ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIALS OF CRUDE EXTRACT AND CURCUMINOIDS FRACTION OF TURMERIC RHIZOMES IN CARBON TETRACHLORIDE-INDUCED LIVER DAMAGE IN WISTAR RATS.

BY

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ABSTRACT

Liver diseases are issues of great concern worldwide. Unfortunately, standard or synthetic drugs used in liver disease therapy are insufficient and have severe side effects, requiring the use of herbal preparations. The present study evaluated the antioxidant and hepatoprotective potentials of crude methanol extract of *C. longa* rhizome and its Curcuminoids fraction in Carbon Tetrachloride (CCl_4)-induced liver damage in Wistar rats. Twenty-seven *(*27) adult Wistar rats of either sex were randomized into 9 groups of 3 animals each. Animals in groups 1 and 3 orally received 1 ml of distilled water and 100mg/kgbw of standard drug (Silymarin), respectively and Two (2) ml of CCl_4 was administered intraperitonially to group 2. Animals in groups 4, 5, and 6 were orally administered doses of 150, 300, and 600 mg/kg b.w of *C. longa* crude extract, respectively, while groups 7,8 and 9 received 75, 150 and 300 mg/kg b.w doses of Curcuminoids fraction respectively for 7 days. CCl_4 was administered 6 hours after the last treatments with the crude extract and Curcuminoids fraction of *C. longa* to induce liver damage in the Wistar rats. Phytochemical, acute toxicity, antioxidant and hepatoprotective monitoring enzymes were evaluated using standard procedures. The liver of the rats was subjected to histopathological studies. Phytochemical screening of the extract shows the presence of Alkaloids (97.24±0.64 mg/g), Saponins (1742.63±1.94 mg/g), Phenols (213.41±1.36 mg/g), Flavonoids (97.24±0.64 mg/g) and Tannins (7.01±0.23 mg/g). The crude methanol extract had LD_{50} value >5000 mg/kg bodyweight. The results obtained showed that the crude extract and curcuminoids fraction of *C. longa* significantly improved Serum Aspartate Transferase (AST) (63.00±9.00 and 48.60±9.00 U/l), Alanine Transferase (ALT) (25.38±2.82 and 19.74±6.58 U/l), Alkaline Phosphatase (ALP) (233.75±41.25 and 178.75±13.75 U/l), Super Oxide Dismutase (SOD) (1.88±0.15 and 2.51±0.92 U/l), Catalase (CAT) (3.99±0.34 and 5.22±1.99 U/l) and Glutathione Peroxidase (GPx) (0.08±0.05 and 1.16±0.00 ug/protein) activity respectively compared to the non-treated group. Protein (66.50±5.70 and 71.25±6.65 U/l) and albumin (3.23±0.80 and 4.00±0.67 mg/dl) for the crude and Curcuminoids fraction respectively were significantly higher compared to the non-treated group. Histopathological studies demonstrated hepatoprotective potentials of *C. longa* crude extract and Curcuminoids fraction against CCl_4 -induced liver damage. From the results of this study, it may be concluded that crude methanol extract and Curcuminoids fraction of *C. longa* rhizome, possess potent antioxidant and Hepatoprotective activity.

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LIST OF ABBREVIATIONS

CCl_4	Carbon tetrachloride
NK	Natural Killer
AST	Aspartate Aminotransferase
SGOT	Serum Glutamate Oxaloacetic Transaminase
ALT	Alanine Aminotransferase
SGPT	Serum Glutamic Pyruvate Transaminase
DHC	Dihydrocurcumin
THC	Tetrahydrocurcumin
HHC	Hexahydrocurcumin
NIIR	National Institute of Industrial Research
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
α- TNF	Alpha Tumor Necrosis Factor
IL-6	Interleukin 6
SOD	Superoxide Dismutase
CAT	Catalase
GPx	Glutathione Peroxidase

GST	Glutathione S-Transferase
ROS	Reactive Oxygen Species
GR	Glutathione Reductase
G-6-PD	Glucose-6-phosphate Dehydrogenase
TGs	Triacylglycerols
NASH	Nonalcoholic Steatohepatitis
ANOVA	Analysis of Variance
SPSS	Statistical Package for Social Science
FHF	Fulminant Hepatic Failure
OECD	Organization for Economic Co-operation and Development
LFT	Liver Function Test

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Plant and plant products had been used throughout human history for various purposes. As a natural defense mechanism against disease and infection, thousands of these products are generated as secondary metabolites by higher plants. These products have pharmacological or biological activity which can be utilized in the discovery and design of pharmaceutical drugs. Plant-derived medicines have played a key role in many cultures ' healthcare, both ancient and contemporary (Newman and Cragg, 2007). The holistic Indian medicine system known as "Ayurveda" primarily utilizes plant-based drugs or formulations to treat multiple illnesses, including cancer, chronic diseases caused by parasites and inflammation. Burhmann *et al*. (2019), showed the significant inhibition of proliferation of cancer cells treated with Calebin A, a non-Curcumin compound in turmeric.

Of at least tens of thousands of global introduced small-molecule drugs, the origins of most can be traced to natural goods (Newman and Cragg 2007). As antioxidants and Hepatoprotective drugs, there are many plant-derived chemicals with powerful antioxidant characteristics that can serve as main developmental compounds (Medina, 2005; Pradhan, 2006).

Turmeric (*Curcuma longa*) is an immemorial medicinal plant, which dates back almost 4000 years. It is not only used as a spice in Southeast Asia, it is used for religious and traditional ceremonies. Its brilliant yellow colour, also known as "Indian saffron," has begun to recognize the importance of modern medicine. *Curcuma longa* as a rhizome, can

be contaminated by heavy metals, including lead. Contaminated *C. longa* is a source of lead exposure in Bangladesh (Jenna, 2019).

Carbon tetrachloride (CCl_4) is a well-known hepatotoxic compound widely used in a wide variety of laboratory animals to cause acute or chronic liver injury (Kaneko *et al.,* 2013). The best characterized model of free radical-mediated liver disease induced by xenobiotics is liver damage caused by CCl_4 in rats (Albano, 2006). A recent study found that CCl_4 causes hepatic damage through reactive oxygen (ROS) species (Albano, 2006). In addition to inhibiting the antioxidant system, elevated production of ROS can generate a state of oxidative stress that leads to cell damage (Koch *et al.,* 2004). This highly toxic radical is responsible for attacks in the cell membrane on unsaturated phospholipid fatty acids leading to lipid peroxidation in the liver cells (Weber, 2003).

The potency or viability of any Hepatoprotective drug is related to its ability to either reduce the harmful effects or maintain the normal hepatic physiological mechanism or functionality that hepatotoxins have altered.

1.2 Statement of Research Problem

One of the main causes of death and morbidity globally is liver illness (James *et al.,* 2020). An estimated 1000 drugs have been discovered to cause liver injury, and this is the most frequent reason for the withdrawal from the market of some approved drugs. Fifty percent of acute liver failure cases are caused by hepatotoxicity of drugs. Drug-induced liver failure can lead to all types of acute and chronic hepatobiliary diseases that occur naturally (Bethesda, 2012).

Oxidative stress is regarded as contributing factor in a multitude of liver dysfunctions or malfunctions that leads to hepatic damage. Cell damage happens when there is an excess of oxygen and nitrogen-derived reactive species or antioxidant deficiency (Medina and Moreno-Otero, 2005; Girish *et al.,* 2008).

1.3 Justification for the Study

The hepatic disease burden throughout the world accounts for the deaths of hundreds of thousands of patients each year. Unfortunately, standard or synthetic drugs used in liver disease therapy are insufficient and have severe side effects, requiring the use of herbal preparations. A significant cause of hepatic dysfunction is drug-induced liver toxicity (Bethesda, 2012). Antioxidant extracts from plants are useful therapeutic approach for treating liver diseases that is safer, cheaper and more efficient for the population impacted. Curcuminoids are *Curcuma longa's* significant biologically active phenolic compound with powerful antioxidant, anti-inflammatory and hepatoprotective potentials (Bethesda, 2012).

1.4 Aim and Objectives of the Study

1.4.1 Aim: The aim of this research is to evaluate the antioxidant and hepatoprotective potentials of crude extract and Curcuminoids fraction from *Curcuma longa* rhizome in Carbon Tetrachloride (CCl_4)-induced liver damage in Wistar rats.

1.4.2 Objectives

The objectives of this study were to:

ii. isolate Curcuminoids fraction from *C. longa* Rhizomes

iii. determine the phytochemical composition of the methanol crude extract of *C. longa* Rhizome.

iv. determine hepatoprotective activity of *C. longa* methanol crude extract and Curcuminoids fraction in CCl_4 -induced liver damage in Wistar Rats.

v. determine the antioxidant potential of *C. longa* methanol crude extract and Curcuminoids fraction in CCl_4 induced liver damage in Wistar Rats.

vi. determine the histopathological impact of *C. longa* methanol crude extract and Curcuminoids fraction in liver damage caused by CCl_4 in Wistar Rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Curcuma longa* (Turmeric)

Curcuma longa is a perennial, herbaceous, rhizomatous plant from the Indian subcontinent, Southeast Asia and Nigeria. This needs temperatures from 20 to 30 °C (68 to 86 ° F) and a substantial amount of annual rainfall to survive (Chang *et al*., 2014). It is roughly 1 m tall (3 ft 3 in). Highly branched, yellow to orange with spicy rhizomes, cylindrical. The leaves are arranged in two rows and rotate. We were divided into sheath, pétiole, and blade of leaf (Tayyem *et al*., 2006). A false stem is built out of the sheaths of the leaf. The petiole is between 50 and 115 cm long (20 to 45 inches). Typically, the simple leaf blades are 76 to 115 cm long and occasionally up to 230 cm (91 in) long. They have a width of 38 to 45 cm (15 to 18 in) and are oblong to elliptical, narrowing at the tip. At the top of the inflorescence, stem bracts are present on which no flowers occur; these are white to green and sometimes tinged reddish-purple, and the upper ends are tapered (Tayyem *et al.,* 2006). It has hermaphrodite flowers, which are zygomorphic and threefold. The three sepals are 0.8 to 1.2 cm (0.3 to 0.5 in) long, fused, and white, and have fluffy hairs; the three calyx teeth are unequal. The three bright-yellow petals are fused into a corolla tube up to 3 cm (1.2 in) long. The three corolla lobes have a length of 1.0 to 1.5 cm (0.4–0.6 in) and are triangular with soft-spiny upper ends. While the average corolla lobe is larger than the two lateral, only the median stamen of the inner circle is fertile. The dust bag is spurred at its base. All other stamens are converted to staminodes. The outer staminodes are shorter than the labellum. The labellum is yellowish, with a yellow ribbon in its center and it is obovate, with a length from 1.2 to 2.0 cm (0.5 to 0.8 in). Three carpels are under a

constant, trilobed ovary adherent, which is sparsely hairy. The fruit capsule opens with three compartments (Tayyem *et al.,* 2006).

In East Asia, the flowering time is usually in August. Terminally on the false stem is an inflorescence stem, 12 to 20 cm (5 to 8 in) long, containing many flowers. The bracts are light green and ovate to oblong with a blunt upper end with a length of 3 to 5 cm (Tayyem *et al.,* 2006).

Today, turmeric is widely grown in the tropics and in different cultures and countries, it goes by different names. In North India, turmeric is commonly referred to as "haldi", a term derived from the Sanskrit word haridra, and in the South it is referred to as "manjal," a word often used in ancient Tamil literature. The term turmeric comes from the Latin word terra erita, referring to the colour of the ground turmeric, which resembles a mineral pigment. It is known in French as terre merite and in many languages simply as "yellow heart". In many cultures, its name is based on the Latin word *curcuma*. In Sanskrit, turmeric has at least 53 different names (Sahdeo and Bharat, 2011), including *anestha* (not offered for sacrifice or homa), *bhadra* (auspicious or lucky), *bahula* (plenty), *dhirgharaja* (long in appearance), *gandhaplashika* (which produces good smell), *gauri* (to make fair), *gharshani* (to rub), haldi (that draws attention to its bright colour), *haridra* (dear to hari, Lord Krishna), *harita* (greenish), *hemaragi* (exhibits golden colour), *hemaragini* (gives the golden colour), *hridayavilasini* (gives delight to heart, charming) and others. In Nigeria it is called *Kurna* in Hausa language (Nair, 2013).

Figure 2.1: *Curcuma longa* Plant

Source: Pinterest.com

Figure 2.2: *Curcuma longa* Rhizome

Source: Live Picture (February, 2019).

2.1.1 Taxonomic classification of *Curcuma longa*

The highest number-only diversity of Curcuma species is in India, with approximately 40 to 45 species. For example, Thailand has 30 to 40 species, but it is much smaller than India. Many tropical Asia countries also have multiple wild Curcuma varieties. Recent studies have also shown that *Curcuma longa's* taxonomy is problematic as only South India specimens can be classified as *C. longa.* There is still a need to establish and validate the phylogeny, relationships, intraspecific and interspecific variability, and even identification of other species and cultivars elsewhere in the world. There is still a need to establish and validate the phylogeny, relationships, intraspecific and interspecific variability, and even identification of other species and cultivars elsewhere in the world. It has been shown that

many species commonly used and marketed as "turmerics" in other parts of Asia belong to several visually different taxa, with overlapping local names (Nair, 2013).

Class:	Liliopsida
Subclass:	Commelinids
Order:	Zingiberales
Family:	Zingiberaceae
Genus:	Curcuma
Species:	*Curcuma longa*

2.1.2 Uses of Curcuma longa rhizome

Turmeric has been used in Asia for thousands of years and is a major part of Ayurveda, Siddha medicine, traditional Chinese medicine, Unani, (Kikusawa *et al.,* 2007) and the animistic rituals of Austronesian peoples. It was first used as a dye, and then later for its supposed properties in folk medicine (Nair, 2013).

Turmeric is one of the key ingredients in many Asian dishes, imparting a mustard-like, earthy aroma and pungent, slightly bitter flavor to foods (Brennan, 2008). It is used mostly in savory dishes, but also is used in some sweet dishes, such as the cake sfouf. In India, turmeric leaf is used to prepare special sweet dishes, patoleo, by layering rice flour and coconut-jaggery mixture on the leaf, then closing and steaming it in a special utensil (Imtiaz, 2016). Mostly turmeric is used in the form of rhizome powder to impart a golden yellow colour (Brennan, 2008). It is used in many products such as canned beverages, baked products, dairy products, ice cream, yogurt, yellow cakes, orange juice, biscuits, popcorn colour, cereals, sauces, and gelatin. It is a principal ingredient in curry powders. It

has numerous uses in East Asian recipes, such as pickle that contains large chunks of soft turmeric, made from fresh turmeric (Brennan, 2008).

Turmeric is used widely as a spice in South Asian and Middle Eastern cooking. Various Iranian khoresh dishes are started using onions caramelized in oil and turmeric, followed by other ingredients (Brennan, 2008). The Moroccan spice mix ras el hanout typically includes turmeric. In South Africa, turmeric is used to give boiled white
rice a golden colour, known as geelrys (yellow rice) traditionally served with bobotie. In Vietnamese cuisine, turmeric powder is used to colour and enhance the flavors of certain dishes, such as bánh xèo, bánh khọt, and mi quang. The staple Cambodian curry paste, kroeung, used in many dishes including amok, typically contains fresh turmeric.
In Indonesia, turmeric leaves are used for Minang or Padang curry base of Sumatra, such as rendang, sate padang, and many other varieties (Brennan, 2008). In Thailand, fresh turmeric rhizomes are used widely in many dishes, in particular in the southern Thai cuisine, such as yellow curry and turmeric soup. Turmeric is used in a hot drink called "turmeric latte" or "golden milk" that is made with milk, frequently coconut milk (Brennan, 2008). The turmeric milk drink known as haldi doodh (haldi means turmeric in Hindi) is a South Asian recipe. Sold in the US and UK, the drink known as "golden mylk" uses nondairy milk and sweetener, and sometimes black pepper after the traditional recipe (which may also use ghee) (Brennan, 2008).

The golden yellow colour of turmeric is due to Curcumin (Merina, 2003). It also contains an orange-coloured volatile oil (NIIR, 2006). Turmeric makes a poor fabric dye, as it is not very light fast, but is commonly used in Indian clothing, such as saris and Buddhist monks's robes (Peter, 2008). It is used to protect food products from sunlight (coded as E100 when

used as a food additive) (NIIR, 2006). The oleoresin is used for oil-containing products. A curcumin and polysorbate solution or curcumin powder dissolved in alcohol is used for water-containing products. Overcolouring, such as in pickles, relishes, and mustard, is sometimes used to compensate for fading. In combination with annatto (E160b), turmeric has been used to colour cheeses, yogurt, dry mixes, salad dressings, winter butter, and margarine (NIIR, 2006). Turmeric also is used to give a yellow colour to some prepared mustards, canned chicken broths, and other foods (Ravindran, 2007).

Turmeric paper, also called *Curcuma* paper or in German literature, Curcuma papier, is paper steeped in a tincture of turmeric and allowed to dry. It is used in chemical analysis as an indicator for acidity and alkalinity (Berger and Sicker, 2009). The paper is yellow in acidic and neutral solutions and turns brown to reddish-brown in alkaline solutions, with transition between pH of 7.4 and 9.2 (Berger and Sicker, 2009). Turmeric and its main bioactive component Curcumin are among the most studied spices in relation to health (Salehi *et al.*, 2019).

2.2 Chemical Composition of *Curcuma longa*

Curcuma longa contains 2 - 9 % Curcuminoids comprising of Curcumin, demethoxycurcumin and bisdemethoxycurcumin belonging to the diarylheptanoids (Salem *et al.,* 2014).

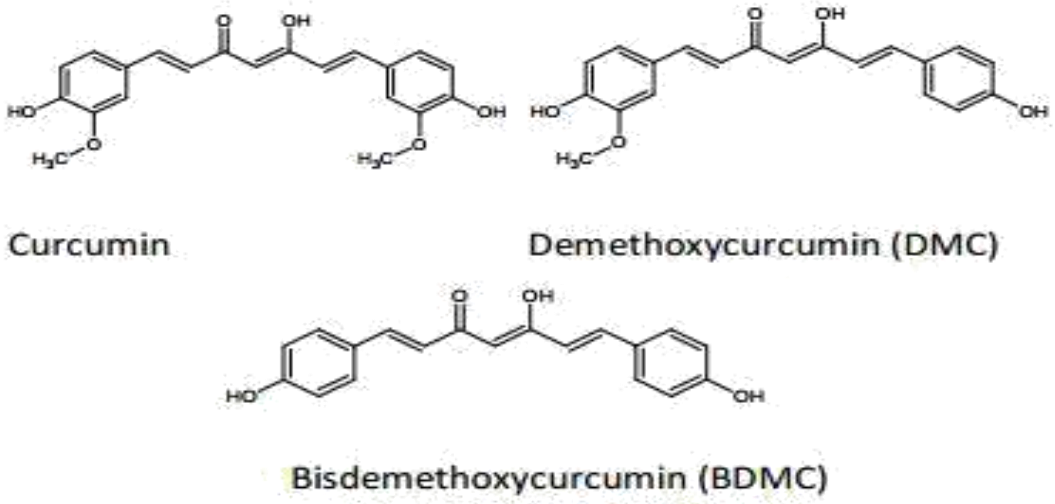

Figure 2.3: The Chemical Structure of Curcuminoids (Huang *et al.,* 2016).

Other compositions include, protein (6.3 %), fat (5.1 %), minerals (3.5 %), carbohydrates (69.4 %) and moisture (13.1 %). The essential oil (5.8 %) obtained by steam distillation of rhizomes has α-phellandrene (1 %), sabinene (0.6 %), cineol (1 %), borneol (0.5 %), zingiberene (25 %) and sesquiterpines (53 %). Curcuminoids (diferuloylmethane) (3–4 %) is responsible for the yellow colour, and comprises Curcuminoids I (94 %), Curcuminoids II (6 %) and Curcuminoids III (0.3 %) (Huang *et al.,* 2016).

2.3 Chemistry of Curcuminoids

Curcuminoid is a Curcumin, demethoxycurcumin, and bisdemethoxycurcumin complex. These are naturally occurring hydrophobic products made of two phenolic rings. In the ortho-oposition, each ring is substituted by methoxy ether functionality and connected to each other by an aliphatic unsaturated heptane linker in para-position with α, β carbon diketone-3 and -5. With a nucleophile such as glutathione, the electrophilic α, β-unsaturated carbonyl groups can react (Scapagnini *et al.,* 2002).

Curcumin tautomerization occurs in a pH-dependent mode. The bis-keto shape prevails in acidic and neutral solutions in this scenario while the enol shape prevails in alkaline solutions. While the heptane-linker's carbon-4 performance as a powerful source of protons was mentioned in the bis-keto form, the enol form works primarily as a source of electrons, the chemical activity responsible for curcumin's antioxidant characteristics (Sharma *et al.,* 2005).

Curcumin is available in the forms of enolic and β-diketone. While stable at acidic pH, instability breaks down into ferulic acid and feruloylmethane at neutral and basic pH. For the most part, the presence of curcumin in a solution is in the form of an enolic (Shenoy *et al*., 2011) and this fact has a significant impact on its radical scavenging capacity. Curcumin's enolic tautomer is considered the significant contributor to its bioactivity and its photophysical and photochemical characteristics (Sharma *et al.,* 2009). Curcumin's stability level is low, and when exposed to adverse physiological situations, degradation occurs (Sharma *et al.,* 2009).

2.4 Biological and Medicinal Potentials of *C. longa* Extracts

The yellow colour is accountable for the Curcuminoids (diferuloylmethane) complex. It has many medicinal properties including chemo-preventive activities of antioxidant, anti-inflammation, antiviral, antibacterial, antifungal, antiparasitary and anticancer (Wang *et al.,* 2015). The potential agents for the prevention and therapy of various cancers including gastrointestinal, breast, lung, melanoma, head and neck, neurological and sarcoma diseases have been shown to be Curcuminoids (Anand *et al.,* 2008; Ravindran *et al.,* 2009; Bar-Sela *et al.,* 2010). Studies have been conducted on a Curcuminoid–essential oil complex, showing that it has therapeutic efficacy in numerous diseases like major depressive

disorders, Alzheimer‟s disease, and rheumatoid arthritis and has potential for widespread application and radioprotective effect in different kinds of cancer (Dhaneshwar *et al.,* 2019). *Curcuma longa*, called the golden spice, has a place in every Indian kitchen. Turmeric and its main bioactive component Curcumin are among the most studied spices in relation to health (Hewlings and Kalman, 2017). For years, researchers have been trying to tap into several medicinal properties of Curcumin. It has powerful killer of cancer cells. Unfortunately, not much research on its potential chemotherapeutic nature has been possible as curcumin is not water-soluble and it is poorly absorbed into the bloodstream. However, instead of looking at dead ends, the researchers at the Biomedical Technology wing of Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram, developed a way to not only solve the issue of water-solubility but also a vehicle to carry it to the treatment site in the body (Oshin, 2020).

The Table 2.1 presents the summary of Biological and medicinal activity of turmeric extracts.

Table 2.1: Biological Activity of *C. longa* and its Compounds.

Compound/extract	Biological activity
Turmeric powder	Wound-healing
Ethanol extract	Anti-inflammatory Hypolipemic, Anti-tumor, Anti-protozoan
Petroleum ether extract	Anti-inflammatory, Antifertility
Alcoholic extract	Antibacterial
Crude ether extract	Antifungal
Chloroform extract	Antifungal
Aqueous extract	Antifertility

Volatile oil	Anti-inflammatory, Antibacterial, Antifungal
Curcuminoids	Antibacterial, Anti-protozoan, Antiviral, Hypolipemic, Hypoglycemic, Anticoagulant, Antioxidant, Anti-tumor, and Anticarcinogenic
Ar-turmerone	Antivenom
MethylCurcuminoids	Anti-protozoan
DemethoxyCurcuminoids	Antioxidant
BisdemethoxyCurcuminoids	Antioxidant
Sodium Curcuminoidsate	Anti-inflammatory, antibacterial

Source: (Bar-Sela *et al.,* 2010).

2.5 Metabolism of Curcuminoids

When administered orally, curcuminoids have poor bioavailability in the human body. It is due to their rapid degradation and poor gastrointestinal tract absorption, resulting in low plasma concentrations and a very low tissue distribution. In the body, curcumin is converted to dihydrocurcumin (DHC), tetrahydrocurcumin (THC), hexahydrocurcumin (HHC), and octahydrocurcumin with reductase activity, and is further decomposed by the action of β-glucuronidase to dihydrocurcumin-glucuronide and tetrahydrocurcumin-glucuronide. Poor absorption was observed in a clinical trial where for an hour oral intake of 3.6 g of standardized extract per day was observed. After intake, the plasma concentration was only 11.1 mmol/L (Sharma *et al.*, 2004). Because of this limitation, different curcumin formulations have been tested to maximize its availability and activity. In addition to oral intake, subcutaneous intake was investigated and proved effective in maintaining curcumin in tissues for a longer period of time (Shahani *et al.,* 2010). For the

treatment of tumors in animals, intravenous intake in the form of nanoparticles has been proved effective. Curcumin in the liposomal form is effective in enhancing the effect of chemotherapy (Wang *et al.,* 2008; Kim *et al.,* 2011; Shih *et al.,* 2015). The use of curcumin on the skin is very effective in treating certain types of psoriasis, wound healing, candidiasis (Prasad *et al.,* 2014). Oral intake in the form of nanoparticles increases the availability of curcumin 5-6 times compared to the standardized extract, as it dissolves better in the digestive system due to increased water solubility and its degradation is longer (Prasad *et al.,* 2014). Nanoparticles are also safe, non-toxic, biodegradable, do not cause body allergies, and can be designed to release time (Sankar *et al.,* 2016). It also increases bioavailability by combining curcumin with piperine, liposomal and phospholipid complexes (Prasad *et al.,* 2014). A hydrophilic carrier dispersed Curcuminoids formula has a bioavailability of 95 percent purified Curcuminoids preparation 46 times (Douglass and Clauatre, 2015).

2.6 Antioxidant Properties of Curcuminoids

As a food additive, curcuminoids have very important antioxidant characteristics. The oxidation of linoleic acid is very slow in the presence of curcuminoids, and when used as a nutritional supplement, the antioxidant impact is about 80 % (Jayaprakasha *et al.,* 2006). It operates in a manner that connects free radicals and a hydrogen atom (responsible for its antioxidant characteristics) is also donated (Jayaprakasha *et al.,* 2006). Curcuminoids have the property of donating electrons to neutralize free radicals through the creation of stable products, breaking a chain reaction of generating free radicals in a living organism. Higher capacity of curcuminoids to capture hydrogen peroxide than commercial antioxidants (BHA, BHT, vitamin E) at the same concentration (20 mM) (Hussein *et al.,* 2016). In

cellular models, Curcumin treatment suppresses free radical production and increases expression of antioxidant enzyme genes (Abrahams *et al.,* 2019). Curcuminoids have a major impact on hepatic oxidative stress in elevated cholesterol diets by decreasing antioxidant liver enzymes (catalase, glutathione peroxidase, and superoxide dismutase), as well as decreasing TNF-α (alpha tumor necrosis factor), IL-6 serum (interleukin 6), liver enzymes, and enzyme markers for heart illness. Curcumin supplementation can decrease cardiovascular issues in patients with hyperlipidemia (Hussein *et al.,* 2016). Curcuminoids have been used as a remedy for many organ and tissue illnesses, most of which are associated with inflammation and oxidative stress, to prevent and treat them. Curcuminoids alleviate oxidative stress, chronic disease inflammation, and regulate most chronic disease-related inflammatory and pro-inflammatory pathways (Anthwal *et al.,* 2014).

2.7 Mechanism of Action of Curcuminoids as Antioxidant

Curcuminoids ' antioxidant activity and free radical reactions are strongly associated with their phenolic O-H and C-H. Curcuminoids ' antioxidant mechanism is based on the phenolic group's H-atom abstraction, not the heptadienone link's core CH_2 group. Curcuminoids, methylcurcuminoids and half-curcuminoids with comparable O-H composition indicate that the two phenolic groups are separate (Priyadarsini *et al.,* 2013).

The chemical structure of Curcuminoids makes it act as a natural free radical scavenger.

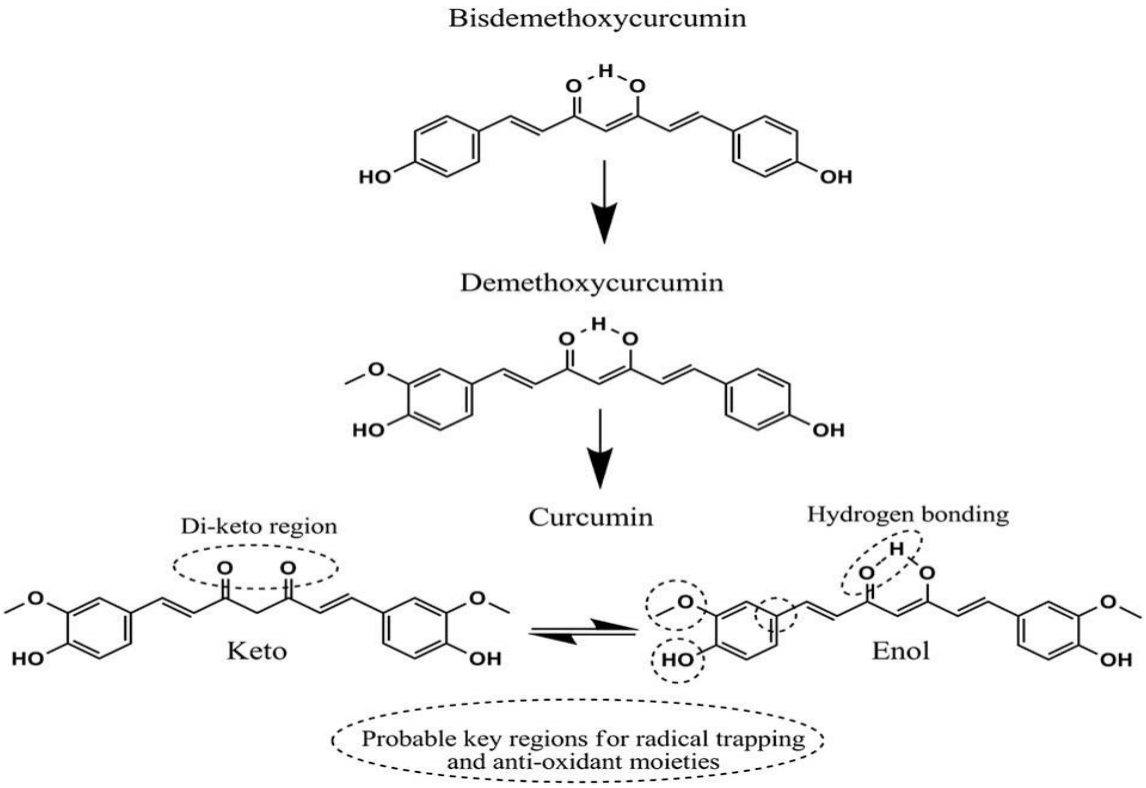

Figure 2.4: The Crucial Sites for the Antioxidant Potential of Curcumin and how it Transforms from Bisdemethoxycurcumin to Curcumin (Irving *et al*., 2011).

2.8 Antioxidant Enzymes

Reactive Oxygen Species (ROS) intracellular concentration relies on the antioxidant system's manufacturing and/or removal. Cells comprises of large amount of antioxidants to avoid or repair the harm induced by ROS and to control the signaling pathways that are redox sensitive. Three of the main antioxidant enzymes found in mammalian cells that are believed to be vital in all oxygen metabolizing cells, viz; superoxide dismutase (SOD), catalase, and substrate-specific peroxidase, glutathione peroxidase (GPx) (Liu, 2004).

2.8.1 Superoxide dismutase

There are three extremely compartmentalized enzymes of SOD. Manganese-containing superoxide dismutase (MnSOD) is situated in the mitochondria; copper and zinc-containing superoxide dismutase (CuZnSOD) is situated in the cytoplasm and nucleus and extracellular SOD (ECSOD0) is extracellularly produced in certain tissues. In a eukaryotic cell, CuZnSOD comprises about 90 percent of complete SOD activity (Liu, 2004). A tiny percentage of this enzyme was discovered in cellular organelles such as lysosomes, peroxisomes, and the nucleus in addition to its main distribution in the cytosol (Sturtz *et al*., 2001). Recently, the presence of CuZnSOD (approximately 2 %) in the intermembrane space of mitochondria (Sturtz *et al*., 2001) has been shown to be important in providing further protection against ROS and in preventing the leakage of superoxide radicals from the mitochondria (Sturtz *et al*., 2001).

2.8.1.1 Chemical reactions

SODs catalyze the disproportionation of superoxide:

$$2\ HO_2 \rightarrow O_2 + H_2O_2$$

In this way, O_2^- is converted into two less damaging species. The pathway by which SOD-catalyzed dismutation of superoxide may be written, for Cu,Zn SOD, with the following reactions :

- Cu^{2+}-SOD + O_2^- → Cu^{+}-SOD + O_2 (reduction of copper; oxidation of superoxide)
- Cu^{+}-SOD + O_2^- + $2H^+$ → Cu^{2+}-SOD + H_2O_2 (oxidation of copper; reduction of superoxide)

The general form, applicable to all the different metal-coordinated forms of SOD, can be written as follows:

- $M^{(n+1)+}$-SOD + O_2^- → M^{n+}-SOD + O_2
- M^{n+}-SOD + O_2^- + $2H^+$ → $M^{(n+1)+}$-SOD + H_2O_2.

Where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).

In a series of such reactions, the oxidation state and the charge of the metal cation oscillates between n and n+1: +1 and +2 for Cu, or +2 and +3 for the other metals.

2.8.2 Catalase

Catalase converts hydrogen peroxide to water and oxygen. Catalase activity is largely found in the peroxisomes of subcellular organelles. Targeted delivery of catalase to the liver through galactosylation suppresses hepatic metastasis and reduces the activity of matrix metalloproteinase (MMP), while a reduction in catalase correlates with the development of malignant phenotype in keratinocytes in the mouse. Catalase also reduces basal and MnSOD-dependent expression of MMPs and deposition of collagen (Nelson *et al.,* 2003).

2.8.2.1 Chemical reactions

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

2.8.2.2 Molecular mechanism of catalase activity

While the complete mechanism of catalase is not currently known the reaction is believed to occur in two stages (Boon *et al.,* 2007):

$$H_2O_2 + \text{Fe (III)-E} \rightarrow H_2O + \text{O=Fe (IV)-E (+)}$$

$H_2O_2 + O{=}Fe\ (IV)\text{-}E\ (+) \rightarrow H_2O + Fe\ (III)\text{-}E + O_2$

Here Fe (IV)-E represents the iron center of the heme group attached to the enzyme. Fe (IV)-E (+) is a mesomeric form of Fe (V)-E, meaning the iron is not completely oxidized to +V, but receives some stabilising electron density from the heme ligand, which is then shown as a radical cation (+)

As hydrogen peroxide enters the active site, it interacts with the amino acids Asn148 (asparagine at position 148) and His75, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, freeing the newly formed water molecule and Fe(IV)=O. Fe(IV)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen (Boon *et al.,* 2007). The reactivity of the iron core can be enhanced by the existence of Tyr358's phenolate ligand in the fifth coordinating place, which can help in Fe(III) to Fe(IV) oxidation. The response effectiveness can also be enhanced by the relationships between His75 and Asn148 and intermediate response (Boon *et al.,* 2007). Catalase can also catalyze multiple metabolites and toxins, including formaldehyde, formic acid, phenols, acetaldehyde and alcohol, through hydrogen peroxide. It does that according to the following reaction:

$H_2O_2 + H_2R \rightarrow 2H_2O + R$

The exact mechanism of this reaction is not known. Any heavy metal ion (such as copper cations in copper (II) Sulfate) can act as a non-competitive inhibitor of catalase (Boon *et al.,* 2007).

2.8.3 Glutathione peroxidase

Cytosolic glutathione peroxidase (GPx, GPx1) is a Selenoprotein that is first defined as an enzyme that protects hemoglobin from red blood cell oxidative degradation. GPx needs several secondary enzymes (Glutathione reductase and Glucose-6-phosphate dehydrogenase) and cofactors (glutathione reduction, NADPH reduction and glucose-6-phosphate) to work at elevated effectiveness (Liu, 2004). As stated above, there are five GPx isoenzymes considered to be a significant enzyme responsible for removing H_2O_2. These enzyme's over-expression protects cells against oxidative harm (Tang *et al.,* 2015) suppresses H_2O_2-induced apoptosis and reverses the pancreatic cancer's malignant phenotype (Liu, 2004).

2.8.3.1 Chemical reactions

The main reaction that glutathione peroxidase catalyzes is:

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

Where GSH is a reduced monomeric glutathione and where GS–SG is a glutathione disulfide. The mechanism involves oxidation by hydrogen peroxide of selenol from a residue of selenocysteine. This process provides the selenenic acid (RSeOH) group derivative. The selenenic acid is then converted back to selenol through a two-step process that begins to form the GS-SeR and water with GSH reaction. A second molecule of GSH reduces the intermediate GS-SeR back to selenol, releasing GS-SG as the by-product. Below is a simplified portrayal (Bhabak and Mugesh, 2010).

$$RSeH + H_2O_2 \rightarrow RSeOH + H_2O$$

$$RSeOH + GSH \rightarrow GS\text{-}SeR + H_2O$$

$$GS\text{-}SeR + GSH \rightarrow GS\text{-}SG + RSeH$$

Glutathione eductase then reduces the oxidized glutathione to complete the cycle:

$$GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+.$$

2.9 Glutathione Sulfhydrate (GSH)

GSH is a tripeptide (γ-glutamylcysteinylglycine), which is commonly distributed in a multitude of tissues. Cysteine's sulfhydryl (thiol) group (SH) acts as a proton donor and is accountable for glutathione's biological activity. An antioxidant, GSH helps safeguard cells against ROS. GSH removes H_2O_2 and responds to CYP450-mediated CCl_4 metabolites with trichloromethyl-free radical (Pompella *et al.,* 2003; lee *et al.,* 2007). GST is a detoxifying enzyme in stage II that plays a major role in cell detoxification and xenobiotics excretion. This enzyme protects cells from toxicants by combining them with GSH, neutralizing their electrophilic locations and making them more water-soluble (Wang *et al.,* 2007). GSH is a cofactor for Glutathione S-Transferase (GST), which is an abundant cellular enzyme in the tissue. The GSH conjugates are further metabolized and excreted into mercapturic acid. Some compounds have been discovered to safeguard the liver that boost GST activity, which metabolizes toxic compounds into non-toxic compounds (lee *et al.,* 2007).

2.10 Mechanism of Action of Antioxidant Enzymes

Many aerobic cellular metabolic processes produce Reactive Oxygen Species (ROS). They include, but are not restricted to, species like superoxide and hydrogen peroxide that respond with different intracellular objectives, including lipids, proteins, and DNA (Cerutti, 2000). Although ROS is produced during ordinary aerobic metabolism, the biological impacts of ROS on these intracellular objectives depend on their concentration, and during oxidative stress there are enhanced levels of these species. Oxidative stress is described as a disruption between the manufacturing of reactive oxygen species (free radicals and reactive

metabolites) and the elimination of antioxidant defenses by protective mechanisms. This imbalance creates harm to significant biomolecules and cells as well as potential organismal effects (Durackova *et al.,* 2010). Increased concentrations of ROS are cytotoxic, while reduced concentrations are needed to regulate several important physiological processes including cell differentiation, apoptosis, cell proliferation, and pathways for redox-sensitive signal transduction. However, enhanced concentrations, including cell death, mutations, chromosomal aberrations, and carcinogenesis, can also lead in ROS-induced harm. Enzymatic and molecular antioxidants such as SOD, CAT, GST, and GSH can suppress the overproduction of reactive cytotoxic molecules induced by CCl4 metabolism (Weber *et al*., 2003; E1-Sayed *et al*., 2006). Conversion of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2), thus forming a vital aspect of cellular antioxidant defense systems.

The SODs transform radical superoxide into hydrogen peroxide and molecular oxygen (O_2), while catalase and peroxidae transform hydrogen peroxide into water and catalase into oxygen and water. The net outcome is the transformation into water of two possibly damaging species, superoxide and hydrogen peroxide. In order to work, SOD and catalase do not need co-factors, while GPx needs not only several co-factors and proteins, but five isoenzymes. Glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) do not operate directly on ROS in the glutathione scheme, but allow the GPx to work (Liu, 2007).

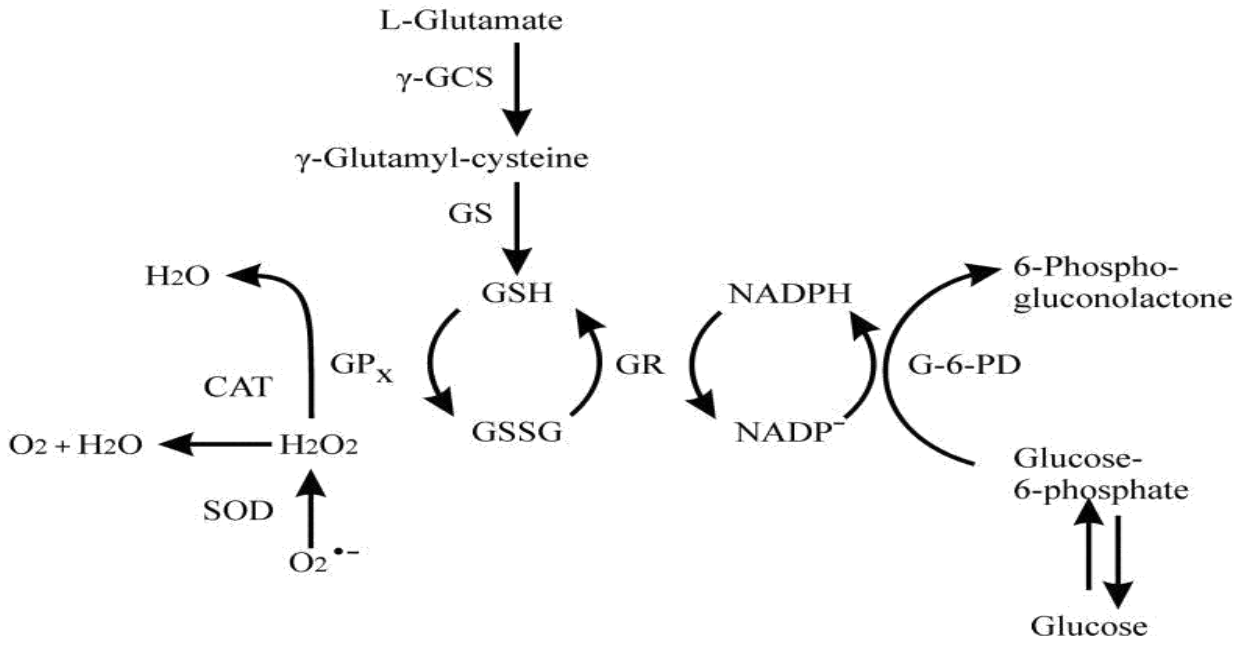

Figure 2.5: Schematic Presentation of Some Antioxidant Enzymes Activities (Sturtz *et al.,* 2001).

The above system demonstrates the three main kinds of antioxidant enzymes in mammalian cells-SOD, catalase, and peroxidase, the most prominent of which is glutathione peroxidase (GPx). The SODs transform $O2^{\bullet-}$ into H_2O_2, while H_2O_2 is converted into water by catalases and peroxidases. If H_2O_2-removal is inhibited, then H_2O_2-mediated harm causes immediate toxicity (Sturtz *et al.,* 2001). GPx needs several secondary enzymes to operate at elevated effectiveness, including glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G-6-PD) and cofactors including glutathione (GSH), NADPH, and 6-phosphate glucose (Okado-Matsumoto, 2001; Sturtz *et al.,* 2001). If GR is inhibited, cells cannot remove H_2O_2 through the mechanism of glutathione peroxidase and increase glutathione disulfide (GSSG) concentrations. If glutathione synthesis is inhibited by either inhibiting gluthatione synthetase (GS) or π-glutamyl cysteine synthetase (π-GCS), glutathione is depleted and H_2O_2 cannot be removed by GPx. Cells also cannot remove

H_2O_2 if catalase is inhibited. Finally, if glucose uptake creating a chemical deprivation of glucose is inhibited, detoxification of hydroperoxide will also be inhibited (Sturtz *et al.,* 2001).

2.11 General Principles for Antioxidant Enzymes Assay

General protocols for antioxidant activity include the measurement of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPx) activity. The SOD converts radical superoxide into hydrogen peroxide and molecular oxygen, while hydrogen peroxide is converted into water by catalase and peroxides. Two toxic species, superoxide radical and hydrogen peroxide, are thus transformed into the harmless water of the product (Christine and Joseph, 2011). Western blots, gels of exercise and assays of activity are different techniques of determining protein and activity in both cells and tissue based on the quantity of protein required for each assay. Other methods, including immunohistochemistry and immunogold, may further assess the concentrations in tissue and cells of the different antioxidant enzymes. In general, it takes 24 to 48 hours to finish these assays (Christine and Joseph, 2011).

2.12 Anatomy and Physiology of the Liver

The liver is the biggest strong organ, the biggest gland and one of the most essential organs that serves as a center for nutrient metabolism and waste metabolite excretion (Ozougwu and Eyo, 2014). Its main role is to regulate the flow and safety of drugs absorbed from the digestive system prior to their allocation to the systemic circulatory system of these drugs. A complete loss of liver function within minutes could lead to death, proving the excellent significance of the liver (Ozougwu, 2014).

2.12.1 Origin of the liver

The cells that ultimately form the adult liver originate from the ventral foregut definitive endoderm during embryogenesis (Watt *et al.,* 2007). The various phases of liver development require the development of skills for liver formation, after which liver specification, hepatic bud formation, development and ultimately differentiation will happen (Burke *et al.,* 2006). The metabolic profile of the youthful liver is far from that of the adult phenotype during the growth of the liver, as well as some length after partus. A lot of metabolic modifications happen in the liver before birth and soon thereafter (Burke *et al.,* 2006). These enable the organism to adapt but also alter its capacity to metabolize xenobiotics to absorb nutrients from food. As the organism matures, an adult metabolic enzyme pattern grows with length. The gene expression pattern of the hepatocytes often returns to a more fetal-like phase during the growth of the hepatocellular carcinoma (Burke *et al.,* 2006). This contributes in some instances to the expression of otherwise discovered metabolic enzymes only during embryogenesis. In many human hepatoma cell lines, which are often used for in vitro toxicology research, this partially fetal-like pattern of expression is also visible (Burke *et al.,* 2006).

2.12.2 General description of the liver

The liver weighs about 1500 g and represents about 2.5 % of adult body weight (Moore and Dalley, 2006). The liver surface is soft and dome-shaped, where it is associated with the concavity of the diaphragm's lower surface. The liver lies primarily in the right upper abdomen quadrant where the thoracic cage and diaphragm hide and protect it. The ordinary liver lies deep on the correct side of the ribs 7–11, crossing the midline to the left nipple (Moore and Dalley, 2006). The liver is divided into four lobes: right, left, caudate, and

quadrate. The biggest are the correct and left lobes, while the narrower caudate and quadrate are later situated. Two ligaments are previously noticeable. The falciform ligament, superiorly, divides the lobes of right and left. The round ligament, which protrudes slightly from the liver, is lower than the falciform ligament. The gallbladder is also noticeable above on the lower part of the right lobe. Much more interesting structures are noticeable subsequently. Superior location of the caudate lobe, is roughly between the lobes of right and left. The sulcus for the lower vena cava is adjacent to the caudate lobe. The porta hepatis, where the hepatic artery and the hepatic portal vein enter the liver, is just below the caudate lobe. The portal vein carries from the digestive system nutrient-laden blood. The bile duct that leads back to the gallbladder is lower than the porta hepatis. For the inferior vena cava, the hepatic vein, where post-processed blood leaves the liver, is discovered to be lower and adjacent to the sulcus. The liver is later kept on by a mesentery mechanism and is also connected via the falciform ligament to the diaphragm. Visceral peritoneum covers most of the liver ((Moore and Dalley, 2006).

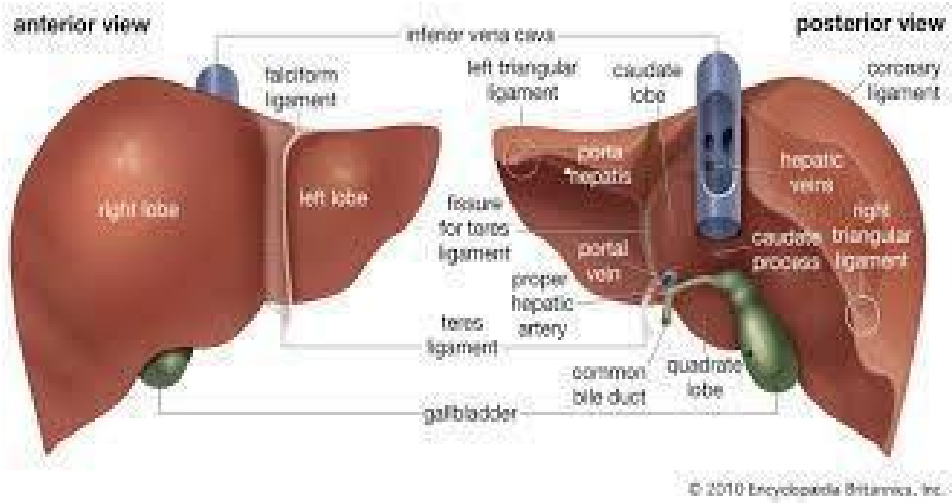

Figure 2.6: Diagram of the Liver Showing the Right and Left Lobes and its Posterior and Anterior Views.
Source: Encyclopedia Britannica, (2010).

2.12.3 Functions of the liver

The liver is generally classified into bile production, bilirubin metabolism, vascular and hematological functions, nutrient metabolism, mitochondrial detoxification, and mineral and vitamin accumulation (Guyton, 2006). These functions has to do with; Bile Secretion, Bilirubin metabolism, Vascular and hematological roles (Essential blood supply), Nutrient metabolism (oxidation of fatty acids, cholesterol / lipoprotein synthesis and ketoacid production, protein–development of amino acid, protein turnover, and transforms galactose /fructose into glucose, gluconeogenesis and contains 100 g of regenerative glycogen), metabolism detoxification of Drugs, mineral and vitamin conservation (e.g Iron, Copper, vitaminutes A, D, E, K and B12 and Glycogen. The liver also performs endocrine functions such as; Vitamin D activation, Thyroxine (T4) transformation to T3, secretes angiotensinogen and metabolizes hormones. Immunological / protective role is carried out by the liver. Its Reticuloendothelial elements include; (Filtering of portal blood from bacteria, essential antigen presentation, phagocytosis by kupffer cells and removal of hemolytic products. Inactivation of toxins and drugs (Phase I (oxidation, elimination and hydrolysis). Phase II (conjugation / cytochrome P450 system) is done by the liver (Guyton, 2006).

2.12.4 Causes of liver diseases

Liver disorders are the most common health hazard found in developing countries due to dietary habits, alcohol ingestion, poor hygiene, unsupervised drug use and smoking. Liver diseases can be non-inflammatory, inflammatory and degenerative. High levels of plasma total cholesterol (LDL-C) and triacylglycerols (TGs) are associated with high risk of atherosclerosis and cardiovascular disease owing to the hepatic ill-function (Dominiczak,

2005; Ekaidem *et al.,* 2007). Hepatotoxicity caused by many toxins; Carbon tetrachloride (CCl_4), thioacetamide, acute or chronic alcohol consumption, various like infections like hepatitis A, B, C and drugs, in which drugs are most common cause. Free radical generations in alcohol use result in development of hepatitis leading to cirrhosis (Anand and Lal, 2016).

2.12.5 Histology of the liver

The basic functional unit of the liver is the liver lobule. A single lobule is about the size of a sesame seed and is roughly hexagonal in shape (Moore and Dalley, 2006). The primary structures found in a liver lobule include: Plates of hepatocytes which forms the bulk of the lobule, Portal triads at each corner of hexagon, Central vein, Liver sinusoids that run from the central vein to the portal triads, Hepatic macrophages (Kupffer cells), Bile canaliculi (little canals‖) formed between walls of adjacent hepatocytes and Space of Disse – a small space between the sinusoids and the hepatocytes. The portal triads consist of three vessels; a hepatic portal arteriole, a hepatic portal venule, and a bile duct. The blood from the arteriole and the venule both flow in the same direction – through the sinusoids toward the central vein, which eventually leads to the hepatic vein and the inferior vena cava. Secreted bile flows in the opposite direction – through the bile canaliculi away from the central vein, toward the portal triad, and exiting via the bile duct. As blood flows through the sinusoids and the space of disse toward the central vein, nutrients are processed and stored by the hepatocytes, and worn out blood cells and bacteria are engulfed by the Kupffer cells (Allen, 2002).

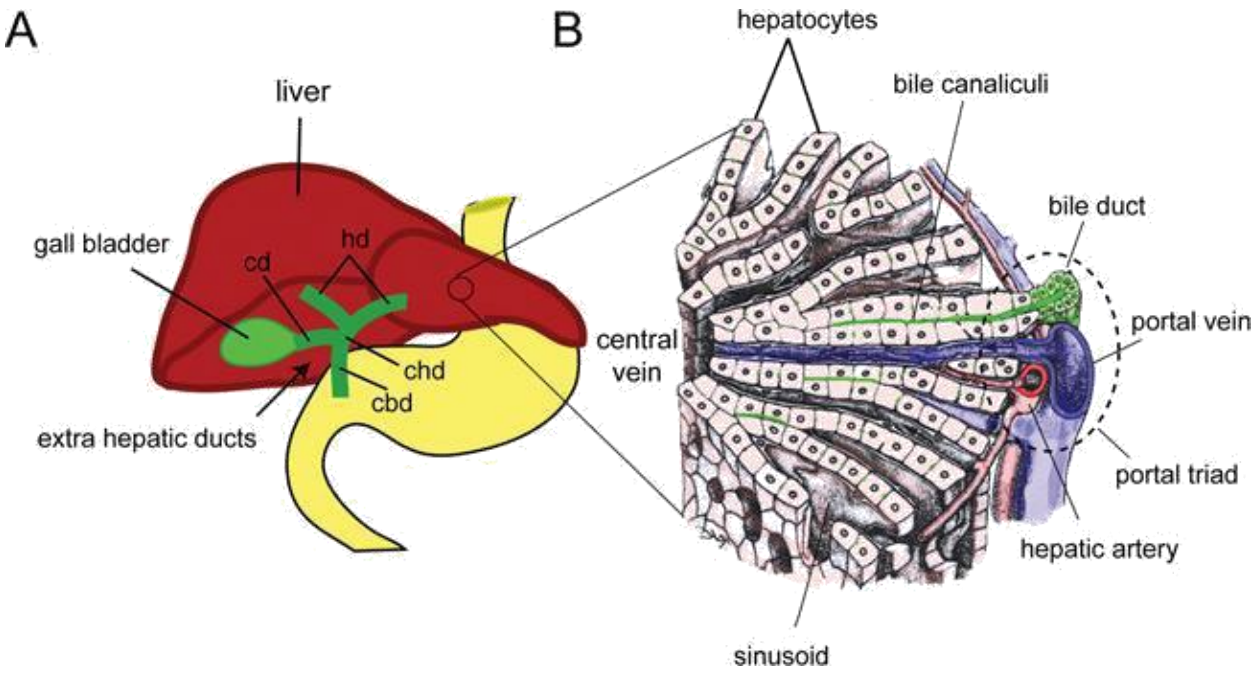

Figure 2.7: Cellular Architecture of the Liver

Source: Zorn, 2013.

(A) The schematic shows an adult liver (red), with the gall bladder and extra hepatic ducts (green), in relation to the stomach and intestine (yellow). The extra hepatic duct system consists of the hepatic ducts, which drain bile from the liver into the common hepatic duct to the gall bladder via the cystic duct and into the duodenum through the common bile duct. (B) A schematic of the cellular architecture of the liver showing the hepatocytes (pink) arranged in hepatic plates separated by sinusoid spaces radiating around a central vein. Bile canaliculi on the surface of adjoining hepatocytes drain bile into the bile ducts (green), which run parallel to portal veins (blue) and hepatic arteries (red) to form the "portal triad" (Zorn, 2013).

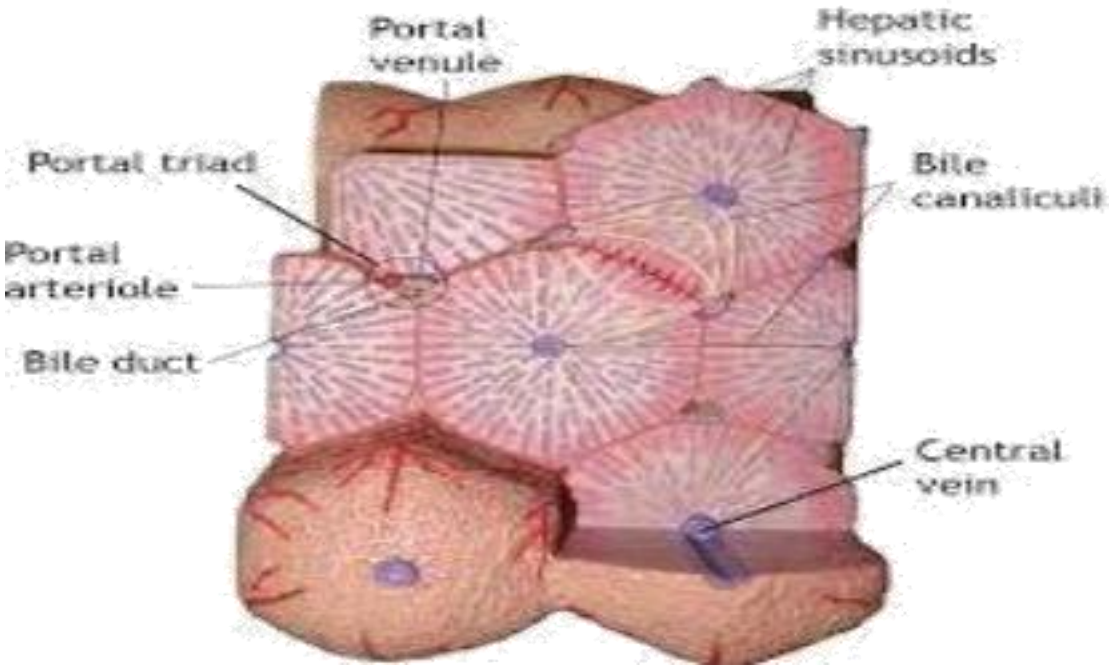

Figure 2.8: Diagram Showing Liver Lobules

Source: Zorn, 2013

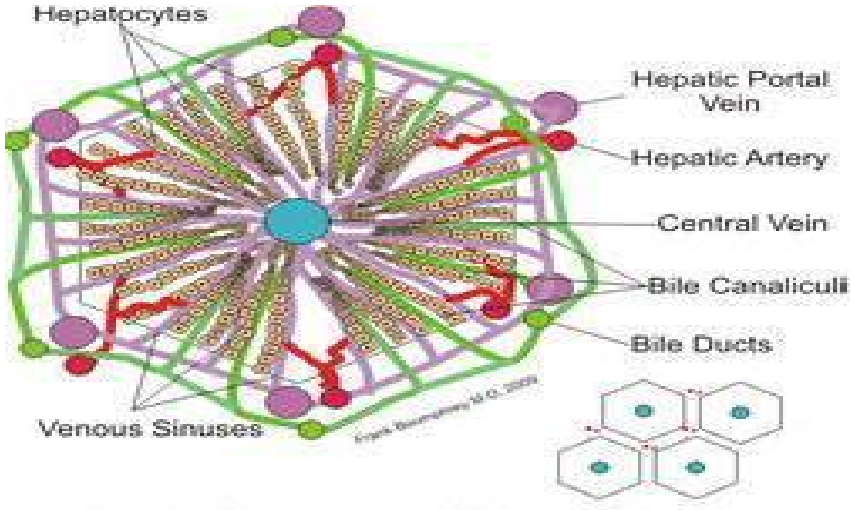

Figure 2.9: Basic Structure of Liver Lobule

Source: Arora, 2012.

The liver is the first site of passage for venous blood arriving from the intestines via vena porta (Butura, 2008). The areas around the influx blood vessels are named periportal. The areas surrounding efflux blood vessels are the perivenous. The periportal area is highly complex and consists of a dense matrix containing collagen where afferent blood vessels are found, together with bile ducts, nerve and lymph (Butura, 2008). Spaces within the matrix contain a variable cell population, such as fibroblasts, hematopoietic cells and inflammatory cells. Also found here are epithelial cells of the bile ducts, endothelial cells of the blood vessels, and smooth muscles of arteries and veins (Kmiec, 2001). The liver lobule consists mainly of plates of hepatocytes and sinusoids, with a light matrix of collagen to form a network between the two. Kupffer cells, as well as fat storing stellate. These types of cells reside mainly in the tissue space between the hepatocyte and the sinusoids. Terminal bile ductules connect here to the bile canaliculi between hepatocystic plates (Kmiec, 2001). The walls of the hepatic sinusoid are lined by three different cell types: the sinusoidal endothelial cells, kupffer cells and stellate cells. Additionally, pit cells, the liver specific Natural Killer (NK) T cells are often present in the sinusoidal lumen (Kmiec, 2001). The main parenchymal mass is normally that of hepatocytes. In rat, the hepatocytes make up about 60 % of liver cell count and the remaining 40 %, non-parenchymal cells only make up for about 6 -7 % of the liver volume while the remaining volume of approximately 23 % is formed by extracellular spaces (Hendriks *et al.,* 2005). The liver is made up of many different cell types, which include; hepatocytes, endothelial cells, kupffer cells (liver resident macrophages) and Stellate cells (liver fat storing cells).

2.12.5.1 Hepatocytes

Hepatocytes account for 60 % of the proteins of the liver and about 80 % of the complete cell volume of the liver. Most of the synthetic and metabolic capacities of the liver are derived from hepatocyte job (Allen, 2002). Hepatocytes are only one cell dense in plates (Singh, 2007). Blood flowing into the hepatic vein within the Disse room moves through both the exposed surface fields of the hepatocyte plates and the hepatocyte extracts toxins and nutrients within the blood (Singh, 2007). As well as detoxifying and excreting cholesterol, steroid hormones and xenobiotic drugs, hepatocytes generate and secrete bile. The mixed functions of monoxidases discovered in hepatocytes metabolize numerous xenobiotics (Singh, 2007). Periportal sort of hepatocytes are often lower, but they have bigger mitochondria and a bigger Golgi device compared to perivial type. On the other side, perive hepatocytes have greater endoplasmic reticulum. Functionally, periportal hepatocytes are more engaged in gluconeogenesis and glycolysis involves perivous (Butura, 2008).

2.12.5.2 Endothelial cells

The endothelial sinusoidal cells line the hepatic sinusoid walls and perform a filtration function due to the presence of fenestrae (Butura, 2008). These cells also show large endocytic capacity for components of the extracellular matrix and immune complexes. They envelop smaller particles in general and may play a role in virus clearance, but do not have phagocytic function. They may also function as cell presenting antigen and secrete some cytokines and eicosanoids (Kmiec, 2001).

2.12.5.3 Kupffer cells

The liver harbors big quantities of Kupffer cells representing the body's biggest resident tissue macrophage population. They are situated within the sinusoid and are in constant contact with gut-derived particles, leading to small but continuous activation of these cells derived from monocytes. A wide variety of inflammatory mediators such as cytokines, reactive oxygen species, eicosanoids and nitric oxide can be secreted upon activation (Friedman, 2008). Kupffer cells have receptors that allow them to bind or bind immunoglobulin-covered cells to complement receptors and phagocytosis cells subsequently (Friedman, 2008).

2.12.5.4 Stellate cells

The liver plays a key role in vitamin A (Retinol) uptake and storage and stores about 95 % of the retinoid found in the body. The primary storing cells of vitamin A are the fat storing perisinusoidal cells of the liver, stellate cells. Inside their cell cytoplasm, they harbor big quantities of retinol and retinylpalmitate in lipid droplets (Friedman, 2008). They are situated in Disse room (between hepatocytes and sinusoid) and tend to come into contact with several sinusoids in general (Friedman, 2008). They also work to control the extracellular matrix turnover and regulate the contractility of the sinusoid. Under stressful circumstances, stellate cells can become activated and converted into myofibroblast–like cells that play a major role in inflammatory fibrotic reaction (Butura, 2008). Not only do stellate cells proliferate when activated, they also generate enhanced quantities of extracellular matrix per cell. One of the most significant signals for activating stellate cells is transforming growth factor beta (TGF β), which results in a greater transcription level of mRNAs coding for extracellular matrix elements such as collagen I, fibronectin, and

proteoglycans (Butura, 2008). Lipid peroxidation products are also a significant stimulus whose impact can be increased in circumstances of oxidative stress (Friedman, 2008).

2.13 Hepatotoxicity

Liver is the principal site for metabolism and excretion in the body. The human liver metabolizes substances by various biochemical pathways including oxidation, reduction, hydration, condensation, hydrolysis, conjugation or isomerization (Reema and Pankaj, 2018). Disorder of any of the afore process may lead to liver cell injury (hepatotoxicity), which in turn leads to many diseases. Such diseases are responsible for higher mortality rates worldwide. Hepatotoxicity can be due to medicines, chemicals, dietary disturbances or herb induced liver damage via hepatotoxins (Reema and Pankaj, 2018). A number of herbal and herbomineral are available in the Ayurveda, the traditional Indian Medicine which have been investigated for their Hepatoprotective potency to treat different types of liver disorders (Reema and Pankaj, 2018).

The liver tolerates maximum role in detoxifying the various toxins present in the food, drinks, drugs and environment. Many risk factors predispose an individual to drug injury such as liver disease, aging, female sex organ and genetics. Liver being a major site of metabolism plays a pivotal role in detoxification of various toxins ingested and or produced during absorption of the food material (Osadebe, *et al.,* 2012; Rane, *et al.,* 2016). The liver usually filters all the blood from the digestive tract, before passing it to the rest of the body to avoid entering of the toxins into the other body system (Dienstag and Isselbacger, 2001). It utilizes metabolic pathways for energy production, metabolism and reproduction to control almost all systems of the body. Liver also synthesizes many complementary

proteins for immune system (Dey *et al*., 2013). Therefore, healthy liver is key to healthy individual.

2.13.1 Mechanism of CCl_4 hepatotoxicity

Carbon tetrachloride (CCl_4) is a highly toxic chemical agent. The toxic effects of CCl_4 on the liver known for years and studied extensively (Bethesda, 2012). The effect of CCl_4 on hepatocytes, depending on dose and exposure time, is manifested histologically as hepatic steatosis e.g fatty infiltration, centrilobular necrosis and ultimately cirrhosis. The damage or death of tissue usually results in the leakage of the enzymes in the affected tissue(s) into the blood stream (Obi *et al.,* 2001).

The molecular mechanism of CCI_4-induced hepatotoxicity has been well documented. The Trichloromethyl free radical (CCL_3), which is formed in the metabolism of CCI_4 through the cytochrome P450 enzyme system, reacts rapidly with molecular oxygen to produce the Trichloromethyl Peroxyl radical (CCI_3O_2) (Weber *et al.,* 2003). This highly toxic radical is responsible for attacks on unsaturated fatty acids of phospholipids present in the cell membrane (Weber *et al.,* 2003). The biotransformation of CCl_4 to metabolites is a cytochrome P450 mediated reaction that initiates lipid peroxidation and resultant tissue damage. The metabolic effect of CCl_4 inside the mitochondria has to do with damaging the Calcium pump by haloalkylation (Albano *et al.,* 2000).

Serum or plasma enzyme levels have been used as markers for monitoring chemically induced tissue damages (Hukkeri, 2002). The enzymes Alanine aminotransferase (ALT; E.C. 2.6.1.2), Aspartate aminotransferase (AST; E.C. 2.6.1.1) and Alkaline Phosphatase (ALP; E.C. 3.1.3.1) are important enzymes that are often employed in assessing liver injury ((Hukkeri, 2002).

2.14 Role of Medicinal Plants in Hepatotoxicity

In Ayurveda, plant materials have been used to protect the liver from injury by various chemicals and dietary agents. Therefore, herbal drugs are safe and have potentials to cure such diseases, so they have gained popularity in recent years. Long term uses of these medicines are cost-effective too. So many medicinal plants, present worldwide, have been mentioned as drugs of hepatoprotective activity and these are extensively used to treat the liver disorders. Various plants and polyherbal have Approximately 160 and other phytochemicals have been claimed to possess antioxidant and hepatoprotective capabilities (Jannu *et al.,* 2012).

2.15 Liver Function Tests (LFT)

Liver function tests (LFT) are a helpful screening tool, which are an effective in diagnosis and monitoring of liver diseases or damage. Some of these tests‟ measures liver performance in its normal functions of producing protein and clearing bilirubin, a blood waste product. Other liver function tests measure enzymes the liver cells release in response to damage or diseases.

2.15.1 Alanine aminotransferases and aspartate aminotransferase

The aminotransferases (transaminases) are the most frequently utilized and specific indicators of hepatocellular necrosis. ALT is found in kidney, heart, muscle and greater concentration in liver compared with other tissues of the body. ALT is purely cytoplasmic catalyzing the transamination reaction (Mauro *et al.,* 2006). Normal serum ALT is 7-56 U/ L (Diana, 2007). Any type of liver cell injury can reasonably increase ALT levels. Elevated values up to 300 U/L are considered nonspecific. Marked elevations of ALT levels greater than 500 U/L observed most often in persons with diseases that affect primarily hepatocytes

such as viral hepatitis, ischemic liver injury (shock liver) and toxin-induced liver damage. Despite the association between greatly elevated ALT levels and its specificity to hepatocellular diseases, the absolute peak of the ALT elevation does not correlate with the extent of liver cell damage (Diana, 2007). Viral hepatitis like A, B, C, D and E may be responsible for a marked increase in Aminotransferase levels. The increase in ALT associated with hepatitis C infection tends to be more than that associated with hepatitis A or B (Mauro *et al.,* 2006). Moreover, in patients with acute hepatitis C serum ALT is measured periodically for about 1 to 2 years (Mauro *et al.,* 2006). Persistence of elevated ALT for more than six months after an occurrence of acute hepatitis is used in the diagnosis of chronic hepatitis. Elevation in ALT levels are greater in persons with nonalcoholic steatohepatitis than in those with uncomplicated hepatic steatosis (Mauro *et al.,* 2006). In a recent study the hepatic fat accumulation in childhood obesity and nonalcoholic fatty liver disease causes serum ALT elevation. Moreover, increased ALT level was associated with reduced insulin sensitivity, adiponectin and glucose tolerance as well as increased free fatty acids and triglycerides (Mauro *et al.,* 2006). Presence of Bright liver and elevated plasma ALT level was independently associated with increased risk of the metabolic syndrome in adults (Tzong-His *et al.,* 2005).

These enzymes- Aspartate Aminotransferase (AST, also known as Serum Glutamate Oxaloacetic Transaminase-SGOT) and Alanine Aminotransferase (ALT, also known as Serum Glutamic Pyruvate Transaminase-SGPT) catalyze the transfer of the amino acids of aspartate and alanine respectively to the á keto group of ketoglutaric acid. ALT is primarily localized to the liver but the AST is present in a wide variety of tissues like the heart, skeletal muscle, kidney, brain and liver (Rosen and Keefe, 2000). Whereas, the AST is present in both the mitochondria and cytosol of hepatocytes, ALT is localized to the

cytosol. The cytosolic and mitochondrial forms of AST are true isoenzymes and immunologically distinct (Green and Flamm, 2002). About 80 % of AST activity in human liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme (Friedman *et al.,* 2003).

Large increases in mitochondrial AST occur in serum after extensive tissue necrosis. Because of this, assay of mitochondrial AST has been advocated in myocardial infarction. Mitochondrial AST is also increased in chronic liver disease. Their activity in serum at any moment reflects the relative rate at which they enter and leave circulation. Of the numerous methods used for measuring their levels, the most specific method couples the formation of pyruvate and oxaloacetate- the products of the aminotransferase reactions to their enzymatic reduction to lactate and malate. Virtually no aminotransferases are present in the urine or bile and hepatic sinusoids are the primary site for their clearance (Friedman *et al.,* 2003).

The AST and ALT levels are increased to some extent in almost all liver diseases. The highest elevations occur in severe viral hepatitis; drug or toxin induced hepatic necrosis and circulatory shock. Although enzyme levels may reflect the extent of hepatocellular necrosis they do not correlate with eventual outcome. In fact, declining AST and ALT may indicate either recovery of poor prognosis in fulminant hepatic failure. The AST and ALT are moderately elevated in acute hepatitis, neonatal hepatitis, chronic hepatitis, autoimmune hepatitis, drug induced hepatitis, alcoholic hepatitis and acute biliary tract obstructions. The ALT is usually more frequently increased as compared to AST except in chronic liver disease. In uncomplicated acute viral hepatitis, the very high initial levels approach normal

levels within 5 weeks of onset of illness and normal levels are obtained in 8 weeks in 75 % of cases (Rosen and Keefe, 2000).

Normal serum AST is 0 to 35 U/L (Diana, 2007). Elevated mitochondrial AST is seen in extensive tissue necrosis during myocardial infarction and also in chronic liver diseases like liver tissue degeneration and necrosis (Thapa and Anuj, 2007). About 80 % of AST activity of the liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme (Thapa and Anuj, 2007). However, the ratio of mitochondrial AST to total AST activity has diagnostic importance in identifying the liver cell necrotic type condition and alcoholic hepatitis (Green and Flamm, 2002). AST elevations often predominate in patients with cirrhosis and even in liver diseases that typically have an increased ALT ((Green and Flamm, 2002).

2.15.1.1 AST/ALT Ratio

The ratio of AST to ALT has more clinical utility than assessing individual elevated levels. A coenzyme pyridoxal-5'-phosphate deficiency may depress serum ALT activity and consequently increases the AST/ALT ratio (Edoardo *et al.,* 2003). The ratio increases in progressive liver functional impairment and found 81.3 % sensitivity and 55.3 % specificity in identifying cirrhotic patients (Edoardo *et al.,* 2003). Whereas mean ratio of 1.45 and 1.3 was found in alcoholic liver disease and post necrotic cirrhosis respectively (Giannini *et al.,* 2002). The ratio greater than 1.17 was found among patients with cirrhosis of viral cause with 87 % sensitivity and 52 % specificity. An elevated ratio greater than 1 shows advanced liver fibrosis and chronic hepatitis C infection (Giannini *et al.,* 2002). However, an AST/ALT ratio greater than 2 characteristically is present in alcoholic hepatitis. A recent study differentiated Nonalcoholic Steatohepatitis (NASH) from alcoholic liver disease

showing AST/ALT ratio of 0.9 in NASH and 2.6 in patients with alcoholic liver disease. A mean ratio of 1.4 was found in patients with cirrhosis related to NASH (Christoph *et al.,* 2007). Wilson's disease can cause the ratio to exceed 4.5 and similar such altered ratio is found even in Hyperthyroidism (Christoph *et al.,* 2007).

2.15.2 Alkaline phosphatase

Alkaline phosphatases are a family of zinc metalloenzymes, with a serine at the active center; they release inorganic phosphate from various organic orthophosphates and are present in nearly all tissues. In liver, alkaline phosphatase is found histochemically in the microvilli of bile canaliculi and on the sinusoidal surface of hepatocytes. Alkaline phosphatase from the liver and bone is thought to be from the same gene but that from intestine and placenta are derived from different genes (Friedman *et al.,* 2003). ALP is present in mucosal epithelia of small intestine, proximal convoluted tubule of kidney, bone, liver and placenta. It performs lipid transportation in the intestine and calcification in bone. The serum ALP activity is mainly from the liver with 50 % contributed by bone (Mauro *et al.,* 2006).

Highest levels of alkaline phosphatase occur in cholesteric disorders. In acute viral hepatitis, alkaline phosphatase is usually either normal or moderately increased. Hepatitis A may present a cholesteric picture with marked and prolonged itching and elevation of alkaline phosphatase. Tumours may secrete alkaline phosphatase into plasma and there are tumour specific isoenzymes such as Regan, Nagao and Kasahara isoenzymes. Elevated serum levels of intestinal alkaline phosphatase have been found in patients with cirrhosis, particularly those with blood group type O, and may be associated specifically with intrahepatic disease as opposed to extrahepatic obstruction (Jansen:Muller, 2000). Hepatic

and bony metastasis can also cause elevated levels of alkaline phosphatase. Other diseases like infiltrative liver diseases, abscesses, granulomatous liver disease and amyloidosis may also cause a rise in alkaline phosphatase. Mildly elevated levels of alkaline phosphatase may be seen in cirrhosis and hepatitis of congestive cardiac failure.

Low levels of alkaline phosphatase occur in hypothyroidism, anaemia zinc deficiency and congenital hypophosphatasia. Wilson‟s disease complicated by haemolysis and FHF may also have very low levels of alkaline phosphatase. Ratio of alkaline phosphatase and bilirubin is low in fulminant Wilson disease. This might be the result of replacement of cofactor zinc by copper and subsequent inactivation of alkaline phosphatase (Jansen and Muller, 2000).

Normal serum ALP is 41 to 133 U/L (Diana, 2007). In acute viral hepatitis, ALP usually remains normal or moderately increased. Elevation of ALP with prolonged itching is related with Hepatitis A presenting cholestasis. Tumours secrete ALP into plasma and there are tumour specific isoenzymes such as Regan, Nagao and Kasahara (Ilham *et al.,* 2008). Hepatic and bony metastasis can also cause elevated levels of ALP. Other diseases like infiltrative liver diseases, abscesses, granulomatous liver disease and amyloidosis may cause a rise in ALP. Mildly elevated levels of ALP may be seen in cirrhosis, hepatitis and congestive cardiac failure (Ilham *et al.,* 2008). Low levels of ALP occur in hypothyroidism, pernicious anaemia, zinc deficiency and congenital hypophosphatasia (Ilham *et al.,* 2008). Transient hyperphosphataemia in infancy is a benign condition characterized by elevated ALP levels of several folds without evidence of liver or bone disease and it returns to normal level by 4 months (Ilham *et al.,* 2008). ALP has been found elevated in peripheral arterial disease, independent of other traditional cardiovascular risk factors (Cheung *et al.,*

2009). Often clinicians are more confused in differentiating liver diseases and bony disorders when they see elevated ALP levels.

2.15.3 Serum bilirubin

Bilirubin is the catabolic product of haemoglobin produced within the reticuloendothelial system, released in unconjugated form which enters into the liver, converted to conjugated forms bilirubin mono and diglucuronides by the enzyme UDP-glucuronyltransferase (Mauro *et al.,* 2006). Normal serum total bilirubin varies from 2 to 21 µmol/L. The indirect (unconjugated) bilirubin level is less than 12 µmol/L and direct (conjugated) bilirubin less than 8µmol/L (Diana, 2007). The serum bilirubin levels more than 17 µmol/L suggest liver diseases and levels above 24µmol/L indicate abnormal laboratory liver tests (Thapa and Anuj, 2007). Jaundice occurs when bilirubin becomes visible within the sclera, skin, and mucous membranes at a blood concentration of around 40 µmol/L (Thapa and Anuj, 2007). The occurrence of unconjugated hyperbilirubinemia due to over production of bilirubin, decreased hepatic uptake or conjugation or both. It is observed in genetic defect of UDP-glucuronyltransferase causing Gilbert\'s syndrome, Crigler-Najjar syndrome and reabsorption of large hematomas and ineffective erythropoiesis. In viral hepatitis, hepatocellular damage, toxic or ischemic liver injury higher levels of serum conjugated bilirubin is seen. Hyperbilirubinemia in acute viral hepatitis is directly proportional to the degree of histological injury of hepatocytes and the longer course of the disease (Thapa and Anuj, 2007). It has been observed that the decrease of conjugated serum bilirubin is a bimodal fashion when the biliary obstruction is resolved (Marshal, 2007). Parenchymal liver diseases or incomplete extrahepatic obstruction due to biliary canaliculi give lower serum bilirubin value than those occur with malignant obstruction of common bile duct but

the level remains normal in infiltrative diseases like tumours and granuloma (Daniel and Marshal, 2007). Raised Serum bilirubin from 20.52 to 143.64 µmol/L in acute inflammation of appendix has been observed (Khan, 2006).

2.15.4 Gamma glutamyl transferase (ϒ-GGT)

GGT is a microsomal enzyme present in hepatocytes and biliary epithelial cells, renal tubules, pancreas and intestine. It is also present in cell membrane performing transport of peptides into the cell across the cell membrane and involved in glutathione metabolism. Serum GGT activity mainly attributed to hepatobiliary system even though it is found in more concentration in renal tissue (Mauro *et al.,* 2006). The normal level of GGT is 9 to 85 U/L (Diana, 2007). In acute viral hepatitis the levels of GGT will reach the peak in the second or third week of illness and in some patients remain elevated for 6 weeks (Giannini *et al.,* 2004).

Increased level is seen in about 30 % of patients with chronic hepatitis C infection (Giannini *et al.,* 2004). Other conditions like uncomplicated diabetes mellitus, acute pancreatitis, myocardial infarction, anorexia nervosa, Gullian barre syndrome, hyperthyroidism, obesity and dystrophica myotonica caused elevated levels of GGT (Giannini *et al.,* 2004). Elevated serum GGT levels of more than 10 times is observed in alcoholism. It is partly related to structural liver damage, hepatic microsomal enzyme induction or alcoholic pancreatic damage (Sugiura *et al.,* 2005). GGT can also be an early marker of oxidative stress since serum antioxidant carotenoids namely lycopene, α-carotene, β-carotene, and β-cryptoxanthin are inversely associated with alcohol-induced increase of serum GGT found in moderate and heavy drinkers (Sugiura *et al.,* 2005). GGT levels may be 2–3 times greater than the upper reference value in more than 50 % of the

patients with nonalcoholic fatty liver disease (McCullough, 2002). There is a significant positive correlation between serum GGT and triglyceride levels in diabetes and the level decreases with treatment especially when treated with insulin. Whereas serum GGT does not correlate with hepatomegaly in diabetes mellitus (McCullough, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals/Reagents and enzymes

3.1.1.1 Chemicals/Reagents

All chemicals used in this work were of analytical grade and they include; Methanol, Acetone, Petroleum Ether, Carbon Tetrachloride (CCL_4), Silymarin (450), Dimethyl sulfoxide (DMSO), Chloroform, Formalin, Ammonium molybdate, Folin-chiocaltu Phenol reagent, and Carrageenan were purchased from E. Merck (Germany). Quercetin potassium ferric cyanide, were purchased from Sigma Co. (St. Louis, MO, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Mayer‟s reagent, Dragendroff‟s reagent, Folin-Ciocalteu reagent, Folin-Denis reagent, Molisch reagent, Ferrous sulphate reagent, Ellman reagent and sulfuric acid were products of Sigma Chemical Co., USA. Glacial acetic acid, Fehling‟s solutions A and B were purchased from Carlo Erba group reagents, Italy.

3.1.1.2 Enzyme kits

Alanine Aminotransferase, Aspartate Aminotransferase, Alkaline Phosphatase, Superoxide Dismutase, Glutathione S-transferase and Catalase enzymes assay kits, were all purchased from Sigma Chemical Co., USA.

3.1.2 Equipment

The equipment used for this study include: Soxhlet extractor, test tubes, beakers, measuring cylinder, weighing balance, steerer, cannular, micro syringe and heating bath.

3.1.3 Plant materials

Rhizomes of *Curcuma longa,* were obtained from Kure market in Minna, Niger State, in the month of February, 2019. The rhizomes were washed, dried at room temperature and pulverized using an electric blender. The powder was sealed and stored in polyethene bag until required for use.

3.1.4 Animals

Wistar rats of either sex weighing between (48-168 g) were obtained from Ahmadu Bello University, Zaria, Kaduna State. The Animals were conveniently housed under standard environmental conditions (Temperature 27±2 °C; 70 % relative humidity; 12 h daylight/night cycle) and had free access to commercial feed pellets and water (Jigam *et al.,* 2011).

3.2 Methods

3.2.1 Extraction of plant material

Extraction of the powdered rhizomes was carried out using Soxhlet extraction method for 6 hours. The extraction of the turmeric crude containing all the components was carried out by weighing 100g of the pulverized turmeric rhizomes using Soxhlet extractor (Rs.22500 Model) and methanol as the extracting solvent. (Mukharjee, 2002). The methanol crude extract of *C. longa* was weighed and stored in the refrigerator until required for use.

3.2.2 Isolation of curcuminoids from *C. longa* rhizomes

Curcuminoids from the pulverized *C. longa* rhizomes was fractionated by first, extracting oleoresin using Soxhlet extractor (Rs.22500 Model) and acetone as the extracting solvent for 6 hours. Curcuminoids was then precipitated using petroleum ether from the oleoresin and filtered using Whiteman filter paper. The filtrate was open-air evaporated and concentrated using water bath. This crude curcuminoids mixture contained curcumin, demethoxycurcumin, bisdemethoxycurcumin. The Curcuminoids fraction of *C. longa* was weighed and stored in the refrigerator until required for use (Revathy *et al.,* 2011).

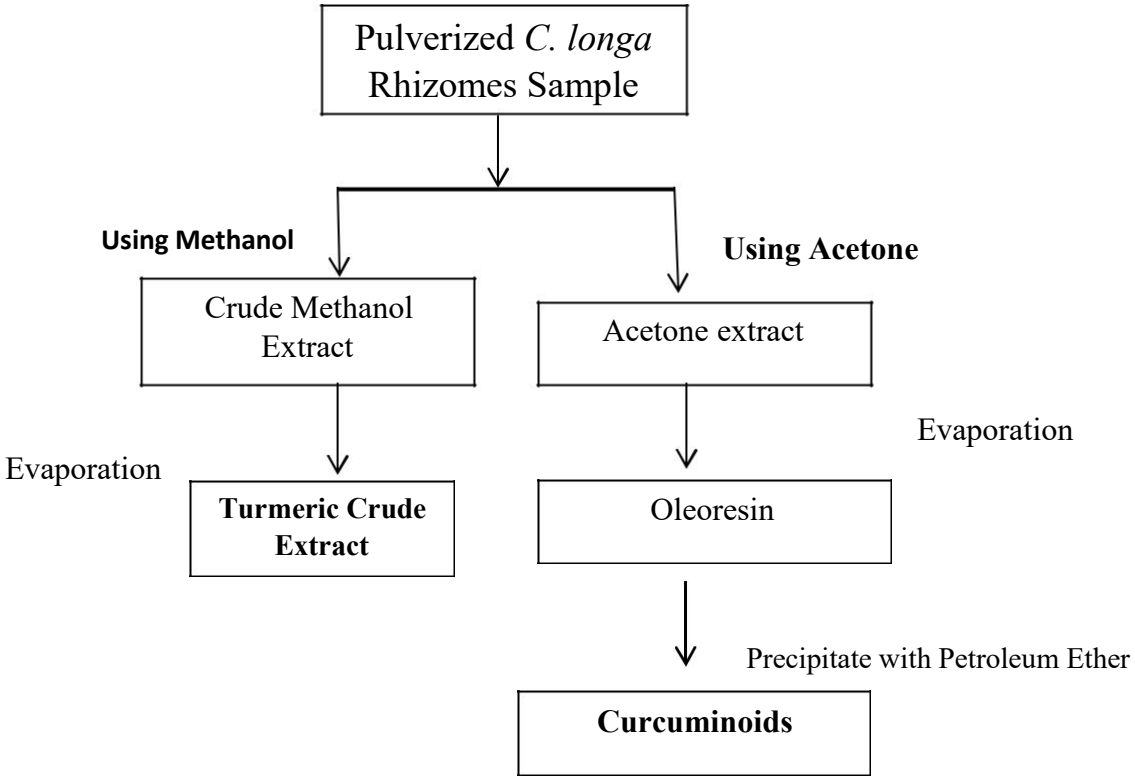

Figure 3.1: Extraction and isolation of Crude and Curcuminoids from *C. longa* *Rhizome.* (Revathy *et al.,* 2011).

3.2.3 Qualitative phytochemical screening

Crude extract of turmeric rhizome was screened for the presence of secondary metabolites. Thus, tests for flavonoids, alkaloids, terpenoids, saponins, steroids, glycosides, tannins and phenols were performed using standard procedures (Sofowora, 2008; Ugochukwu *et al.,* 2013).

3.2.3.1 Test for flavonoids (Alkaline reagent test)

One gram of the extract was treated with few 3 drops of 20 % sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids.

3.2.2.2 Test for alkaloids (Wagner's reagent)

One gram of the extract was mixed with 2 mL of 1 % HCl and heated gently. Wagner‟s reagent was then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

3.2.2.3 Test for terpenoids

One gram of the extract was mixed with 0.5 mL of acetic anhydride and 5 mL of concentrated H_2SO_4 was carefully added to the mixture which formed a layer. The formation of interface with reddish brown or bluish green indicates the presence of terpenoids.

3.2.2.4 Test for saponins

One gram of the extract was mixed with 5 mL of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

3.2.2.5 Test for steroids

Acetic anhydride (2 mL) was added to 1 g of the extract. The appearance of a colour change from violet to blue or green indicates the presence of steroids.

3.2.2.6 Test for tannins (Braymer's test)

One gram of the extract was treated with 10 % ferric chloride solution and observed for formation of blue or greenish colour solution.

3.2.2.7 Test for phenols (Ferric chloride test)

One gram of the extract was treated with aqueous 5 % ferric chloride and observed for formation of deep blue or black colour.

3.2.3 Quantitative phytochemicals screening

Quantitative phytochemicals analysis of the plant extract was carried out using standard methods for the estimation of phyto-constituents.

3.2.3.1 Determination of total flavonoids

The diluted sample (0.5 mL) was added into test tube containing 1.5 mL of methanol. 0.1 mL of 10 % $AlCl_3$ solution and 0.1 mL sodium acetate ($NaCH_3COO^-$) were added, followed by 2.8 mL of distilled water. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm. The amount of 10 % $AlCl_3$ was substituted by the same amount of distilled water in blank. Quercetin was used to establish the calibration curve (Chang *et al.,* 2002).

3.2.3.2 Determination of total alkaloids

Three milliliters of the sample extract was added to 3 mL of H_3PO_4 (88 %) and 1.5 mL (0.5 %) of methanol to determine the alkaloid concentration. The resulting solution was warmed at 50 °C for 15 minutes and read at 574 nm. This absorbance was interpolated in the standard curve (Oloyede, 2005).

3.2.3.3 Determination of saponins

The extract (0.5 g) was added to 20 mL of 1N HCl and was boiled for 4 hours. After cooling it was filtered and 50 mL of petroleum ether was added to the filtrate for ether layer and evaporated to dryness. Five milliliter of acetone ethanol was added to the residue and 0.4 mL of each was taken into 3 different test tubes. Exactly 6 mL of ferrous sulphate reagent was added into them followed by 2 mL of concentrated H_2SO_4. It was thoroughly mixed after 10 minutes and the absorbance was taken at 490 nm. Standard saponin was used to establish the calibration curve (Oloyede, 2005).

3.2.3.4 Determination of total phenolic content

The extract (300 µL) was dispensed into test tube (in triplicates), to which was added 1.5 mL of commercial Folin-Ciocalteu reagent (diluted ten times) followed by sodium carbonate solution (7.5 w/v). The reaction mixture was mixed, allowed to stand for 30 minutes at room temperature before the absorbance was measured at 765 nm against a blank prepared by dispensing 300 µL of distilled water instead of sample. Total phenolic content was expressed as garlic acid equivalent (GAE) in mg/g material (Chang *et al.,* 2002).

3.3 Acute toxicity test of methanol crude extract of *C. longa* rhizome

Acute toxicity study was carried out using the method of (Lorke, 1983). This method involves two phases. In the first phase, nine rats randomly divided into three groups of three rats per group were given 10, 100 and 1000 mg extract/kg. bw orally (via a cannula), respectively. The rats were observed for signs of adverse effects and death for 24 hours. In the second phase of the study, the procedure was repeated using nine rats randomly divided into three groups of one rat each, higher doses; 1600, 2900 and 5000 mg turmeric crude extract/kg. bw, were given, respectively. The rats were also observed for signs of toxicity and mortality for 24 hours.

3.4 Experimental design for determination of antioxidant and hepatoprotective activity of *C. longa* rhizome crude extract and curcuminoids fraction.

All treatments were administered daily at exactly 10:00 am for a period of 7days. CCl_4 was administered once at a dose of 2 mL/kgbw 6.00 hours after the administration of the extract on the 7^{th} day, and the animals were sacrificed 24 hours after CCl_4 administration (Shenoy *et al.,* 2011). The grouping and treatments are shown in the Table 3.1

Table 3.1: Experimental Design and Animal Grouping

GROUP	TREATMENTS
1	Normal control Administered 1 mL distilled water
2	Untreated (2 mL/kgbw CCl_4)
3	(Positive standard) 100 mg/kgbw Silymarin
4	150 mg/kgbw *C. longa* rhizome crude extract
5	300 mg/kgbw *C. longa* rhizome crude extract
6	600 mg/kgbw *C. longa* rhizome crude extract
7	75 mg/kgbw Curcuminoids fraction
8	150 mg/kgbw Curcuminoids fraction
9	300 mg/kgbw Curcuminoids fraction

0.9 % DMSO in distilled water was used as negative control. The extract dosage was prepared by first dissolving in 0.9 % dimethyl sulfoxide (DMSO) in distilled water. The extract, fraction and the drug were given orally, while CCl_4 was administered intraperitonially.

3.5 Blood collection and serum preparation

The blood sample was collected 24 hours after treatment with CCl_4 according to the method of Shittu *et al*. (2014), following mild chloroform anesthesia of the rats, the blood obtained through direct cardiac puncture into K^+ EDTA (Ethylene Di-amine Tetra-Acetic acid) bottles for estimation of hematological parameters. Another portion of the blood was collected and allowed to clot for 30 minutes and then centrifuged at 3000 rpm for 15 minutes to separate the serum from other blood components. The serum was used for other analyses.

3.6 Biochemical parameters

3.6.1 Determination of antioxidant activity of *C. longa* crude extract and curcuminoids fraction

The activity of Superoxide Dismutase, Catalase and Glutathione transferase were determined from the blood samples collected of the sacrificed animals after experimental period to determine the antioxidant activity of the crude extract and fraction, using the assay kit.

3.6.1.1 Superoxide Dismutase (SOD) activity

The method of Misra and Fridovich (1972) was used to determine SOD activity in tissues.

Principle

Superoxide dismutase (SOD) inhibits the auto oxidation of epinephrine at pH 10.2. Superoxide radical ($O^{\cdot -2}$) generated by xanthine oxidase reaction causes the oxidation of epinephrine to adenochrome and the yield of adenochrome produced increases per $O^{\cdot -2}$ introduced with increasing pH (Valerino and McCormack, 1971) and also increases with increasing concentration of epinephrine. These results led to the proposal that auto oxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving super oxide radical and hence inhibitable by SOD.

Procedure

One hundred micro litre of the diluted sample was added to 1000 µL of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reaction was started by addition of 150 µL of freshly prepared 0.3mM epinephrine to the mixture. The reference

cuvette contained 1000 µL buffer, 150 µL of epinephrine and 100 µL of distilled water. The increase in absorbance at 480nm was monitored every 0.5 min for 2.5 min.

Calculations:

Where A0 = absorbance after 0.5 min

A5 = absorbance after 2.5 min

3.6.1.2 Catalase activity

The method of Sinha (1972) was used to determine catalase (CAT) activity.

Principle

This method relies on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of hydrogen peroxide. The chromic acetate generated is then quantified colourimetrically at 570 – 610 nm. CAT is allowed to split hydrogen peroxide over specific minutes after which the reaction is terminated by the addition of dichromate/acetic acid mixture. The hydrogen peroxide left unsplited is quantified by measuring chromic acetate colourimetrically after heating the reaction mixture.

Procedure

The assay mixture is made up of 1 mL of H_2O_2 solution (800 µmoles) and 1.25 mL of phosphate buffer (0.1M, pH 7.4). 1 mL of appropriately diluted enzyme preparation (1:5) was rapidly mixed with the reaction mixture by gentle swirling. A 1 mL aliquot of the reaction mixture was withdrawn and added to 2 mL dichromate/acetic acid reagent and the absorbance read at 60 sec intervals for 3 minutes at 570 nm.

C ________________________________

Where: OD = optical density

Δ OD = change in optical density

3.6.1.3 Glutathione peroxidase activity

The activity of Glutathione peroxidase (GPx) in tissues was determined by the using the method of Rotruck *et al*. (1973).

Principle

GPx catalyzes the splitting of H_2O_2 and other peroxides (-OOH) with concomitant oxidation of reduced glutathione (GSH).

$$H_2O_2 + 2GSH \xrightarrow{GP} H_2O + GSSG$$

The method is based on the ability of GPx to utilize µmol of GSH to decompose µmol of H_2O_2. The reaction is allowed to proceed for a specified period (in minutes) and then terminated by the addition of trichloroacetic acid. The residual GSH in the reaction mixture is then quantified by the addition of Ellman"s Reagent (5", 5"-dithiobis- (2-nitrobenzoic acid), DTNB).

Procedure

The reaction was started by the addition of 200 µL of H_2O_2 (10 mM) to a reaction mixture already containing 800 µL Tris-HCl buffer (0.1 M pH 7.4), 400 µL GSH (2mM), 200 µL sodium azide (10 mM) and 50 µL of diluted sample made up to 2 mL with distilled water. The assay mixture was incubated at 37 ^{o}C for 5 minutes after which the reaction was terminated by the addition of 500 µL of TCA (10 %). After centrifuging the assay mixture

at 4000 x g for 10 minutes, 250 µL of the supernatant was added to 1000 µL of DTNB (0.004 %) and 250 µL of disodium hydrogen phosphate (0.3 M) solution. The colour developed was read at 420 nm, a reaction mixture without enzyme was used as the control. The glutathione peroxidase activity was expressed as Units per milligram protein (Units/mg protein).

Calculations:

GPx activity

Where: 307.32 = molecular weight of GSH

3.7 Determination of hepatoprotective activity of turmeric crude extract and curcuminoids fraction on liver marker enzymes

Blood samples collected sacrificed animals after experimental period will be subjected to Liver Function Test (LFT), which determines the activity of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP), amount of albumin, total protein and bilirubin. (Vogel *et al.,* 2002).

3.7.1 Aspartate aminotransferase activity

Aspartate amino-transferase (AST) is also known as Glutamate Oxaloacetate transaminase (GOT). The enzymes mediate reactive systems in which amino group is transferred from an amino acid to an oxo-acid without formation of ammonia as an intermediate. This is measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4 – dinitrophenyl-hydrazine based on the procedure of Reitman and Frankel (2015).

Principle

The enzymatic rate method is used to measure the AST activity in serum or plasma. In the reaction, the AST catalyzes the reversible transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. The oxaloacetate is then reduced to malate in the presence of malate dehydrogenase with the concurrent oxidation of NADH to NAD. The system monitors the rate of change in absorbance at 340 nm over a fixed-time interval. The rate of change in absorbance is directly proportional to the AST activity in the sample. AST measurements are used in the diagnosis and treatment of certain types of liver and heart diseases.

α – oxoglutarate + L –aspartate $\xrightarrow{\textbf{GOT}}$ glutamiate + oxaloacetate

Procedure

Zero point zero five millilitre of sample was added to 0.25 mL of Buffer (Phosphate buffer), L – aspartate, α – Oxoglutarate while the blank contained only 0.25 mL of 2, 4 – dinitrophenylhydrazine. This was mixed and allowed to incubate for exactly 30 minutes at 37 ^{o}C, after which 0.25mL of the buffer was added to the mixture. 0.05 mL of distilled water was added to the blank, this was mixed and allowed to stand for 20 minutes at room temperature before 2.5 mL of NaOH was added to both mixtures. Absorbance was read at 546 nm (530 – 550 nm) after 5 minutes. The activity of AST in the sample was extrapolated off the standard curve.

3.7.2 Alanine aminotransferase activity

Alanine transaminase (ALT) or Glutamate pyruvate transaminase (GPT). It Catalyze the formation of pyruvate and glutamate from alanine and α – oxoglutarate. This was assay using the procedure of Reitman and Frankel (2015).

Principle

Cytosolic glutamic-pyruvate transaminase (GPT) catalyzes the reversible reaction of alanine and 2-oxoglutarate (alpha-ketoglutarate) to form pyruvate and glutamate. The active form of the enzyme is a dimer, and is inferred to have a molecule of pyridoxal phosphate associated with each monomer. This reaction allows the synthesis of alanine from intermediates of glucose metabolism in a well-fed person. Under fasting conditions, alanine, derived from protein breakdown, can be converted to pyruvate and used to synthesize glucose via the gluconeogenic pathway in liver, or fully oxidized via the TCA cycle in other tissues. The enzymatic rate method measures ALT activity in serum or plasma. In the reaction, ALT catalyzes the reversible transamination of L-alanine and α-ketoglutarate to pyruvate and L-glutamate. The rate of change in absorbance is directly proportional to the ALT activity in the sample (Yang *et al*., 2002).

$$\alpha - \text{Oxoglutarate} + \text{alanine} \xrightarrow{\text{GPT}} \text{L-glutamate} + \text{pyruvate}$$

Procedure

Zero point zero five millilitre of sample was added to 0.25 mL of phosphate buffer, L – aspartate, α – Oxoglutarate while the blank contained only 0.25 mL of 2, 4 – dinitrophenylhydrazine. This was mixed and allowed to incubate for exactly 30 minutes at 37 ^{O}C, after which 0.25 mL of the buffer was added to the mixture. 0.05 mL of distilled water was added to the blank, this was mixed and allowed to stand for 20 minutes at room temperature before 2.5 mL of NaOH was added to both mixtures. Absorbance was read at 546 nm (530 – 550 nm) after 5 minutes. The activity of AST in the sample was extrapolated off the standard curve.

3.7.3 Alkaline phosphatase (ALP)

Alkaline phosphatase (ALP) is known as orthophosphoric monoester phosphohydroxylase. The optimum pH of alkaline phosphatase (ALP) varies from 8.5-10.0 and is influenced by the type of substrate employed, concentration of the type and concentration of activation and finally, the buffer employed.

Principle

P-Nitrophenyl phosphate + H_20 $\xrightarrow{ALP}$ P- Nitrophenol + Inorganic phosphate

The working reagent was prepared by dissolving substrate R2 into 10 mL of R1. 1 mL of working reagent was pipetted into each test tube with the addition of 0.20 mL of plasma. The mixture was mixed gently, initial absorbance (A1) taken at 1 min and absorbance was taken every minute for additional 3 minutes at 405 nm. The difference between absorbance (ΔA) and the average absorbance differences per minute (ΔA / min) were calculated

Calculation:

ALP activity (U/L) = (Δ0D/min) x 2750.

3.7.4 Total proteins

Total protein test kit (Randox Laboratories Ltd, Crumlin, UK), was used for the estimation of plasma total protein. Cupric ions in alkaline medium interact with protein peptide bonds, hence, resulting in the formation of a coloured biuret complex (Weichselbaum, 1946).

The content of the test kit is R1a (10 mL of bottled R1 (biuret reagent) in 40 mL distilled water (dH_2O)), R21 (10 mL of bottled R2 in 40 mL dH_2O), and protein standard. The reaction mixture contained 1.0 mL of R1a and 0.02 mL of plasma for sample‟s test tubes, while blank and standard test tubes contain 1.0 mL of R1a and 0.02 mL of dH_2O or 0.02

mL of standard respectively. Each test tube was mixed and incubated at 25^{o}C for 30 minutes. The absorbance of the standard (Abs standard) and samples (Abs samples) were measured against reagent blank at 546 nm and protein concentration was calculated as follows:

3.8 Histopathological studies of the liver of experimental animals

The histopathological analysis of the liver excised from the experimental animals was carried out in the Histopathology laboratory, University of Ilorin, Nigeria. The organ was cut and placed in an embedded paraffin block. Thereafter, it was fixed with 10 % formalin for 1 hour and afterwards dehydrated with methanol (70, 90 and 100 %) at different concentrations in ascending concentration and at different times in order to remove water from the tissues. Thereafter, clearing with xylene was done for 2 to remove alcohol and prepare the tissue for waxing. Embedding was done using paraplast wax by impregnating blocks with molten wax at 60^{o}C for 3 hours. slicing was done at 5 microns using a microtome. The slide was dyed for 20 minutes on hot plate. Afterwards, dewaxing and hydration were done using xylene and various percentage of alcohol respectively. Thereafter, staining was done with Cole‟s hematoxylin for 10 minute to stain the nucleus while eosin was used to stain the cytoplasm for 3 minutes. Dehydration was once again carried out in alcohol and alcohol cleared with xylene. A mounting medium, dibutylphthalate xylene (DPX) was dropped on the tissue section and viewed through the microscope.

3.9 Data analysis

Data obtained in this study were analyzed using the statistical software SPSS 18.0, 2008. Numerical data were presented as Mean ± Standard Error. The significance of the mean difference between two independent groups was determined using Student‟s t-test and one-way Analysis of Variance (ANOVA) while multiple comparisons were used when comparing more than two groups. A p-value<0.05 was considered significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Percentage yields of methanol crude extract and curcuminoids fraction of *C. longa.*

The Percentage yields of methanol crude extract of *C. longa* and Curcuminoids fraction precipitated from oleoresin acetone extract of *C. longa* are given in Table 4.1. The percentage yields of the extracts were 13.12 % and 1.70 % respectively.

Table 4.1: Percentage Yields of Methanol Crude Extract and Curcuminoids Fraction of *C. longa*

Plant Sample	Powdered sample (g)	Weight of extract (g)	%Yield
C. longa crude extract	200	26.24	13.12
C. longa Curcuminoids fraction	200	3.40	1.70

4.1.2 Phytochemical composition of crude extract of *Curcuma longa*

4.1.2.1 Quantitative phytochemical composition of methanol crude extract of *C. longa*

The quantitative phytochemical screening of methanol crude extract from *C. longa* rhizome, detected the presence of alkaloid, flavonoids, tannins, phenols and saponins. The results of quantitative phytochemical screening of crude extract of *C. longa* are given in Table 4.2. The concentrations of phytochemicals detected were; Saponins (1742 mg/100g) was found to be the highest followed by phenols (213 mg/100g), then Alkaloids and Flavonoids (97.4 mg/100g each). Tannins (7.01 mg/100g) was found to have the least quantity.

Table 4.2: Quantitative Phytochemical Composition of Methanol Crude Extract of *Curcuma longa*

Parameters	Concentration (µg/mg)
Phenols	213.41 ± 1.36^{c}
Flavonoids	97.24 ± 0.64^{a}
Tannins	7.01 ± 0.23^{d}
Alkaloids	97.24 ± 0.64^{a}
Saponins	1742.63 ± 1.94^{b}

Data are presented in Mean ± Standard Error of Mean of triplicate determinations. Values along the same column with different superscripts are significantly different ($p < 0.05$).

4.1.3 Safe dose of methanol crude extract of *C. longa*

The results of safe dose determination of crude methanol extracts are shown in Table 4.3. There was no mortality within 24 hours after oral administration of 10, 100 and 1000 mg/kg/ body weight of extract to the rats in the first phase. The Turmeric extract had a safe dose of 1000 mg/kg bw, since higher doses of (1600, 2900 and 5000 mg/kg bw) showed some sign of toxicity; Restlessness for about 20 minutes was observed after the rats were given 1600 mg/kg bw. Similarly, rubbing of mouth on the cage surface, with redness of the eye which lasted for about 5 minutes was observed at exactly 2900 mg/kg bw dosage. Hyper breathing and redness of the eye which lasted for an hour was observed at exactly 5000 mg/kg bw dose.

Table 4.3: Acute Toxicity Profile of *Curcuma longa* Crude Methanol Extract

Treatment	Dose (mg/kg bw)	No of animals	Mortality	Sign of toxicity
Phase 1	10	3	0/3	Animals were calm and devoid of unusual behavior
	100	3	0/3	Animals were calm and devoid of unusual behavior
	1000	3	0/3	No sign of toxicity was observed in any of the animals
Phase 2	1600	3	0/3	No sign of toxicity was observed in any of the animals
	2900	3	0/3	Rubbing of the mouth on the surface of the cage for about 5 minutes, redness of the eye
	5000	3	0/3	Hyper breathing, redness of the eye which last for an hour

LD_{50}>5000mg/kg bw. Safe dose is 1600 mg/kg bw.

4.1.3 Effects of administered compounds on the body weight of the Wistar rats

During the seven (7) days of experimental study, significant changes in the body weight of the rats was determined. The weight of CCl_4 administered rats significantly ($p<0.05$) increased throughout the experimental period, similarly rats treated with standard drug (Silymarin) as well as the normal control rats had a progressive increase in body weight (118.33±15.17 and 103.00±9.71). Administration of *C. longa* crude extract at 600 mg/kg bw reduced the body weight to a greater extent than that at 150 and 500 mg/kg body weight respectively. Furthermore, Administration of 75, 150 and 300 mg/kg bw Curcuminoids extract indicates significant reduction in the body weight of rats, however, the reduction was dose dependent as can be seen in Table 4.4.

Table 4.4: Effects of Methanol Crude Extract of *Curcuma longa* Rhizome, Curcuminoids Fraction, Silymarin and CCl_4 on Body Weight of the Rats.

GROUP	Dosages (mg/kg bw)	Day 0	Day 4	Day 6
Normal		92.33±17.25[a]	96.67±8.76[a]	103.00±9.71[b]
Negative	100mg/kg bw	93.00±13.50[a]	99.00±16.09[b]	108.00±1.00[c]
Standard	2mg/kg bw	104.00±16.50[b]	109.33±14.67[c]	118.33±15.17[d]
Crude extract	150mg/kg bw	91.33±24.88[a]	96.67±14.85[a]	111.33±13.90[c]
Crude extract	500mg/kg bw	73.67±4.48[e]	86.66±35.96[a]	92.33±33.63[a]
Crude extract	600mg/kg bw	72.67±8.17[e]	87.67±4.33[a]	89.67±6.74[a]
Curcuminoids	75mg/kg bw	112.67±8.17[c]	92.67±4.33[a]	87.67±6.74[a]
Curcuminoids	150mg/kg bw	122.33±2.67[d]	106.33±5.69[b]	96.33±5.67[a]
Curcuminoids	300mg/kg bw	104.33±34.80[b]	95.33±14.67[a]	91.33±15.16[a]

Data are presented in Mean ±Standard Error of Mean of triplicate determination. Values along the same column for each enzyme with different superscripts are significantly different. ($p < 0.05$)

4.1.5 Hepatoprotective activity of crude extract of *Curcuma longa* and curcuminoids fraction on some liver function biomarkers in CCl_4 induced hepatoxicity in Wistar rats

Hepatoprotective activity of methanol crude extract of *Curcuma longa* and Curcuminoids using serum AST, ALT, ALP, Protein and albumin as markers in CCl_4 induced hepatotoxic rats is shown in Figures; 4.3, 4.4, 4.5, 4.6 and 4.7 respectively. The liver function biomarker enzymes (AST, ALT and ALP) in CCl_4 induced group of rats increased significantly ($p<0.05$) when compared with the normal control and positive standard groups. However, there was significant difference observed between the total protein and albumin of CCl_4 induced rats when compared with the positive standard group, but insignificantly ($p>0.05$) when compared to normal control groups.

In the extract treated groups, 75 mg/kgbw dose of extract indicated insignificant difference ($p>0.05$) in AST, ALT and ALP activities when compared with the normal control group. However, the total protein and albumin showed significant difference ($p<0.05$) when compared with the normal control group. 150 mg/kg bw dose of Curcuminoids extract showed significant difference ($p<0.05$) in ALT, ALP, Total protein and Albumin when compared with the normal control group, but insignificantly different ($p>0.05$) when in AST activities.

The 300 mg/kg bw dose of Curcuminoids showed insignificant difference ($p>0.05$) in the liver marker enzymes (AST, ALT and ALP) when compared with the normal control group. However, the total protein and albumin were comparable with the normal control group.

The 150, 300 and 600 mg/kg bw doses of crude extract indicated insignificant difference ($p>0.05$) in ALT and ALP activities compared to normal control. However, there was significant difference between the three doses (150, 300 and 600 mg/kg bw) of crude extract in AST activities when compared with the normal control group.

The total protein, 150and 600 mg/kgbw exhibited insignificance ($p>0.05$) when compared with the normal control group, but significantly different at the dose of 300 mg/kg bw when compared with the normal control group.

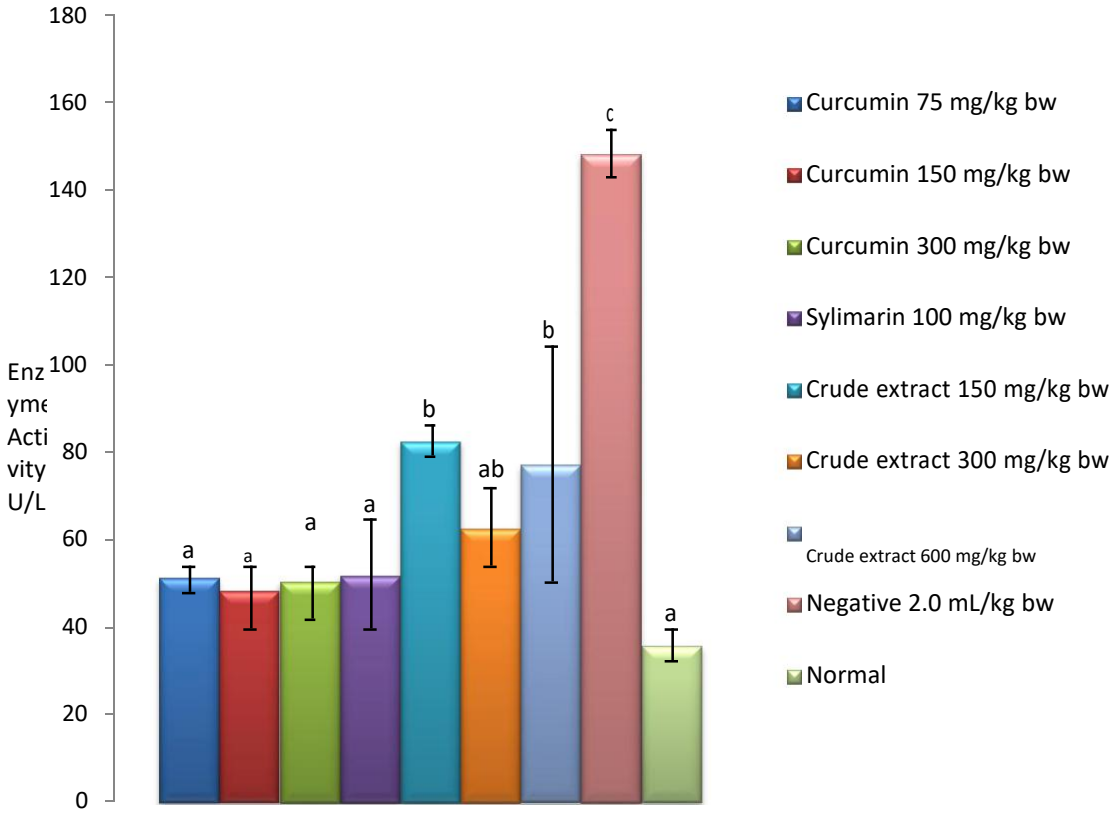

Figure 4.1: Effect of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Serum Aspartate Aminotransferase (AST) in CCl_4-induced Hepatoxicity in Wistar Rats.

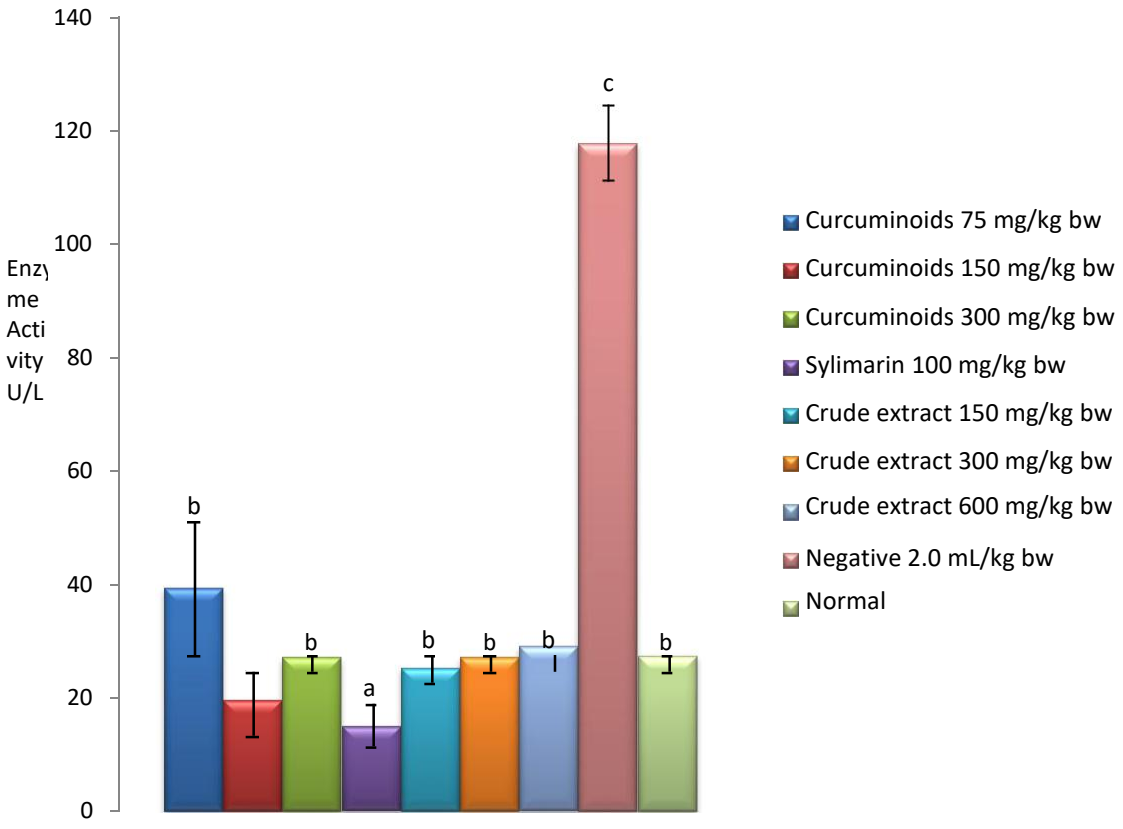

Figure 4.2: Effect of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Serum Alanine Aminotransferase (ALT) in CCl_4-induced Hepatoxicity in Wistar Rats.

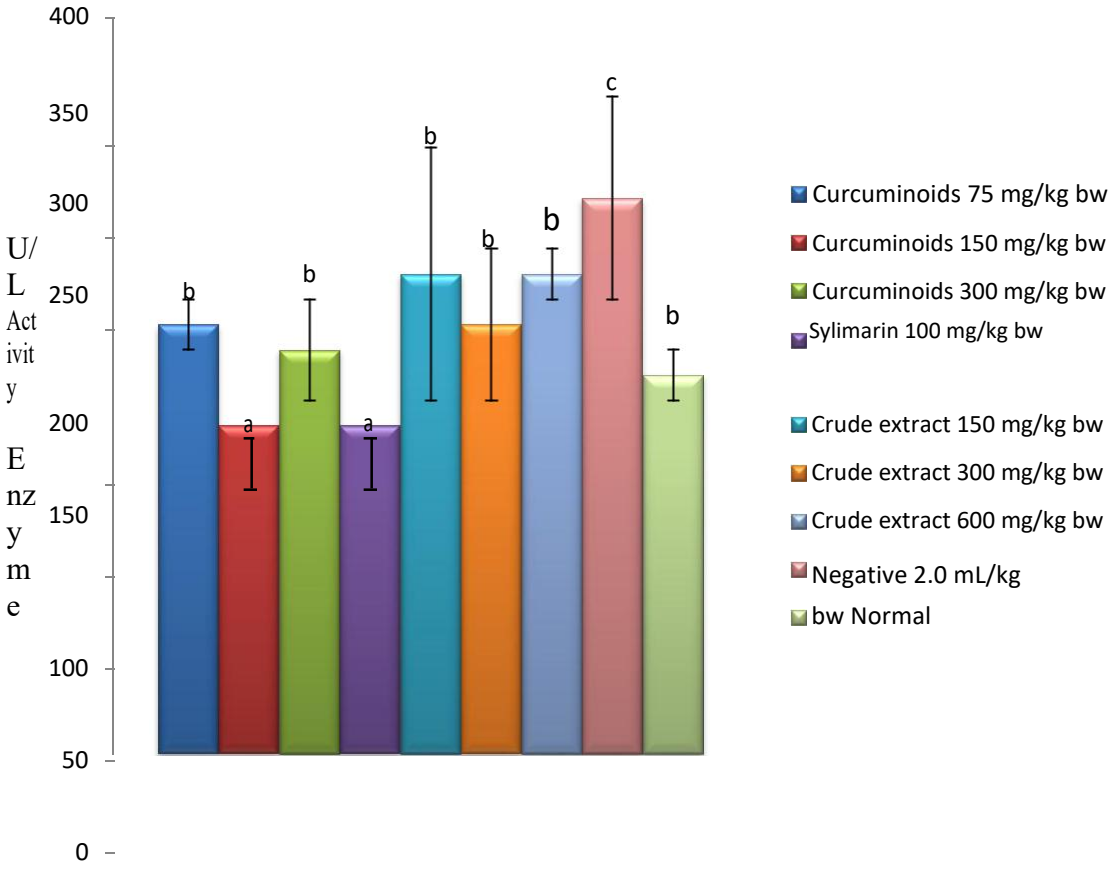

Figure 4.3: Effect of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Serum Alkaline Phosphatase (ALP) of CCl_4-induced Hepatoxicity in Wistar Rats.

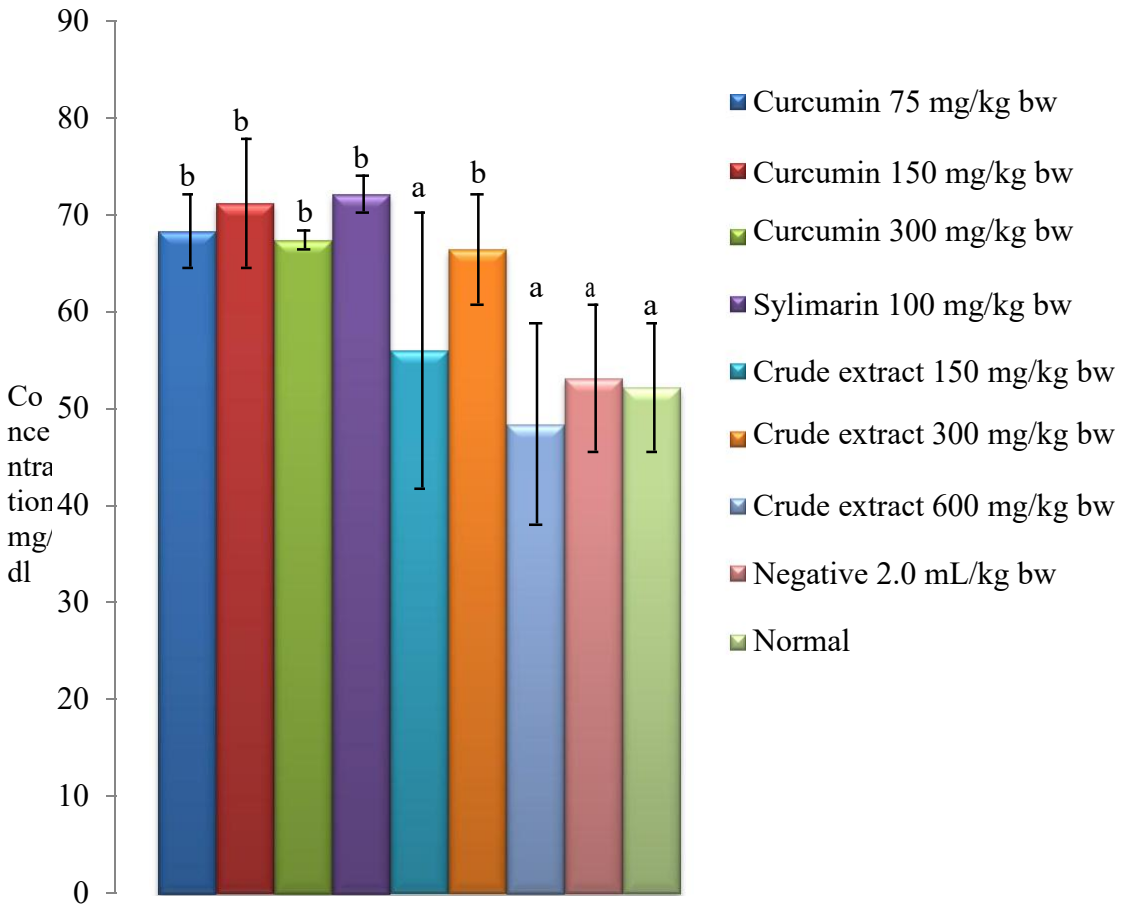

Figure 4.4: Effect of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Serum Protein in CCl_4-induced Hepatoxicity in Wistar Rats.

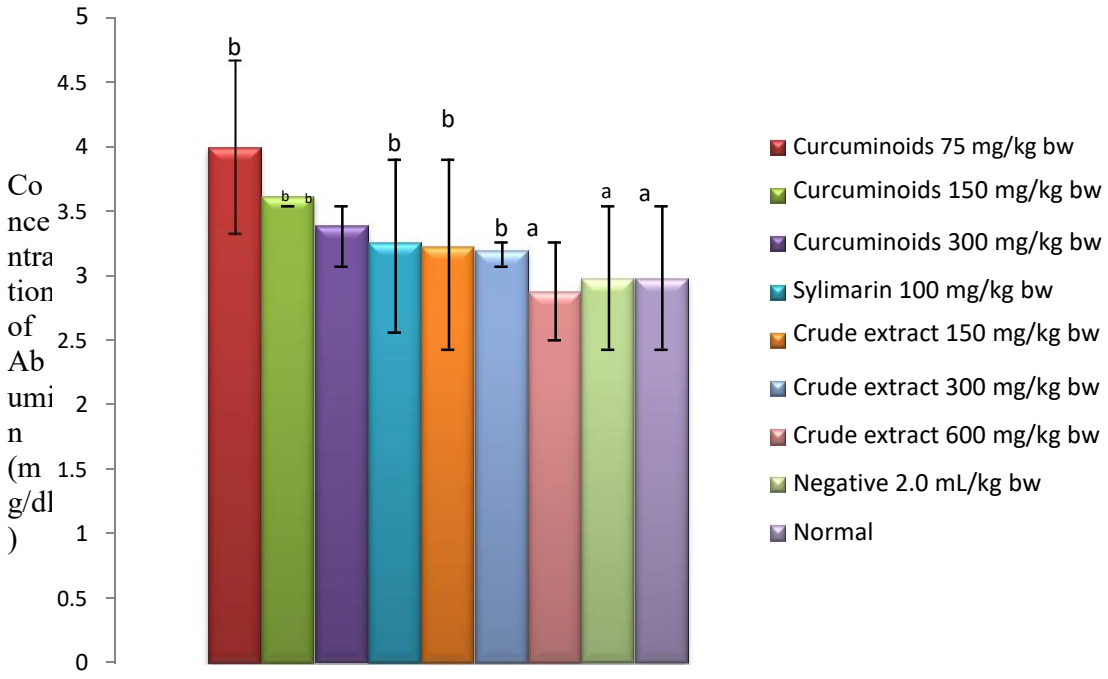

Figure 4.5: Effect of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Serum Albumin in CCl_4-induced Hepatoxicity in Wistar Rats.

4.1.6 Antioxidant activity of crude extract of *Curcuma longa* and Curcuminoids fraction on some marker enzymes in CCl_4-induced hepatoxicity in Wistar rats.

Antioxidant activity of methanol crude extract of *Curcuma longa* and Curcuminoids fraction on some marker enzymes in CCl_4 induced hepatotoxic rats is shown in Figures 4.8, 4.9 and 4.10. The rats that were dosed CCl_4 only exhibited significant decrease ($p<0.05$) in the SOD and GPx activity when compared with the normal control, positive standard and extract treated group. Similarly, the activities of SOD and GPx in the extract treated group increased significantly, compared to CCl_4 administered rats and normal control group.

However, in the CAT activities, there was significant difference ($p<0.05$) in the rats that were dosed with CCl_4 when compare with the positive standard and extract treated group of rats, but no significance difference when compare with the normal control group. The crude extract at the doses of 150 and 300 mg/kgbw, showed insignificant difference ($p>0.05$) when compared to positive standard and the Curcuminoids fractionat 75, 150 and 300 mg/kgbw. However, a significant difference ($p<0.05$) was observed in the crude extract at a dose of 600 mg/kg bw when compared to the positive standard group of rats and the Curcuminoids fraction at all doses of 75, 150 and 300 mg/kg bw respectively.

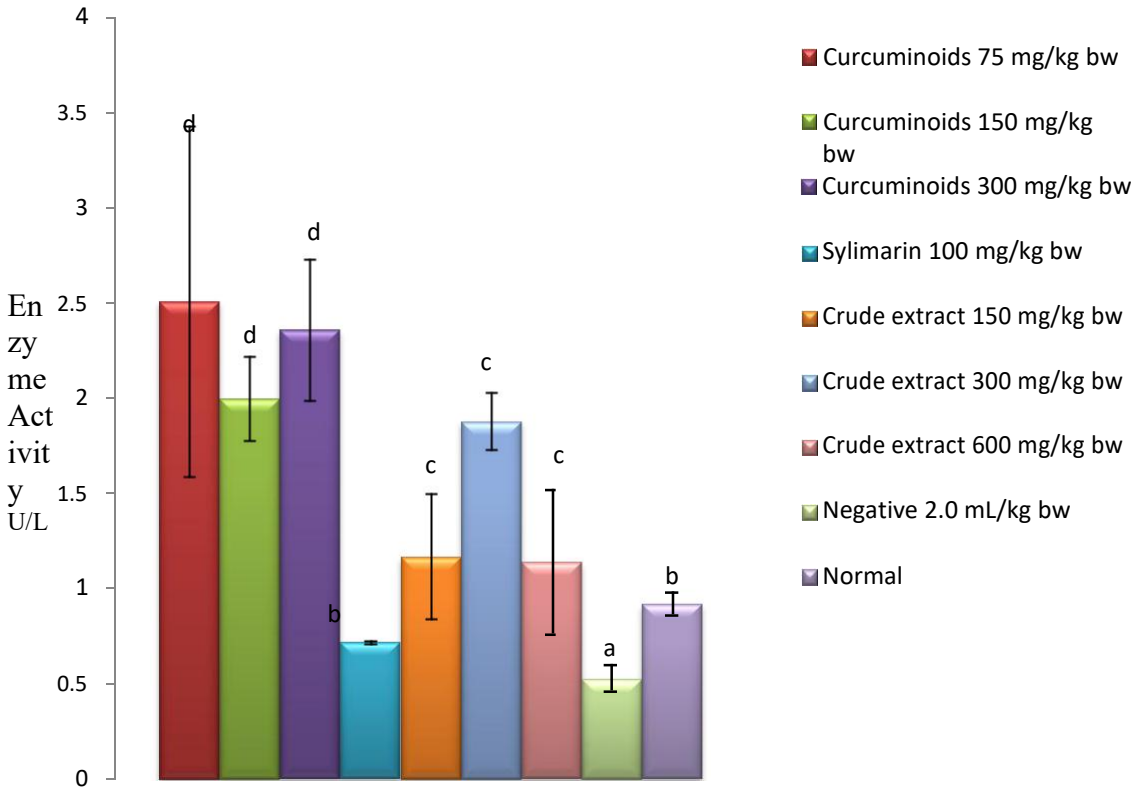

Figure 4.6: Effects of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Superoxide Dismutase (SOD) Activity in the Serum of CCl_4-induced Hepatoxicity in Wistar Rats.

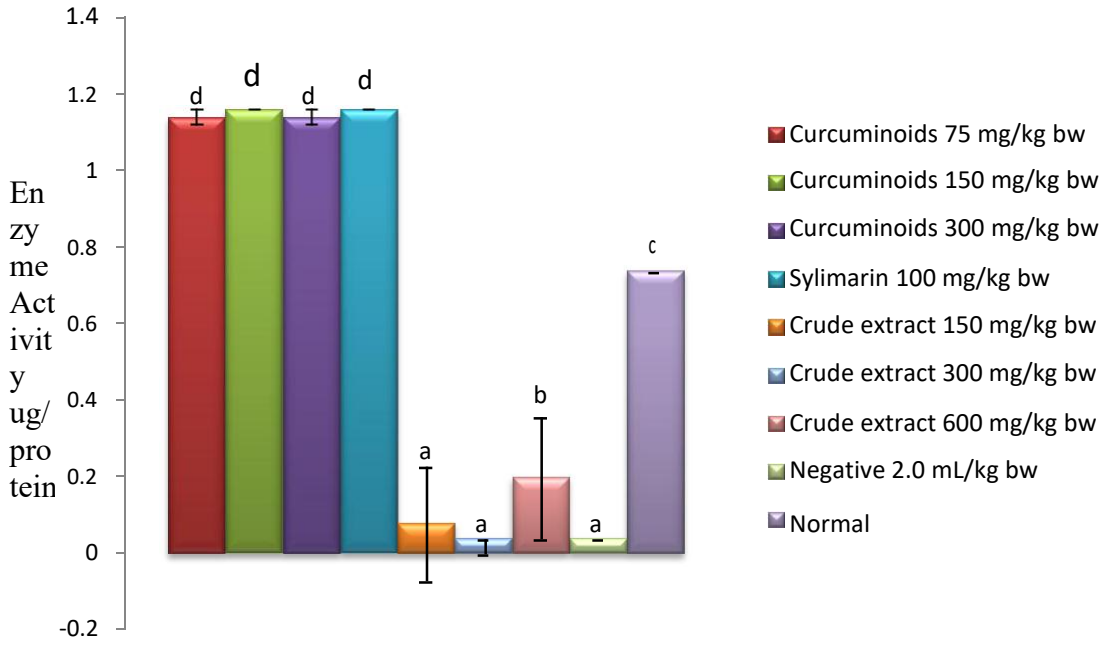

Figure 4.7: Effects of Methanol Crude Extract of *Curcuma longa* and Curcuminoids on Glutathione Peroxidase (GPx) Activity in the Serum of CCl_4-induced Hepatoxicity in Wistar Rats.

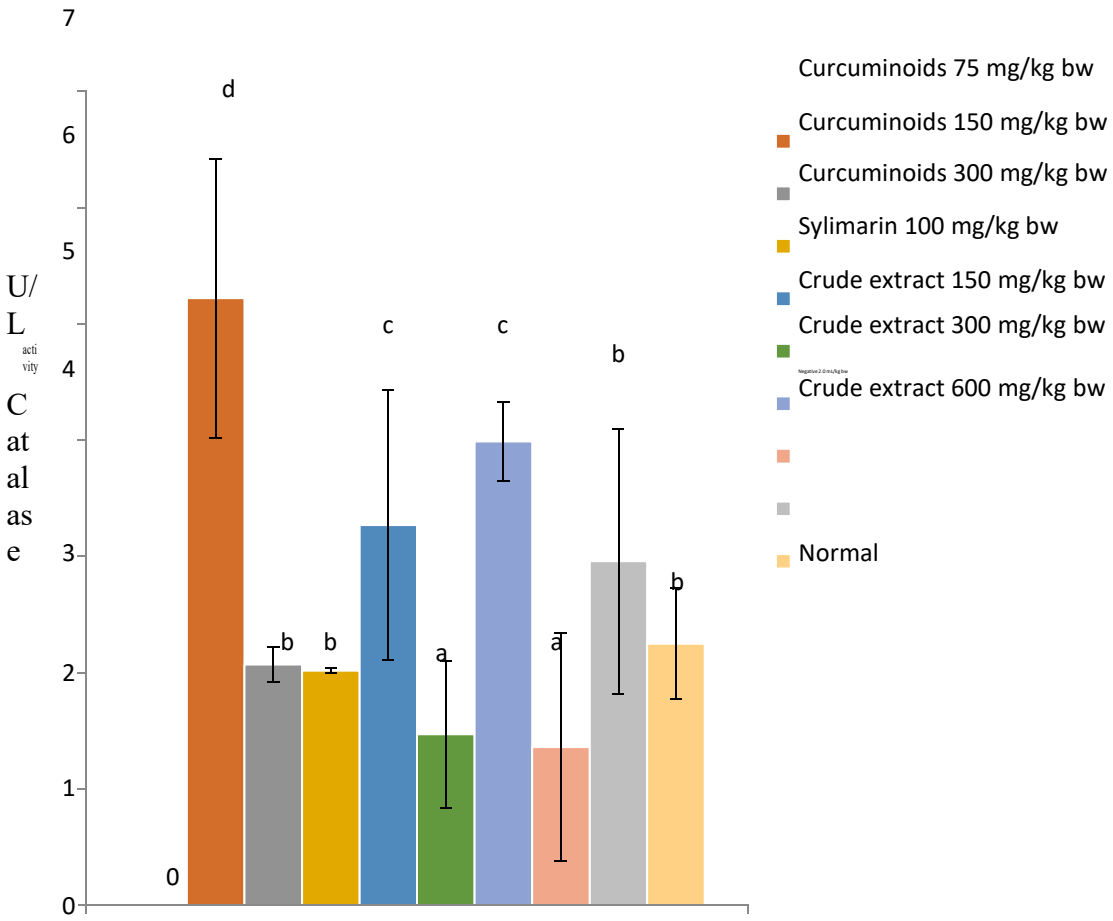

Figure 4.8: Effects of Methanol Crude Extract of *Curcuma longa* and Curcuminoids Fraction on Catalase (CAT) Activity in the Serum of CCl_4-induced Hepatoxicity in Wistar Rats.

4.1.7 Histopathology of the liver

The effect of Curcuminoids and crude extract is shown in Table 4.6, Plate I and II. The central vein of the liver became less congested at higher doses of the crude extract and Curcuminoids when compared with the Control. The Integrity of the Hepatocytes improved as the administered concentrations/doses of the extract and Curcuminoids increased when compared with the control. There was an improvement in the cellular morphology as the Curcuminoids concentration increased when compared with the untreated group. The photomicrographs of the liver sections of CCl_4 intoxicated rats after treatments with crude extract and Curcuminoids of *Curcuma longa,* showed pronounced central vein congestion and degenerative changes, poor integrity of the hepatocytes and gross distortion in general appearances, while the groups treated with Silymarin and the normal control showed no central vein congestion, normal integrity of the hepatocytes, normal general features of the liver and no degenerative changes. The groups treated with 150 mg/kg bw and 300 mg/kg bw doses of Curcuminoids fraction, showed no central vein congestion, normal integrity of the hepatocytes, normal general features of the liver and no degenerative changes, but 75 mg/kg bw dosage, showed mild central vein congestion, mild integrity of the hepatocytes, good general appearance with no distortion and mild degenerative changes. The crude extract of *C. longa* showed pronounced central vein congestion, poor integrity of the hepatocytes, pronounced distortion in general appearances and pronounced degenerative changes 150 mg/kg bw, while the crude extract showed, mild central vein congestion, mild distortion in the integrity of the hepatocytes, mild distortion in general appearances and mild degenerative changes at 300 mg/kg bw dosage. The crude extract at 600 mg/kg bw dose showed no central vein congestion, good and no distortion in the integrity of the hepatocytes, mild distortion in general appearance and mild degenerative changes.

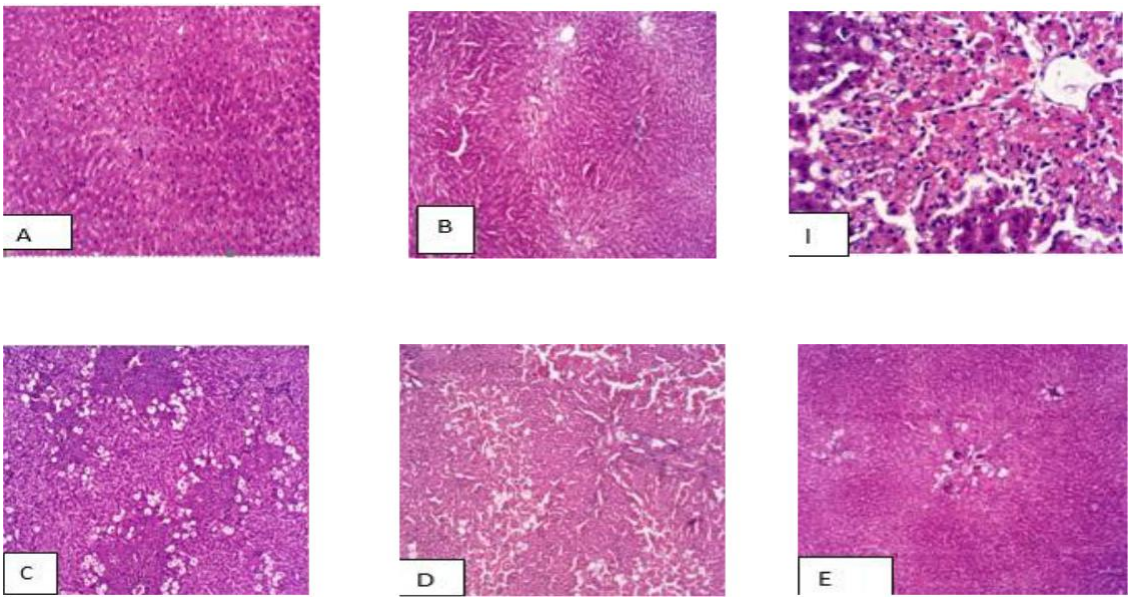

Plate I: Photomicrograph of liver sections of CCl_4 intoxicated rats after treatments with Curcuminoids of *Curcuma longa*. Magnification 400x. A: Control, B; 100 mg/kg bw Sylimarin, , I: Untreated control, C; 75 mg/kg bw Curcuminoids, D: 150 mg/kg bw Curcuminoids, E: 300 mg/kg bw Curcuminoids.

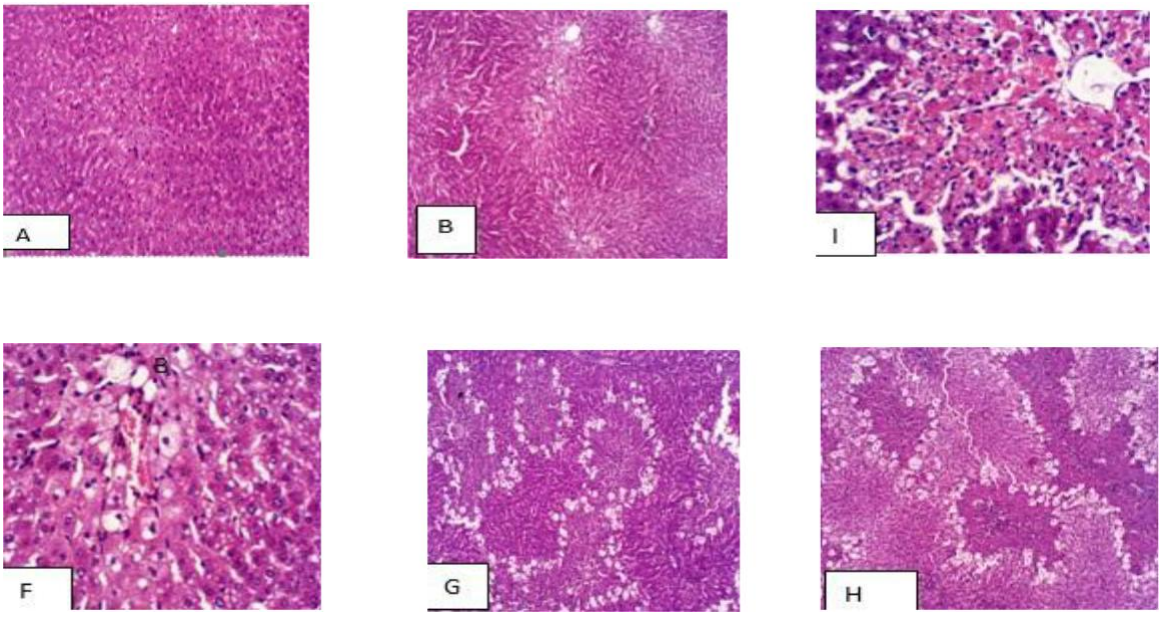

Plate II: Photomicrograph of liver sections of CCl_4 intoxicated rats after treatments with crude extract of *Curcuma longa*. Magnification 400x. A: Control, B; 100 mg/kg bw Sylimarin, I: Untreated control, F: 150 mg/kg bw crude extract, G: 300 mg/kg bw crude extract, H: 600 mg/kg bw crude extract.

4.2 Discussion

This study establishes the presence of high quantity of phytochemicals, such as alkaloids, flavonoids, phenols and saponins in *C. longa* rhizome, which is an indication of its potential for medicinal and therapeutic treatment, because the pharmacological impacts of plants are based on these compounds (Jigam *et al.,* 2009). Phytochemicals usually have medicinal potentials and serve as a blueprint for powerful drug synthesis in some instances (Jigam and Atunde, 2001). Especially the steroidal alkaloids are used in medicine and they also display significant pharmacological activity (Davis and Heywood, 1963).

Phenols are known to be capable of inhibiting particular inflammatory enzymes and preventing illness (Okwu, 2004). Tannins are reported to have haemostatic and astringent physiological characteristics that accelerate wound healing and inflamed mucus membrane. Their powerful anti-oxidant capacity and stabilization are typical of the role (Ogunleye and Ibitoye 2003). Saponins, often referred to as natural detergents owing to their foamy nature, also possess anti-carcinogenic characteristics, immune modulation and cell proliferation regulation, as well as reducing activity of cholesterol (Jimoh and Oladji, 2005).

Acute toxicity measures an experimental animal's toxicological reaction to single or instant exposure to a sample substance. Measuring an unidentified substance's acute toxicity is the first guide to pharmacological and toxicological research (Lorke, 1983). Methanol crude extract of *C. longa* rhizome had a high safety bracket, as no death occurred within 24 hours of extract administration.

In selecting herbal medicine for use in health care, one fundamental and extremely regarded criterion is safety. Plant extracts should be safe for consumption and not only effective.

Therefore, the need to understand their toxic potential is strongly connected with plant extract screening for their operations against microorganisms or disease conditions.

Methanol crude extract and Curcuminoids fraction from *C. longa* had an excellent antioxidant and hepatoprotective potentials against hepatic damage induced by CCl_4 in the rats. The antioxidant and hepatoprotective activity of the methanol crude extract and Curcuminoids from *C. longa* may be due to phytochemicals such as flavonoids, phenols, and alkaloids.

The effectiveness of any hepatoprotective drug depends mainly on its ability to either reduce the damaging impacts or maintain the ordinary physiological hepatic system that has been changed by hepatotoxin.

Carbon tetrachloride (CCl_4) is the most common hepatotoxin used in the liver disease or harm experimental study of liver (Shenoy *et al.,* 2011). The serum concentrations of these marker enzymes; AST, ALT, and ALT represent or projects the liver's physiological state. The concentrations of these enzymes alter as a result of liver distortion caused by organ cell injury induced by toxic metabolites and illnesses.

The findings of this research indicate that CCl_4 caused a significant rise in marker enzyme serum concentrations (AST, ALT, and ALP) in the group of rats that received CCl_4 alone. Increased serum ALP, ALT, AST as obtained in this study are indicative of cellular leakage and loss of functions of the membrane integrity. The increased enzyme levels in the plasma of CCl_4–treated rats suggest that, the toxicant was able to reach the liver and induce a detectable damage within 24 hours. The toxicity of CCl_4 to the liver of the rats is largely as a result of the active metabolite, trichloromethyl radical (Johnson and Kruening, 1988).

The result of this study suggests that, seven days prophylactic administration of methanol crude extract of *C. longa* and Curcuminoids fraction of *C. longa* at different doses caused a decline in the hepatotoxity of CCl_4. This is evidenced in the marked decrease in serum AST, ALT and ALP level relative to the group treated with CCl_4 alone. The sharp increase in protein and Albumin concentration shown in result of this study is an indication of the hepatoprotective activity of the crude extract and fraction of *C. longa.*

Liver function testing (LFT) is a useful screening instrument for detecting hepatic dysfunction. Since the liver performs a variety of functions, no single test is enough to provide a complete estimate of liver function (Rosen and Keefe, 2000). The most frequently used and particular indicators of hepatocellular necrosis are aminotransferases. In acute hepatitis, neonatal hepatitis, chronic hepatitis, autoimmune hepatitis, drug-induced hepatitis, alcoholic hepatitis, and acute biliary tract obstructions, AST and ALT are mildly elevated (Rosen and Keefe, 2000). Except in chronic liver disease, the ALT is generally improved more frequently than in AST. The very elevated original concentrations in uncomplicated acute viral hepatitis approach ordinary concentrations within 5 weeks of disease onset (Park *et al.,* 2000).

The outcome of this research suggests that pre-administration of crude extract and Curcuminoids fraction of *C. longa* to the rats with various doses induced a decrease in the hepatotoxicity of CCl_4. This is apparent in the marked decline in AST, ALP, and ALT serum levels relative to the group treated with CCl_4 alone. The dose of Curcuminoids at 150 mg/kgbw appears to have greater liver protection than other doses of crude extract and fraction of *C. longa.*

Hukken *et al.* (2002) indicated that CCl4-induced liver injury correctly represents the elevation in the plasma level of cytoplasmic and mitochondrial enzymes. This rise in enzyme concentrations (AST, ALP and ALT) was ascribed to the damaged structural integrity under the impact of CCl4. Significant decrease in levels of AST, ALP and ALT as a result of prophylactic treatment with *C. longa* crude extract and Curcuminoids fraction is almost definitely suggestive of protecting the hepatocyte membrane's structural integrity.

The increase in enzyme levels in rats treated with crude extract and Curcuminoids fraction of *C. longa* as shown in the findings, suggest that they have antioxidant characteristics. This is evident in the result of the study that showed the rats dosed with CCl_4 only exhibiting significant decrease ($p<0.05$) in the SOD, CAT and GPx activity and increase in the treated group significantly.

The reduction in concentration of these marker enzymes; SOD, CAT and GPX, observed in this study may be related to the excessive production of free radicals generated by the metabolism of CCl_4. The antioxidant enzymes are inactivated or exhausted by lipid peroxides and/or reactive oxygen species which results in decreased activities of the enzymes in CCL_4 toxicity. An increase in the level of the marker enzymes observed with various doses of crude extract and curcuminoid fraction of *C. longa* pre-treatment is in agreement with the study by Campos *et al.* (1989).

Histopathological studies demonstrated hepatoprotective potentials of *C. longa* crude extract and Curcuminoids fraction against CCl_4 -induced liver damage. There was significant reduction in the pathological changes in the liver of the experimental rats treated with *C. longa* crude extract and Curcuminoids fraction compared to the non-treated group.

It has been reported that *C. longa* that curcumin induced recovery from hepatic injury involves reduction apoptosis of activated cells (Poli and Parola, 2001).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This study showed that methanol crude extract of *C. longa* and Curcuminoids (Curcumin, demethoxycurcumin and bisdemethoxycurcumin) fractions of *C. longa* rhizome have significant antioxidant, hepatoprotective and high safety margins. Plants with these pharmacological properties may be good supplements for prophylactic and curative treatments. Therefore, the crude extract and the fraction of *C. longa* are potential sources of new plant-based antioxidants and hepatoprotective agents that may be considered as supplement to drugs against hepatic damage.

5.2 Recommendations

The significant antioxidant and hepatoprotective effects of crude methanol extracts of *C. longa* rhizome and the Curcuminoids fraction suggest that:

i. It warrants further detailed studies and clinical trials, utilizing bioassay guided fractionations under varied pharmacological conditions in order to discover unexploited therapeutic efficacy.

ii. A drug development programme should be undertaken to develop modern drugs from rhizomes of the plants having medicinal applications

iii. As the global scenario is now changing towards the use of non-toxic plant products, having traditional medicinal use, development of modern drugs from *C. longa* should be emphasized for the control of various diseases.

iv. Relevant authorities or agencies like, National Agency for Food and Drug Administration and Control (NAFDAC), Standard Organization of Nigeria (SON),

Food and Agriculture Organization (FAO) and others, should create awareness on the use of Turmeric and its components as safe for dietary regimens to combat diseases and spice.

v. Regarding its constituent‟s bioavailability, modern drug delivery systems and formulations e.g Nanoparticles, Liposomal and Phospholipid complexes should be developed. Turmeric can be mixed with natural and safe Piperine-containing substances e.g pepper, for increased bioavailability.

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APPENDICES

Appendix A

Hepatoprotective Activity of Crude Extract of *Curcuma longa* and Curcuminoids on Some Liver Function Biomarkers in CCL_4 Induced Hepatotoxic Rats

	Dosage (mg/kg bw)	AST (U/L)	ALT (U/L)	ALP (U/L)	PROTEIN (U/L)	ALBUMIN (mg/dL)
Curcuminoids	75	51.60±3.60^{a}	39.48±11.56^{b}	233.75±13.75^{b}	68.40±3.80^{b}	4.00±0.67^{b}
Curcuminoids	150	48.60±9.00^{a}	19.74±6.58^{a}	178.75±13.75^{a}	71.25±6.65^{b}	3.62±0.03^{b}
Curcuminoids	300	50.80±9.00^{a}	27.26±2.82^{b}	220.00±27.50^{b}	67.45±0.95^{b}	3.39±0.32^{b}
Silymarin	100	52.20±12.60^{a}	15.04±3.76^{a}	178.75±13.75^{a}	72.20±1.90^{b}	3.26±0.70^{b}
Crude extract	150	82.80±3.60^{b}	25.38±2.82^{b}	261.25±68.75^{b}	56.05±14.25^{a}	3.23±0.80^{b}
Crude extract	300	63.00±9.00^{ab}	27.26±2.82^{b}	233.75±41.25^{b}	66.50±5.70^{b}	3.20±0.12^{b}
Crude extract	600	77.40±27.00^{b}	29.14±2.22^{b}	261.25±13.75^{b}	48.45±10.45^{a}	2.88±0.38^{a}
Negative	2.0 mL/kg bw	148.60±5.40^{c}	117.86±6.58^{c}	302.50±55.00^{c}	53.20±7.60^{a}	2.98±0.96^{a}
Normal		36.00±3.60^{a}	27.52±1.88^{b}	206.25±13.75^{b}	52.25±6.65^{a}	2.98±0.96^{a}

Data are presented in Mean ±Standard Error of Mean of triplicate determination. Values along the same column for each enzyme with different superscripts are significantly different. ($p< 0.05$)

ALP = Alkaline phosphatase AST = Aspartate amino transferase ALT = Alanine amino transferase

Appendix B

Effects of Crude Extract of *Curcuma longa* and Curcuminoids on Some Antioxidant Enzymes Activity in the Serum of CCL_4 Induced Hepatotoxic Rats

	Dosage (m g/kg bw)	SOD (U/L)	GPx (ug/protein)	CAT (U/L)
Curcumin	75	2.51 ± 0.92^{d}	1.14 ± 0.02^{d}	5.22 ± 1.99^{d}
Curcumin	150	2.00 ± 0.22^{d}	1.16 ± 0.00^{d}	2.07 ± 0.51^{b}
Curcumin	300	2.36 ± 0.37^{d}	1.14 ± 0.02^{d}	2.02 ± 0.02^{b}
Sylimarin	100	0.72 ± 0.01^{b}	1.16 ± 0.00^{d}	3.27 ± 1.16^{c}
Crude extract	150	1.17 ± 0.33^{c}	0.08 ± 0.05^{a}	1.47 ± 0.63^{a}
Crude extract	300	1.88 ± 0.15^{c}	0.04 ± 0.04^{a}	3.99 ± 0.34^{c}
Crude extract	600	1.14 ± 0.38^{c}	0.20 ± 0.16^{b}	1.36 ± 0.98^{a}
Negative	2.0 mL/kg bw	0.53 ± 0.07^{a}	0.04 ± 0.00^{a}	2.96 ± 1.14^{b}
Normal		0.92 ± 0.06^{b}	0.74 ± 0.00^{c}	2.25 ± 0.48^{b}

Data are presented in Mean ±standard Error of Mean of triplicate determination. Data followed by different superscript alphabet along the same column were significantly different ($p<0.05$)

Appendix C

Histopathological Profile of Liver Section of CCl_4 Intoxicated Rats Treated with Crude Extract and Curcuminoids from *Curcuma longa* with Reference to the Control

Experimental Groups	Central vein congestion	Integrity of Hepatocytes	General appearances	Degenerative Changes
150 mg/kg bw crude extract	~~III~~ (PRONOUNCED)	II (Poor)	II (Gross/pronounced distortion)	~~III~~ (PRONOUNCED)
300 mg/kg bw crude extract	III (MILD	III (MILD	III (Mild distortion	III (MILD
600 mg/kg bw crude extract	---- (NIL)	IIII (Good with no distortion))	III (Mild distortion)	III (Mild distortion)
75 mg/kg bw Curcuminoids	III (MILD	III (MILD	IIII (Good with no distortion))	III (MILD
150 mg/kg bw Curcuminoids	---- (NIL)	~~III~~ (Excellent/Normal)	III (Mild distortion)	---- (NIL)
300 mg/kg bw Curcuminoids	---- (NIL)	~~III~~ (Excellent/Normal)	~~III~~ (Excellent/Normal)	---- (NIL)
100 mg/kg bw Silymarin	---- (NIL)	~~III~~ (Excellent/Normal)	~~III~~ (Excellent/Normal)	---- (NIL)
Untreated	~~III~~ (PRONOUNCED)	II (Poor)	II (Gross/pronounced distortion)	~~III~~ (PRONOUNCED)
Control	---- (NIL)	~~III~~ (Excellent/Normal)	~~III~~ (Excellent/Normal)	---- (NIL)