STUDIES ON *IN-VIVO* ANTIOXIDANT AND HEPATOPROTECTIVE PROPERTIES OF *CITRUS SINENSIS* (L.) OSBECK PEEL EXTRACT IN WISTAR RATS

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FACULTY OF PHARMACEUTICAL SCIENCES

AHMADU BELLO UNIVERSITY, ZARIA

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By

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NIGERIA

FEBRUARY, 2014

DECLARATION

I declare that the work in this thesis entitled **Studies on *In-Vivo* Antioxidant and Hepatoprotective Properties of *Citrus sinensis* Peel Extract** has been carried out by me in the **Department of Pharmacology and Therapeutics**. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Ikeokwu Amarachukwu Linda

Signature Date

CERTIFICATION

I declare that the work in this thesis entitled **Studies on *in-vivo* antioxidant and hepatoprotective properties of *citrus sinensis* peel extract** by **Amarachukwu Linda Ikeokwu** meets the regulations governing the award of the degree of Masters Degree in Pharmacology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Citrus sinensis is the commonest, most widely cultivated and consumed citrus fruit. Large amounts of peel wastes are formed when citrus is consumed or processed; and suitable methods have to be adopted to utilize these waste materials for conversion into value-added products. Flavonoids, the major component of citrus peel have been reported to exert antioxidant and hepatoproective activity. This study was designed to investigate the *in-vivo* antioxidant and hepatoprotective properties of the methanol extract of citrus fruit peel in Wistar rats. Qualitative and quantitative phytochemical screening was carried out on extracts of three different citrus species fruit peels for comparison and selection, after which *C. sinensis* fruit peel extract was selected for further studies because it had the highest flavonoid content. Oxidative stress and liver damage was induced in rats using 2 ml/kg subcutaneous injection of CCl_4 every 72 h for 10 days. Biochemical parameters were assessed as indices of oxidative stress and liver damage. Acute toxicity studies suggests that the LD_{50} (oral route) is above 5,000 mg/kg. The extract showed significant protection (at $P<0.001$) against the depletion of liver tissue catalase levels observed in the CCl_4 treated group at 500 mg/kg (89% protection) comparable to that of the standard drug sylimarin (84% protection). The extract reduced liver enzymes significantly (at $P<0.001$ for alanine aminotransferase and aspartate aminotransferase; and $P<0.05$ for alkaline phosphatase as well as $P<0.05$ for bilirubin) similar to sylimarin. Furthermore, the extract also had similar protective effect to sylimarin on oxidative stress induced ischemia in kidney tissues. Urea levels were significantly ($P<0.05$) reduced from 6.86±0.80 mmol/L observed in the CCl_4 treated group to 3.72±0.58 mmol/L by the 500 mg/kg extract. Preliminary

phytochemical screening of methanol extract of *C. sinensis* fruit peels revealed the presence of flavonoids, phenols, tannins, saponins, steroids, carbohydrates and cardiac glycosides; with total flavonoids and phenol content of 17.3 mg QE/g and 13.6 mg GAE/g respectively. The pathological evaluations of liver sections of rats treated with *C. sinensis* peel extract showed mild distortion revealing less damage comparable to the control and sylimarin treated groups. Thus, it can be concluded that the methanol extract of *C. sinensis* peel is relatively safe orally in rats and possess antioxidant activity that may be effective in the management of liver damage and possibly other oxidative stress induced diseases. Thus, supporting the development of the biologically active compounds from *C. sinensis* as antioxidants for the management of hepatic disorders.

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ABBREVIATIONS

AIDS: Acquired Immune Deficiency Syndrome

AFP: α-Fetoprotein

Alb: Albumin

ALP: Alkaline phosphatase

ALT: Alanine transaminase

AST: Aspartate transaminase

ATP: Adenosine Triphosphate

BHT: Butylated hydroxytoluene

BLT: British Liver Trust

CAT: Catalase

CCl_4: Carbon tetrachloride

CBil: Conjugated Bilirubin

CE: Cholesterol esterase

$CHCl_3$: Chloroform

Chol: Cholesterol

CLD: Chronic

Creat: Creatinine

CO: Cholesterol oxidase

CYP450: Cytochromes P450

DNA: Deoxyribonucleic acid

EASL: European Association for the Study of the Liver

EDTA: Ethylene Diamine Tetra-Acetate

FAO: Food and agriculture organization of the united nations

GGT: Gamma Glutamyl Transferase

GPO: G-3-phosphate Oxidase

GPx: Glutathione peroxidase

GK: Glycerol kinase

GSH: reduced glutathione

HBV: Hepatitis B virus

HBeAg: e antigen

HBsAg: hepatitis B surface antigen

HBcAg: hepatitis B core antigen

HCC: Hepatocellular Carcinoma

HCV: Hepatitis C virus

HDV: Hepatitis D virus

HEV: Hepatitis E virus

HIV: Human Immunodeficiency Virus

HGV: Hepatitis G virus

H_2O_2: hydrogen peroxide

HOCl: hypochlorous acid

IgA: Immunoglobulin A

IgM: Immunoglobulin B

IgG: Immunoglobulin C

α INF: Interferon α

LOOH: lipid peroxide

MDA: Malondialdehyde

mg GAE/g: mg Gallic Acid Equivalent per gram of extract mg

mg QE/g: mg Quercetin Equivalent per gram of extract

MRI: Magnetic Resonance Imaging

NADPH: reduced Nicotinamide Adenine Dinucleotide Phosphate

NAFLD: Nonalcoholic Fatty Liver Disease

NASH: Nonalcoholic Steatohepatitis

NPSH: nonprotein thiol compound

5'-NT: 5'-nucleotidase

OFRs: Oxygen free radicals

POD: Peroxidase

p.o.: per oral

PT: Prothrombin Time

RBCs: Red blood cells

RLW: Relative Liver Weight

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

s.c: Subcutaneous

SOD: Superoxide Dismutase

TBA: Thiobarbituric acid

TBARS: thiobabitturic acid-reacting substances

TBil: Total Bilirubin

TP: Total protein

Trigly: Triglyceride

UNESCO: United Nations Educational, Scientific, and Cultural Organization

WHO: World Health Organization

%WL: Percentage Weight Loss

CHAPTER ONE

1. INTRODUCTION

1.1 Preamble

During normal metabolism, body cells use oxygen and in the process generate highly reactive molecules known as free radicals capable of abstracting electrons (oxidation) from neighbouring cells (Moreira da Silva *et al*., 2010). Oxidative stress is created when excessive free radicals react with proteins, cell walls and DNA causing damage to cell structures that are critical to the immune system (Xiuzhen *et al.*, 2007). Free radicals can also be produced by inflammatory response, stress and environmental pollution. When these free radicals are not remove and cell damage is not repaired, cell death occur leading to higher propensity for disease (Halliwell, 1992).

Many drugs, toxic substances and infectious organisms are associated with hepatotoxicity due to their ability to generate free radicals and to cause a disturbance in hepatocyte biochemistry (Fernandez-Checa and Kaplowitz, 2005). The free radicals generated are so high that they overpower the natural scavenger system leading to hepatic damage in the form of jaundice, cirrhosis and fatty liver (Wolf, 1999).

Antioxidants are compounds that help prevent cellular damage caused by free-radical (Jacob, 1995). Fruits, vegetables and medicinal herbs are the richest sources of antioxidant compounds (Sies *et al.*, 1992). Many antioxidant compounds extracted from plant sources (phytochemicals) have been identified as free radical or active oxygen scavengers (Ramarathnam *et al.*, 1995). Hence, interest has increased

considerably in finding naturally occurring antioxidants, which can protect the human body from free radicals and retard the progress of many diseases (Prior, 2003).

In many parts of the world, citrus fruits particularly *Citrus sinensis* (sweet orange) have always remained part of human diet for many years. In recent times, however, they have assumed greater importance in diets of both urban and rural dwellers. The increased interest in their consumption is not only due to their sweet refreshing properties but also as a result of increased knowledge of their nutritional and medicinal values (Ezejiofor *et al.,* 2011). *C. sinensis* fruit and its juice have several beneficial, nutritive and health properties (Okwu and Emenike, 2006). Citrus fruits and juices have long been recognized to contain secondary metabolites including antioxidants such as ascorbic acid, flavonoids, phenolics and pectin that are important to human nutrition and health (Gorinstein *et al.,* 2002).

Large amounts of waste materials are generated when citrus fruits are consumed or processed into juice or into canned fruit products. In current citrus industry, emphasis are laid only on orange fruits harnessed and marketed fresh or as processed (and canned) juice, while fruit peels produced in great quantities during the process are mainly discarded as waste. For this reason, researchers have focused on the utilization of citrus products and by-products (Kubo *et al*., 2005; Wu *et al*., 2007). Thus, this research seeks to evaluate some of the purported medicinal properties of Citrus fruit peels (Liu *et al*, 1998; Idu *et al*, 2009).

1.2 Statement of Research Problem

Oxidative stress is critically involved in the pathogenesis of different chronic diseases (Hadi *et al*., 2000) which among others include liver diseases, neurological disorder, arthritis, inflammation (Quambo *et al.*, 1998; Atawodi, 2005), cardiovascular diseases, diabetes mellitus (both types 1 and 2), thyroid abnormalities (broadly hypo- and hyperthyroidism), atherosclerosis, neurodegenerative disorders and certain types of cancer (Halliwell, 1996). These diseases are among the most prevalent diseases around the world and are responsible for a large portion of morbidity and mortality out of all health problems (Tiwari, 2004).

Many toxic substances and infectious organisms generate free radicals (Fernandez-Checa and Kaplowitz, 2005). Likewise, metabolism of certain drugs like paracetamol, produce free radicals, which cause liver damage (Miller and Rice-Evans, 1997). Free radicals cause damage to nucleic acid, proteins, and membrane lipids. The liver may be damaged by free radicals through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury (Halliwell *et al.,* 1992, Wang and Jiao, 2000).

The liver is a vital organ of paramount importance involved in the maintenance of metabolic functions and detoxification (Ramachandra *et al.,* 2007). It is the key organ regulating homeostasis in the body and is involved in almost all the biochemical pathways related to growth, immunity, nutrient supply, energy provision and reproduction (Ward and Daly, 1999). Thus, a healthy liver is a crucial factor for overall health and well-being of an individual. However, liver diseases remain one of the serious health problems (Halliwell *et al.,* 1992, Wang and Jiao, 2000). Hepatotoxicity is a growing concern of modern society. The increasing incidence of

industrial pollutions, occupational hazards and unhealthy lifestyle options such as alcoholism, cigarette smoking, substance abuse and consumption of fatty foods, have contributed to the morbidity and mortality due to liver disease (Scott Luper, 1998).

Drug induced liver damage is a health problem worldwide and is expected to increase as the number of drugs being consumed increases (Ozougwu, 2011). It is a major health issue that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies (Saleem *et al.*, 2008). Drug induced liver injury is the most commonly cited reason for withdrawal of already approved drugs from the market (Butura, 2008). Due to its unique role in metabolism and close relationship with the gastrointestinal tract, the liver is susceptible to injury from drugs and other substances (Jaeschke *et al.*, 2002). According to the United States Acute Liver Failure Study Group, drug-induced liver injury accounts for more than 50% of acute liver failure, with hepatotoxicity caused by overdose of paracetamol accounting for 39% and idiosyncratic liver injury triggered by other drugs accounting for about 13% (Holt, and Ju, 2006). Drug-induced liver injury is responsible for 5% of all hospital admissions (Ostapowicz *et al.*, 2002) and 50% of all acute liver failures and mimics all forms of acute and chronic liver disease (Kaplowitz, 2001).

Liver disease is a major cause of death in the world, creating a serious health concern (EASL, 2012). Chronic liver disease (CLD) is a major world health problem with a high mortality rate (Zucker, 2003). Liver cirrhosis and drug induced liver injury accounts for the ninth leading cause of death amongst the western and developing countries populations (Mohamed *et al.*, 2010). In the United States, Chronic liver

disease and cirrhosis were responsible for 31,802 deaths with a mortality rate of 10.3% in 2010 (Sherry *et al.,* 2012). The number of people who die from liver disease in England is rising (from 9,231 in 2001 to 11,575 in 2009). This translates to a 25% increase in liver disease deaths between 2001 and 2009. Another survey reported that 16,087 people in the UK died from liver disease in 2008 (British Liver Trust, 2009), a 4.5% increase compared to 2007 (Patel *et al*., 2008). This is in contrast to other major causes of death, which have been declining (Effiong *et al*., 2012).In the European union, the prevalence of liver diseases is estimated to be approximately 6% translating to about 29 million people, with hepatitis, the most common liver disease affecting over 10 million of this population (EASL, 2012).

Although data on the mortality of liver diseases in Africa and Nigeria is scarce, there are reports that liver diseases are common in Africa and account for high morbidity and mortality. Hospital-based analyses indicate that acute viral hepatitis, chronic hepatitis, cirrhosis and hepatocellular carcinoma are responsible for at least 12% of medical admissions and over 20% of hospital mortality in many parts of Africa (Eldryd *et al*, 2004). In Nigeria, Chronic liver disease is second to Human Immunodeficiency Virus (HIV) infection as a cause of death in the medical wards of the University of Jos Teaching Hospital (Mandong *et al.,* 2002). Liver diseases accounted for 8.8% of all deaths in the Adult medical wards of University of Ilorin Teaching Hospital (UITH), Ilorin over a 10 year period (Chijioke and Kolo, 2009). With reports from several parts of the world indicating a rise in liver problems, the British Liver Trust (BLT) estimates that the disease could kill more than 60,000 people in the next decade (Patel *et al*., 2008).

Despite the increasing burden of liver diseases, available treatment options are problematic. The effectiveness of orthodox medicines such as interferon, colchicine, penicillamine, and corticosteroids are inconsistent in the treatment of liver diseases and are often accompanied by debilitating adverse reactions. All too often the treatment is worse than the disease (Rutherford, 2013b). These drugs only provide symptomatic relief, without significant changes to the progression of the disease (Owais *et al.*, 2006). They are laden with high relapse rate and are expensive. Ironically, some of these drugs have hepatotoxic side effects e.g. azathiopurine (an immunosuppressant) can cause cholestatic jaundice (Ramagnuolo *et al.*, 1998), colchicine can cause liver failure (Favari and Perez-Alvarez, 1997), e.t.c. The development of new pharmacological agents that can overcome these barriers has become a major goal in drug research and development. Herbal drugs play a role in the management of various liver disorders most of which speed up the natural healing processes of the liver (Subramoniam *et al*., 1998), making the plant kingdom a major target in the search of new drugs and lead compounds to treat these debilitating disorders. These herbal medicines are often prescribed without any scientific basis. A proper evaluation of these herbal medicines with pertinent emphasis on established pharmacological and toxicological paradigms is imperative in order to determine their efficacy, safety and possibly their mechanism(s) of action (Gupta *et al*., 2005).

Citrus is one of the most important commercial fruit crops grown in all continents of the world (Tao *et al.*, 2007). *C. sinensis* (Sweet orange) constitutes about 60% of total citrus yields and is keeping a stable development (Shan, 2006). It is the commonest and the most widely cultivated and consumed citrus fruit in Nigeria (Odbanjo and Sangodoyin, 2002). The fruits are mainly used by juice processing industries while

the peels are generally wasted. Since the juice yield is less than half of the fruit weight, very large amounts of peel wastes are formed every year (Manthey and Grohmann, 2001).

Peel waste which are highly perishable and seasonal, is a problem to the processing industries and pollution monitoring agencies. Thus, the peel of *C. sinensis* is considered as one of the major factors that hamper the development of the juice industry. In Florida alone, *C. sinensis* processing yields about 1.2 million tons of peel waste. Nigeria produces 0.3 million tons out of the 36.0 million tons world production of dry peel waste and has the potential to produce more.

Though Nigeria is not well noted for the exportation of *C. sinensis* fruits, she has the potential to produce more for both local and international markets. Presently, the local processing of citrus fruits is on the increase to meet increasing local demands for fruit juice that was previously met by large-scale importation (Odbanjo and Sangodoyin, 2002). This also means quantum increases in the volume of waste derived from its fruit juice production. There is always an increased attention in bringing useful products from waste materials and *C. sinensis* wastes are no exceptions. Suitable methods have to be adopted to utilize them for the conversion into value-added products (Nand, 1998).

1.3 Justification

A balance between oxidants and antioxidants is known to exist under physiological conditions and small changes in oxidant and/or antioxidant levels may disturb this balance (Sibille *et al.*, 2004). Antioxidants may offer resistance against oxidative

stress by scavenging free radicals, inhibiting lipid peroxidation and by other mechanisms and thereby help in preventing free radical induced diseases (Miller and Rice-Evans, 1997). The antioxidant activities of natural products are related to the total phenolics, flavonoids and other bioactive compounds present (Scarlbert and Garry, 2000; Ayoola *et al.*, 2004).

There is a growing interest in herbal remedies because of their effectiveness, minimal side effects and relatively low cost (Gupta *et al.,* 2005). Herbal medicines have been used traditionally worldwide for the prevention and treatment of various diseases. According to the WHO, 80% of the world population use plant-based remedies as their primary form of healthcare (Subramoniam *et al*., 1998). In developing countries of Africa where about 200,000 to 300,000 known plant species are found, and where about 85% of the population relies on traditional medicine as the cornerstone of their healthcare delivery, the United Nations encourages the inclusion of herbal medicine of proven safety and efficacy in their healthcare delivery program (UNESCO, 1998; Atawodi, 2005).

The consumption of the decoction of citrus fruit peel as tea for various medicinal purposes has become popular around the world. This practice has its origin from ancient Chinese medicine for the treatment and prevention of a number of ailments. In the Chinese tradition citrus fruit peel is used for the treatment of influenza, liver problems, gallstones, rheumatoid arthritis, high blood pressure, hyperlipidemia and arteriosclerosis, myocardial infarction and for the prevention of thrombosis. It is also used as anti-emetic, anti-diarrheal, antacid, for the inhibition of the growth of a variety of digestive tract cancers, e.t.c. (Liu *et al*, 1998).

Oboh and Ademosun (2006) had reported that citrus fruits have about 65 % of their total weight as by-products rich in phenolic antioxidants. Li *et al*., (2006), reported that the major component of citrus peel is flavonoids, especially the polymethoxyflavones (PMFs). Generally flavonoids have been reported to exert wide range of biological activities which they exert as antioxidants, free radical scavengers and chelators of divalent cation (Cook and Samman, 1996; Chebil *et al*., 2006; Middleton *et al.,* 2000). Similarly, Gorinstein *et al.,* (2002) found that the total phenolics content in some citrus peels were 15 % higher than those in fruits. Flavonoids and other phenolics of plant origin have been reported to have roles as scavengers and inhibitors of lipid peroxidation (Miller and Rice-Evans, 1997).

Some workers (Gorinstein *et al.,* 2002; Ghasemi *et al.,* 2009) reported the *in-vitro* antioxidant activity of the citrus peels. However, there is important distinction between the *in-vitro* antioxidant effectiveness of a substance and its ability to prevent oxidation *in-vivo*; because of biochemical and other endogenous factors that come into play (Garry, 1999). On the other hand, recent studies have shown that plants under family Rutaceae like *Murraya koenigii, Citrus limon, Citrus aurantium* and *Tephrosia purpure* have been reported to possess hepatoprotective activity (Gupta, 2009; Bhavsar, 2007; Jain, 2006; Karaca, 2005). It is therefore imperative that *in-vivo* antioxidant properties of citrus peel be investigated for their efficacy in protecting the body from the adverse effects of free radicals as is the case with the damaging effect of Carbon tetrachloride (CCl_4) on the liver

1.4 Theoretical Framework

Carbon tetrachloride is reduced by cytochromes P450 (CYP450) to form a highly toxic trichloromethyl radical. This radical can dismutate to form chloroform ($CHCl_3$) or can react with dioxygen and/or cellular macromolecules to produce lipid peroxidation and disrupt cellular processes (McGregor and Lang, 1996). This causes the swelling and necrosis of hepatocytes and results in the release of cytosolic enzymes into the blood (Wargovich *et al.,* 2001). Excessive production of the reactive species manifests in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., culminating into severe hepatic injury (Singh *et al.,* 1998).

CCl_4 causes oxidative stress in rats as seen by increased lipid peroxidation accompanied by altered antioxidant status. CCl_4 causes a marked rise in lipid peroxidation level in liver and kidney tissues, where glutathione, catalase, superoxide dismutase levels are decreased (Manna *et al.,* 2009). Peroxidative damage to cell membranes affects the integrity and function of the membrane, compromising the cell's ability to maintain ion gradients and membrane phospholipid asymmetry (Usoh *et al.,* 2012). Thus, the transport function of hepatocytes which are the most predominant cells of the liver is impaired in CCl_4-induced hepatic injury leading to leakage of enzymes (Usha *et al*., 2008), and so serum levels of many biochemical markers like aspartate transaminase (AST) and alanine transaminase (ALT) are elevated. Moreover, the liver is the main organ which regulates many important metabolic functions. Hepatic injury is directly associated with these altered metabolic functions (Mitra *et al.,* 1998). Hence, serum alkaline phosphatase (ALP), triglycerides, cholesterol and bilirubin are also elevated (Merlin and Parthasarathy, 2011). Hence, CCl_4 is an acceptable animal model for analyzing antioxidant and

hepatoprotective activity. Its trichloromethyl radical (•CCl_3 -) induced toxicity in rat's liver closely resembles human cirrhosis (Al-Shabanah *et al.,* 2000).

Therefore, CCl_4 was used to induce oxidative stress and liver damage in this study. The presence or absence of antioxidant properties of the extract will be tested by assessing *in-vivo* antioxidant enzymes such as catalase and superoxide dismutase (Merlin and Parthasarathy, 2011) as well as the assessment of lipid peroxidation in microsomes prepared from liver by spectrophotometric estimation of thiobabitturic acid-reacting substances (TBARS) (Varshney and Kale, 1990). On the other hand, the presence or absence of hepatoprotective activity of the extract will be assessed using first-line test parameters often measured in patients presenting with liver diseases .i.e. determination of serum levels of bilirubin (total and conjugated fractions), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum levels of albumin, total protein and alkaline phosphatase (Kobos and Roche, 2004).

1.5 Aim and Objectives

The aim of the present study is to investigate the *in-vivo* antioxidant and hepatoprotective properties of citrus fruit peel in Wistar rats. The specific objectives are:

1. To carry out some qualitative and some quantitative phytochemical screening on the methanol extract of three citrus fruit peels (*C. sinensis*, *C. aurantiifolia*, and *C. medica*) for comparison and selection of one species for further studies.

2. To determine the acute oral lethal dose (LD_{50}) of the methanol extract of selected species (*C. sinensis*) peel in Wistar rats.
3. To determine the antioxidant activity of the methanol extract of selected species (*C. sinensis*) peel in Wistar rats.
4. To determine the hepatoprotective activity of the methanol extract of selected species (*C. sinensis*) peel in Wistar rats.

1.6 Research Hypothesis

Citrus peel extract has antioxidant activity and/or hepatoprotective activity

CHAPTER 2

2. LITERATURE REVIEW

2.1 Free Radicals, Oxidative Stress and Antioxidants

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Although oxidation reactions are crucial for life, they can also be damaging. Oxidation reactions can produce free radicals, which start chain reactions that induce oxidative stress which damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves (Bjelakovic *et al*., 2007).

2.1.1 Free radical

A free radical is a molecule, or a molecular fragment that has a free, or unpaired electron. The free radicals are capable of reacting indiscriminately with any molecules with which they come in contact. Once radicals are formed they can either react with another radical or with a nonradical molecule by various interactions. If two radicals meet they can combine their unpaired electron, thus forming a covalent bond. However, most molecules found *in vivo* are nonradicals. In this case a radical might donate its unpaired electron to the other molecule, or might take one electron from it, thus transforming its radical character. Thus, a new radical is formed. This means that a radical generates another radical, leading to a chain reaction (Nonhebel *et al*., 1979).

Many types of free radical species can be formed by various biological reactions (for example, phenolic and aromatic species during xenobiotic metabolism as a part of natural drug detoxification mechanisms) (Jasmina *et al*., 1999).

2.1.1.1 Free radical-generating systems in normal processes in-vivo

Free radicals are molecular species which are both essential and harmful for aerobic cells. OFRs and other free radicals are constantly formed in the human body by normal metabolic processes, as the reduction of oxygen to water by the mitochondrial electron transport chain (Rice-Evans, 1994). Free radicals are involved in several normal biological processes *in vivo*. Examples of such normal biological processes include:

a. they are part of the cascade of events in the antimicrobial action of the phagocytic cells via NADPH-oxidase, in the arsenal of defense cells (neutrophils, monocytes, macrophages, eosinophils) with which human body is equipped. This process is central to the human antimicrobial defense system, and intended to damage the membranes, DNA and other cellular component of the invading organism (Jasmina *et al.,* 1999).
b. Free radicals can act as regulatory molecules in biochemical processes; for example, lymphocytes and fibroblasts constantly generate small amounts of superoxide radical as growth regulators.
c. Other nonphagocytic cells, such as endothelial cells and arterial smooth muscle cells, can be stimulated to release superoxide. Nitric oxide from endothelial cells is involved in the regulation of vascular tone, inducing the relaxation of smooth muscle cells. Macrophage-derived nitric oxide has been implicated in the killing of tumor cells and bacteria.
d. Free radicals are also involved in the mechanism of action of certain enzymes, for example, ribonucleoside diphosphate reductase, cytochrom P-450 and prostaglandin synthase (Jacobson, 1996).

e. The observations that a wide variety of normal and malignant cell types generate and release superoxide and hydrogen peroxide suggest that a role for such a species as cellular "messengers" is feasible (Burdon, 1994).

Thus, free radicals are involved, in a controlled fashion, in many normal biochemical mechanisms *in vivo,* but they became highly reactive if not tightly controlled (Jasmina *et al*., 1999).

2.1.2 Oxidative stress

"Oxidative stress" occurs when, there is a profound disturbance in the prooxidant-antioxidant balance in favor of former, leading to lipid peroxidation, denaturation of proteins or enzymes or mutagenic damage to nucleic acid (Sies, 1991). Oxidative stress may be due to endogenous (sources mentioned above) or exogenous sources of free radicals (Rice-Evans, 1994).

Increased radical input arises exogenously from:

(i) ionizing radiation,

(ii) excess availability of transition metals,

(iii) side effects of drugs and toxic chemicals, and

(iv) oxygen excess and increased oxygen concentration.

Oxidative stress accompanying disease state may arise from endogenous factors:

(i) phagocyte recruitment and activation at the site of injury,

(ii) leakage from the disrupted mitochondrial electron chain,

(iii) delocalization of transition metal ion and leakage of haem protein,

(iv) decreased protective capacity including reduced antioxidant enzymes,

(v) reduced levels of antioxidant, and

(vi) leakage or destruction of antioxidants.

These factors may be mutually interactive and lead to generation of radical species. Due to their highly reactive nature they can readily combine with other molecules, such as enzymes, receptors, and ion pumps, causing oxidation directly, and inactivating or inhibiting their normal function (Davies, 1993: Jacobson, 1996; Jasmina *et al*., 1999). Some of the products of oxygen free radicals attack on other molecules can interfere with nucleic acid function, generating alterations in the base sequence with the potential for mutations, leading to cancer or germ-line mutations (Burdon, 1994).

Changes in normal proteins and other structures by free radical species can also generate novel immunogenic structures (Davies, 1993). One of the most destructive effects of oxygen free radicals is the initiation of lipid peroxidation which can result in runaway reactions. If not terminated, this chain reaction will lead to destruction of the cell membranes, breakdown compartmentalization and release of lysosomal enzymes and subsequent autolysis. Termination of this chain of events can only be achieved by the reaction going to completion or by chain-breaking antioxidants that destroy the free radicals produced (Jasmina *et al*., 1999).

2.1.3 Antioxidants

Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause (Sies, 1997). As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is

being intensively studied. Plants and animals maintain complex systems of multiple types of antioxidants (Bjelakovic *et al.*, 2007). To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Jacob, 1995). These components are listed below accordingly:

I. Endogenous Antioxidants

a. Enzymatic

Superoxide dismutase
Catalase
Glutathione peroxidase
Glutathione reductase

b. Non-Enzymatic

i. Metal Binding Proteins
Albumin (copper)
Ceruloplasmin (copper)
Metallothionein (copper)
Ferritin (iron)
Myoglobin (iron)
Transferrin (iron)

ii. Metabolic antioxidants
Bilirubin
Thiols, e.g., glutathione, lipoic acid, N-acetyl cysteine
NADPH and NADH
Ubiquinone (coenzyme Q10)
Uric acid
L-ariginine,
melatonin
lipoic acid

II. Exogenous Antioxidants

Dietary Antioxidants and Phytoconstituents/phytonutrients
Vitamin C
Vitamin E
Beta carotene and other carotenoids e.g., lycopene and lutein
Polyphenols, e.g., flavonoids, flavones, flavonols, and proanthocyanidins (Percival, 1998)

2.1.3.1 Antioxidant defence mechanism

Antioxidants may exert their activity by several mechanisms which include:

1. Suppressing the production of active species by reducing hydroperoxides and H_2O_2,
2. by sequestering metal ions,
3. termination of chain reaction by scavenging active free radicals and
4. by repairing and/or clearing damaged cells.
5. Some antioxidants induce biosynthesis of other antioxidants or defense enzymes (Tiwari , 2001; Tiwari , 2004).

Therefore antioxidants synthesized in body or supplied from outside like phytoconstituents plays important role to protect the body from free radical induced injury. Typical examples are given below:

- *Superoxide dismutase (SOD);* is an important endogenous antioxidant enzyme that acts as the first line defense system against reactive oxygen species (ROS) which scavenges superoxide radicals to form H_2O_2.

- *Catalase;* is an endogenous antioxidant enzyme present in all aerobic cells that facilitates the removal of hydrogen peroxide (H_2O_2) to form water and oxygen.

- *Glutathione peroxidase (GPx);* present in the cytoplasm of the cells removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH).

- *Glutathione reductase (GR;)* is a flavoprotein enzyme, which regenerates GSH from oxidized glutathione in the presence of NADPH.

- *GSH;* is a tripeptide and a powerful antioxidant present within the cytosol of cells and is the major intracellular nonprotein thiol compound (NPSH). Thiol (SH) groups

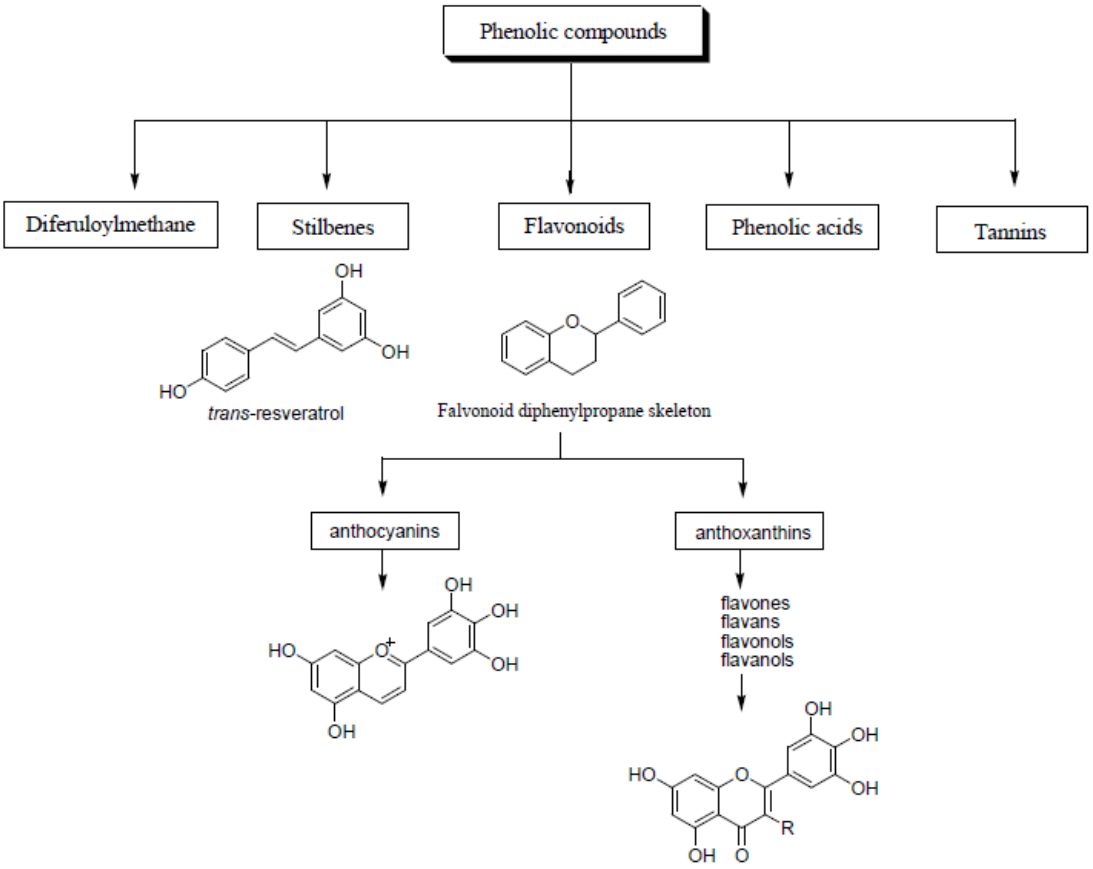
present in GSH reacts with H_2O_2 and OH• radical and prevent tissue damage. GSH is capable of scavenging ROS directly or enzymatically via GPx.

- *Vitamins C and E* are exogenous antioxidants but are also non-enzymatic endogenous antioxidants because they exist within normal cells. They react with free radicals to form radicals themselves which are less reactive than the radicals. They break radical chain reactions by trapping peroxyl and other reactive radicals (Sen *et al.*, 2010).

2.1.3.2 Phytonutrients

A number of other dietary antioxidant substances exist beyond the traditional vitamins discussed above. Many plant-derived substances, collectively termed "phytochemicals," are becoming increasingly known for their antioxidant activity (Briviba and Sies, 1994). Dietary polyphenols are the most abundant antioxidants in human diets (Butterfield *et al.*, 2002). They represent a group of secondary metabolites which widely occur in fruits and vegetables. Dietary polyphenols exhibit many biologically significant functions, such as protection against oxidative stress and degenerative diseases. Experimental data indicate that most of these biological actions can be attributed to their intrinsic antioxidant capabilities (Chebil *et al.*, 2006**).**

Figure 2.1 Classification of dietary polyphenols.



(Xiuzhen *et al.*, 2007)

With over 8,000 structural variants, polyphenols denote many substances with aromatic ring(s) bearing one or more hydroxyl moieties. They are subdivided into groups by the number of phenolic rings and of the structural elements that link these rings (Butterfield *et al.*, 2002), see figure 2.1 above.

In plants, flavonoids serve as protectors against a wide variety of environmental stresses while, in humans, flavonoids appear to function as "biological response modifiers" (Percival, 1998). Flavonoids have been reported to exert wide range of biological activities. These includes: anti-inflammatory, antibacterial, antiviral, antiallergic (Cushnie and Lamb, 2005; Cook and Samman, 1996), cytotoxic

antitumour, treatment of neurodegenerative diseases, vasodilatory action (Williams *et al.*, 2004; Tsuchiya, 2010). In addition flavonoids are known to inhibit lipid-peroxidation, platelet aggregation, capillary permeability and fragility, cyclo-oxygenase and lipoxygenase enzyme activities. They exert these effects as antioxidants, free radical scavengers, chelators of divalent cation (Cook and Samman, 1996; Chebil *et al.*, 2006; Middleton, 2000). Hence flavonoids may inhibit several oxidative stress induced diseases.

2.1.4 Oxidative stress induced diseases

Oxygen derived free radical reactions have been implicated in ageing process and in the pathogenesis of many human diseases including;

- Neurodegenerative disorder like alzheimer's disease, parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, memory loss and depression.
- Cardiovascular disease like atherosclerosis, ischemic heart disease, cardiac hypertrophy, hypertension, shock and trauma.
- Pulmonary disorders like inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease.
- Diseases associated with premature infants, including bronchopulmonary, dysplasia, periventricular leukomalacia, intraventricular hemorrhage, retinopathy of prematurity and necrotizing enterocolitis.
- Autoimmune disease like rheumatoid arthritis.
- Renal disorders like glomerulonephritis and tubulointerstitial nephritis, chronic renal failure, proteinuria, uremia.

- Gastrointestinal diseases like peptic ulcer, inflammatory bowel disease and colitis.
- Tumors and cancer like lung, leukemia, breast, ovary, rectum cancers etc.
- Eye diseases like cataract and age related diseases of the retina, maculopathy.
- Diabetes
- Skin lesions
- Immunodepression.
- Liver disease, pancreatitis.
- AIDS.
- Infertility (Sen *et al.,* 2010).

These diseases are among the most prevalent diseases around the world with high morbidity and mortality (Tiwari, 2004). However, the focus for this study shall be liver disease.

2.2 The Liver

The liver is a large organ weighing about 1.5kg in an average adult (Beckett, 2001). It is found in all vertebrates and some invertebrates. In humans, the liver is located in the upper right portion of the abdomen, just below the diaphragm, overlying the gallbladder.

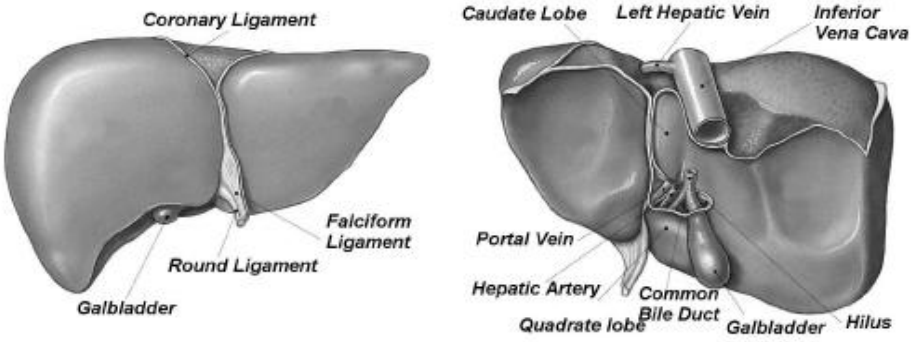


Figure 2.2: The liver.

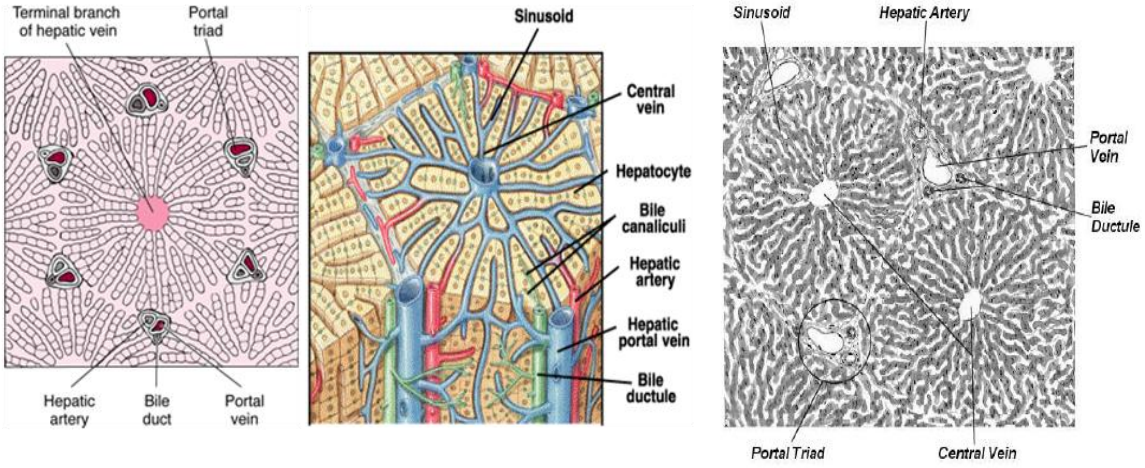


Figure 2.3: Organization of the liver. The liver is organized into lobules around terminal branches of the hepatic vein. Between the lobules are portal triads. Each triad consists of branches of a bile duct, portal vein, and hepatic artery (Herrine, 2012).

2.2.1 Structure of the liver

The liver is wedge-shaped, dark reddish in colour and divided into 4 lobes: right, left, caudate, and quadrate. The right and left lobes are the largest, while the caudate and quadrate are smaller and located posteriorly. These lobes are further divided into lobules which are the basic functional unit of the liver (Allen, 2002). A single lobule is about the size of a sesame seed and is roughly hexagonal in shape (see figure 2.2 above).

The liver has 5 cell types: hepatocytes, Kupffer cells, sinusoidal endothelial cells, bile duct epithelial cells, and Ito cells.

1) Hepatocytes represent 60% of the liver's cells, and about 80% of the liver's total cell mass. Most of the liver's synthetic and metabolic capabilities stem from the hepatocytes. Hepatocytes are arranged in plates only a single cell thick. Blood flowing toward the hepatic vein passes both exposed surface areas of the hepatocyte plates, and toxins and nutrients within the blood are extracted by the hepatocytes.
2) Kupffer cells are macrophages that reside in the sinusoids. These cells help clear out old red blood cells and bacteria. They also break down heme (the iron-containing pigment in hemoglobin) into bilirubin.
3) Sinusoidal endothelial cells are fenestrated (Latin for "windows"), meaning they have large pores that allow most proteins to pass freely through the sinusoidal endothelium into where they can make direct contact with hepatocytes. The pores are also bi-directional, meaning that proteins created by the liver and other substances stored or processed by the liver can also be passed back into the blood.
4) Bile duct epithelial cells line the interlobular bile ducts within the portal triads.
5) Ito cells are found in the space of disse. They are important because when the liver is injured, the Ito cells transform into cells that produce collagen, which leads to liver fibrosis. If this occurs on a large scale, it can lead to cirrhosis of the liver.

2.2.2 Liver Functions

The liver performs over 500 metabolic functions that can be generally classified into 3 groups;

1) *Storage:* The liver balances the supply of nutrients with demand. For example, the liver stores glucose as glycogen, and converts it back to glucose when needed. The liver can convert excess glucose and amino acids into fatty acids for storage. If the supply of glycogen is depleted, the liver can also synthesize glucose from amino acids, lactate, and glycerol (Allen, 2002). Other nutrients stored in the liver include iron and vitamin A (Nelson and Cox, 2005).

2) *Synthesis:* The liver synthesizes many proteins that circulate in the blood. These include albumin, coagulation factors, alpha1-antitrypsin, very low density lipoprotein, insulin-like growth factors, thrombopoietin, and so forth. It also produces glucose from certain amino acids, lactate or glycerol (gluconeogenesis), glycogen from glucose (glycogenesis) and glucose from glycogen (glucogenolysis). The liver synthesizes cholesterol and produces bile which acts as a detergent, and breaks fats down into smaller components so they can be digested in the small intestine. Bile also provides a way for the liver to remove wastes, including bilirubin, cholesterol, and toxins. Finally, the liver can synthesize non-essential amino acids when needed by the body (Allen, 2002).

3) *Biotransformation and elimination:* The liver is central to the metabolism of virtually every foreign substance. Most drugs and xenobiotics are lipophilic, enabling them to cross the membranes of intestinal cells (Weinshilboum, 2003). Toxins are detoxified by the liver's ability to metabolize lipophillic compounds (Nelson and Cox, 2005). Drugs are rendered more hydrophilic by biochemical processes in the hepatocyte, yielding water-soluble products that are excreted in urine or bile (Weinshilboum, 2003). This hepatic biotransformation involves

oxidative pathways, primarily by way of the cytochrome P-450 enzyme system (Guengerich, 2001). However, the reaction to which some xenobiotics are subjected to in the liver actually increases their toxicity (Granner *et al*, 2003). The liver also processes excess amino groups into urea and other products to be excreted by the kidney.

Thus, the liver serves as the body's distribution centre, exporting nutrients in the correct proportion to other organs, smoothing out fluctuations in metabolism caused by intermittent food intake and processing products for elimination (Nelson and Cox, 2005). Failure of these metabolic functions represents the basic pathophysiology of all forms of liver disease (Allen, 2002).

The liver has protective mechanisms against disease. It can regenerate itself by repairing or replacing injured tissue. Furthermore, it has many cell units responsible for the same task. If one area is injured, other cells will perform the functions of the injured section indefinitely or until the damage has been repaired. However, these mechanisms may be overpowered (C-Health fact sheet, 2013).

2.2.3 Diseases of the liver

Liver disease (also referred to as hepatic disease) is a term that describes significant pathological changes in the anatomy and function of the liver. Based on duration, diseases of the liver may be chronic, subacute, acute or fulminant. A chronic disorder lasts for more than 6 months; a subacute disorder lasts for 3 to 6 months; while an acute disorder occurs over a period less than 3 months. A very severe disorder that

leads to liver failure within 6 weeks is termed fulminant (Allen, 2002). Chronic liver disease is more prevalent than acute liver disease (McLaughlin, 2000).

There are different types of liver disease which can result from a wide variety of insults such as infections, autoimmune disorders, drugs and toxins (Herrine, 2012). They include fatty liver disease, alcoholic liver disease, hemochromatosis, cirrhosis, hepatitis, Wilson's disease, primary sclerosing cholangitis, primary biliary cirrhosis, Budd-Chiari syndrome (BCS), Hepatic veno-occlusive disease (VOD) and Gilbert's syndrome, just to mention a few. The focus here will be the two most common forms: hepatitis and cirrhosis (C-Health fact sheet, 2013).

2.2.3.1 Hepatitis

Hepatitis refers to inflammation of the liver (Allen, 2002). Hepatitis which is the most common type of liver disease (Rutherford, 2013a) has a number of potential causes, both infectious and non-infectious. Alcohol, prescription medications, and drug abuse are predominant noninfectious causes, while viruses and bacteria are important infectious etiologic factors. Fulminant hepatitis refers to sudden, severe liver dysfunction which may lead to hepatocellular necrosis as well as hepatic encephalopathy. Secondary hepatitis may occur as a sequela of other disease entities such as mononucleosis, syphilis, and tuberculosis. Viral and drug induced hepatitis are examples of primary hepatitis (Golla *et al.*, 2004).

1) Acute viral hepatitis; is caused by specific hepatotrophic viruses that have diverse modes of transmission and epidemiology. The individual prevalence for each virus varies based on geographical location and risk factors. Most cases of acute viral hepatitis are self limiting, resolving spontaneously 4 to 8 weeks after symptom onset,

some however progress to chronic hepatitis, while some rare cases progress to fulminant hepatitis (Rutherford, 2013a). The different types of virus that cause viral hepatitis are:

Hepatitis A virus (HAV) is a single-stranded RNA virus surrounded by a protein capsid. Transmission is primarily by the oral-fecal route. Various methods of spread are through contact of an infected person, traveling to an endemic region, and ingestion of contaminated food or water. Hepatitis A is typically a self-limiting disease and usually causes mild illness characterized by sudden onset of non-specific symptoms.

Hepatitis B virus (HBV) is a DNA virus with a nuclear capsule enveloped by an outer lipid layer containing hepatitis B surface antigen (HBsAg). The nuclear capsule contains other viral components, including the hepatitis B core antigen (HBcAg), the e antigen (HBeAg), DNA, and DNA polymerase. HBV replicates within the hepatocyte through an intermediate step of reverse transcription mediated by viral polymerase. Sexual contact, intravenous drug use, and transfusion of blood and blood products are common modes of transmission (Golla *et al.*, 2004). Transmission can also take place via saliva. A younger individual who becomes infected with HBV has a higher risk for developing chronic HBV and hepatocellular carcinoma. Approximately 90% of adult HBV-infected patients experience a full recovery, but 5-10% of the patients will develop chronic hepatitis with complications such as cirrhosis and hepatocellular carcinoma (DePaola, 2003).

Hepatitis C virus (HCV) is a blood-borne, single stranded RNA flavivirus encoding for a capsid protein, two envelope proteins, and some nonstructural proteins. People at

increased risk include hemophiliacs, dialysis patients, and intravenous drug users. Other modes of transmission are sexual, perinatal, and idiopathic. Though the primary mode of transmission is through blood, HCV has also been detected in saliva. 85% of patients with HCV will develop chronic hepatitis (Wisnom and Siegel, 2003). Chronic HCV infection is the major cause of cirrhosis and hepatocellular carcinoma.

Hepatitis D virus (HDV) is a defective RNA virus that uses the HBV surface antigen as a viral envelope. HDV can occur as a coinfection or superinfection in individuals infected with HBV, which may then progress to severe fulminant infection. Transmission of HDV can occur via infected blood or blood products and is primarily seen in intravenous drug users and hemophiliacs. HDV may also be transmitted through sexual activity.

Hepatitis E virus (HEV) is a nonenveloped, singlestranded RNA virus that is highly unstable due to the lack of a lipid membrane. Transmission, similar to HAV, is by the oral-fecal route. The primary method of spread is through contaminated drinking water and occurs mostly in places with inadequate sanitary precautions. The infection is usually self-limiting, although there have been cases of fulminant hepatitis and chronic disease. In pregnant patients infected with HEV, there is a higher risk of fulminant hepatitis.

Hepatitis G virus (HGV) is caused by two isolated viruses that appear to be almost identical. They are single-stranded RNA viruses similar to HCV (Golla *et al.*, 2004). HGV is transmitted through blood and blood products, sexual activity, and perinatal contact. Due to similar transmission routes, HGV may be found in association with other hepatitis viruses, especially HCV. Individuals at risk are organ transplant and

blood transfusion recipients, IV drug users, dialysis patients, and health care workers with exposure to blood and chronic HCV hepatitis patients (Wisnom and Siegel, 2003). Although the rate of remission is low, little evidence exists that HGV causes significant liver damage, even with persistent viremia (Golla *et al.,* 2004).

2) Chronic Hepatitis: Common causes of chronic hepatitis include hepatitis B and C viruses (discussed above), autoimmune mechanisms (autoimmune hepatitis), and drugs. Some patients present with cirrhosis or its complications (eg, portal hypertension). Hepatitis B virus (HBV) and hepatitis C virus (HCV) are frequent causes of chronic hepatitis. Hepatitis A and E viruses are not causes.

Many drugs, including isoniazid, methyldopa, nitrofurantoin and acetaminophen can cause chronic hepatitis. The mechanism varies with the drug and may involve altered immune responses, cytotoxic intermediate metabolites, or genetically determined metabolic defects. Other causes of chronic hepatitis include alcoholic hepatitis and nonalcoholic steatohepatitis (Rutherford, 2013b).

Autoimmune hepatitis

Autoimmune hepatitis is a chronic inflammatory disease. Although the etiology is not clear, it is thought that environmental and viral factors may cause alterations in cellular markers on hepatocytes leading to an autoimmune response in genetically susceptible individuals. Autoimmune hepatitis may lead to liver cirrhosis (Golla *et al.,* 2004).

2.2.3.2 Hepatic cirrhosis

Cirrhosis is the irreversible end result of fibrous scarring; normal hepatic architecture is replaced with interconnecting bands of fibrous tissue. Normal function is disturbed by the resulting inadequate blood flow and ongoing damage to hepatocytes. Liver fibrosis and cirrhosis are diseases marked by an increase in total liver collagen, as well as other matrix proteins that are disruptive to liver architecture and function (Golla *et al.*, 2004). Any chronic liver disease can eventually lead to cirrhosis. On the other hand cirrhosis can eventually lead to hepatocellular carcinoma and liver failure (Rutherford, 2013b) (see figure 2.3, below).

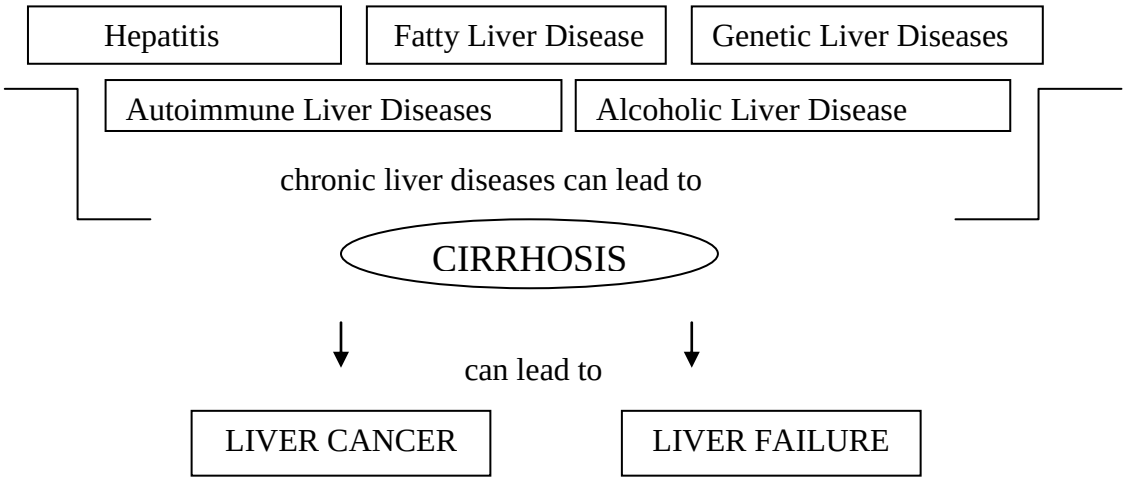


Figure 2.4 Chronic liver diseases can eventually lead to cirrhosis. On the other hand cirrhosis can eventually lead to liver cancer and liver failure.

The most common etiologic factors resulting in cirrhosis are hepatitis B, hepatitis C, and excessive alcohol consumption. Other potential causative factors are immune-mediated damage (as with autoimmune mediated hepatitis discussed above), genetic abnormalities (e.g hematochromatosis), and nonalcoholic fatty liver disease (NAFLD) (Golla *et al.*, 2004). Discussion of some of these causes of cirrhosis and hepatocellular carcinoma will follow.

1) Fatty liver disease; is an excess buildup of fat in the liver, more than 5 to 10 percent of the liver's total weight. This condition is most common in those who are overweight, diabetic or have metabolic syndrome. If unrelated to alcohol consumption, it is known as nonalcoholic fatty liver disease (NAFLD) or nonalcoholic steatohepatitis (NASH). Otherwise, it is known as alcoholic liver disease. Excess liver fat can lead to inflammation, progressing to cirrhosis in 20 percent of patients. Treatment includes weight loss, exercise, diabetes control, regulating blood pressure and cholesterol, limiting or eliminating alcohol consumption and abstinence from smoking (Cleveland Clinic Foundation, 2008).

2) Alcoholic Liver Disease; comes in 3 major varieties: alcoholic fatty liver, alcoholic hepatitis, and alcoholic cirrhosis. All 3 can occur alone or together.

a. *Fatty liver* is the most common, and the least harmful. It can occur within days of moderate to heavy drinking. Fat accumulates in the cytoplasm of liver cells, causing the liver to swell, sometimes to large proportions. Fatty liver often has no symptoms, and can disappear as quickly as it appears.
b. *Alcoholic hepatitis* can exist as either acute or chronic conditions. Acute hepatitis can often cause death, and the chronic form often leads to cirrhosis. However, alcoholic hepatitis is also potentially reversible, if recovery occurs and the patient abstains from drinking.
c. *Alcoholic cirrhosis*, like all forms of cirrhosis, is often life-threatening. The disease is characterized by regenerative nodules of hepatic tissue completely surrounded by fibrous scar tissue. The scar tissue grows faster than liver cells can regenerate, and the growing network of scar tissue inhibits blood flow as described earlier. Once

cirrhosis develops, the risk of liver cancer elevates substantially, even if the patient abstains from drinking for several years.

3) Hemochromatosis: This is a condition in which too much iron is contained in the body. It is the most common genetic disease in the United States among people of European origin (Golla *et al.*, 2004), but does not have to be inherited. In hemochromatosis, the body cannot absorb iron as effectively, and also cannot detect when iron levels are too high. Harmful levels of iron can be accumulated in the body as it is not expelled from the body easily. This excess iron is then absorbed into the body's organs, particularly the liver (Allen, 2002). Iron damages the body through its promotion of oxidation, increasing the level of free radicals in the body. The iron accumulation may lead to diabetes mellitus, cardiomyopathy, and cirrhosis.

4) Hepatocellular Carcinoma (HCC); is the most common primary liver malignant cancer. It is one of the most common cancers in the world (Allen, 2002). HCC is the sixth most common cancer in men, and the eleventh most common cancer in women in the United States (Golla *et al.*, 2004). HCC is even more common in East Asia and sub-saharan Africa (Herrine, 2012). This cancer is a devastating disease with five-year survival rates around 2%. The most common etiologic factors in HCC are HBV and HCV. Other causes are hemochromatosis and alcoholic liver disease. In this condition, α_1-antitrypsin levels may be elevated. The incidence of HCC is rising, and this trend is expected to continue for years (Golla *et al.*, 2004). Environmental carcinogens such as fungal aflatoxins are believed to contribute to the high incidence of HCC in subtropical (Herrine, 2012).

2.2.4. Symptoms and complications of liver disease

During early stages of most liver disease symptoms are usually masked, and any signs or symptoms can be vague and easily confused with other problems. Unfortunately, there are often no signs of liver disease until the damage is considerable. Early symptoms of liver damage include fatigue, weakness, malaise, loss of appetite, nausea and vomiting, loss of weight, pain or discomfort in the abdomen (the area below the ribs, including the stomach area), itch, small red veins that resemble the body of a spider (spider naevi), enlarged and tender liver, dark urine/grey pale faeces and loss of libido (Thursz, 2011). Symptoms and complications that indicate more severe damage include:

1) Jaundice: This is a visible sign of liver problems. It appears as a yellowish discoloration of the skin and whites of the eyes (sclerae) caused by the abnormal buildup of bilirubin in the blood. Urine is dark because of bilirubin excreted through the kidneys. Increase in bilirubin may be as a result of inflammation or other abnormalities of the liver cells, blockage of bile ducts, and breakdown of a large number of red blood cells (this occurs with malaria).

2) Hepatomegaly: This refers to enlargement of the liver. Usually, a slightly enlarged liver is not associated with any symptoms, while a grossly enlarged liver may present with symptoms of abdominal discomfort or "feeling full."

3) Cholestasis: This refers to reduced or blocked bile flow. Symptoms of cholestasis may include jaundice, chills and bone loss.

4) Portal hypertension and variceal bleeding: Scarring of the liver inhibits blood flow. This leads to increased pressure in the portal vein (portal hypertension) and blood may be forced through more delicate veins (varices) which burst under the pressure *(Pereira, 2011)*. Portal hypertension can lead to the growth of collateral vessels that will bypass the liver and connect directly to the general circulation, thereby conveying substances that are usually removed by the liver into the general circulation. With portal hypertension there may be distended abdominal cavity and bleeding of the varicose veins (variceal bleeding) at the lower end of the oesophagus and in the stomach lining (Eldon, 2012b).

5) Ascites and peripheral oedema: Ascites is the build-up of fluid in the abdominal cavity, caused by fluid leaks from the liver and the intestine. Peripheral oedema is swelling in the ankles and legs which are very common in people with advanced cirrhosis. Ascites can cause dismfort and shortness of breath (Pereira, 2011). Ascites due to liver disease usually accompanies other liver disease complications like portal hypertension.

6) Hepatic encephalopathy: This is the deteriation of brain function as a result of buildup of toxic substances which are normally removed by the liver. It can range from mental confusion, sleep disturbance, subtle personality changes and poorer performances in tests, to problems in movement and speech, and can progress to a loss of consciousness, where it can be life-threatening.

7) Bleeding: The liver synthesizes blood clotting components. There is the risk of severe bleeding when the liver is not functioning effectively.

8) Kidney problems: With advanced cirrhosis and complications such as encephalopathy, jaundice and bleeding problems, there is the risk of a serious complication called hepatorenal syndrome, which is kidney failure in liver disease (Pereira, 2011).

9) *Liver failure*: This is a life-threatening condition that occurs when large parts of the liver become damaged beyond repair and the liver is no longer able to function. The most common causes of chronic liver failure include hepatitis B, hepatitis C, chronic alcohol consumption, cirrhosis, hemochromatosis, and malnutrition. While The causes of acute liver failure include acetaminophen overdose, viruses including hepatitis A, B, and C (especially in children) and reactions to certain prescription and herbal medications (Kimball, 2012).

2.2.6 Diagnosis of liver disease

Tests and procedures used to diagnose liver problems include blood tests, imaging tests and tests of liver tissue.

2.2.5.1 Blood tests.

Serum liver biochemical tests are the major blood tests for diagnosis of liver diseases. They include:

(1. Tests for cellular injury - alanine aminotransferase, aspartate aminotransferase.

(2. Tests for cholestasis, or duct injury - alkaline phosphatase, gamma-glutamyltransferase, and bilirubin.

(3. Tests for synthetic function - prothrombin time and serum proteins.

(4. Other blood tests

Tests for cellular injury

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST); are perhaps the most commonly used indicators of liver damage (Canadian Liver Foundation, 2012). ALT and AST leak from damaged cells; thus, are sensitive indicators of liver injury (Eldon, 2012b). AST is found in highest concentration in heart compared with other tissues of the body such as liver, skeletal muscle and kidney. Whereas ALT is mainly found in liver compared with other tissue of the body (Shivaraj *et al*., 2009). Thus, ALT is a more specific indicator of liver damage (Canadian Liver Foundation, 2012).

Normal serum ALT is 7-56 U/ L while normal serum AST is 0 to 35 U/L (Shivaraj *et al*., 2009). In acute injury to the liver, as in viral hepatitis, the level of the ALT and AST may be used as a general measure of the degree of liver inflammation or damage. In chronic liver disease, this is not the case, for these enzymes may be entirely within the normal range even in the presence of cirrhosis (Canadian Liver Foundation, 2012). 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 (Shivaraj *et al*., 2009).

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. The ratio increases in progressive liver functional impairment (Shivaraj *et al*., 2009). The AST/ALT ratio is ≤1 in the majority of cases of acute hepatocellular injury. An AST/ALT ratio >1 may

be seen in alcoholic liver disease, drug-induced liver injury, malignancy or cirrhosis. In acute alcoholic liver disease, the ratio is often >2 (Bpac better medicine, 2007).

Tests for cholestasis, or duct injury

Bilirubin; the pigment in bile, is produced from the breakdown of heme proteins, mostly from the heme moiety of hemoglobin in senescent RBCs. Unconjugated (free) bilirubin which is insoluble, is conjugated in the liver with glucuronic acid to form the more water-soluble bilirubin diglucuronide (Eldon, 2012b).

Jaundice occurs when bilirubin becomes visible within the sclera, skin, and mucous membranes at a blood concentration of around 40 μmol/L. 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 8 μmol/L. The serum bilirubin levels more than 17 μmol/L suggest liver diseases and levels above 24 μmol/L indicate abnormal laboratory liver tests (Shivaraj *et al*., 2009).

Unconjugated hyperbilirubinemia (indirect bilirubin fraction > 85%) reflects increased bilirubin production (eg, in hemolysis) or defective liver uptake or conjugation (eg, in Gilbert syndrome). Such increases in unconjugated bilirubin are usually < 5 times normal (to < 6 mg/dL [<100 μmol/L]) unless there is concurrent liver injury.

Conjugated hyperbilirubinemia (direct bilirubin fraction > 50%) results from decreased bile formation or excretion (cholestasis). When associated with other liver function test abnormalities, a high serum bilirubin indicates hepatocellular dysfunction.

Alkaline phosphatase (ALP); Increased levels of this hepatocyte enzyme suggest cholestasis (Eldon, 2012b) and is the most frequently used test to detect obstruction in the biliary system (Canadian Liver Foundation, 2012). Normal serum ALP is 41 to 133 U/L (Shivaraj *et al*., 2009). Although this enzyme is found both in the liver and bile, and leaks into the bloodstream in a manner similar to that described for the ALT and AST, ALP is also found in other organs such as bone, placenta, and intestine. For this reason, it is often useful to measure another enzyme not found in these organs, either gamma-glutamyl transpeptidase (GGT) or 5'-nucleotidase (5'-NT), along with ALP when the origin of the elevated alkaline phosphatase is not clear. Abnormalities of the 5'-NT or GGT would then suggest liver or biliary tract disease (Canadian Liver Foundation, 2012).

Gamma Glutamyl Transferase (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 is mainly attributed to hepatobiliary system even though it is found in more concentration in renal tissue. The normal level of GGT is 9 to 85 U/L. Increased levels are seen in liver diseases. Other conditions like uncomplicated diabetes mellitus, acute pancreatitis, myocardial infarction, anorexia nervosa, Gullian barre syndrome, hyperthyroidism, obesity and dystrophica myotonica also cause elevated levels of GGT. The primary usefulness of GGT is limited in ruling out bone disease as GGT is not found in bone.

5’ Nucleotidase (5'-NT); is a glycoprotein generally disseminated throughout the tissues of the body. It is localised in cytoplasmic membrane and catalyzes the release

of inorganic phosphate from nucleoside-5-phosphates. The normal range established is 0 to 15 U/L (Shivaraj *et al*., 2009). Increases in levels of this enzyme are as sensitive as alkaline phosphatase for detecting cholestasis and biliary obstruction but are more specific, almost always indicating hepatobiliary dysfunction (Eldon, 2012b).

Tests for synthetic function

Prothrombin (PT); is a valuable measure of the liver's ability to synthesize fibrinogen and vitamin K–dependent clotting factors: factors II (prothrombin), V, VII, and X. PT may be expressed in time (sec) or, preferably, as a ratio of the patient's measured PT to the laboratory's control value. Changes can occur rapidly because some of the involved clotting factors have short biologic half-lives (eg, 6 h for factor VII). Abnormalities indicate severe hepatocellular dysfunction, a bad signal in acute liver disorders. In chronic liver disorders, an increasing PT indicates progression to liver failure. The PT does not increase in mild hepatocellular dysfunction and is often normal in cirrhosis.

Serum proteins: Hepatocytes synthesize most serum proteins, including α- and β-globulins, and albumin. These proteins usually increase in response to damage (eg, inflammation) to various tissues, so that elevations may not specifically reflect liver disorders.

Serum albumin; commonly decreases in chronic liver disorders because of an increase in volume of distribution (eg, due to ascites), a decrease in hepatic synthesis, or both. Values < 3 g/dL (< 30 g/L) suggest decreased synthesis, caused by advanced cirrhosis (the most common cause), alcoholism, chronic inflammation or protein

undernutrition. Hypoalbuminemia can also result from excessive loss of albumin from the kidneys (ie, nephrotic syndrome), gut (eg, due to protein-losing gastroenteropathies), or skin (eg, due to burns or exfoliative dermatitis). Because albumin has a half-life of about 20 days, serum levels take weeks to increase or decrease (Eldon, 2012b).

Total protein: includes the total of albumin and other plasma proteins (mostly immunoglobulins). Changes in the total protein are non specific as there may be increases and decreases in different components. For example chronic severe inflammatory liver disease may be associated with a low albumin and other liver globulins, but raised levels of immunoglobulins (Bpac better medicine, 2007). Hepatocytes also make proteins that aid in the diagnosis of specific disorders:

- α_1-Antitrypsin (absent in α_1-antitrypsin deficiency)
- Ceruloplasmin (reduced in Wilson's disease)
- Transferrin (saturated with iron in hemochromatosis)
- Ferritin (greatly increased in hemochromatosis)

Tests for synthetic function of the liver may include tests for serum cholesterol and triglycerides where additional tests for metabolic and secretional capacity of the liver are required; as these may be elevated in liver disease (Merlin and Parthasarathy, 2011).

Other blood tests

These are highly specialized tests which may be used to indicate more specifically the presence of certain liver diseases or inherited conditions that affect the liver. For example:

Viral serologic tests: specific antibodies, proteins, and nucleic acids used to indicate the presence of viral hepatitis include:

1- Immunoglobulin M antibody to Hepatitis A Virus for acute Hepatitis A infection.

2- Hepatitis B Surface Antigen (HBsAg) and hepatitis B Virus DNA (HBV DNA for hepatitis B

3- Antibody to Hepatitis C Virus (anti-HCV) and hepatitis C Virus RNA (HCV RNA) for hepatitis C virus.

4- Serologic testing and antibody to Hepatitis D virus (anti-HDV) is used to detect infection of Hepatitis D virus.

5- Diagnosis of Hepatitis G virus is based on detection of HGV RNA in serum one to four weeks after infection. Serum anti-HGV indicates past infection.

Ammonia: Elevated ammonia levels occur in hepatic encephalopathy.

Immunologic tests: In chronic liver disorders, serum immunoglobulins often increase. Levels increase slightly in acute hepatitis, moderately in chronic active hepatitis, and markedly in autoimmune hepatitis. Different immunoglobulins are usually very high in different disorders:

- IgM in primary biliary cirrhosis
- IgA in alcoholic liver disease

- IgG in autoimmune hepatitis

Antimitochondrial antibodies are heterogeneous antibodies which are positive, usually in high titers, in > 95% of patients with primary biliary cirrhosis. They are also occasionally present in autoimmune hepatitis and drug-induced hepatitis. Antinuclear and/or anti-smooth muscle antibodies may indicate the presence of autoimmune hepatitis.

α-Fetoprotein (AFP): Slight elevations of AFP should prompt consideration of primary hepatocellular carcinoma (HCC). Serum AFP generally correlates with tumor size, differentiation and metastatic involvement.

Serum iron: Elevations in the serum iron, transferrin saturation and ferritin may indicate the presence of hemochromatosis.

Ceruloplasmin: A deficiency of ceruloplasmin is usually seen in patients with a copper metabolism disorder called Wilson disease.

α_1-antitrypsin: A low level of α_1-antitrypsin may indicate the presence of lung and/or liver disease in children and adults due to α_1-antitrypsin deficiency (Canadian Liver Foundation, 2012).

2.2.5.2 *Imaging Tests*

Imaging is essential for detecting focal liver lesions (eg, abscess, tumor). It is limited in detecting and diagnosing diffuse hepatocellular disease (eg, hepatitis, cirrhosis).

Ultrasonography; is the least expensive, safest, and most sensitive technique. It is the procedure of choice for differentiating intrahepatic from extrahepatic causes of jaundice and detecting liver masses

Computed Tomography (CT); is commonly used to identify hepatic masses, particularly small metastases, with an accuracy of about 80%. It is considered the most accurate imaging technique. CT can detect fatty liver and the increased hepatic density that occurs with iron overload.

Magnetic Resonance Imaging (MRI); is superior to CT and ultrasonography for diagnosing diffuse liver disorders (eg, fatty liver, hemochromatosis) and for clarifying some focal defects (eg, hemangiomas) (Eldon, 2012c).

2.2.5.3 Liver biopsy

Liver biopsy provides histologic information about liver structure and evidence of liver injury (type and degree, any fibrosis); this information can be essential not only to diagnosis but also to staging, prognosis, and management. Although only a small core of tissue is obtained, it is usually representative, even for focal lesions.

Liver biopsy is usually done percutaneously at the bedside or with ultrasound guidance. Ultrasound guidance is preferred because its complication rate is slightly lower and it provides opportunity to visualize the liver and target focal lesions. Gross examination and histopathology are often definitive (Eldon, 2012d).

2.2.5 Prevention of liver disease

Cirrhosis or hepatitis can be prevented in the following way:

- Getting hepatitis vaccine or an immunoglobulin shot to prevent hepatitis A or B.
- Eating balanced diet.
- Abstinence from drinking alcohol or drinking in moderation. Alcohol should be avoided when taking acetaminophen.
- Practicing proper hygiene. Such as washing hands thoroughly after using the bathroom or before touching any food.
- Adhering to officially recommended safety precautions in handling blood or blood products and in healthcare for health workers.
- Avoiding the sharing of any personal toiletry items, including toothbrushes and razors.
- Practicing safe sex.
- Using medications as directed and avoiding illegal intravenous drugs.
- Avoiding exposures to toxic substances and practicing officially recommended standards for occupational safety (Kimball, 2012).

2.2.6 Treatment of liver disease

The aim of treatments of liver disease is usually to stop disease progression, reverse any existing damage (if possible) and to manage symptoms or treat any life-threatening complications.

2.2.6.1 Stopping the progression of liver disease

- Viral Hepatitis

Anti-viral medications are available to treat infections such as hepatitis B and hepatitis C. This is an area of active research and drug development. Today many

treatments offer improve outcomes, by clearing or controlling the virus to slow any decline in the condition of the liver (Pereira, 2011).

HBV: Antiviral treatment is indicated for patients with elevated aminotransferase levels, clinical or biopsy evidence of progressive disease, or both. Treatment may be very expensive as it may need to be continued indefinitely. Stopping treatment prematurely can lead to relapse, which may be severe. Six antiviral drugs are available:

- Entecavir (a nucleoside analogue) daily at 1 mg or adefovir (a nucleotide analogue) daily at 10 mg orally is usually used as first-line treatment. Combination therapy has not proved superior to monotherapy. Adefovir may cause renal dysfunction.
- Tenofovir (300 mg daily) has superior efficacy and comparable safety.
- Lamivudine is a nucleoside analogue given at 100 mg orally once daily. It has few adverse effects compared to other antiviral drugs used to treat HBV infection.
- Interferon α (usually IFN-α2b) at a dose of 5 million IU subcuteaneously once daily. 10 million IU subcuteaneously 3 times a week for 4 months can be used. The drug is available as injection and is often poorly tolerated. Pegylated IFN-α2 can also be given. Dosage is 180 μg subcuteaneously once a week. Adverse effects are similar to those of INF-α but may be less severe.
- Telbivudine is a new drug that has greater efficacy than lamivudine but has high rates of resistance.

HCV: For chronic hepatitis due to HCV, treatment is indicated if aminotransferase levels are elevated and biopsy shows active inflammatory disease with evolving fibrosis. Treatment aims to permanently eliminate HCV-RNA (sustained response),

which is associated with permanent normalization of aminotransferase and cessation of histologic progression. Combination therapy with pegylated IFN-α (IFN-α2b at 1.5 μg/kg or IFN-α2a 180 μg) subcuteaneously once a week plus ribavirin (500 to 600 mg per oral twice daily) is given.

- Autoimmune hepatitis

Corticosteroids, with or without azathioprine, prolong survival. Prednisone is usually started at 30 to 40 mg *per* oral daily, and then tapered to the lowest dose that maintains aminotransferases at normal or near-normal levels. Concomitant azathioprine at 1 to 1.5 mg/kg *per* oral daily may be added, especially if low-dose prednisone fails to maintain suppression (Rutherford, 2013b).

- Alcoholic liver disease and non-alcoholic fatty liver disease

The most effective way to treat alcoholic liver disease and non-alcoholic fatty liver disease is to make lifestyle changes, such as abstinence from alcohol, diet improvement and regular exercise (Pereira, 2011).

For alcoholic liver disease, drugs used should only supplement other interventions. Opioid antagonists (naltrexone or nalmefene) and drugs that modulate γ-aminobutyric acid receptors (baclofen or acamprosate) appear to have a short-term benefit by reducing the craving and withdrawal symptoms. Disulfiram inhibits aldehyde dehydrogenase, allowing acetaldehyde to accumulate; thus, drinking alcohol within 12h of taking disulfiram causes flushing and has other unpleasant effects. However, disulfiram has not been shown to promote abstinence and consequently is recommended only for certain patients.

Corticosteroids (eg, prednisolone 40 mg/day po for 4 weeks, followed by tapered doses improve outcome in patients who have severe acute alcoholic hepatitis and who do not have infection, gastroinestinal bleeding, renal failure, or pancreatitis (Eldon, 2012e).

Other conditions may be managed by slowing down disease progression:

- Regularly removing a quantity of blood from a vein (venesection) in the iron overload condition, haemochromatosis.
- Wilson's disease, a condition where copper builds up in the body, can be managed with drugs such as D-penicillamine (750–1500 mg/day administered in two or three divided doses) which bind copper for excretion in urine (Pereira, 2011).

2.2.6.2 Reversing the damage from liver disease

Fatty deposits in the liver or inflammation may be reversed once the cause of the damage is removed. Scarring such as fibrosis may also be improved. Once cirrhosis has developed, damage is more permanent, but removing the cause should prevent any further progression (Pereira, 2011). However, Antioxidants (eg, *S*-adenosyl-l-methionine, phosphatidylcholine, metadoxine) show promise in ameliorating liver injury during early cirrhosis but require further study (Eldon, 2012e).

2.2.6.3 Managing symptoms/treating complications of liver disease

Symptoms such as itching in cholestatic hepatitis can be relieved by administration of oral cholestyramine 8 g once or twice daily. Another aspect of treatment is to deal

with the complications which occur in more advanced stages of liver damage. These include:

- Portal hypertension and variceal bleeding: Medicines including beta blockers may be prescribed to reduce the risk and the severity of any bleed.
- Ascites and peripheral oedema: The main treatments are restriction of salt intake and the use of diuretics.
- Hepatic encephalopathy: The main treatment is lactulose which acts as a laxative and also helps the body remove the toxins that build up when the liver is failing.
- Bleeding: Vitamin K and plasma may be given as a treatment in medical emergencies.
- Kidney problems: For most patients, a liver transplant is needed.
- Liver cancer: The aim is to detect and treat the cancer as early as possible. Treatment can involve chemotherpy, radiotherapy or surgery (Pereira, 2011).
- Liver failure: If detected early enough, acute liver failure caused by an overdose of acetaminophen can sometimes be treated and its effects reversed. Likewise, if a virus causes liver failure, supportive care can be given at a hospital to treat the symptoms until the virus runs its course. In these cases, the liver will sometimes recover on its own. However, for liver failure that is the result of long-term deterioration, the initial treatment goal may be to save whatever part of the liver that is still functioning. If this is not possible, a liver transplant is required (Kimball, 2012).

2.3 Drug-Induced Liver Injury (DILI)

Hepatotoxicity is liver injury caused by foreign compounds (xenobiotics). These include prescribed and nonprescribed therapeutic agents (drugs), including herbal medicines, and a vast array of other organic and inorganic substances that may be ingested deliberately or accidentally and that may contaminate the environment, workplace, or home (Chitturi and Farrell, 2007). This section concentrates on drug-induced liver injury, in which hepatotoxicity is caused by drugs used either in medical practice or those used by individuals for therapeutic, nutritional, or recreational purposes; the latter including drugs of abuse. Although passing reference is made to other hepatotoxins. Chemicals that cause liver injury are called hepatotoxins (Friedman *et al*., 2003).

Adverse hepatic events caused by drugs can be considered to be either predictable (high incidence) or unpredictable (low incidence). Drugs that produce predictable liver injury, such as acetaminophen, usually do so within a few days and are generally a result of direct liver toxicity of the parent drug or its metabolites. Unpredictable events manifest as overt or symptomatic disease and can occur with intermediate (1–8 weeks) or long (1 year) periods of latency. A typical example of the former is phenytoin, and an example of the latter is isoniazid. The majority of adverse drug-induced hepatic events are unpredictable (Kaplowitz, 2004).

2.3.1 Mechanism of liver injury

Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Metabolism of chemicals takes place largely in the liver, which accounts for the organ's susceptibility to metabolism-dependent, drug-induced injury. The pathogenesis of drug-induced liver injury usually involves the participation of a

toxic drug or metabolite that either elicits: (1. an immune response or (2. directly affects the biochemistry of the cell. In either case, the resultant cell death is the event that leads to the clinical manifestation of hepatitis (Kaplowitz, 2004).

2.3.1 .1 Biochemical mechanism of liver injury

The drug metabolites can be electrophilic chemicals or free radicals that undergo or promote a variety of chemical reactions, such as the depletion of reduced glutathione; covalently binding to proteins, lipids, or nucleic acids; or inducing lipid peroxidation. All of these have consequent direct effects on organelles such as the mitochondria, the endoplasmic reticulum, the cytoskeleton, microtubules, or the nucleus. Dysfunction of the mitochondria releases excessive amount of oxidants which, in turn, injure hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP2E1 also leads to oxidative stress (Jaeschke *et al.,* 2002). They may also indirectly influence cellular organelles through the activation and inhibition of signaling kinases, transcription factors, and gene-expression profiles. The resultant intracellular stress leads to cell death caused by either cell shrinkage and nuclear disassembly (apoptosis) or swelling and lysis (necrosis).

Hepatocyte death is the main event that leads to liver injury, although sinusoidal endothelial cells or bile duct epithelium may also be targets (Kaplowitz, 2004). Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside liver. This promotes further liver damage (Patel *et al*., 1998). Non-parenchymal cells such as Kupffer cells, fat storing stellate cells, and leukocytes (i.e. neutrophil and monocyte) also have roles in the mechanism (Kaplowitz, 2004).

2.3.1 .2 Immune-mediated mechanism of liver injury

Sensitization of liver-specific cytokines can occur, thereby causing cytokine-induced hepatotoxicity. Alternatively, the reactive metabolite may covalently bind to or alter liver proteins, such as cytochrome P450 enzymes, leading to an immune response and to immune-mediated injury. This immune-mediated, drug-induced hepatitis is usually characterized by fever, eosinophilia, or other allergic reactions that distinguish it from non-immune-mediated drug-induced hepatitis.

The mechanism for the induction of the immune-mediated drug reaction is not clear, but it may involve a hapten-like action. Generally, low-molecular-weight organic chemicals or drugs are not immunogenic, but they may become so when they are bound to a macromolecule, such as a protein. If a drug metabolite is able to act as a hapten, it would covalently bind to a liver protein and, subsequently, alter that protein. This altered protein would then be perceived as foreign by the immune system, resulting in an autoimmune attack on normal hepatocellular constituents (Kaplowitz, 2004).

2.3.2 Risk factors for drug-induced liver injury

The factors that increase the risk of drug-induced liver injury may include dose/duration of treatment, blood levels, age, gender, coincidental metabolic disorders or genetic predisposition to hypersensitivity reactions, concomitant exposure to other drugs or environmental agents, and underlying liver disease. Some examples are summarized in Table 2.2.

Table 2.1 Risk Factors for the Incidence and Severity of Drug-Induced Liver Diseases

Risk factor	Representative agents	Importance

Age	Isoniazid, nitrofurantoin, halothane, troglitazone	Age >60 years increases frequency and severity
	Valproic acid, salicylates	More common in children
Gender	Halothane, minocycline, nitrofurantoin, dextropropoxyphene	More common in women, especially chronic hepatitis
	Amoxicillin–clavulanate, azathioprine	More common in men
Dose	Acetaminophen; some herbal medicines	Risk of hepatotoxicity depends on blood levels
	Anticancer drugs; perhexiline, tacrine, oxypenicillins, dantrolene	Partial relationship to dose
	Methotrexate, vitamin A	Total dose, dose frequency, and duration of exposure influence risk of hepatic fibrosis
Genetic factors	Halothane, phenytoin, sulfonamides	Multiple cases in families, in vitro test results
	Amoxicillin–clavulanate	Strong human leukocyte antigen association
	Valproic acid	Familial cases, association with mitochondrial enzyme deficiencies
Other drug reactions	Isoflurane, halothane, enflurane Erythromycin, other macrolide antibiotics Diclofenac, ibuprofen Sulfonamides, cyclo-oxygenase 2 inhibitors	Cross-sensitivity reported between these classes of drugs

Concomitant drugs	Acetaminophen	Isoniazid, zidovudine, and phenytoin lower hepatotoxic dose threshold and increase severity
	Valproic acid	Other anticonvulsants increase risk
Excessive alcohol use	Acetaminophen hepatotoxicity Isoniazid, methotrexate	Lowers dose threshold, worsens outcome Increases risk of liver injury, hepatic fibrosis
Nutritional status		
Obesity	Halothane, tamoxifen, methotrexate	Increases risk of liver injury, nonalcoholic steatohepatitis, or hepatic fibrosis
Fasting	Acetaminophen	Increases risk of hepatotoxicity
Liver disease	Hycanthone, pemoline Antituberculosis chemotherapy, ibuprofen	Increases risk of liver injury Increases risk of liver injury with chronic hepatitis B and C
Other diseases		
Diabetes mellitus	Methotrexate	Increases risk of hepatic fibrosis
HIV/AIDS	Sulfonamides (cotrimoxazole)	Increases risk of hypersensitivity
Renal failure	Tetracycline, methotrexate	Increases risk of liver injury, hepatic fibrosis
Organ transplantation	Azathioprine, thioguanine, busulfan	Increases risk of vascular toxicity

(Chitturi and Farrell, 2007)

The risk of developing hepatotoxicity involves a complex interplay between the chemical properties of a drug, use of concomitant drugs or alcohol, nutritional status age, sex, underlying diseases (e.g., HIV or diabetes), and genetic factors. Genetic

factors include genes that control the handling of the drug (metabolism, detoxification, and transport), as well as those that influence cell injury and repair. Additionally, genetic polymorphisms with functional effects occur with many of the genes that encode drug-metabolizing enzymes and drug transporters. However, whether a genetic polymorphism of a drug-metabolizing enzyme has clinical relevance depends on its functional role in the metabolism of a drug. Familial sensitivity to the toxic effects of metabolites has also been shown, which indicates that these may be inherited defects in the defense against specific drug-related injuries (Kaplowitz, 2004).

2.3.3 Biochemical Patterns of liver injury

Biochemically, 3 types of liver injury are generally noted:

Table 2.2 Biochemical Patterns of liver injury

Type of injury:	**Hepatocellular**	**Cholestatic**	**Mixed**
ALT	≥ Twofold rise	Normal	≥ Twofold rise
ALP	Normal	≥ Twofold rise	≥ Twofold rise
ALT: ALP ratio	High, ≥5	Low, ≤2	2-5
Examples (Mumoli *et al.*, 2006)	Acetaminophen Allopurinol Amiodarone HAART NSAID	Anabolic steroid Chlorpromazine Clopidogrel Erythromycin Hormonal contraceptives	Amitryptyline, Enalapril Carbamazepine Sulfonamide Phenytoin

*2.3.3.1 Hepatocellular***:** This implies direct damage to hepatic cells with subsequent release of hepatocellular constituents. Certain drugs exclusively induce hepatocellular hepatotoxicity which generally manifests as malaise and right upper quadrant abdominal pain. This is associated with marked elevation in aminotransferase levels (ALT, AST, or both), which may be followed by hyperbilirubinemia in severe cases. If hepatocellular liver injury is accompanied by jaundice, impaired hepatic synthesis, and encephalopathy, chance of spontaneous recovery is low, and liver transplantation would have to be considered.

2.3.3.2 Cholestatic: Certain drugs exclusively or predominantly induce cholestasis. Several of such drugs are associated with hypersensitivity-type reactions. The specific immunological targets of these hypersensitivity-type adverse reactions are poorly understood. However, given that the predominant histological features are portal inflammation and biliary injury, they are likely to be related to the bile duct. It is possible that toxic metabolites undergoing canalicular excretion react with macromolecules in the duct cells or undergo further metabolism within these cells, resulting in ductal injury (Kaplowitz, 2004). Cholestatic hepatotoxicity is characterized by development of pruritus and jaundice accompanied by marked elevation of serum alkaline phosphatase levels. Usually, this type of injury is less serious than severe hepatocellular syndromes, but recovery may be protracted.

2.3.3.3 Mixed: In these clinical syndromes, neither aminotransferase nor alkaline phosphatase elevations are clearly predominant. Symptoms may also be mixed (Herrine, 2012).

2.3.4 Histopathological patterns of drug-induced liver injury

Specific histopathological patterns of liver injury from drug-induced damage are:

2.3.4.1 Zonal necrosis

This is the most common type of drug-induced liver cell necrosis where the injury is largely confined to a particular zone of the liver lobule. It may manifest as a very high level of ALT and severe disturbance of liver function leading to acute liver failure. Causes include Paracetamol and carbon tetrachloride

2.3.4.2 Hepatitis

In this pattern, hepatocellular necrosis is associated with infiltration of inflammatory cells. There can be three types of drug-induced hepatitis. (a) Viral hepatitis is the most common, where histological features are similar to acute viral hepatitis (e.g. halothane, isoniazid and phenytoin). (b) In focal or non-specific hepatitis, scattered foci of cell necrosis may accompany lymphocytic infiltration (e.g. aspirin).

(c) Chronic hepatitis is very similar to autoimmune hepatitis clinically, serologically, and histologically (e.g. methyldopa and diclofenac).

2.3.4.3 Cholestasis

Liver injury leads to impairment of bile flow and cases are predominated by itching and jaundice. Histology may show inflammation (cholestatic hepatitis) or it can be bland (without any parenchymal inflammation). On rare occasions, it can produce features similar to primary biliary cirrhosis due to progressive destruction of small bile ducts (Vanishing duct syndrome).

Causes:

(a) Bland: Oral contraceptive pills, anabolic steroid, androgens

(b) Inflammatory: Allopurinol, carbamazepine

(c) Ductal: Chlorpromazine, flucloxacillin

2.3.4.4 Steatosis

Hepatotoxicity may manifest as triglyceride accumulation which leads to either small droplet (microvesicular) or large droplet (macrovesicular) fatty liver. There is a separate type of steatosis where phospholipid accumulation leads to a pattern similar to the diseases with inherited phospholipid metabolism defects (e.g. Tay-Sachs disease)

Causes:

(a) Microvesicular: Aspirin (Reye's syndrome), ketoprofen, tetracycline (especially if expired)

(b) Macrovesicular: Acetamenophen, methotrexate

(c) Phospholipidosis: Amiodarone, total parenteral nutrition

2.3.4.5 Granuloma

Drug-induced hepatic granulomas are usually associated with granulomas in other tissues and patients typically have features of systemic vasculitis and hypersensitivity. More than 50 drugs have been implicated. Some examples are allopurinol, phenytoin, isoniazid, quinine, penicillin and quinidine.

2.3.4.6 Vascular lesions

These result from injury to the vascular endothelium.

Causes:

Venoocclusive disease: Chemotherapeutic agents, bush tea

Peliosis hepatitis: Anabolic steroids

Hepatic vein thrombosis: Oral contraceptives

2.3.4.7 Neoplasm

Neoplasms have been described with prolonged exposure to some medications or toxins. Hepatocellular carcinoma, angiosarcoma, and liver adenomas are the ones usually reported. Causes include vinyl chloride, combined oral contraceptive pill, anabolic steroid and arsenic (Chitturi and Farrell, 2007).

2.3.5 Diagnosis of drug-induced liver injury

Presentation varies widely, ranging from absent or nonspecific symptoms (eg, malaise, nausea, anorexia) to jaundice, impaired hepatic synthesis, and encephalopathy. Early recognition of DILI improves prognosis. Assessment of drug-induced liver injury generally involves:

(1. Identification of characteristic patterns of laboratory abnormalities
(2. Exclusion of other causes

Identification of a potential hepatotoxin and a pattern of liver test abnormalities that is characteristic of the substance (its signature) make the diagnosis likely. Because there is no confirmatory diagnostic test, other causes of liver disease, especially viral, biliary, alcoholic, autoimmune, and metabolic causes, has to be excluded. Drug rechallenge, although it can strengthen evidence for the diagnosis is usually avoided.

2.3.6 Prevention of drug-induced liver injury

Efforts to avoid DILI begin during the drug development process, although apparent safety in small preclinical trials does not ensure eventual safety of the drug after it is in widespread use. Postmarketing surveillance, now increasingly mandated by drug agencies, can call attention to potentially hepatotoxic drugs. Routine monitoring of liver enzymes has not been shown to decrease the incidence of hepatotoxicity. Use of pharmacogenomics may allow tailoring of drug use and avoidance of potential toxicities in susceptible patients.

2.3.7 Treatment of drug-induced liver injury

Management emphasizes drug withdrawal, which, if done early, usually results in recovery. In severe cases, especially if patients have hepatocellular jaundice and impaired liver function, liver transplantation may be required. Antidotes for DILI are available for only a few hepatotoxins; such antidotes include *N*-acetylcysteine for acetaminophen toxicity and silymarin or penicillin for *Amanita phalloides* toxicity (Steven and Herrine, 2013b).

2.4 Herbal Medicine and Hepatoprotection

Hepatoprotectives are a class of therapeutic agents that includes many synthetic and natural products that protect against hepatic damage induced by various infections, drugs and toxins. In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage or help regeneration of hepatic cell (Chaterjee, 2000). However, herbal drugs play a role

in the management of various liver disorders most of which speed up the natural healing processes of the liver (Subramoniam *et al*., 1998). Hepatoprotective herbal drugs contain a variety of chemical constituents like phenols, flavanoids, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, organic acids, lipids, alkaloids and xanthines (Ozougwu, 2011).

Silymarin is a flavonoid extract from the seeds of milk thistle, which exhibit good hepatoprotective effect against a broad spectrum of acute and chronic liver diseases. Silymarin has been recorded to protect against and treat many liver diseases caused by exposure to different toxicants. It is also effective in protection against carbon tetrachloride toxicity (Abdel *et al.*, 2009).

A decoction of *C. sinensis* or other citrus fruits peels are popularly taken around the world for the treatment of various ailments including liver problems. This practice has its origin from ancient Chinese medicine (Liu *et al*, 1998). A proper scientific evaluation of medicinal plants such as this is important to the discovery of novel drugs; assess efficacy and toxicity associated with the use of either herbal preparations or conventional drugs of plant origin (Fraschini *et al*, 2002).

2.4.1 *Citrus sinensis*

2.4.1.1 Names and biological classification

Common name: Sweet orange

Local name: Orombo, *Osan* (Yoruba); *Oloma* (Ibo); *lemun zaki* (Hausa); *Sokoro*, (Efik); *Anumei* (Esan) (Idu *et al*, 2009)

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Superdivision	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Subclass	*Rosidae*
Order	*Sapindales*
Family	*Rutaceae*
Genus	*Citrus* L.
Species	*Citrus sinensis* (L.) Osbeck

2.4.1.2 Description

C. sinensis belongs to the family Rutaceae which contains a large number of species (more than 400). The plant probably originated in Southeast Asia (the specific epithet "sinensis" refers to its Chinese origin) and is widely grown in tropical and subtropical climates for its sweet fruit (Orwa, *et al*, 2009). It has been the most cultivated tree fruit in the world since 1987. In 2010, 68.3 million tonnes of *C. sinensis* fruits were grown worldwide (FAO Statistics, 2011).

C. sinensis is an ever-green medium-size tree that grows 6-13 m high with enclosed conical top and mostly spiny branches. The trees have thin, smooth, and gray-brown to greenish bark. Its dark green and glossy leaves are smooth, oval in shape and 5-15 x 2-8 cm in dimension. The leaves have a distinctive fragrance often similar to that of the fruit. The broad calyx flowers are small and saucer-shaped. They are waxy, greenish-white in colour and even more fragrant. They have 5 petals and are 1.3-2.2

cm long. This plants bears 4-12 cm spherical fruits that are reddish-green to yellowish-green in colour, consisting of a leathery peel (6 mm thick). The tightly adherent peels protect the inner juicy pulp, which is divided into segments that may not contain seeds, depending on the cultivar (Orwa, *et al*, 2009). Its seeds are pale whitish to greenish, flattened, and angular (Manner *et al*, 2006).

2.4.1.3 Important Uses

I. Food

C. sinensis is mainly planted for its fruit juice. The inner juicy pulp part of the fruit is a good source of vitamin C. They are consumed fresh or made into juice, or jelly (Orwa, *et al*, 2009). Generally, citrus fruits are suggested as part of a healthy diet because of its high vitamin C content and its lycopene and flavonoids, which are known to reduce prostate and breast cancer risk, reduce viral effects and inflammation, and improve capillary activity and cholesterol levels (Manner *et al*, 2006). Nevertheless, *C. sinensis* leaves and peels are boiled to make tea (Orwa, *et al*, 2009).

II. Ethnomedicinal

C. sinensis is a very useful medicinal plant in china and its usefulness has become popular around the world (Liu *et al*, 1998). Its twig or stem is chewed for vomiting, toothache and teeth cleaning among the Esan Tribe of Edo State, Nigeria. In the South-western part of Nigeria the twigs, stem bark and fruits are used in the treatment of dysentery, toothache, fever and headache. They are also used as antimicrobial, anthelmintic, and antiscorbutic agents (Idu *et al*, 2009).

In traditional Pacific island medicine a leaf decoction with salt is taken orally for digestive tract ailments, nerve disorders, fever, asthma, blood pressure, general fatigue and vomiting. Crushed leaves or fruit juice is massaged into the skin to relieve itching. Macerated root, leaf or fruit peel is taken orally for urethritis and macerated fruit peel or bark decoction is taken orally for liver ailments. Fruit juice or leaf decoction with sugar is taken orally for cold and loss of appetite, while crushed leaf decoction as a bath relieves headache and rheumatism. Broken bones are massaged with roasted fruit. Leaf oil is used for its carminative, antispasmodic and sedative properties (Orwa, *et al*, 2009). A leaf decoction is used against mouth sores in infants. A bark decoction is used to treat postpartum sickness, serious flu, and internal injuries (Manner *et al*, 2006).

III. Others

C. sinensis and other citrus plants are among the most important honey plants in many parts of the world. In California, for example, citrus has been said to constitute 25% of honey production. The pulp and other by-products from juice production are used as cattle feed. *C. sinensis* is sometimes planted as an amenity tree in gardens. Its fragrant flowers are very pleasant in home gardens (Manner *et al*, 2006). The fruit peels, leaves and flowers contain fine essences of oils that are used in manufacture of cosmetics and medicinal applications (Orwa, *et al*, 2009).

2.4.1.4 Research Reports on C.sinensis

Previous study by Ashok *et al* (2011) identified the antibacterial activity of *C. sinensis* peels extracts obtained from different solvents against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumonia and Salmonella typhi*. Methanolic extract of *C. sinensis* peel showed good *in-vitro* antioxidant activity and potent antibacterial activity against both gram positive and gram negative bacteria (Dayanand *et al.*, 2011).

Oral administration of either *C. sinensis* leaves or peels extracts to rats with ovariectomy-induced bone loss (OVX) produced significant increase in serum calcium level when compared to that of untreated OVX rats (Nagwa *et al.*, 2011). Parmar and Kar (2008) evaluated an extract of *C. sinensis* (CS) peel for efficacy in L-thyroxine (L-T[4]) induced tissue lipid peroxidation (LPO), hyperthyroidism and hyperglycemia in mice. However, it decreased the concentration of different serum lipids such as total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and very low-density lipoprotein cholesterol. Administration of *C. sinensis* extract in hyperthyroid animals revealed the ameliorating potential of *C. sinensis* extract against various adverse effects of hyperthyroidism. It appeared that the test extract primarily acted through its antioxidative/free radical-scavenging, antithyroidal and HDL-C stimulating properties. Oliveira *et al.*, (2005) showed that the *C. sinensis leaf* acetone extracts exerts a negative inotropic effect probably by reducing the calcium inward current and by opening some potassium membrane channels.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection and identification of plant material

Commercially grown *Citrus sinensis* (L.) Osbeck, *Citrus aurantiifolia* (Christm.) Swingle, and *Citrus medica* (L.) Burm.f. were purchased from the Faculty of agriculture Ahmadu Bello University, Zaria, in February, 2012. The fruits were authenticated and assigned voucher numbers 1432, 1440 and 4828 respectively by M. S. Gallah at the Herbarium Section of the Department of Biological sciences, Ahmadu Bello University, Zaria.

3.1.2 Experimental animals

Wistar albino rats of either sex weighing 110 to 150 g obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria were used for the study. The animals were maintained on standard animal feed and clean tap water *ad libitum*, and allowed to adapt to the laboratory environment for two weeks in order to acclimatize. Ethical guidelines on handling of experimental animals of the Ahmadu Bello University, Zaria, Nigeria, were strictly followed as approved by the relevant committee.

3.1.3 Drugs, chemicals and reagents

North West Life Science Specialties (NWLSS^{TM}) antioxidant kits; Enzopak biochemical kits, sylmarin capsules (Uni Pharma, Cairo Egypt), 99.9% CCl_4 (BDH

chemicals, USA), Folin-Ciocalteu reagent (BDH chemicals, USA) and methanol (Sigma Chemical Co. USA) were used.

3.2 Methods

3.2.1 Preparation of plant material

The whole peels of collected fruits were separated and shade dried at room temperature (27 – 30ºC) to constant weight over a period of 7 days. Dried whole peels were coarsely powdered using a mortar and pestle and further pulverised using an electric blender.

3.2.2 Extraction of Plant material

The pulverized plant materials were separately extracted with aqueous methanol (methanol: water, 70: $30^{v}/_{v}$) for 72 hours at room temperature by maceration. The residues were filtered and concentrated over a water bath at 60°C. The percentage yield of each was calculated. Solutions of extracts were freshly prepared for each study.

3.2.3 Phytochemical screening

3.2.3.1 Preliminary phytochemical analysis (Qualitative analysis):

The extracts were subjected to preliminary phytochemical screening following the standard procedure of Sofowora (1993) as described by Halilu, *et al*, (2012) with modifications. Briefly:

1) Test for alkaloids

One gramme of extract was stirred in 10 ml distilled water and then filtered. The filtrate was divided into 3 portions and the following reagents were used to test for the presence of alkaloids.

a) Mayer's Test: The filtrate was treated with few drops of Mayer's reagent (Potassium Mercuric Iodide).The formation of a yellow coloured precipitate indicated the presence of alkaloids.

b) Wagner's Test: The filtrate was treated with few drops of Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate indicated the presence of alkaloids.

c) Dragendroff's Test: The filtrate was treated with few drops of Dragendroff's reagent (solution of Potassium Bismuth Iodide). The formation of reddish precipitate indicated the presence of alkaloids.

2) Test for Flavonoids

a) Shinoda's Test: The extract was dissolved in 2 ml of distilled water and warmed. 4 drops of concentrated hydrochloric acid were then added to 1ml of the solution followed by addition of some magnesium ribbons. Immediate appearance of orange colour denoted flavones, red crimson colour denoted flavonols, and pink magenta colour denoted flavones.

b) Sodium hydroxide test: The other 1ml solution was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicated the presence of flavonoids.

3) Test for Tannins

a) Lead acetate test: To 1 ml of extract dissolved in distilled water, 2 drops of lead subacetate solution was added. A coloured precipitate indicated the presence of tannins.

b) Bromine water test: 1 ml of extract solution was treated with 3 drops of bromine water. Non-formation of coloured precipitate indicated the presence of hydrolysable tannins.

4) Test for Saponins

a) Frothing Test: 0.5g of extract was dissolved in 5ml distilled water and shaken in a test tube for 5 mins. The occurrence of a honeycombs froth, which lasts for about 45 minutes, indicated the presence of saponins.

b) Hemolysis Test: 0.5g of extract was dissolved in 5ml distilled water. 2 drops of fresh whole blood was added to the solution. Immediate hemolysis of blood indicated the presence of saponins.

5) Test for phytosterols / Triterpenoids

1g of extract was dissolved in distilled water and then treated with chloroform. The liquids were separated using separating funnel. The chloroform portion was collected and then divided into 2 portions and was used for:

a) Salkowski's Test

A few drops of Conc. Sulphuric acid was added to the filtrate by the wall of the test tube. The appearance of golden yellow colour indicated the presence of triterpenes.

b) Libermann Burchard's test

A few drops of acetic anhydride were added to the filtrate in a test tube, followed by the addition of Conc. Sulphuric acid by the wall of the test tube. The formation of brown ring at the junction indicated the presence of phytosterols.

6) Test for Phenolic compounds

Ferric chloride test: 1g of extract was dissolved in distilled water. Few drops of a solution of ferric chloride were added. The appearance of blue - black colour indicated the presence of tannins.

7) Test for of Anthraquinones

Borntrager's Test: 1g of extract was dissolved in distilled water and fractionated with petroleum ether. The petroleum ether fraction was concentrated and shaken with 5mls of 25% ammonia solution. A cherry-red colour of the alkaline solution indicated anthraquinones in an oxidized form.

8) Test for Cardiac Glycosides

Keller- Kiliani's Test

1g of extract was dissolved in 20mls of distilled, 1ml of strong lead sub-acetate solution was added to precipitate pigments, which were filtered off. The filtrate obtained was shaken with equal volume of chloroform and allowed to separate into two layers in a separating funnel. The chloroform layer was removed and evaporated to dryness over a water bath. The residue was dissolved in 3ml of ferric chloride in glacial acetic acid, and then transferred to a dry test tube. Few drops of concentrated sulphuric acid were added by the wall of the test tube. On standing, a brown colour at the interphase (due to deoxysugars) and a pale green colour in the upper layer (due to steroidal nucleus) is a preliminary test for Cardiac Glycosides.

9) Test for carbohydrates

1g of extract was dissolved in 5mls of distilled water and filtered. The filtrate was divided into 2 portions and was used to test for the presence of carbohydrates using the following reagents:

a) Molisch's Test: The filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube. The formation of the violet ring at the junction indicated the presence of carbohydrates.

b) Fehling's Test: The filtrate was hydrolysed with dil. HCl, and then neutralized with alkali and warmed with Fehling's A & B solutions. The formation of red precipitate indicated the presence of reducing sugars.

3.2.4.2 Quantitative phytochemical screening

Determination of total flavonoid content: The method adapted by Bationo *et al.,* (1994) was used for flavonoid determination. Briefly, 2ml of 2% $AlCl_3$ (in methanol) was mixed with 1.25 ml of extract (0.1 mg /ml in methanol). The absorbance were read after 10 min at 415 nm, and the levels of flavonoids in the extract was determined from the regression equation of a standard curve that was obtained from a dilution series of quercetin in methanol.

Determination of total phenol content: Total soluble phenols in the extract was determined with Folin-Ciocalteu reagent using gallic acid (50-250 µg) as a standard phenolic compound. 1.0 ml of extract solution containing 1.0 mg extract was diluted with 46 ml of distilled water in a volumetric flask. 1.0 ml of Folin-Ciocalteu reagent was added and the content of the flask mixed thoroughly. 3 min later 3.0 ml of 20% sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of the blue color that developed was read at 760

nm. The concentration of total phenols was expressed as gallic acid equivalents in mg/g of dry extract (Lobo *et al*, 2011).

3.2.4 Acute toxicity test

The two-phase method described by Lorke (1983) was used for this study. Briefly, in the first phase, three groups of three animals each were given 10, 100 and 1000 mg/kg of *C. sinensis* extract by the oral route. The rats were observed for signs and symptoms of toxicity and mortality over a period of 24 h. In the second phase, four rats were administered 1,200, 1,600, 2,900 and 5,000 mg/kg of the extract and observed for another 24 h post administration. A total of 13 rats were used for the study.

3.2.5 *In vivo* Antioxidant and hepatoprotective activity

3.2.5.1 Selection of doses

Dose levels were chosen in such a way that the middle dose was one-tenth of the maximum dose during acute toxicity studies, and a low dose, which was half of the one-tenth dose. The high dose was twice that of one-tenth dose (Gopalakrishnan and Rajameena, 2012).

3.2.5.2 Experimental design

Forty-two male and female rats were divided into seven groups, each group consisting of six animals. Administrations were done by oral (p.o.) and subcutaneous (s.c.) routes following the method of Suja *et al.*, (2004) as described by Palanivel *et al.*, (2008). Modification on the dose of sylimarin used was made from (Tukur, 2012).

Group	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**

Distilled Water (ml/kg, p .o.)	1		1				
Normal Saline (ml/kg, s .c.)	1						1
CCl_4 (ml/kg, s.c. 72 h)		2	2	2	2	2	
Sylimarin (mg/kg, p.o.)		100					
C. sinensis Extract (mg/kg, p.o.)				250	500	1,000	1,000

Table 3.1 Experimental design for 10 days (6 rats per group)

3.2.5.3 Animal Sacrifice and Blood/Organ Collection: Twenty four hours after the last administration of standard or test compound, the rats were weighed and sacrificed by decapitation following mild chloroform anesthesia. The blood of each animal was collected into clean dry test tube and allowed to clot. Each animal was dissected and the liver collected immediately with rinsing in double portions of ice-cold normal saline, weighed and kept in separate transparent polythene bags.

3.2.5.4 In vivo Antioxidant activity

A portion of each liver was homogenized in ice-cold phosphate buffer saline (0.1 mM, pH 7.4) so as to provide a 10% homogenate solution. These homogenates were centrifuged at 3,000 g for 10 minutes to obtain supernatants that were immediately stored in deep freezers at -20°C until required for analysis. The 10% liver homogenate were used for superoxide dismutase, catalase, glutathione peroxidase and thiobarbituric acid reactive substances tests – see Appendix I.

3.2.5.5 Assessment of hepatoprotective activity

The serum from clotted blood of each animal was collected after about 3 hrs, following centrifugation at 3,000 g for 15 minutes. Serum was used for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, direct bilirubin, total protein, albumin, cholesterol, triglyceride, urea and creatinine tests - see Appendix II.

3.2.5.6 Organ/body weight

Organ/body weight changes were expressed as relative liver weight and percentage weight loss.

3.2.6 Histopathological studies

A portion of liver was initially fixed in 10% buffered neutral formalin for 48 hour and then with bovine solution for 6 hour for histopathological studies. Paraffin sections were taken at 5 mm thickness processed in alcohol-xylene series and was stained with alum hematoxylin and eosin. The sections were examined microscopically for histopathology changes.

3.2.7 Statistical Analysis: All experimental results were expressed as mean ± S.E.M. Data was analyzed using one way analysis of variance (ANOVA) and Student t-test. The values of $p<0.05$ were considered statistically significant.

CHAPTER FOUR

4. RESULTS

4.1 Extraction: The extracts obtained from the three plants were semi-solid, sticky, and brown with different types of sweet smell. The extracts were completely soluble in water. Yields of 20.96% ($^{w}/_{w}$), 20.6% ($^{w}/_{w}$) and 32.96% ($^{w}/_{w}$) were obtained for *C. sinensis*, *C. aurantiifolia* and *C. medica* respectively.

4.2 Phytochemical Screening

Phytochemical screening revealed the presence of flavonoids, tannins, saponins, phenols, steroids, carbohydrates and cardiac glycosides in peels of all three citrus fruit species (Table 4.1).

Table 4.1: Phytochemical constituents of *C. sinensis*, *C. aurantiifolia* and *C. medica* peels.

Phytoconstituent Tested	*C. sinensis*	*C.aurantifolia*	*C.medica*
Tannins	+	+	+
Alkaloids	—	—	—
Saponins	+	+	+
Flavonoids	+	+	+
Steroids	+	+	+
Carbohydrates	+	+	+
Phenolic compounds	+	+	+
Cardiac Glycosides	+	+	+
Anthraquinones	—	—	—

Key: + = Present ;— = Not Detected

4.2.1 Determination of total phenolics and flavonoid content

4.2.1.1 Determination of total flavonoid content: Total flavonoid content in the extracts were determined from a regression equation of a quercetin standard curve ($y = 0.0057x + 0.0144$, $R^2 = 0.9997$) and expressed as mg Quercetin Equivalent per gram of extract (mg QE/g) (Fig. 4.1). The total flavonoid content of *C. sinensis*, *C.*

aurantiifolia and *C. medica* extracts were 17.3, 14.2 and 5.9 mg QE/g respectively. Thus, *C. sinensis* extract revealed the highest total flavonoid content.

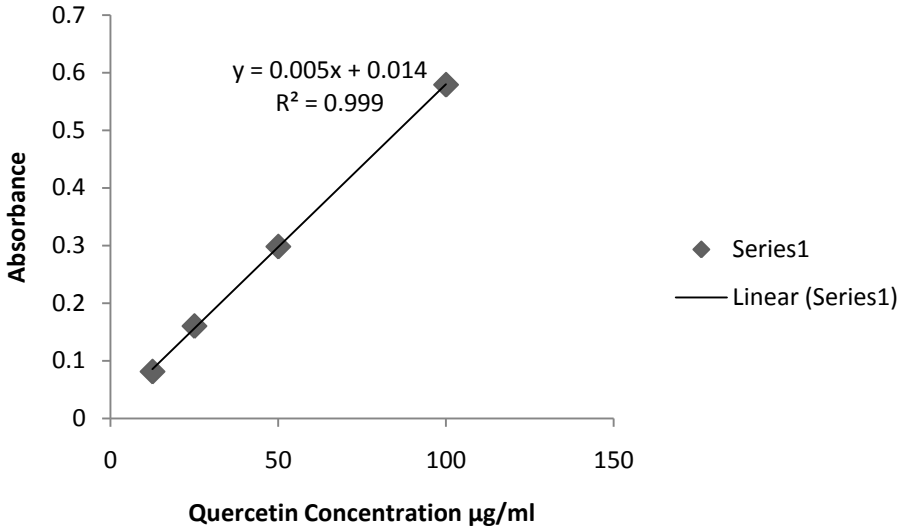


Fig. 4.1: Standard curve for total flavonoid estimation with quercetin

4.2.1.2 Determination of total phenol content: Total phenol content in the extracts were determined from a regression equation of a gallic acid standard curve (y = 0.0016x + 0.1433, R^2 = 0.958) and expressed as mg Gallic Acid Equivalent per gram of extract (mg GAE/g) (Fig. 4.2). The total phenol content of *C. sinensis*, *C. aurantiifolia* and *C. medica* extracts were 13.6, 41.7 and 26.1 mg GAE/g respectively. Thus, *C. aurantiifolia* extract revealed the highest total phenol content.

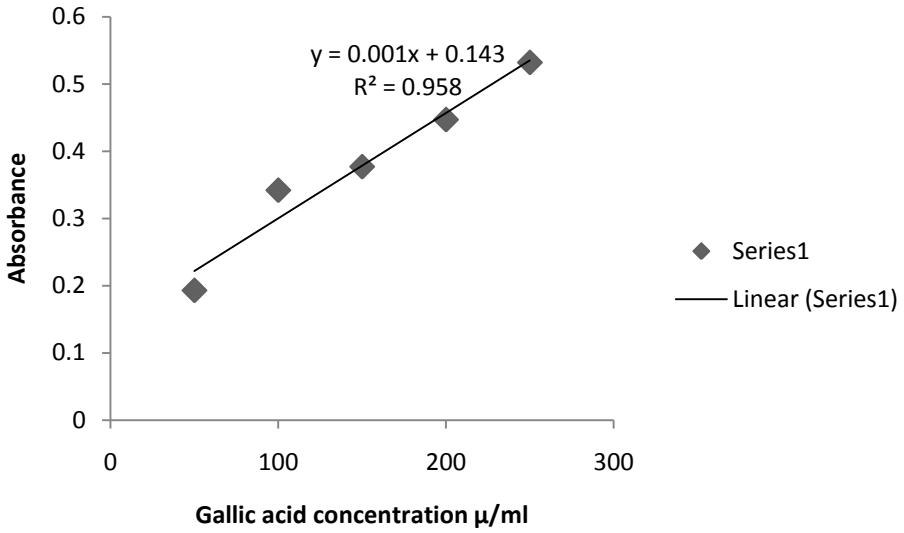


Fig. 4.2: Standard curve for total phenol estimation with gallic acid

4.3 *In vivo* Antioxidant and hepatoprotective activity

4.3.1 Acute Toxicity Test

No death was recorded for all the animals that received 1,000, 2,000, 4,000 and 5,000 mg/kg *C. sinensis* extract. As no mortality was observed at the maximum dose of 5,000 mg/kg, the middle dose for the study was 500 mg/kg ($^{1}/_{10}{}^{th}$), while the two other doses were 1,000 mg/kg and 250 mg/kg.

4.3.2 Antioxidant Activity of *C. sinensis* peel extract in CCl_4-induced hepatotoxicity in rats.

C. sinensis peel extract showed significant and dose dependent antioxidant activity (at $P<0.001$) against the depletion of liver tissue catalase (CAT) levels caused by CCl_4 toxicity (Table 4.2). Catalase was reduced to 41.8±1.7iu/l in the CCl_4 group compared to 66.4±1.6 iu/l observed in the normal group. This was then brought to relatively normal levels of 63.8±5.2 iu/l (89% protection) in the 500 mg/kg and 58.8±5.0 iu/l

(69% protection) in the 1,000 mg/kg of the extract compared to 62.6±1.1iu/l observed in the sylimarin group. There was only a minimal effect at the 250 mg/kg dose. However, a decrease in catalase level was observed in the group that received only 1,000 mg/kg extract compared to the normal control group.

Patterns similar to that observed for CAT levels were also observed for superoxide dismutase (SOD) and glutathione peroxidase (GPx), despite the observation that the extract at 1,000 mg/kg dose worsened the negative effect of CCl_4 on liver tissue GPx levels. However, the effects of CCl_4, the extract and the standard drug on these two antioxidant enzymes were not statistically significant.

On the other hand, thiobarbituric acid reactive substances (TBARS) levels did not show antioxidant protection. TBARS level was lower in the CCl_4 treated group meaning lower levels of lipid peroxidation products compared to the normal group.

Table 4.2: Antioxidant effect of *C. sinensis* peel extract on endogenous antioxidant enzymes and lipid peroxidation in CCl_4-induced hepatotoxicity in rats.

	CAT (u/L)	**TBARS (µmol/mg)**	**SOD (u/L)**	**GPx (u/L)**
Control	66.4±1.6**	2.18±0.12*	2.40±0.08	47.6±2.9
CCl_4	41.8±1.7^{++}	1.28±0.02^{+}	1.82±0.07	43.6±3.3
250 mg/kg C.S. + CCl_4	43.0±0.6^{++} (5)	1.38±0.18	2.02±0.12 (36)	48.4±2.7 (120)
500 mg/kg C.S. + CCl_4	63.8±5.2** (89)	2.14±0.26*	2.34±0.17 (90)	48.8±0.7 (130)
1,000 mg/kg C.S. + CCl_4	58.8±5.0 (69)	1.84±0.17*	2.18±0.21 (62)	35.6±8.5
100 mg/kg Sylimarin + CCl_4	62.6±1.1** (84)	2.18±0.12*	2.50±0.15* (117)	48.0±2.5 (110)
1,000 mg/kg C.S.	52.8±2.3	1.64±0.18	2.28±0.08	45.0±1.4

CAT: Catalase, TBARS: thiobabitturic acid-reacting substances, SOD: Superoxide Dismutase, GPx: Glutathione peroxidase, C.S.: *C. sinensis*

Values are mean±S.E.M, n=5. Values in parenthesis indicate percentage antioxidant protection. Values are statistically significant at **P<0.001 and *P<0.05 compared to CCl_4 group; and at $^{++}$P<0.001, $^{+}$P<0.05 compared to control group (ANOVA followed by Student t-test).

4.3.3 Assessment of Hepatoprotective Activity

4.3.3.1 Effect of C. sinensis peel extract on liver enzymes in CCl_4-induced hepatotoxicity in rats.

C. sinensis fruit peel extract reduced liver enzymes significantly (P<0.001 for ALT and AST; and P<0.05 for ALP) just like sylimarin at the 500 mg/kg dose compared to the CCl_4 treated group (Table 4.3). At this dose, 100% protection was observed for ALT and AST while 87% protection was observed for ALP. At 1,000 mg/kg dose the level of protection was reduced for all three liver enzymes. Minimal protection was observed at 250 mg/kg dose with ALT (27%) and AST (36.3%) levels whereas there was none with ALP levels at this dose compared to the normal control group. However, there was a slight increase in ALP levels in the group that received only 1,000 mg/kg of extract compared to the normal control group. This increase was not observed in ALT and AST levels.

Table 4.3: Effect of *C. sinensis* peel extract on CCl_4-induced alteration in liver enzymes.

	ALT (u/L)	**AST (u/L)**	**ALP (u/L)**
Control	64.8±7.3**	44.2± 6.1**	59.0± 5.2*
CCl_4	291.4±26.5^{++}	199.2±25.7^{++}	158.2±24.5^{+}
250 mg/kg C.S. + CCl_4	230.2±57.1 (27)	143.0±36.8 (36.3)	162.4±31.9^{+} (0)
500 mg/kg C.S. + CCl_4	41.0±17.4** (100)	35.2±12.7** (100)	72.4±11.8* (87)
1,000 mg/kg C.S. + CCl_4	112.8±40.7* (79)	83.4±24.1* (75)	76.2±18.7* (83)
100 mg/kg Sylimarin + CCl_4	37.6±15.5** (100)	44.4±15.5** (100)	71.6± 9.8* (87)
1,000 mg/kg C.S.	66.2± 6.4**	44.4± 2.7**	73.0± 4.5*

ALP: Alkaline phosphatase, ALT: Alanine transaminase, AST: Aspartate transaminase, C.S.:*C. sinensis*

Values are mean±S.E.M, n=5. Values in parenthesis indicate percentage hepatoprotection. Values are statistically significant at**P<0.001 and *P<0.05 compared to CCl_4 group; and at $^{++}$P<0.001, $^{+}$P<0.05 compared to control group (ANOVA and Student t-test).

4.3.3.2 Effect of C. sinensis peel extract on bilirubin and serum proteins in CCl_4-induced hepatotoxicity in rats.

In line with previous results, *C. sinensis* peel extract caused significant reduction of serum bilirubin (at P<0.001 for total bilirubin and P<0.05 for conjugated bilirubin) similar to sylimarin at the 500 mg/kg dose compared to the CCl_4 treated group (table 4.4). This positive effect of the extract was less at the 1,000 mg/kg dose. However, the effects of CCl_4, the extract and the standard drug on total protein and albumin were not statistically statistically different from the control group.

Table 4.4: Hepatoprotective effect of *C. sinensis* peel extract on serum bilirubin and proteins in CCl_4-induced hepatotoxicity in rats.

	TBil (mmol/L)	**CBil (mmol/L)**	**TP (g/L)**	**Alb (g/L)**
Control	11.1± 1.6**	5.2 ± 1.0*	67.4±1.5	31.8±1.5
CCl_4	89.1±14.5^{++}	45.7±10.4^{+}	61.4±1.2	33.2±1.7
250 mg/kg C.S. + CCl_4	86.4±21.0^{+}	31.5± 8.9*	62.0±1.1	34.0±2.9
500 mg/kg C.S. + CCl_4	8.3± 1.8**	4.5±1.5*	65.0±1.9	31.2±1.8
1,000 mg/kg C.S. + CCl_4	27.4±16.2*	13.9±9.5*	62.8±2.3	33.2±2.5
100 mg/kg Sylimarin + CCl_4	10.4± 1.1**	5.4±0.8*	68.8±0.7	33.0±1.5
1,000 mg/kg C.S.	14.3± 2.9*	7.4±0.8*	65.4±0.9	31.6±2.1

Alb: Albumin, TP: Total protein, CBil: Conjugated Bilirubin, TBil: Total Bilirubin, C.S.: *C. sinensis*

Values are mean±S.E.M, n=5. Values are statistically significant at **P<0.001 and *P<0.05 compared to CCl_4 group; and at $^{++}$P<0.001, $^{+}$P<0.05 compared to control group (ANOVA followed by Student t-test).

4.3.3.3 Effect of C. sinensis peel extract on kidney function, organ/body weight and lipid profile in CCl_4-induced hepatotoxicity in rats.

Urea levels were significantly increased to 6.86±0.80 mmol/L in the CCl_4 treated group from 2.98±0.29 mmol/L of the normal control group. *C. sinensis* peel extract reduced this effect of CCl_4 to near normal values of 3.72±0.58 mmol/L at the 500 mg/kg dose and 3.54±0.54 mmol/L at the 1,000 mg/kg. Sylimarin reduced urea to normal levels in the standard group. Similarly, creatinine was increased from 49.4±5.3 µmol/L observed in the normal control group to 76.4±10.4 µmol/L observed in the CCl_4 treated group. This was then reduced to near normal values of 45.2±1.9 µmol/L, 50.7±12.5 µmol/L and 49.6±1.9 µmol/L with 500 mg/kg extract dose, 1,000 mg/kg extract dose and 100m/kg sylimarin respectively. With Cholesterol, triglyceride levels and organ/body weight changes neither CCl_4 nor extract showed statistically significant difference from the control. However, an interesting pattern closely resembling that of previous results of this study can be observed in RLW and %WL values among the different groups (see table 4.5).

Table 4.5: Effect of *C. sinensis* peel extract on kidney function, organ/bodyweight and lipid profile in CCl_4-induced hepatotoxicity in rats

	Urea (mmol/L)	Creat (µmol/L)	Chol (mmol/L)	Trigly (mmol/L)	RLW (%)	%WL
Control	2.98±0.29*	49.4±5.3	2.32±0.06	0.88±0.09	3.90±0.12	-9.93±2.06
CCl_4	6.86±0.80^{+}	76.4±10.4	2.44±0.04	0.82±0.08	4.73±0.23	5.06±4.11
250mg/kg C.S. + CCl_4	7.16±1.28*	77.6±17.6	2.52±0.12	0.80±0.16	4.55±0.28	11.57±2.63
500mg/kg C.S. + CCl_4	3.72±0.58*	45.2±1.9	2.56±0.12	0.92±0.15	4.77±0.17	5.75±3.67
1,000mg/kg C.S. + CCl_4	3.54±0.54*	50.7±12.5	2.44±0.08	0.80±0.09	4.72±0.19	5.10±2.76
100 mg/kg Sylimarin + CCl_4	2.80±0.29*	49.6±1.9	2.56±0.13	1.24±0.13	4.94±0.34	5.78±4.74
1,000 mg/kg C.S.	3.86±0.44	61.0±5.2	2.56±0.10	0.88±0.12	3.38±0.15	-5.61±3.92

Creat: Creatinine, Chol: Cholesterol, Trigly: Triglyceride, RLW: Relative Liver Weight, %WL: Percentage Weight Loss, C.S.:*C. sinensis*

Values are mean±S.E.M, n=5. Values are statistically significant at *P<0.05 compared to CCl_4 group; and at $^{+}$P<0.05 compared to control group (ANOVA followed by Student t-test).

4.3.3.4 Histopathology Profile

Histopathology profile of group I (Control) showed normal hepatic architecture (Plate I). Meanwhile, CCl_4 treated animals exhibited severe hepatotoxicity indicated by intense adipocyte infiltration (fatty liver), lymphocyte hyperplasia, complete loss of liver parenchyma caused by marked hepatocellular necrosis and vascular congestion caused by marked peri-portal inflammation (Plate II). All extract and sylimarin treated groups showed reduction in hepatic damage in line with biochemical analysis results. 250 mg/kg C.S. extract treated group showed the least reduction in hepatic damage although there was no peri-portal inflammation with this dose. Contrary to results of biochemical analysis, 1,000 mg/kg C.S. extract treated group showed less damage than the 500 mg/kg C.S. treated group. The histopathological profile of the 1,000 mg/kg C.S. extract treated group showed only mild adipocyte infiltration and mild hepatocellular necrosis. Nevertheless, the C.S. extract only (1,000 mg/kg) group showed mild hepatocellular necrosis and mild lymphocyte hyperplasia compared to the normal group. Table 4.6 shows a summary of histopathological changes observe in all the groups.

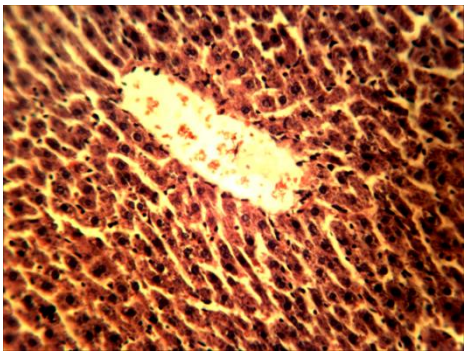


Plate I: Control

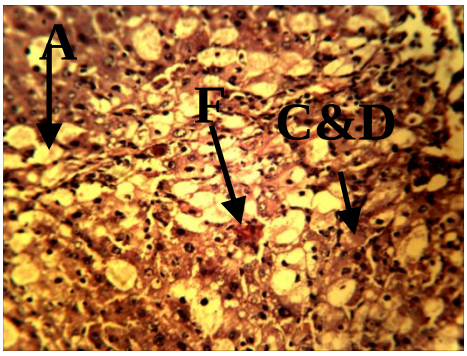


Plate II: CCl_4

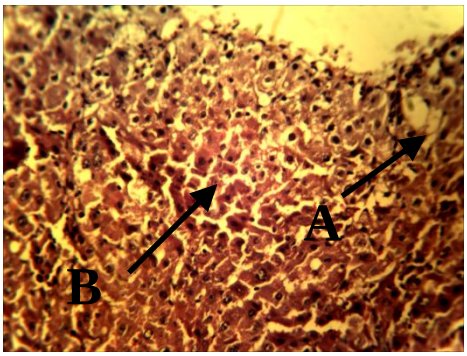


Plate III: Sylimarin + CCl_4

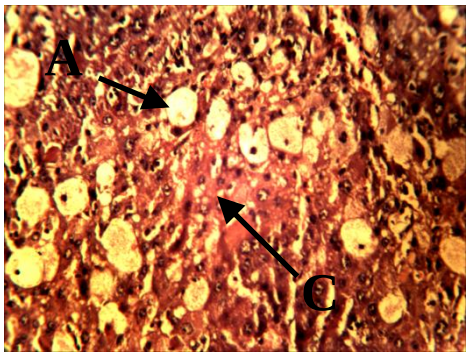


Plate IV: 250 mg/kg C.S. +CCl_4

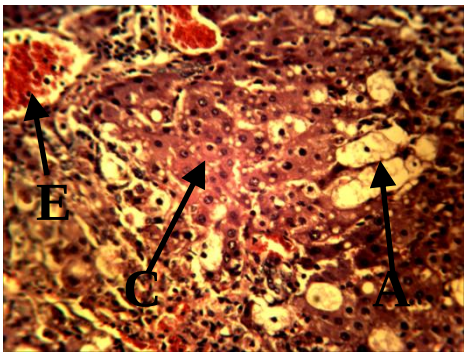


Plate V: 500mg/kg C.S. + CCl_4

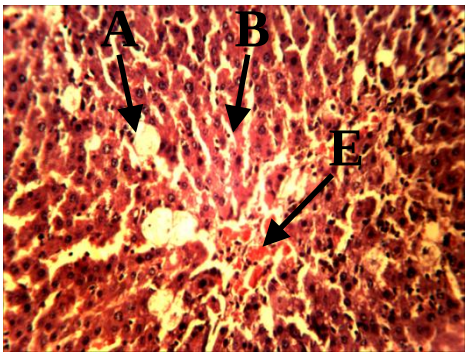


Plate VI: 1,000 mg/kg C.S. + CCl_4

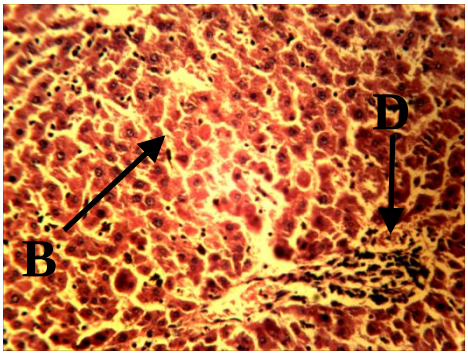


Plate VII: 1,000 mg/kg C.S. only

A- Adipocyte infiltration
B- Hepatocellular necrosis
C- Loss of liver parenchyma
D- lymphocyte hyperplasia
E- peri-portal inflammation
F- Vascular congestion

× 250 magnification.
Haemotoxylin and Eosin staining technique.

Table 4.6: Hepatoprotective effect of *C. sinensis* extract on histopathological changes observed in CCl_4-induced hepatotoxicity in rats.

	Hepatocytes	**Adipocytes**	**Portal tract**
Control	-	-	-
CCl_4	Marked hepatocellular necrosis with complete loss of liver parenchyma and lymphocyte hyperplasia.	Intense adipocyte infiltration.	Marked peri-portal inflammation with vascular congestion.
250 mg/kg C.S. +CCl_4	Marked hepatocellular necrosis with loss of liver parenchyma	Intense adipocyte infiltration.	-
500 mg/kg C.S.+CCl_4	Moderate hepatocellular necrosis with loss of liver parenchyma	Moderate adipocyte infiltration.	Mild peri-portal inflammation.
1,000 mg/kg C.S.+CCl_4	Mild hepatocellular necrosis	Mild adipocyte infiltration.	Moderate peri-portal inflammation.
100 mg/kg Sylimarin + CCl_4	Mild hepatocellular necrosis	Mild peri-capsular adipocyte infiltration.	-
1,000 mg/kg C.S.	Mild hepatocellular necrosis with lymphocyte hyperplasia	-	-

C.S.- *C. sinensis*

CHAPTER FIVE

5. DISCUSSION

Oxidative stress is created when excessive free radicals react with proteins and cell membranes thereby causing damage to cell structures (Xiuzhen *et al.,* 2007). This damage is critically involved in the pathogenesis of different diseases (Hadi *et al*., 2000) including liver disease (Quambo *et al.,*1998; Atawodi, 2005). The liver is a vital organ involved in the maintenance of metabolic functions and detoxification in the body (Ramachandra *et al.,* 2007). This organ is susceptible to injury from drugs and other substances (Jaeschke *et al.,* 2002).

Carbon tetrachloride (CCl_4) causes oxidative stress in rats accompanied by altered antioxidant status resulting to a marked rise in lipid peroxidation levels in liver and kidney tissues, whereas catalase and superoxide dismutase levels are decreased (Manna *et al.,* 2009). CCl_4 becomes toxic upon activation mainly through CYP2E1. After CCl_4 bioactivation, the resulting trichloromethyl ($CCl_3^{\bullet}$) radical binds covalently to CYP 2E1, either to the active site of the enzyme or to the heme group, thereby causing suicide inactivation (Uzma *et al.,* 2011). The radical can dismutate to form chloroform ($CHCl_3$) or can react with dioxygen and/or cellular macromolecules to produce lipid peroxidation and disrupt cellular processes (McGregor and Lang, 1996). This causes the swelling and necrosis of hepatocytes resulting to release of cytosolic enzymes into the blood (Wargovich *et al.,* 2001). Excessive production of the reactive

species manifests in tissue thiol depletion, lipid peroxidation, plasma membrane damage etc., culminating into severe hepatic injury (Singh *et al.,* 1998). The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. Both silymarin and *C. sinensis* extract used in this study decreased CCl_4 induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytes cell membrane or regeneration of damaged liver cells.

The standard drug silymarin is a flavonolignan from *Silybum marianum* (milk thistle) consisting of four flavonolignan isomers namely- silybin, isosilybin, silydianin and silychristin. Silymarin has clinical applications in alcoholic liver diseases, liver cirrhosis, Amanita mushroom poisoning, viral hepatitis, toxic and drug induced liver diseases and in diabetic patients. Silymarin is orally absorbed and is excreted mainly through bile as sulphate conjugates. It acts by antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating mechanisms. Though silymarin does not have antiviral properties against hepatitis virus, it promotes protein synthesis, helps in regenerating liver tissue, controls inflammation, enhances glucuronidation and protects against glutathione depletion. Silymarin offers good protection in various toxic models of experimental liver diseases in laboratory animals (Pradhan and Girish, 2006).

Preliminary phytochemical screening of the methanolic extracts of *C. sinensis*, *C. aurantiifolia* and *C. medica* revealed the same type of phytochemical constituents, which include flavonoids, tannins, saponins, steroids, and cardiac glycosides in all

three extracts. Quantitative phytochemical screening to determine the flavonoid contents of the three extracts suggest *C. sinensis* peel exract to contain the highest quantity of flavonoid, which agrees with the results of Ghasemia *et al., (*2009), thus supporting selection of *C. sinensis* for this study. Flavonoids which are the most widely studied group of the phenolic compounds have been reported to exert a wide range of biological activities such as antioxidants, free radical scavengers and chelators of divalent cation (Cook and Samman, 1996; Chebil *et al.,* 2006; Middleton, 2000). *C. sinensis* peel extract had the highest flavonoid content but the lowest total phenolic content compared to *C. aurantiifolia* and *C. medica* peels, suggesting that most of the phenolic compounds in its peel extract may be flavonoid compounds, which further collaborates with the choice of *C. sinensis* for this study.

Generally the data presented in this study revealed that the methanol extract of *C. sinensis* may contain some biologically active compounds with potential antioxidant and hepatoprotective properties. The oral LD_{50} is estimated to be greater than 5,000 mg/kg suggesting that it is relatively safe when administered orally to rats. Furthermore, the increase in CAT, SOD and GPx content of liver tissues observed in the extract treated group suggests antioxidant activity of *C. sinensis.* The reason for higher levels of TBARS which is a measure of the lipid peroxidation product malondialdehyde (MDA) in the normal group compared to the toxin group is not clear. However, this may be attributed to the controversial use of TBARS as a marker for lipid peroxidation particularly that MDA can be formed during eicosanoid metabolism. Besides, the analytical methods for measuring MDA are prone to artifactual errors (Janero *et al.,* 1990).

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues with the highest activity in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals. Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. Decrease in enzyme activity of CAT is a sensitive index in free radical induced hepatocellular damage (Palanivel *et al.,* 2008) as seen in the significant elevation of AST, ALT and ALP levels alongside the depletion of CAT enzyme observed in the CCl_4 treated group compared to the control group. *C. sinensis* peel extract caused a significant increase in hepatic CAT activity and thus reduced free radical induced oxidative damage to liver. Hence, there was a significant restoration of AST, ALT and ALP levels with administration of the peel extract in a dose dependent manner.

When associated with other liver function test abnormalities, a high serum bilirubin indicates hepatocellular dysfunction. Conjugated hyperbilirubinemia results from decreased bile formation or excretion. (Eldon, 2012b). The significant increase in total bilirubin and conjugated bilirubin observe in the CCl_4 treated group was reduced to normal levels in both silymarin and extract groups. This suggests restoration of hepatocellular function either by cellular membrane stabilization and protection of hepatocytes or healing of hepatic parenchyma and regeneration of hepatocytes. Effective control of bilirubin and ALP suggests an early improvement in the secretary mechanism of the hepatic cells (Palanivel *et al.,* 2008).

Protective mechanisms of the liver involve regeneration with repair or replacement of injured tissue. Furthermore, the liver has many cell units responsible for similar

functions such that other cells perform the functions of the injured sections indefinitely or until the damage has been repaired (C-Health fact sheet, 2013). This means that the liver is able to maintain a degree of functional capability depending on the extent of damage. This was demonstrated by the observation that there was no effect on the levels of total protein, albumin, Cholesterol and triglyceride with 2ml/kg CCl_4 subcutaneous administration at every 72hrs used in this study.

Hepatorenal syndrome which is kidney failure in liver disease is a common complication that occurs with advanced cirrhosis and its other complications such as encephalopathy, jaundice and bleeding problems (Pereira, 2011). CCl_4 causes depletion of glutathione, catalase and superoxide dismutase in liver and kidney tissues (Manna *et al*., 2009). Similar to report of previous workers (Ozturk *et al*., 2012; Turgut *et al*., 2008), silymarin had a protective effect on ischemia and reperfusion injury in the kidney tissues caused by CCl_4 in the results of this experiment. The kidney function test results, urea and creatinine level, were increased by CCl_4 and reduced by silymarin. Although similar to the results of Ozturk *et al.,* (2012), differences in creatinine levels were statistically insignificant. Increase in urea and creatinine levels observed in the CCl_4 treated group were reduced to near normal values by *C. sinensis* peel extract. The same rate of reduction in these biomarkers of kidney function in the extract and sylimarin groups indicates that *C. sinensis* peel extract has similar protective effect to sylimarin on kidney tissue.

Fatty deposits and other hepatic changes that accompany liver diseases and hepatotoxicity leads to increase in liver size which is usually seen as increase in liver to body weight ratios and can be calculated as relative liver weight (RLW)

percentages of laboratory animals (Uzma *et al.*, 2011). Although there was no statistically significant difference among groups in values of relative liver weight (RLW) and percentage weight loss (%WL), an interesting pattern closely resembling that of previous results can be observed. There was increase in liver weight among the rats in the CCl_4 treated group that lost weight during the course of the experiment. Although there was no improvement in these parameters among the extract and sylimarin group in terms of values, the relative similarities in RLW percentages between the CCl_4 and extract/sylimarin treatment groups can be understood. Perhaps, rat livers of the extract/sylimarin treatment groups having more functional capacity than the CCl_4 group had more metabolite content (such as glycogen) more than the CCl_4 group, while the CCl_4 group had more fat and fluid content caused by damage. This two-way variation can lead to similar liver to body weight ratios as observed in the results obtained.

Besides, the 3.38±0.15%RLW and -5.61±3.92 %WL (that is, 5.61±3.92 % weight gain) results of the 1,000 mg/kg extract only group compared to the 3.90±0.12%RLW and -9.93±2.06%WL of the normal control group reveals that the 1,000 mg/kg extract only group did not put on weight normally during the course of the experiment. Hence, the extract may have caused weight loss in ways other than that caused by liver damage. This observation is further clarified by the 11.57±2.63% WL of the 250 mg/kg being more than the 5.06±4.11 %WL of the CCl_4 group. This could mean that this lowest dose extract which had shown little or no hepatoprotection in previous results made the rats lose weight caused by liver toxicity of CCl_4 in addition to that caused by the extract.

Histology of the liver sections of control animals showed normal hepatic cells with well-preserved lobular pattern and visible central vein. On the other hand, the liver sections of CCl_4 -treated rats showed massive fatty changes, necrosis, ballooning degeneration and lymphocytes hyperplasia with loss of cellular boundaries. The histological architecture of liver sections of the rats treated with *C. sinensis* peel extract showed more or less normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration almost comparable to the control and silymarin treated groups. These results again show the hepatoprotective effect of the exract.

Nevertheless, it is important to note that the photomicrograph of the 1,000 mg/kg extract only group showed mild hepatotoxic changes. This further clarifies the reduction of the antioxidant and hepatoprotective properties of the extract observed at this dose in the biochemical analysis results. However, the photomicrograph of the 1,000 mg/kg C.S. + CCl_4 group showed better restoration of hepatic architecture compared to the 500 mg/kg C.S. + CCl_4 group, in contrast to biochemical analysis results. It is therefore possible that at 1,000 mg/kg, the extract offers better protection from the toxic effects of CCl_4, but some other toxic phytoconstituent(s) causes other forms of hepatoxicity other than the type caused by CCl_4 as seen in the photomicrograph of the CCl_4 group. Saponins and their derivatives from some plants have been documented to have hepatotoxic effect (Ilodigwe *et al*.2012).

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CHAPTER SIX

6. SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Preliminary phytochemical screening of the methanolic extracts of *C. sinensis*, *C. aurantiifolia* and *C. medica* peels revealed the same type of phytochemical constituents. However, *C. sinensis* peel extract showed the highest concentration of flavonoids and was selected for further studies. The hepatoprotective activity of methanol extract of *C. sinensis* peel evidenced by reversal of altered values of various biochemical markers was dose dependent in a similar pattern with its antioxidant activity. These results were further supported by the histological pictures obtained.

6.2 Conclusion

The local use of different citrus species peels for similar medicinal purposes was supported by the results of the phytochemical screening which revealed similar phytoconstituents in all three species tested; and the oral LD_{50} result of *C. sinensis* peel extract suggests that it is a safe oral remedy.

The results of this study supports the research hypothesis, since *C. sinensis* peel extract showed both hepatoprotective and antioxidant activity. The mechanisms of hepatoprotective activity of the extract may be by free radical scavenging and antioxidant activity. Flavonoids have been shown to have antioxidant and hepatoprotective activities (Merlin and Parthasarathy, 2011). It is therefore possible that the observed antioxidant and hepatoprotective activity of *C. sinensis* peel may be due to the presence of flavonoids and/or any other phtytoconsituent.

6.3 Recommendation

Further studies are needed to confirm and establish the possible mechanisms of the antioxidant and hepatoprotective activities detected. Also, further studies for isolation, purification and characterization of active principle(s) are necessary.

From the results of this study, there are indications that *C. sinensis* peel extract has phytochemicals that may be toxic with high dose or chronic administration. Hence, there is need for further and thorough studies on the toxicity profile of *C. sinensis* peel to ascertain its safety.

Finally, with the results obtained from this study, it has become imperative that *C. sinensis* peel be tested for efficacy in the treatment or management of other free radical induced diseases, especially those for which it has traditional uses e.g. diabetes, high blood pressure, hyperlipidemia and digestive tract diseases.

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APPENDIX I

In Vivo Antioxidant Activity Test Procedures and Principles

Superoxide dismutase (SOD) assay

Kit Content Assay

Buffer	1 X 30 ml
Hematoxylin	1 vial
Sample Dilution Buffer	1 X 30 ml

Reagent Preparation

The kit was allowed to warm up to room temperature completely before using.

Buffer: The bottle was shaken and open to air repeatedly up to five times before use so as to saturate the content with O_2.

Hematoxylin Solution: Hematoxylin was reconstituted with 1.2 ml (1200 µL) of distilled water.

Assay Procedure

1. 920 µL of *Assay Buffer* was pipetted into each cuvette for assay.

2. 40 µL of *Assay Buffer* was added for blank and 40 µL of *Sample* for test. This was mixed and incubated for two minutes.

3. 40 µL *Hematoxylin* was then added to start the auto-oxidation reaction.

4. The cuvette content was quickly mixed and absorbance was recorded immediately at 560 nm and then at every 7 seconds interval for 5minutes.

Test Principle: The method is based on monitoring the autooxidation rate of hematoxylin (HTH) as originally described by Martin *et al*., (1987). Briefly, in the presence of SOD enzyme at specific assay pH, the rate of auto-oxidation is inhibited and the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. Sample SOD activity is determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample and expressed as traditional McCord Fridovich "cytochrome c" units. The basic principal of the assay is shown schematically by the following equation:

$$O_2 + HTH_2 \xrightarrow{SOD} H_2O_2 + HT\ (Abs560\uparrow)$$

Data Analysis

1. auto-oxidation rates were determined by regression of the uninhibited linear portion of the rate curves. The equation for this linear regression was expressed as:

$$Y = aX + b,$$

where ‘a’ is the decomposition rate or (ÄAbs560 nm/min)

Average rates were calculated from triplicate assays.

2. The ratio of sample rate and blank rate was obtained using the equation

$$Ratio_{s/b} = Rate_s/Rate_b$$

where ‘$Rate_s$’ is the average auto-oxidation rate of a sample, and ‘$Rate_b$’ is the average autooxidation rate of the blank.

3. Percentage for SOD inhibition of baseline (blank) reaction rate was calculated as:

$$\% \text{ Inhibition} = (1 - Ratio_{s/b}) \times 100\%$$

4. SOD activity measured using the classical “cytochrome c reduction” method has been found to be approximately 1.25 times measurable percent inhibition of hematoxylin auto-oxidation as have been established. The SOD concentration in classical McCord Fridovich “cytochrome c reduction” unit activity was therefore calculated as:

$$[\text{SOD U/ml}]_s = 1.25 \times (\% \text{ Inhibition})$$

This value represents the SOD activity in the sample added to the reaction mixture not the final concentration in the sample.

5. Thus the SOD concentration in the original sample (“cytochrome c reduction” U/ml) was calculated using the equation:

$$[\text{SOD U/ml}]_{os} = [\text{SOD U/ml}]_s \times (\text{Sample Dilution Factor})$$

Catalase (CAT):

Kit Contents:

Sample Dilution Buffer	1 X 30 ml bottle
Assay Buffer	1 X 30 ml bottle
Hydrogen Peroxide (H_2O_2) Reagent	1 Vial

Catalase Standard 1 vials

Reagent Preparation

Working H_2O_2 Reagent: Entire contents of H_2O_2 Reagent was added to Assay Buffer and labeled as Working Assay Buffer. The vial was washed three times with the mixed solution and combined to the Working Assay Buffer to assure full transfer of contents. Catalase standards and samples were both assayed using this Working Assay Buffer.

Catalase Standard: Just before use, 1.0 ml of Sample Dilution Buffer was added to the Catalase standard vial making the resultant solution 150 U/ml in catalase activity. This was then diluted further in additional four microcentrifuge tubes by mixing following:

Standard no.	Sample Dilution Buffer (µL)	150 U/ml Standard (µL)	Standard conc. (U/ml)
1	0	400	0.0
2	100	300	37.5
3	200	200	75
4	300	100	112.5
5	400	0	150

Assay Procedure

1. 1,000 µL of Sample Dilution Buffer was pipetted into a clean cuvette and placed in the reference cuvette holder. This was used to zero the instrument at 240 nm wavelength.

2. 950 µL of Working Assay Buffer was pipetted into a clean semi-micro UV cuvette.

3. 50 µL of diluted standard or sample was added into the cuvette and mixed as quickly as possible.

4. Absorbance was recorded immediately at 240 nm and after every 2 second for 15 seconds.

Test Principle: Catalase Activity Assay is based on the method of Beers and Sizer (1952). Catalase enzyme activity can be measured by monitoring the consumption of H_2O_2 substrate at 240 nm. Accordingly, one unit of catalase activity is classically defined as the amount of enzyme that will decompose 1.0 KMole H_2O_2 substrate (starting concentration = 10.3 mM) per minute at pH 7.0 and 25°C.

$$\underset{\text{(Absorbance at 240 nm}\downarrow\text{)}}{2H_2O_2} \xrightarrow{\text{Catalase}} \underset{\text{(no Absorbance at 240 nm)}}{2H_2O + O_2}$$

Data Analysis

1. The standard curve was plotted to obtain the slope (a) and intercept (b) of the curve by regression: $Y = aX + b$

or

$$Rate = Slope[Catalase_{ds}] + Intercept$$

2. Rewriting and rearranging the equation, the catalase concentration of diluted samples $[Catalase_{ds}]$ added to the reaction mixture was calculated as:

$$[Catalase_{ds}] = (Rate - Intercept)/Slope$$

where 'Rate' is the decomposition rate (VA240 nm/min) of the sample.

3. Catalase concentration of the original sample $[Catalase_{os}]$ was then calculated by multiplying the diluted sample result by the dilution factor:

$[Catalase_{os}] = [Catalase_{ds}] * df$

***Glutathione peroxidase(GPx)*:**

NADPH Reagent: β-Nicotinamide adenine dinucleotide phosphate and GSH, reduced; 2 bottles powder.

NADPH Diluent: Glutathione Reductase in buffer with stabilizer and 4 mM $NaNO_3$; 2 x 6 ml.

Assay Buffer: Phosphate buffer with EDTA and 4 mM $NaNO_3$, pH 7.0; 1 x 12ml H_2O_2 Reagent 3% Hydrogen Peroxide

Reagent Preparation

NADPH Reagent: The entire content of one NADPH Diluent bottle was added to one NADPH bottle. The cap on the vial containing the reconstituted NADPH was secured and mixed briefly by inverting the bottle, labeled "Working NADPH" and then kept on ice.

H_2O_2 Reagent: 3% hydrogen peroxide as supplied was diluted 1:100 (e.g .02 ml 3% H_2O_2 to 2 ml Assay Buffer) and labeled as "Diltution 1". "Dilution 1" was further diluted 3:100 (e.g. 0.3 ml Dilution 1 to 10 ml Assay Buffer) and labeled as Working H_2O_2.

Assay Procedure

1. All reagents were brought to room temperature.

3. 200 µL of diluted sample was pipetted into each cuvette for assay.

4. 200 µL of *Working NADPH* was added to each cuvette.

5. 200 µL of *Working H_2O_2* was added to each cuvette.

6. After 1 minute, absorbance at 340 nm was monitored for 5 minutes with a recording interval of 30 seconds.

7. GPx activity was calculated from the net rate.

Data Analysis

The GPx concentration, expressed as mU/ml, was calculated using the GPx activity definition equation:

$$[GPx] = \frac{2(mRate_s - mRate_b) \times V_{Rxm}}{6.22 \times Vs} \times df$$

where

$mRate_s$ = -1,000 × ΔA340/min of sample

$mRate_b$ = -1,000 × ΔA340/min of blank

6.22 = NADPH 340 nm millimolar absorption coefficient at 1 cm path length.

V_{Rxm} = Volume of Reaction Mixture

Vs = Volume of Sample

2 = Correction for 2moles GSH oxidized to 1mole GSSG per mole NADPH oxidized.

df = Sample dilution factor

Test Principle: Glutathione Peroxidase Assay is an adaptation of the method of Paglia and Valentine (1967). Glutathione Peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP+ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GPx is limiting, the decrease in absorbance at 340 nm is directly proportional to the GPx concentration.

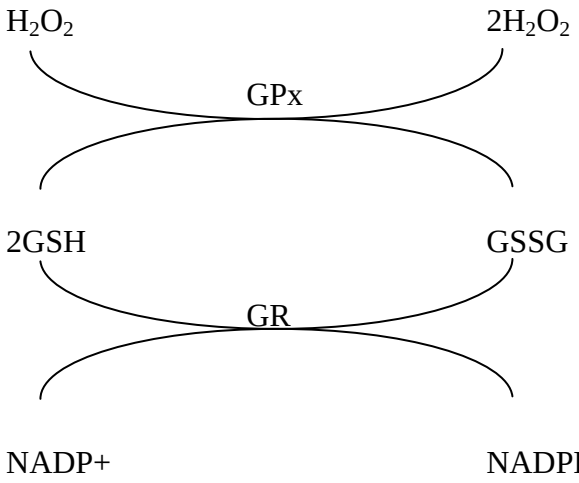


Figure 7.1 Recycling of reduced glutathione (GSH).

GPx activity is reported as units based on the definition: 1 unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H_2O_2) of 1.0 μmole GSH to GSSG, per minute at 25 ºC, pH 7.0.

***Thiobarbituric Acid Reactive Substances (TBARS)*:**

Kit Contents

TBA Reagent:	2-Thiobarbituric acid (TBA), dry powder, 5 bottles
BHT Reagent:	Butylated hydroxytoluene in ethanol, 2.0 ml vial
Acid Reagent:	1M Phosphoric acid, 5 x 10 ml bottles
Assay Buffer:	Phosphate buffer, pH 7.0 with EDTA, 125 ml bottle
Calibrators:	Tetramethoxypropane in a stabilizing buffer at 4,3,2,1 and 0μM Malondialdehyde (MDA) equivalents; 2.5 ml each

Reagent Preparation

TBA Reagent: 10.5 ml deionized water was added to the TBA bottle and mixed using magnetic stirrer until TBA dissolved.

Assay Procedure

1. 10 µl BHT Reagent was pipetted into test tubes for assay
2. 250 µl Calibrator or sample was added to each test tube.
3. 250 µl Acid Reagent was added each test tube.
4. 250 µl TBA Reagent was added each test tube.
5. The reaction mixture was vortexed vigorously and incubated for 60 minutes at 60°C in a water bath and then centrifuged at 10,000 x*g* for 2-3 minutes
8. Each reaction mixture was transferred to a cuvette and spectra recorded from 400-700 nm.
9. 3rd derivative analysis was performed to obtain results.

Test Principle: The assay is based on the reaction of MDA with TBA; forming an MDA-TBA2 adduct that absorbs strongly at 532 nm (Botsoglou, 1994).

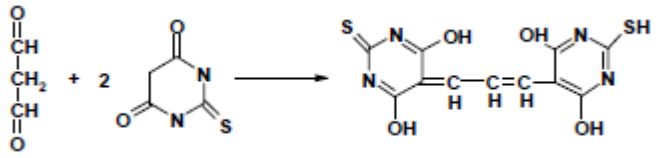


Figure 7.2 Reaction between MDA and TBA.

Butylated hydroxytoluene (BHT) and EDTA are added to the sample and reaction mixture to minimize oxidation of lipids that contribute artifactually during sample processing and the TBA reaction. The temperature of the reaction mixture is also reduced to minimize the decomposition of lipid hydroperoxides. Because much of the MDA is protein bound, mostly as a Schiff base, the pH of the reaction is optimized to facilitate hydrolysis of the MDA. Additionally, the reaction mixture is subjected to derivative spectrophotometric analysis that resolves the problem of the variable and nonlinear baseline observed when attempting to measure the A532 absorbance in various biological samples.

APPENDIX II

Assessment of hepatoprotective activity procedures and principles

Test for aspartate aminotransferase (AST)

Principle: L-Aspartate and α-Ketoglutarate react in the presence of AST in the sample to yield oxaloacetate and L-glutamate. The oxaloacetate is reduced by malate dehydrogenase (MDH) to yield L-malate with the oxidation of NADH to NAD+. The reaction is monitored by measurement of the decrease in absorbance of NADH at 340 nm. The rate of deduction in absorbance is proportional to AST activity in the sample.

Procedure: 1 ml *Working Reagent* was pipetted into test tubes followed by 0.05ml of *sample*. The reaction mixture was mixed immediately. First absorbance of test was read at exactly 1 minute and thereafter at 30, 60, 90 and 120 seconds at 340 nm. The mean change in absorbance per minute (ÄA/min) was determined and test results calculated thus:

Serum AST activity (IU/L) = ÄA/min. x F

Where

F = 3376 (based on the millimolar extinction coefficient of NADH at 340 nm).

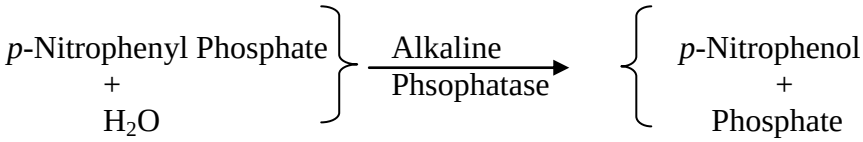
Test for alanine aminotransferase (ALT)

Principle: Alanine and α-ketoglutarate are first converted to glutamate and pyruvate which is converted by lactate dehydrogenase to make lactate and NAD+. The conversion of the NADH to NAD+ product, measured at 340 nm, is proportional to the level of ALT enzyme in the sample.

Procedure: same as for AST above.

Test for alkaline phosphatase (ALP)

Principle: Alkaline Phosphatase in a sample hydrolyses *p*-nitrophenyl phosphate into *p*-nitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405 nm due to liberation of *p*-nitrophenol is proportional to the alkaline phosphatase activity.



Procedure: 1.0 ml buffered substrate was pipetted into test tubes followed by sample 0.02 ml. This was mixed and absorbance read at 30, 60, 90 and 120 seconds at 405 nm. The mean change in absorbance per minute (Δ A/min) was used to calculate alkaline Phosphatase activity:

Alkaline Phosphatase activity (IU/L) = Δ A/min x F

Where F = 2713 (calculated on the basis of molar extinction coefficient for p-nitrophenol and ratio of total assay volume to sample volume).

Test for total bilirubin and direct bilirubin

Principle: Bilirubin reacts with diazotized sulphanilic acid to form an azo compound the colour of which is measured at 546 nm (530-560 nm) and is proportional to the concentration of Bilirubin. For Total Bilirubin the reaction is accelerated by caffeine reagent. The readings for total bilirubin are taken after 5 mins. incubation & the readings for direct bilirubin are taken after 3 mins. incubation.

Procedure for Bilirubin: 50 µl of sample was pipetted into sample blank and sample test tubes. 100 µl of Working Solution 1 was added to sample test tubes and100 µl

Solution 2 was added to sample blank followed by 1.0 ml of Solution 3 in both. The reaction mixture was mixed properly and incubated for 5 mins. at room temperature and absorbance read at 546 nm against sample blank.

Procedure for Direct Bilirubin: 50 µl of sample was pipetted into sample blank and sample test tubes. 100 µl of Working Solution 1 was added to sample test tubes and100 µl Solution 2 was added to sample blank followed by 1.0 ml of Solution 3 in both. The reaction mixture was mixed properly and incubated for 5 mins. at room temperature and absorbance read at 546 nm against sample blank.

Calculation: Serum Bilirubin (mg/dl) = (Abs. of sample-Abs. of sample blank) × 26.312

Test for total protein

Principle: In an alkaline medium, protein reacts with the copper in Biuret reagent causing an increase in absorbance. The increase in the absorbance, at 540 nm due to formation of the coloured complex, is directly proportional to the concentration of protein.

Procedure: 2.5ml reagent was pipetted into blank, standard and sample test tubes followed by 0.01ml Standard solution in standard and 0.01ml sample in sample test tubes. The reaction mixture was properly mixed and allowed to stand at room temperature for 10 minutes. Absorbance of standard and test was then read at 540 nm against reagent blank.

$$\text{Total Protein Concentration (gm/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 5$$

Test for albumin

Principle: In an acidic medium, albumin binds with bromocresol green (BCG) causing a shift in the absorption spectra of the yellow BCG dye. The blue green colour formed is directly proportional to the albumin present when measured at 630 nm.

Procedure: 2.5ml reagent was pipetted into blank, standard and sample test tubes followed by 0.01ml Standard solution in standard and 0.01ml sample in sample test tubes. The reaction mixture was properly mixed and allowed to stand at room temperature for 10 minutes. Absorbance of standard and test was then read at 630 nm against reagent blank.

$$\text{Albumin Concentration (gm/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 5$$

Test for cholesterol

Principle: The cholesterol esters are hydrolysed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidised by cholesterol oxidase (CO) to cholesten 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4- aminoantipyrine and phenolic compound in the presence of peroxidase to yield a coloured complex which is read at 505 nm. The intensity of colour produced is directly proportional to the concentration of total cholesterol in the sample.

$$\text{Cholesterol esters} \xrightarrow{\text{CE}} \text{Cholesterol + fatty acids}$$

$$\text{Cholesterol + O2} \xrightarrow{\text{CO}} \text{Cholesterol-4-en-3-One + } H_2O_2$$

$$\left.\begin{array}{l} 2H_2O_2 + 4\text{AAP} \\ \text{+ Phenolic compound} \end{array}\right\} \xrightarrow{\text{POD}} \text{Quinoneimine dye + } 4H_2O$$

Procedure: 1.0ml cholesterol-L reagent was pipetted into blank, standard and sample test tubes followed by 0.01ml Cholesterol-Standard in standard and 0.01ml of sample in test tubes. The reaction mixture was properly mixed and incubated for 10 minutes at 37^{o}C. Absorbance of standard and test was then read at 505 nm against reagent blank.

$$\text{Cholesterol Concentration (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 200$$

Test for triglycerides

Principle: Lipase hydrolyses triglycerides sequentially to Di & Monoglycerides and finally to Glycerol. Glycerol kinase (GK) using ATP as PO4 source converts Glycerol liberated to Glycerol-3-phosphate (G-3-Phosphate). G-3-phosphate Oxidase (GPO) oxidizes G-3-phosphate formed to Dihydroxy acetone phosphate and hydrogen peroxide is formed. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidize 4-aminoantipyrine and TOOS (N-ethyl-N-Sulphohydroxy propyl-m Toluidine) to a purple coloured complex. The absorbance of the coloured complex is measured at 546 nm which is proportional to Triglycerides concentration.

Procedure: 1.0ml reagent was pipetted into blank, standard and sample test tubes followed by 0.01ml Standard solution in standard and 0.01ml sample in sample test tubes. The reaction mixture was properly mixed and incubated for 15 minutes at 37^{o}C. Absorbance of standard and test was then read at 546 nm against reagent blank.

$$\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 200$$

Test for urea

Principle: Urease breaks down urea in to ammonia and carbon dioxide. In alkaline medium, ammonia reacts with hypochlorite and salicylate to form

dicarboxyindophenol, a coloured compound. The reaction is catalysed by sodium nitroprusside. The intensity of colour produced is measured photometrically at 578 nm.

Procedure: 0.01ml standard, 0.01ml sample and 0.01ml distilled water were pipetted into standard, sample and blank test tubes respectively. 1.0 ml Working reagent was added, properly mixed and incubated for ten minutes at room temperature. 1.0 ml Working colour reagent-C was then added properly mixed and incubated for ten minutes at room temperature. After this, absorbance of standard and test was read at 578 nm against reagent blank.

$$\text{Urea Concentration (gm/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times 40$$

To convert urea concentration (mg/dl) to mmol/lit:

Urea concentration (mg/dl) x 0.167 = mmol/lit.

Test for creatinine

Principle: Creatinine present in the serum or urine reacts with alkaline picrate to form a colored complex. The rate of formation of colored complex is directly proportional to creatinine concentration. This rate of reaction (intensity of color produced) is measured photometrically at 510 nm and is compared with that of the standard.

Procedure: 1.0ml reagent was pipetted into standard and sample test tubes followed by 0.1ml Standard solution in standard and 0.1 ml sample in sample test tubes. The reaction mixture was properly mixed. Absorbance of standard (ST) and Test (TS) was read at 20 seconds (ST1, TS1) and again at 80 seconds (ST2, TS2) at 510 nm, against distilled water.

$$\text{Creatinine concentration (mg/dl)} = \frac{\text{TS2-TS1}}{} \times 2$$

ST2-ST1

To convert (mg/dl) to micromole/liter:

micromole/liter = (mg/dl) x 88.5