that grow on food and food crops.

Aflatoxin-producing members of *Aspergillus* are common and widespread in nature and are widely distributed in agricultural products. They can colonize and contaminate grain before harvest or during storage. Host crops are particularly susceptible to infection by *Aspergillus* following prolonged exposure to a high humidity environment or damage from stressful conditions such as drought, a condition which lowers the barrier to entry.

The native habitat of *Aspergillus* is in soil, decaying vegetation, hay, and grains undergoing microbiological deterioration. It also invades all types of organic substrates

whenever conditions are favorable for its growth. Favorable conditions include high moisture content (at least 7%) and high temperature.

Crops which are frequently affected include cereals (maize, sorghum, pearl millet, rice, wheat), oilseeds (peanut, soybean, sunflower, cotton), spices (chilli peppers, black pepper, coriander, turmeric, ginger), and tree nuts (almond, pistachio, walnut, coconut, brazil nut). In addition, some food products of agriculture also fall victim to aflatoxin. They include fermented beverages made from grains, milk, cheese, fruit juices and numerous other agricultural commodities.

Aflatoxins are toxic and among the most carcinogenic substances identified (Hudler, 1998). No animal species is immune to the acute toxic effects of aflatoxins including humans; however, humans have an extraordinarily high tolerance for aflatoxin exposure and rarely succumb to acute aflatoxicosis.

Chronic, subclinical exposure does not lead to symptoms as dramatic as acute aflatoxicosis. Children, however, are particularly affected by aflatoxin exposure which leads to stunted growth and delayed development (Aguilar *et al.*, 1993).

Chronic exposure also leads to a high risk of developing liver cancer, as aflatoxin metabolite can intercalate into DNA and alkylate the bases through its epoxide moiety. Human epidemiology and experimental animal studies have provided a statistical association between aflatoxins and the threat of liver cancer (Abdel-Wahhab *et al.*, 2006). The degree to which aflatoxins contribute to this disease may be influenced by a number of human health factors, including hepatitis B virus (HBV) infection, nutritional status and age, as well as the extent of aflatoxin exposure (Abdel-Wahhab and Aly, 2003). This is thought to cause mutations in the *p53* gene, an important gene in preventing cell cycle progression when there are DNA mutations, or signaling apoptosis. These mutations seem to affect some base pair locations more than others: for example, the third base of codon 249 of the p53 gene appears to be more susceptible to aflatoxin-mediated mutations than nearby bases. It has been reported that AFB1 exerts liver-specific carcinogenicity by inducing a guanine (a purine) to thymine (a pyrimidine) substitution at codon 249 on the P53 gene (Hsu *et al.*, 1991).

AFB1 is metabolized by the mixed-function oxidase system to a number of hydroxylated metabolites and to AFB1 8, 9- epoxide, which binds to DNA, forming covalent adducts. It is also known to produce membrane damage through increased lipid peroxidation (Galvano *et al.*, 2001).

Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid because fungal growth in foods is not easy to prevent. Even though heavily contaminated food supplies are not permitted in the market place in developed countries, concern still remains for the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the food supply.

1.5 THE INCIDENCE OF LIVER DISEASE

African populations are confronted with the development of chronic diseases whose treatment and or management results in more economic problems. Hepatitis, as well as other hepatic diseases, belongs to these diseases which are not so familiar to African traditional medicine.

There are various causes of acute and chronic liver injury. The results of various insults to the liver may manifest themselves quite differently in terms of histology, clinical presentation, serology, and long term consequences. Many of these injuries do not result in long term damage to the liver. However, the injurious agents that result in long term effects may lead to inflammation, fibrosis and disruption of hepatic architecture with attempts of regenerative nodule formation which is referred to as cirrhosis.

Inflammation of the liver is characterized by the presence of inflammatory cells in the tissue of the liver (Beckingham, 2001). The condition can heal on its own or can progress to fibrosis (scarring) and cirrhosis. Inflammation of the liver may be acute (lasting for less than six months) or chronic (persisting longer). Causes may include infections or toxins; notably alcohol, some medications, plants and industrial solvents; also viruses and autoimmune diseases.

Persistent inflammation of the liver seems to be a risk factor for cirrhosis in spite of the fundamental etiology (Caldwell *et al.*, 1999; Du and Wang, 1998) . Worldwide, cirrhosis of the liver is the 16th leading cause of death and is responsible for hundreds of thousands of deaths each year (W.H.O., 2003). Cirrhosis onset is often asymptomatic with mild clinical symptoms, and individuals with subclinical cirrhosis can lead relatively normal lives for many years. Diagnosis of cirrhosis, generally requiring histopathologic review of a liver biopsy specimen, is scarcely performed in many resource-constrained settings.

Research in developed countries suggest that chronic infection with Hepatitis B and C viruses (Corrao *et al.*, 1998; Tsai *et al.*, 1994; Tsai *et al.*, 2003), excessive alcohol intake (Corrao *et al.*, 1998), hereditary factors (Gershwin *et al.*, 2005), obesity (Poonawala *et al.*, 2000), smoking (Tsai *et al.*, 2003), and occupational exposure to vinyl chloride (Mastrangelo *et al.*, 2004), are all associated with cirrhosis of the liver.

Chronic liver disease is the 12th cause of death in the USA with hepatitis C being the most common cause (Younossi *et al.*, 2011). Hepatitis C virus (HCV) infection is prevalent in approximately 2% of the world's population and is the leading cause of acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (Davis *et al.*, 2003). Cirrhotic persons, however, are at high risk for liver decomposition and, irrespective of the cause, have a high risk for developing hepatocellular carcinoma (HCC). Meanwhile, the WHO in 2003 reported hepatocellular carcinoma as a leading cause of cancer death worldwide.

Non-alcoholic fatty liver disease (NAFLD) (especially common in children and adolescents) is becoming another major cause of chronic liver disease in the western world. In a study conducted in the US, the leading causes of NAFLD were obesity (putting most people at risk of liver diseases) and AIDS with other causes (alcoholism, HCV and HBV) remaining relatively constant between 1988 and 2008 in the USA (Younossi *et al.*, 2011).

Non-alcoholic fatty liver disease is recognized to be a hepatic manifestation of metabolic disorders (Marchesini *et al.*, 2001) and is characterized by hepatocellular steatosis, apoptosis, inflammatory infiltration and fibrosis (Matteoni *et al.*, 1999). It has been

demonstrated that cholesterol overload results not only in metabolic disorders but also hepatic fibrosis and inflammation (Jeong *et al.*, 2005).

It is also reported that chronic infection with hepatitis B virus (HBV) is common in sub-Saharan Africa, with hepatitis C virus (HCV) infection also being present (McMahon, 2005). Dietary exposure to aflatoxin, primarily through ingestion of contaminated maize and groundnuts, is also rife (Turner *et al.*, 2005). This is mainly because of the lack of clinical and research infrastructure. Thorough investigation into the etiology and characteristics of chronic liver disease in sub- Saharan Africa has been inadequate. Despite wide availability of treatment for hepatitis B virus (HBV) infection, it has remained a common cause of end stage liver disease in the tropics (Mutaal *et al.*, 2010).

A study conducted in a hospital in Accra, Ghana, between 1996 and 2002 has shown that out of 22,394 persons who died and passed through autopsies, 1,176 (5.25%) suffered from liver diseases. The study also revealed that liver diseases had been rising at a rate of about 0.3 per cent annually in Ghana with more men suffering and dying from liver diseases than women. In addition, tests conducted during blood donations indicated that cases of Hepatitis was increasing in the country (Yeboah, 2009).

There is therefore the growing need to research into liver diseases and hepatoprotective remedies especially from natural sources since the orthodox remedies available have little alleviation to offer (Orhan *et al.*, 2007).

1.5.1 Plants and Possible Hepatoprotection

Liver protecting plants contain a variety of chemical constituents like phenols, coumarins, monoterpenes, glycosides, alkaloids and xanthenes (Bhawna and Kumar, 2009).

Glycyrrhizin, a popular hepatoprotective drug, is known to prevent the development of hepatocellular carcinoma (HCC), but glycyrrhizin is usually administered intravenously. Drugs that are effective by oral administration are convenient for patients for long-term administration, and development of more effective drugs than glycyrrhizin is preferable

(Okamoto *et al.*, 2001). The search for more effective drugs from natural sources is paramount.

A number of plants and traditional formulations are available for the treatment of liver diseases (Rai, 1994; Schuppan *et al.*, 1999). As regards, six hundred (600) commercial herbal formulations with claimed hepatoprotective activity are being sold all over the world. Around a hundred and seventy (170) phytoconstituents isolated from hundred and ten (110) plants belonging to fifty-five (55) families have been reported to possess hepatoprotective activity (Girish *et al.*, 2009). In India, more than 93 medicinal plants are used in different combinations in the preparations of forty (40) patented herbal formulations. However, only a small proportion of hepatoprotective plants as well as formulations used in traditional medicine are pharmacologically evaluated for their safety and efficacy (Subramoniam and Pushpangadan, 1999).

In the quest to investigate these claims, pharmacological evaluations are necessary for all plant-based hepatoprotectants. For instance, the leaf decoction of *Byrsocarpus coccineus* (Connaraceae) which is drunk for the treatment of jaundice in West African traditional medicine was found to be hapatoprotective against CCl_4-induced hepatotoxicity in rats (Akindele *et al.*, 2010).

Evaluation of *in vivo* hepatoprotective activity of the aqueous extract of *Artemisia absinthium* L. which has been used for the treatment of liver disorders in traditional Uighur medicine (western China) was done in experimental mice against immunologically-induced liver injury (by injection of endotoxin-LPS) (Amat *et al.*, 2010).

The fruit pulp of Adansonia digitata commonly called baobab is said to exhibit significant hepatoprotective activity and consumption of the fruit may play an important part in human resistance to liver damage. The mechanism of liver protection may be due to the presence of triterpenoids, β-sitosterol, β-amyrin palmitate and ursolic acid in the fruit pulp (Adewusi and Afolayan, 2010).

Silymarin

Amongst the many plant hepatoprotective agents, silymarin is a standardized plant extract obtained from the seeds of milk thistle (*Silybum marianum)* silymarin, a mixture of flavanoid complexes, is the active component of milk thistle plant that protects liver and kidney cells from toxic effects of drugs, including chemotherapy (Post-White *et al.*, 2007).

It is composed of a mixture of four isomeric flavonolignans: silibinin, isosilibinin, silydianin and silychristin. It contains about 60% polyphenol silibin and is used as a hepatoprotective agent (Boigk *et al.*, 1997). Silymarin is widely used for protection against various liver diseases in Europe and around the world.

In addition to its free radical scavenging properties, silymarin increases antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and inhibits lipid peroxidation (Zhao *et al.*, 2000).

It is reported to offer protection against various chemical hepatotoxins such as CCl_4, and alcoholic liver (Crocenzi and Roma, 2006). *In vivo* studies have demonstrated that silymarin exerts a protective effect in hepatocytes exposed to a range of molecules, including tert-butyl hydroperoxide, phenylhydrazine, paracetamol and carbon tetrachloride (Halim *et al.*, 1997; Valenzuela and Guerra, 1985). Treatment with silymarin resulted in a statistically significant decrease in transaminase levels in four studies compared with baseline levels, and in one compared with placebo in patients with chronic viral hepatitis.

Due to its proven hepatoprotectiveness, silymarin is being used as one of the standard agents for comparison in the evaluation of hepatoprotective effects of *Spathodea campanulata*.

1.6 JUSTIFICATION, AIMS AND OBJECTIVES

1.6.1 Justification of Project

Traditional plant-based medicines have several claims with or without scientific proof. They however still put forth a great deal of importance to the people living in developing countries and also lead to discovery of new drug candidates for a variety of diseases that threaten human health. Available biomedical evidence suggests that approximately 80% of Africans rely on traditional healthcare practitioners and medicinal plants for their daily healthcare needs (Abere *et al.*, 2010). African indigenous herbal medicines are widely used throughout the African continent, despite an apparent lack of scientific evidence for their quality, safety and efficacy (Johnson *et al.*, 2007).

Spathodea campanulata is widely used for the treatment of various ailments but little or no interest has been shown in its safety or otherwise. Interestingly, the tree whose leaves and bark are used so widely in treatment of many ailments has seeds which when fresh are edible but are used to make arrow poison to kill animals when dried (Abbiw, 1990). As to whether the other parts, specifically the stem bark, which are mostly used possesses this toxicity is currently unknown. *Spathodea campanulata* has been used for a period in West Africa and Asia mostly for stomach troubles (Mendes *et al.*, 1986). However, there are no toxicity reports on the use of the plant.

Considering the high patronage of the infusion of the bark of *Spathodea campanulata*, there is the urgent need for *in vivo* toxicological studies to assess the safety of the extract and assess the effect on other target organs like the liver, since toxicants usually exert their effect on target organs in the body. In addition, the presence of some phytochemicals such as β-sitosterol and oleanolic acid, which are known to protect against oxidative stress (Awad *et al.*, 2008), makes modulation of hepatic injury by *Spathodea campanulata* worth investigating.

1.6.2 Aims

Because there is no scientific data available on the safety of *Spathodea campanulata* stem bark extract, the aim of this study was to investigate *in vivo,* the potential toxicity and the hepatoprotective activity of the aqueous stem bark extract of *Spathodea campanulata* stem bark extract.

1.6.3 Specific Objectives

The aqueous stem bark extract of *Spathodea campanulata* shall be evaluated for its:

- Effect on whole blood, blood cells and on serum biochemical parameters
- Effect on the liver and kidneys
- Effect on diuresis and urine electrolytes
- Effect on cytochrome P450 enzymes
- Effect on liver function and morphology in CCl_4-induced toxicity in rats
- Effect on liver function and morphology in paracetamol-induced toxicity in mice
- Effect on liver function and morphology in Aflatoxin B1-induced totoxicity in rats

Spathodea campanulata aqueous stem bark extract shall be characterized and possible mechanism of hepatoprotection investigated.

CHAPTER 2

PLANT COLLECTION, EXTRACTION AND PHYTOCHEMICAL ANALYSIS

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Collection

The stem bark of *Spathodea campanulata* was collected at Kotei, a community near the Kwame Nkrumah University of Science and Technology campus on the 9th of October, 2008 at about 6:00 am. The samples were ascertained by Dr. Kofi Annan of the Department of Pharmacognosy, of Kwame Nkrumah University of Science and Technology, Kumasi. They were then sun-dried for eight days.

A voucher specimen has been deposited in the Faculty of Pharmacy Herbarium: KNUST/HM1/2012/L062.

2.1.2 Preparation of Extract

The collected stem bark was washed thoroughly under running water, cut into smaller pieces and air dried for eight days. The dried stem bark was pulverized with a hammer-mill into powder which was then extracted by decoction (boiled in distilled water for fifteen minutes). The decoction was then filtered, allowed to cool and frozen. It was then kept at approximately -20^{o}C for transport to the Centre for Scientific Research into Plant medicine, Mampong Akwapim in the Eastern Region, Ghana, where it was freeze-dried. The freeze-dried extract (a brown powder), herein referred to as SCE, *Spathodea* or extract was then collected into a glass container and stored in a decicator. SCE was reconstituted prior to use in the study. The final yield was 15.2% w/w.

2.2 PHYTOCHEMICAL SCREENING

Phytochemicals are chemical compounds that occur naturally in plants. The term is generally used to refer to those chemicals that may affect health, but are not established as essential nutrients.

The presence of tannins, alkaloids, flavonoids, general test for glycosides (reducing sugars), anthraquinones, sterols and saponins were tested by simple qualitative methods (Trease and Evans, 1989).

2.2.1 Chemicals/ Reagents

Sulphuric acid, sodium hydroxide, fehling's solution (A and B), hydrochloric acid (HCl), Mayer's reagent, Dragendorff's reagent, ferric chloride, Ammonia solution, chloroform and dichloromethane were purchased from BDH, Poole, England; distilled water was prepared in the department of Biochemistry, Kwame Nkrumah University of Science and Technology, Kumasi.

2.2.2 Methodology

Glycosides

SCE (0.5g) was warmed in a test tube with 5ml of dilute (10%) sulphuric acid on a water bath at 100^{o}C for 2 minutes. The mixture was filtered to obtain the supernatant or filtrate. Using 5% solution of NaOH (noting the volume of NaOH added), the acid extract was neutralized. 0.1 ml of Fehling's solution A and them Fehling's solution B were added to the mixture until alkaline and heated on the water bath for 2minutes. This was labelled as test 1.

In a control test, 5ml of water instead of sulphuric acid was warmed with 0.5g of *Spathodea* on the water bath. Water was added in place of NaOH to an equal volume with test 1. Fehling's solutions A and B were added.

The intensity of red precipitate was compared in the two instances.

Alkaloids

To about 0.5g of SCE, 5ml of 1% aqueous HCl was added and stirred. The mixture was warmed on a steam bath and filtered. 1ml of the filtrate was transferred into each of two test tubes. To one tube, a few drops of Mayer's reagent were added; to the other, a few drops of Dragendorff's reagent. Both tubes were observed for yellowish or brown precipitate.

Tannins

About 0.5g of *Spathodea* was dissolved in 10ml of distilled water and filtered. 1% aqueous $FeCl_3$ was added. The resultant mixture was observed for any change in colour. The same process was repeated with only water to serve as control.

Saponins

0.5g of extract was shaken vigorously with water in a test tube. The solution was observed for the persistence or otherwise of froth for over 5minutes even on warming.

Flavonoids

A strip of filter paper was dipped into a liquid extract prepared by dissolving the *Spathodea* (0.5g) in water (10mls). The filter paper was dried and exposed to ammonia solution. A change of colour to an intense yellow which disappears when exposed to fumes of HCl or dilute HCl would confirm a positive test.

Anthraquinones

About 0.2g of *Spathodea* was boiled with 2ml dilute sulphuric acid and 2ml of 5% aqueous ferric chloride in a test tube for 5mins. The mixture was filtered while still hot and the filtrate cooled. The filtrate was extracted with an equal volume of dichloromethane. The lower organic layer was separated and shaken with half its volume of dilute ammonia.

Sterols

A 0.5g of *Spathodea* was dissolved in 2ml of chloroform in a test tube. Concentrated sulphuric acid was carefully added down the side of the tube to form a lower layer. The interface was observed for any change in colour.

2.3 RESULTS OF PHYTOCHEMICAL SCREENING

The phytochemistry screening of the aqueous freeze-dried extract of *Spathodea campanulata* stem bark showed the presence for sterols, alkaloids, tannins and reducing sugars. Tests were negative for antraquinones, glycosides, saponins and flavonoids (Table 2.1).

Table 2.1: Phytochemical screening of the aqueous stem bark extract of *Spathodea campanulata.*

TEST	RESULTS
Sterols	+
Anthraquinones	-
Glycosides	-
Alkaloids	+
Saponins	-
Flavonoids	-
Tannins	+
Reducing Sugars	+

(+) Present and (-) Absent

2.4 DISCUSSION

The preliminary phytochemical screening showed that the aqueous stem bark extract of *Spathodea campanulata* contained tannins, reducing sugars, sterols and alkaloids. This is in confirmation to work done by Ngouela (1988) who found the stem bark to contain sterols as well as triterpenes. The presence of many biologically active phytochemicals such as triterpenes, flavonoids, alkaloids, steroids, tannins and glycosides in various plant extracts may be responsible for their respective pharmacological properties (Agarwal and Rangari, 2003; Liu *et al.*, 1996; Mbagwu *et al.*, 2007; Narendhirakannan *et al.*, 2007; Singh *et al.*, 2002). Alkaloids, which are nitrogen-containing low molecular weight substances, are a major class of plant secondary metabolites that show a wide variety of chemical structures and biological activities. Some are used in both modern and traditional medicine. For instance, vincristine and taxol are widely used as anticancer drugs and morphine (in some countries) is an indispensable analgesic in clinical medicine (Otani *et al.*, 2005). Alkaloidal compounds isolated from different parts of several medicinal plants have been reported to be responsible for some pharmacological properties (Duwiejua *et al.*, 2002; Whitehouse *et al.*, 1994). Sterols, specifically phytosterols, more commonly known as plant sterols, have been shown in clinical trials to block cholesterol absorption sites in the human intestine, thus helping to reduce cholesterol in humans (Ostlund *et al.*, 2003). Reducing sugars, with aldehyde or ketone group, in solution is able to act as a reducing agent. Presence or absence of specific phytochemicals in a plant can explain the benefits or dangers the plant contributes when ingested. The presence of sterols, reducing sugars, tannins and alkaloids may confer several pharmacological activities on the stem bark of the plant which may explain the effect of the aqueous stem bark extract observed in traditional medicine.

2.5 CONCLUSION

In conclusion, preliminary phytochemical screening of the aqueous stem bark extract of *Spathodea campanulata* showed that it contains tannins, reducing sugars, sterols and alkaloids.

CHAPTER 3

SAFETY EVALUATION OF SCE IN RODENTS

3.1 INTRODUCTION

The aqueous stem bark extract of *Spathodea campanulata* is used in Ghana to treat dysentery, and stomach ache (Mendes *et al.*, 1986). It is also used to treat insulin and non-insulin dependent diabetes in Rwanda (Niyonzima *et al.*, 1990). It has also been found to contain alkaloids and tannins amongst other phytochemicals. However, some plant extracts containing tannins also contain alkaloids that have toxic side effects in man (Atta Ur and Zaman, 1989; Nyarko *et al.*, 2005). Toxicity testing is therefore a matter of necessity. Chronic use of some medications, including herbal medicines known to contain flavonol compounds may modulate cytochrome P450 (CYP) isozymes activities (Beckmann-Knopp *et al.*, 2000).

In addition, scientific testing will serve to provide scientific validation for the use of these plant based products in the management of various conditions traditionally.

Following the recommendation that all natural products used in therapeutics must be subjected to safety tests by the same methods for new scientific drugs (Agbedahunsi *et al.*, 2004; Franzotti *et al.*, 2000; Khalil *et al.*, 2006; Witaicenis *et al.*, 2007), the aqueous extract of *Spathodea campanulata stem bark,* locally used in Ghana for various conditions, such as for different kinds of stomach upset and skin problems, is assessed for safety in rodents. The present study focuses on its acute and sub-acute toxicity, its effect on whole blood and key biochemical parameters in the blood plasma as well as its effect on the kidney in the experimental animals.

3.2 MATERIALS AND METHODS

Animals

The animals used in the study, Sprague-Dawley rats (180-220g) and ICR mice (20-25g), were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon and maintained in the Animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The animals were housed in groups of 6 in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, KNUST, Kumasi, Ghana.

Drugs/ Chemicals

Formalin, ethylene diamine tetra acetic acid (EDTA) and sodium dihydrogen phosphate were purchased from Sigma (St. Louis, MO, U.S.A.); Furosemide was purchased from Bandy Chemist, Kumasi, Ghana.

3.2.1 Toxicity Testing in Rats

Acute Toxicity

Healthy Sprague–Dawley (SD) male rats weighing 180-220g were divided and housed in four groups of five rats each for 5 days before the start of experiment. All animals had free access to water and food except for a 12 hours fasting period before oral administration of the extract.

Animals in group A received distilled water (1ml p.o) and served as control. *Spathodea* was dissolved in distilled water and administered by gavage at single doses of 1250, 2500, 5000 mg/kg respectively to groups B, C and D. The general behaviour of rats was observed in observational cages continuously for one hour (1hr) after the treatment and then intermittently for 4 hours, and thereafter over a period of 24 hours for any signs of toxicity and deaths, and the latency of death (Twaij *et al.*, 1983). The amount of food consumed per animal as well as water consumed was also noted over 1, 2, 4, 6 and 24h after administration of extract.

Sub-Acute toxicity

Spathodea (1250, 2500 and 5000 mg/kg *p.o*) was administered to male Sprague-Dawley rats (180-220g), 5 per group, daily for 14 consecutive days. Group A, the control, received 1ml of distilled water daily. Group B, C and D were treated with extract (1250, 2500 and 5000 mg/kg *p.o.*) respectively daily. The extract was prepared such that not more than 2 ml was given orally. The animals were monitored closely for signs of toxicity. Appearance and behaviour pattern were monitored daily and any abnormalities registered.

The rats were euthanized on the fifteenth day by cervical dislocation, the jugular vein was cut and blood flowed freely. An amount of 1.5 ml of blood was collected in a vial containing 2.5 µg of ethylene diamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay and 2.5 ml of the blood was collected into plain vacutainer tubes without anticoagulant. The blood was centrifuged at 500 *g* for 15 min and serum was collected and stored at – 80ºC until assayed for biochemical parameters.

Effect of extract on organ weights in rats

Selected organs including the spleen, liver, kidney and stomach were excised quickly, trimmed of fat and connective tissue, blotted dry and weighed on a balance. Body weight of

the rats was taken on day 0 and 15. The organ-to-body weight index (OBI) was calculated as the ratio of organ weight and the animal body weight (at the end of the experiment) x 100.

Effect of SCE on haematological parameters

Haematological parameters including red blood cell count (RBC), white blood cell count (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and Mean corpuscular haemoglobin concentration (MCHC) were determined by an automatic analyzer (Sysmex XT-2000 L CELL-DYN 1700, Abbot Diagnostics Division, Abbot Laboratories, Abbot Park, Illinois, USA).

Effect of SCE on Serum Biochemistry

Parameters that were determined include: levels of the liver enzymes- aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), as well as serum determination of total bilirubin (T-BIL), direct bilirubin (D-BIL), indirect bilirubin (I-BIL), total-protein, albumin, blood urea nitrogen (BUN) and creatinine. These were performed using an automatic analyzer ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfied, RI, USA).

Histopathological Analysis

Portions of the tissue from liver and kidney were used for histopathological examination. Tissues were fixed in 10% buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera.

3.2.2 Toxicity testing in Mice

Acute Toxicity

Healthy male ICR mice weighing 20-25g were divided and housed in groups of 5 per cage for 5 days before start of experiment. All animals had free access to water and food except for a 12 hours fasting period before oral administration of the extract.

The *Spathodea* extract was dissolved in distilled water and administered by gavage at single doses of 1250, 2500, 5000 mg/kg. The general behaviour of mice was observed continuously for one hour (1 h) after the treatment and then intermittently for 4h, and thereafter over a period of 24h for any signs of toxicity and deaths, and the latency of death (Twaij *et al.*, 1983).

Sub-Acute Toxicity

S. campanulata (1250, 2500 and 5000 mg/kg *p.o*) was administered to male ICR mice (20-25g), seven per group, daily for 14 consecutive days. Group A, the control, received 1 ml kg^{-1} *p.o* of saline daily. Group B, C and D were treated with extract (1250, 2500 and 5000 mg/kg *p.o.*) respectively daily. The extract was prepared such that not more than 0.25 ml was given orally. The animals were monitored closely for signs of toxicity. Appearance and behaviour patterns were monitored daily and any abnormalities registered.

The mice were euthanized on the fifteenth day by cervical dislocation, the jugular vein was cut and blood flowed freely. An amount of 1 ml of blood was collected in a vial containing 2.5 µg of ethylene-diamine tetra acetic acid (EDTA) as an anticoagulant for haematological assay.

Effect of extract on organ weights in Mice

Selected organs; liver and kidneys were excised quickly, trimmed of fat and connective tissue, blotted dry and weighed on a balance. Body weight of the mice was taken on day 0

and 15. The organ-to-body weight index (OBI) was calculated as the ratio of organ weight and the animal body weight x 100.

Effect of SCE on haematological parameters of Mice

Haematological parameters including red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and Mean corpuscular haemoglobin concentration (MCHC) were determined by an automatic analyzer (Sysmex XT-2000lCELL-DYN 1700, Abbot Diagnostics Division, Abbot Laboratories, Abbot Park, Illinois, USA).

Histopathological Analysis

Portions of the tissue from liver and kidney were used for histopathological examination. Tissues were fixed in 10% buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera.

3.2.3 Diuretic Effect of SCE in Rats

The diuretic activity of SCE was evaluated according to the method described by Ratnasooriya and Jayakody, (2004) with few modifications.

Rats weighing 150 to 200g were put into five groups of five. Animals were deprived of water but not food for 18 hours. Urinary bladders were emptied by gentle compression of their pelvic area and by pulling their tails. Each animal was then orally administered 10ml of

isotonic saline solution (NaCl, 0.9%) to impose uniform water load. Forty-five minutes later, the rats were orally treated with their respective doses/drugs in 1 ml of distilled water. Group A received 1 ml of distilled water and served as the control. Group B-E received Furosemide 15 mg/kg, SCE (625, 1250 & 2500) mg/kg respectively. Each rat was individually placed in a metabolic cage and cumulative urine output was measured, using a measuring cylinder, at 1, 2, 4, 6 and 24 hours. The colour and turbidity of urine was followed. Electrolyte levels in urine were measured on collection with Biotecnica BT 3000 Targa chemistry analyzer (Biotecnica Instrument, Diamond Diagnostics - USA). The water as well as food consumption of the animals was also noted.

Analysis of Data

Data were presented as mean ± SEM. The presence of significant differences among means of groups was determined by one-way ANOVA using GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA). Significant difference between pairs of groups was calculated using the Newman-Keuls Multiple Comparision Test.

3.3 RESULTS

3.3.1 Toxicity studies in Rats

Acute Toxicity

A single oral dose of *Spathodea campanulata* stem bark extract did not cause any deaths neither did the rats show any signs of toxicity at dose levels used (1250, 2500, 5000mg/kg). The LD_{50} (rat, oral) is therefore estimated to be beyond 5000mg/kg body weight.

Sub-acute toxicity

The rats did not show any observable signs of toxicity or change in general behaviour. When the extract was given at a dose of 1250, 2500, 5000 mg/kg per day for 14 days, rats remained alert with no motor or neurological changes, no adverse gastrointestinal tract disorders, respiratory distress or locomotor changes was observed in any of the animals. The extract however appeared to have slight diarrheal properties upon continuous administration to the rats as stools from rats treated with the extract were slightly more hydrated than that from the control animals.

Effect of SCE on body and some target organ weight in rats

There were no significant changes in the body weight of rats that survived at the end of the experiment, (Figure 3.1) neither were the relative weights of some selected target organs affected (Table 3.1).

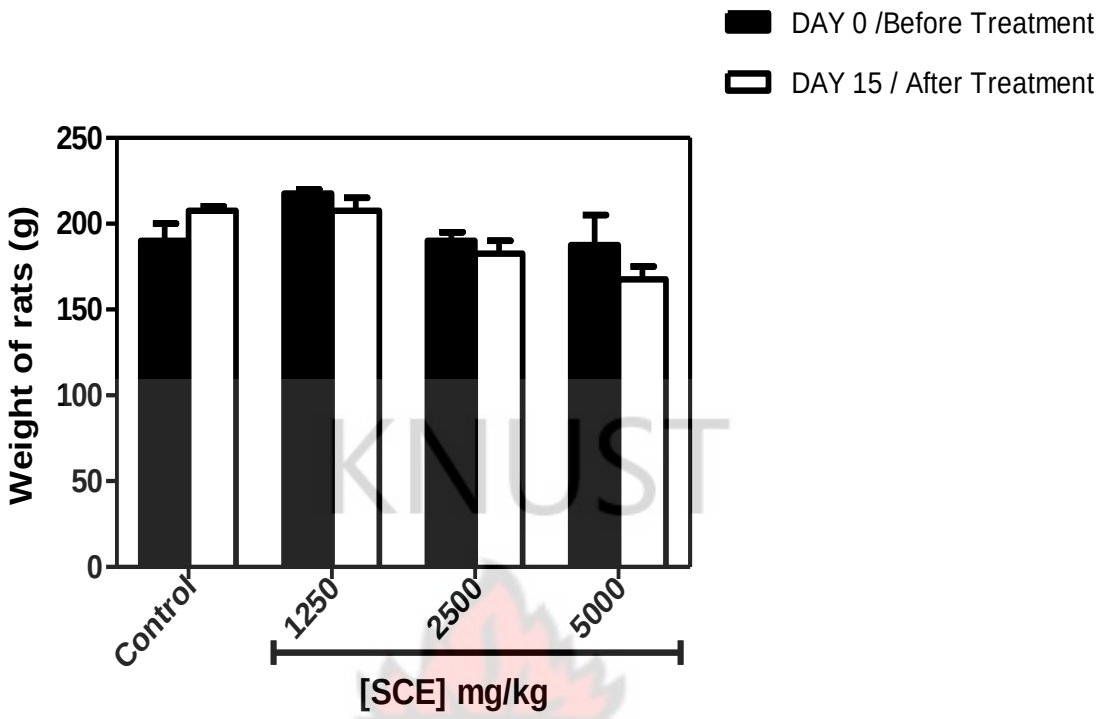


Figure 3.1: Weight of rats taken before and after 14-day treatment with *Spathodea campanulata* stem bark extract

Effect on Haematological parameters

The effect of sub-acute oral administration of *SCE* on the haematological parameters is presented in Tables 3.2 and 3.3. Haematological values of treated rats were not significantly different from those of the control group for all parameters measured at any dose (1250-5000), (Table 3.2). The differential white blood cell counts in the treated groups were not different from that of the control group either (Table 3.3).

Effect on Serum Biochemistry

Biochemical profiles of the treated animals are presented in Table 3.4 and Fig 3.2. The sub-acute oral administration of *Spathodea* (up to a dose of 5000mg/kg body weight) did not cause any significant changes in the serum proteins and bilirubin as well as some electrolytes (Potassium and Chloride), Blood Urea Nitrogen (BUN), Creatinine, Cholesterol, and Glucose. However, there was a significant decrease ($p<0.05$) of the serum sodium ion levels in the 2500mg/kg and 5000mg/kg doses compared to the control. The levels of the liver marker enzymes, (AST and GGT) of treated animals were not significantly different from control. In the case of ALT and ALP, however, levels in rats decreased in the first two doses (1250 and 2500 mg/kg) but ALP levels increased, significantly (up to 582.5) ($p<0.05$), in the 5000mg/kg dose compared to the control (Fig 3.2).

Table 3.1: Effect of *SCE* on organ weights of rats treated with the extract for 14 days.

Organ	Relative organ weight			
	Control	**1250mg/kg**	**2500mg/kg**	**5000mg/kg**
Liver	3.2600±0.3279	2.6060±0.2061	3.0930±0.2519	3.1320±0.2619
Kidneys	0.7098±0.1240	0.5822±0.0055	0.6223±0.0307	0.8513±0.0862
Spleen	0.2058±0.0058	0.2742±0.0048	0.2578±0.0238	0.2656±0.0656
Stomach	1.0600±0.4682	0.7246±0.0103	0.8367±0.0611	1.1250±0.0750
Heart	0.4104±0.0645	0.3624±0.0236	0.4003±0.0125	0.3719±0.0218

Values are expressed as mean± SEM (n=5) compared to the control by the Newman Keuls test.

Table 3.2: Effect of *SCE* on the haematological indices of Sprague dawley rats treated for two weeks.

	CONTROL	1250 mg/kg	2500 mg/kg	5000 mg/kg
RBC	6.75 ± 1.26	6.80 ± 0.34	6.83 ± 0.095	4.94 ± 0.74
HGB	11.93 ± 2.19	12.70 ± 0.30	12.15 ± 0.05	8.85 ± 1.35
HCT	38.00 ± 7.46	38.90 ± 0.30	38.45 ± 0.25	27.50 ± 3.60
MCV	55.63 ± 0.74	57.35 ± 2.45	56.35 ± 1.15	56.00 ± 0.90
MCH	17.60 ± 0.15	18.70 ± 0.50	17.80 ± 0.30	17.95 ± 0.05
MCHC	31.60 ± 0.60	32.65 ± 0.55	31.50 ± 0.00	32.00 ± 0.60
PLT	607.3 ± 99.49	564.0 ± 100	584.0 ± 99.00	586.0 ± 90.00

Values are expressed as means ± SEM (n=3), compared to the control by the Newman Keuls test

Table 3.3: Effect of *SCE* on the differential white blood cell count of Sprague dawley rats treated for two weeks.

	Wbc10^3/ul	**Neutrophil(%)**	**Eosinophil (%)**	**Monocyte (%)**	**Lymphocyte (%)**
CONTROL	3.903±1.87	41.73±9.42	0.9667±0.167	6.20±2.91	51.10±11.20
1250mg/kg	2.87±0.30	30.35±1.55	1.75±1.75	3.50±1.20	64.25±1.15
2500mg/kg	4.95±0.63	28.00±1.60	1.00±0.10	3.55±1.45	67.45±3.15
5000mg/kg	3.23±1.38	35.15±20.55	0.10±0.10	15.30±10.10	49.35±10.65

Values are expressed as means ± SEM (n=3), compared to the control by the Newman Keuls test.

Table 3.4: Effect of *SCE* on the biochemical parameters of Sprague Dawley rats treated for two weeks.

	CONTROL	1250mg/kg	2500mg/kg	5000mg/kg
K	5.927±0.596	5.420±0.310	5.235±0.385	6.075±0.035
Na	147.30±1.856	141.5±1.50	134.0±3.00*	135.0±1.00*
Cl	101.40±1.732	100.0±0.0	99.40±2.60	98.20±0.40
BUN	57.00±16.64	28.50±6.50	33.00±11.00	71.00±7.00
CRE	1.81±0.263	1.240±0.11	1.530±0.25	2.340±0.13
CHO	115.70±19.34	98.00±16.00	112.0±3.00	114.5±9.5
T. PRO	5.83±0.617	6.90±0.40	6.40±0.20	5.25±0.25
ALB	3.50±0.306	4.40±0.30	3.90±0.10	3.25±0.15
ALT	91.33±10.48	42.50±12.50*	59.50±2.50*	79.50±14.50
AST	174.70±25.76	173.5±2.5	182.0±5.00	143.5±31.50
GGT	3.67±1.453	5.50±2.50	6.50±4.50	5.50±0.50
GLUCOSE	107.00±2.082	89.50±14.50	111.5±10.50	77.50±11.50

Values are expressed as means ± SEM (n=3), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test.

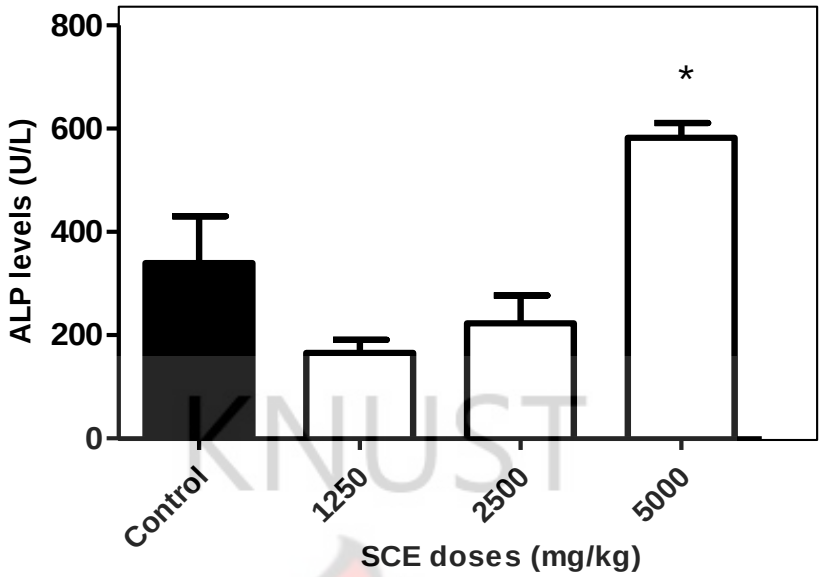


Figure 3.2: ALP levels of rats after 14-day treatment with SCE. Bars represent means ± SEM (n=3). * indicates significance ($p<0.05$) compared to the control group (ANOVA).

Histopathological Analysis

Livers and kidneys from the saline treated (control) group had normal appearance and histology.

Generally, there were no observable changes in architecture of livers and kidneys of treated animals compared to the control. The histology was consistent with the normal ALT, AST, bilirubin, creatinine and BUN levels in the serum.

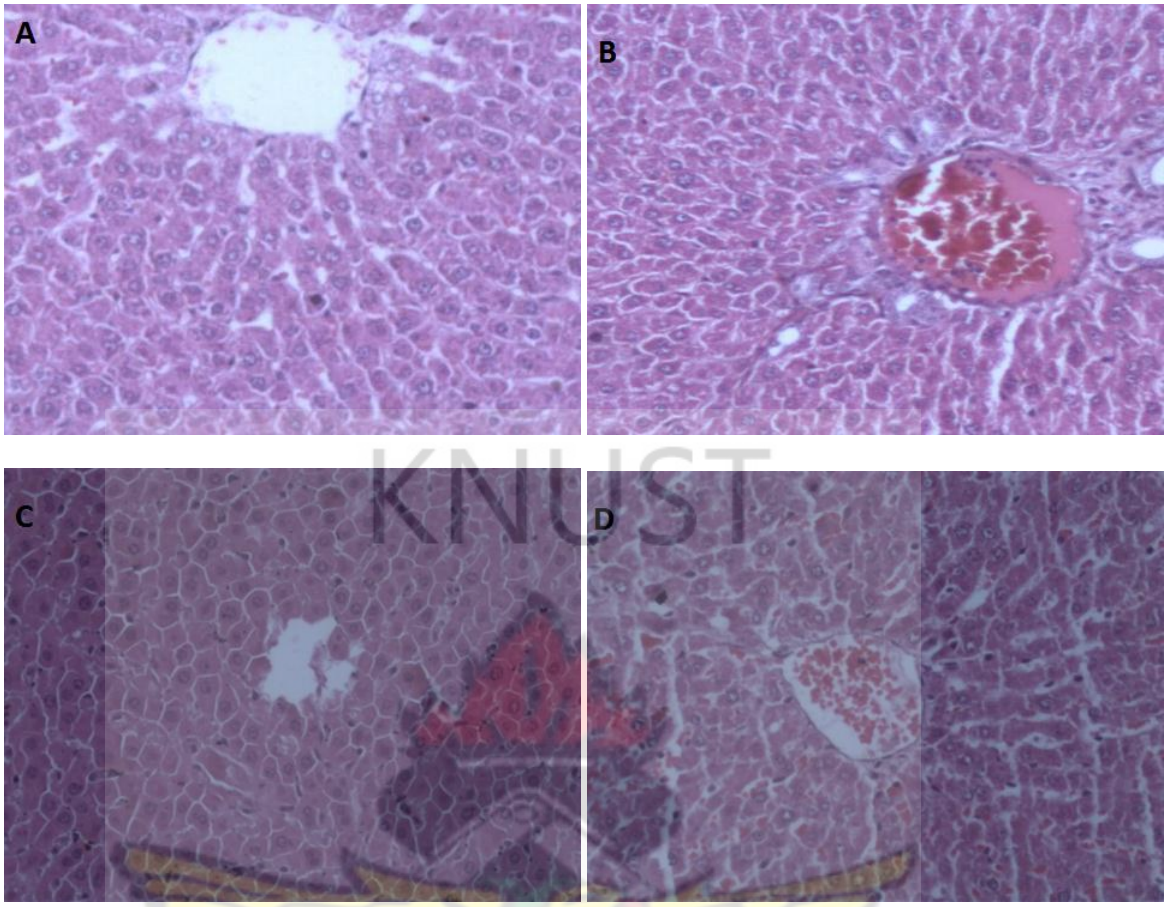


Figure 3.3: Photomicrograph (x 400) showing histopathological slides of the livers of *SCE* treated rats. (A) Control, (B) 1250mg/kg, (C) 2500mg/kg, (D) 5000mg/kg

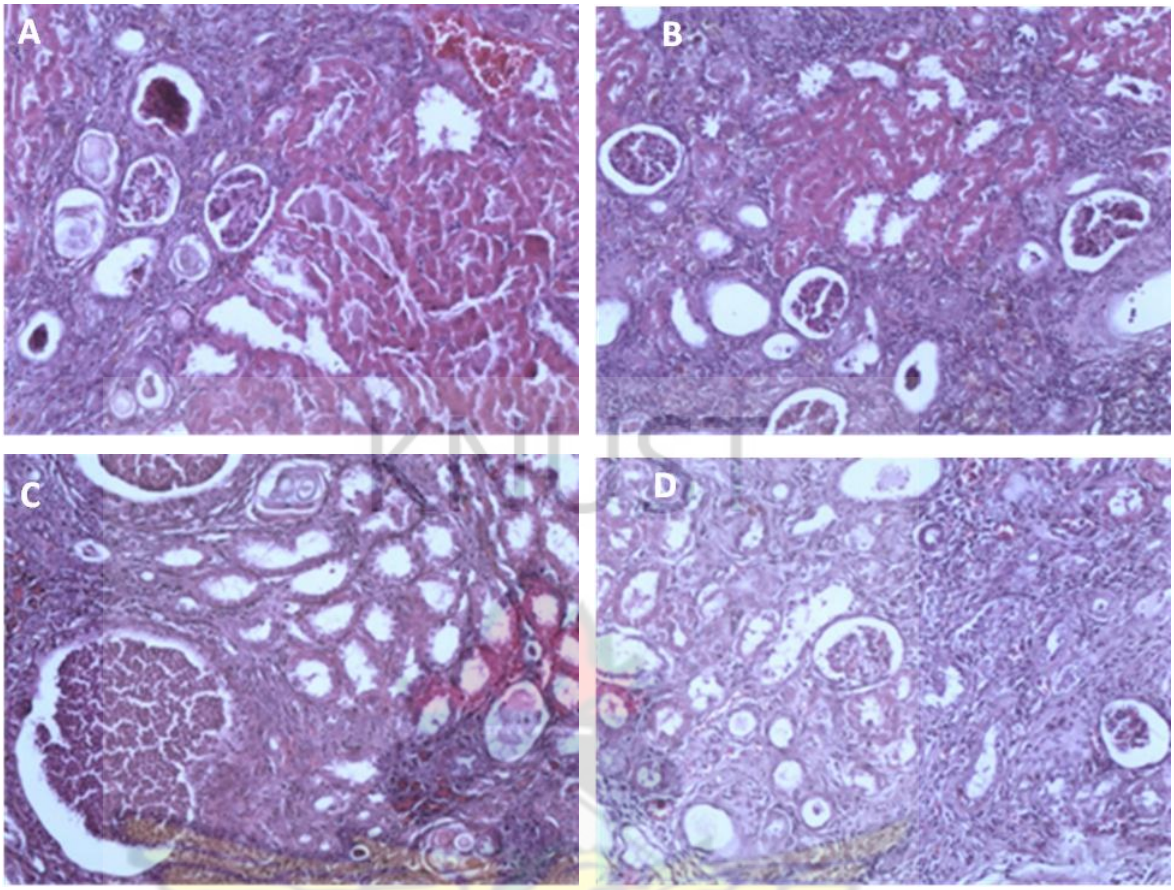


Figure 3.4: Photomicrograph (x400) showing histopathological slides of the kidneys of *SCE* treated rats. (A) Control, (B) 1250mg/kg, (C) 2500mg/kg, (D) 5000mg/kg

3.3.2 Toxicity studies in mice

Acute Toxicity

A single oral dose of *Spathodea campanulata* stem bark extract did not cause any deaths neither did the rats show any signs of toxicity at any dose level (1250, 2500, 5000mg/kg). The LD_{50} (mice, oral) is therefore estimated to be beyond 5000mg/kg body weight.

Effect of SCE on body and some target organ weight in mice

The mice did not show any observable signs of toxicity or change in general behaviour. When the extract was given at a dose of 1250, 2500, 5000 mg/kg per day for 14 days, the mice remained alert with no motor or neurological changes, no gastrointestinal tract disorders, respiratory distress or locomotor changes was observed in any of the animals.

There were no significant change in the body weight of animals at the end of the experiment (Figure 3.5) neither were the relative weights of some target organs affected (Table 3.5). Similarly, the haematology of mice was not significantly affected by treatment with the extract (Table 3.6).

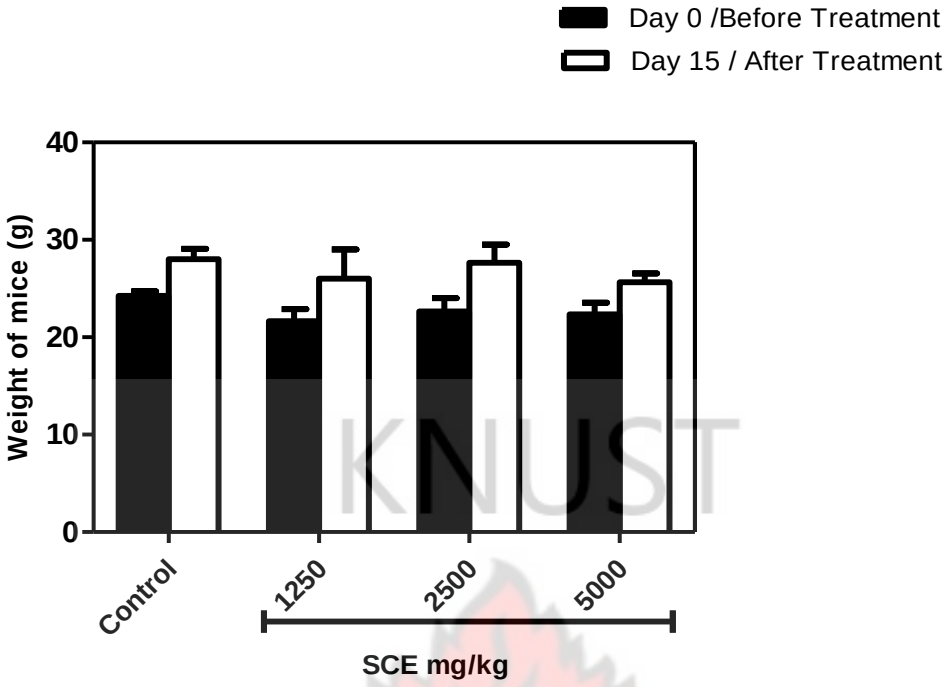


Figure 3.5: Weight of mice taken before and after 14-day treatment with *Spathodea campanulata* stem bark extract.

Table 3.5: Effect of 14 day treatment with *SCE* on organ weight of mice

Organ	**Relative organ weight**			
	Control	**1250 mg/kg SCE**	**2500 mg/kg SCE**	**5000 mg/kg SCE**
Liver	3.953 ± 0.06369	4.093 ± 0.1567	3.983 ± 0.08645	4.075 ± 0.1548
Kidneys	1.149 ± 0.04641	1.130 ± 0.04273	1.040 ± 0.07915	1.097 ± 0.08344

Values are expressed as mean± SEM (n=5) compared to the control by the Newman Keuls test.

Table 3.6: Effect of *SCE* on the haematological indices of ICR mice treated for two weeks.

	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT
CONTROL	9.467±1.419	9.560±0.2714	14.88±0.1702	46.98±0.4516	49.25±1.065	15.58±0.3860	31.65±0.4368	1399±192.1
1250 mg/kg SCE	7.850±1.150	9.995±0.5950	15.70±1.200	48.90±2.500	48.95±0.4500	15.70±0.3000	32.10±0.8000	1085±120.5
2500 mg/kg SCE	8.690±1.190	9.255±0.2550	14.37±0.2728	47.70±0.7000	48.50±0.9000	15.10±0.6000	31.45±0.2500	1183±168.0
5000 mg/kg SCE	7.007±0.6375	9.483±0.1862	14.90±0.2646	48.23±1.048	50.90±0.8145	15.70±0.05773	30.90±0.4163	1379±82.50

Values are expressed as means ± SEM (n=3), compared to the control by the Newman Keuls test.

Histopathological Analysis

From the histopathological observations, it could be seen that SCE did not show any observable toxicity to the kidneys even at the highest dose compared to the control. In the liver also, the extract at the highest dose (5000 mg/kg) caused slight fatty liver, (Figure 3.6 & 3.7).

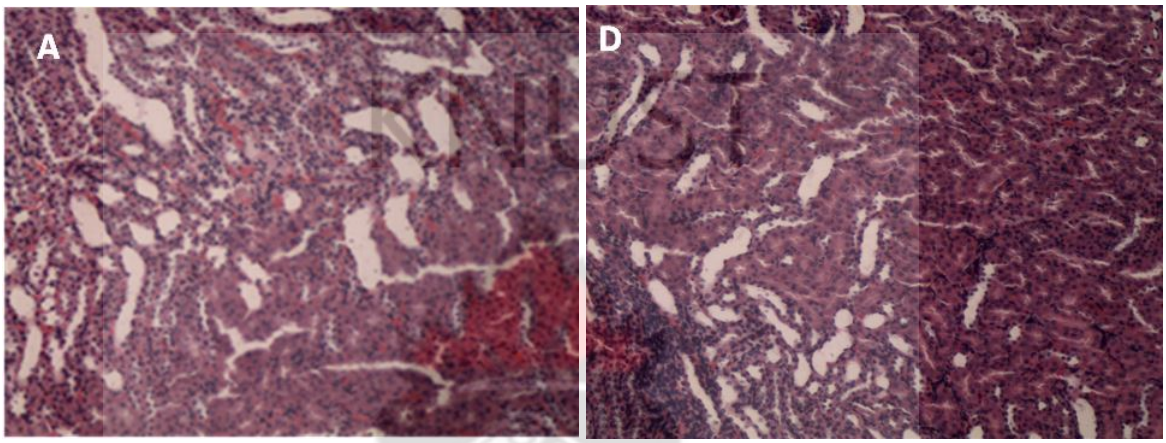


Figure 3.6: Photomicrograph (x40) showing histopathological slides of the kidneys of *SCE*-treated mice. (A) Control and (D) the highest dose 5000mg/kg.

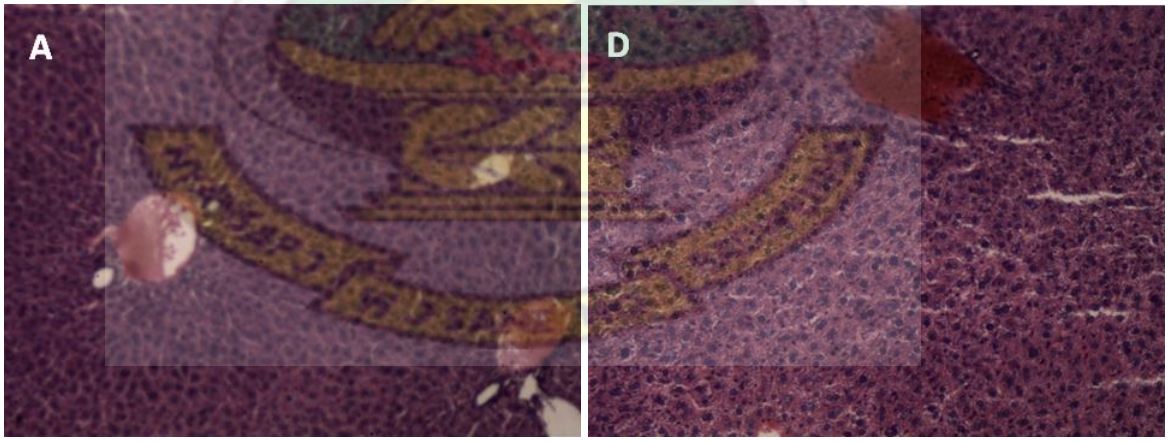


Figure 3.7: Photomicrograph (×400) showing histopathology of the livers of Spathodea-treated mice. (A) Control and (D) the highest dose, 5000mg/kg.

3.3.3 Diuretic Effect in Rats

Effect on Urine output

As observed in figure 3.8, an increase in urine output over a period of 24 hours after a single dose of SCE (100, 625, 1250, 2500 mg) was observed only in the 2500mg/kg dose. The urine output was more than two times (over 100%) higher than the control group in the highest dose. However, there was no dose dependent increase from the lower doses compared to the control. The urine output in the furosemide group was increased as expected (about 50% more) of that of the control group.

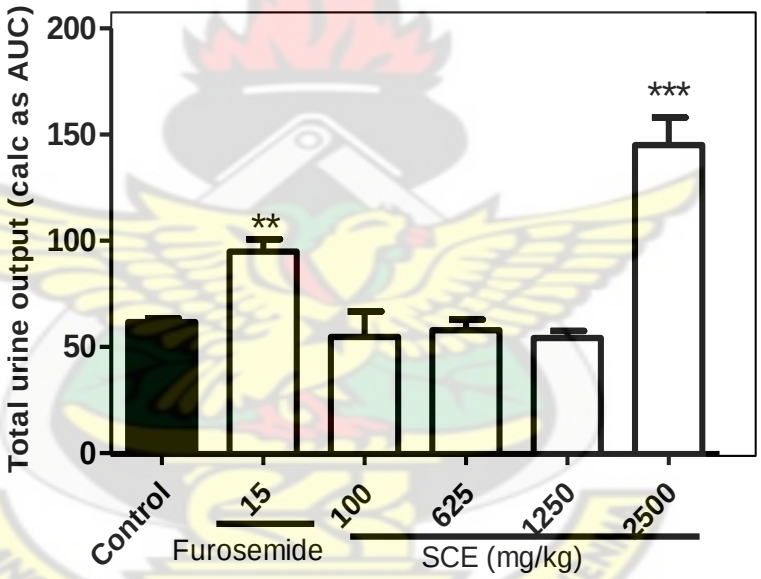


Figure 3.8: Effect of SCE single dose treatment over 24 hours on urine output in SD rats (100, 625, 1250, or 2500) or Furosemide. Cumulative values are reported as mean±SEM for five rats in each group. **p < 0.01; ***p < 0.001; compared to controls (ANOVA) followed by Student's Newman-Keuls multiple comparison test.

Effect on Urine Electrolyte

As indicated in figures 3.9 and 3.10, a single oral dose of furosemide, increased the urine sodium and potassium (from 964.85 to 2506.5 and 2992 to 8440 respectively; $p<0.05$ and $p<0.01$) as expected. There appears to be a dose-dependent increase of urine sodium and potassium levels in SCE-treated rats though not statistically significant for sodium. In the urine potassium levels (which followed a dose dependent increasing trend) however, the increase in the highest dose (1250 mg/kg) was statistically significant ($p<0.01$) to a similar extent as in furosemide, compared to the control.

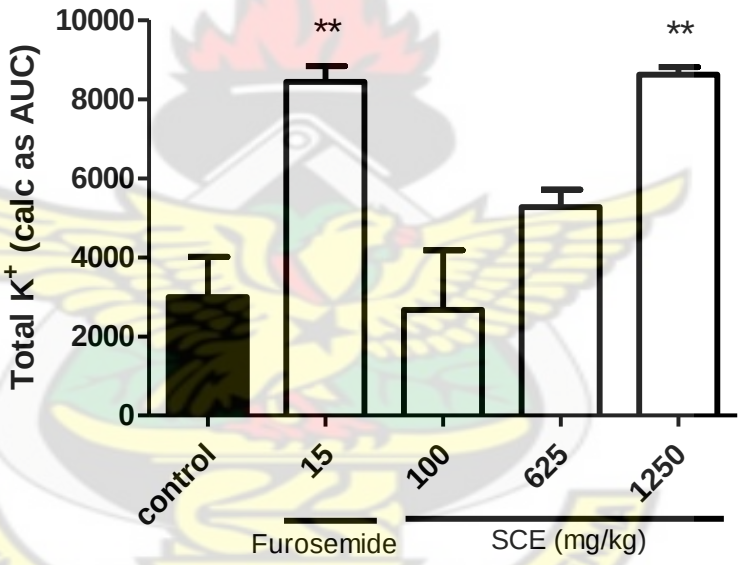


Figure 3.9: Effect of SCE (100, 625, 1250 mg/kg) and furosemide (15 mg/kg) on K^+ concentration in urine over 24 hours (defined as the area under the curve (AUC)) of diuresis in treated rats. Values are reported as mean ± SEM for five rats in each group. *p < 0.05; **p < 0.01; compared to controls using Student's Newman-Keuls multiple comparison test.

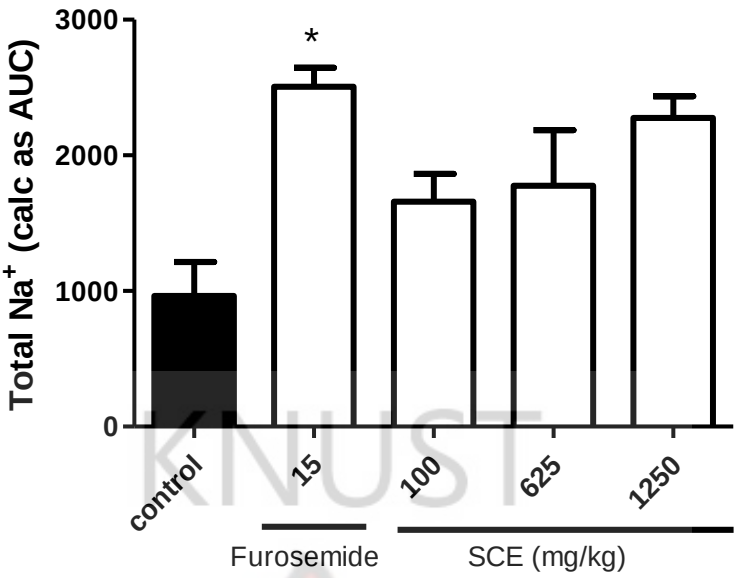


Figure 3.10: Effect of SCE (100, 625, 1250 mg/kg) and furosemide (15 mg/kg) on Na^+ concentration in urine over 24 hours (defined as the area under the curve (AUC)) of diuresis in treated rats. Values are reported as mean ± SEM for five rats in each group. *p < 0.05; compared to control using Student's Newman-Keuls multiple comparison test.

3.4 DISCUSSION

The general toxicity study was done in two rodent species; rats and mice. This is because of possible differences between species in drug metabolism and susceptibility to toxic effects.

The study has established that a single dose of the aqueous extract by the oral route up to a dose of 5000 mg/kg when observed over a period of 24 hours (acute toxicity) did not cause any mortality or alter the behavioural pattern of either rats or mice. Compared with the control group, rats as well as mice were alert with no motor or neurological changes, no gastrointestinal tract disorder, and respiratory distress. The weights of treated male rats and mice were not significantly different from those of the control groups as it is in the case of toxicity where there is a general decrease in the weight in the animals.

The results of acute toxicity testing in this study indicate that the aqueous stem bark extract of *Spathodea campanulata* is fairly non-toxic. In addition the oral LD_{50} in rats is estimated to be beyond 5000mg/kg. Similarly, the oral LD_{50} in mice is estimated to be beyond 5000mg/kg.

A daily administration of *SCE* (1250, 2500, 5000 mg/kg) in male rats for 14 days did not produce signs of toxicity at any dose level.

The extract at the highest dose of 5000mg/kg, given orally for 14 days, did not cause any observable toxic effects in neither rats nor mice. There were no significant changes in the weights of animals though there was a marginal decrease in weight of treated rats compared to those of the control. This decrease in weight is rather not significant though is worth noting. The weight of some target organs; the liver, stomach, heart, spleen and kidneys were also not affected. The extract did not affect the integrity of the blood of the animals. Certain medicinal herbal preparations or conventional drugs negatively affect various blood components. For example, some flavonoids including those isolated from herbs cause haemolytic anaemia and thrombocytopaenia (Nyarko *et al.*, 2005).

The haemoglobin content, red blood cell count, haematocrit, mean corpuscular volume as well as the platelet count of treated animals (rats and mice) showed no variation when compared with those of their control groups. To ascertain whether the defense system of the animals had been compromised, WBC count, as well as the various percentages of the types of WBCs was measured. These were also not affected by the 14 days administration of the extract. Serum biochemical parameters as specific markers for some target organs (AST, ALT for liver; BUN, Creatinine and electrolytes for kidney function, etc.) showed no significant differences between treated groups and control except in the case of the Na^+ concentration which decreased with increasing doses and the alkaline phosphatase levels which increased in the highest dose in rats. Damage to or effect on target organs often results in increase in clinical chemistry parameters such as serum enzymes like ALP, AST and ALT and analytes like total and conjugated bilirubin (in the case of the liver), urea and creatinine (in the case of the kidney) (Akdogan *et al.*, 2003). The ALP levels increased significantly in the highest dose and decreased relatively in the least and middle dose compared to the control. This could be due to a hepatobiliary condition such as primary biliary cirrhosis. With the exception of growing animals or animals with bone disease (where an increase in ALP is due to the osteoblasts' production of it), the elevated serum ALP activity is mostly attributed to hepatobiliary origin (Ramaiah, 2007).

High levels of ALP exist in cells that are rapidly dividing or are otherwise metabolically active. However, ALP levels reach spectacular levels in primary biliary cirrhosis, in conditions of disorganized hepatic architecture (cirrhosis), and in diseases characterized by inflammation, regeneration, and obstruction of intrahepatic bile ductules (Witthawaskul *et al.*, 2003). The finding was however not supported in the whole blood analysis where parameters like the platelets and WBC counts were normal. In addition, when the bilirubin and GGT levels were considered, it revealed that the bile ducts of the rats could not have been obstructed since the GGT and bilirubin are better indicators. Increase in ALP activity is also associated with the administration of drugs such as corticosteroids and anticonvulsants (Meyer and Harvey, 2004). In rats, the serum ALP activity has been reported to increase

rapidly following a meal and thus cannot be reliable alone to detect cholestasis (Barton *et al.*, 2000). It is known that on a per gram basis, intestinal mucosa in the rat has higher ALP activity than the liver (Amacher, 2002). Thus, food consumption should be taken into consideration while interpreting ALP values in the rats, especially since decreased food consumption and body weight routinely noted in toxicology studies results in decreased ALP. Other parameters measured indicate that SCE neither caused damage to cardiac or skeletal muscle nor imposed any hepatic excretory dysfunction (cholestasis) or cause any damage to the bone marrow. Although the increased ALP at 5000 mg/kg in rats seems to be an isolated case, the decreasing trend of AST and ALT levels in rats, cannot be overlooked. Clinically, decreased AST and ALT levels have no documented relevance if not in a patient already having a hepatic dysfunction; whereas an increase suggest a hepatic leakage or acute liver damage (Chand *et al.*, 2011).

The histology of the livers showed no signs of toxicity in the rats at all doses used contrary to the suggestion of cholestasis by the increased ALP. In the mice however, fatty liver is suspected in the highest dose (5000 mg/kg) whereas there were no histological changes in the kidneys of either rats or mice.

On the other hand, the decrease in sodium ions (hyponatremia) in the rats treated with the highest dose calls for attention.

Hyponatremia, is a state of relative water excess due to stimulated arginine vasopressin (AVP) and fluid intake greater than obligatory losses with serum sodium concentration of 135 mmol/L or less (Almond *et al.*, 2005). It is the most frequent electrolyte disorder encountered in hospitalized patients. This suggests that SCE may have caused fluid retention evident in the low levels of sodium. Hyponatremia may be hypotonic caused by osmols such as glucose (in the case of diabetics) and mannitol. However, SCE has been shown to possess hypoglycemic property in the present study and also by Niyonzima *et al* (1999). The water retention therefore is not likely to be caused by the presence or activity of glucose. The hyponatremia could therefore be linked to antidiuretic hormone (ADH) activity which could

be stimulated by an unknown agent present in SCE. ADH controls re-absorption of molecules in kidney tubules. Sodium loss (possibly through diuresis) which leads to volume depletion serves as a sign for ADH release. As a result, an increase in re-absorption of molecules will stimulate water retention which will lead to dilution of blood sodium. Vasopressin receptor agonist can be used to treat hyponatremia and congestive heart failure or liver cirrhosis (Palm *et al.*, 2006). It is proposed that SCE could be a diuretic.

Investigations of this proposal showed that lower doses of the extract (≤ 2500 mg/kg) which are relatively safe are also diuretic.

A standard diuretic, furosemide, was compared with the extract. The effect of a single administration of a dose of either extract or furosemide was monitored over a period of 24 hours.

Furosemide produced a marked diuretic effect at a dose of 15 mg/kg indicated by a significant increase in urine output. The extract produced a marked increase in urine output at the highest dose (2500 mg/kg). This sudden increase in urine output caused by the extract was higher than the urine output due to furosemide. The result of the present study shows that a single dose of SCE (2500 mg/kg) observed over 24 hours causes a significant increase in urine volume in rats suggesting that SCE might have diuretic properties as proposed.

There is obvious correlation between urine volume and electrolyte content in urine. It appears that SCE not only increased urine output (volume and frequency) but also enhanced sodium ion (Na^+) excretion. At all dose levels, Na^+ concentration in urine was higher than in the control group but lower than in the Furosemide™ treated group.

In addition, potassium ion (K^+) excretion was dose-dependently increased in the SCE-treated group with time. Interestingly, the K^+ output was even higher in SCE treated groups than in furosemide-treated group after 4 hours suggesting a lasting and profound diuretic effect of the extract. The duration of action with furosemide is brief (less than 4 h when orally administered and 3 h in case of intravenous administration) (Sadki *et al.*, 2010).

SCE may inhibit Na and K ion reabsorption similar to loop diuretics such as furosemide. This could support the use of the plant in traditional medicine, to treat 'kidney trouble' (Abbiw, 1990). With the kind of kidney problem traditionally treated with SCE not specified, it is possible to attribute this use to the diuretic property of the plant.

Multiple diuretic classes are also available for use in chronic kidney disease (CKD) depending on their mode of action. They may also be employed for secondary uses in antihypertensive therapy since most antihypertensive drugs stimulate renal tubular sodium reabsorption increasing extra cellular fluid volume and causing hyponatremia.

3.5 CONCLUSION

Results from the present study found no obvious toxicity in both rats and mice during a 14 day treatment period neither were there any adverse effects seen histopathologically in either the liver or kidneys. It can be concluded that SCE is relatively safe at doses below 5000 mg/kg and possess some diuretic effect in experimental rats. These findings may provide some justification for the use of the SCE to treat 'kidney problems' traditionally.

Further studies are however required to elucidate the mechanism of diuresis of the extract and the active compounds responsible for diuresis.

However, the decreasing trend of ALT and AST after treatment with SCE will be followed in subsequent chapters to see if it is of any consequence.

CHAPTER 4

HEPATOPROTECTIVE ACTIVITY OF SCE AGAINST CCL_4-INDUCED HEPATOTOXICITY

4.1 INTRODUCTION

In the previous chapter, an aqueous stem bark extract of *Spathodea campanulata* was found to be relatively safe and a diuretic. However, the treatment of rats with the extract also caused a decrease in serum aminotransferases at lower doses. This finding, though clinically insignificant, prompts a research into the possible protective ability of the extract on the liver. The hypothesis that the extract can reduce elevated AST and ALT will be tested in this chapter using carbon tetrachloride to induce toxicity to the liver.

The carbon tetrachloride (CCl_4) model of hepatotoxicity is an extensively investigated model. It mimics oxidative stress in many pathophysiological situations (McGregor and Lang, 1996).

Carbon tetrachloride, a known hepatotoxin, is a simple molecule which when administered to a variety of species, causes centrilobular hepatic necrosis (zone 3) and fatty liver. It is a very lipid-soluble compound and is consequently well-distributed throughout the body; but despite this, its major toxic effect is on the liver, irrespective of the route of administration (Timbrel, 2009).

CCl_4 intoxication in various studies has demonstrated that CCl_4 causes free radical generation in many tissues such as liver, kidney, brain, heart lung and blood (Ganie *et al.*, 2011). Carbon tetrachloride (CCl_4) is biotransformed by the cytochrome P450 enzymes to the trichloromethyl (CCl_3') radical, a hepatotoxic metabolite. Covalent binding of this radical to proteins initiates a cascade of events leading from hepatic compromise to cell necrosis.

The decreasing trend in alanine amino transferase (ALT) and alkaline phosphatase (ALP) in the toxicity testing suggested that *SCE* may have hepatoprotective potential. In addition, it is known to be rich in antioxidants such as oleanolic acid (Mbosso *et al.*, 2008) and β-sitosterol which has been shown to protect against oxidative stress by reducing the level of free radicals and increasing the level of antioxidant enzymes (Awad *et al.*, 2008).

However, a literature search revealed that no studies had been done on the possible effectiveness of the aqueous stem bark extract of *Spathodea campanulata* against liver disorders.

In this chapter, the hepatoprotective properties of the aqueous stem bark extract against CCl_4 induced liver damage in Sprague-Dawley rats is reported.

4.2 MATERIALS AND METHODS

Animals

The animals used in the study, Sprague-Dawley rats (180-220g), were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon and maintained in the Animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The animals were housed in groups of 6 in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, KNUST, Kumasi, Ghana.

Drugs and Chemicals

Formalin, ethylene diamine tetra acetic acid (EDTA) and sodium dihydrogen phosphate were purchased from Sigma (St. Louis, MO, U.S.A.), Silymarin, named 'Legalon 70' (from Bukwang Param, Seoul, Korea), was purchased from Bandy chemist in Kumasi, Ghana.

4.2.1 Prophylactic Studies

The prophylactic studies were done according to the method described previously (Bremanti, 1978), with a few modifications. This is where the animal group(s) received the test drug or control for a period of time before the hepatotoxin was administered to them.

Thirty male rats were divided into six (6) groups (n=5). *Spathodea* (625, 1250, 2500mg/kg) and silymarin (50mg/kg) were fed orally to the first four groups of rats respectively, 48, 24, and 2h before CCl_4 (20% mixed with liquid paraffin, 1ml/kg p.o.) administration. Group E was given only water (vehicle) and F received the same dose of CCl_4 serving as the toxin control (Bremanti, 1978; Chandan *et al.*, 2008). 24 hours after CCl_4 administration, all animals were anesthetized. Blood samples were collected from a common carotid into EDTA and dry centrifuge tubes. After blood collection, animals were sacrificed and livers were removed for tissue studies.

The EDTA anticoagulated blood was tested for total platelet count whereas the coagulated blood in the centrifuge tubes were centrifuged and the serum were tested for liver markers; total protein (TP), albumin (Alb), globulin (Glob), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Gamma glutamyl transferase (GGT) and Alkaline phosphatase (ALP).

Livers removed were blotted of blood and preserved in 10% buffered formalin (pH 7.2) and dehydrated through a series of ethanol solutions, embedded in paraffin and routinely processed for histological analysis. Sections of 2 µm thickness were cut and stained with haematoxylin-eosin for examination. The stained tissues were observed through an Olympus microscope (BX-51) and photographed by a chare-couple device (CCD) camera.

Histopathological analysis was done on the fixed livers.

4.2.2 Curative Study

In the curative studies, toxins are administered to rats for a period of time before treatment with the test drug or standard drug is administered to obliterate the effect of the toxin in the livers of the animals. Lower doses of extract were found to be effective in the preliminary studies and so were used.

Six groups (A-F) of five rats each were used for the curative CCl_4-induced hepatotoxicity model. Liver injury was produced by administration of CCl_4 (20% in liquid paraffin, 1ml/kg p.o per day for five consecutive days) to five groups (B-F). *Spathodea* (100, 300, 625, mg/kg), silymarin (50mg/kg) and vehicle (distilled water) were fed to the five groups of animals 2, 24 and 48h after the last dose of CCl_4 administered (Chandan *et al.*, 2008). Group A served as the normal control and so received only distilled water throughout the experiment. 2hours after the final dose of extract, silymarin or distilled water, all animals were sacrificed. Blood samples were collected from a common carotid into dry centrifuge tubes. After blood collection, animals were sacrificed and livers were removed for tissue studies. The coagulated blood in the plane tubes were centrifuged and the serum was tested for liver markers such as total protein (TP), albumin (Alb), globulin (Glob), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Gamma glutamyl transferase (GGT) and Alkaline phosphatase (ALP).

Livers were removed blotted of blood and preserved in 10% buffered formalin. Histopathological analysis was done on the H/E stained mounted slide preparations of the livers.

4.3 RESULTS

4.3.1 Prophylactic Study

Effect of SCE pre-treatment against CCl_4 toxicity on body weight of rats.

There were no significant changes in the relative weights of the animals at all levels of treatment (Fig 4.1).

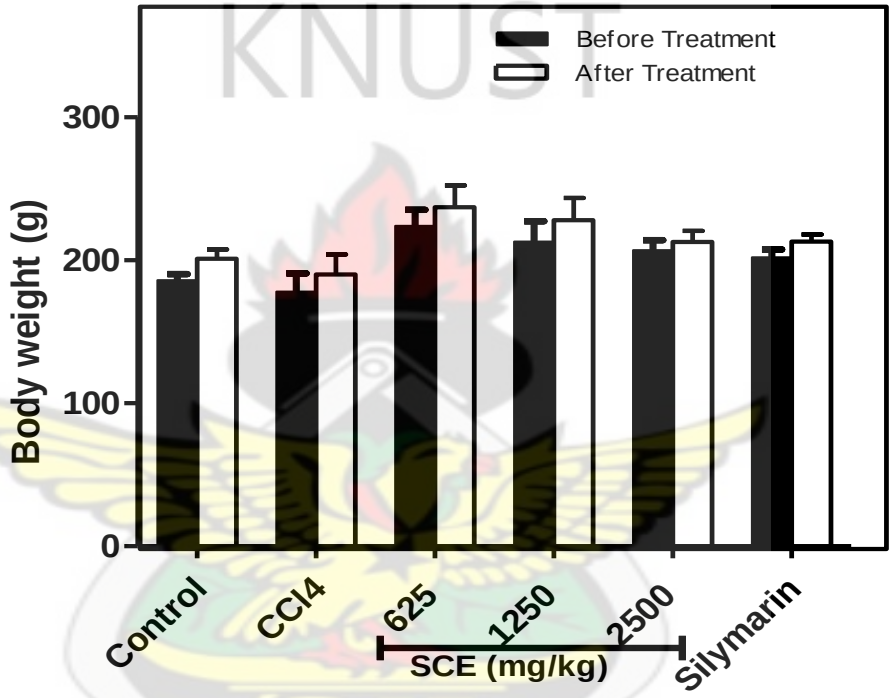


Figure 4.1: The effect of SCE pre-treatment against CCl_4 intoxication, on body weight of rats. Values are expressed as means± SEM (n=5).

Effect of SCE pretreatment against CCl_4 intoxication on Relative liver weight of rats

The relative liver weights of the rats (fig. 4.2) treated with carbon tetrachloride increased significantly, as expected, compared to the control ($p<0.01$). This increase was however reversed or prevented by pretreatment with SCE at the 625 mg/kg dose (close to normal). Liver weights of animals in the 1250 and 2500 mg/kg of SCE as well as silymarin-treated groups were not significantly reduced after a marked increase caused by CCl_4 compared to the control.

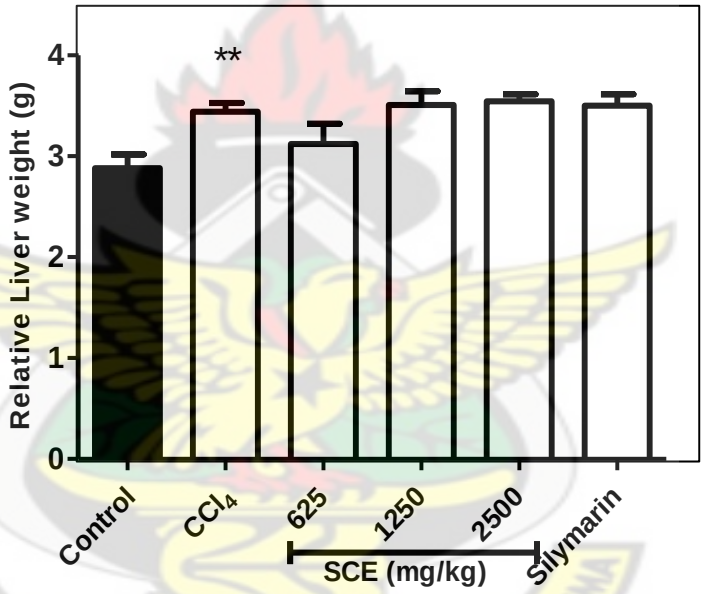


Figure 4.2: Effect of, *SCE* pre-treatment against CCl_4 intoxication, on relative liver weight of rats. Values are expressed as means± SEM (n=5). Animal were treated with extract or silymarin for three days before they were treated with CCl_4 *p.o.*

Effect of SCE pretreatment against CCl_4 intoxication on serum marker Enzymes

The effect of SCE and silymarin pre-treatment on serum enzymes of CCl_4 intoxicated rats is shown in figures 4.3-4.5 and table 4.1. The administration of a single dose of CCl_4 significantly increased the levels of AST ($p<0.01$) and ALT ($p<0.001$) (Fig 4.3 & 4.4), compared to the control by ANOVA followed by Newman Keuls test. Treatment with different doses of *Spathodea* or silymarin significantly reduced the elevated parameters; AST, ALT, and GGT. This decrease was not dose dependent; the 625mg/kg dose had a higher reducing power which resulted in values closer to the normal control. There was a general increase in GGT levels which was not reversed in the other treatment groups. The levels in the 625mg/kg dose group however decreased significantly when compared to that of the toxin control, followed by silymarin at 50 mg/kg. Protein and albumin levels were not affected by the treatment with CCl_4. The bilirubin levels were also not affected by the treatment with the hepatotoxin, Table 4.1. ALP levels however were significantly increased by the hepatotoxin treatment. This increase was not affected by treatment with extract.

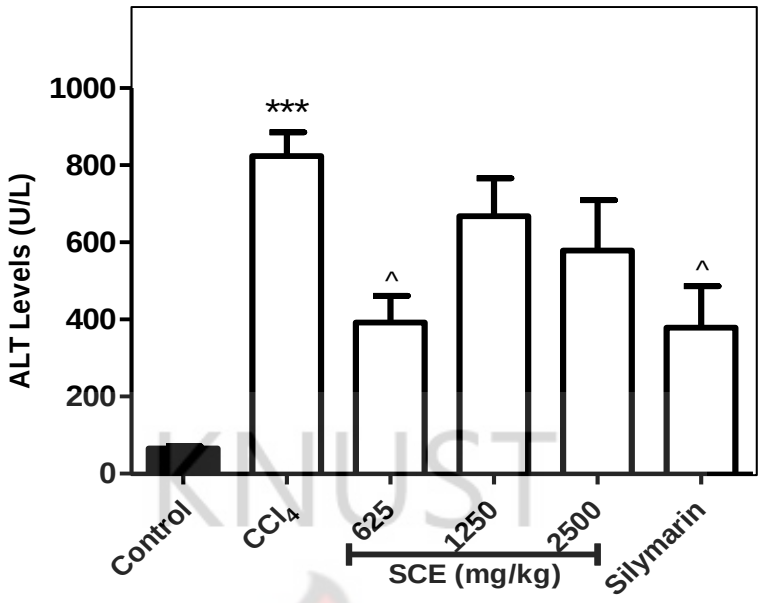


Figure 4.3: ALT levels of rats pre-treated with SCE or silymarin before CCl_4 intoxication. *** indicates significance ($p<0.001$) compared to the control and ^ indicates ($p<0.05$) compared to the CCl_4 (ANOVA). Values are presented as the mean ± SEM (n=5).

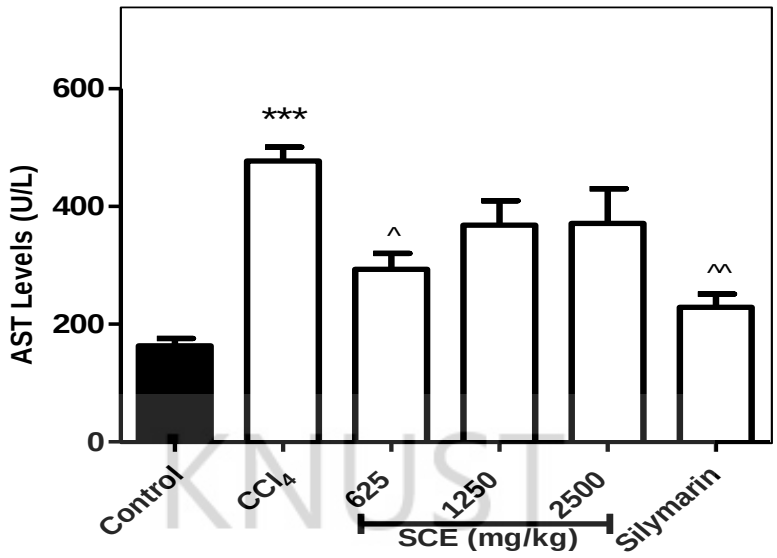


Figure 4.4: AST levels of rats pre-treated with SCE or Silymarin before CCl_4 intoxication. Values are presented as the mean± SEM (n=5). ***indicates significance ($p<0.001$) compared to control and ^ indicates significance compared to the CCl_4 group, ^ ($p<0.05$), ^^ ($p<0.01$) (ANOVA).

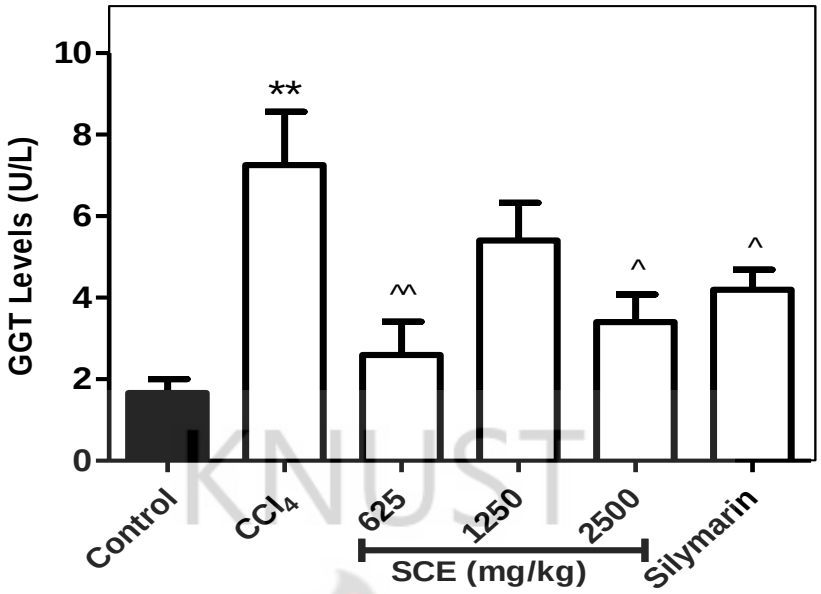


Figure 4.5: GGT levels of rats pre-treated with SCE or Silymarin before CCl_4 intoxication. Values are presented as the mean± SEM (n=5). ** indicates significance ($p<0.01$) compared to the control, ^ indicates significance compared to the CCl_4 toxin group; ^ ($p<0.05$), ^^ ($p<0.01$) (ANOVA).

Table 4.1: Effect of SCE on Protein, Bilirubin, Alkaline Phosphatase and Platelet count of rats treated with extracts and silymarin before CCl_4 intoxication.

	Vehicle control	Toxin control	625mg/kg	1250mg/kg	2500mg/kg	Silymarin
Total protein	8.300 ± 0.7616	9.500 ± 0.1683	9.280 ± 0.08602	8.940 ± 0.2379	9.620 ± 0.1281	9.200 ± 0.1761
Direct bilirubin	0.0260 ± 0.0260	0.0650 ± 0.03948	0.0740 ± 0.0460	0.0340± 0.02135	0.0560± 0.0560	0.0640 ± 0.04665
Total bilirubin	4.560 ± 0.3982	4.80 ± 0.5788	4.640 ± 0.2619	5.00 ± 0.1871	5.060 ± 0.3108	4.640 ± 0.4925
Albumin	4.760 ± 0.1166	4.673 ± 0.0250	4.400 ± 0.07071	4.440 ± 0.1208	4.680 ± 0.05831	4.480 ± 0.0800
Platelets	412.6 ± 65.47	384.4 ± 48.41	640.6 ± 157.7	410.0 ± 62.79	495.2 ± 82.56	345.2 ± 50.79
ALP	278.4 ± 9.244	463.5 ± 12.69*	403.0 ± 22.03	470.8 ± 125.0	389.8 ± 29.35	454.0 ± 83.77

Values are presented as mean ± SEM (n=5).

Effect of pretreatment with lower doses of SCE on CCl_4-induced Hepatotoxicity

From previous results of the prophylactic treatment, SCE appears to be more protective at lower doses. However, at 100 and 300 mg/kg it did not significantly protect the animals against the impending effect of CCl_4 as evident in the ALT marker.

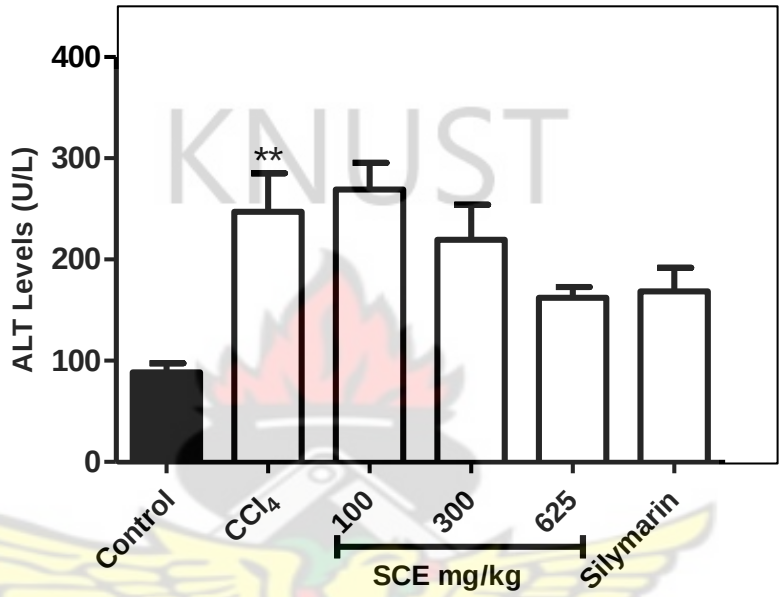


Figure 4.6: ALT levels of rats pre-treated with low doses of SCE (100, 300, 625 mg/kg) before CCl_4 intoxication.

** indicates significance ($p<0.01$) and compared to the control (ANOVA). Values are presented as the mean ± SEM (n=5).

Histopathological Examination of livers pre-treated with SCE/ Silymarin before CCl_4 treatment

Liver sections taken from untreated CCl_4-injured rats had more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous septa than healthy control group (Fig 4.7). Histopathological analysis showed that Silymarin as well as the extract (at 625mg/kg) markedly improved the degree of hepatic fibrosis in CCl_4-injured rats. Treated groups displayed delicate fibrous septa, and lower collagen levels than untreated CCl_4-injured group.

There was mild cellular degeneration, necrosis and inflammation in the 625 dose. The 1250 mg dose also showed mild cellular degeneration, necrosis and inflammation. In the 2500mg dose, the cellular degeneration was more pronounced with mild necrosis and fibrosis. The silymarin group suffered mild inflammation and cellular degeneration with very mild necrosis. In the toxin control, there was moderate cellular degeneration and necrosis, higher than all treatment groups, with inflammation and fibrosis.

The clear vacuoles contained lipid; however, histological fixation caused it to be dissolved and hence only empty or clear spaces are seen which represent steatosis.

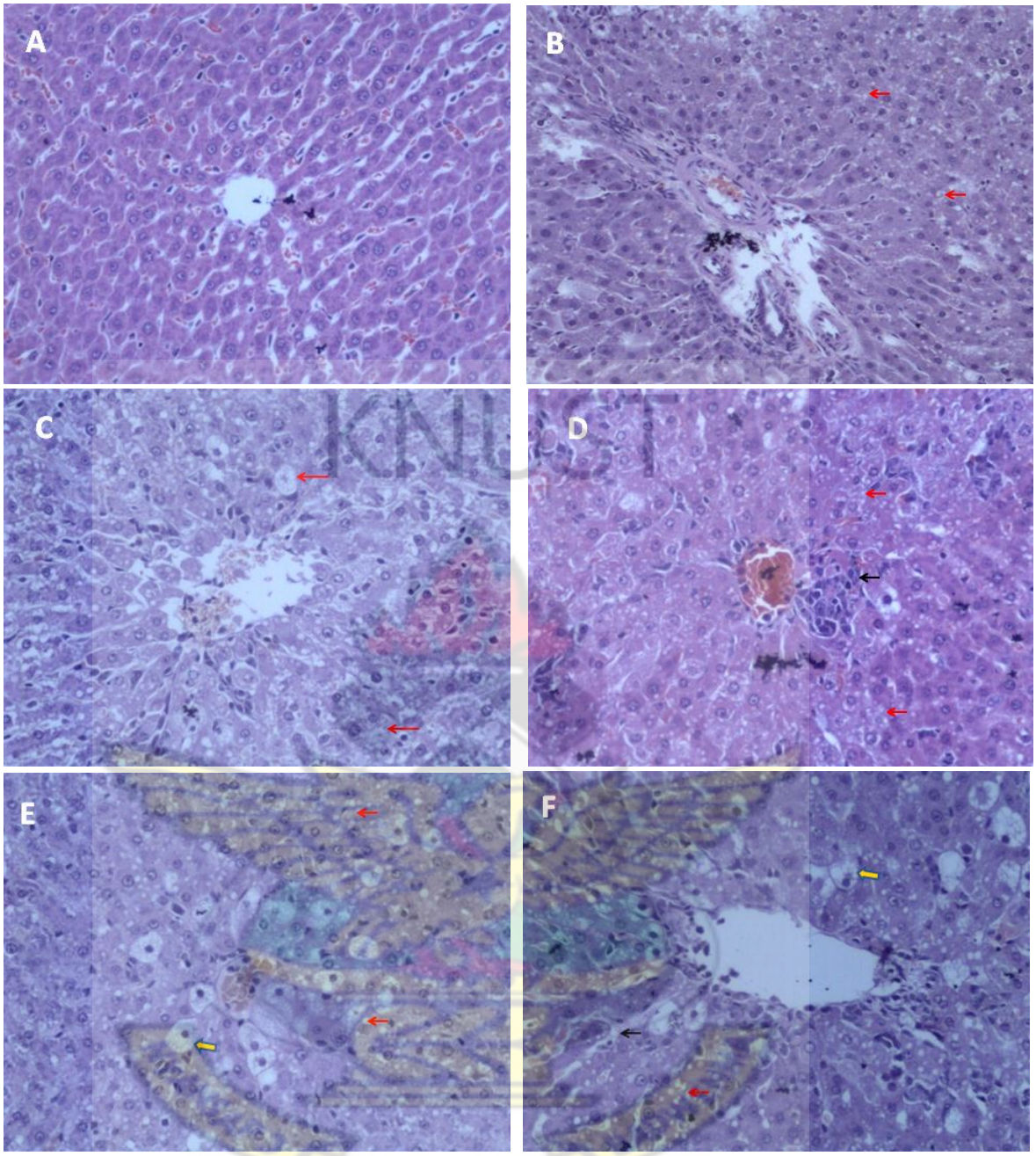


Figure 4.7: Photomicrograph (x 400) showing histopathological profile of the livers of rats in SCE prophylactic treatment. Sections of the liver after treatment with *Spathodea campanulata* stem bark extract / Silymarin before CCl_4 intoxication. (A) Normal Control, liver from rat treated with saline shows normal cellular architecture with distinct hepatic cells, (B) 625mg/kg, (C) 1250mg/kg, (D) 2500mg/kg, (E) Silymarin, (F) CCl_4 Control.(⇦) Indicates macrovesicular steatosis, (←) indicates steatosis (fatty liver), (←) indicates fibrous septa.

4.3.2 Curative Study

Effect of SCE on body weight of CCl_4-intoxicated rats

There were no significant changes in the weights of the animals at all levels of treatment either after five days intoxication or further treatment with the extract or silymarin.

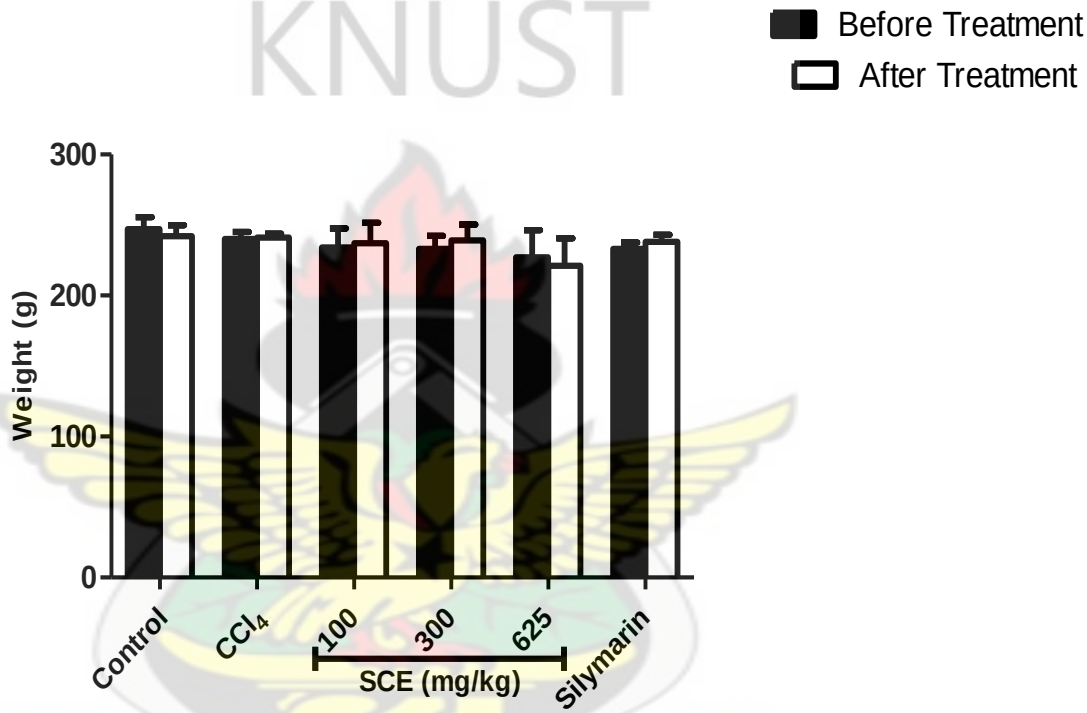


Figure 4.8: Effect of *Spathodea campanulata* extract on body weight of CCl_4-treated rats. Values are presented as the mean± SEM (n=5).

Effect of SCE on the liver weight of CCl_4-treated rats

Treatment of rats with CCl_4 for five consecutive days significantly ($p<0.01$) increased the liver weights of the rats as expected. This increase was however not significantly reversed by treatment with extract (at any dose level) and silymarin.

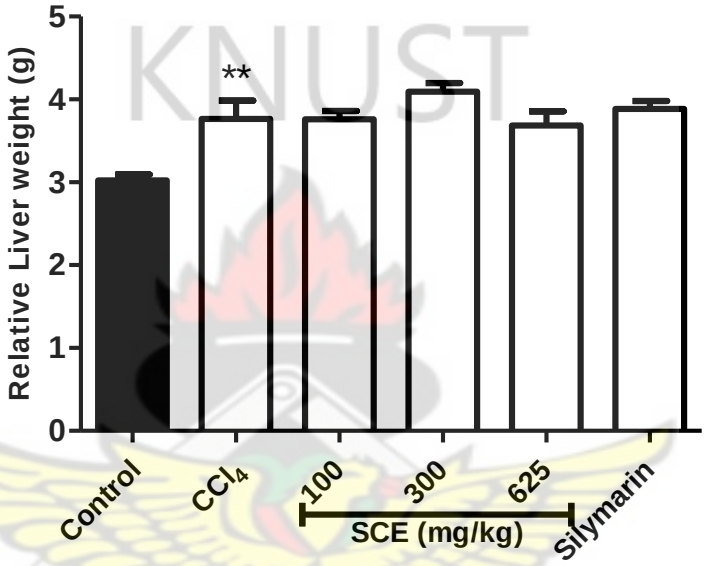


Figure 4.9: Effect of *Spathodea campanulata* stem bark extract on the liver weight of rats pre-treated with CCl_4. ** indicates significance ($p<0.01$) compared to the control. Values are presented as the mean ± SEM (n=5).

Effect of SCE on serum enzymes in CCl4-treated rats

The effect of SCE on CCl_4-induced hepatotoxicity is shown in Figs 4.9 & 4.10 and Table 4.2. CCl_4 treatment increased the AST, ALT and total bilirubin levels in the serum of rats. Administration of *Spathodea* and silymarin significantly reduced the AST, ALT and total Bilirubin induced by five (5) consecutive doses of CCl_4 compared to the normal control (ANOVA, followed by Newman Keuls test). The decrease was more profound in the lower dose treatment of extract. Although BUN levels were significantly increased in the CCl_4 group, treatment with extract and silymarin did not significantly decrease the elevated BUN (Table 4.2). GGT and Total proteins were not significantly affected by treatment with the toxin. Blood urea nitrogen (BUN) was also increased significantly in the serum of the CCl_4 treated group. This increase was however not reversed by treatment with the extract at any dose level or silymarin.

Table 4.2: Effect of SCE and Silymarin on some biochemistry parameters of rats pre-treated with CCl_4.

PARAMETER	Normal Control	Toxin Control	100mg/kg	300mg/kg	625mg/kg	Silymarin
GGT	0.0 ± 0.100	1.960± 0.625	1.940± 1.018	1.940± 0.498	2.920± 1.058	2.540± 1.364
Total Protein	61.80±1.235	66.96± 1.739	60.64± 2.179	61.58± 2.127	77.62± 1.573	72.34± 1.725
Total Bilirubin	1.50 ± 0.100	3.740±0.419***	1.660± 0.093^^^	2.220± 0.2518^^	3.620± 0.447	2.420± 0.153^^
BUN	5.222± 0.2217	10.42±0.425***	7.646± 0.558	11.52± 1.423	9.266± 0.503	8.752± 0.769

Values are expressed as mean ± SEM (n=5), *** indicates significance ($p<0.001$) compared to the control, ^^^ ($p<0.001$) and ^^ ($p<0.01$) compared to the CCl_4 control.

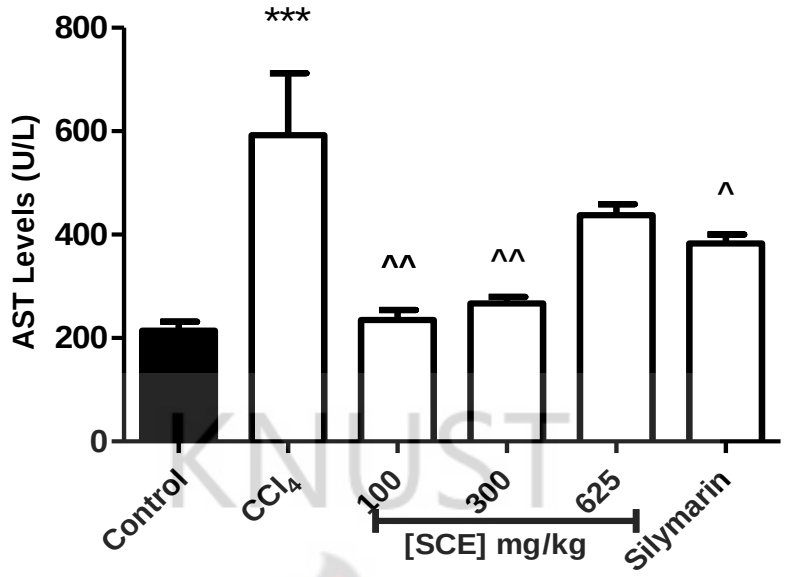


Figure 4.10: Effect of SCE on the AST levels of rats pre-treated with CCl_4. Values are expressed as the mean ± SEM (n=5). *** indicates significance ($p<0.001$) compared to the control; and ^^ indicates significance ($p<0.01$) and ^ indicates ($p<0.05$) compared to the CCl_4 (toxin) control.

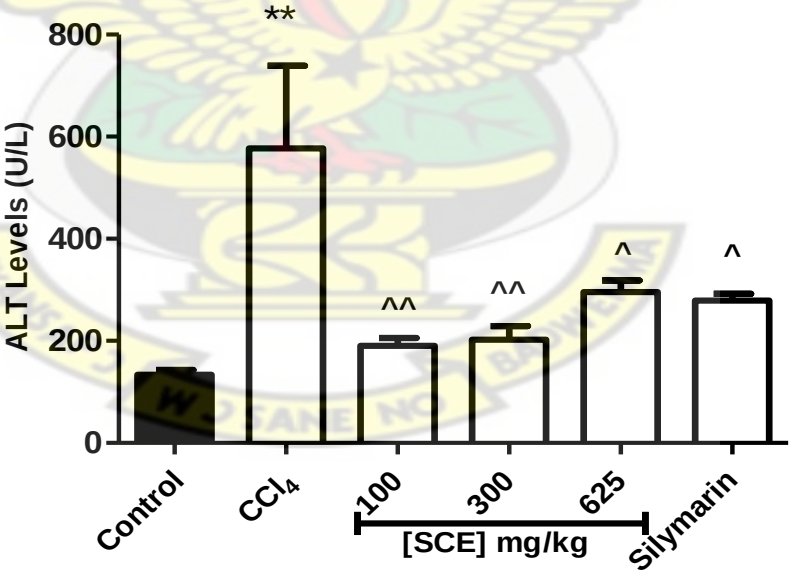


Figure 4.11: Effect of SCE on the ALT levels of rats pre-treated with CCl_4. Values are expressed as the mean ± SEM (n=5). ** indicates significance ($p<0.01$) compared to the normal control; ^^ indicates significance ($p<0.01$) and ^ indicates ($p<0.05$) compared to the toxin control.

Histopathological Analysis of livers of rats treated with SCE following CCl_4 intoxication

Liver sections from CCl_4-injured rats had more inflammatory infiltration, steatosis and coagulative necrosis than healthy control group (figure 4.12). The extract at 100 and 300 mg/kg respectively did not show significant improvement over the effect of CCl_4 on the liver. Though the extent of steatosis was a little lower than in the CCl_4 group, liver architecture (coagulative necrosis) was not improved. There was very mild cellular degeneration, necrosis and inflammation in the 625 mg/kg dose however. Histological analysis showed that the extract (at 625mg/kg) markedly improved the degree of steatosis in CCl_4-injured rats. This was very close to the control and the standard drug (silymarin) treated group which showed very little fatty infiltration.

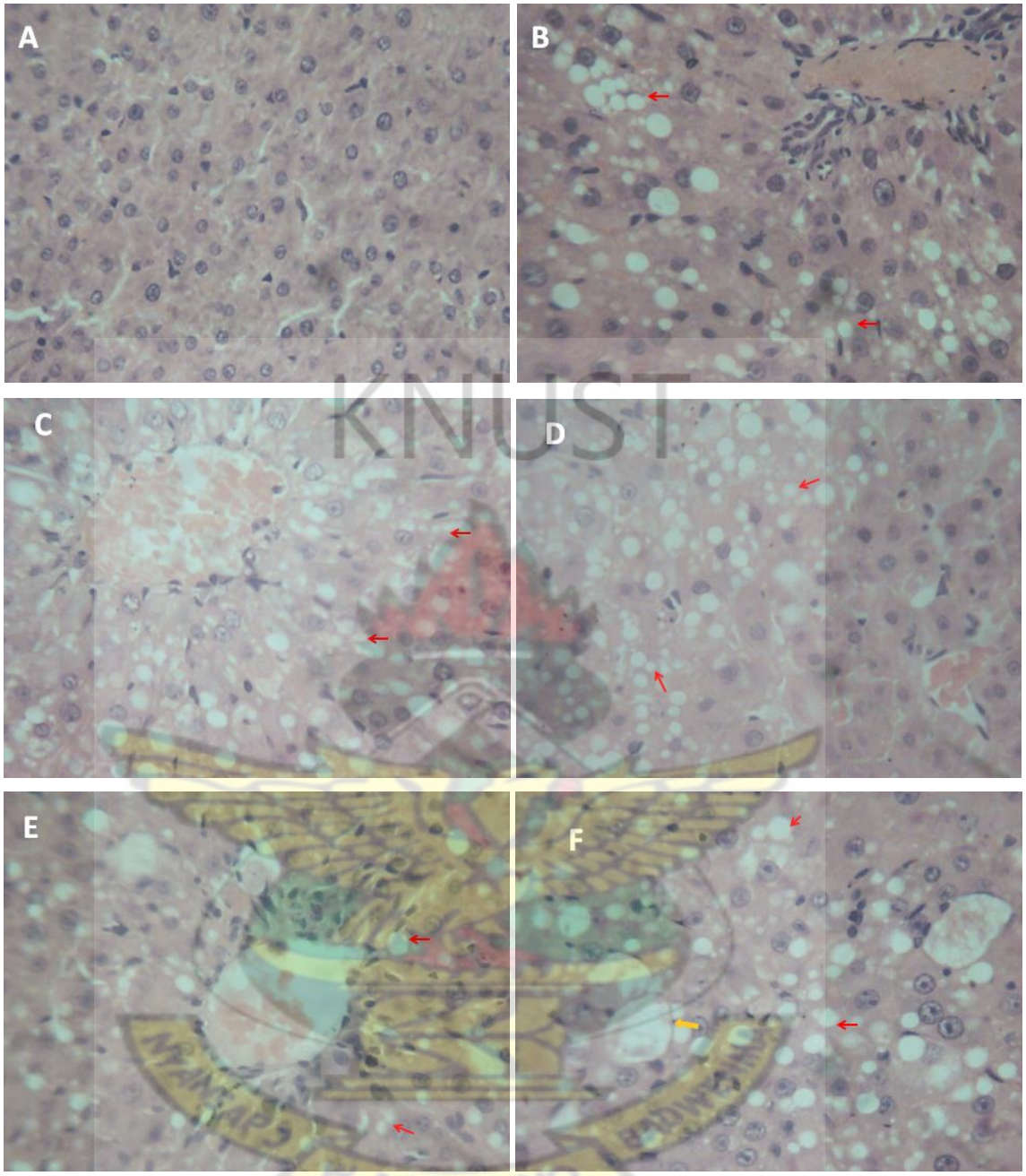


Figure 4.12: Photomicrograph (x 40) showing histopathological profile of the livers of rats treated with CCl_4 followed by SCE treatment.

(A) Normal Control, liver from rat treated with distilled water shows normal cellular architecture with distinct hepatic cells, (B) 100mg/kg, (C) 300mg/kg, (D) 625mg/kg, (E) Silymarin, (F) CCl_4 Control. (⇨) Indicates macrovesicular steatosis, (→) indicates steatosis (fatty liver).

4.4 DISCUSSION

The liver is a multipurpose organ in the body involved in the regulation of internal chemical environment. Therefore damage to the liver inflicted by a hepatotoxic agent is of critical consequence.

The changes associated with CCl_4-induced liver damage are comparable to that of acute viral hepatitis (Rubinstein, 1962) therefore $CC1_4$-mediated hepatotoxicity was employed as the experimental model for liver injury. Having been extensively studied, CCl_4 is now established to be accumulated in hepatic parenchymal cells and metabolically activated by cytochrome P-450 dependent monooxygenases to form a trichloromethyl free radical ($CC1_3$). This radical alkylates cellular proteins (including cytochrome P-450) and other macromolecules (Bishayee *et al.*, 1995) with a concurrent attack on polyunsaturated fatty acids in the presence of oxygen to produce lipid peroxides (Bishayee *et al.*, 1995; Recknagel, 1983) leading to liver damage. Hepatotoxic compounds such as CCl_4 are known to cause marked elevation in serum transaminases.

The administration of CCl_4 (20%, 1ml/kg) to rats, either in the pre-treatment or curative studies, did not cause the death of any of the animals neither did it affect any physical properties (body weight and general behavior). There was a significant increase however in the weight of the livers after treatment with CCl_4. Generally, a CCl_4 intoxicated liver is expected to enlarge and increase in weight (Uemitsu *et al.*, 1986). This is due to the infiltration of fatty acids and glycerols into the hepatocytes upon damage to cell membranes. The increased liver weight was however not significantly affected by treatment with the extract.

In agreement with results obtained in similar investigations (Bishayee *et al.*, 1995), the treatment with CCl_4 in the present study elicited a significant increase in the levels of serum transaminases (ALT, AST and GGT). This was evident in both prophylactic and curative studies. Treatment with the extract however significantly decreased the serum transaminases levels which were elevated by CCl_4 treatment.

Administration of the extract to rats before intoxication was aimed at conditioning the hepatocytes so as to accelerate regeneration of parenchymal cells, thus protecting against

membrane fragility and decreasing the leakage of marker enzymes into the circulation. A significant effect was however achieved only by the 625 mg/kg dose; not higher, not lower.

It appears that the 100 and 300 mg/kg doses of the extract were too low to condition the hepatocytes or protect them from an impending danger (prophylactically) and the 1250 and 2500 went through possible interactions or activation resulting in a lack of hepatoprotective activity. As observed in the toxicity studies, higher doses of the extract appeared to be toxic while lower doses appeared to be safe.

The liver has the capacity to regenerate when left alone after acute toxicity (Timbrel, 2009); consequently, a single dose before curative treatment was overcome by regeneration (data not shown) before treatment was ended. In view of this, the rats were given five consecutive doses of CCl_4 to ensure that toxicity to the liver is sustained before treatment with the extract was started.

The repeated doses of CCl_4 caused a significant increase in the AST, ALT and total bilirubin levels. The lower doses of extract (100, 300, 625 mg/kg) significantly reduced the AST and ALT. This supports the efficacy of the extract as suggested by the prophylactic study.

Although both AST and ALT are commonly thought of as liver enzymes because of their high concentrations in hepatocytes, only ALT is markedly specific for liver function. ALT is specifically produced in the hepatocytes, making it relatively more specific to hepatic function compared to AST, which is present in a variety of tissues such as liver, muscle and red blood cells. Consequently muscle injury/trauma (intra muscular injections, severe restraint during handling) and haemolysis can lead to significant AST elevations (Ramaiah, 2007). Again, AST has a shorter half-life of about 12 hours whiles ALT has a half-life of about 60 hours (Meyer and Harvey, 2004). Nevertheless, serum levels of AST and ALT rise and fall in parallel (Rose *et al.*, 2007).

The potent dose of extract in the prophylactic studies, 625mg/kg, significantly decreased the elevated ALT, AST and GGT, whereas higher doses appeared to compound the elevation.

Gamma glutamyl transferase (GGT) levels were raised after the administration of CCl_4 in the prophylactic treatment. As earlier mentioned, GGT and ALP are employed to detect impaired bile flow (cholestasis); Following cholestasis, impaired bile flow and certain drugs

result in the increased synthesis (induction) of these enzymes which occurs within hours and they are released into circulation by unknown mechanisms (Ramaiah, 2007). Treatment with SCE (625mg/kg) markedly reduced the level of GGT better than the standard drug, silymarin.

Total serum bilirubin can also be employed to detect cholestasis in addition to the GGT and ALP and also as a measure of hepatocellular damage (Ramaiah, 2007). In the curative study, total bilirubin levels in the serum of the rats increased significantly in the CCl_4 treated group. This increase was reduced almost to normalcy when treated with the low doses of extract and better reduced than when treated with the reference drug (silymarin at 50 mg/kg).

Contrary to expectations, the lower doses which significantly restored serum AST and ALT did very little to restore the architecture of the liver to normal. Steatosis in the liver caused by CCl_4 was not healed by treatment with the extract. Though the degree of damage is slightly lower in the 625 mg/kg dose compared to the CCl_4 control, it can be said that the extract had little effect in replenishing the injury that the tissue suffered as compared to the effect in the prophylactic treatment. This can be attributed to the repeated dosing with CCl_4 before treatment with extract. Again, the treatment with the extract for a few days was not sufficient to remedy the sub-chronic effect of CCl_4. In this case, treatment with the extract for a longer period might have a greater effect as anticipated.

Among the many drugs for liver injury, silymarin is the most clinically popular for patients and is known to have hepatotherapeutic and anti-fibrotic properties (Kim *et al.*, 2007). Silymarin has also been proven effective in several research fields, such as protecting against genomic injury, increasing hepatocyte protein synthesis, decreasing the activity of tumor promoters and stabilizing mast cells. According to many authors however, it has a low bioavailability (Comoglio *et al.*, 1995; Giacomelli *et al.*, 2002).

The results of the study suggest that SCE is both proactive and responsive in protecting against CCl_4 induced hepatotoxicity. It is however more bioactive when given to treat an existing damage to the liver. This means that, at doses lower than 625 mg/kg, SCE is effective in arresting or dealing with the damage rather than being stored for a later use.

Between one and three hours after dosing with carbon tetrachloride, triglycerides accumulate in hepatocytes, detectable as fat droplets, and there is continued loss of enzyme activity in the endoplasmic reticulum (Timbrel, 2009). Deregulation of the system can perpetuate inflammatory processes that lead to tissue damage and organ dysfunction (McPhee *et al.*, 1997). As expected, liver sections from untreated CCl_4 –injured rats had higher degree of inflammation and steatosis than the treated groups. Rats that received 625mg/kg, the least dose in the prophylaxis study, showed mild cellular degeneration, necrosis and inflammation. About the same was expressed in the 1250mg/kg treated group. In the highest dose however, cellular degeneration was more pronounced, comparable to the untreated CCl_4 group, with mild necrosis and fibrosis. This confirms the possible negative drug interaction of the extract at higher doses.

The silymarin treated animals showed very mild inflammation and cellular degeneration and fibrosis.

Studies under light microscope confirms the curative efficacy of *Spathodea* against CCl_4 induced liver damage as evident by the reversal of centrilobular necrosis, macro-vesicular fat accumulation (steatosis) and inflammation in hepatic parenchymal all of which were caused by CCl_4 administration.

4.5 CONCLUSION

This study has demonstrated that the aqueous stem bark extract of *Spathodea campanulata* is able to protect or reverse carbon tetrachloride induced hepatotoxicity in rats.

CHAPTER 5

EFFECT OF *SPATHODEA CAMPANULATA* AQUEOUS STEM BARK EXTRACT ON OTHER HEPATOTOXICANTS

5.1 INTRODUCTION

The previous chapter demonstrated the hepatoprotective effect of *Spathodea campanulata* stem bark extract against carbon tetrachloride-induced hepatotoxicity. Though this model is extensively studied and mimics many patterns of hepatotoxicity, other hepatotoxicants need to be looked at to ascertain the effect of the extract as a hepatoprotective agent.

Undeniably, many hepatotoxic agents are encountered in daily life. They range from drugs which are abused through contaminations in food and drink to industrial chemicals. Since SCE has shown potency against an industrial chemical, carbon tetrachloride, an investigation into other household hepatotoxic agents has been necessitated. A famous hepatotoxic drug is paracetamol. At the prescribed dose, paracetamol serves as an analgesic and antipyretic agent (Rumack, 2004). An abuse however can lead to dangerous consequences to the liver and ultimately put the individual's life at risk (Moyer *et al.*, 2010).

It is a drug whose toxicity is based on bioactivation by cytochrome P450 to form the reactive metabolite N-acetyl P- benzoquinone imine (NAPQI) (Pelkonen and Raunio, 1997), covalent binding and on the generation of oxygen-free radicals and depletion of GSH (Ali *et al.*, 2001). This chain of events will result in oxidative stress which plays a central role in the hepatic damage caused by paracetamol. Antioxidants have been tested as alternative treatment against paracetamol toxicity (Avila *et al.*, 2011). One of such antidote (which has been accepted for treatment) is n-acetyl cysteine (NAC) (James *et al.*, 2003). N- acetyl cysteine prevents N-acetyl-p-benzo-quinone imine (NAPQI) from binding to hepatic macromolecules in the early phase of paracetamol intoxication (<8 h) (Kandis *et al.*, 2010). It may exert its effect by acting as a glutathione precursor or alternatively as a sulfate precursor (Singh *et al.*, 2006). Upon deacetylation, NAC becomes L-cysteine, entering cells where it may serve as a precursor for GSH synthesis. Indeed, NAC has been shown to prevent GSH depletion and/or to increase hepatic GSH levels.

However, species difference has been postulated to affect degree of susceptibility to paracetamol-induced hepatotoxicity. Mice and humans have been said to be more susceptible to paracetamol toxicity as opposed to rats (Kedderis, 1996). In a study by Davis and his team in 1974, it was found out that whereas the rats were not very sensitive

to the hepatotoxicity, both mice and hamsters proved to be very sensitive (Davis *et al.*, 1974). Therefore, studies with relevance to humans could be done in mice or hamsters.

A new common cause of liver disease especially in developing countries is aflatoxin from *Aspergillus*. Epidemiological data suggests that aflatoxin B1 (AFB1) contributes to the high incidence of primary liver cancer in the populations which are exposed to high levels, especially in Africa (Asare *et al.*, 2007) and Asia where food storage conditions are poor (Knight *et al.*, 1999).

Aflatoxin B1 (AFB1) is one of the most potent known liver carcinogens (Smela *et al.*, 2002) in both experimental animals and humans (Kensler *et al.*, 1999). Children, however, are predominantly affected by aflatoxin exposure, which leads to stunted growth and hindered development (Abbas, 2005).

Southern China and sub-Saharan Africa have the highest dietary AFB1 exposure, making it and hepatitis B virus (HBV) (also common in these regions) the major causes of cancer mortality in these geographic areas (Staib *et al.*, 2003). Epidemiological evidence indicates that there is a synergistic interaction between AFB1 exposure and HBV infection on the induction of hepatocellular carcinoma (HCC) (Smela *et al.*, 2002). Where AFB1 dietary exposure (by consuming mycotoxin- contaminated foods like corn, rice, and peanuts) and chronic viral hepatitis were found, a point mutation at the third position of codon 249 resulting in a G:C to T:A transversion was common in hepatocellular carcinoma (HCC) (Bressac *et al.*, 1991), which is the fifth most commonly occurring cancer worldwide (Ranchal *et al.*, 2009)

A large dose of AFB1 causes periportal necrosis and bile duct epithelial cell injury (Luyendyk *et al.*, 2002). The harmful effects of AFB1 have been attributed to the metabolism of this mycotoxin to reactive metabolites that can bind to cellular macromolecules. Initially, an epoxidation reaction, catalyzed by certain cytochrome P450 (CYP 450) enzymes, especially hepatic CYP1A2 and CYP3A4, converts AFB1 to the DNA-reactive AFB1 exo-8,9-oxide (Adams *et al.*, 1996).

Exposure of humans to inflammogens like lipopolysaccharide which potentiate AFB1 is common (Luyendyk *et al.*, 2002). Concurrent inflammation has been postulated to be a potentially important risk factor for hepatotoxic effects of AFB1 and other xenobiotic agents

(Barton *et al.*, 2001; Thrasher and Crawley, 2009). Nevertheless, some therapeutic drugs and antioxidants, e.g., oltipraz, related dithiolethiones, and various triterpenoids, protect from both acute toxicity and carcinogenesis (Maxuitenko *et al.*, 1998). These agents induce several hepatic glutathione S-transferases (GST) as well as aldo-keto reductases (AKR) which are thought to contribute to protection (Roebuck *et al.*, 2009). Glutathione S-transferases (GSTs) can conjugate the reactive epoxide with reduced glutathione, thus preventing the formation of DNA adducts which can lead to mutations (Knight *et al.*, 1999). mediated by the induced glutathione S-transferase activity (Liu *et al.*, 1995).

Rat is the most sensitive species for aflatoxin tumorigenesis and liver is the most sensitive site (Choy, 1993).

5.2 METHODOLOGY

Animals

The animals used in the study, male ICR mice (25-30g) and Sprague-Dawley rats (180-220g), were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon and maintained in the Animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The animals were housed in groups of 6 in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, KNUST, Kumasi, Ghana.

Chemicals

N-acetyl cysteine was purchased from Sigma (St. Louis, MO, U.S.A.), Paracetamol powder was purchased from LETAP Pharmaceutical company, Accra, Ghana, Silymarin, named 'Legalon 70' (from Bukwang Param, Seoul, Korea), was purchased from Bandy chemist in Kumasi, Ghana.

5.2.1 Hepatoprotective activity against Paracetamol-induced hepatotoxicity

The effect of prophylactic treatment of SCE on the paracetamol induced hepatotoxicity was investigated according to the method described by Ali *et al* (2001).

Forty eight mice were put in six groups of eight mice each for the experiment. Groups one (1) to four (4) received three doses of extract (100, 300, 625 mg/kg) and NAC (300 mg/kg) respectively for five consecutive days. Groups five (5) and six (6) served as the normal and toxin controls respectively receiving distilled water. Two hours after the last treatment of all animals (with the exception of the normal control which received normal saline) received 600 mg/kg of paracetamol suspension in distilled water orally. Three hours after administration of paracetamol, animals were anesthetized. Blood samples were collected from a common carotid into dry centrifuge tubes. After blood collection, animals were sacrificed and livers were removed for tissue studies. The coagulated bloods in the plain tubes were tested for liver markers. Liver slices fixed for 12 hours in Bouin's solution were processed for paraffin embedding following standard micro techniques (Galigher and Kozloff, 1971). 5µm sections of liver stained with alum Haematoxylin and Eosin were observed microscopically for histopathological changes.

5.2.2 Hepatoprotective activity against AFB1

The prophylactic hepatoprotective activity of SCE against AFB1 was done as described by Liu *et al* (1995).

For the preliminary tests, three groups of three male rats were treated as tests 1, 2, 3 and nine rats of the same sex and age were treated as control. Test 1, 2 and 3 animals each received

1mg/kg of aflatoxin B1 orally. Animals were observed for a period of 24 hours. At the end of 24 hours, animals in all four groups were weighed and test 1 animals as well as three animals from the control were sacrificed. Their blood and livers were harvested. Tests 2 and 3 were sacrificed 72 hours and 6 days respectively each along with three animals from the control. Serum enzyme levels were assessed in the blood of the animals sacrificed and their livers weighed.

Hepatoprotective activity of SCE against AFB1 was assessed later by treating four groups of five animals each as follows: Group A served as control and received normal saline. Group B, C and D received normal saline, SCE (625mg/kg) and silymarin (50mg/kg) for four consecutive days. Two hours after the last treatment, animals in groups B, C and D received 1.5 mg/kg AFB1 orally. All animals were sacrificed after twenty four hours. The weight of animals before and after treatment was noted as well as the serum biochemical parameters relevant to the liver were also measured.

5.2.3 Analysis of Data

The results of hepatoprotective activities against paracetamol and AFB1 are expressed as mean ± SEM. Results were statistically analysed using one-way ANOVA, followed by the Newman-Keuls Multiple Comparison Test. $P<0.05$ were considered to be significant.

5.3 RESULTS

5.3.1 Effect of SCE on Paracetamol-induced hepatotoxicity

Effect on some serum parameters

The effect of SCE on paracetamol-induced hepatotoxicity is seen in Figures 5.1-5.3. The extract showed protective effect on the liver against paracetamol induced hepatotoxicity in mice. This is shown in the levels of serum AST, ALT and Total protein (Figures 5.1 – 5.3). 600 mg/kg paracetamol increased the levels of the serum AST ($p< 0.001$) and ALT ($p<0.001$). These were reduced profoundly by pretreatment with extract and n-acetyl cysteine ($p<0.001$ or $p<0.01$). The same treatment with paracetamol significantly decreased the serum levels of total proteins ($p<0.01$) which were restored by treatment with extract and n-acetyl cysteine ($p<0.05$).

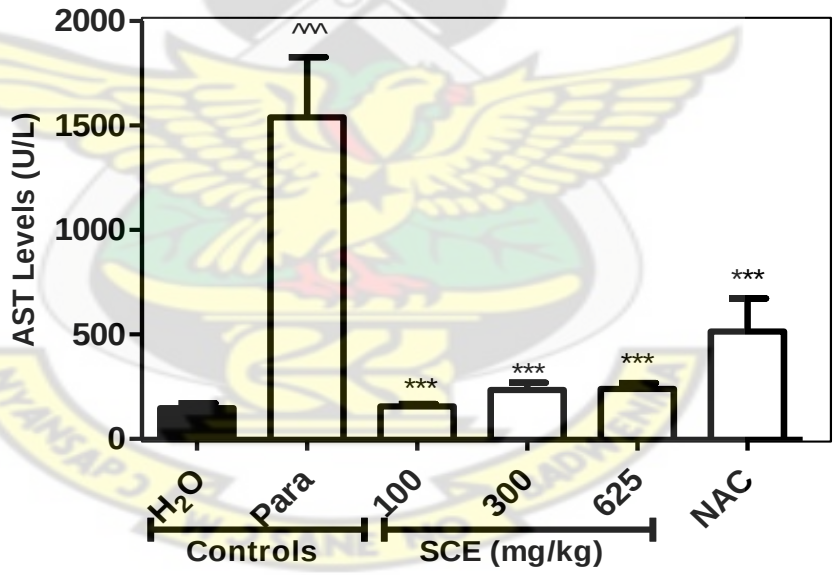


Figure 5.1: Serum AST levels of mice pretreated with SCE before paracetamol intoxication. Values are expressed as mean ± SEM (n=8). ^^^ indicates significance ($p<0.001$) compared to the normal control; *** indicates significance ($p<0.001$) compared to the paracetamol control.

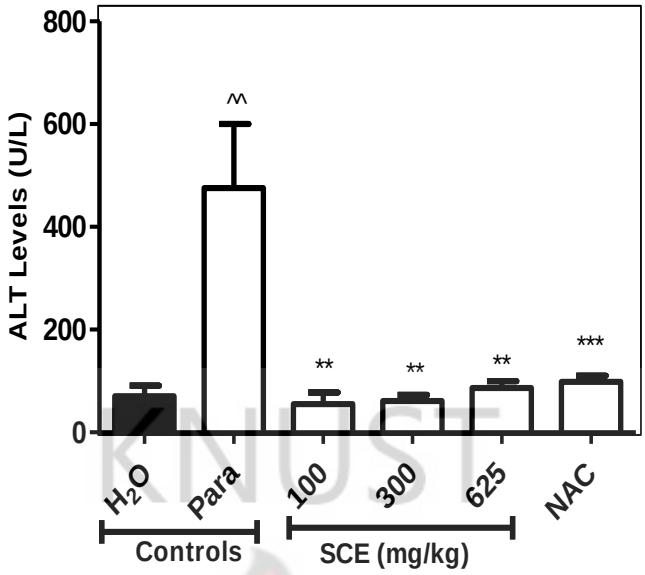


Figure 5.2: Serum ALT levels of mice pretreated with SCE before paracetamol intoxication. Values are expressed as mean ± SEM (n=8). ^^ indicates (p<0.01) compared to the normal control; ** indicates significance (p<0.01) compared to the paracetamol control.

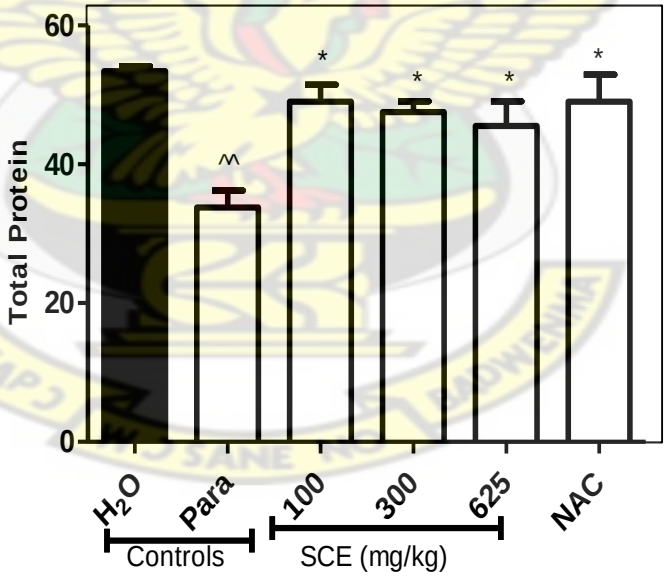


Figure 5.3: Total protein in serum of mice pretreated with SCE before paracetamol intoxication. Values are expressed as mean ± SEM (n=8). ^^ indicates significance (p<0.01) compared to the normal control; * indicates significance (p<0.05) compared to the paracetamol control.

Effect of SCE on the histopathology of mice treated with Paracetamol

The histopathology of mice treated with extracts or silymarin before paracetamol intoxication is shown in figure 5.4.

Liver sections taken from paracetamol treated mice had more inflammatory infiltration, hepatocyte coagulation and fibrous septa compared to controls. Qualitative and quantitative histology analysis showed Silymarin as well as the extract (at 625mg/kg) markedly improved the degree of hepatic fibrosis in paracetamol treated mice. Treated groups (Para+ extract/silymarin) displayed delicate fibrous septa, and lower collagen levels than the untreated (Para) group.

The 625 mg/kg dose suffered cellular degeneration, necrosis and inflammation to a lesser extent than the silymarin group which suffered more inflammation and fatty liver in zone 3 of the hepatocytes.

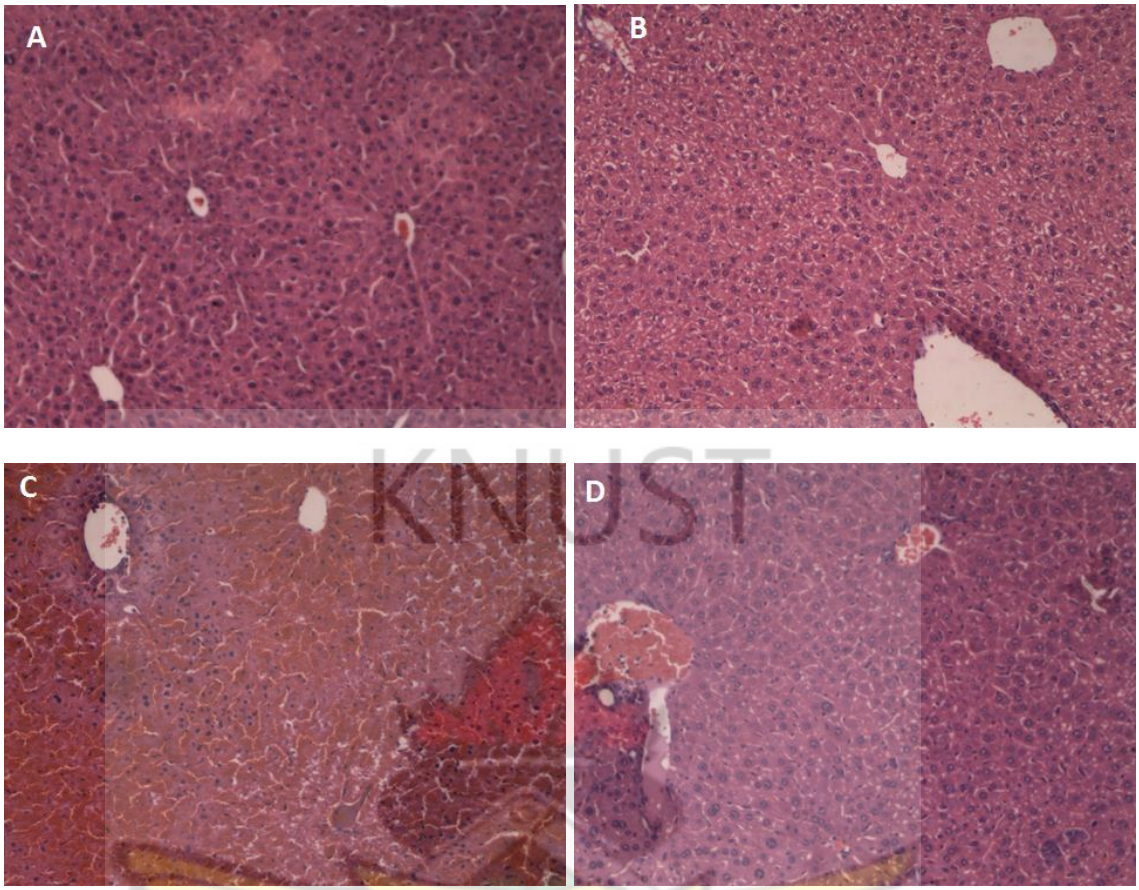


Figure 5.4: Histopathological slides of livers of mice treated with paracetamol after *Spathodea* or silymarin treatment (40×). (A) Normal control, (B) Paracetamol control, (C) Silymarin and (D) SCE at 625mg/kg.

5.3.2 Effect of SCE on AFB1 induced hepatotoxicity

Preliminary tests

Treatment of animals with 1 mg/kg aflatoxin B1 caused a decrease in their weight which was restored as the animals recovered. It was also realized that the effect of the AFB1 on the animals diminished with time (data not shown). The effect of the AFB1 was maximal after 24 hours and started to diminish after 24 hours. Serum AST and ALT levels were increased maximally at 24 hours and started to diminish afterwards.

Effect on serum biochemistry parameters

An oral administration of AFB1 (1.5 mg/kg) caused a significant increase in serum AST and ALT ($p<0.01$, $p<0.001$ respectively) (Figure 5.5 & 5.6). Total bilirubin levels were not significantly affected though there was a slight increase in AFB1 treated animals. There were also no significant effect on the total protein and albumin levels. Treatment with the extract also reduced the elevated transaminases significantly ($p<0.05$ for AST and $p<0.001$ for ALT).

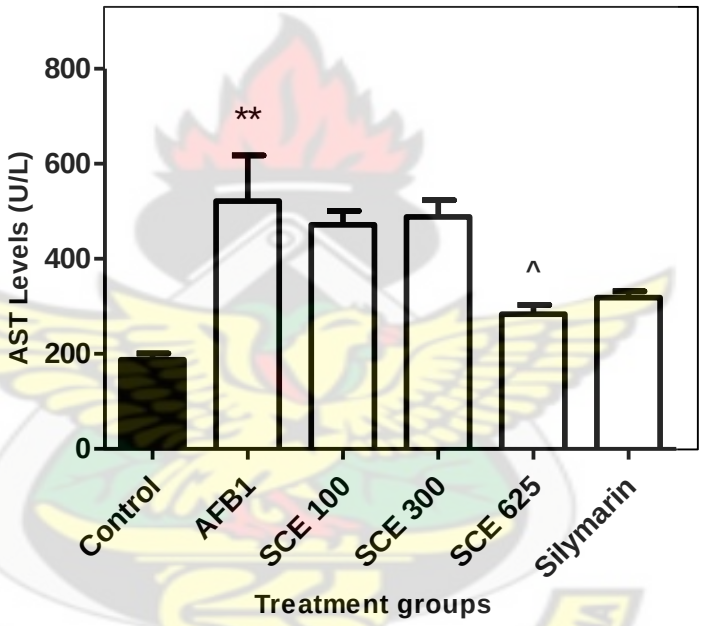


Figure 5.5: Effect of SCE pretreatment on the AST levels of rats treated with AFB1. Values are expressed as the mean ± SEM (n=5). ** indicates significance ($p<0.01$) compared to the normal control; ^ indicates significance ($p<0.05$) compared to the AFB1 control.

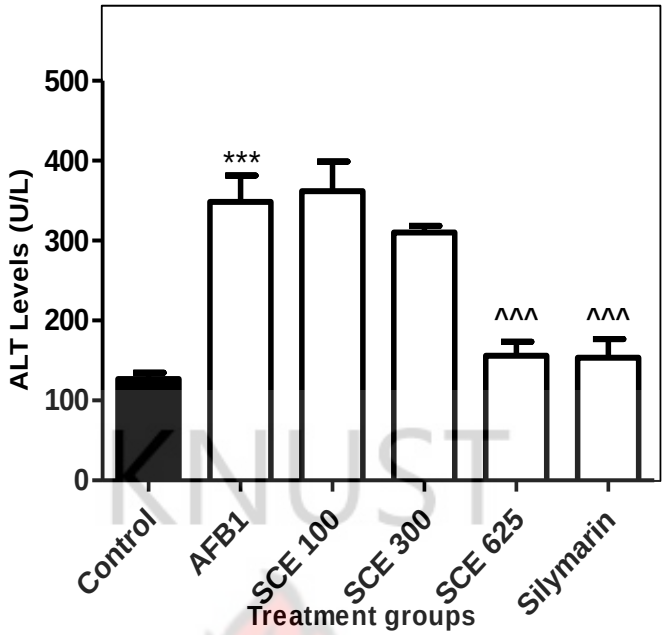


Figure 5.6: Effect of SCE pretreatment on the ALT levels of rats treated with AFB1. Values are expressed as the mean ± SEM (n=5). *** indicates significance ($p<0.001$) compared to the normal control; ^^^ indicates significance ($p<0.001$) compared to the AFB1 control.

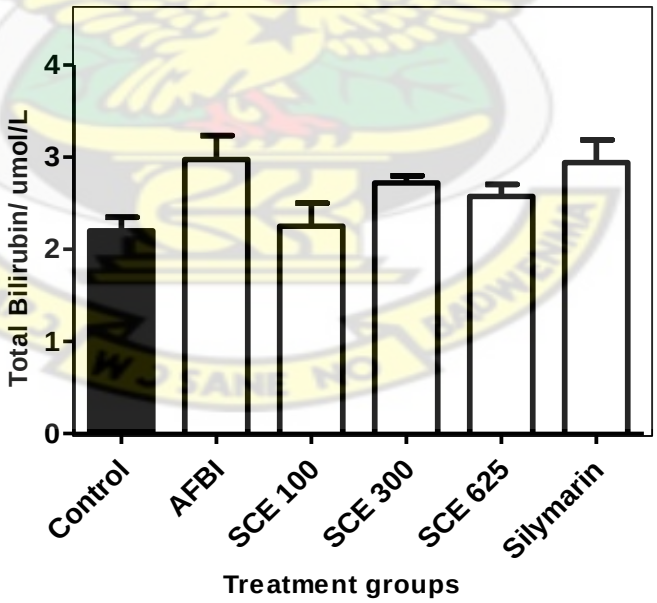


Figure 5.7: Effect of SCE pretreatment on the total bilirubin levels of rats treated with AFB1. Values are expressed as the mean ± SEM (n=5).

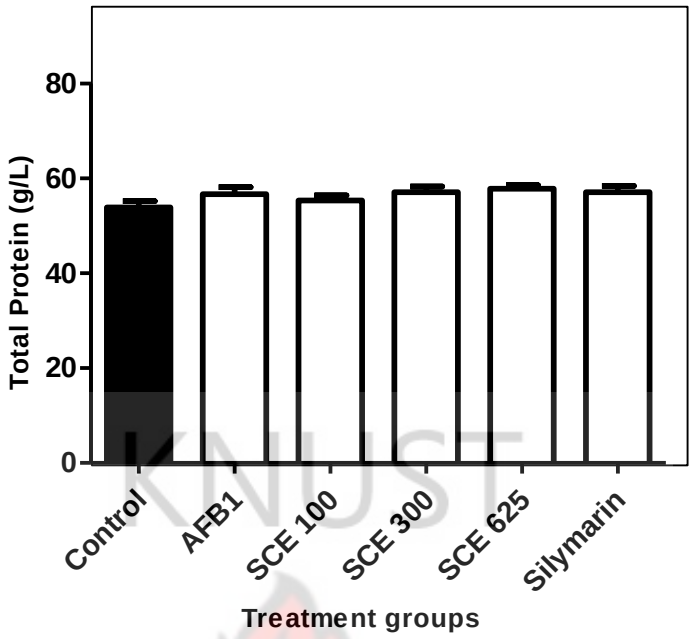


Figure 5.8: Effect of SCE pretreatment on the total protein levels of rats treated with AFB1. Values are expressed as the mean ± SEM (n=5).

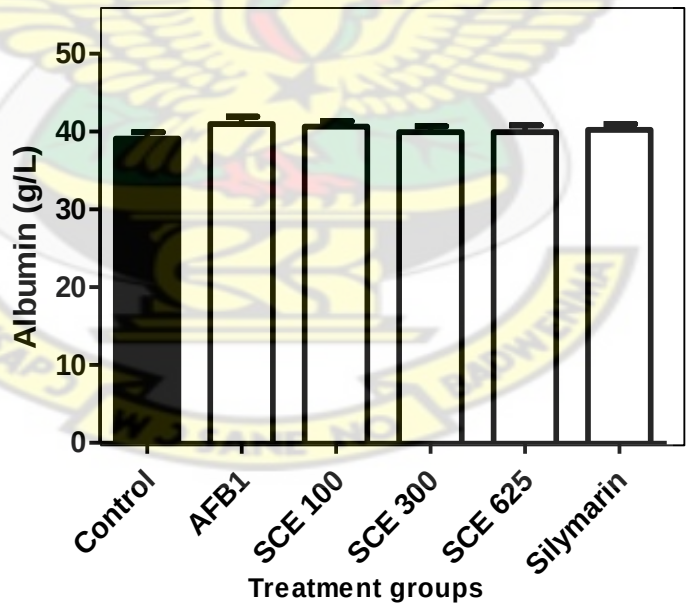


Figure 5.9: Effect of SCE pretreatment on the albumin levels of rats treated with AFB1. Values are expressed as the mean ± SEM (n=5).

Effect on body weight

There were no significant changes in the body weight of treated rats at the end of the experiment though there appears to be a general decline in weight of treated animals (Figure 5.10) neither were the relative weights of some selected target organs affected

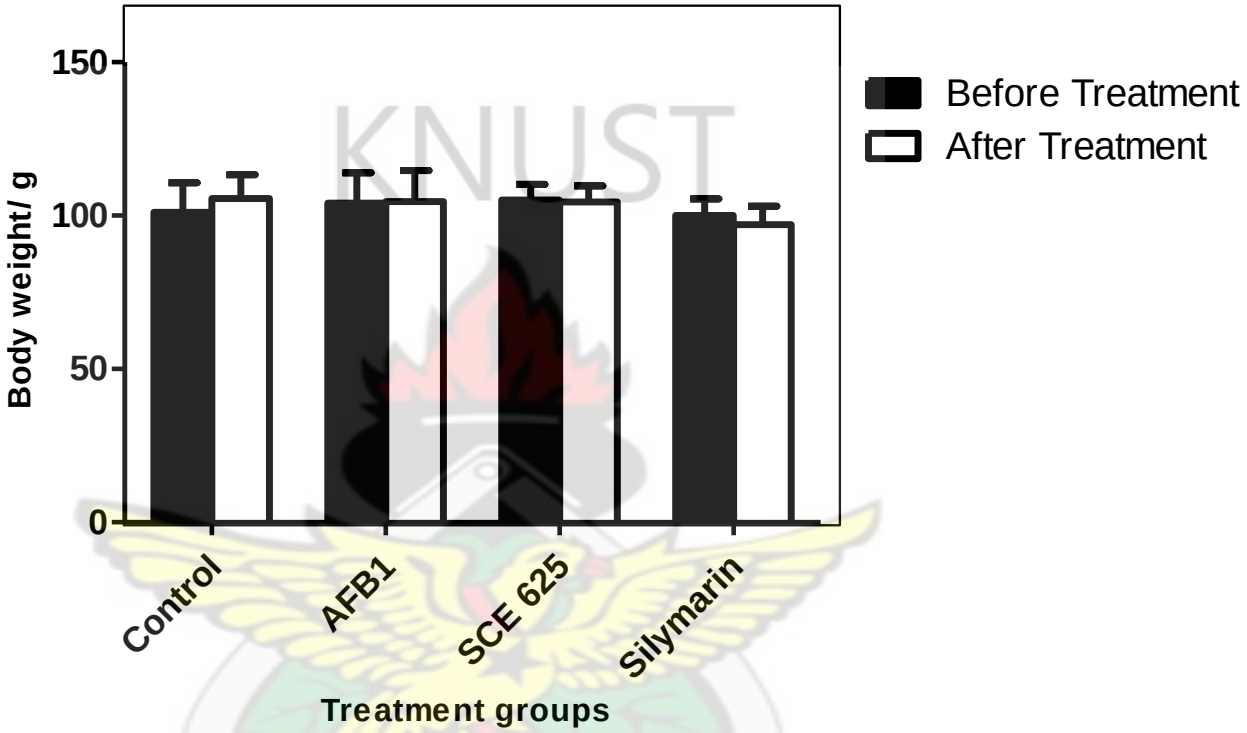


Figure 5.10: Weight of rats taken before and after AFB1 treatment with *Spathodea campanulata* stem bark extract or silymarin. Values are expressed as mean ± SEM.

5.4 DISCUSSION

In the present study, the ability of SCE to prevent drug-induced and micotoxin-induced hepatotoxicity was investigated. A single toxic dose each of paracetamol and aflatoxin B1 was used to induce liver damage.

Paracetamol is an extensively used antipyretic-analgesic drug which produces acute hepatic damage when taken in overdose (Lee, 2004). It is established that, a fraction of paracetamol is converted via the cytochrome P450 pathway to a highly toxic metabolite; N– acetyl–p–benzoquinone imine (NAPQI) (Dahlin *et al.*, 1984; Moyer *et al.*, 2010) which is normally conjugated with glutathione to be excreted in the urine. Overdose of paracetamol depletes glutathione stores through conjugation, leading to a buildup of NAPQI (James *et al.*, 2003) which covalently binds with cellular macromolecules leading to hepatotoxicity (Mitchell *et al.*, 1973b). Mitochondrial dysfunction and the development of acute hepatic necrosis are consequently potentiated.

Hepatic parenchymal cell injury was estimated by measuring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum. It was established that a single dose of paracetamol had caused injury to the parenchymal cell resulting in a leakage of the aminotransferases. These enzymes (ALT, AST) are released into circulation when there is hepatic necrosis making them measureable in serum (Jollow *et al.*, 1973). High levels of AST obtained indicate liver damage caused by viral hepatitis, cardiac infarction and muscle injury (Vezzoli *et al.*, 2011). ALT, a more specific marker for liver damage was also elevated. Significant elevation of AST and ALT indicates cellular leakage and loss of functional integrity of parenchymal cell in the liver. This was however prevented by pretreatment with the extract at all doses employed. The results agrees 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).

Injury to intrahepatic bile ducts was also estimated by measuring serum activities of gamma glutamyl transferase (GGT) and syntheses ability was estimated by a measure of the total protein and bilirubin. Paracetamol administration caused a significant decrease in serum total protein when compared to control. Pretreatment with the extract however ameliorated significantly the damage to the parenchymal cell compared to the control group. This demonstrates that the extract is acting in a way to restore the synthetic ability

of the liver which was injured by the administration of paracetamol. Extensive vascular degeneration and centrilobular necrosis was produced in the hepatocytes by paracetamol. The livers of mice treated with paracetamol showed gross vascular degeneration, fatty liver and necrosis. This was prevented by pretreatment by SCE indicated by the absence or minimization of necrosis and vascular degeneration. The efficacy of a hepatoprotective drug is dependent on its ability to reduce the harmful effect or restore the normal hepatic physiology that has been altered.

When SCE was compared with n-acetyl cysteine, which is the standard treatment for paracetamol overdose (Polson and Lee, 2005b), it was observed that SCE protected the animals as much as did the n-acetyl cysteine.

In addition to drug-induced hepatotoxicity, aflatoxin-induced hepatotoxicity was also a choice model investigated. The secondary metabolites from *aspergillus flavus* are known to cause hepatocellular carcinoma (Zhu *et al.*, 1989). The possible protective effect of SCE against AFB1 was investigated.

Since hepatic enzymes are restricted (especially ALT) in periportal hepatocytes, where they are involved in amino acid metabolism, they are good biochemical markers for early acute hepatic damage (Adedara *et al.*, 2010b). This is because these transamination reactions and their serum activities apparently increase as a result of cellular membrane damage and leakage (Das *et al.*, 2010).

Treatment of animals with a single dose of AFB1 caused increase in serum transaminases (ALT and AST). The observed increase in serum enzymes may have been caused by a leakage of these enzymes from the periportal regions. A large dose of AFB1 is known to cause periportal necrosis and bile duct epithelial cell injury (Luyendyk *et al.*, 2002). Once the hepatocytes were bruised, leakage of hepatic content into blood was made possible. Pretreatment with SCE inhibited the negative effect the aflatoxin could have had on the liver. The increase in serum transaminases was significantly prevented. Compared to the control, there was a very low toxic effect of the aflatoxin. Once the injury to the hepatocytes is healed, the leakage is stopped. This may be the case of the animals treated with the extract, as serum enzymes were reduced to an appreciable level compared to the control.

AFB1 treatment however had no significant effect on the synthetic ability of the liver as total proteins and albumins were not affected. This may be due to the animals receiving only a single dose of AFB1. Repeated dosing of animals with AFB1 has been shown to cause a decrease in the weight of animals and protein levels in serum (Liu *et al.*, 1995). The AFB1-treated animals either decreased slightly in weight or maintained their weight failing to increase. The decrease in weight may be linked to the decreased synthesis ability of the liver resulting in a decreased albumin or total protein content. It may also be linked to a loss of appetite in the animals. This is because in the preliminary tests, the animals which received aflatoxin appeared to eat less over the first 24 hours after intoxication (data not shown). As a result, animals failed to increase in weight as compared to the control group which increased in weight as expected with growth.

The effect with the extract treatment was similar in the silymarin treated group. Silibin, a constituent of silymarin is known to induce enzymes involved in the metabolism of AFB1 (Kohno *et al.*, 2002). Higher phase II enzyme activity are expected to be released in rats fed with silibin; mainly glutathione S-transferase.

The possibility of AFB1 to generate ROS during metabolism has been postulated (Angeli *et al.*, 2010). The ability of SCE and silymarin (as reference) to attenuate the hepatotoxic effect of AFB1 could be due to the antioxidant property these compounds may have since aflatoxin is known to exert its toxicity through the induction of free radicals (Madhusudhanan *et al.*, 2006). Polyphenols for example are known to be soluble chain-breaking inhibitors of the peroxidation process, acting as scavengers of intermediate peroxyl and alkoxyl radicals and chelating metal ions (Domitrovic *et al.*, 2009). Prevention of DNA oxidation is also achieved by these polyphenols mainly by quenching free radicals and modulating biometabolism enzymes (Angeli *et al.*, 2010). It will therefore not be surprising that amongst the constituents that SCE, polyphenols will be named.

In the preliminary tests, it was observed that the effect of AFB1 diminished with increasing time. Thus the best time to evaluate the effect was 24 hours after administration of a single dose of AFB1. Preliminary treatment with SCE attenuated the toxic effect the aflatoxin had on the livers of the rats.

5.5 CONCLUSION

Spathodea campanulata stem bark extract possesses hepatoprotective activity against paracetamol and aflatoxin B1 induced hepatotoxicity. This protective effect may be due to antioxidant properties the extract may possess.

CHAPTER 6

POSSIBLE MECHANISMS OF HEPATOPROTECTION BY *SPATHODEA CAMPANULATA* AQUEOUS STEM BARK EXTRACT

6.1 INTRODUCTION

The previous chapters have established the hepatoprotective activity of *Spathodea campanulata* stem bark extract (SCE). The extract caused a decrease in serum enzymes and restored the architecture of the liver, which were otherwise elevated and distorted respectively by the hepatotoxicants administered. However, the specific mechanism by which this extract protected the liver against the injuries produced by the hepatotoxicants has not been established.

When xenobiotic are ingested, they are identified by the body as foreign substances which must be eliminated. The elimination process involves metabolism where these xenobiotic are chemically transformed. The end product may be therapeutic or toxic.

The basic purpose of drug metabolism in the body is to make drugs more water soluble and thus more readily excreted in the urine or bile. One common way of metabolizing drugs involves the alteration of functional groups on the parent molecule (e.g., oxidation) via the cytochrome P450 enzymes which are predominant in the liver (Cupp and Tracy, 1998).

The cytochrome P450 enzymes are a super family of enzymes that metabolize a number of endogenous substances and xenobiotic. Isozymes of the mono-oxygnases are targets for modulation by various medicines, including herbal medicines (Beckmann-Knopp *et al.*, 2000). Xenobiotic biotransformation is one of the major determinants of genotoxic effects of chemicals.

Specifically, some of the products of biotransformation are known to act in oxidative stress against the liver. For example, hepatic CYP2E1 has been shown to generate reactive oxygen species upon activation of CCl_4 and other xenobiotic (Gonzalez, 2005; Montoliu *et al.*, 1995) including ethanol (Comoglio *et al.*, 1995). Some models of liver disease suggest a crucial involvement of oxidative stress in liver failure (Das *et al.*, 2005; Lorenzi *et al.*, 2011; Recknagel, 1983; Rector *et al.*, 2010).

In addition, evidence from some human studies suggests that chronic inflammation of the liver caused by diseases such as hepatitis causes up-regulation of the CYP1A2 and CYP3A4 isoforms of CYP450 (Kirby *et al.*, 1996).

All the toxicants used in this research (CCl4, paracetamol, aflatixin B1) are known to undergo metabolic activation by the cytochrome P450 enzymes (Gonzalez, 2005; Imaoka *et al.*, 1992; Raucy *et al.*, 1989) which is responsible for the bulk of their damaging effects. A closer look at the mechanism of CCl_4 hepatotoxicity affords several points of intervention. Carbon tetrachloride is first activated by CYP 450 (specifically 2E1) to the trichloromethyl radical which then either abstracts a hydrogen atom from a suitable donor (a lipid for example) or gets oxidized to the trichloromethyl peroxy radical which has its own oxidative pathway (Fig 6.1). The production of reactive radical metabolites is the start of events such as lipid peroxidation, thought to be the cause of the damage.

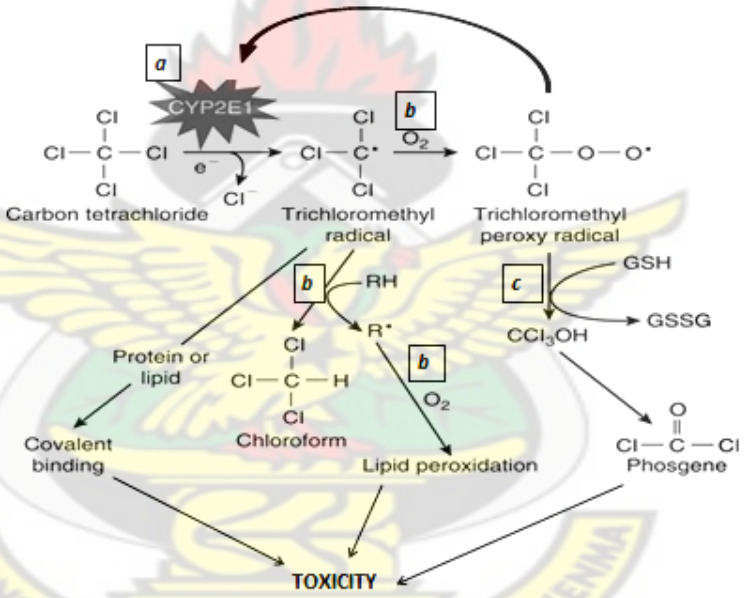


Figure 6.1: Proposed possible points of intervention of CCl_4 hepatotoxicity by SCE.

Interventions at point: *'a'* represents inhibition of CYP 450, *'b'* represents mopping up of free radicals and prevention of lipid peroxidation whereas *'c'* represents enhancement of antioxidant enzyme system.

Adopted originally from Timbrel (2009) and modified.

Paracetamol, normally eliminated from the body by conjugation with sulphate or glucuronide, has a proportion metabolized via cytochrome P450 to a metabolite NAPQI, especially in overdose (Moyer *et al.*, 2010). NAPQI conjugates preferably with reduced glutathione (Fig 6.2) and depletes it in a short period. Although GSH is present in the liver at high concentrations relative to other peptides, it becomes depleted, leaving the remaining toxic metabolite to bind covalently to liver proteins, leading to liver necrosis (Hassoun and Periandri-Steinberg, 2010).

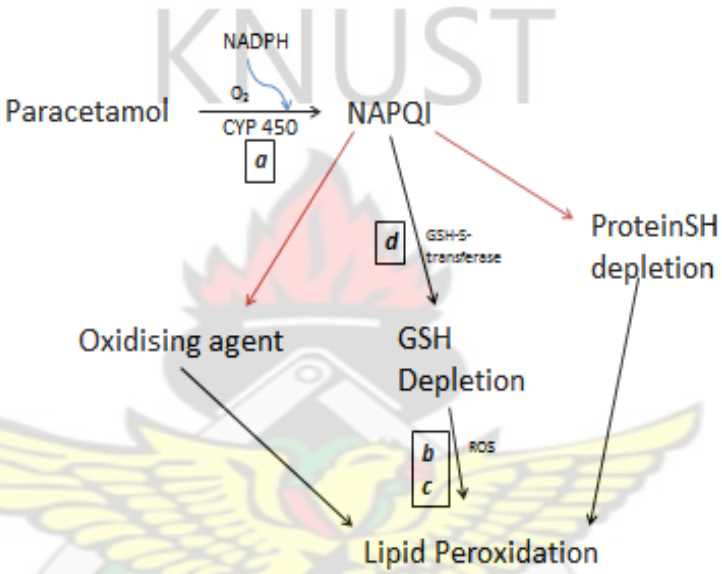


Figure 6.2: Possible points of intervention of paracetamol hepatotoxicity by SCE.

Once GHS is depleted, the cells are vulnerable to reactive oxygen species (ROS) which will produce other free radicals and result in lipid peroxidation.

Free radicals are well known reactive molecules, unstable and very reactive chemical species, which have an unpaired electron in their structure (Chacko *et al.*, 2007), mainly derived from univalent reduction of oxygen. The concept of biological free radicals is partially responsible for turning oxygen into a hazard. These pose challenges to the cellular morphology and functional integrity causing a decrease in membrane fluidity, loss of enzymes, receptor activity and injury to membrane proteins leading to cell inactivation and disease conditions (Sivakumar *et al.*, 2010)

The most important free radicals are the radical derivatives of oxygen. Increased oxidative stress may result from over-production of precursors to reactive oxygen radicals and/or decreased efficiency of inhibitory and scavenger systems. The stress then may be augmented and propagated by an autocatalytic cycle resulting in tissue damage and cell death (Hunt *et al.*, 1993). Cell damage will also result in elevated production of reactive oxygen species.

Most hepatoprotective agents or drugs have been postulated to act via the antioxidant protection mechanism either by enhancing the antioxidant enzyme system or by acting as antioxidants themselves. Notable among them is silymarin, a mixture of antioxidant flavonolignans (silibin and silibinin) extracted from the medicinal plant *Silybum marianum*. It is said to prevent lipid peroxidation in some experimental models (Soto *et al.*, 1998). Also, N-acetyl cysteine which is the current treatment for paracetamol toxicity is a known antioxidant (Kuvandik *et al.*, 2008; Rumack, 2002; Yagmurca *et al.*, 2007). It inhibits N-acetyl-p-benzo-quinone imine (NAPQI) from binding to macromolecules pertaining to the liver in the early phase of paracetamol intoxication (<8 h) (Kandis *et al.*, 2010). It may exert its effect by acting as a glutathione precursor or alternatively as a sulfate precursor. The protective actions of N-acetylcysteine (NAC) after paracetamol overdose are primarily by replenishment of hepatic glutathione stores and direct detoxyfication of NAPQI (San-Miguel *et al.*, 2006).

A hepatoprotective drug such as SCE, which has been compared to silymarin and NAC, could be acting in one or more similar way(s) to restore metabolic function and morphology. Along with many pharmacological researches done on *Spathodea*, the antioxidant properties of the stem bark extract has been demonstrated by Houghton and his team. They reported that *Spathodea campanulata* stem bark extract can scavenge DPPH (2, 2- diphenyl-l-picrylhydrazyl) showing a strong reactive oxygen species scavenging activity (Houghton *et al.*, 2005). However, other antioxidant activities are not documented.

Since SCE demonstrates hepatoprotection, and has DPPH scavenging activity (Houghton *et al.*, 2005), one important investigation will be to analyze the antioxidant protection, both *in vitro* and *in vivo*, alongside other specific mechanism(s) by which it may protects the liver from various hepatotoxicants.

6.2 MATERIALS AND METHODS

Animals

The animals used in the study, Sprague-Dawley rats (150-200g) and ICR mice (25-30g) were purchased from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. They were kept and maintained in the Animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The animals were housed in groups of 6 in stainless steel cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAFCO, Tema), given water *ad libitum*. The studies were conducted in accordance with internationally accepted principles for laboratory animal use and care (EEC directive of 1986: 86/609 EEC). Approval for this study was obtained from the Ethical Review Committee of the College of Health Sciences, KNUST, Kumasi, Ghana.

Chemicals and Reagents

Methanol, Ferric Chloride, n-Propyl gallate, sodium carbonate, ethanol, sodium chloride, Tannic acid, Potassium ferricyanide, Hydrochloric acid, sulphuric acid, sodium hydroxide, glacial acetic acid, formic acid, glycerol, calcium chloride, were obtained from BDH, Poole, England; DPPH, Trichloroacetic acid, Folin-Ciocalteu phenol reagent, Tris-HCl, Phosphate buffered saline (PBS), sodium deoxycholate, Triton X-100, Sodium dodecyl sulphate, EDTA, Bovine Serum Albumin, Phenobarbital, Phenyl methane sulphonyl fluoride (PMSF), sodium dithionite, reduced glutathione (GSH), β-NADPH, glutathione reductase, sodium azide, cytochrome c from horse heart, nitroblue tetrazolium chloride (NBT), xanthine oxidase, xanthine sodium salt, GPx-positive control and sucrose were obtained from sigma Aldrich, Germany. Hydrogen peroxide, Bells®, was obtained from Kama health services, Kumasi, Ghana.

6.2.1 In vitro Antioxidant properties of SCE

Total Phenolics content

The entire soluble phenols present in the total crude extract (0.1, 0.3, 1, 3 mg ml in distilled water) was measured by colorimetric assay using the Folin-Ciocalteu's phenol reagent as described by (Benklebia, 2005) with some modifications using tannic acid (0.01, 0.03, 0.1, 0.3 mg/ml in distilled water) as standard.

Principle:

Phosphomolybdate-phosphotungstate salts of Folin-Ciocalteau's reagent are reduced by phenolic compounds in alkaline medium giving a blue coloration, the intensity of which can be quantified spectrophotometrically at 760nm. Absorbance increases with increasing phenol content.

Experimental design:

The test drug (1 ml) was added to 1 ml Folin-Ciocalteu's reagent (diluted tenfold in distilled water).The content of the test tube was mixed and allowed to stand for five minutes at 25˚C in an incubator. 1 ml of 2% Sodium carbonate solution was added to the mixture. The reaction mixture was then allowed to stand for 2 hours at 25˚C in an incubator. It was then centrifuged at 3000 rpm for 10 minutes to get a clear solution. The absorbance of the supernatant was then determined at 760 nm using a Shimadzu 1240 UV-VIS spectrophotometer (Shimadzu Scientific Instruments).

Distilled water (1 ml) was added to 1 ml Folin-Ciocalteu's reagent (diluted ten-fold in distilled water) and processed in the same way as done for the test drugs and used as control with only distilled water serving as the blank.

Four replicates were used. Data was presented as linear regression of concentration of tannic acid against change in absorbance and a column graph of concentration of extract (SCE) against the total phenol, expressed as tannic acid equivalent using GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA).

Reducing power

The reducing power of the crude extract of *Spathodea* (0.1, 0.3, 1, 3 mg/ml) was determined by its ability to reduce Fe^{3+} to Fe^{2+} (Oyaizu, 1986) according to the method described in literature by (Amarowicz *et al.*, 2005) with modifications using tannic acid (0.01, 0.03, 0.1,0.3 mg/ml) as standard.

Principle:

The method measures the ability of a test compound to reduce Fe^{3+} to Fe^{2+}. The resultant Fe^{2+} then reacts with ferricyanide ion to form a Prussian blue complex with maximum absorbance at 700 nm. The intensity of blue complex formed is directly proportional to the absorbance of the solution and reducing power of the test substance in solution.

$$Fe^{3+} \rightarrow Fe^{2+}$$

$$K_3Fe(CN)_6(aq) + Fe^{2+}(aq) \rightarrow KFe[Fe(CN)_6] + 2K^+(aq)$$

Experimental design:

The Tannic acid/extract solution (1 ml) was mixed with 2.5 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide solution [$K_3Fe(CN)_{6(aq)}$] in a test tube. The mixture was incubated at 50˚C for 20 min. Trichloroacetic acid solution (TCA) (1.5ml of 10%) was added to the incubated mixture, and centrifuged at 3000 rpm for 10 minutes.

The supernatant (2.5 ml) was mixed with 2.5ml distilled water and 0.5 ml of 0.1% ferric chloride solution ($FeCl_{3\ (aq)}$) in a test tube. The absorbance was then measured at 700 nm using Shimadzu 1240 UV-VIS spectrophotometer (Shimadzu Scientific Instruments).

Distilled water (1 ml) was added to 2.5ml sodium phosphate buffer and 2.5 ml potassium ferricyanide [$K_3Fe(CN)_6$] in a test tube. This mixture was taken through the same procedure as for the test drugs and used as blank.

Six replicates were used. Data was presented as concentration-absorbance curves and the EC_{50} (concentration that gives 50% of maximal response) computed using GraphPad Prism for Windows version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

6.2.2 In vivo antioxidant activity of SCE

Animals used for these experiment (ICR mice and SD rats) were treated in each case with toxin and extract as in the prophylaxis (SOD and GPx assay) or curative (lipid peroxidation) methods of the hepatoprotective investigations (Tsai *et al.*, 2010). The livers of the treated animals were then harvested at the end of the treatment period, washed in ice cold phosphate buffer, blotted dry and stored in a freezer at -80^{o}C till they were used for the assay.

Lipid Peroxidation

Preparation of tissue homogenate:

Livers from the treated rats (as in CCl_4 curative) were taken, blotted and homogenized in phosphate buffered saline (1/10 weight /volume) and centrifuged at 10000g for 10min at 4 ^{o} C. The supernatant was collected and stored in a freezer (-80ºC) to be used for the assay.

Experimental design:

Lipid peroxides (measured as thiobarbituric acid reactive substance, TBARS) in liver homogenate were estimated using thiobarbituric acid reactive substances as by the method of Ohkawa and colleagues (Ohkawa *et al.*, 1979).

To 0.2ml of tissue homogenate, 0.2ml of 8.1% SDS, 1.5ml of 20% Acetic acid and 1.5ml of 0.8% TBA were added. The mixture was diluted to 4ml with distilled water and heated in a water bath at 95^{o}C for 60min using a glass ball as a condenser. The mixture was left to cool. 1ml of water and 5ml of n-butanol/pyridine mixture were added and shaken vigorously. The

tube with its content was then centrifuged at 4000rpm for 10min, the organic layer was taken and its absorbance measured at 532nm in a Shimadzu UV mini-1240, UV-vis spectrophotometer.

The procedure was repeated using 1,1,3,3-tetramethoxypropane as standard. The level of lipid peroxidation was expressed as moles of TBA reactants/100g of wet tissue.

Superoxide dismutase (SOD) activity

The determination of superoxide dismutase activity was done according as it is outlined in Current Protocols in Toxicology, 2005.

Preparation of tissue homogenate:

Excised livers from treated rats or mice were perfused with PBS containing 0.16 mg/ml of heparin. They were then weighed, minced and homogenized in cold isotonic buffer (10mM Tris-HCl pH 7.4, 200mM mannitol, 50mM sucrose, 1mM EDTA) in a 10 ml/g of tissue ratio.

The homogenized sample was centrifuged at 1000×g at 4ºC for 10 minutes to isolate the crude nuclear fractions. The supernatant was further centrifuged at 3000×g at 4ºC for 10 minutes, at 20000×g for 20 minutes and 144000×g for 90 minutes. The final supernatant which is the cytosolic fraction was stored for the assay of SOD.

Principle:

The test for SOD measures the dismutation of $O_2^{\cdot}$ into O_2 and H_2O_2. SOD is measured as the inhibition of the rate of cytochrome c by the superoxide radical observed at 550nm.

Cytochrome $C_{(oxidized)} + O_2^{\cdot} \longrightarrow$ Cytochrome $C_{(reduced)} + O_2$

Experimental design:

The SOD assay was done as seen in the work of (McCord and Fridovich, 1969).

The reaction cocktail was prepared by mixing 125 ml PBS (pH 7.8), 5 ml EDTA (10.7 mM), 5 ml cytochrome c (1.1mM), 250 ml xanthine salt solution (0.108mM) and 115 ml distilled water. The pH was then adjusted to 7.8 at 25ºC.

Xanthine oxidase enzyme solution, at a concentration of 0.05U/ml, was prepared in phosphate buffered saline just before use.

In a cuvette, 2.8 ml reaction cocktail was mixed with 0.2 ml distilled water and absorbance at 550 nm was read for five minutes. This served as the blank.

For the uninhibited reaction, 2.8 ml reaction cocktail was added to 0.1 ml distilled water and 0.1 ml xanthine oxidase enzyme solution. Again the absorbance for five minutes was read at 550 nm.

For the inhibited reaction, 2.8 ml reaction cocktail was added to 0.1 ml xanthine oxidase enzyme solution and 0.1 ml of the sample to be tested. The content was immediately mixed by inversion and the increase in absorbance at 550 nm was recorded for five minutes.

Calculations:

$$\% \text{ Inhibition} = \frac{\Delta A/\text{min (Uninhibited)} - \Delta A/\text{min (Inhibited)}}{\Delta A/\text{min (Uninhibited)} - \Delta A/\text{min (Blank)}} \times 100$$

$$\text{Volume activity in units/ ml enzyme} = \frac{\% \text{ Inhibition} \times \text{dilution factor}}{50\% \times 0.1}$$

50% = inhibition of the rate of cytochrome c reduction

= volume (ml) of enzyme used

$$\text{Specific activity in units/ mg protein} = \frac{\text{Volume activity in units/ ml enzyme}}{\text{mg protein / ml enzyme sample}}$$

One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system using xanthine salt solution and xanthine oxidase at pH 7.8 at a temperature of 25ºC in a 3 ml reaction volume. The xanthine oxidase should produce an initial (uninhibited) ΔA of 0.025 ± 0.005 per minute.

Glutathione peroxidase activity

The determination of glutathione peroxidase activity was done according as it is outlined in current protocols in toxicology, 2005.

Preparation of tissue homogenate:

Liver of treated animals were removed and perfused with PBS. The perfused liver was them homogenized as 20% homogenate (tissue weight/volume of buffer) in ice-cold buffer (Tris-HCl). To obtain the post-nuclear fractions for the GPx activity measurements the homogenate was centrifuged at 100000×g for 10 minutes. The supernatant was collected and stored for the assay.

Principle:

The decline in the absorbance at 340nm monitors the consumption of NADPH in the following reaction catalyzed by glutathione reductase:

$H^+ + GSSG + NADPH \longrightarrow 2GSH + NADP^+$

This reaction is driven by the formation of GSSG, coupling the glutathione peroxidase reaction to the change in absorbance monitored in the assay.

Experimental design:

For a 2ml assay, 1.26 ml of assay buffer (50mM sodium phosphate, pH 7.0), 0.2 ml of 10mM GSH, 0.2 ml of 2mM NADPH, 0.02 ml sodium azide and 0.02 ml of 100U/ml glutathione reductase were mixed in a cuvette.

For the blank, 0.2 ml homogenizing buffer was added to the mixture in the cuvette whiles 0.2 ml of clarified sample was added for the test. The content of the cuvette was mixed and allowed to equilibrate for 5 minutes.

Hydrogen peroxide (0.1 ml) was then added, quickly mixed to start the reaction and the change in absorbance measured at 340nm for 5 minutes.

For the glutathione peroxidase activity in the coupled assay, the NADPH consumption rate is used to calculate activity. The absorbance per minute calculated was converted to enzyme units.

$(\text{Abs/min} \div 6.22\times10^{3}) \div 1000 = \text{mol NADPH consumed/min}$

The value 6.22×10^{3} is the optical density of a 1M solution of NADPH in a path length of 1cm. The divisor of 1000 corrects the NADPH consumption from per liter to the actual assay volume of per ml.

The enzyme activity units value was then divided by the amount of protein in the assayed sample aliquot, expressed in mg, to yield the sample specific activity.

6.2.3 Effect of SCE on cytochrome p450 enzyme

Effect of SCE on liver Cytochrome P450 enzymes in Mice and Rats

ICR mice weighing 25-30g and SD rats weighing 150-200g were used for the experiment. Animals were divided and housed five in a group for five days before treatment.

Four separate groups each of rats and mice were used. One group (A) of both rat and mice received distilled water and served as control whiles the other groups (B, C and D) received SCE (625 mg/kg), Ketoconazole (100 mg/kg) and Phenobarbital respectively (100 mg/kg) daily *p.o* for seven consecutive days. On the eighth day, the animals were sacrificed by cervical dislocation.

Total CYP450 was also measured in animals treated with CCl_4 or paracetamol and SCE as in the prophylactic studies described earlier.

Livers were removed, blotted of blood and homogenized in a 0.25M sucrose solution at a ratio of 5 ml/g of tissue. Phenyl methane sulphonyl fluoride (PMSF) was added to inactivate serine proteases and the homogenate was centrifuged at 600g for 5min. Supernatant was further centrifuged at 12000g for 10min, and the second supernatant further centrifuged at 100,000g for 1hr. The resultant pellets were suspended in 0.25M sucrose. Protein concentration was adjusted to 20 mg/ml using the Lowry method of protein assay. The P450 level was then determined by forming the P450-CO complex CO in a CO chamber leaving a non-CO complex control outside the chamber. The difference in absorbance at 450 and 490 was measured in a microplate reader.

P450 levels were calculated as $[P450]_{(mM)} = (\Delta Apc - \Delta Ap) / 91$.

Where:

ΔApc = change in absorbance of the P450-CO complex
ΔAp = change in absorbance of the non-CO control
91 = extinction coefficient

6.3 RESULTS

6.3.1 In vitro Antioxidant properties

Total Phenolics Assay

The total phenol content of tannic acid (0.01,-0.3 mg/ml) expressed as absorbance, increased with increasing concentration (r^2 =0.97) (Figure 6.3). SCE (0.1-3.0 mg/ml) also showed a dose-dependent increase in phenolic content (0.0009 ± 0.00006 to 0.0856 ± 0.0012) expressed as tannic acid equivalent (Figure 6.4). The content of total phenols in the extract was however found to be lower than that of the tannic acid.

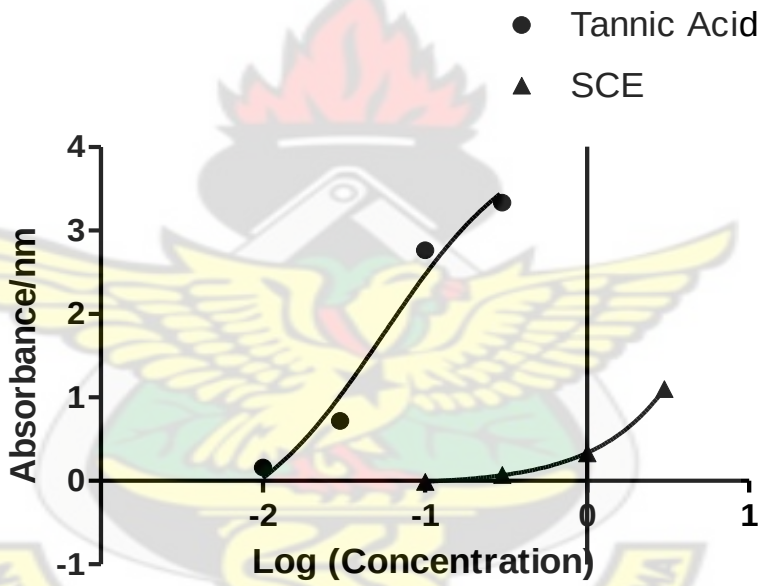


Figure 6.3: Log (concentration) against absorbance of tannic acid and SCE.

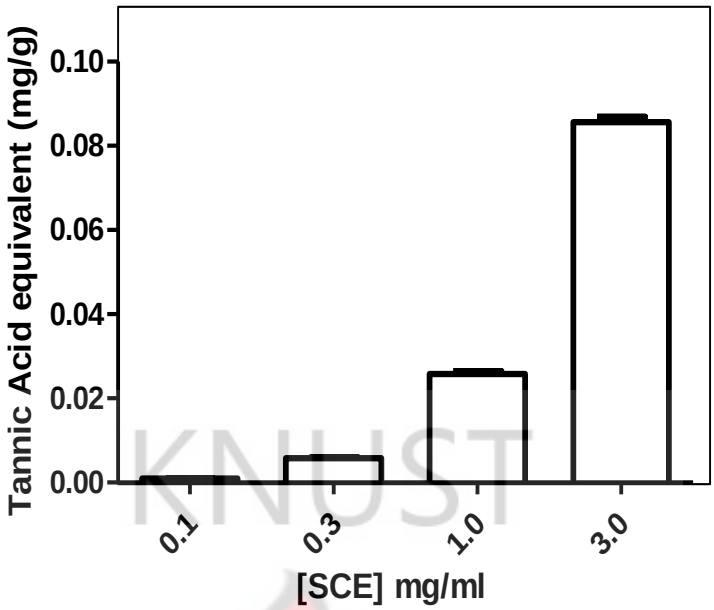


Figure 6.4: Total phenolic content of SCE (0.1-3.0 mg/kg) expressed as tannic acid equivalent (mg/g extract). Each point represents the mean ± SEM (n=4).

Reducing Power

The extract at 0.1- 3 mg/ml and the standard antioxidant tannic acid at 0.01 -0.3 mg/ml dose dependently reduced Fe^{3+} to Fe^{2+} resulting in dose-dependent increase in absorbance (Figure 6.5). The EC_{50} showed that the extract (with EC_{50} of 1071 mg/ml) is more than 100 fold less potent than the standard drug Tannic acid (EC_{50} of 10.87 mg/ml). However the extract shows potential in being able to reduce Fe^{3+} to Fe^{2+} at higher concentration.

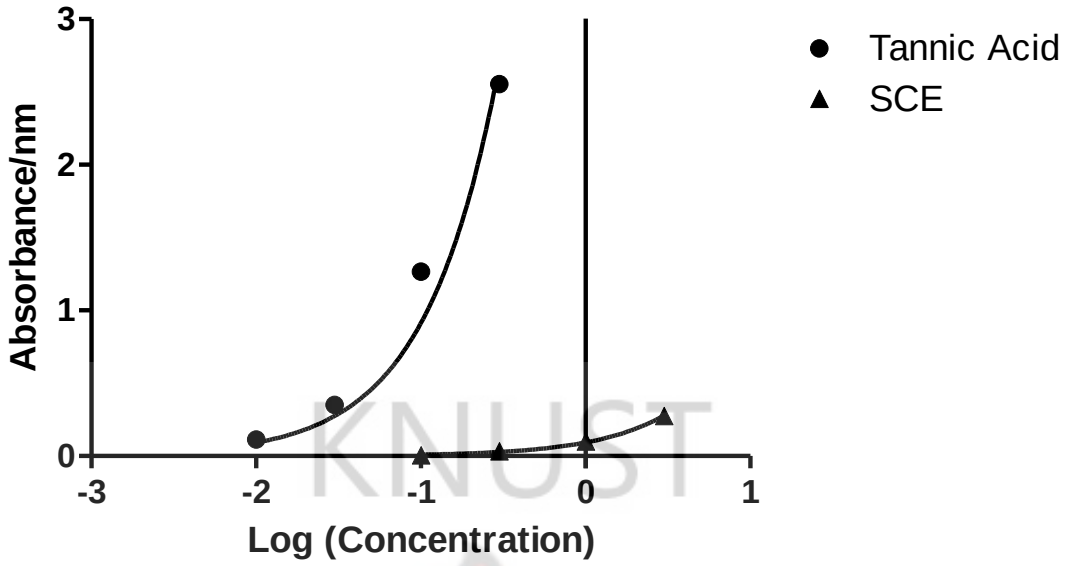


Figure 6.5: Reducing power of SCE (0.1-3.0 mg/kg) compared with tannic acid (0.01-0.3 mg/kg). Each point represent the mean ± SEM (n=3).

6.3.2 In vivo Antioxidant Activity

Lipid Peroxidation

TBA reactive substance level (Figure 6.6) increased significantly in the carbon tetrachloride-treated animals relative to the control: about six folds ($p<0.001$). The increase was reversed to near normal with the treatment with the extract at all three doses compared to the control, ($p<0.001$).

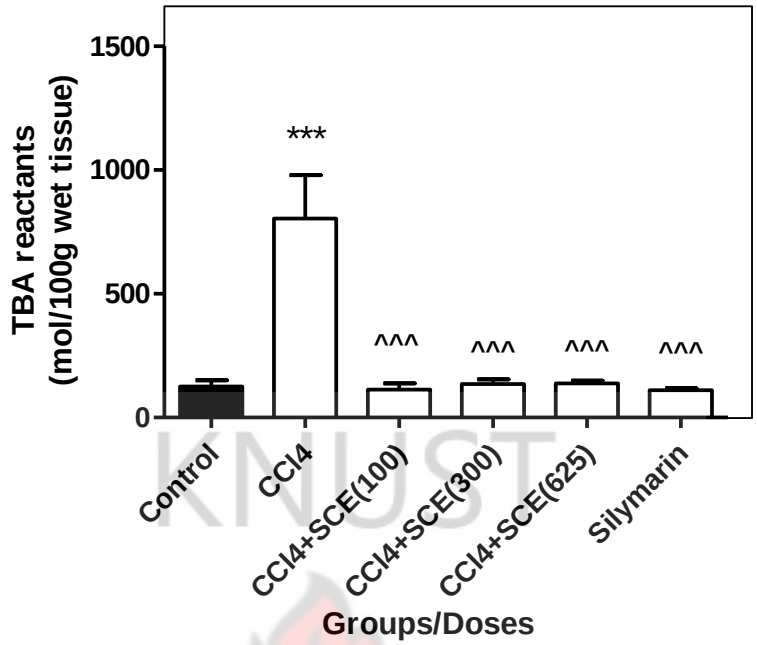


Figure 6.6: Changes in TBARS level in normal and treated rats.

Values represent mean ± SEM for five animals in each group. Values are statistically significant at *** $p<0.001$ when compared with the control and ^^^ $p<0.001$ when compared with the CCl_4 control.

Superoxidation Dismutase Activity

The measure of total SOD units (Fig 6.7 and 6.8) showed that treatment with carbon tetrachloride had insignificant effect of superoxide dismutase. As a result, the extract and silymarin had no effect in restoring SOD. Paracetamol however caused a tremendous decrease (about three fold) in the SOD units. This was restored by the extract and N-acetyl cysteine to near normal rates.

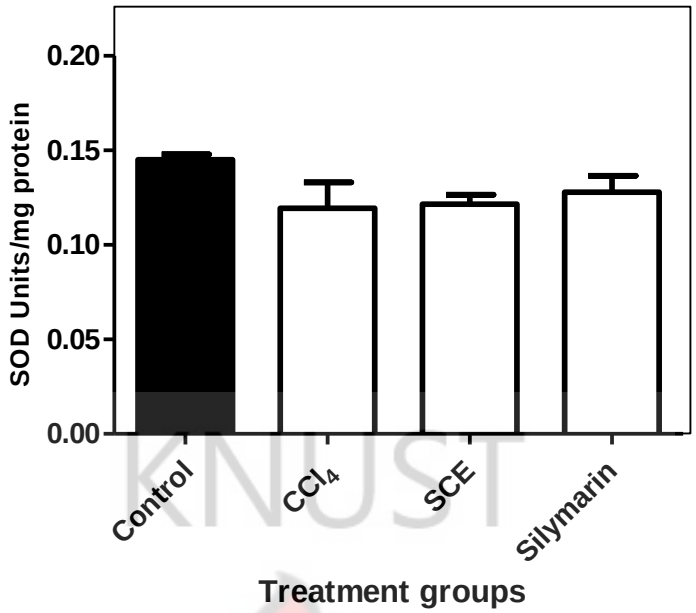


Figure 6.7: Effect of *Spathodea campanulata* extract on SOD units of CCl_4 treated rats.

Values are presented as the mean± SEM (n=5). Treatment groups were not significantly different from control.

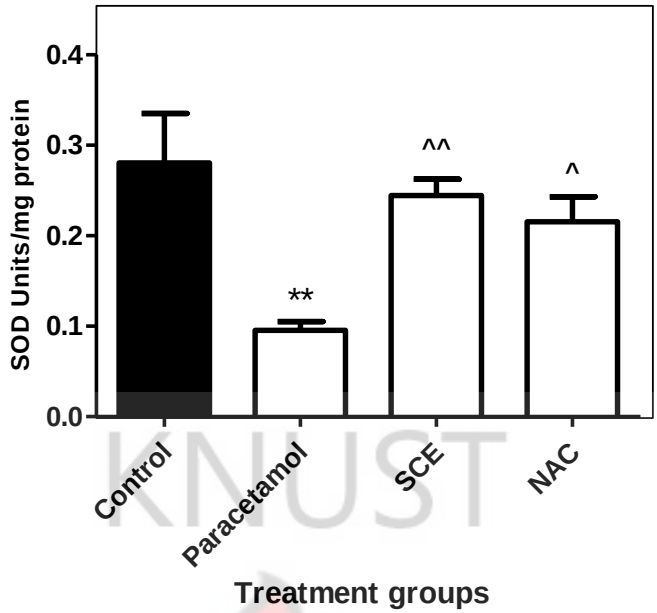


Figure 6.8: Effect of SCE on SOD units of paracetamol treated mice.
Values are presented as the mean ± SEM (n=5). ** indicates significant decrease ($p<0.01$) compared to the control; ^^ indicates significance ($p<0.01$) and ^ indicates ($p<0.05$) compared to the paracetamol group.

Glutathione Peroxidase activity

Treatment of mice with paracetamol significantly decreased GPx activity about eight folds compared to control ($p<0.001$). Treatment with SCE was able to significantly restore the activity of GPx similar to the control and NAC (control drug) ($p<0.001$). In the treatment of rats with CCl_4, there was a decrease in GPx activity which wasn't significant compared to the control. Although the decrease was not significant, treatment with the extract and the standard drug appeared to have restored the decrease to normal levels.

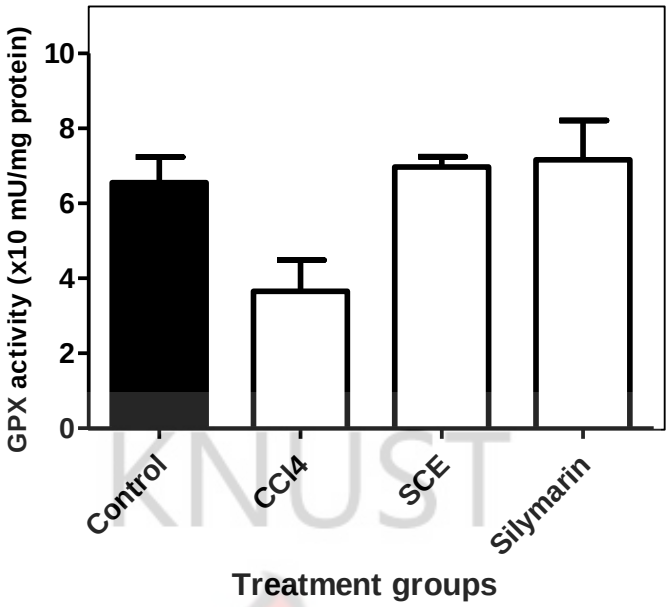


Figure 6.9: Effect of SCE on glutathione peroxidase activity (GPx) in the livers of rats treated with CCl_4. Values are presented as the mean ± SEM (n=5).

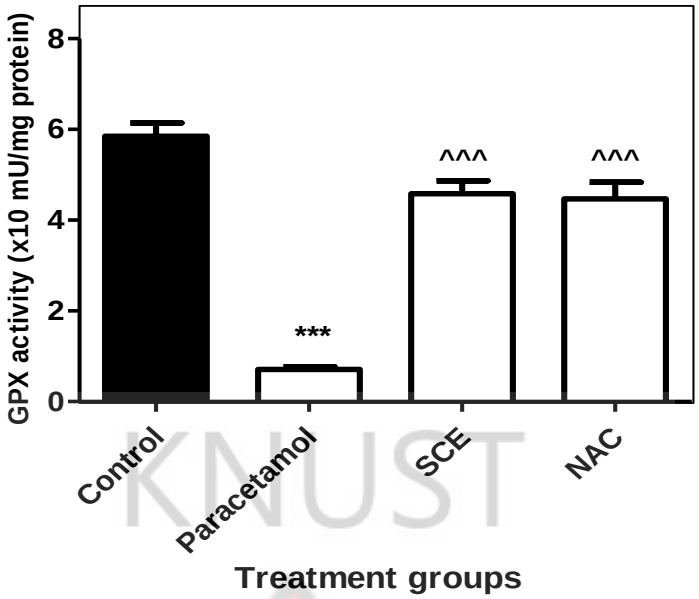


Figure 6.10: Effect of SCE on glutathione peroxidase activity (GPx) of the livers of mice treated with paracetamol.

Values are presented as the mean ± SEM (n=8). *** indicates significant decrease ($p<0.001$) compared to the control; and ^^^ indicates significant increase ($p<0.001$) compared to the paracetamol treated control.

6.3.3 Effect of SCE on total CYP 450 enzymes

Total cytochrome P450 content in the livers of rats/mice treated with SCE

A seven days treatment of rodents with extract resulted in a pronounced decrease in the total cytochrome P450 enzymes in the liver of these animals (Fig 6.11 and 6.12). The decrease is comparable to ketoconazole which is a known inhibitor of cytochrome p450. Phenobarbital also increased the total cytochrome P450 enzymes compared to the control.

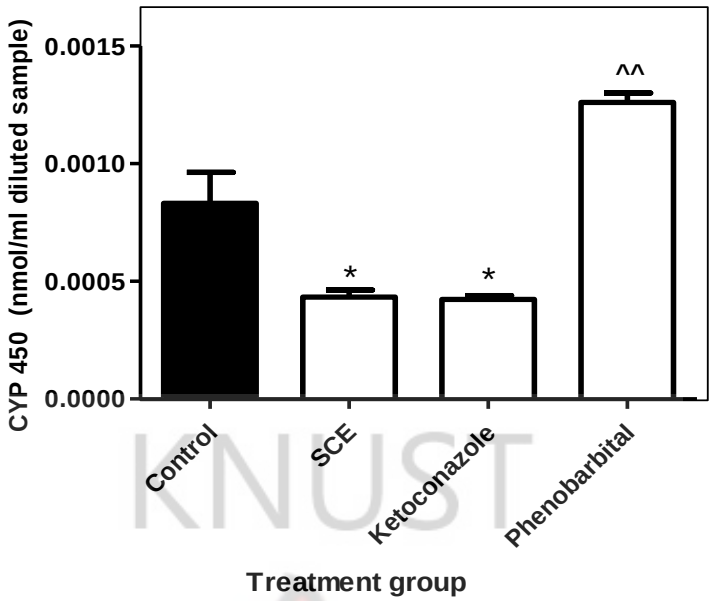


Figure 6.11: Effect of SCE treatment on total liver cytochrome P450 content in rats.

Values are presented as the mean ± SEM (n=5). * indicates significant decrease ($p<0.05$) compared to the control; and ^^ indicates significant increase ($p<0.01$) compared to the control.

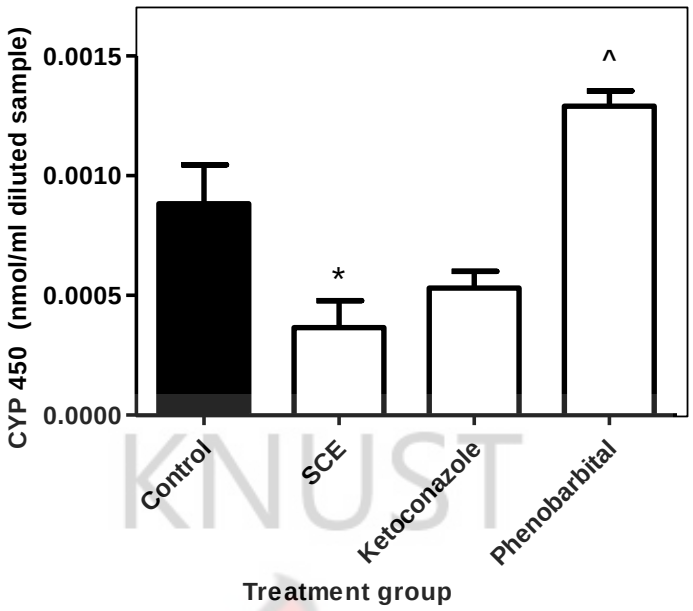


Figure 6.12: Effect of SCE on cytochrome P450 content in the livers of mice.

Values are presented as the mean ± SEM (n=8). * indicates significant decrease ($p<0.05$) compared to the control; and ^ indicates significant increase ($p<0.05$) compared to the control.

Effect of SCE on total CYP450 in the presence of CCl_4

Treatment of rats with CCl_4 caused a significant decrease in total cytochrome P450 content. SCE also cause a significant decrease in the CYP 450 content. The extent of decrease is lower than in the treatment with CCl_4 alone. Silymarin decreased the total CYP 450 further than CCl_4 and extract+CCl_4.

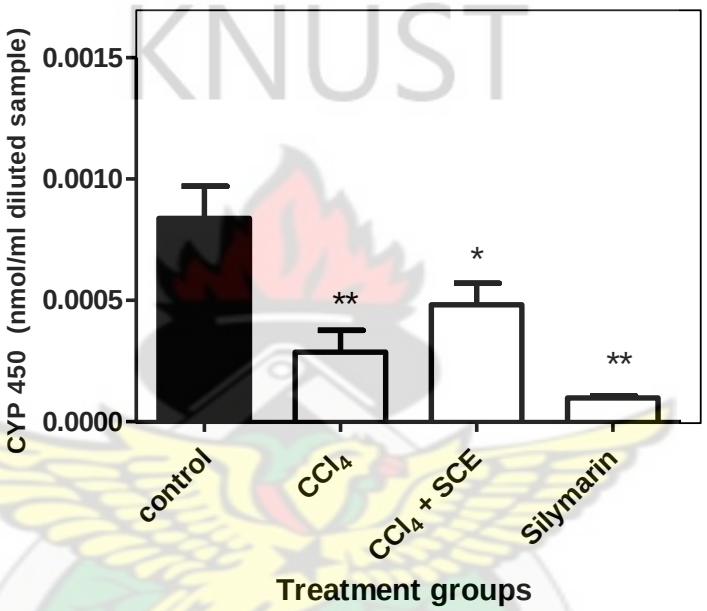


Figure 6.13: Effect of SCE on total cytochrome P450 in the presence of CCl_4.

Values are presented as the mean ± SEM (n=8). ** indicates significant decrease ($p<0.01$) compared to the control; and * indicates significance ($p<0.05$) compared to the control.

Effect of SCE on total CYP450 in the presence of paracetamol

Treatment of mice with paracetamol resulted in up-regulation of the total cytochrome P450 in the liver. The extract decreased slightly the elevated total CYP450. This decrease was however statistically insignificant. NAC however did not affect the total cytochrome P450 levels in livers of animals treated with paracetamol.

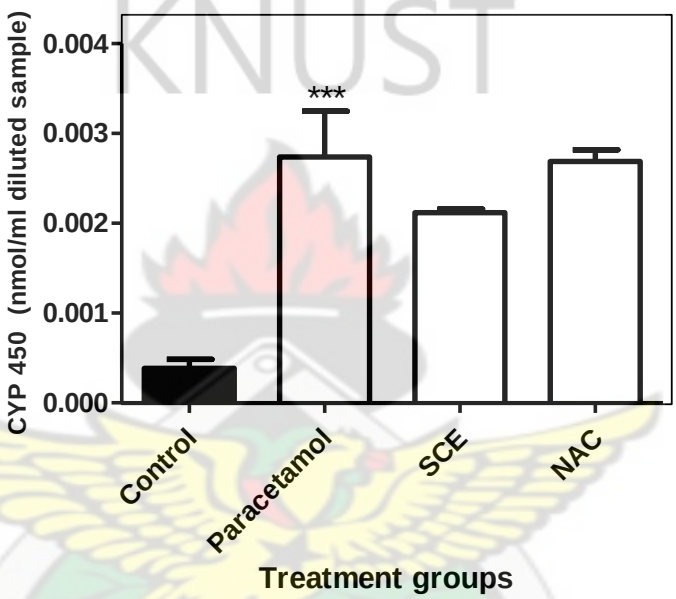


Figure 6.14: Effect of SCE on total cytochrome P450 in the presence of paracetamol. Values are presented as the mean ± SEM (n=8). *** indicates significant increase ($p<0.001$) compared to the control.

6.4 DISCUSSION

The preceding chapters provided clear evidence that SCE protects the livers of rodents from some specific hepatotoxicants. However the mechanism of SCE-induced hepatoprotection was left to be investigated.

In the present study, the mechanism of hepatoprotection by SCE was investigated in paracetamol and carbon tetrachloride acute liver injury models. Two possible mechanisms were followed. In the first possible mechanism, the antioxidant characteristics of SCE were investigated using *in vitro* and *in vivo* models.

An antioxidant is defined as any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate (Halliwell, 1995).

The reducing power and total phenolic content were tested in the *in vitro* antioxidant experimental models. An examination of whether SCE can reduce Fe^{3+} to Fe^{2+} gave a positive result. The reduction of Fe^{3+} to Fe^{2+} is a property of antioxidants. Antioxidant compounds are able to donate electrons to reactive radicals converting them into more stable and unreactive species (Cetinkaya *et al.*, 2011). This is called the reducing power of the antioxidant. The extract was also shown to have an appreciable level of phenolic compounds in the total phenolic test. Phytochemicals such as flavonoids and other polyphenolic constituents of plants have been reported to be effective radical scavengers and inhibitors of lipid peroxidation (Dai and Mumper, 2010; Ebrahimzadeh *et al.*, 2010). Phenolics are the most widespread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelators. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al.*, 1995). The presence of phenols together with the reducing ability of the extract in addition to the DPPH scavenging activity (Houghton *et al.*, 2005) shows that SCE has antioxidant properties which may be responsible for its hepatoprotective activity. Antioxidants protect tissues against oxidative stress; polyphenols act as antioxidants by scavenging reactive oxygen and nitrogen as well as chelating redox-active transition metal ions inhibiting them from

catalyzing free radical formation (Domitrovic *et al.*, 2009). Antioxidant enzymes such as SOD and GPx are also induced to scavenge reactive oxygen species which are induced by the hepatotoxic agents.

To further confirm antioxidant activity as the mode of hepatoprotection, *in vivo* antioxidant properties were investigated in the presence of the hepatotoxicants.

Because the partially reduced forms of oxygen, during oxidation in a living system, are cytotoxic, protective antioxidant enzymes (including superoxide dismutase (SODs), catalase, and glutathione peroxidases) usually exist to convert these reduced forms of oxygen to water. Superoxide dismutase is an enzyme which has an overall effect to lower the steady-state concentration of superoxide in the liver cells (Valko *et al.*, 2007).

During CCl_4 hepatotoxicity the reactive metabolite, trichloromethyl radical, abstracts hydrogen atoms, or binds to proteins and other macromolecules leading to oxidative stress. In oxidative stress, superoxide dismutase activity is depressed as evident in the CCl_4-treated group.

Pretreatment with SCE was able to ameliorate the oxidative stress as demonstrated by the increase in SOD activity in the SCE treated group similar to the control group (figure 6.8).

SOD catalyzes the reaction:

$O_2 + O_2 + 2H \rightarrow H_2O_2 + O_2$

Using reduced glutathione as the reducing agent, glutathione peroxidases also convert hydrogen peroxide (H_2O_2) to water. Consequently, the oxidative stress is either reduced or eliminated. Hence, GSH constitutes the first direct line of defense against free radical activity in the cells and is a critical determinant of tissue susceptibility to oxidative damage.

The test for glutathione peroxidase (GPx) activity confirmed that oxidative stress was produced by the hepatotoxicants. The decrease was more significant in the paracetamol induced hepatotoxicity than in the CCl_4 induced hepatotoxicity (Figure 6.9 and 6.10). This can be attributed to the fact that GSH is not depleted in CCl_4 toxicity (Shimuzu *et al.*,

1989) as it is in paracetamol-induced hepatotoxicity (Knight *et al.*, 2001). The decrease in GPx activity in the case of paracetamol hepatotoxicity was also restored by pretreatment with the extract.

On the other hand, lipid peroxidation (LPO) is a major consequence in CCl_4-hepatotoxicity. The TBA reactive substance test showed an incidence of lipid peroxidation by carbon tetrachloride treatment. Lipids undergo peroxidation in the presence of reactive oxygen species, which interfere with the structural appearance of these lipids and make them deleterious in the process. To regulate lipid peroxidation, there is a defense system involving antioxidant enzymes that play an imperative role in scavenging reactive oxygen species (Mahboob *et al.*, 2001). The susceptibility of an organism to free radical stress and peroxidative damage is related to the balance between the free radical burden and the adequacy of antioxidant defenses. Treatment with the extract however decreased the extent of lipid peroxidation caused by CCl_4 and restored the antioxidant protection of the living cell. Lipid soluble antioxidants may therefore play a role in reducing lipid peroxidation.

LPO however is not a critical event in the mechanism of paracetamol induced liver injury and, therefore, lipid-soluble antioxidants are ineffective in reducing paracetamol-induced liver injury (Knight *et al.*, 2002). Thus, water-soluble radical scavengers, for example, GSH may be more promising as therapeutic agents in preventing the progression of paracetamol-induced liver injury (James *et al.*, 2003).

The extract may therefore have precursors to or constitute some lipid soluble antioxidants as well as have significant regenerative or de novo synthesis effect on glutathione peroxidase (GPx) and reduced glutathione (GSH).

Throughout literature, bioactivation has been postulated to play a critical role in hepatotoxicity. Many hepatotoxicants including CCl_4, nitrosamines, and paracetamol require metabolic activation, particularly by liver cytochrome p450 enzymes to form their reactive toxic metabolites, which in turn cause liver injury both in experimental animals and humans. The culprit isoenzyme in the p450 family is the 2E1 in both CCl_4 and paracetamol toxicity (Badger *et al.*, 1996; Bremanti, 1978; Dai and Cederbaum, 1995). Alterations in the activity of CYP2E1 can therefore affect the susceptibility to hepatic injury from CCl_4. CYP2E1 exhibits a unique ability to potentiate an iron catalyzed Fenton-

type reaction and an increased rate of microsomal lipid peroxidation (Dai *et al.*, 1993; Ekstrom and Ingelman-Sundberg, 1989).

Liver injury induced by CCl_4 is the best-characterized system of the xenobiotic-induced hepatotoxicity and is a commonly used model for screening the hepatoprotective activity of drugs (Brautbar and Williams, 2002). The trichloromethyl radical formed during CCl_4 metabolism is capable of binding to lipids, initiating lipid peroxidation and damaging the liver.

Also, paracetamol is bioactivated by cytochrome P450 to NAPQI which is known to be both an oxidizing agent and an arylating agent (James *et al.*, 2003). NAPQI binds to cytosolic GSH and depletes them in the process.

The major peroxide detoxification enzyme, GSH peroxidase, functions very inefficiently under conditions of GSH depletion (James *et al.*, 2003). GPx therefore is expected to be inhibited when GSH has been depleted and the opposite is also true. In addition, during formation of NAPQI by cytochrome p450, the superoxide anion is formed, with dismutation leading to hydrogen peroxide formation (Dai and Cederbaum, 1995). It is safe to say therefore that oxidative stress in the liver has its genesis in bioactivation.

Consequently, total cytochrome p450 content in the liver was assayed. In both mice and rats given the extract, there was a strong inhibition of CYP450 comparable to ketoconazole. In the event where CYP450 is inhibited, binding of CCl_4 as well as paracetamol to the cytochrome P450 enzymes is reduced. This probably leads to decreased or altered bioactivation resulting in low potentiation of injury to the liver and its environment.

In the presence of carbon tetrachloride, the effect of the extract on the total cytochrome P450 enzymes was not statistically different from the CCl_4-only treated group. This is possible because the trichloromethyl peroxy radical which is formed by oxidation of carbon tetrachloride is destructive to cytochrome P450 enzymes. The decrease in the total cytochrome p450 in the CCl_4 only and CCl_4+extract are not significantly different from each other.

It is also known that mitochondrial permeability transition (MPT) occurs with formation of superoxide (Nagababu *et al.*, 1995). In the event of permeable mitochondria and damaged endoplasmic reticulum by oxidative stress, the peroxide is able to destroy the p450 enzymes. Inflammation itself could inhibit CCl4-induced hepatotoxicity by the suppression of cytochrome P-450 activities (DiSilvestro and Carlson, 1992).

The total cytochrome p450 levels in animals that received paracetamol alone remained significantly increased (Figure 6.14). This agrees with literature that paracetamol up-regulates CYP450 (Das *et al.*, 2010). As expected, the treatment with extract resulted in a decrease in the total cytochrome p450 enzymes. The extent of paracetamol-induced hepatotoxicity is reduced in the presence of enzyme inhibitors such as ketoconazole and isoniazid (Walubo *et al.*, 2004). It is likely that the extract acts in a similar manner as ketoconazole; that is inhibition of cytochromes 2E1 as well as 3A.

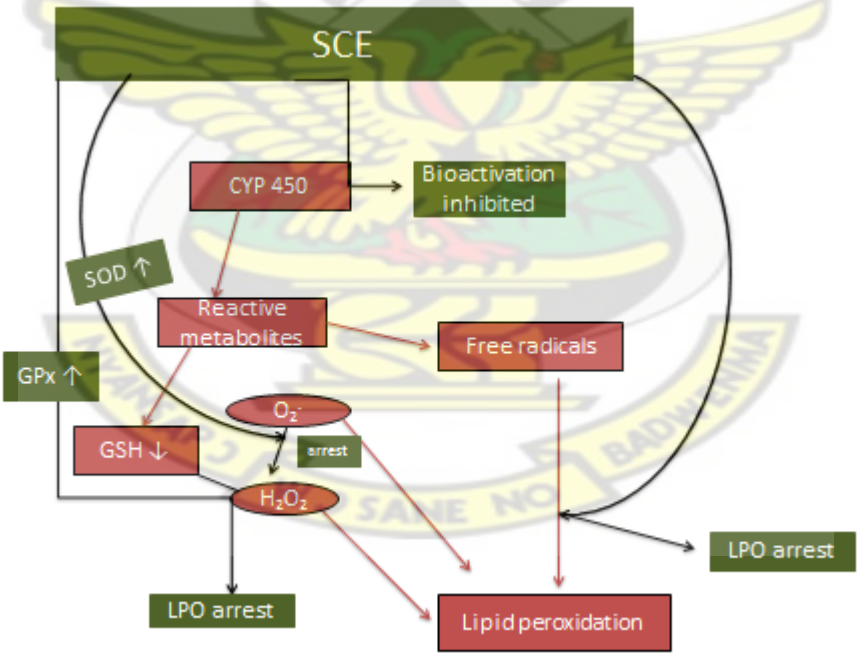


Figure 6.15: Schematic diagram showing the points of intervention by SCE in hepatotoxicity arrest.

Note: Green filled shapes represent the activity of SCE whiles red filled shapes represent the pathway of hepatotoxicants.

In this study, the extract exhibited protective effects by interfering with CCl_4 and paracetamol –mediated oxidative stress through decreased bioactivation and production of free radical derivatives evidenced by the decreased cytochrome p450 activity and increased glutathione peroxide and superoxide dismutase activity (Figure 6.15).

6.5 CONCLUSION

SCE exhibits its hepatoprotective potential by modulating activity levels of enzymes and metabolites governing liver function and by helping in maintaining cellular integrity of hepatocytes. Against CCl_4 and paracetamol-induced hepatotoxicity, SCE interferes with generation and reactions of primary radicals (CCl_3/CClOO and NAPQI) and mops up free radicals derived from oxidative stress cause by the primary radicals.

CHAPTER 7

GENERAL DISCUSSION AND SUMMARY OF FINDINGS

7.1 GENERAL DISCUSSION

There is a call by the WHO on developing countries to improve health delivery by the primary health care approach; meanwhile, the use of herbal medication is recognized as an integral part of the primary health care system.

An important aspect of evaluating herbal medications should be a screening for possible toxicity. Unfortunately, toxicity testing of herbal products is either limited or non-existent in the populations where these herbal medicines are used. *Spathodea campanulata* is a tree which is used as a herbal medication. The tree, distributed through Guinea to Angola, Sudan and Uganda (Joly, 1985), is also native to Ghana and is popular in the Akan communities and regions almost as a panacea. Apart from the leaves, flowers and seeds which are used for the treatment of several ailments, the stem bark is also very prominent in traditional medicine and thus attracts pharmacological interest.

There are several formulations of *Spathodea campanulata* stem bark for the treatment of some ailments. For example, a decoction is used to treat dysentery and stomach ache (Mendes *et al.*, 1986), as well as kidney troubles and diabetes while the pulp is used for wound healing (Niyonzima *et al.*, 1990)

Though there is no evidence of any adverse effect in the use of this traditional medicine, there is also no guarantee of safety of the stem bark decoction since there is no monitoring of adverse reactions on herbal medications in general. The absence of scientific data to support its safety or otherwise and the popularity of the stem bark decoction of *Spathodea campanulata* in Ghanaian traditional medicine prompted a safety evaluation which was not reported.

The hypothesis that the use of SCE may not be entirely safe was investigated. Since traditionally, the decoction is taken orally, it was decided that the evaluation be done on the aqueous stem bark extract. The results from this study showed that SCE is relatively safe at low doses. This assertion in SD rats and ICR mice, though cannot be extrapolated to humans, however reduces anxiety to the use of the plant extract to some extent since it did not cause death in the animals treated. This assertion is supported first by the LD_{50} value greater than 5000 mg/kg in both rats and mice by the oral route. Again, the continuous treatment for 14

days had no adverse effects on the animals. The integrity of blood was not compromised by the treatment with the extract neither was there any negative effect on target organs both according to histology and biochemistry. The highest dose (5000 mg/kg) however caused a peculiar increase in the serum ALP level raising questions as to whether there could be a possible induction of biliary cirrhosis (Ramaiah, 2007) or that it was due to normal metabolic activity. A normal GGT level in all dose levels however questions the possible biliary cirrhosis since it (GGT) is a more specific marker. This left the possibility more to normal metabolic activity; if not, the course is unknown. However, since the highest dose showed potential toxicity, it will be safer to use the drug at doses lower than 5000 mg/kg. The same dose also caused a decrease in serum sodium ions, a condition known as hyponatremia. This is due to stimulated arginine vasopressin (AVP) and fluid intake greater than obligatory losses (Almond *et al.*, 2005) and this could have been induced by the extract. A look at the direct effect of the extract on the urinary tract became necessary and the diuretic property of the extract was therefore investigated. The results showed a positive diuretic effect on the treated rats. This may account for the low serum Na^+ levels observed earlier. A diuretic is any drug that elevates the rate of urination and thus provides a means of forced diuresis. Though there are several categories of diuretics which act distinctly, they all increase the excretion of water from bodies. Diuretics are known for their several advantages in several oedematous disease states. They are used to treat heart failure, liver cirrhosis, hypertension and some kidney diseases (Sadki *et al.*, 2010). Interestingly, one of the traditional uses of SCE is for the treatment of 'kidney trouble' (Abbiw, 1990). The kind of kidney trouble however has not been named but perhaps diuresis from SCE has been contributing a great deal to solving it.

Additionally, there was also an interesting decline of hepatic enzymes (AST, ALP and ALT) observed especially at lower doses (1250 and 2500 mg/kg) of SCE in the 14 days treatment in rats. Although this decline has no documented clinical significance, it raised questions to a positive effect of the extract on the liver. This led to the hypothesis that "the use of SCE could have positive effect on the liver".

An agent with positive effects on the liver is worth looking into since the causes of liver diseases over the years have varied. Apart from the viral causes of liver diseases (hepatitis), there are other forms of liver diseases caused by different chemicals and toxins; there is diet

and behavioural (NAFLD), addiction (alcoholic liver), mycotoxin contaminations (aflatoxin) or even drug-induced hepatic injury (abuse of drug). These factors are not all easily avoidable subjecting the liver to inflammation at least once in a while. This makes a search for other hepatoprotective drugs of higher potency, higher accessibility and affordability urgent.

Interestingly, almost all of these types of hepatic injury progress and terminate in fibrosis or cirrhosis at which point there is little help to offer to save the patient. Persistent inflammation of the liver appears to be a risk factor for cirrhosis in spite of the fundamental etiology (Caldwell *et al.*, 1999; Du and Wang, 1998). Unpredictable hepatic reactions, which are idiosyncratic, can be viewed as either immune-mediated hypersensitivity or non-immune reactions (Davies, 1985).

In Ghana and most parts of Africa, the economic status has made conventional drugs relatively expensive to the ordinary citizen. The search for a less expensive alternative is therefore of much consequence especially since the incidence of liver disease is on the increase, not to mention alcohol consumption and obesity especially among the youth.

According to World Health Organization (WHO) estimates, alcohol is the fourth leading risk factor for death and disability globally, almost at par with tobacco (WHO, 2010). Estimates from the WHO Global Alcohol Database (WHO, 2011) and specific surveys show that though high proportions of Africans do not drink, consumers among them tend to consume high volumes of alcohol. Alcohol when ingested is metabolized by the liver. This leads to the formation of molecules whose further metabolism in the cell results in reactive oxygen species (ROS) production. Alcohol also stimulates the activity of enzymes called cytochrome P450, which contribute to ROS production (Bailey and Cunningham, 2002). It can also alter the levels of certain metals in the body, thereby facilitating ROS production or reduce the levels of antioxidants (the agents that can eliminate ROS). The resulting state of the cell, known as oxidative stress, can lead to cell injury.

In this study, SCE has been shown to be hepatoprotective against three hepatotoxic agents namely; carbon tetrachloride, paracetamol and aflatoxin B1. The choice of the three was made based on occurrence (incidence) and understanding.

Carbon tetrachloride is an extensively studied hepatotoxic agent. When administered to a variety of species, it causes centrilobular necrosis and fatty liver (Timbrel, 2009). These characteristics are recognized in most types of hepatic damage. Primarily, hepatotoxicity is depicted by an increase in serum AST and ALT. This is because these enzymes are localized in periportal hepatocytes, where they are involved in amino acid metabolism; transamination reactions and their serum activities most probably increase as a result of cellular membrane damage and leakage (Adedara *et al.*, 2010b; Kaplan, 1993). ALP and GGT may also be increased in the case of hepatobiliary damage (Gershwin *et al.*, 2005). CCl_4 is known to cause an increase in the serum enzymes and a decrease in the total protein and albumin levels. This is as a result of the leakage of these enzymes into circulation when the hepatocytes are damaged. Again, CCl_4 depresses the synthetic function of the liver leading to reduced levels of serum albumin and therefore reduced total protein. Due to the damage to the hepatocytes, fatty infiltration from the cells causes fatty liver.

The study showed that, SCE can attenuate CCl_4-hepatotoxicity; better as a curative agent than in prophylaxis. The extract restored the elevated serum enzymes and up-regulated total proteins to near normal. It also improved on, as well as protected, the architecture of the liver by reducing the level of fibrosis and necrosis caused by CCl_4. The restored enzymes and architecture strongly suggests that the extract could be hepatoprotective against carbon tetrachloride. These observations were similar in both prophylactic and curative treatment with the extract. The curative treatment however attenuated to a greater extent the hepatotoxicity caused by carbon tetrachloride. This suggests that the defense system may be more responsive to injury than proactive though SCE is favoured in its ability to boost the defense mechanism of the system.

Another hepatotoxic risk which has stirred interest in many scientists is paracetamol overdose. Continuous use of lower doses has also been postulated to be a risk factor (Rumack, 2004). This has led to several researchers looking in this direction. Paracetamol at higher doses causes centrilobular necrosis that can be fatal (Gujral *et al.*, 2002). It acts by depleting reduced glutathione stores making the cells susceptible to damage by ROS. Susceptibility to paracetamol hepatotoxicity is greater in mice than in rats (Kedderis, 1996), thus SCE was tested against paracetamol in mice. The extract, as it did in CCl_4-induced damage, again protected the liver of the treated mice. This resulted in serum enzymes and proteins being restored to levels closer to the control. The architecture of the liver was also

protected from necrosis caused by paracetamol overdose. The absence of fatty droplets in the livers of SCE-treated mice showed that the hepatocytes were not damaged by the paracetamol due to SCE pretreatment. A slight difference in the serum parameters compared to the control suggests that the hepatocytes were not completely protected from the impending danger either. Pretreatment with the extract may have boosted the protective ability of the system against the impending danger. This fortification however was limited. In the presence of the danger, the fortification did not prove to be enough resulting in the slight injury. This possibly suggests that treatment of paracetamol toxicity when it happens is more reasonable. However, curative treatment also has to be done as soon as possible since the lag between an overdose and death is short. Though recommendable, the prophylactic use of SCE needs further investigation. This is the same with the accepted treatment for paracetamol hepatotoxicity, n-acetyl cysteine (James *et al.*, 2003). Pretreatment with n-acetyl cysteine before paracetamol toxicity affords little protection compared to post or curative treatment buttressing the point that the immune system is more responsive than proactive to injury. On the other hand, mice did not survive long enough for post treatment with the toxic dose chosen. Comparatively however, the extract appeared to be more hepatoprotective than NAC when given prophylactically.

Drug abuse and addictions may be dealt with completely, but dealing with contamination especially in food and drink is not constant. For example, getting rid of aflatoxin contamination has been a global fight for a long time and continues to pose a challenge. This pre informed the choice of aflatoxin as a test hepatotoxin against SCE.

Aflatoxin B1 is one of the most carcinogenic mycotoxins in nature (Smela *et al.*, 2002). Exposure to aflatoxin contamination is usually through diet. Diet contamination with aflatoxin is commonest in Asia and Africa (Staib *et al.*, 2003). With the global trade in agriculture, mycotoxin contamination has become more of a reality for animal producers worldwide (Zhao *et al.*, 2010).

A high dose of aflatoxin B1 is known to cause hepatocellular apoptosis and necrosis (O'Brien *et al.*, 2000), elevated serum enzymes and DNA aberrations (Barton *et al.*, 2001) and bile duct epithelial cell injury (Luyendyk *et al.*, 2002).

In this study, pretreatment with the extract profoundly reduced the serum AST and ALT both of which were elevated in the AFB-only treated group. Though a single dose of aflatoxin

was found to elevate serum enzymes, the same dose did not affect the protein level or the hepatobiliary path. Although it may take several dosing of aflatoxin to depress protein synthesis, persistent inflammation of the liver with small doses of aflatoxin puts the liver at risk of hepatic damage.

The results of the experiments performed strongly suggest that SCE has hepatoprotective potential *in vivo*. The study has so far shown that the extract could be protecting the hepatocytes, as well as the hepatobiliary pathway. This proposition is strengthened by the fact that serum enzymes specific to the liver (ALT) and others which were markedly elevated by the administration of the hepatotoxic agents was reduced significantly by treatment with SCE. Pretreatment with the extract as well as curative treatment protected the livers of the animals from toxicity from these toxic agents. The synthetic ability of the liver was restored, evident by the change in albumin and total protein upon treatment with the extract and toxicants consecutively as opposed to the toxicants alone. Though the architecture of the liver was damaged by these hepatotoxic agents, evident by the observation of fatty liver and cirrhosis, treatment with the extract significantly healed the liver of the insult.

Note worthily, SCE is acting in some mechanisms to attenuate the insults caused by these hepatotoxic agents investigated. It was therefore necessary to shed light on which mechanism by which it is working to protect the liver against the above mentioned insults.

Carbon tetrachloride, paracetamol and aflatoxin B1 are all known to undergo metabolic activation in order to achieve their toxic effects on the liver (Gonzalez, 2005; Imaoka *et al.*, 1992; Raucy *et al.*, 1989). The involvement of cytochrome P450 cannot be overlooked as they specifically metabolize these agents. Specific cytochrome P450s are responsible for specific bioactivation. For carbon tetrachloride, it is clear that it is CYP 2E1 while in the case of paracetamol, several p450 isoenzymes may metabolise it but the most relevant is 2E1 (Gonzalez, 2005; Raucy *et al.*, 1989). It is similar in the case of aflatoxin B1 where CYP 1A, 1B (Zhou *et al.*, 2006) and others are involved in metabolic activation (Imaoka *et al.*, 1992).

Bioactivation does not seem to be the only cause of the potentiation process. Covalent binding of these metabolites results in a cascade of reactions leading to hepatotoxicity.

Oxidative stress is one mechanism that has been postulated to be important in the development of hepatotoxicity (Jaeschke *et al.,* 2002). Free radical mediated processes have been implicated in pathogenesis of most of the hepatic diseases.

For instance, the trichloromethyl radical from carbon tetrachloride metabolism oxidizes lipids or covalently binds to proteins and exert its toxic effects. Lipid peroxidation is thought to be the major step in carbon tetrachloride hepatotoxicity (Chandan *et al.,* 2008). NAPQI, the reactive metabolite from paracetamol metabolism, reacts with glutathione (GSH) spontaneously or catalyzed by glutathione- S-transferases to form a GSH-adduct, which is mainly excreted into bile. Once GSH is depleted by NAPQI, oxidative stress takes over the cell.

Lipid peroxidation and oxidative DNA damage are the principal manifestations of aflatoxin B1-induced toxicity which could be mitigated by antioxidants (Souza *et al.,* 1999).

The probability that SCE must have antioxidant properties had to be analysed using *in vitro* and *in vivo* models. The extract exhibited the ability to reduce phosphomolybdate-phosphotungstate salts of Folic Ciocateu. Against Fe^{3+}, the extract was able to reduce it to Fe^{2+}. Both properties show the ability of the extract to reverse oxidation of molecules caused by the injury to the cells. The antioxidant property thus should be involved in the SCE intervention of hepatotoxicity. Moreover, preliminary phytochemical studies revealed the presence of tannins, sterols, alkaloids and reducing sugars. These phytochemicals have antioxidant properties which could be responsible for the activity seen. In addition, β-sitosterol, a typical example of the constituents of SCE has been shown to protect the DNA against oxidative stress by increasing the level of typical antioxidant enzymes (Awad *et al.*, 2008).

The effect of this antioxidant property was therefore assessed *in vivo* where the effect on GSH system showed that SCE may be working to replenish the depleted GSH in the case of paracetamol overdose. This must also account for the dismutation of the superoxide anion produced as a result of oxidative stress since the SOD activity was higher in animals treated with the extract and hepatotoxic agent. It has been reported that glutathione (GSH) plays an important role in the detoxification of ethanol (Adedara *et al.*, 2010b) and ethanol ingestion

has been reported to decrease the levels of the antioxidant glutathione in lung tissue (Moss *et al.*, 2000).

The extract also exhibited a reducing effect on lipid peroxidation. The effect on lipid peroxidation seems to sum up the antioxidant protective ability of SCE since most of the oxidative stress will lead eventually to lipid peroxidation.

Many chemicals can damage mitochondria, an intracellular organelle that produces energy. Its dysfunction releases excessive amount of oxidants which, in turn, injure hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP 2E1 also leads to oxidative stress (Jaeschke *et al.*, 2002). Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside liver. This promotes further liver damage (Patel *et al.*, 1998). Non-parenchymal cells such as Kupffer cells, fat storing stellate cells, and leukocytes (neutrophil and monocyte) also have role in the mechanism.

In these studies, cytochrome P450 inhibition by the extract appeared to be the rate-determining step for protection of the liver. This was confirmed by the total cytochrome P450 assay where, a seven days treatment with SCE at 625 mg/kg significantly inhibited total cytochrome P450 in both rat and mice liver. Furthermore, the total cytochrome P450 content which is increased in paracetamol hepatotoxicity was inhibited by treatment with the extract.

Overall, the studies have shown that the absence of adverse effects is not synonymous to safety, since routine use of SCE at higher doses could result in potential harm. However, SCE has the potential to be a useful hepatoprotective agent. These findings agree with the hypotheses.

7.2 SUMMARY OF FINDINGS

It is concluded from these studies that the aqueous stem bark extract of the traditional medicinal plant *Spathodea campanulata* (Bignonaceae), SCE, which is patronized for its anti-stomach ache, anti-dysentery and wound healing properties in Ghana, is potentially nontoxic at lower doses and potentially toxic at higher doses. In addition to the potential safety of SCE, it also induces diuresis in rats. SCE increases urine output and urine

electrolytes. Furthermore, it also has hepatoprotective activity against carbon tetrachloride, paracetamol and aflatoxin B1. The hepatoprotective activity of the agent involves deactivation of ROS by the free radical scavenging ability and reducing ability of the antioxidants present. Cell recovery induced by the extract may be due to induction or restoration of GSH protective system and superoxide dismutase. Again the hepatoprotective ability of SCE may be a consequence of CYP 450 inhibition. At active dose, it inhibits total cytochrome P450 monoxygenase in a similar way as ketoconazole. This inhibition appears to be constant whether in the presence of the injury or not.

Overall, the findings of the studies strongly suggest that SCE is hepatoprotective and may have been protective to those who have used it orally as a treatment for other ailments and perhaps enhanced or protected their liver.

On the basis of this study, the use of the extract prophylactically may be beneficial especially in areas where aflatoxin contamination in food is a problem.

7.3 CONCLUSION

Collectively, the results indicate that the aqueous stem bark extract of *Spathodea campanulata* is fairly nontoxic and may exhibit hepatoprotective activity at lower doses by enhancing antioxidant protection in the cell and interfering with bioactivation of hepatotoxic agents.

FUTURE WORK

Recommended future work is to look at the specific effect of SCE on specific isoenzymes of the cytochrome P450 monooxygenase as well as to isolate the chemical compound(s) responsible for hepatoprotection.

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