PROTECTIVE EFFECT OF *Pterocarpus mildbraedii* HARMS EXTRACT ON PROPANIL-INDUCED HEPATOTOXICITY AND ALTERATIONS IN APOPTOTIC-RELATED PROTEINS IN WISTAR RATS

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ABSTRACT

One of the probable causes of liver diseases is exposure to environmental chemicals. Agrochemicals containing propanil are known to induce hepatic toxicity. *Pterocarpus mildbraedii* leaf is used in traditional medicine to treat various disorders without scientific justification. This study was designed to investigate the protective role of extract of *Pterocarpus mildbraedii* against propanil-induced hepatotoxicity in rats.

Pterocarpus mildbraedii leaves, purchased from Oyingbo market, Lagos State, were authenticated at the University of Lagos Herbarium (LUT/5913). Powdered leaf was extracted in soxhlet, using dichloromethane:methanol (1:1), to yield crude extract of *Pterocarpus mildbraedii* (PME). Sixty-four male Wistar rats (130-160 g), comprising of eight groups (n=8) were used for these experiments. Rats were treated orally with normal saline (control), PME (100 mg/kg), PME (200 mg/kg), PME (400 mg/kg), propanil (200 mg/kg), PME (100 mg/kg) + propanil (200 mg/kg), PME (200 mg/kg) + propanil (200 mg/kg) and PME (400 mg/kg) + propanil (200 mg/kg) for seven consecutive days. Hepatic tissues and serum were assayed for markers of hepatic damage, oxidative stress, inflammation, and apoptosis. Aspartate aminotransferase (AST), lactate dehydrogenase (LDH), bilirubin (BIL), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO) and nitric oxide (NO) were assayed by spectrophotometry. Inducible Nitric Oxide Synthase (iNOS), Cyclooxygenase-2 (COX-2), Nuclear factor kappa B (NF-κB), Caspase 3, Caspase 9, Bax, Bcl-2 expressions were measured using immunohistochemistry. Tumour suppressor p53, Bcl-2 antagonist of cell death (Bad), NF-κB, inhibitor of total nuclear factor-kappa B α (IκB α), stress activated protein kinase/ C Jun NH_2-terminal kinase (SAPK/JNK), p38 mitogen-activated protein kinase (p38) and signal transducer and activator of transcription 3 (STAT 3) were assessed by ELISA. Histopathology of liver was determined by microscopy and apoptosis by TUNEL assay. Data were analysed using ANOVA at $\alpha_{0.05}$.

The yield of PME was 41.9%. Administration of propanil significantly increased AST (132.10±6.32 U/L), LDH (85.70±6.60 U/L), BIL (1.15±0.16 mg/dL), SOD (0.97±0.05 U/mg protein), MDA (1.03±0.08 µgMDA/mg protein), MPO (4.98±0.12 µmol/min/mg protein) and NO (0.38 µmol/mg protein) relative to control (115.90±8.65, 32.84±9.39, 1.15±0.16, 0.38± 0.01, 0.40±0.11, 2.47±0.10 and 0.19± 0.05, respectively). Pre-treatment of propanil-exposed rats with

PME (200 mg/kg) significantly decreased LDH (83%), BIL (50%), SOD (50.5%), MDA (33.1%), MPO (63.3%) and NO (59.5%). Further, propanil administration decreased the levels of GSH (2.98±0.24 µg/mg protein) and CAT (52.7±0.24 µmol H_20_2 consumed/min/g tissue) when compared with the controls (2.04±0.09 and 51.00±0.51). However, intervention with PME restored these serum biochemical indices and antioxidant parameters back to normal values. Expressions of iNOS, COX-2, NFκB, Caspase 3, Caspase 9 and Bax were higher in the propanil group relative to control. Levels of signaling mediators p38 (81.28±7.70), STAT 3 (88.80±4.40) and NF-κB (72.76± 5.30) were lower, while SAPK (125.39±9.30), IκB α (115.83±5.60) and Bad (112.48±4.70) were higher in propanil-treated rats relative to control value set at 100. TUNEL-positive nuclei and severe periportal fibrosis were observed in tissues following propanil exposure. However, pre-treatment with PME significantly attenuated the observed propanil-induced inflammation and apoptosis.

Pterocarpus mildbraedii extract protected against propanil-induced hepatotoxicity via mechanisms that involved its antioxidant, anti-inflammatory and anti-apoptotic properties.

Keywords: *Pterocarpus mildbraedii*, Propanil, Hepatotoxicity, Apoptosis, Anti-inflammatory

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To my King, my Creator, my Sustainer, Upholder, my Provider, my Defender, my Strong Tower, my Redeemer, my Saviour, my Captain, my Fountain, my Enabler, my Comforter and my Source, I say "All Glory, Honour, Adoration and Thanksgiving be Unto You" .

Chiagoziem Anariochi Otuechere

CERTIFICATION

I certify that OTUECHERE CHIAGOZIEM ANARIOCHI carried out the work under my supervision in the Drug Metabolism and Toxicology Unit of the Department of Biochemistry, University of Ibadan, Nigeria.

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DEDICATION

This work is dedicated to the memory of my late parents Sir Godfrey and Reginah Otuechere for laying the foundation with their love, care and sacrifice.

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ABBREVIATIONS

AIF	Apoptosis inducing factor
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
AP-1	Activator protein-1
APAF1	Apoptotic protease activating factor 1
ARE	AU rich element
ASK1	Apoptosis signal-regulating kinase 1
ATF	Activating transcription factor
ATM	Ataxia-telangiectasia mutated gene
ATP	Adenosine-5'-triphosphate
AST	Aspartate aminotransferase
Bad	Bcl-2 associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-Cell lymphoma
BH	Bcl-2 homology domain
BSA	Bovine Serum Albumin
cAMP	Cyclic adeonosine monophosphate
CAS	Caspase (Cysteinyl Aspartic Acid-protease)
CAT	Catalase
CDNB	1-Chloro-2-4-Dinitrobenzene

COX	Cyclooxygenase
CYT	Cytochrome
DAB	Diaminobenzidine
DCM	Dichloromethane
DD	Death domain
DED	Death effector domain
DISC	Death Inducing Signaling Complex
DIABLO	Direct IAP binding protein with low PI
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNPH	2, 4-Dinitrophenylhydrazine
DPPH	1,1-diphenyl-2-picrylhydrazyl
DR	Death Receptor
DTNB	5', 5'-Dithiobis (2-Nitrobenzoic Acid)
dUTP	2 de-oxy uridine 5 triphosphate
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular regulated kinase
FADD	FAS associated death domain
FasL	Fas ligand
GAPDH	Glyceraldehyde-3-Phossphate Dehydrogenase
γ-GT	Gamma-Glutamyl transferase
GR	Glutathione reductatse
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
GST	Glutathione-S-transferase
HGF	Hepatocyte growth factor
IAP	Inhibitor of apoptosis protein
IFN	Type 1 interferon
IκB	Inhibitor of Nf-κB
IKK	IκB kinase

IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun NH2-terminal kinase
LDH	Lactate Dehydrogenase
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MAPK	Mitogen-activated protein kinase
MKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAPK/ERK kinase
MKP	MAPK phosphatase
MK-2	MAPK-activated protein kinase-2
NFƙB	Nuclear factor-ƙB
NO	Nitric oxide
oxLDL	Oxidised LDL
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered salt solution
PCD	Programmed cell death
PRP	Propanil
PME	*Pterocarpus mildbraedii* extract
p38	p38 MAPK
PI3K	Phosphatidylinositol 3-kinase
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
TBA	Thiobabituric Acid
TBARS	Thiobarbituric Acid-reactive substances
TCA	Trichloroacetic Acid
TMB	Tetramethylbenzidine

TNF	Tumor Necrosis Factor
TRADD	TNF-Receptor Associated Protein with Death Domain
TUNEL	Terminal de-oxy nucleotidyl dUTP nick end labeling

CHAPTER ONE

1.0 INTRODUCTION

Hepatotoxicity can be defined as injury to the liver that is associated with impaired liver function caused by exposure to a drug or another noninfectious agent, including industrial or environmental chemicals (Navarro and Senior, 2006). In the last four decades, the adverse effects of environmental chemicals on the liver have become increasingly frequent. The level of exposure varies from minute quantities to very high doses because the liver is subject to potential damage from an enormous array of pharmaceutical and environmental chemicals. Injury may result from direct toxicity to hepatocytes or biliary epithelial cells, causing necrosis, apoptosis, or disruption of cellular function through hepatic conversion of a xenobiotic to an active toxin or through immune mechanisms, usually by a drug or a metabolite acting as a hapten to convert a cellular protein into an immunogen (Sener *et al.,* 2005).

Numerous diseases and environmental factors can affect liver function resulting in gallstones, liver steatosis, fibrosis, cirrhosis, and even cancer. By clearing xenobiotics through biotransformation (in most instances by detoxification or sometimes by production of active or even more toxic metabolites), the liver itself can be injured by these xenobiotics. Due to its high blood perfusion rate and high metabolic capacity, the liver is continuously exposed to high levels of xenobiotics and to their metabolites. Fortunately, the liver has a high capacity of regeneration and ability to repair any underlying damage. Generally speaking, hepatotoxicity occurs when liver regeneration capabilities are exhausted and cell damage ensues. Furthermore, the liver's role as the immediate recipient of orally absorbed chemicals, plus its abundance of oxidative metabolism, makes it a common target for more toxic metabolic products when detoxifying and protective mechanisms are overwhelmed. Hepatic injury due to xenobiotic exposure involves inflammation, oxidative stress, and lipid peroxidation reactions that can result in mitochondrial damage and cell death. The resulting pathological alterations of the endogenous substances such as proteins, nucleic acids, and lipids can harm the proper functioning of the liver (Hong *et al.,* 2009; Krieger, 2010).

From time in memorial, pesticides have been used in agriculture to enhance food production by eradicating unwanted weeds, insects and controlling disease vectors. But the use of pesticides is fraught with health hazards. The contribution of pesticides to the etiology of

liver diseases has been well-documented (Jain *et al.,* 2011; Sayim, 2007; Tripathi and Srivastav, 2010). For example, in animal studies, rats exposed to the azole fungicides (cyproconazole, epoxiconazole and prochloraz), the pyrethroid insecticide, Prallethrin, and 2,4-Dichlorophenoxyacetic acid based herbicide presented with oxidative damage and liver injury (Heise *et al.,*2014; Mossa *et al.,* 2013; Tayeb *et al.,* 2010). Other pesticides, such as imidacloprid (Arfat *et al.,* 2014) and Fenitrothion (Zeid and Khalil, 2014) have also been reported to induce hepatic injury using the mice and fish models of animal experimentation respectively. Propanil (PRP) is an acylanilide herbicide used to control barnyard grass, broadleaf weeds, and for the post-emergent treatment of rice (Moore *et al.,* 1998). The widespread use of the herbicide on rice and wheat crops means that humans could be at risk of high level exposure. Earlier reports by McMillan and coworkers (2008) indicated that exposure to PRP had been associated with toxicity in humans and other mammals. Furthermore, it had been demonstrated that propanil could potentially induce cytotoxicity and nephrotoxicity in vitro (Rankin *et al.,* 2008). A recent study reported that propanil induced dose-dependent histopathological changes in the liver and kidney tissues of exposed mice (Cakici and Akat, 2013). However, the molecular events of liver toxicity in response to PRP intoxication remain unknown.

Reactive oxygen species (ROS) play an important role in the toxicity of pesticides and environmental chemicals. Pesticides may induce oxidative stress by generation of ROS and alteration in antioxidants or ROS-scavenging enzyme systems. Elevation in the levels of ROS results in the oxidation of cellular components and unsaturated fatty acids have been shown to be vulnerable molecules. Induction of oxidative stress by pesticides as a possible mechanism of toxicity has also been a focus of research for the past decade (Banerjee *et al.,* 1999; Abdollahi *et al.,* 2004). Apart from affecting the cellular proteins, lipids and DNA, ROS have a very crucial role in inducing apoptosis in the mitochondria. Conditions of excessive oxidative stress can result in cellular apoptosis or necrosis. Apoptosis has been implicated as a major mechanism of cellular death due to pesticide-induced toxicity (Li *et al.,* 2014; Hossain and Richardson, 2011). Among the different apoptotic pathways, the mitochondrial pathway seems to play a predominant role. In this process, the generation of excessive ROS leads to changes in mitochondrial membrane potential, an increase in cytochrome C release from the damaged mitochondria and the activation of caspase-3.

Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeogu *et al.*, 2005). The main advantages of natural bioactive molecules are their mild side effects on the body in comparison to chemically synthesized drugs. Several plant extracts have been examined for use in a wide variety of liver disorders. *Glycosmis pentaphylla* protects membrane integrity in mice hepatocytes (Nayak *et al.*, 2011), *Silybum marianum* (source of silymarin) seed oil has a protective efficacy against carbon tetrachloride-induced toxicity and oxidative stress in murine liver (Hermenean *et al.*, 2015). Epidemiological studies have shown that the consumption of vegetables and fruits can protect humans against oxidative damage by inhibiting or quenching free radicals and ROS. Many plants including fruits and vegetables are recognized as sources of natural antioxidants that can protect against oxidative stress and thus play an important role in the chemoprevention of diseases that have their aetiology and pathophysiology in ROS (Atawodi, 2005).

Pterocarpus Mildbraedii species belong to the family of papilionaceae and they occur throughout the tropics. The Nigerian species are trees with bright yellow and usually alternate leaflets (Keay *et al.*, 1964). The fully expanded and older leaves of *Pterocarpus* species were found to have saponins, tannins, phenols and flavonoids. The presence of phenolic compounds and flavonoids in their leaves indicate their medicinal value. Gill (1992) has already reported the therapeutic, antiseptic, antifungal, bactericidal properties and anti-diabetic effects of the vegetable, but no detailed study on their specific composition and structure has been conducted. In order to expedite the utilization of these plants and their byproducts, information on their active constituents and mechanisms of action are essential. This research, therefore aims at exploring the biological mechanisms of this leafy vegetable with a view to validating folkloric claims.

1.1.0 RATIONALE AND OBJECTIVES

Liver diseases have become a global problem, accounting for thousands of mortalities yearly. Damage to liver is always associated with cellular necrosis and an increase in the serum

levels of many biochemical markers such as aspartate amino transferase, alanine aminotransferase, alkaline phosphatase , bilirubin, albumin (Latha *et al.,* 2009). Several chemicals and other environmental agents have been implicated in the etiology of liver diseases. Among them is propanil (3, 4-dichloropropioanilide) classified as an acylanilide herbicide which belongs to the class of phenylamides. It is used to control barnyard grass, broadleaf weeds and for the post-emergent treatment of rice (Moore *et al.,* 1998). The World Health Organization recognized propanil as slightly hazardous in terms of human risk (WHO, 2004a). The widespread use of the herbicide on rice and wheat crops means that individuals in agriculture are particularly at risk of high level exposure. Earlier reports by McMillan and co-workers (1991) indicated that exposure to propanil has been associated with toxicity in humans and other mammals. Furthermore, it had been demonstrated that propanil has the potential to induce cytotoxicity and nephrotoxicity *in vitro* (Rankin *et al.,* 2008). Antioxidants had been proven to play an important role in the regulation of a vast array of physiological and pathological processes. They principally contribute to the protection of cells and tissues against deleterious effects of reactive oxygen species and other free radicals. The plant kingdom is a huge reservoir of bioactive molecules, many of which are yet to be exploited for various pharmaceuticals and other industrial applications. Medicinal plants are of great importance to the health of individuals and communities. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeogu *et al.,* 2005). The main advantages of natural bioactive molecules are their mild side effects on the body in comparison to chemically synthesized drugs. Natural products and their active principles as sources for new drug discovery and treatment of diseases have attracted attention in recent years. Vegetables serve as indispensable constituents of the human diet supplying the body with minerals, vitamins, protein and energy. They are useful in reducing the risk of cancer and organ diseases since they are low in fat, high in dietary fiber, and rich in folic acid, vitamin C, potassium and magnesium, as well as containing a host of other phytochemicals (Gbile and Adesina, 1986). Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries (Zakaria, 1991). The screening of plant extracts have been of great

interest to scientists for the discovery of new drugs effective in the treatment of several diseases.

The rationale and objectives of this present study therefore are to:

1. To assess the extent of disruption of the antioxidant defense system following treatment with propanil.
2. To examine the possible protective effect of *Pterocarpus mildbraedii* extract in a rat model of propanil-mediated liver dysfunction.
3. To elucidate the molecular mechanism(s) by which PME protects against PRP-induced liver toxicity in rats.
4. To evaluate the free radical scavenging and antioxidant activities of PME *in vitro.*
5. To fractionate and isolate various compound(s) from the leafy vegetable of *Pterocarpus mildbraedii.*
6. To correlate the biochemical alterations induced by propanil in blood and kidney and liver tissues with overall morphology.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.0 PUBLIC HEALTH IMPACT OF PESTICIDES

The use of chemicals in modern agriculture has significantly increased productivity. But it has also significantly increased the concentration of pesticides in food and in the environment, with associated negative effects on human health (Richter, 2002). According to the United States Environmental Protection Agency (US EPA 2014), a pesticide is defined as "any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest". Although pesticides are often restricted to insecticides, the term 'pesticide' also applies to herbicides, fungicides, and various other substances used for controlling pests. Exposure to pesticides can occur directly from occupational, agricultural and household use, and indirectly through the diet. The high risk groups exposed to pesticides include production workers, formulators, sprayers, mixers, loaders and agricultural farm workers. Children may be more susceptible to the effects of pesticides due to increased exposure via food and breast milk, underdeveloped detoxification pathways, and longer life expectancy in which to develop diseases with long latency periods (Lam *et al.,* 2015; Cohen, 2007).The lipophilic and persistent nature of these environmentally stable compounds and their ability to bio-magnify through the food chain (Barber *et al.,* 2005; Jaga and Dharmani, 2003) are primary reasons for the continued exposure of these pesticides in the general population. The body defense mechanism, especially the liver, is responsible for the detoxification of these xenobiotic compounds through metabolism by cytochrome P450 enzymes, including CYP3A and CYP2B families. Lethal bioaccumulation of pesticide concentration due to high dose or chronic exposure could overwhelm the liver detoxification capacity and cause toxicity (Wahlang *et al.,* 2013; Zhang *et al.,* 2002).

The adverse human health effects associated with these compounds, even at low concentrations, are diverse and include, but are not limited to, reprotoxic, hepatotoxic, neurotoxic, nephrotoxic and carcinogenic actions (Jamal *et al.,* 2015; Wilson *et al.,* 2014; Navaranjan *et al.,* 2013; Tayeb *et al.,* 2010; Rankin *et al.,*, 2008). Infants are extremely vulnerable to pre and postnatal exposure to endocrine disruptor pesticides, resulting in a wide range of adverse health effects including possible long-term impacts on intellectual function and delayed effects on the central nervous system functioning (Eskenazi *et al.,* 2006, Ribas-Fito *et al.,* 2003). Pesticide exposure is recognized as an important environmental risk factor associated with cancer development. The epidemiology of pesticide exposure and cancer in humans has been studied globally in various settings. Insecticides, herbicides, and fungicides

are associated with hemopoetic cancers, cancers of the prostate, pancreas, liver and other body systems (Jaga and Dharmani, 2005).

2.1.1 PROPANIL, USAGE AND APPLICATION

Propanil (3, 4-dichloropropionanilide) is a selective acylanilide herbicide used widely in rice cultivation in many parts of the world. It may be the most extensively used herbicide for rice production worldwide (Moore and Farris, 1997) and is ranked within the top 20 pesticides used for agriculture in the United States (Kiely *et al.*, 2004). Unfortunately, acute self-poisoning leading to severe poisoning and death has been reported with propanil, particularly in Asia where subsistence farming is more common (Hori, 2002). Propanil is made industrially by nitration of 1, 2-dichlorobenzene, reduction with catalytic hydrogen, then reaction with acetyl chloride (Wyatt *et al.*, 2008).

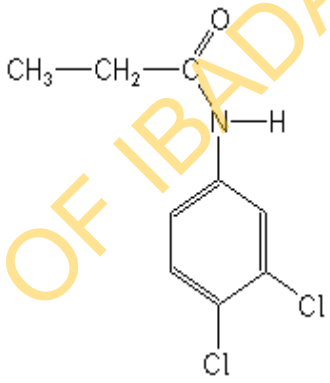


Figure 1: Structure of propanil

2.1.2 METABOLISM OF PROPANIL

Propanil is hydrolyzed *in vivo* to 3, 4-dichloroaniline (DCA) which is in turn oxidized to 3, 4-dichlorophenylhydroxylamine which is a potent inducer of methaemoglobin (McMillan *et al.*, 1991). These reactions are similar to those of dapsone which are well characterized: the severity of methaemoglobinaemia relates to the amount of dapsone's hydroxylamine metabolite, which varies with dose and cytochrome P450 activity (Bluhm *et al.*, 1999). We therefore expect that there will be a proportional relationship between the concentration of Propanil and its metabolites and clinical toxicity, although this has not yet been confirmed in humans.

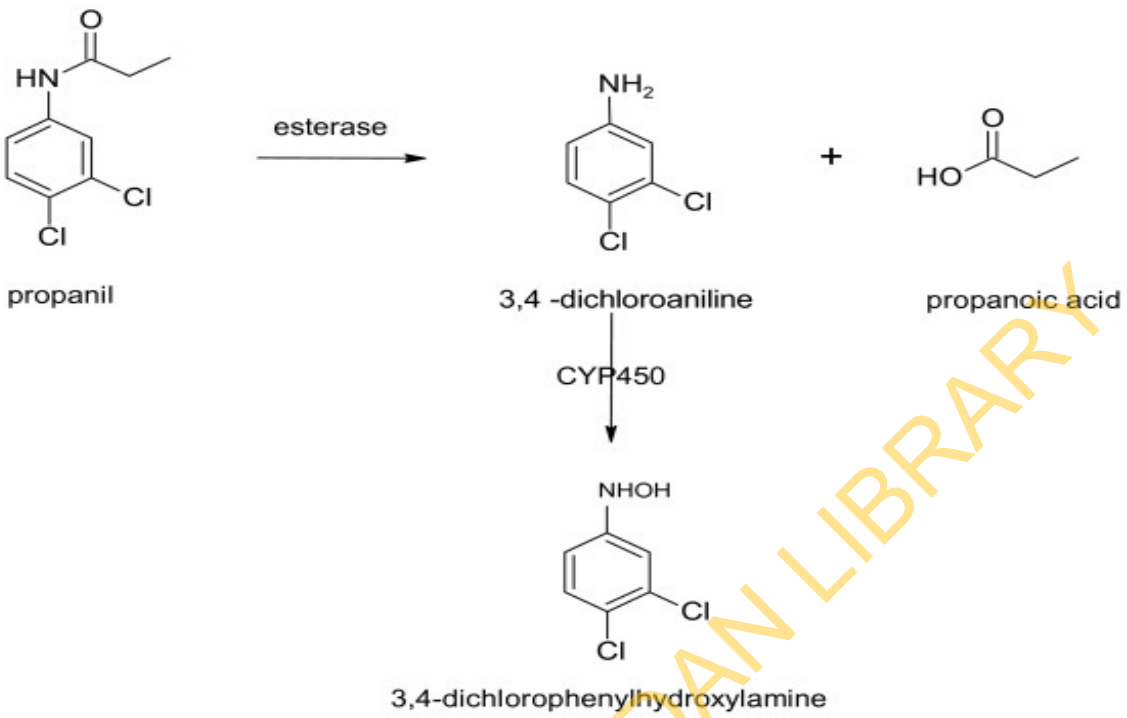


Figure 2: Metabolism of propanil *in vivo* **(**Mcmillan *et al.*, 1991).

Propanil (CAS No. 709-98-8) is also classified as a chloroaniline herbicide and known as DCPA, propanide and ***N***-(3, 4-dichlorophenyl) propanamide. It is stable in solution at pH 3 – 9 but may be subject to hydrolysis to 3, 4-dichloroaniline and propionic acid outside this range, although this has been debated. If hydrolysis occurs to a significant extent in the acid medium of the gastrointestinal tract there may be a diminished importance for esterases for metabolism of propanil. The specific esterases that hydrolyze propanil to DCA have not been identified but are known to be inhibited by paraoxon and sodium fluoride.

2.1.3 TOXICOLOGICAL EFFECTS OF PROPANIL

Propanil exposure has been associated with toxicity in humans and other mammals. The acute toxicity of propanil is expressed primarily by methemoglobin formation, which leads to cyanosis in occupationally exposed humans who work in pesticide manufacturing, formulation or packaging, since propanil can be absorbed into the system through inhalation, ingestion and by dermal exposure (Kimbrough, 1980). It has been reported that toxicity of propanil may be

mediated by its enzymatic conversion to 3, 4, dichloroaniline (Rose *et al.*, 2002). Apart from toxicities to target organs such as the liver, spleen and the testes, propanil is very irritating to the eye causing irritation, conjunctivitis, photophobia and even severe lesions if splashed in the eye (Lefevre, 1980). Santillo *et al* (1995) measured several parameters indicative of liver functions in rats and found that propanil treatment resulted in elevated serum levels of the enzymatic indicators of liver function, aspartate aminotransferase and alkaline phosphatase (Santillo et *al.*, 1995).

More recently, Rankin *et al.*, (2008) demonstrated that propanil has the potential to induce cytotoxicity and nephrotoxicity in vitro. Propanil is toxic by ingestion and slightly toxic by dermal absorption (Occupational Health Services, Inc. 1991). It is readily absorbed into the body through ingestion, inhalation or dermal exposure. It may cause central nervous system (CNS) depression. CNS effects include headache, dizziness, drowsiness, and confusion. Other symptoms include dark urine and blood (from the formation of methemoglobin), acetanilide in the urine, chills, cyanosis (also from methemoglobin), and jaundice. Death from respiratory failure may occur. Ingesting Propanil may result in a burning sensation and irritation of the mouth, throat and gut, accompanied by gagging, coughing, nausea or vomiting. Ingestion may also cause stupor, dizziness, fever, drowsiness and blue lips and fingernail. Inhalation of vapors can irritate the nose and throat and cause drowsiness, slurred speech, headache, nausea, dizziness, stupor and unconsciousness (WSSA Herbicide Handbook Committee, 1989). Propanil has also been reported to caused chloracne and hyperkeratinosis (thickening and roughening of the skin) in unprotected factory workers. Sensitization, irritation and dermatitis are possible (Gilden, 2010). Furthermore, propanil has been reported to affect the immune system at organ, cellular, and molecular levels. In an elegant review by Salazar and co-workers (2008) propanil was shown to cause thymic atrophy and splenomegaly, as well as decreasing the developing T-and B-cell populations in the thymus and bone marrow. The pesticide also suppressed the ability of macrophages to phagocytose, kill pathogenic bacteria, and produce inflammatory cytokines in NK cells. Propanil also affected the respiratory burst in macrophages, by inhibiting reactive oxygen and nitrogen species production. The review reported that some of the molecular mechanisms responsible for propanil's effect include alterations in NF-κB activity and intracellular Ca^{2}+ signaling. T-cell cytotoxic activity and cytokine production were also inhibited by propanil. In another study assessing the

immunotoxicity of propanil in occupationally exposed agricultural workers, the herbicide inhibited phytohemagglutinin-induced IFN-gamma and IL-10 production, while LPS-induced TNF-production was not affected indicating a direct effect of propanil on selected immune parameters (Corsini *et al.*, 2007).

2.2.0 LIVER STRUCTURE AND FUNCTION

The liver plays a key role in metabolism of nutrients and various xenobiotics, such as food additives, drugs or environmental pollutants such as pesticides. It is the largest gland in human body, harbouring important processes associated with regulation of carbohydrate, lipid, amino acid and hormone metabolism, the synthesis and degradation of plasma proteins, the storage of vitamins and metals, the secretion of bile and finally with xenobiotics metabolism. In all mammalian species, the liver is located in the anterior/upper right part of the abdominal cavity below the diaphragm. It is composed of the right lobe (largest in humans), the median lobe (quadrate in humans), left lobe, and caudate lobe. The livers of all laboratory mammals, primates, and humans, except the rat, have a gall bladder (Banes and Marek, 2005; Iatropoulos and Williams, 1996).

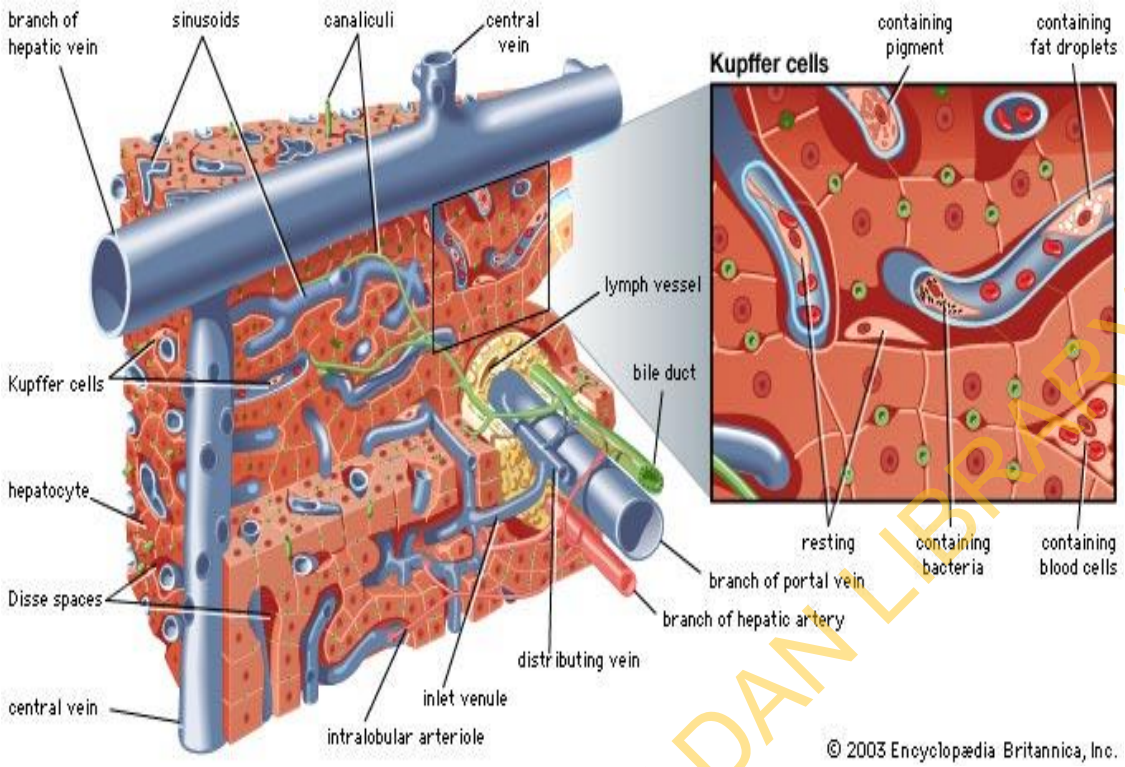


Figure 3: Structure of the liver showing views of hepatic lobules and cell types **(**Encyclopaedia Britannica, Inc.; www.britannica.com)

2.2.1 The Liver Cells

The principal cellular population found in liver is hepatocytes, the parenchymal diploid or polyploid cells. Based on their localization in lobule, hepatocytes can be divided among

periportal and perivenous cells, which differ in their metabolic activity, amount of specific intracellular organelles and a spectrum of other liver cell populations located in their close vicinity. The hepatocellular parenchyma accounts for 60% of the total cell population and 80% of the total volume of the organ, with the lobular parenchyma representing approximately 93%, the hepatic veins 4% and the portal triads 3% of the hepatic parenchyma. Non parenchyma cells comprise 30 to 35% of the total number of liver cells but only 6% of the total liver volume. Almost half of the non-parenchyma cells are fenestrated endothelial cells. These cells represent the major cell population found in sinusoids and they mediate communication between hepatocytes and inner space of sinusoids, as well as prevent pathogen infiltration into the liver parenchyma. The remainder consists of phagocytic Kuppfer cells, extra luminal fat-storing cells of Ito, biliary epithelial cells, pit cells (or natural killer cells). Kuppfer cells are liver macrophages activated by gut-derived bacterial endotoxins, which are characterized by high phagocytic, endocytic and secretory activities, important also for paracrine interactions between hepatocytes and hepatic stellate cells (HSCs). HSCs, also known as Ito cells, possess many functions, including vitamin A and lipid storage. Upon liver damage, these cells participate in initiation and progression of liver fibrosis and thus may contribute to the development of liver cancer. Lymphocytes are also distributed in liver tissue with yet another cell population, so-called pit cells, which have characteristic features of natural killer cells (NK) and thus exert cytotoxic activity against tumor cells (Kmiec, 2001; Desmet *et al.,* 1999; McCuskey and Sipes, 1997).

2.2.2 Liver Regeneration

The liver is a target of several toxic compounds and infectious agents, but it has an awesome capacity for self- regeneration. The cellular sources of liver regeneration often depend on the nature of insult. If there is loss or gain of liver mass, such as through liver injury or pregnancy, respectively, compensatory proliferation or apoptosis of cells allow restoration of original liver/body mass ratio once the stimulus is removed. Derangement of this homeostasis and loss of liver function due to parenchymal injury may lead to multi-organ failure and death (Michalopoulos, 2010). This proliferative response to loss of parenchymal function is known as liver regeneration or compensatory hyperplasia and hypertrophy, where

the cells in the remaining portion proliferate and/or increase in size to restore the original liver mass (Miyaoka *et al.*, 2012).

Several models of liver regeneration and growth exist. The most widely used model is the two-thirds partial hepatectomy (PH) model. In this model, the two largest lobes of the multi-lobular rodent liver, which approximates 70% parenchymal mass, is used. When these two lobes are excised via a straightforward surgical procedure (Mitchell and Willenbring, 2008), the cells of the remaining lobes restore liver mass over the course of 1-2 weeks. With this model, phenomena associated with minor (~30% PH) to severe (~90% PH) parenchymal loss could be studied simply by removing one less or one more lobe of the liver. In addition, chemical injury models, which cause hepatocyte injury and death, by activating an inflammatory response in addition to a regenerative response, also exist. Carbon tetrachloride and acetaminophen are commonly used to induce hepatocyte injury and death selectively in the pericentral zone (Jaeschke *et al.*, 2011; DeCicco *et al.*, 1998). They require metabolic activation by cytochrome P450 (CYP) enzymes, a process which often generates toxic free radicals. The CYP-expressing hepatocytes die first, creating a centrilobular distribution of injury and death. The major advantage in using a hepatotoxin model to study regenerative response after parenchymal injury is that it more closely approximates the regenerative response that occurs in common human hepatic diseases, including the damage and inflammatory infiltrate.

2.2.3 Xenobiotics and liver metabolism

Xenobiotic metabolism involving the excretion of chemicals from the body is one of the major roles of the liver. Ironically, these detoxification processes may be also accompanied with increased toxicity of reactive metabolites. Drugs, food additives and environmental pollutants are examples of xenobiotics which could be converted by the liver to their toxic metabolites.

On exposure to xenobiotics, the liver of vertebrates manages to eliminate such foreign compounds as early as possible. This is accomplished by making use of the normally existing biochemical mechanisms in the tissue. Certain enzymes and other endogenous biomolecules which are actually meant for the metabolism of endogenous substrates may be utilized for this purpose. Biotransformation begins with the transient formation of a reactive intermediate, whose lifetime is long enough to allow an attack on cellular components. This occurs when a

reactive intermediate (generally radicals or electrophiles such as a carbonium ion) is formed and reacts rapidly with cellular macromolecules (such as unsaturated lipids, proteins, nucleic acids), thus leading to their degradation and finally to cellular necrosis. The first step of the metabolic process yields a primary metabolite, which can, in some cases, accumulate in the cell and react with cellular components before being transformed. The final metabolites, when in excess, may accumulate and react with cellular macromolecules (Macherey and Dansette, 2008).

Xenobiotics enter the cells by either passive or protein-assisted membrane transport and they are metabolized by two principal groups of enzymes. The phase I enzymes which include monooxygenases, such as CYP enzymes, catalyze hydroxylation, deamination, dehalogenation, epoxidation or peroxidation reactions. The phase II (also called the conjugation phase) enzymes, catalyze conjugation metabolites obtained from phase 1 reactions with the donors like uridine diphosphate (UDP)-glucuronate (glucuronosylation), adenosine-3´-phosphate-5´-phosphosulfate (PAPS) (sulfatation) or glutathione, there-by creating water-soluble complexes, which are more efficiently excreted from the body. Activities of detoxification enzymes depend on many factors, such as age, gender, genetic factors/polymorphisms, or by previous exposure to various chemicals thus resulting in drug or toxic response individuality.

However, phase III biotransformation is a more newly coined descriptor that refers to active membrane transporters that function to shuttle drugs and other xenobiotics across cellular membranes (Omiecinski *et al.*, 2011; Nebert and Dalton, 2006).

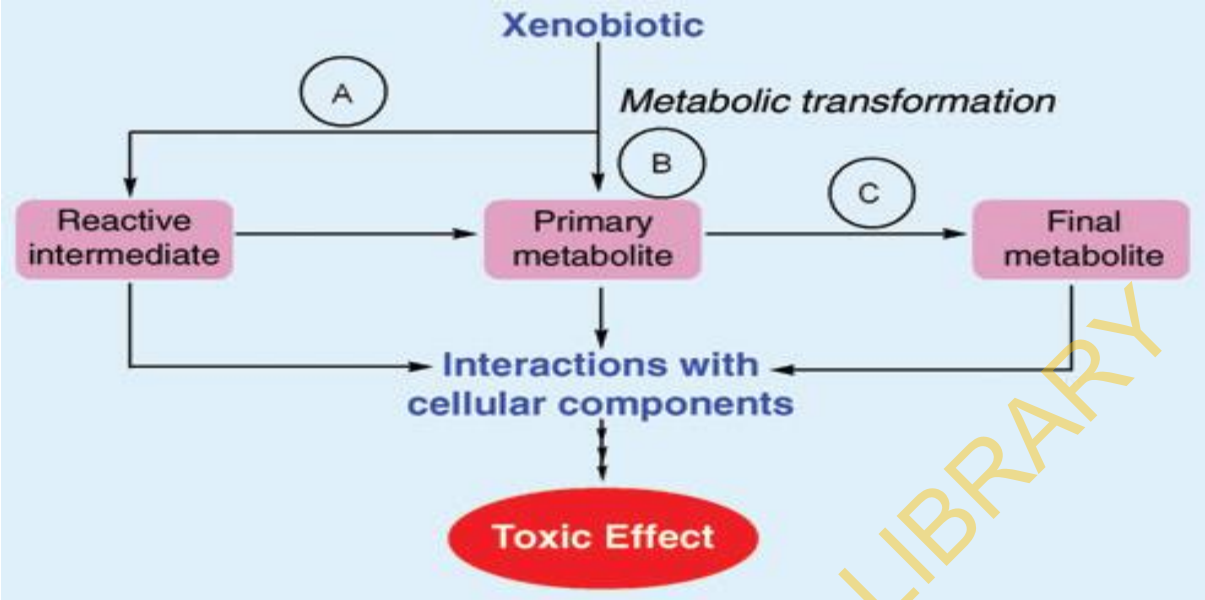


Figure 4: Xenobiotic transformation (Macherey and Dansette, 2008).

2.2.4 Metabolic Bioactivation by Cytochrome P450 Enzymes

The most frequent mechanism of hepatocellular injury involves production of injurious metabolites by the cytochrome P450 system. This family of enzymes is located in the smooth endoplasmic reticulum of hepatocytes primarily, although they are also found in many other cells of the body. A major role of cytochrome P450 enzymes is to metabolize lipid soluble chemicals into water-soluble compounds for excretion from the body in bile or urine. In the first step of this 2-step biotransformation process, termed phase 1, chemicals are bioactivated to a high energy reactive intermediate molecule, in preparation for the second step, phase 2, which involves formation of covalent bonds with polar molecules such as glucuronic acid. Conjugation forms a water-soluble metabolite that can be excreted. However, in some circumstances, such as over dosage, the high-energy reactive metabolites can form adducts that are covalent bonds with other cellular constituents such as proteins and nucleic acids. In acute toxicity, adducts can form with essential cellular enzymes leading to cell injury or death. The site of toxic cellular injury within the hepatic acinus reflects the site of bioactivation of the chemical. For instance, carbon tetrachloride is metabolized by the cytochrome P450 system to $CCl_3^{\bullet}$, a free radical that induces cell membrane injury. Lesions induced by carbon tetrachloride are most severe in the periacinar (centrilobular) areas, because this is the area where the smooth endoplasmic reticulum is most abundant, and, therefore, where the active

form of the chemical is present in greatest concentration. Consequently, the centrilobular region of the hepatic lobule is by far the most common site of acute toxic injury. Acetaminophen toxicity is another and more commonly encountered example of this mechanism of liver injury (Zhang *et al.*, 2002).

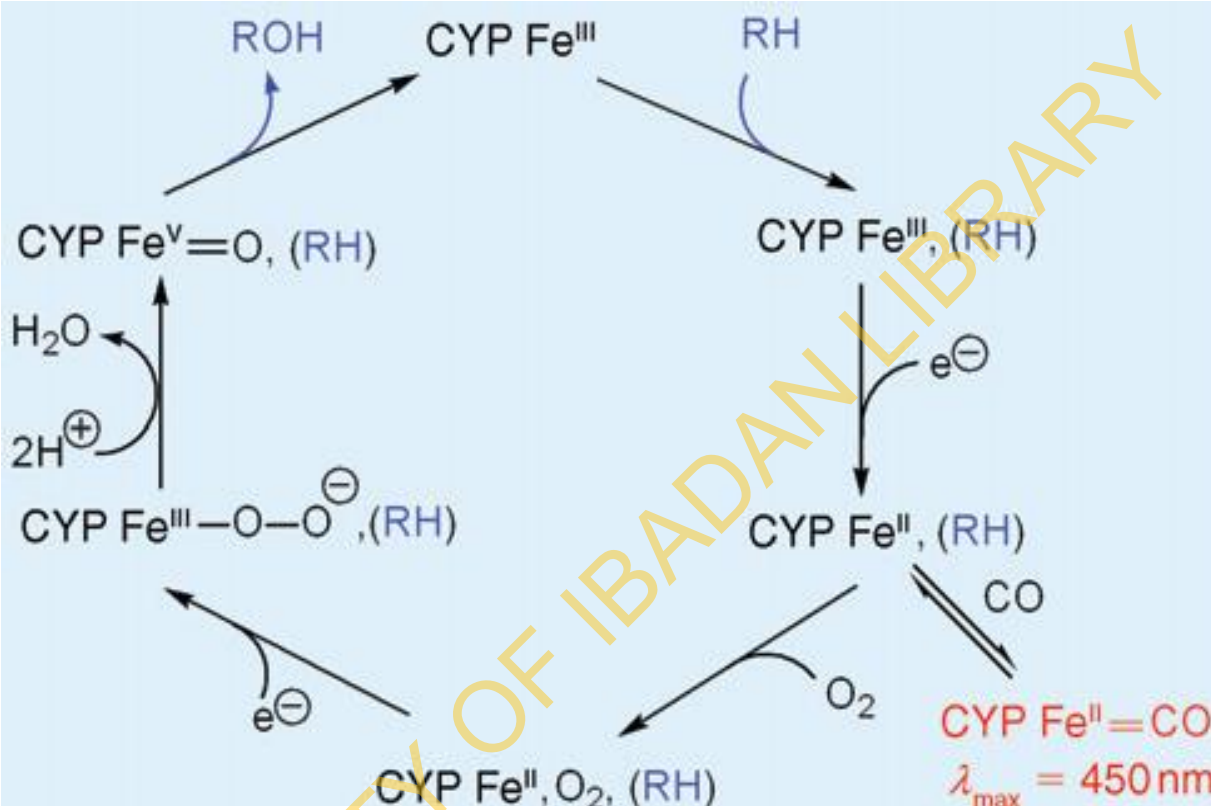


Figure 5: Catalytic cycle of cytochrome P450 (CYP) monooxygenase (Macherey and Dansette, 2008).

2.3.0 Experimental Models of Hepatotoxicity

Some drugs and chemicals can be employed as inducers of hepatotoxicity in the testing of various hepatoprotective agents. Although individual analgesics rarely induce liver damage due to their widespread use, Non-Steroidal Anti-inflammatory Drug (NSAIDS) have emerged as a major group of drugs exhibiting hepatotoxicity. Both dose-dependent and idiosyncratic reactions have been documented (Manov *et al.*, 2006). Aspirin and phenylbutazone are associated with intrinsic hepatotoxicity; idiosyncratic reaction has been associated with

ibuprofen, sulindac, phenylbutazone, piroxicam, diclofenac and indomethacin. Glucocorticoids, so named due to their effect on the carbohydrate mechanism, promote glycogen storage in the liver. An enlarged liver is a rare side effect of long-term steroid use in children. The classical effect of prolonged use both in adult and paediatric population is steatosis. Isoniazid is one of the most commonly used drugs for tuberculosis; it is associated with mild elevation of liver enzymes in up to 20% of patients and severe hepatotoxicity in 1-2% of patients (Sarich *et al.*, 1999). Fungi or moulds such as *Aspergillus flavus* and *Aspergillus parasiticus* produce toxins that have a number of toxic effects, particularly hepatotoxicity (Adedara *et al.*, 2010a). Other toxicants with negative effect on the liver include arsenic, carbon tetrachloride, and vinyl chloride.

2.3.1 Carbon Tetrachloride-Induced Hepatotoxicity

Carbon tetrachloride, once used extensively in dry cleaning and even as an anesthetic, continues to be relevant as a model substance to elucidate the mechanisms of action of hepatotoxic effects such as fatty degeneration, fibrosis, hepatocellular death, and carcinogenicity. CCl_4 is activated by cytochrome (CYP) 2E1, CYP2B1 or CYP2B2, and possibly CYP3A, to form the trichloromethyl radical, $CCl_3^{\cdot}$. This radical can bind to cellular molecules (nucleic acid, protein, lipid), impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration (steatosis). Adduct formation between $CCl_3^{\cdot}$ and DNA is thought to function as initiator of hepatic cancer. This radical can also react with oxygen to form the trichloromethylperoxy radical $CCl_3OO^{\cdot}$, a highly reactive species. $CCl_3OO^{\cdot}$ initiates the chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, in particular those associated with phospholipids. This affects the permeabilities of mitochondrial, endoplasmic reticulum, and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis, which can contribute heavily to subsequent cell damage (Weber *et al.*, 2003). It has also been postulated that liver cell injury induced by carbon tetrachloride involves initially the metabolism of carbon tetrachloride to trichloromethyl free-radical by the mixed function oxidase system of the endoplasmic reticulum (Brattin *et al.*, 1985).

2.3.2 Paracetamol-Induced Hepatotoxicity

Paracetamol is a safe and effective analgesic/antipyretic drug when used at therapeutic levels (Rumack, 2004). Paracetamol induced liver injury is a classic case of Drug-induced liver injury (DILI) and accounts for nearly half of acute liver failure in the United States (Larson *et al.*, 2005). It can be modeled in more than one strain of rodents (Mehendale, 2005) and has a clear dose-dependency in both animals and humans. Paracetamol hepatotoxicity has been related with several cases of cirrhosis, hepatitis and suicide attempts. Notably, oxidative stress plays a central role in the hepatic damage caused by paracetamol and antioxidants have been tested as alternative treatment against paracetamol toxicity (Avila *et al.*, 2011). The analgesic, also known as acetaminophen, causes a potentially fatal, hepatic centrilobular necrosis when taken in overdose. Acetaminophen was metabolically activated by cytochrome P450 enzymes to a reactive metabolite that depleted glutathione (GSH) and covalently bound to protein. It was shown that repletion of GSH prevented the toxicity. This finding led to the development of the currently used antidote *N*-acetylcysteine. The reactive metabolite was subsequently identified to be *N*-acetyl-*p*-benzoquinone imine (NAPQI). Although covalent binding has been shown to be an excellent correlate of toxicity, a number of other events have been shown to occur and are likely important in the initiation and repair of toxicity (James *et al*., 2003).

2.3.3 Aflatoxin B1 induced liver injury

AFB1 is a mycotoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* which are common and widespread in nature. The mycotoxin is found in foodstuffs, including corn, rice, oil seeds, dried fruits and peanuts that have been improperly stored in hot, humid and unsanitary conditions (Baydar *et al.*, 2005). It is also found in the milk, meat and eggs of farm animals that feed on aflatoxin-contaminated foods (Bennett and Klich, 2003; Fink-Gremmels, 1999). Approximately 4.5 billion people are at risk of chronic exposure to aflatoxin-contaminated food. According to the United States Food and Drug Administration, AFB1 is considered to be an unavoidable contaminant of food, but nevertheless can be minimized (Williams *et al*., 2004). There are four aflatoxins (aflatoxin B1, B2, G1 and G2) that are known to be carcinogenic to both humans and animals, of which aflatoxin B1 is the most potent hepatotoxic and hepatocarcinogenic agent. Chronic exposure also leads to a high risk of developing liver cancer, as aflatoxin metabolite can intercalate into DNA and alkylate the bases through its epoxide moiety. Human epidemiology and experimental animal studies have

provided a statistical association between aflatoxins and the threat of liver cancer (Abdel-Wahhab *et al.*, 2006). AFB1 is metabolized by the mixed-function oxidase system to a number of hydroxylated metabolites and to AFB1 8, 9- epoxide, which binds to DNA, forming covalent adducts. It is also known to produce membrane damage through increased lipid peroxidation (Galvano *et al.*, 2001). Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Such exposure is difficult to avoid because fungal growth in foods is not easy to prevent. Even though heavily contaminated food supplies are not permitted in the market place in developed countries, concern still remains for the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the food supply.

2.3.4 Thioacetamide model of hepatoxicity

Thioacetamide (C_2H_5NS; TAA), an organosulfur compound is formally used in leather processing, laboratories, textile and paper industries. TAA is a model hepatotoxicant, consumed to induce acute and chronic liver injury due to its effects on protein synthesis, RNA, DNA and gamma-glutamyl transpeptidase activity. TAA undergoes a two-step bioactivation to sulfine, and afterward to sulfene, a reactive metabolite. Sulfine is accountable for the enlargement of nucleoli, increase in nuclear volume and intracellular concentration of Ca^{++}, change in cell permeability, and inhibition of mitochondrial activity. At the same time sulfene is responsible for the release of nitric oxide synthase and NF-κB directing to centrilobular necrosis, protein denaturation and lipid peroxidation. Furthermore, it impairs the urea cycle and the activity of ornithine aminotransferase. Prolonged oral intake of this compound directs to macro liver nodules, liver cell adenomas, cholangiomas and hepatocarcinomas, histologically similar to that caused due to viral hepatitis infection (Akhtar and Sheikh, 2013). The acute liver injury induced by a necrogenic dose of thioacetamide is characterized by a severe perivenous necrosis (Cascales *et al.*, 1992). The necrosis develops as a consequence of the biotransformation of TAA through the microsomal flavin-dependent monooxygenase. The reactive metabolites responsible for TAA hepatotoxicity are the radicals derived from thioacetamide-*S*oxide and the reactive oxygen species derived as subproducts in the process of microsomal TAA oxidation; both of wich can deplete reduced glutathione leading to oxidative stress (Bautista *et al.*, 2011). Furthermore, in rats, single doses cause centrilobular necrosis

accompanied by increases in plasma transaminases and bilirubin. To elicit these effects, TAA requires oxidative bioactivation, leading first to its S-oxide (TASO) and then to its chemically reactive S, S-dioxide (TASO (2)), which ultimately modifies amine-lipids and proteins. In a confirmatory study, TAA was non-toxic to isolated hepatocytes up to 50 mM for 40 hours; however, TASO was highly toxic to isolated hepatocytes as indicated by LDH release, cellular morphology, and vital staining with Hoechst 33342/propidium iodide (Hajovsky *et al.*, 2012).

2.3.5 D-galactosamine Induced Hepatotoxicity

D-galactosamine (D-GalN) is a specific hepatotoxic agent metabolized exclusively in hepatocytes, which reduces the intracellular pool of uracil nucleotides, thus inhibiting the synthesis of RNA and proteins. When administered in combination with a low dose of lipopolysaccharide (LPS), D-GalN highly sensitizes animals to develop lethal liver injury, showing biochemical and metabolic changes akin to fulminant hepatic failure. D-GalN and the LPS-induced liver failure model takes advantage of the ability of a transcriptional inhibitor D-GalN to potentiate the toxic effects of LPS, producing typical hepatic necrosis and apoptosis followed by fulminant hepatitis. In addition, D-GalN is known for inducing the features of acute hepatitis in rats. The toxic effect of D-GalN is connected with an insufficiency of UDP-glucose and UDP-galactose and the loss of intracellular calcium homeostasis. These changes affect cell membranes and organelles and the synthesis of proteins and nucleic acids. After D-GalN application, the location of proteoglycans is changed in the rat liver. D-GalN also inhibits the energy metabolism of hepatocytes (Ferenčõkov *et al.*, 2003). Recent studies also demonstrated that oxidative stress and the inflammatory response played a pivotal role in D-GalN-induced acute liver injury (Pushpavalli *et al.*, 2010).

2.3.6 Chloroform model of hepatotoxicity

Chloroform ($CHCl_3$, CAS No. 67-66-3), also called trichloromethane or methylchloride, is a volatile organic compound that is used as a solvent and cleanser for plastic compounds as well as an acrylic adhesive. Chloroform is a ubiquitous atmospheric and water contaminant. Beside its extensive use as a solvent in industrial processes, it is formed as a by-product during the chlorination of water intended for human use and paper bleaching. Due to its volatility, chloroform can be easily released from waste or chlorinated waters into the

atmosphere or in the ambient air. Therefore, a large part of the human population may be chronically exposed to chloroform from different sources, although drinking water has been considered the main one. Recently, routes of exposure other than oral consumption of chlorinated water have been evaluated as relevant. Indeed, some indoor activities, such as showering or bathing as well as cooking and housekeeping, may significantly contribute to total chloroform body burden through dermal and inhalation exposure (Kang *et al.*, 2014; Backer e*t al.,* 2000). The hepatotoxicity of $CHCl_3$, was reported to be due to phosgene-mediated cellular GSH depletion or increased amounts of covalent binding to hepatocellular macromolecules alkanes can be enhanced by pretreating the animals with inducers of the cytochrome P-450 system like phenobarbital or various alcohols and ketones (Purushotham *et al*., 1988).

2.3.7 Ethanol- induced hepatic injury

Excessive alcohol consumption is associated with fatty liver, and if persistent, it can lead to alcoholic steatohepatitis, liver fibrosis and cirrhosis. Alcohol-induced liver injury progresses from fatty infiltration and follows a pernicious course of inflammation leading to irreversible damage. Significant changes occur in host defense mechanisms after consumption of alcohol, including modified reticuloendothelial function as well as altered immune, lymphocyte, granulocyte and platelet functions. It is also well known that chronic ethanol ingestion produces fatty liver, hepatomegaly, alcoholic hepatitis, fibrosis, and cirrhosis (O'Shea *et al.,* 2010; Thurman *et al.,* 1999). In 6-8 weeks old male albino rats, given ethanol at a dose of 0.5 gm/100 gm/day for 8 weeks, a statistically significant increase in the mean enzyme levels, liver weight and volume were observed in the ethanol treated group compared to the controls. Furthermore, morphological studies indicated that hepatocytes contained large number of cytoplasmic vacuoles, pyknotic nuclei, and lymphocytic infiltration in treated animals (Habib-ur-Rehman *et al.,* 2011). In a mice model of toxicity, acute EtOH administration was reported to cause prominent hepatic microvesicular steatosis with mild necrosis , an elevation of serum ALT activity, lipid peroxidation levels, and increased hepatic TNF production (Song *et al*., 2006).

2.4.0 MECHANISMS OF HEPATOTOXICITY

Toxic injury occurs in the liver more often than any other organ. This can be attributed to the fact that virtually all ingested substances that are absorbed are first presented to the liver and that the liver is responsible for the metabolism and elimination of many substances. According to Lee (2003) liver injury can be categorized by a number of systems. Some systems are based on the histological lesion produced (i.e., inflammation, necrosis, cholestasis), others the type of injury (i.e., cytotoxic, cholestatic, mixed), but the advent of a newer understanding that gives a view of injury at the molecular level has given rise to a more focused scheme for understanding and categorizing liver injury.

2.4.1 Canalicular and Cholestatic Injury

Cholestasis can be produced by chemicals that damage the structure and function of the bile canaliculi. A key component of bile secretion involves a series of ATP-dependant export pumps such as the canalicular bile salt transporter, that moves bile salts and other transporters that export other bile constituents from the hepatocyte cytoplasm to the lumen of the canaliculus. Some drugs bind these canalicular transporter molecules and lead to the arrest of bile formation or movement within the lumen of the canalicular system (Trauner *et al.*, 1998). In rare circumstances affected individuals (~1%) develop progressive destruction of cholangiocytes leading to "vanishing bile duct syndrome" (Lewis, 2000). Additional investigation is needed to elucidate the mechanism of destruction of cells of the biliary tree. Secondary injury can result as bile salts have a detergent action that can damage cell membranes and injure biliary epithelium or hepatocytes in areas of cholestasis. Another mechanism leading to cholestasis involves disruption of actin filaments situated around the bile canaliculi preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments such as phalloidin and those that affect calcium homeostasis and cellular energy production can generate this type of injury.

2.4.2 Mitochondrial Injury

Chemicals that damage mitochondrial structure, enzymes or DNA synthesis can disrupt β-oxidation of lipids and oxidative energy production within the hepatocytes (Bissel *et al.*, 2001). Prolonged interruption of β-oxidation leads to micro vesicular steatosis within

hepatocytes. Mild insult leads to macro vesicular steatosis. In severe cases micro vesicular steatosis, hepatic failure and death can result. Some drugs may inhibit β-oxidation (aspirin, valproic acid, and tetracycline) and others may disrupt oxidative phosphorylation alone or in addition (bile acids, amiodarone) depleting the hepatocytes of energy. Certain antiviral dideoxynucleoside analogues can disrupt mitochondrial DNA synthesis through inhibition of DNA polymerase gamma, leading to depletion of mitochondrial DNA and mitochondria leading to hepatocyte death.

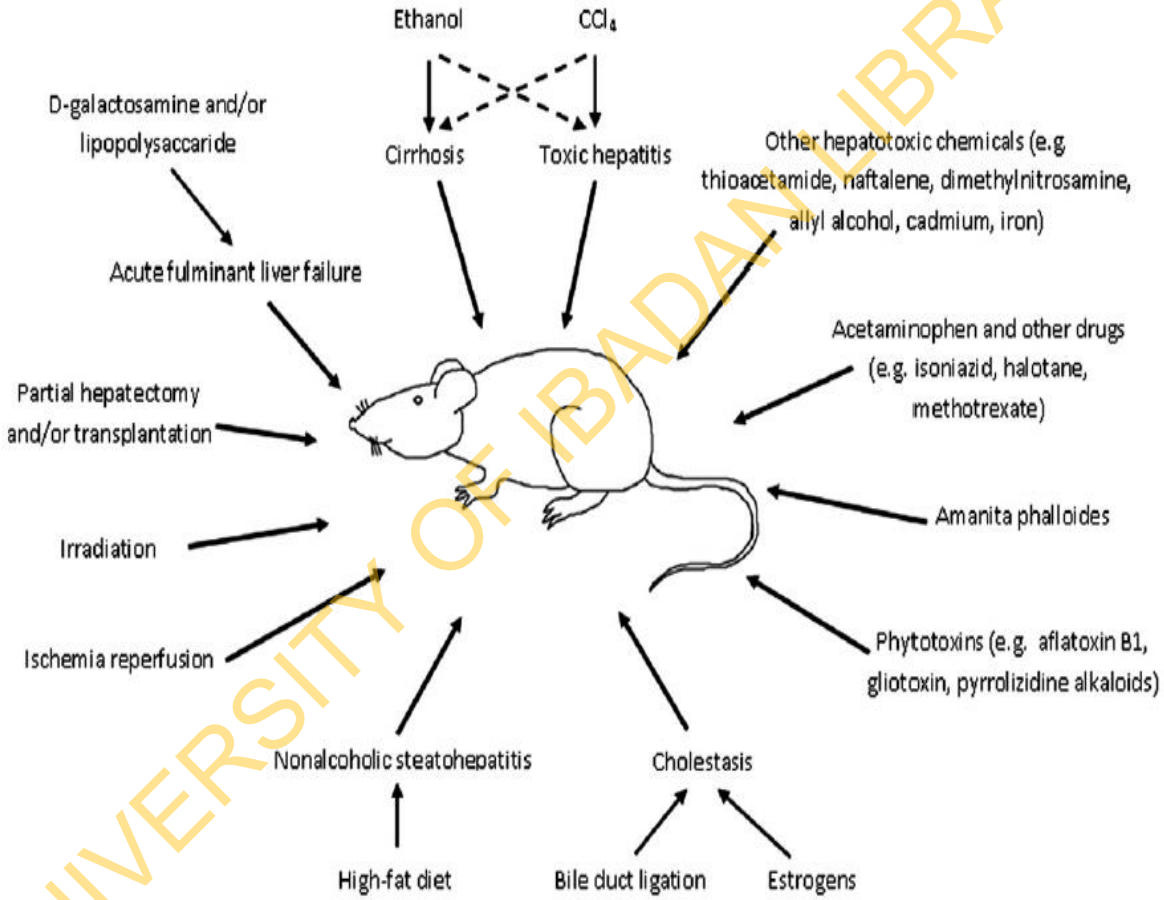


Figure 6: Mechanisms of hepatotoxicity (Farghali *et al.*, 2015)

2.5.0 APOPTOSIS

The term apoptosis was coined by Kerr and co-workers in 1972 to describe a morphologically distinct form of cell death (Kerr *et al.*, 1972). Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in

tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or toxic agents (Norbury and Hickson, 2001). Apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that link the initiating stimuli to the final demise of the cell.

2.5.1 Morphological Features of Apoptosis

During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy. With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Pyknosis, the most characteristic feature of apoptosis, is as a result of chromatin condensation (Kerr *et al.,* 1972). Apoptosis is characterised by a series of typical morphological events, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells. Internucleosomal fragmentation of genomic DNA has been the biochemical hallmark of apoptosis for many years (Kerr *et al.,* 1972; Elymore, 2007). Extensive plasma membrane blebbing also occurs and cell fragments are separated into apoptotic bodies, without compromising the intergrity of the organelle. The apoptotic bodies are phagocytosed and degraded by macrophages, parenchymal cells, or neoplastic cells and within the phagolysosomes. Inflammatory reactions are not associated with the process of apoptosis because apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; and the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka *et al.,* 2003). Apoptosis has been characterized by several biochemical criteria, including different kinetics of phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane, changes in mitochondrial membrane permeability, release of intermembrane space mitochondrial proteins, and caspase-dependent activation and nuclear translocation of a caspase-activated DNase resulting in internucleosomal DNA cleavage. Identification of these morphological and biochemical markers of apoptosis makes it possible to distinguish it from other forms of cell death (Krysko *et al.,* 2008). Apoptotic shrinkage, disassembly into apoptotic bodies and engulfment of individual cells characteristically occur without associated inflammation, which would be the consequence of releasing intracellular contents into tissues. Recent studies indicate that proteolytic cleavages of a set of key proteins by activated caspase proteases play a

role in the accomplishment of apoptotic morphology (Martin and Green, 1995). Although the exact mechanism of how the degradation of these proteins results in apoptotic morphology remains unknown, many target proteins of caspases participate in the formation and regulation of the membrane-associated cortical microfilament cytoskeleton, which is an important determinant of the cell shape.

2.5.2 Stimulation of Apoptosis

In the course of apoptotic death intact cell organelles and cell membranes are fragmented into small membrane-bound bodies. Cellular DNA is cleaved by endonucleases to 120–180 base pair fragments. Classically apoptosis can be triggered through two basic mechanisms in the hepatocyte; interactions between death ligands (Fas-ligand, TNF) and death receptors (Fas and TNFR-1) that trigger caspase 8 activation or damage to mitochondrial inner membranes releasing cytochrome ***c*** that binds to Apaf-1 activating it, leading to downstream activation of caspase 9. These pathways are not completely separate as a protein named Bid that is activated by caspase 8 can cause mitochondria to release cytochrome ***c***. Certain chemicals may be able to trigger apoptosis by direct stimulation of pro-apoptotic pathways in the hepatocytes. Alternatively, apoptosis can be stimulated by several other pathways including immune-mediated events such as those discussed above that lead to the release of tumor necrosis factor alpha or activation of Fas pathways. Chemicals that damage mitochondria can also stimulate apoptosis through the release of cytochrome ***c***. Cholestasis can also stimulate apoptosis through the action of pro-apoptotic bile acids such as glycodeoxycholic acid (Bissel *et al.*, 2001; Jaeschke *et al.*, 2002). Additional pathways may involve triggering of apoptosis by protein kinase C activation and mitochondrial injury. In addition, bioactivation by the cytochrome P450 system can produce reactive molecules that engender oxidative stress which can then be a stimulus to induce synthesis of Fas ligand and increase the susceptibility of hepatocytes to apoptosis.

2.5.3 Mechanisms of Apoptosis

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-

dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage (Martinvalet *et al*., 2005).

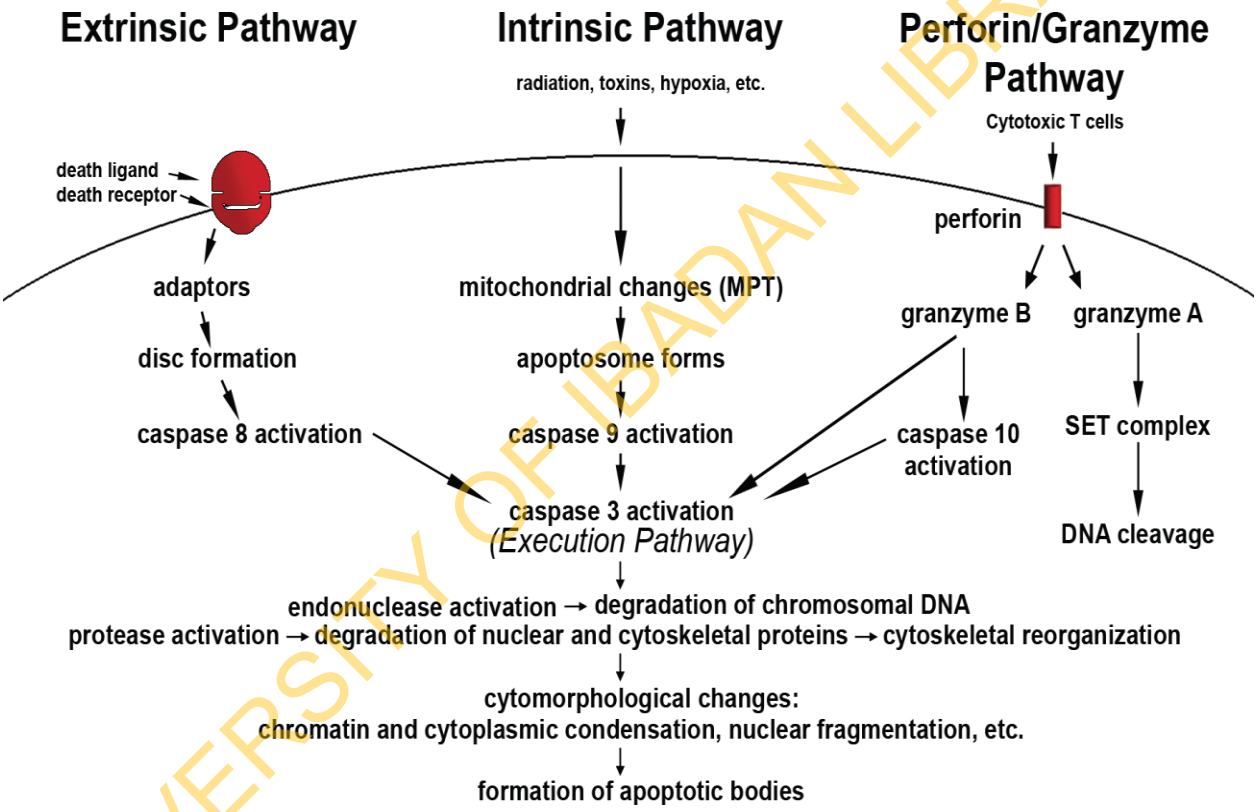


Figure 7: Schematic representation of major events in apoptosis (Elmore, 2007)

2.5.4 Caspase activation

Caspases are widely expressed as zymogens in most cells and once activated can often activate other pro-caspases, leading to the initiation of a protease cascade. This proteolytic cascade, in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and culminating in rapid cell death. Caspases have proteolytic activity and are able to

cleave proteins at aspartic acid residues. Different caspases could also have different specificities involving the recognition of neighboring amino acids. Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. About ten major caspases have been identified and they are broadly categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Rai *et al.*, 2005).

2.5.5 The Intrinsic or Mitochondrial pathway of apoptosis

The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis (Elymore, 2007). Other stimuli that initiate this pathway positively include: radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. These stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the inter membrane space into the cytosol eg cytochrome c, Smac/DIABLO. It is these proteins that activate the caspase-dependent mitochondrial pathway. For instance, Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an "apoptosome". The clustering of procaspase-9 in this manner leads to caspase-9 activation. The second group of pro-apoptotic proteins, AIF, endonuclease G and CAD, are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell has committed to die. AIF translocates to the nucleus and causes DNA fragmentation into ~50–300 kb pieces and condensation of peripheral nuclear chromatin (Elymore, 2007; Saelens *et al.*, 2004, Joza *et al.*, 2001).

2.5.6 Intracellular regulators of apoptosis

Apoptotic pathways are regulated by numerous intracellular factors. The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family

of proteins (Cory and Adams, 2002; Antonsson and Martinou, 2000). The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be divided into anti-apoptotic members, such as Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and pro-apoptotic members, such as Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. It is generally believed that the ratio of pro-apototic to anti-apoptotic Bcl-2 family members is critical in determining whether the cell will undergo apoptosis. The main mechanism of action of the Bcl-2 family of proteins appears to be the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability. Furthermore mitochondrial damage in the Fas pathway of apoptosis is mediated by the caspase-8 cleavage of Bid. Serine phosphorylation of Bad is associated with 14-3-3, a member of a family of multi-functional phosphoserine binding molecules. When Bad is phosphorylated, it is trapped by 14-3-3 and sequestered in the cytosol but once Bad is unphosphorylated, it will translocate to the mitochondria to release cytochrome C. The tumor suppressor protein p53 has a critical role in regulation of the Bcl-2 family of proteins; however the exact mechanisms have not yet been completely elucidated (Elymore, 2007; Schuler and Green, 2001; Antonsson and Martinou, 2000).

2.6.0 INFLAMMATION AND LIVER DISEASES

2.6.1 Role of Nitric Oxide and inducible nitric oxide (iNOS)

Nitric oxide (NO) is a highly reactive, diffusible gas that is produced by many tissues, and it exerts a range of physiological and pathophysiological effects. Despite its diverse and complicated roles, certain patterns of the effect of NO on the pathogenesis and progression of liver diseases are observed (Iwakiri and Kim, 2015).

NO is synthesized by nitric oxide synthase (NOS) through a series of redox reactions involving L-arginine (the main substrate), oxygen and nicotinamide adenine dinucleotide phosphate. There are 4 major isoforms of NOS: endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and mitochondrial nitric oxide synthase. Following synthesis by NOS, the half life of endogenously generated NO is extremely short, about 1 s. Thus, endogenous NO production is intimately regulated by the activity of NOS. The generated NO molecule has a large diffusion coefficient and can therefore freely penetrate cellular membranes in an autocrine or paracrine manner. NO regulates a wide range of cellular activities by binding to metal ions, proteins, lipids, and

guanine nucleotides. Within the cell, NO stimulates the conversion of guanosine 5' triphosphate (GTP) to cyclic guanosine 3' 5'monophosphate (cGMP), thereby regulating calcium balance through the cGMP dependent protein kinase pathway. The end products of NO metabolism *in vivo* are nitrate (NO_3) and nitrite (NO_2) and these serve as indirect measure of the total NO concentration. In liver biology, eNOS and iNOS are major players, whereas the role of nNOS is little known (Iwakiri and Kim, 2015; Wang et al., 2013).

Overproduction of NO in the liver has been implicated as an important event in animal models of inflammation and injury in the liver. Some of the cellular activities of NO which may lead to hepatotoxicity include down-regulation of cytochrome P450, depletion of protein and DNA syntheses, and the induction of apoptosis and necrosis. Nitric oxide also inhibits catalase activity, suggesting that it may alter the detoxification of cytotoxic oxygen radicals. Furthermore, NO combines with the superoxide radical, forming the potent oxidizing agent, peroxynitrite, which can react directly with sulfhydryl residues in cell membranes leading to lipid peroxidation and genotoxicity (Gardner et al, 1998). In liver biology, eNOS and iNOS are major players, whereas the role of nNOS is little known (Iwakiri and Kim, 2015). It is believed that the cytokines TNF-α and IL-1β synergistically activate iNOS expression in the liver. Additionally, a cross-talk exists between iNOS expression and the transcription factor NF-κB (Ma et al., 2008).

2.6.2 Role of Myeloperoxidase

Neutrophils are professional phagocytes, whose main function is to sense and destroy pathogenic organisms. In addition, they are the most prominent leukocytes in acute inflammatory reactions and contribute to host tissue injury in a number of inflammatory conditions, including ischemia-reperfusion injury, sepsis, and vasculitis. Importantly, being the key cellular component of the acute inflammatory response, neutrophils are thought to contribute to the initiation and shaping of the immune response (Serhan and Savill, 2005; Nathan, 2006).

One of the principal molecules released after recruitment and activation of phagocytes is myeloperoxidase (MPO), an important enzyme involved in the generation of reactive oxygen species. MPO is highly expressed by neutrophils and as such widely used as a neutrophil marker. In the presence of physiological chloride concentrations, MPO reacts with hydrogen

peroxide to catalyze formation of hypochlorous acid/hypochlorite ($HOCl/OCl^-$) and other oxidizing species. These oxidants may contribute to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates, including DNA, lipids, and protein amino groups. In the absence of physiological chloride concentrations, the MPO-H_2O_2 system can also form reactive nitrogen species that may initiate lipid peroxidation or form protein tyrosine residues, another posttranslational modification found in many pathological conditions (Klebanoff, 2005; Davies *et al*., 2008; Hazen *et al*., 1999).

Myeloperoxidase may contribute to tissue injury by several mechanisms. HOCl is a potent oxidant that attacks nucleophilic amino groups; generating reactive aldehydes and chloramines. MPO also reacts with unsaturated lipids to form chlorohydrins. Lipid peroxidation and protein cross-linking can be catalyzed by tyrosyl radical, which results from the oxidation of tyrosine by myeloperoxidase. In addition, HOCl has been shown to inactivate the protease inhibitor α-1-antitrypsin and to activate latent neutrophil collagenase (Brown *et al.*, 2001).

2.6.3 Role of Nuclear Factor-kappa B (NF-κB)

Oxidative stress and inflammation are highly prevalent in major liver diseases, such as viral hepatitis, alcoholic and non-alcoholic steatohepatitis, liver cirrhosis, and liver cancer. Being a master regulator of inflammation and cytoprotection, NF-κB signaling is tightly regulated by the inhibitor of nuclear factor-kappaB (IkappaB) kinase 1 (IKK1) and IKK2. In unstimulated cells, NF-kB dimers p65 and p50 are sequestered in cytosol by inhibitor of kBα (IkBα). The IKK complex has two catalytic subunits, IKK2 and IKK1, which activate NF-κB via the classical and alternative pathways, respectively.

In the classical pathway, upon activation by stimuli such as tumor necrosis factor α (TNFα), IKK2 is phosphorylated, which in turn phosphorylates IkBα. This leads to degradation of IkBα, nuclear translocation of p65/p50, and induction of NF-κB–dependent genes, including inflammatory genes TNF-α and interleukin-6 (IL-6). The alternative NF-κB pathway is activated by distinct ligands via phosphorylation of IKK1, leading to processing of p100 and release of p52 and RelB into nucleus, resulting in induction of unique subsets of NF-kB–dependent genes by RelB/p52 and RelB/p50 dimers (Hayden *et al*., 2012; Liu *et al.*, 2012). NF-κB mediates both proinflammatory and antiapoptotic responses to inflammatory mediators,

ensuring that hepatocytes are protected from cell death while appropriate inflammatory and immune responses are initiated. The dual function of the NF-κB pathway requires a delicate balance, since too little or too much NF-κB activation may have a negative impact on the liver owing to increased inflammation or insufficient protection from cell death. Accordingly, the NF-κB pathway is causally involved in many aspects of chronic liver disease, such as hepatitis and hepatocarcinogenesis (Luedde and Schwabe, 2011).

2.6.4 Role of cyclooxygenases-2 (COX-2)

The cyclooxygenase (COX) pathway is responsible for the conversion of arachidonic acid into prostaglandins (PGs), the most widely recognized mediators of inflammation. PGs synthesis in mammals is carried out by the expression of 2 forms of cyclooxygenases. COX-1 is constitutively expressed in most tissues and has a narrow specificity for substrates, preferentially using arachidonic acid and releasing PGs that are involved in the physiological action of these lipid mediators. In humans, COX-1 is found constitutively expressed in a wide range of tissues including the kidney, lung, stomach, small intestine and colon. COX-1 is considered a housekeeping enzyme responsible for maintaining basal prostaglandin levels important for tissue homeostasis. In contrast, most tissues do not normally express COX-2 constitutively, notable exceptions including the central nervous system, kidneys and seminal vesicles. However, the stimulation of COX-2 expression in Src-transformed fibroblasts endothelial cells and monocytes treated with the tumour promoter tetradecanoyl-phorbol-acetate or lipopolysaccharide led to the notion that COX-2 is an inducible enzyme that produces prostaglandins during inflammatory and tumorigenic settings. In particular, COX-2 is a key executor of uncontrolled inflammation and its inhibition represents a major target in the treatment of inflammatory disorders (Martín-Sanz *et al*., 2010; Greenhough *et al*., 2009).

COX-2 inhibitors have been shown to have liver protective effects. Kolaviron and berberine have been reported to suppress Cox-2 expressions in both dimethyl nitrosamine and carbon tetrachloride models of hepatotoxicity in rodents (Domitrović *et al.*, 2011; Farombi *et al*., 2009).

2.7.0 FREE RADICALS, REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

2.7.1 Types of Free radicals and Reactive oxygen species

Free radicals are well known reactive molecules, unstable and very reactive chemical species, which have an unpaired electron in their structure mainly derived from univalent reduction of oxygen. A free radical species may be highly reactive and can initiate chain reactions by extracting an electron from a neighboring molecule to complete its own orbital (Chacko *et al.*, 2007, Marks *et al.*, 1996). Their principal danger comes from the damage they can do when they react with significant cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs. The most common forms of free radical species are the oxygen-centered, carbon-centered (R., RCOO.) and the nitrogen-centered (NO., ONOO-) (Halliwell, 1994). Majority of free radical species are also formed when chemicals are metabolized to one reactive intermediate or the other. The disruption of the metabolism of molecular oxygen during electron transport in the mitochondria has been identified as a source of these species called reactive oxygen species (ROS). Examples of ROS are superoxide anion (O_2-•), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), hydroperoxyl radicals (HO_2) and singlet oxygen ($^1O_2$). O_2-• can be used to form other reactive species such as hydroxyl radicals, hydrogen peroxide and hydroperoxyl radicals. The hydroxyl radical is the most potent oxidant encountered in biological systems and can react readily with a variety of molecules, such as lipids, DNA and proteins. Hydroxyl radicals are formed via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + \cdot OH + OH^-$$

Interaction of H_2O_2 with superoxide can also lead to the formation of •OH (Haber-Weiss Reaction): $O_2^{-\cdot} + H_2O_2 \Rightarrow O_2 + HO^- + \cdot OH$

Hydrogen peroxide, technically, is not an oxygen free radical but rather, a non-radical ROS. It is a secondary product of one-electron reduction of $O_2^{-\cdot}$, catalyzed by the enzyme superoxide dismutase : $O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \Rightarrow H_2O_2 + O_2$ (Dismutation reaction)

In addition to the oxygen-centered radicals, carbon-centered radicals can arise following hydrogen extraction from unsaturated bonds in fatty acids during lipid peroxidation or metabolism of certain xenobiotics. In addition, nitrogen-centered radicals occur naturally in mammalian organisms and are utilized in numerous physiological processes. For instance nitric oxide (NO·) is a signaling molecule for the immune system, it is important to many metabolic processes and is a paracrine messenger. An excess of NO·, however, can result in cytotoxicity and tissue injury, particularly if it reacts with O_2-· to form peroxynitrite ($ONOO^-$) which can

induce a chain reaction of events, such as direct biological damage by excessively oxidizing –SH groups and also leading to the production of nitric dioxide, which is a powerful initiator of lipid peroxidation (Halliwell 1994; Younes 1999).

2.7.2 OXIDATIVE STRESS

To avert free radical damage, the body has a defense system of antioxidants. Antioxidants are molecules which can safely relate with free radicals and stop the chain reaction before vital molecules are damaged. There are several enzyme systems within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidant are vitamin E, beta carotene, and vitamin C. The body cannot manufacture these micronutrients; hence they must be supplied in the diet. The balance between antioxidants and pro-oxidants is a serious concept for sustaining a healthy biological system. The problem of free radicals causes imbalance in mechanisms between pro-oxidants and antioxidants in the body. When free radicals are in excess or antioxidant defenses are compromised, ROS can react with fatty acids, proteins, and nucleic acids and change their function. This imbalance, leading to oxidative stress, has been implicated in several human diseases like atherosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's disease and Parkinsonism (Stowe and Camara, 2009; Halliwell, 1994). Pesticides are known to cause free radical-mediated toxicity in organisms via production of ROS leading to an imbalance between the oxidative and antioxidant indices. There are indications that idiosyncratic hepatotoxicity induced by pesticides may be mediated by oxidative stress, characterized by enhanced levels of ROS (Mansour *et al.,* 2009; Rai *et al.,*2009; Muniz *et al.,* 2008; Zeljezic *et al.,* 2008).

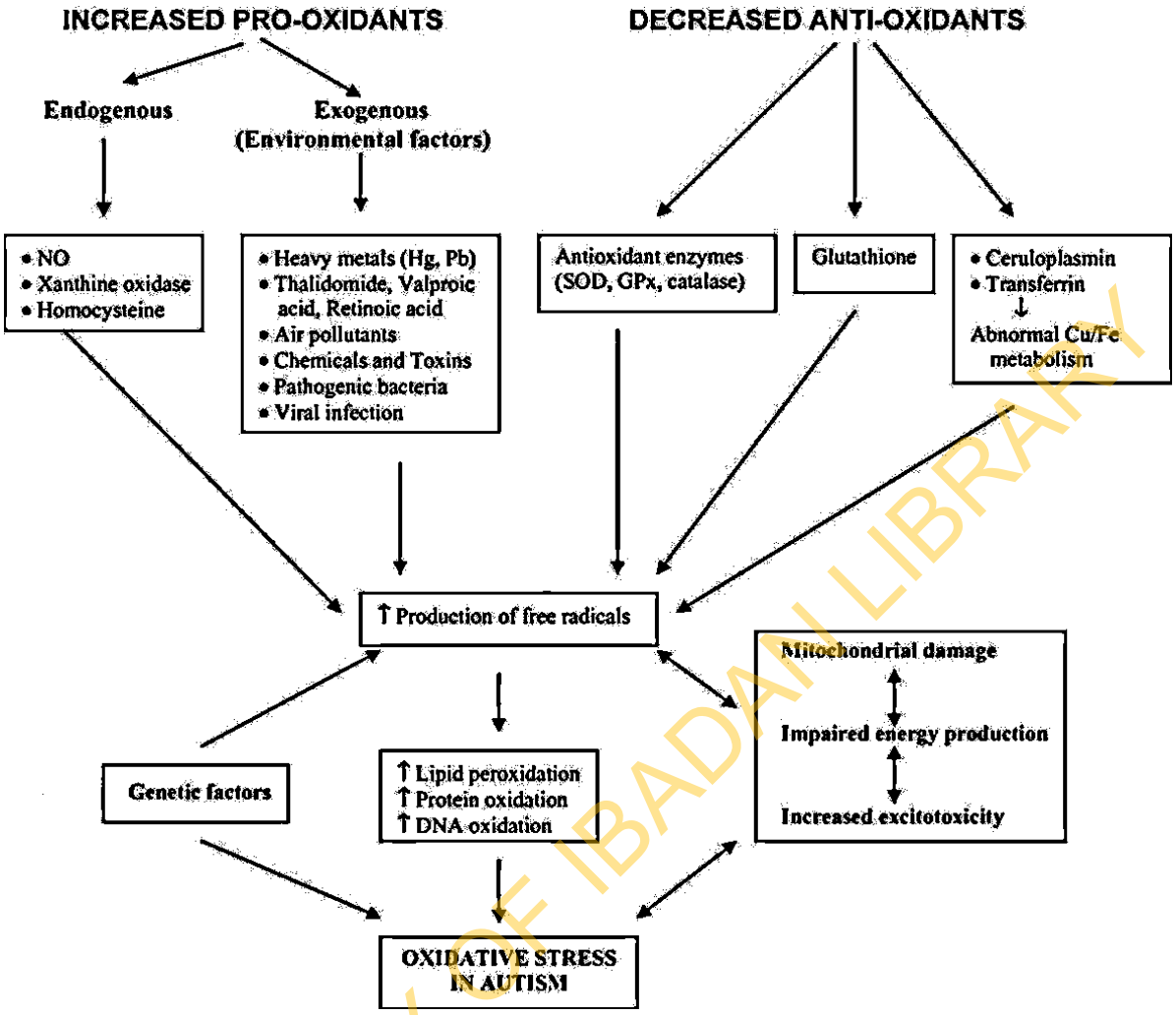


Figure 8: Schematics relating free radicals, oxidative stress and antioxidants

2.8.0 ANTIOXIDANTS

Anti-oxidants are substances capable of mopping up free radicals thereby preventing them from causing cellular damage. Studies have showed that a number of plants and phytocompounds such as flavonoids, isoflavone, flavones, anthocyanins, catechin, and isocatechin possess antioxidant activity with a direct relationship being correlated between

their antioxidant activities and phenolic contents (Ivanova *et al.*, 2005; Savikin *et al.*, 2009). Antioxidant activity of phenolics is mainly due to their redox potential which allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelators. Phytochemicals exerting antioxidant actions are largely being recognized as beneficial to human health and disease prevention, possibly by interfering in the processes involved in reactive oxygen and nitrogen species mediated pathologies including coronary diseases, cancer, age-related degenerative brain disorders (Aruoma *et al.*, 2003).

2.8.1 Antioxidant Defense System

There are two main categories of antioxidants, those that prevent the generation of free radicals and those that intercept any free radicals already generated. They exist in both the aqueous membrane compartment of cells and can be enzymes or non-enzymes (Surapaneni and Vishnu, 2009).

2.8.2 Enzymatic antioxidant defense system

Cellular defense mechanisms against superoxides include a series of linked enzyme reactions which remove the toxic radicals and repair radical induced damage, thus preventing the cell from oxidative stress. These antioxidant enzymes include: **Superoxide dismutase (SOD)** which catalyzes the conversion of superoxide anion into hydrogen peroxide (H_2O_2) and oxygen (O_2). The H_2O_2 formed is relatively less toxic than the superoxide itself, although, also toxic to cells and is removed by another antioxidant defense enzyme, **Catalase (CAT).** This enzyme is situated in the peroxisomes of eukaryotic cells, catalyzing the reaction;

$$H_2O_2 \longrightarrow H_2O + O_2$$

Glutathione peroxidase (GPx) is another group of enzymes in this defense system, which contains selenium as an essential component. This enzyme makes use of glutathione as substrate and functions also to degrade H_2O_2 and reduce organic peroxides to alcohols. This enzyme acts upon the reduced form of glutathione (GSH) to produce oxidized glutathione (GSSG). Other antioxidant enzymes include: glutathione reductase, glutathione S- transferase, hemoxygenase, iodothyronine deiodinases, selenophosphate synthetase and thioredoxin reductase (Litwack, 2008).

2.8.3 Non-enzymatic antioxidant defense system

This includes small endogenous antioxidant molecules such as glutathione (GSH), coenzyme Q (CoQ) and uric acid. Other exogenous antioxidants, such as tocopherols (Vitamin E), ascorbate (Vitamin C), Vitamin A and carotenoids and some metals, essential for the function of antioxidant enzymes, are of dietary origin.

Gluthatione: is a low molecular weight thiol-containing tripepetide (ɣ-glutamyl cysteinyl-glycine) present in virtually all living cells in high concentrations. Apart from functioning as a co-substrate for glutathione peroxidases and transferases, glutathione can react directly with free radicals. In these reactions, the thiol group loses a hydrogen atom to generate a thiyl radical which combines to form glutathione disulfide.

CoQ or ubiquinone: is a redox-active and lipophilic substance present in most cellular membranes. It consists of a quinone head attached to a chain of isoprene units numbering 9 or 10 in different mammal species. CoQ10 has a fundamental role in cellular bioenergetics as a co-factor in the mitochondrial electron transport chain (respiratory chain) and is therefore essential for the production of ATP. CoQ10 functions as a mobile redox agent shuttling electrons and protons in the electron transport chain. CoQ is highly efficient in preventing lipid, protein and DNA oxidation and it is continuously regenerated by intracellular reduction systems. CoQ10 in its reduced form, ubiquinol, is a potent lipophilic antioxidant that is capable of recycling and regenerating other antioxidants such as tocopherol and ascorbate.

Ascorbic acid: this is also known as vitamin C and is a water-soluble vitamin. It reacts with oxygen radicals to generate semidehydroascorbate radical ($AH^{\cdot}$**)**

$$^{\cdot}OH + AH_2 \longrightarrow H_2O + AH^{\cdot}$$

$$^{\cdot}O_2^{-} + H^{+} \longrightarrow H_2O_2 + AH^{\cdot}$$

The semidehydroascorbate thus formed disproportionate to generate ascorbate (AH_2) and dehydroascorbate (A). It is interesting to note that ascorbic acid may exhibit pro-oxidant properties under certain conditions, but their antioxidant capacity outweighs these pro-oxidant inclinations.

α-tocopherol also known as vitamin E is a lipophilic antioxidant, whose characteristic chromanol group reacts with peroxyl and alkoxyl radicals, thus inhibiting the propagation of peroxidative chain reactions.

$$ROOO^{\cdot} + \text{VIT E-OH} \longrightarrow ROOH + \text{Vit-E-O}$$

Vitamin E-radical is reduced by either ascorbic acid or glutathione to regenerate the active forms of α-tocopherol.

$$\text{Vit E-O}\mathring{\text{O}}^{\cdot} + GSH \longrightarrow \text{VIT E-OH} + GS^{\cdot}$$

2.9.0 GLOBAL USE OF HERBAL REMEDIES

The use of herbal medicinal products and supplements has increased tremendously over the past three decades with not less than 80% of people worldwide relying on them for some part of primary healthcare. Although therapies involving these agents have shown promising potential with the efficacy of a good number of herbal products clearly established, many of them remain untested and their use are either poorly monitored or not even monitored at all (Ekor, 2013). The use of herbal medicines and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare settings. This past decade has obviously witnessed a tremendous surge in acceptance and public interest in natural therapies both in developing and developed countries, with these herbal remedies being available not only in drug stores, but now also in food stores and supermarkets. It is estimated that up to four billion people (representing 80% of the world's population) living in the developing world rely on herbal medicinal products as a primary source of healthcare and traditional medical practice which involves the use of herbs is viewed as an integral part of the culture in those communities (WHO, 2004; Bandaranayake, 2006).

Herbal remedies have a therapeutic effect and are acceptable interventions for diseases and symptoms. Interestingly, demand for medicinal plants is progressively rising in industrialized nations as it is in developing countries The World Health Organisation (WHO) estimates that about 80% of the developing world's population meets their primary healthcare needs through traditional medicine (Jadeja *et al.*, 2011; Abere *et al.*, 2010). In folklore medicine in Nigeria, *Rauwolfia vomitoria* (Afzel) is used for treating hypertension, stroke,

insomnia and convulsion (Amole *et al.*, 2009). The seeds of *Citrus parasidi* Macfad are effective in treating urinary tract infections that are resistant to the conventional antibiotics (Oyelami *et al.*, 2005), dried seeds of *Carica papaya* L. is effective in the treatment of intestinal parasitosis (Okeniyi *et al*., 2007). The analgesic and anti-inflammatory effects of *Garcinia kola* Heckel is known to enhance its use for osteoarthritis treatment (Adegbehingbe *et al*., 2008).

2.9.1 MEDICINAL PLANTS AND THEIR HEPATOPROTECTIVE EFFECTS

Liver diseases, which are still a global health problem, may be classified as acute or chronic hepatitis (inflammatory liver diseases), hepatosis (non inflammatory diseases) and cirrhosis (degenerative disorder resulting in liver fibrosis). Unfortunately, treatments of choice for liver diseases are controversial because conventional or synthetic drugs for the treatment of these diseases are insufficient and sometimes cause serious side effects. Except for vaccines and inter-feronα-2b, which concern only viral infections, modern medicine is quite limited in preventing or treating hepatic diseases; the only drugs available are cholagogues, choleretics, and drugs for cholesterolic lithiasis, N-acetylcysteine and flavolignanes obtained from *Silybum marianum.* This limitation of therapeutic options gives considerable interest to the search for active compounds from plants traditionally used for these diseases (Evans, 2002).

Since ancient times, mankind has made use of plants in the treatment of various ailments because their toxicity factors appear to have lower side effects. Many of the currently available drugs were derived either directly or indirectly from medicinal plants. Recent interest in natural therapies and alternative medicines has made researchers pay attention to traditional herbal medicine. In the past decade, attention has been centered on scientific evaluation of traditional drugs with plant origin for the treatment of various diseases. Due to their effectiveness, with presumably minimal side effects in terms of treatment as well as relatively low costs, herbal drugs are widely prescribed, even when their biologically active constituents are not fully identified (Asadi-Samani *et al.*, 2015). Several plants have been tested against a wide spectrum of liver diseases. Natural products, including herbal extracts, could significantly contribute to recovery processes of the intoxicated liver. According to reliable scientific information obtained from the research on medicinal plants, plants such as *Silybum marianum, Phyllanthus* species (amarus, niruri, emblica), and *Glycyrrhiza glabra* have been widely and most of the

time fruitfully applied for the treatment of liver disorders, exerting their effects via antioxidant-related properties (McBride *et al.*, 2012; Tatiya *et al.*, 2012; Hu *et al.*, 2008). Liver protective plants have also been reported to contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes. Recent experience has also shown that plant drugs are relatively non-toxic, safe and even free from serious side effects (Bhawna and Kumar, 2010). Green leafy vegetables are used since ancient periods as source of food as they contain many nutrients and minerals which are helpful in maintaining human health and represent an important proportion of foods with medicinal value. Green leafy vegetables have been identified as good sources of natural antioxidants such as tocopherols, vitamin C and polyphenols which are responsible for maintaining good health and protect against coronary heart diseases and cancer (Kumar *et al*., 2013). There are several studies of the liver protective effects of leafy vegetables commonly consumed in Nigeria. Oboh (2005) investigated the hepatoprotective property of ethanolic and aqueous extracts of *T. occidentalis* leaf against garlic induced-oxidative stress in rat hepatocytes. The hepatoprotective effect of aqueous extract of *Talinum triangulare* (Water Leaf) on Carbon tetrachloride (CCl_4) induced liver damage in Wistar rats has also been reported. In this study, the vegetable, at a dose of 600 mg/kg, elicited reduction in steatosis and degeneration of the liver tissue after exposure to CCl_4 (Adefolaju *et al.*, 2008).

2.9.2 *Pterocarpus mildbraedii*

Pterocarpus mildbraedii (Harms) belongs to the Papilionaceae family which comprises of about 60 species found mainly in the tropics. It is a tree between 15 to 35m tall with smooth, grey or pale brown bark, exuding red gum when cut, and small, rounded crown. The Nigerian species are trees with bright yellow and usually alternate leaflets (Bosch, 2004). *Pterocarpus mildbraedii* has traditional names which include “oha” (Igbo), “urube” (Edo), “mkpafere” (Ibibio) and “osun ure” (Yoruba). The leaves of P. *mildbraedii* are used for soup making in Nigeria. Over the years, tribes in Eastern and Southern Nigeria have used the leaf extracts from *P. mildbraedii* in the treatment of headaches, pains, fever, convulsions, respiratory disorders and as antimicrobial agents (Agoha *et al.*, 1976). The leaves have also been reported to protect against degenerative diseases and diabetes. The fully expanded and older leaves of

Pterocarpus species were found to have alkaloids, saponins, tannins, phenols and flavonoids. The presence of these secondary metabolites in their leaves indicate their medicinal value. Several authors have reported on the analgesic, anti-inflammatory and antibacterial properties of vegetables rich in the above phytochemicals (Akinyeye *et al.*, 2010; Akpanyung *et al.*, 1995, Harbone, 1993). Gill (1992) has already reported the therapeutic, antiseptic, antifungal, bactericidal properties and anti-diabetic effects of the vegetable, but no detailed study on their specific composition and structure has been conducted. A study on the proximate composition of the leaves revealed high content of minerals such as Phosphorus, Calcium, Sodium, Zinc, Potassium, Magnesium and Iron, indicating a possible pharmacological effect in bone-related ailments (Akinyeye *et al.*, 2010; Omale and Ugwu, 2011). Aqueous extract of *Pterocarpus mildbraedii* has been shown to possess some antioxidant activity which apparently suggests its usefulness in the treatment of diseases especially the liver-borne diseases (Odukoya *et al.*, 2007). In a recent investigation on the nutritional value of three leafy vegetables popularly consumed in the Eastern part of Nigeria, high fibre and protein contents and lowered lipid and carbohydrate caloric values were reported for *Pterocarpus mildbraedii,* suggesting its potential to check type 2 diabetes, obesity and heart diseases (Okon and James, 2015). In another experiment, the dichloromethane extract of the leaves was shown to possess free radical scavenging activity and showed strong cytotoxicity against human myeloid leukemia(HL-60), hepatocellular carcinoma (SMMC-7721), lung carcinoma (A549), breast adenocarcinoma (MCF-7) and colon cancer (SW480) cell lines (Iweala *et al.*, 2015).

Figure 9: *Pterocarpus mildbraedii* Tree

2.10.0 EXTRACTION AND ISOLATION OF NATURAL PRODUCTS

Extraction of natural products from crude material is one of the simple steps towards isolation whilst on the other hand isolation process of natural products remains a tough, extensive and a monotonous task (Mahler and Thomason, 2005). Spectroscopic methods

coupled with good separation techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. Sound strategies have helped in the isolation and characterization of many bioactive molecules (Rios *et al*., 1991), Nowadays, bioassay-guided fractionation of medicinal plants is a routine feature in the attempt to isolate bioactive components from natural sources (Wei *et al*., 2008).

2.10.1 Extraction

In practice, as soon as the material is collected, in the case of plants, it needs to be identified by a taxonomist so as to ascertain the correct identity of the material. Various parts of the plant or the whole plant are collected (leaves, flowers, stem, wood, bark, root, root bark, etc.) and dried quickly in drying cabinets because fresh material has much water and this can lead to degradation of the components of the plants by microbes. Good ventilation conditions or high speed fans can be used (Wei *et al*., 2008). Once the material has been dried to constant weight, it is ground up to smaller particles and extracted using solvents of different polarities. The extraction process could either use one solvent or by stepwise extraction in which the same material is extracted using different solvents normally from non-polar to polar solvents.

There are different techniques which can be applied to extract natural products from crude samples; these include:

Maceration

Here the plant material is extracted in solvents of differing polarity at room temperature, by leaving the plant material soaked in the selected solvents and/or shaking at room temperature and this allows for maximum extraction of most components.

Decoction

The plant material is boiled with the solvent usually under reflux. This method allows for extraction of a large number of metabolites, from the most insoluble material like the waxes to the lipophilic natural products.

Continuous extraction

Perhaps the most widely and commonly used technique for the extraction of natural products. The polarity gradient of the solvent is applied. Although some components may be destroyed in the process, it is still the best method of extraction used in natural product chemistry.

Infusion

In this technique hot liquid 'solvent' is poured on the plant material and this is different from decoction where the plant material is boiled with the solvent.

2.10.2 Partial purification and fractionation

Once the extraction is complete, the extract is usually concentrated under vacuum. The activity within the obtained extract can then be demonstrated by bioassay methods using both the crude and the fractionated or semi-purified extracts. Fractionation has the added advantage of getting to the biologically active material faster. There are techniques which can be applied to partially purify the desired components by removing some of the undesired components. The techniques which can be used are solvent/solvent partition and precipitation. Solvent/solvent partition needs two immiscible liquids and separates the components according to solubility which can be polarity or charge. This method relies on the ability of the components to be either soluble in water or the organic phase (Wei et al, 2008). In precipitation/precipitation an excessive amount of the solvent e.g. ethanol, ammonium sulphate, lead etc. is added to the crude material to and some components in the crude material will precipitate.

2.11.0 Chromatographic techniques

For the separation of compounds within the extract, chromatographic techniques are employed. Chromatographic techniques have been instrumental in the separation of natural products. Chromatography is a process whereby a mixture of solutes may be resolved into components by exploiting differences in affinity of the solutes for particles of an insoluble matrix over which a solution of the components is passing. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase (Wei *et al.*, 2008). There are different types of chromatographic techniques which can be utilised to separate compounds. Here, three chromatographic techniques will be discussed.

2.11.1 Thin layer chromatography

Thin layer chromatography (TLC) is one of the fastest and most widely used chromatographic techniques in the separation of natural products. TLC is mostly used for

phytochemical analysis of plant extracts and to check purity of isolated compounds. TLC employs glass or aluminium plates pre-coated with the sorbent (e.g., silica gel) to varying thickness depending on the amount of the sample to be loaded. The compound mixture is loaded on plates at around 1-2 cm from the bottom of the plate and lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are available for visualization of the separated materials. TLC has the advantage of being a highly cost-effective qualitative technique since a large number of samples can be analysed simultaneously.

2.11.2 Preparative thin layer chromatography

Preparative thin layer chromatography (PTLC) is a technique which is usually employed to isolate bioactive natural compounds after column chromatography. PTLC uses the same principles as those of TLC; the difference is only that PTLC has a thick stationary phase compared to TLC. This gives it the advantage of having large quantity of sample loaded on plates as a band and then developed in the chosen solvent system. After developing the plates they can be analysed using non-destructive detection e.g., UV and/or destructive chromogenic spray by exposing only a small portion of the plate. The band(s) with the compound(s) of interest now can be removed using a spatula or cut out with scissors. The compound can be cleaned by filtration, size exclusion in column chromatography, centrifugation, crystallisation etc. The purity of the compound(s) is checked using TLC or High Performance Liquid Chromatography (HPLC).

2.11.3 Column chromatography

Column chromatography (CC) is a popular technique which is used for fractionation and isolation of bioactive natural compounds. This technique is usually employed after solvent/solvent partition. To fractionate or isolate bioactive compounds the stationary phase normally used is silica gel with the mobile being the solvent(s) of choice. There are eluting techniques which can be used which are isocratic elution and gradient elution. Isocratic elution employs only one mobile phase while gradient elution employs a sequence of mobile phases usually in order of polarity, increasing for normal phase chromatography and decreasing polarity for reverse phase chromatography. Gradient elution is normally employed when

isolating and/or fractionating natural bioactive compounds from crude samples. After elution fractions collected are analysed using chemical tests, TLC, bioassays etc. to identify fractions of interest, similar fractions are grouped together for future work (Gurib-Fakim, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 CHEMICALS

Technical grade propanil was obtained from Harvest Field Industries Limited, Lagos, Nigeria. Epinephrine, 1-chloro-2, 4-di nitrobenzene (CDNB), 5',5'- Dithiobis-2-nitrobenzoic

acid (DTNB), Reduced Glutathione (GSH), Hydrogen peroxide (H_2O_2), 1,1-Dipheny-2-picrylhydrazyl (DPPH) radical, Thiobarbituric acid (TBA), Gallic acid were obtained from Sigma Chemical Company (USA). Metaphosphoric acid was obtained from MRS (U.K.). Sulphanilic acid (4-Aminobenzenesulphonic acid) was obtained from Surechem products Ltd (England).ɣ-Glutamyl transferase (ɣ-GT), Lactate dehydrogenase(LDH), Alanine aminotransferse(AST), Aspartate aminotransferase (AST), Alkaline Phosphatase, Bilirubin, Urea, Creatinine, Total Cholesterol, and Triglyceride assay kits were obtained from Randox Laboratories (Crumlin, U.K.). Sodium acetate, Magnesium chloride, Trichloroacetic acid (TCA), Ammonium molybdate, Ferrous sulphate, Potassium dichromate, Glacial acetic acid, Ethylenediamine tetra acetic acid (EDTA), Sodium chloride, Ascorbic acid, Sodium hydroxide, Zinc sulphate, Hydrochloric acid, Sulphuric acid were obtained from BDH (Poole, U.K.) and Hopkins and Williams. Rabbit monoclonal Bax, Bcl-2, caspase-3, caspase-9, Cox-2, iNOS and NF-κB antibodies were obtained from Abcam (UK). Path-Scan ELISA kits measuring phospho-IκB-α, total nuclearfactor-kappa B (NF-κB), phospho-NF-κB, phospho-Jun N-terminal kinase (JNK), phospho-p38, phospho-STAT3 levels, phosphor- p53, total p53, phospho Bad and total Bad were obtained from Cell Signaling Technology, USA. TUNEL kit was purchased from Promega, USA. All other chemicals used were of analytical grade and were obtained from Sigma-Aldrich, St. Louis, USA.

3.2.0 PLANT EXTRACTION

Leaves of *Pterocarpus mildbraedii* were obtained from Oyingbo market in Lagos State, Nigeria. They were taxonomically authenticated and documented with the Voucher number LUT/5913 at the Herbarium of the Department of Botany, University of Lagos, Lagos, Nigeria. The leaves were air-dried, blended and packed into soxhlet extractor and extracted with two solvents dichloromethane and methanol (1:1). The fraction obtained was then concentrated in vacuo to yield extract. This extract was dissolved in physiological saline and administered by gavage to rats.

3.3.0 EXPERIMENTAL ANIMALS

Adult rats of the Wistar strain, weighing 120 g – 160 g were obtained from the primate colony of the Department of Veterinary Anatomy, University of Ibadan. Rats were fed on

commercial pelleted diet (Ladokun Feeds Ibadan, Nigeria) and drinking water *ad libitum*, maintained under standard laboratory conditions and subjected to natural photoperiod of 12 h light/12 h dark cycle.

3.3.1 Collection of samples

Rats were sacrificed by cervical dislocation and blood samples collected by cardiac puncture into centrifuge tubes; centrifuged at 3000 *g* for 10 minutes in a laboratory centrifuge to obtain the serum. Tissue was excised, transferred into ice-cold 0.25 M sucrose solution, blotted with clean tissue paper and weighed. Tissue was homogenized in phosphate buffer (0.1 M, pH 7.4), centrifuged at 4,500*g* and the supernatants used for the various biochemical measurements.

3.3.2 Preparation of liver lysates

Liver tissues weighing 100 mg were homogenized in 1 mL lysis buffer; 20 mM Tris pH=7.5, 150 mM Nacl, I mM ethylene diamine tetraacetate, 1 mM ethylene glycol bis(2 amino ethyl) N, N, N', N' tetraacetic acid, 1% Triton X-100, 2.5 mM pyrophosphate, 1mM ß-glycerophosphate, 1 mM sodium vanadium oxide, 1µg/mL leupeptin. Samples were then centrifuged at 10,000 *g* for 15 minutes at 4^{o}C and supernatants collected and stored at -20^{o}C.

3.4.0 EXPERIMENTAL DESIGN FOR THE *IN VIVO* EXPERIMENTS

3.4.1: Effect of propanil on biochemical and oxidative stress indices in the liver and kidney of rats

30 male rats were randomly distributed into 5 groups of 6 animals in each group. Group A: Control received olive oil while the treatment groups were administered as follows:
Group B (25 mg/kg); Group C (100 mg/kg); Group D (150 mg/kg) and Group E (200 mg/kg). Doses are based on acute oral LD_{50} values of propanil in rats are between 367-2500 mg/kg. Using the upper limit of the reported LD_{50} value, we arrived at the following doses 0.01 LD_{50}, 0.04 LD_{50}, 0.06 LD_{50}, and 0.08 LD_{50} respectively. Animals were orally administered propanil (mixed with olive oil) once daily for 7 days. Biochemical analyses of kidney and liver function indices were assessed.

3.4.2 Effect of *Pterocarpus mildbraedii* extract on propanil-induced liver toxicity in rats

40 male albino rats distributed into eight groups of five animals each were treated by oral intubation, once daily, for 7 days : Group A: Control, received normal saline ; Group B: 100 mg/kg *Pterocarpus mildbreadii* extract (PME); Group C: 200 mg/kg PME; Group D: 400 mg/kg PME; Group E: Propanil (200 mg/kg); Group F: Propanil (200 mg/kg)+ PME 100 mg/kg; Group G: Propanil (200 mg/kg)+ PME 200 mg/kg; Group H: Propanil (200 mg/kg)+ PME 400 mg/kg. At the end of treatment, samples were collected for biochemical analyses.

3.4.3 Effect of *Pterocarpus Mildbraedii* extract on propanil-induced inflammation in rats

Propanil was dissolved in olive oil and administered to rats orally at a dose of 200 mg/kg body weight for 7 days. PME was simultaneously administered with PRP in the PME + PRP group in equal doses of 200 mg/kg body weight. The control group was administered with normal saline (1 mL/kg body weight). At 1 day after the last day of treatment, lysates obtained from the liver were used for the ELISA analysis of NF-κB p65, phospho NF-κB p65 (ser 536), phospho SAPK/JNK (Thr 183/Tyr 185), phospho-p38 (Thr 180/Tyr 182), phospho STAT 3(Tyr 705) and phospho IκBα (ser 32) protein levels. The protein levels of COX-2, iNOS and NF-κB were also determined using immunohistochemistry.

3.4.4 Effect of *Pterocarpus mildbreadii* extract on propanil-induced apoptosis in rats

24 adult male rats of initial body weights between 125- 150 g were randomly divided into four groups with six animals per group and their initial body weights were recorded. Propanil was dissolved in olive oil and administered orally for 7 days as shown below:

Control: 1 mg/kg body weight/day normal saline

Group 1: 200 mg body weight of PRP only

Group 2: 200 mg/kg body weight of PME only

Group 3: 200 mg/kg body weight of PME + 200mg body weight of PRP

After treatment for 7 days, animals were killed by cervical dislocation. Liver samples for ELISA were harvested, weighed, and homogenized in lysis buffer. While the samples for immunohistochemistry were fixed in 4% phosphate buffered formalin. The protein levels of

p53, phospho- p53 (ser 15), Bad and phospho Bad were determined by sandwich ELISA. The protein expressions of caspase 3, caspase 9, Bax and Bcl-2 were also determined using immunohistochemistry. Apoptotic cell death was observed using TUNEL assay.

3.5.0 EXPERIMENTAL DESIGN FOR PRELIMINARY PHYTOCHEMICAL SCREENING AND CHARACTERISATION OF *Pterocarpus mildbraedii* LEAVES

3.5.1 Preliminary phytochemical screening assays

Phytochemical tests were carried out for the plant extract. The crude extract was screened for the presence or absence of secondary metabolites which include tannins, alkaloids, phenolic compounds, saponins, terpenoids using standard procedures with minor modifications where necessary **(**Trease and Evans, 2002).

3.5.2 Test for Tannins

About 0.5 g each portion will be stirred with about 10 mL of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 mL of the filtrate occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins.

3.5.3 Test for Terpenoids

A little of each portion of extract will be dissolved in ethanol. To it 1 mL of acetic anhydride was added followed by the addition of conc. H_2SO_4. A change in colour from pink to violet showed the presence of terpenoids (Sofowora, 1993).

3.5.4 Test for Saponins

One gram of each portion was boiled with 5 mL of distilled water and filtered. To the filtrate, about 3 mL of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins (Sofowora, 1993).

3.5.5 Ferric chloride test for Flavonoids

About 0.5 of each portion of extract will be boiled with distilled water and then filtered. To 2 mL of the filtrate, few drops of 10% ferric chloride solution will then be added. A green-blue or violet coloration confirms the presence of a phenolic hydroxyl group (Trease and Evans, 2002).

3.5.6 Test for Alkaloids

Few quantity of the each portion will be stirred with 5 mL of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 1mL was taken individually into 2 test tubes. To the first portion, few drops of Dragendorff's reagent were added; occurrence of orange-red precipitate was taken as positive. To the second 1 mL, Mayer's reagent was added and appearance of buff-coloured precipitate will be an indication for the presence of alkaloids (Sofowora, 1993).

3.6.0 *IN VITRO* ANTIOXIDANT ASSAYS

The antioxidant assays carried out are: Total Phenolic Content, Total Flavonoid Content and 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) assay.

3.6.1 Estimation of Total Phenolic Content

The total phenolic content of PME was determined according to the method described by Yen *et al.*, (2001). 0.1 g of the extract was dissolved in 5 mL of 0.3% HCl in methanol/water (60:40, v/v). The resulting solution (100 µl) was added to 2 mL of 2% Na_2CO_3. After 2 minutes, 50% Folin-Ciocalteau reagent (100 µl) was added to the mixture, which was then left for 30 minutes. Absorbance was measured at 750 nm using a spectrophotometer. A standard curve was prepared using Gallic acid as standard. Total phenolic content of the extract was therefore expressed as (+)-Gallic acid equivalents.

3.6.2 Determination of total flavonoid content

Total flavonoid content was determined by the aluminum chloride colorimetric method, using Gallic acid (10-100 mg/L) as a standard. The principle of this technique is the formation of complexes between $AlCl_3$ and the keto or hydroxyl groups of flavones, flavonols, and flavonoids (Chang *et al.*, 2002). Briefly, 500 µL of the 1:20 diluted and filtered extract (at an original concentration of 100 mg/mL in methanol) was mixed with 1.5 mL of 95% methanol, 100 µl of 10% aluminum chloride ($AlCl_3$), 100 µl of 1 M potassium acetate and 2.8 mL of distilled water. The mixture was incubated at room temperature for 40 minutes and the absorbance was measured at 415 nm. The total flavonoid content was expressed as Gallic acid equivalents mg /g dry weight.

3.6.3 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) assay

The effect of the PME on 1, 1-diphenyl-2-picrylhydrazyl (DPPH.) radical was estimated according to the method of Hatano *et al* (1988). The relatively stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH·) free radical is used for antioxidant activity measurement of lipid soluble compounds. It is known that a freshly prepared DPPH· solution exhibits a deep purple colour with an absorption maximum at 517 nm. PME (50-500 µg) in 4 mL of distilled water was added to a methanolic solution of DPPH (1 mM, 1 mL). The mixture was shaken and left to stand at room temperature for 30 minutes. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Gallic acid (50 µg) was used as standard. The radical scavenging activity (RSA) was calculated as percentage of DPPH· discolouration.

3.7.0 FRACTIONATION AND EXTRACTION OF PLANT MATERIALS

Leaves of *Pterocarpus mildbraedii* were air dried and blended. The pulverized plant material weighing 235.7 g was packed into soxhlet extractor and extracted with two solvents dichloromethane and methanol (1:1). The fraction obtained was then concentrated *in vacuo* to yield extract to yield a black syrupy extract weighing 99.1g. Column chromatography was carried out on silica gel (70-230 and 240-300 mesh size, Merck), Merck alumina (70-230 mesh) and LH-20 Sephadex. Thin-Layer Chromatography (TLC) was performed on Merck pre-coated silica gel 60 F_{254} on aluminum foil for establishing the purity of compounds. Spots on TLC plates were examined with a UV lamp operating at a wavelength of 366nm for fluorescence and 254nm for florescence quenching spots.

3.7.1 Extraction and synthesis of Methyl Esters from PME

Methyl esters were produced from the oil of *Pterocarpus mildbraedii* using a two-step reaction system. The first step involved the use of 2% sulphuric acid in methanol and secondly transesterification reaction using KOH as catalyst. The oil was first esterified using 2% sulphuric acid in methanol at 70°C to convert the free fatty acid content to methyl esters. The esterification was carried out for 2 h, and the progress of the reaction was monitored using TLC to check the conversion of the free fatty acids to esters. The resultant product was extracted with ethyl acetate, washed with water until free of acid, passed over sodium sulphate, and concentrated using a rotary evaporator. The esterified oil was finally transesterified using 1% KOH in methanol at 70°C. The methyl esters formed were extracted with ethyl acetate, washed

free of KOH, dried over sodium sulphate, and concentrated in a rotary evaporator. The resulting Fatty acid methyl esters (FAME) were subjected to GC-MS analysis.

3.7.2 Fourier Transform Infrared / Gas Chromatography-Mass Spectrometry

The gas chromatography (GC) and mass spectrometry (MS) analyses were realized using GCMS-QP2010 ULTRA. Gas chromatograph equipped with a fused silica capillary column Rxi-1MS 30 m x 0.25 mm x 0.25 µm (Restek) on ultrapure helium gas at 3.0 mL/min and coupled to a mass detector (mass spectrometer). The injector and interface were operated at 250°C. The oven temperature was raised from 100 °C (3 min) to 250°C at a heating rate of 7°C/min and then held isothermally at that temperature. Sample solution (1 µl) was injected in a split mode (ratio 1:10). The GC-MS interface was at a temperature 260°C. The MS was operated at an ionization voltage of 70 eV (electron impact) over an acquisition mass range. The constituents of the sample were identified based on their linear retention indices and comparing their MS spectral with data obtained from the National Institute of Standard and Technology, USA (NIST, 2011). The relative proportions of the constituents were percentages from the GC peak areas. The infrared spectra were recorded on Shimadzu 8400s, (Shimadzu corporation, Kyoto, Japan) Fourier Transform-Infrared (FTIR) Spectrometer using KBr pellet.

3.8.0 BIOCHEMICAL ASSAYS

3.8.1 Serum Creatinine Determination

PRINCIPLE

Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration (Jaffe *et al*., 1987).

REAGENT COMPOSITION

Contents	Initial concentrations of solutions
CAL standard	

R1a. picric acid	35mmol/l
R1b. sodium hydroxide	0.332mmol/l

PROCEDURE

A working reagent was prepared by mixing equal volumes of reagents R1a and R1b. 1mL of working reagent and sample were pipetted into a cuvette. Likewise, 0.2 mL of sample was added to 2 mL of standard. After mixing, the absorbance A_1 of the standard and sample and after 30 seconds read. Exactly two minutes later, absorbance A_2 of standard and sample were determined at 492nm.

3.8.2 Blood Urea Nitrogen Estimation

PRINCIPLE

Urea in serum is hydrolyzed to ammonia in the presence of urease, the ammonia is the measured spectrophotometrically by Berthelot's reaction.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

NH_3 + hypochlorite + phenol → Indophenol (blue compound)

REAGENT COMPOSITION

Contents	Initial concentration of solutions
R1. EDTA	116 mmol/l
Sodium nitroprussside	6 mmol/l
Urease	1 g/l
R2. Phenol (diluted)	120 mmol/l

R3. Sodium hypochlorite (diluted)	27 mmol/l
Sodium hypochloride	0.14N
CAL standard	

PREPARATION OF REAGENTS

1. **Phenol**

   Contents of bottle R2 was diluted with 660 mL of distilled water and mixed thoroughly.

2. **Sodium hypochlorite**

   Contents of bottle R3 was diluted with 750 mL of distilled water and mixed thoroughly.

PROCEDURE

Sample (10 µl) and Reagent 1(100 µl) were pipetted into test tubes, mixed and incubated at 37◦C for 10 minutes. 2.5 mL each of Reagents 2 and 3 was added, mixed immediately and incubated at 37◦C for 15 minutes. Absorbance of the sample (A_{sample}) and standard ($A_{standard}$) was read against the blank at 546 nm.

3.8.3 Determination of Alanine aminotransferase (ALT) activity

PRINCIPLE

ALT activity was determined based on the method of Reitman and Frankel (1957).

α- Oxoglutarate + L-alanine $\xrightarrow{\text{ALT}}$ L-glutamate + Pyruvate

Alanine Aminotransferase is measured by monitoring the concentration of pyruvate hydrazine formed with the substrate 2, 4-dinitrophenylhydrazine.

REAGENT COMPOSITION

CONTENTS	INITIAL CONCENTRATIONS OF SOLUTIONS
R1. Buffer	
Phosphate buffer	100 mmol/l, pH 7.4
L-alanine	200 mmol/l
α-oxoglutarate	2.0 mmol/l
R2. 2,4-dinitrophenylhydrazine	2.0 mmol/l

PROCEDURE

Sample (0.1 mL) was mixed with 0.5 mL of Solution R1 and mixture incubated for 30 minutes at 37°C. 0.5 mL of solution R2 was added and the mixture was allowed to stand for exactly 20 minutes at 20 to 25◦C. 5 mL NaOH was added, followed by incubation for exactly 30 minutes at 37°C. The absorbance of sample (A_{sample}) was read against the reagent blank after 5 minutes at 546nm. The activity of ALT was obtained by plotting a standard curve.

3.8.4 Determination of Aspartate aminotransferase (AST) activity

PRINCIPLE

AST activity was determined based on the method of Reitman and Frankel (1957).

α-Oxoglutatarate + L-aspartate $\xrightarrow{\text{AST}}$ L-glutamate + oxaloacetate

AST is measured by monitoring the concentration of oxaloacetate hydrazine formed with 2, 4-dinitrophenylhydrazine.

REAGENT COMPOSITION

CONTENTS	INITIAL CONCENTRATION OF SOLUTIONS
R1 buffer	
Phosphate buffer	100mmol/l, pH 7.4
L-aspartate	100mmol/l
α-oxoglutarate	2.0mmol/l
R2 2,4-dinitrophenylhydrazine	2.0mmol/l

PROCEDURE

0.1 mL of sample was mixed with 0.5 mL of Solution R1 and mixture incubated for 30 minutes at 37°C. 0.5mL of Solution R2 was added and the mixture was allowed to stand for exactly 20 minutes at 20 to 25◦C. 5mL NaOH was added, followed by incubation for exactly 30 minutes at 37°C. The absorbance of sample (A_{sample}) was read against the reagent blank after 5 minutes at 546nm. The activity of AST was obtained by plotting a standard curve.

3.8.5 Determination of Alkaline phosphatase activity

PRINCIPLE

This is an optimized standard method according to the recommendations of the Deutsche Gesellschat für Klinische Chemie.

$$p-\text{nitrophenylphosphate} + H_2O \xrightarrow{\textbf{ALP}} \text{phosphate} + p-\text{nitrophenol}$$

REAGENT COMPOSITION

CONTENTS	CONCENTRATION IN THE TEST
R1a Buffer	

Diethanolamine buffer $MgCl_2$	1 mol/l, pH 9.8 0.5 mmol/l
R1b Substrate p-nitrophenylphosphate	10 mmol/l

PROCEDURE

For the semi-micro determination of ALP activity, 0.02 mL of sample was added 1mL of the reagent (R1a+R1b) in 1 cm light cuvette**.** The mixture was monitored kinetically, read after 0, 1, 2 and 3 minutes. Absorbance was read at 405nm against air. ALP activity was then calculated using the following formula: U/I= $2760 \times \Delta A \frac{nm}{min}$.

3.8.6 Direct Bilirubin estimation

PRINCIPLE

Colorimetric method based on that described by Jendrassik and Grof (1938). Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total Bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

REAGENT COMPOSITION

Contents	Initial concentration of solutions
R1. Sulphanilic acid	29 mmol/l
Hydrochloric acid	0.17N
R2. Sodium nitrite	38.5 mmol/l
R3. Caffeine	0.26 mmol/l
Sodium benzoate	0.52 mmol/l

R4. Tartrate	0.93 mmol/l
Sodium hydroxide	1.9N

PROCEDURE

Reagents R1+ R2+R3 and sample were allowed to stand for 10 minutes at 20-25◦C. Then R4 was added, and mixture allowed to stand for 5-30 minutes at 20-25◦C. Absorbance of the sample was read against the sample blank (A_{DB}) at 578nm. Direct Bilirubin (mg/dl) = 14.4 × A_{DB}

3.8.7 Determination of γ--Glutamyl transferase (γ-GT) activity

γ- GT activity was determined following the procedure of Szasz (1969).

PRINCIPLE

The principle is based on the fact that γ-GT catalyzes the transfer of the glutamyl group from a glutamyl peptide to an amino acid of another peptide, glycylglycine, to yield a product 5-amino-2-nitribenzoate, which absorbs UV-light at 405nm, thus making a direct kinetic assay possible.

L-γ-glutamyl-3-p-nitroanilide + glycylglycine $\xrightarrow{\gamma - GT}$ L-γ-glutamyl- glycylglycine + 5-amino-2-nitribenzoate

The amount of 5-amino-2-nitribenzoate liberated is proportional to the γ-GT activity.

REAGENTS

Reagent 1 Buffer	Tris buffer pH 8.6	100 mmol/l
Reagent 2	Glycylglycine	100 mmol/l
Substrate	L-γ-glutamyl-3-carboxy-4-nitroanilide	3 mmol/l

PROCEDURE

The working reagent was prepared by dissolving one tablet of R2 substrate into 15 mL R1 buffer. To 1 mL of this reagent was added 0.10 mL of sample. After 1 minute the absorbance was read at 404nm every minute for 2 minutes. The difference between the absorbance and the average absorbance difference per minute (ΔAbs. /min) can be calculated using the equation γ-GT (U/I) = ΔAbs. /min × 1190.

3.8.8 Determination of Lactate Dehydrogenase activity

PRINCIPLE

LDH catalyzes the oxidation of lactate to pyruvate in the presence of NAD, which is subsequently reduced to NADH. The rate of NADH formation measured at 340nm is directly proportional to serum LDH activity (Wahlefeld, 1983).

L-lactate + NAD → pyruvate + NADH + H^+

REAGENTS

The working reagent was prepared by mixing five (5) volumes of R1 with one (1) volume of R2 in a disposable container. After combining R1 and R2 as directed, the reagent contained: Lithium lactate 55 mmol/l, NAD formation 7.5 mmol/l; buffer (8.95); stabilizers and preservatives.

PROCEDURE

Working reagent (1.0mL) was pipetted into appropriate tubes and pre-warmed at 37◦C for at least five (5) minutes. Sample (25 µl) was added to the reagent, mixed and incubated at 37°C for 1 minute. After 1 minute, absorbance was read at 340 nm. This procedure was repeated every minute for the next 2 minutes. The average absorbance difference per minute (ΔAbs/min), which gives the LDH activity, is calculated thus: $\Delta Abs./\min \times 6592$.

3.8.9 TOTAL CHOLESTEROL ESTIMATION

PRINCIPLE

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

Cholesterol ester + H_20 $\xrightarrow{cholesterol\ esterase}$ Fatty acids

Cholesterol + O_2 $\xrightarrow{cholesterol\ oxidase}$ cholestene-3-one + H_2O_2

$2H_2O_2$ + phenol + 4-aminopyrine $\xrightarrow{peroxidase}$ quinoneimine + $4H_2O$

REAGENTS

Contents	Initial concentration of solution
R1 Reagent	
4-aminoantipyrine	0.30mmol/l
Phenol	6mmol/l
Peroxidase	≥ 0.5 U/mL
Cholesterol esterase	≥ 0.15 U/mL
Cholesterol oxidase	≥ 0.0.1U/mL

Pipes buffer	80mmol/l; pH 6.8
CAL standard	

PROCEDURE

Sample (10 µl) was mixed with reagent 1 and incubated for 5 minutes at 37◦C. The absorbance of the sample (A_{sample}) was then measured against the reagent blank within 60 minutes at 500nm. Using a standard: Concentration of cholesterol in sample is calculated as: $\frac{A_{sample}}{A_{standard}} \times$ standard concentration (mg/dl).

3.8.10 TRIGLYCERIDES ESTIMATION

PRINCIPLE

The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinone imine formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

Triglycerides + H_2O $\xrightarrow{lipases}$ Glycerol + fatty acid

Glycerol + ATP $\xrightarrow{GK}$ Glycerol-3-phosphate + ADP

$2H_2O$ + 4-Aminophenazone + 4-chlorophenol → Quinoneimine +HCl + $4H_2O$

REAGENTS

Contents	Concentrations in the test
R1a Buffer Pipes buffer	40 mmol/l, pH 7.6

4-chlorophenol	5.5 mmol/l
Magnesium ions	17.5 mmol/l
R1b Enzyme reagent	
4-aminophenazone	0.5 mmol/l
ATP	1.0 mmol/l
Lipases	≥ 150 U/mL
Glycerol kinase	≥0.4 U/mL
Glycerol-3-phosphate oxidase	≥ 1.5 U/mL
Peroxidase	≥ 0.5 U/mL
CAL standard	

PROCEDURE

Sample (10 µl) was mixed with the enzyme reagent which was reconstituted by adding 15 mL of Reagent 1. The mixture was incubated for 5 minutes at 37◦C. The absorbance of the sample (A_{sample}) was then measured against the reagent blank within 60 minutes at 500nm. Using a standard: Concentration of Triglyceride in sample was calculated as: $\frac{A_{sample}}{A_{standard}} \times$ standard concentration (mg/dl).

3.8.11 Determination of superoxide dismutase activity

The level of superoxide dismutase (SOD) activity was determined by the method Misra and Fridovich (1972).

PRINCIPLE

Adrenaline (epinephrine) at pH 10.2 makes this reaction basic for a simple assay for this enzyme. Superoxide (O_2) radical generation by Xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced increased with increasing pH and also increased with increasing concentration of epinephrine.

REAGENTS

1. Carbonate buffer (0.05 M, pH 10.2)

Na_2CO_3 (14.3g) and 4.2g of $NaHCO_3$ were dissolved in 900mL of distilled water. The pH was adjusted to 10.2 and then made up to 1L.

2. Adrenaline (0.3 mM)

Adrenaline (13.7 mg) was dissolved in 200 mL distilled water and then made up to 250 mL. This solution was prepared just after the experiment.

PROCEDURE

Sample (1 mL) was diluted in 9 mL distilled water to make 1 in 10 dilutions. An aliquot (0.2 mL) of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 mL of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion . The referenced cuvette contain 2.5 mL buffer, 0.3 mL of substrate (adrenaline) and 0.2 mL of water. The increase in absorbance at 480nm was monitored every 20 seconds for 150 seconds.

CALCULATIONS

Increase in absorbance per minute= $\frac{A_s - A_0}{2.5}$

% inhibition= $\frac{\text{increase in absorbance of sample}}{\text{increase in absorbance of blank}} \times 100$

1 unit inhibition= Amount of SOD to cause 50%

3.8.12 Determination of catalase activity

Catalase activity was determined according to the method of Clairborne (1995).

PRINCIPLE

This method is based on the fact that dichromate in acetic acid to chromatic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced in measured colorimetrically at 570-610nm. Since dichromate has absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromatic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate /acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

REAGENT

1. **Dichromatic solution (5%)**

   $K_2Cr_2O_7$ (5g) was dissolved in 80mL of distilled water and made up to 100mL with same.

2. **Hydrogen Peroxide(0.2M)**

   H_2O_2 (0.67g) was mixed with distilled water in a 100mL volumetric flask and the solution made up to the same mark with same.

3. **Dichromate/ acetic acid**

   This reagent was prepared by mixing 5% of solution of $K_2Cr_2O_2$ with glacial acetic acid (1:3 by volume) and it is stable for about one month.

4. **Phosphate buffer (0.01M, pH 7.0)**

   $Na_2HPO_4.12H_2O$ (3.5814g) and 1.19g $NaH_2PO_4.2H_2O$ dissolved in 900mL of distilled water. The pH adjusted to 7.0 and distilled water added to make up 1L.

PROCEDURE

Colorimetric determination of H_2O_2

Different amounts of H_2O_2, ranging from 10 to 100 µmoles were pipetted into small test tubes and 2 mL of dichromate/acetic acid was added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of

chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3 mL and the optical density measured with a spectrophotometer at 570 nm. The concentrations of the standard were plotted against absorbance.

Test tube	1	2	3	4	5	6	7
$H_2O_{2(mL)}$	0.0	0.1	0.2	0.3	0.4	0,5	0.6
Dichromate/acetic acid(mL)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water(mL)	1.00	0.90	0.80	0.70	0.60	0.50	0.40

Determination of Catalase activity in sample

Sample (1 mL) was mixed with 9 mL of distilled water to give a 1 to 10 dilution of sample. The assay contained 4mL of H_2O_2 solution (800 µl), 5mL of phosphate buffer in a 10mL flat bottom flask. 1mL of properly diluted enzyme preparation was rapidly mixed with the mixture by a gentle swirling motion. The reaction was run at room temperature. A 1 mL portion of the reaction mixture was withdrawn and blown into 2 mL dichromate/ acetic acid reagent at 60 seconds, the hydrogen peroxide contents of the withdrawal sample was determined by checking the absorbance at 570nm.

CALCULATION

Catalase activity can be calculated by plotting the standard curve and then extrapolating from the curve the concentration of the H_2O_2 remaining.

$$H_2O_2\ consumed = 800\mu moles - H_2O_2\ remaining$$

$$\text{Catalase activity} = \frac{H_2O_2\ \text{consumed}}{\text{mg of protein}}$$

3.8.13 Determination of glutathione -s-transferase activity

Glutathione-S-transferase (GST) activity was determined according to Habig *et al.*, (1974).

PRINCIPLE

The principle is based on the fact that all known glutathione -S-transferase demonstrate a relatively high activity with 1-chloro-2, 4-dinitrobenzene as the second substrate. Consequently, the conventional assay for glutathione -S-transferase activity utilizes 1-chloro-2, 4-dinitrobenzene as substrate. When this substance is conjugated with reduced glutathione, this absorption increase at the new wavelength of 340nm provides a direct measurement of the enzymatic reaction.

REAGENTS

1. **1-chloro-2, 4-dinitrobenzene (20 nM)**

   1-chloro-2, 4-dinitrobenzene (CDNB 10.1mg) was dissolved in 3mL of ethanol

2. **Reduced glutathione (0.1 M)**

   Reduced glutathione (GSH, 61.5mg) was dissolved in 2mL of 0.1m phosphate buffer (pH 6.5).

3. **Phosphate buffer (0.1 M, pH6.5)**

   This was prepared by dissolving 4.96 g of dipotassium hydrogen phosphate and 9.73 g of potassium dihydrogen in distilled water. The pH was adjusted to pH 6.5 and the volume made up to 1000 mL.

PROCEDURE

The medium for the estimated was prepared as showed in the table and the reaction was allowed to run for 60 seconds each time before the absorbance was read against the blank at 240nm. The temperature was maintained at approximately 31◦C. The absorbance was measured using UV-1650pc spectrophotometer.

GLUTATHIONE-S-TRANSFERASE ASSAY MEDIUM

Reagent	Blank	Test (µl)
Reduced glutathione (0.1M)	30(µl)	30 (µl)
CDNB (20mM)	150(µl)	150 (µl)
0.1M phosphate buffer, pH 6.5	2.82mL	2.79 mL

PMF	_____	30 (µl)

CALCULATION

GST activity= $\frac{\text{Absorbance/min}}{9.6} \times \frac{1}{0.03\text{mg/protein}}$

Where 9.6 min^{-1} cm^{-1} is the extinction coefficient of CDNB.

3.8.14 Determination of reduced glutathione level

The method of Beutler *et al.*, (1963) was followed in estimating the level of reduced glutathione (GSH).

PRINCIPLE

The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable (yellow) colour when 5'5'-Dithiobis-(2-nitrobenzoic acid). Ellman's reagent with the reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412nm.

REAGENTS

1. GSH working standard

GSH (40 mg) was dissolved in 0.1M phosphate buffer 7.4, and made up to 100 mL with the same.

2. Phosphate buffer (0.1M, pH 7.4)

a. First 0.1M $Na_2HPO_4.12H_2O$ was prepared by dissolving 14 g in 400 mL of distilled water.

b. 0.1M $Na_2HPO_4.2H_2O$ was prepared by dissolving 1.5603 g in 100 mL of distilled water.

Finally 0.1M phosphate buffer was prepared by adding 200 mL of (a) to 100 mL of (b) and the pH to 7.4 with the drops of concentration HCl or NaOH as the case may be. This is stable

indefinitely unless mold forms. If crystal develop during storage at 4◦C heating may dissolve these.

3. Elman's reagent (5'5'-Dithiobis-(2-nitrobenzoate) DNTB)

This was prepared by dissolving 40 mg of Elman's reagent in 0.1M phosphate buffer and made up to 100 mL.

4. Precipitating solution

Prepared from 1.67g metaphosphate acid, 0.2g disodium or dipotassium ethylene diaminetetraacetic acid (EDTA) and 30 g NaCl per 100 mL of distilled water and stored at 4◦C. It is stable for 3 weeks. The EDTA was added to prevent difficulties that might arise where water supply contains appreciable concentration of metallic ions.

PROCEDURE

A sample (0.2 mL) was added to 1.8 mL distilled water and 3.3 mL of the precipitating solution was mixed with the sample. The rate of addition was not critical. The mixture was then allowed to stand for approximately 5 minutes and the filtered. 1 mL of the filtrate was added to 4mL of 0.1M phosphate buffer. Finally 0.5 mL of Ellman's reagents was added. A blank was prepared with the 4mL of the 0.1 mL phosphate buffer, 1mL of diluted precipitating solution (3 parts to 2 parts of distilled water) and 0.5 mL of the Ellman's reagent. The solution was kept at room temperature for 15 minutes and read at 412 nm. The optical density was measured at that wavelength and the estimate was obtained from GSH standard.

PREPARATION OF GSH STANDARD CURVE

Tube No	1	2	3	4	5	6	7
GSH Stock Solution(mL)	0.0	0.05	0.1	0.2	0.3	0.4	0.5
Phosphate Buffer(mL)	0.5	0.45	0.4	0.3	0.2	0.1	0.0
Ellman's reagent(mL)	4.5	4.5	4.5	4.5	4.5	4.5	4.5

GSH Concentration(µg)	0	20	40	80	120	160	200

The standard curve was plotted and the concentration of remaining GSH was obtained from the curve by extrapolation.

3.8.15 DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substance (TBARS) according to the method of Varshney and Kate (1990).

PRINCIPLE

Under acidic condition, malondialdehyde (MDA) produced from the peroxidation of membrane fatty acid and food products react with the chromogenic reagent, 2-thiobarbituric acid (TBA), to yield a pink coloured complex with maximum absorbance at 532 nm and fluorescence at 553 nm. The pink chromophore is readily extractable into organic solvent such as butanol.

REAGENTS

1. **Trichloroacetic acid (TCA, 30%)**

TCA (9 g) was dissolved in distilled and made up to 100 mL with same.

2. **Thiobarbituric acid (0.75%)**

This is prepared by dissolving 0.225 g of thiobarbituric acid (TBA) in 0.1M HCl up to 20 mL with same.

3. **Tris-KCl buffer (0.15M, pH 7.4)**

KCl (1.12 g) and 2.36 g of Tris Base were dissolved separately in distilled water and made up to 100 mL with same. The pH was then adjusted to 7.4.

PROCEDURE

An aliquot (0.4 mL) of the liver PMF was mixed with 1.6mL of Tris-KCl buffer to which 0.5 mL of 30% TCA was added. 0.5 mL of 0.75% TBA was added and placed in a water bath for 45 minutes at 80◦C. This was then cooled in ice and centrifuged at 3000 g. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi

(1980). Lipid peroxidation in units /mg protein or gram tissue was computed with a molar extinction coefficient of $1.56\times10^{-5}M^{-1}\,cm^{-1}$.

CALCULATION

$$\text{MDA (units/mg/protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{1.56 \times 10^{5} \times \text{volume of sample} \times \text{mg of protein}}$$

3.8.16 Determination of Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was assessed by measuring the H_2O_2-dependent oxidation of *o*-dianisidine according to the method of according to Bradley *et al* (1982).

PRINCIPLE

Myeloperoxidase (MPO) is a lysosomal enzyme present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. MPO, which is released during inflammation, is an oxidative enzyme present in phagocyte. This heme enzyme uses the oxidizing potential of superoxide and hydrogen peroxide to convert chloride ion to hypochlorous acid and other ROS. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury. In this assay, the ability of MPO to oxidize o-dianisidine in the presence of H_2O_2 to generate the oxidized product, which was then measured spectrophotometrically served as the basis for this assay.

REAGENTS

1. 50 mM Phosphate buffer (pH6.0)

This was prepared by dissolving 2.18 g of dipotassium hydrogen phosphate and 1.70 g of potassium dihydrogen phosphate in 250 mL of distilled water and the pH adjusted to 6.0.

2. 0.005% H_2O_2

Make a preliminary dilution by dissolving 0.1 mL of 30% H_2O_2 solution with 100 ml of distilled water. From this diluted solution pipette 2.5 ml and dissolve in 150 ml of buffer.

3. O-dianisidine

0.129mg/mL was dissolved in the buffer.

4. To the above mixture was added 0.086 µl/ml of concentrated HCl.

PROCEDURE

0.1mL of sample was mixed with 50 mM phosphate buffer pH6.0 containing 0.129 mg/ml O-dianisidine and 0.0005% H_20_2 in a final volume of 3 ml. Absorbance change at 460 nm was measured. One unit of MPO activity was defined as that degrading one micro mole of peroxide/minute at 25^o C.

CALCULATION

MPO (µmol/min/mg protein) $= \frac{\text{Absorbance } \Delta 460 \text{ /min} \times \text{volume of mixture}}{0.0113 \times \text{volume of sample} \times \text{mg of protein}}$

3.8.17 Determination of Nitric oxide concentration

Serum/liver nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as index of nitric oxide ($NO^{\cdot}$) production. Quantitation was based on the Griess reaction according to the method of Bryan and Grisham (2007).

PRINCIPLE

Nitric oxide (NO) is a ubiquitous intercellular messenger molecule with important cardiovascular, neurological, and immune functions. NO is a short-lived, reactive free radical that participates in a variety of reactions. NO-mediated activation of soluble guanylate cyclase is responsible for signal transduction and for most of its physiological roles; however, excess of NO can exert cytotoxic effects. This may involve both i) direct toxicity, *e.g.*, the reaction of NO with iron-containing enzymes of the respiratory cycle and of the DNA synthetic pathway, and ii) the interaction of NO with free radicals like superoxide ion (O_2^-) to form peroxynitrite ($ONOO^-$), which is a potent oxidizing molecule capable of eliciting lipid peroxidation and cellular damage. NO is produced in mammalian cells by a group of isoenzymes collectively termed NO synthases (NOS). All forms of NOS catalyze the conversion of L-arginine to L-citrulline in an NADPH-dependent manner, producing NO from the terminal *N*-guanidino group of L-arginine (Menaka *et al*., 2007).

PROCEDURE

300 µl of Griess reagent was added to 600 µl of distilled water, and 100 µl of sample was added. The reaction mixture was stood for 30 minutes and absorbance read at 550 nm.

Calibration curve at various concentrations was prepared by diluting stock 20 mmol/L solutions of $NaNO_2$ with distilled water.

3.8.18 Determination of total protein concentration

The principle was based on Biuret reaction as described by Gornall *et al.* (1949).

PRINCIPLE

The Biuret reaction involves a reagent containing copper (cupric) ions in alkaline solution. Molecules containing 2 or more peptide bonds associate with the cupric ions to form a coordination complex that imparts a purple colour to the solution with A_{max} = 540 nm. The purple colour of the complex can be measured independently of the blue colour of the reagent itself with a spectrophotometer or colorimeter.

REAGENTS

1. Normal Saline (0.9% w/v NaCl)

NaCl (2.7 g) was dissolved in distilled water and made up to 300 mL with the same. This was stored at 4^oC.

2. Sodium Hydroxide (0.2 M)

NaOH (8.0 g) dissolved in distilled water and the solution made up to a litre.

3. Stock Bovine Serum Albumin (BSA, standard)

BSA (7.4 mg) dissolved in 0.9% NaCl and made up to 100 mL so that the final concentration gives 7.4 mg/100 mL.

4. Biuret reagent

$CuSO_4.5H_2O$ (3 g) and sodium potassium tartarate (5 g) were dissolved in 500 mL of 0.2M NaOH. Potassium iodide (100 mL) was added and the solution made up to a litre with 0.2M NaOH. Potassium iodide was added to the reagent to prevent precipitation of Cu^{2+} ions.

Preparation of standard curve

Make serially diluted solutions of your standard protein in clean test tubes as follows:

Test tubes	1	2	3	4	5	6
mL of standard protein	0	0.2	0.4	0.6	0.8	1
mL of distilled water	1.0	0.8	0.6	0.4	0.2	0

Mix properly after adding the distilled water. Now add 4mL each of biuret reagent to each of the contents of the test tubes 1-6. Mix well after each addition of the biuret reagent. Allow the tubes to stand for 30 minutes and then read the absorbance at 520nm in spectrophotometer. Your blank is tube 1. Use this to set the instrument at zero before reading tubes 2-6.

Now into 7 introduce 0.1 mL of sample add 0.9mL distilled water followed by 4 mL of biuret reagent. Allow to stand for 30 minutes and read the absorbance in a spectrophotometer.

CALCULATIONS

Total protein content= $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{concentration of standard}$

3.9.0 Histology

Livers from rats of all the groups were fixed in 10% formaldehyde, dehydrated in graded alcohol and embedded in paraffin. Fine sections were obtained, mounted on glass slides and counter-stained with hematoxylin-eosin (H&E) for light microscopic analyses. The slides were coded and examined by a histopathologist who was ignorant about the treatment groups.

3.10.0 MOLECULAR ASSAYS

3.10.1 Immunohistochemical Analysis

The liver tissue specimens were fixed in neutral formalin solution (10%). Tissue specimens were embedded in paraffin wax and sectioned (thickness, 5 µm). Paraffin sections were deparaffinized in xylene, hydrated, and then placed in phosphate buffered saline (PBS; pH 7.6). Antigen retrieval was performed by boiling for 15 min in citrate buffer (0.01 M). Sections were treated with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity, rinsed with deionized water, and then washed with PBS. Sections on the slides were treated with 130 µl appropriately diluted biotinylated secondary antibody (using the antibody dilution buffer) and incubated in a humidified chamber at room temperature for 30 min. After another wash, sections were incubated with appropriately diluted biotinylated secondary antibody at 23 °C in a moist chamber for 1 h. Detection of the antibody was performed using a streptavidin –Horse Radish Peroxidase detection system with Diaminobenzidine(DAB) as the chromogen. Sections were counterstained with Mayer's hematoxylin, dehydrated, and then cover-slipped with Permount. Rabbit monoclonal to COX-2, iNOS, Caspase-9, Caspase-3, Bcl-2 and Bax (Abcam, UK) were used for these studies.

3.10.2 Detection and quantification of apoptotic-related proteins and inflammatory transcription factors by ELISA

Sandwich ELISA apoptosis array was used to detect the presence of elements of the apoptotic cascade in liver lysate (described in 3.3.2). The PathScan® Apoptosis Multi-target Sandwich ELISA kit (Cell Signaling Technology, Beverly, MA) was used as per the manufacturer's protocol. Briefly, liver lysates (diluted to 250 pg/µl in the sample diluent supplied by the manufacturer) were then added to a 96-well plate pre-bound with primary antibodies to various protein antigen targets, and incubated for 2 hours at 37°C. The wells were then incubated for one hour with the corresponding detection antibodies and HRP-linked secondary antibodies. The supplied 3,3',5,5'-tetramethylbenzidine (TMB) substrate was then added to each well and incubated for 10 minutes at 37°C, after which the reaction was stopped using the stop solution provided. The absorbance of the ELISA reactions was read at 450 nm using a spectrophotometer. The magnitude of the reaction absorbance was directly proportional to the quantity of the bound target protein (phospho-IκB-α, total nuclear factor- kappa B (NF-

κB), phospho-NF-κB, phospho-Jun N-terminal kinase (JNK), phospho-p38, phospho-STAT3 levels, phospho- p53, total p53, phospho-Bad and total Bad).

3.10.3 TUNEL Assay for the detection of apoptotic cells

Preparation of reagents for TUNEL assay

1. **4% paraformaldehyde**

   4 mL of paraformaldehyde was made up to 100 mL of PBS

2. **0.3% H_2O_2**

   0.3 mL of H_2O_2 was made up to 100 mL with distilled water.

Protocol for TUNEL assay

Apoptotic cells were detected using the DeadEnd Colorimetric Apoptosis Detection System (Promega Corp., Madison, WI, USA). The system end-labels the fragmented DNA of apoptotic cells using a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeled (TUNEL) assay. It consists of incorporation of biotinylated nucleotides at the 3'-OH DNA ends using the terminal deoxynucleotidyl transferase, recombinant (rTDT) enzyme. Horseradish peroxidase-labeled streptovadin is then bound to these biotinylated nucleotides, which are then detected using the peroxidase substrate, hydrogen peroxide and the stable chromogen diaminobenzidine (DAB).

Briefly, the tissue sections were deparafinized, rehydrated and fixed in 4% paraformaldehyde solution in PBS. Proteinase K (20 µg/mL) treated for 15min was followed be a second fixation in 4% paraformaldehyde solution in PBS. The sections were incubated with equilibration buffer (20 mM potassium cacodylate, pH 6.6; 25 mM Tris-HCl, pH 6.6. 0.2mM DTT, 0.25 mg/mL BSA, 2.5 mM cobalt chloride) for 10 min. Subsequently; the sections were allowed to react with rTDT reaction mixture (98µl equilibration buffer, 1 µl rTDT enzyme) for 1 h at 37^{o}C in a humidified chamber. The slides were immersed in 2x SCC for 15 minutes to stop the reaction and the endogenous peroxidase was blocked by incubating the sections with 0.3% H_2O_2 in PBS for 5 minutes. After incubation with streptavidin-HRP solution for 30 min at room temperature, slides were stained with DAB- H_2O_2 in the dark. Positive nuclei stained dark and were visualized under a fluorescence microscope. Negative controls with TUNEL assay were performed according to the instructions provided by the manufacturer.

3.11.0 DATA ANALYSIS

All values are mean ± standard error of mean. Differences between the groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Graph Pad Prism software. Values are regarded as significantly different at $P < 0.05$.

CHAPTER FOUR

4.0 EXPERIMENTS AND RESULTS

4.1.0 EXPERIMENT 1: EFFECT OF PROPANIL ON BIOCHEMICAL AND OXIDATIVE STRESS INDICES IN THE LIVER AND KIDNEY OF RATS

INTRODUCTION

Pesticides are ubiquitous in the environment and have significant economic, environmental and public health impact. Their usage helps to improve human nutrition through greater availability, longer storage life and lower costs of food. Pesticides also reduce human labor requirements and attendant risk of work-related injury. However, widespread use of pesticides due to their low bio-accumulation brings a hazardous risk of exposure to mammalian systems because of optimum residual level and steady dissipation rate (Abdou and El Mazoudy, 2010; Jain *et al*., 2011).

Propanil (3, 4-dichloropropioanilide), an acylanilide herbicide, has widespread use on rice and wheat crops meaning that humans could be at risk of high level exposure (Moore *et al.*, 1998). Because of its persistence in the environment, human and aquatic animals are at risk of exposure. In this regard, PRP and its more toxic metabolite, 3, 4-dicloroaniline, have been identified in the blood of teleost fish and factory workers (Moraes *et al*., 2009, Morse and Baker, 1979). Many studies have shown biochemical and oxidative effects of PRP in the

blood of mice, rats and in various organs of aquatic organisms such as brain, muscles and liver (Sancho *et al.*, 2009, Moraes *et al.*, 2009). Specifically, PRP was found to increase the activities of alanine aminotransferase, lactate dehydrogenase and gamma-glutamyltransferase in European eel *Anguilla anguilla* (Sancho *et al.*, 2009); MDA levels in rats (Otuechere *et al.*, 2012) and Catalase activity in liver of teleost fish (Moraes *et al.*, 2009). It is now known that different toxic compounds cause oxidative stress in organisms. The effect of propanil on tissue susceptibility to lipoperoxidation, suggestive of the herbicide's ability to cause tissue damage, is believed to be one of the molecular mechanisms involved in pesticide-induced toxicity as well as a major contributor to the loss of cell function under oxidative stress conditions (Santillo *et al.*, 1995, Sevgiler *et al.*, 2007). The present study was undertaken to evaluate the effect of PRP on the biochemical indices and antioxidant systems of rat liver and kidney.

PROCEDURE

30 male rats of about 8 weeks old were divided into 5 groups of 6 each. Group A rats received olive oil for 7 days and served as control. Groups B, C, D and E rats were administered 25 mg/kg, 100 mg/kg, 150 mg/kg, and 200 mg/kg propanil, respectively, for 7 days (the rationale for the dose calculation was shown on page 48). Each rat was weighed before being killed. The liver and kidney were used for the assessment of histological changes and biochemical analyses. Serum biochemical markers were carried out to determine the concentrations of alanine and aspartate aminotransferases, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase, bilirubin, cholesterol, triglycerides, urea and creatinine using diagnostic kits from Randox Laboratories Limited, Crumlin, United Kingdom while estimation of tissue total protein, superoxide dismutase, catalase, glutathione s-transferase, glutathione and lipid peroxidation were quantified as described on pages 53-74.

RESULTS

Organ and relative organ weights

The absolute and relative weights of organs of control and PRP-treated rats are shown in Table 1A. The kidney organ and relative weights did not change significantly in the course of the experiment, compared with control. However, there was a dose-dependent increase in the absolute and relative liver weight compared with control rats.

Table 1A: **Effect of propanil (PRP) on organ weights and relative organ weights of liver and kidney in rats**

Group	Liver weight (g)	Relative liver weight (g/100g body weight	Kidney weight (g)	Relative kidney weight (g/100g body weight)
Control	4.72±1.03	2.34±0.17	1.14 ±0.09	0.58 ± 0.05
25 mg/kg	5.34±0.34	2.73±0.06	0.99 ±0.03	0.48 ±0.01
100 mg/kg	5.28±0.79	2.77±0.13	0.97 ±0.02	0.49 ±0.01
150 mg/kg	5.98±0.52	3.25±0.08	1.11±0.08	0.56 ±0.04
200 mg/kg	6.22±0.50	3.51±0.08	1.08 ±0.04	0.55 ±0.02

Values are expressed as mean ± SEM for five rats per group.

Table 1B: Serum clinical parameters in rats following 7 days treatment with Propanil

Parameters	Control	25mg/kg	100mg/kg	150mg/kg	200mg/kg
AST, U/I	4.96 ±2.5	7.23±3.40	10.43±1.6	9.04±2.50	10.48±4.2*
ALT, U/I	1.95±1.20	3.75±2.20	3.44±1.2	3.28±2.20	3.06±1.00
ALP, U/l	114.82±12.0	118.39±23.1	151.19±12.7	159.31±39.0	153.18±9.7
LDH, U/l	52.52±10.64	202.20±28.93	165.08±52.64	286.14±87*	342.54±32.2*
γ -GT , U/l	4.63±1.26	3.09±1.80	2.21±1.10	1.93±1.30	3.09±1.30
Bilirubin mg/dl	15.90±0.05	19.0±0.07*	17.7±0.05*	17.6±0.13*	17.40±0.14*
Cholesterol mg/dl	16.87±4.56	20.33±4.95	24.15±3.64	28.76±5.80	48.78±8.10*

Triglycerides, mg/dl	28.78±11.35	19.05±3.69	21.95±13.04	21.60±11.18	25.98±4.29
Urea, mg/dl	20.60±0.05	23.71±0.04*	22.33±0.07*	21.80±0.37*	23.38±0.04*
Creatinine, mg/dl	35.00±0.01	43.00±0.02*	43.00±0.02*	43.00±0.01*	69.00±0.07*

Values are expressed as mean ± SEM for five rats in each group. *Significantly different from control ($p<0.05$).

Serum Clinical Indices

Table 1B reveals that administration of PRP at various doses increased the AST, ALT, ALP and LDH activities when compared with the corresponding group of control animals. AST activity increased by 110% and 111% at 100 mg/kg and 200mg/kg respectively; ALT activity increased by 76%, 66% and 57% at 100 mg/kg, 150 mg/kg and 200 mg/kg respectively; ALP activity increased by 32%, 39% and 33% at 100 mg/kg, 150 mg/kg and 200 mg/kg respectively; LDH activity increased by >200% at all treatment doses. PRP, at 200 mg/kg, also induced significant increases of 9% and 189% in the levels of bilirubin and cholesterol, respectively, when compared with their corresponding group of control animals. PRP produced a significant increase ($p<0.05$) in serum creatinine and BUN concentrations, at all doses, compared with the control rats. However, γ –GT activity remained unaffected in all treatment groups when compared with controls.

Table 1C: Total protein, antioxidant system, MDA formation in the liver and kidney of Propanil-treated rats

Parameters	Control	25mg/kg	100mg/kg	150mg/kg	200mg/kg
Total Protein(liver)[a]	4.20 ±0.05	3.80±0.05	2.75±0.04**	2.25±0.04**	2.90±0.03**
Total protein(kidney)[a]	3.90±0.06	3.32±0.08*	3.42±0.21*	4.40±0.09	3.73±0.13
SOD(liver)[b]	60.00±0.17	63.00±0.19	116.00±0.24*	109.00±0.18*	97.00±0.18*
SOD(kidney)[b]	24.16±4.70	32.51±6.01	72.70±7.02*	72.73±14.26*	71.96±14.23*
CAT(liver)[c]	121.10±12.6	113.10±14.7	118.70±31.60	103.50±26.60	77.90±12.03
CAT(kidney)[c]	130.50±13.90	129.40±16.70	95.40±25.4	61.70±12.90*	60.50±9.40*

GST(liver)[d]	19.50±0.08	22.50±0.08	23.20±0.03	25.90±0.05	30.10±0.20
GST(kidney)[d]	23.73±3.00	27.98±5.50	30.45±6.20	28.92±6.90	48.40±5.40*
GSH(liver)[e]	21.73±3.60	19.24±3.70	15.58±4.23	21.32±4.84	27.23±3.18
GSH(kidney)[e]	18.71±0.90	17.35±0.31	17.51±0.49	18.00±0.39	17.41±0.81
MDA(liver)[f]	37.80±2.60	43.27±3.40	64.12±3.02*	66.40±7.50*	75.00±8.80**
MDA(kidney)[f]	39.90±2.43	56.9±6.75	52.50±2.90	56.80±5.00	67.90±5.10*

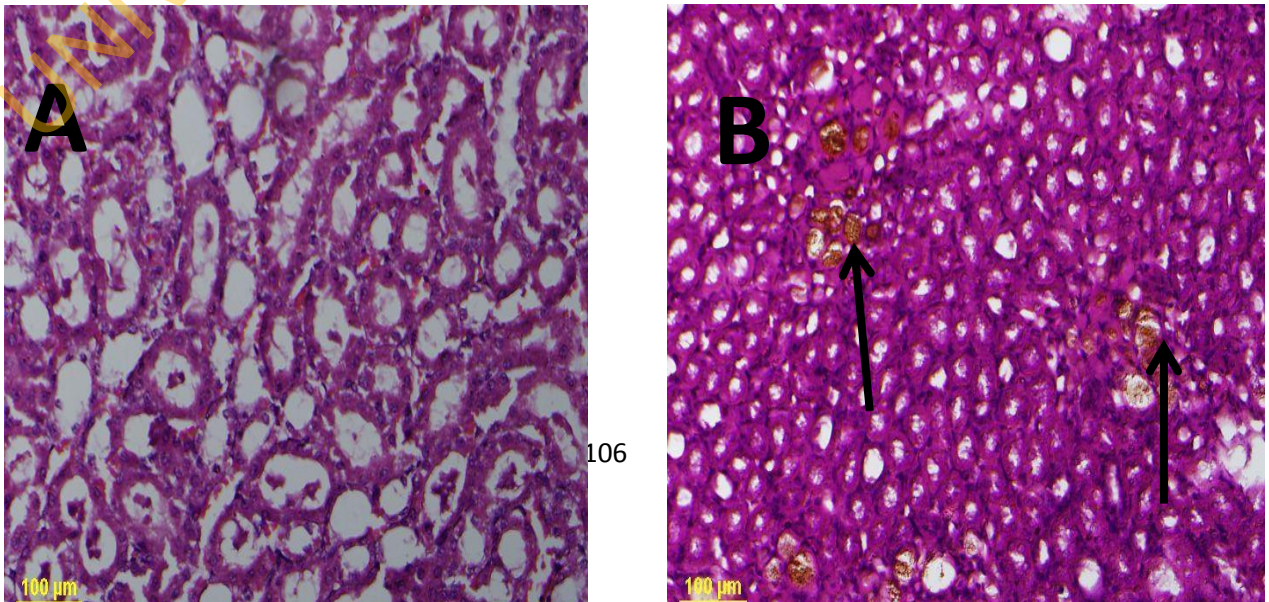
Values are expressed as mean ± SEM for five rats in each group. *Significantly different from control ($p<0.05$).

**Significantly different from control ($p<0.001$). [a]mg/g tissue, [b]Units/mg protein, [c]µmoleH_20_2consumed/min/mg protein, [d]µmol/min/mg protein, [e]µg/mg protein, [f]µgMDA/mg protein.

The effect of PRP on antioxidant systems and MDA formation in organs is illustrated in Table 1C. Treatment with PRP was associated with a decrease in protein levels in both renal and hepatic organs. These changes were significant in the liver at 100 mg/kg, 150 mg/kg and 200 mg/kg when compared with compared with the control. These changes were however not significant in the kidney when compared with the control group. Superoxide dismutase (SOD) activity significantly increased in the liver by 93%, 81% and 57% and also, in the kidney, by

200%, 201% and 198% at 100mg/kg, 150 mg/kg and 200 mg/kg respectively when compared with control rats. The activities of catalase (CAT) in liver decreased by 2%, 15% and 36%; kidney CAT showed a 27%, 53% and 54% decline in activity at 100mg/kg, 150mg/kg and 200mg/kg respectively when compared with control rats. Whereas hepatic glutathione-S-transferase (GST) activity was elevated by 18%, 32% and 54%, renal GST was increased by 56%, 48% and 103% at 100 mg/kg, 150 mg/kg and 200 mg/kg respectively when compared with control rats. In the kidney, reduced glutathione (GSH) levels decreased by 6%, 4% and 7% in the 100 mg/kg, 150 mg/kg and 200 mg/kg treatment groups, respectively, when compared with control rats. Hepatic GSH levels remained unaffected in all treatment groups. Administration of PRP at doses of 25 mg/kg, 100 mg/kg, 150 mg/kg and 200 mg/kg increased MDA levels in the liver by 14%, 69%, 76% and 98% and in the kidney by 43%, 32%, 42% and 70% respectively compared with normal animals.

Photomicrographs showing renal and hepatic morphology in all treatment groups are shown in Figures 1A and 1B. Kidney and liver sections from rats in control group were essentially normal. The PRP-treated kidney showed focal haemorrhages between the tubules of the cortex at 150 mg/kg and 200 mg/kg. Severe portal congestion was observed at 25 mg/kg, and in the highest dose group, periportal fibroplasia with severe mononuclear infiltration of the cells were seen in the liver cells.



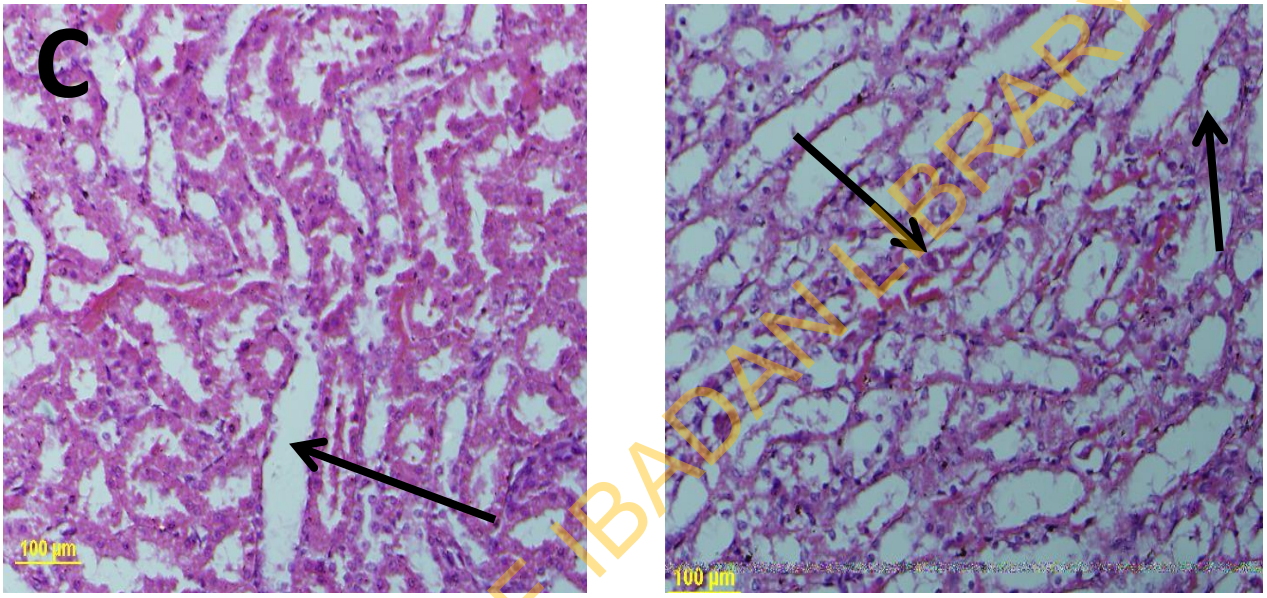
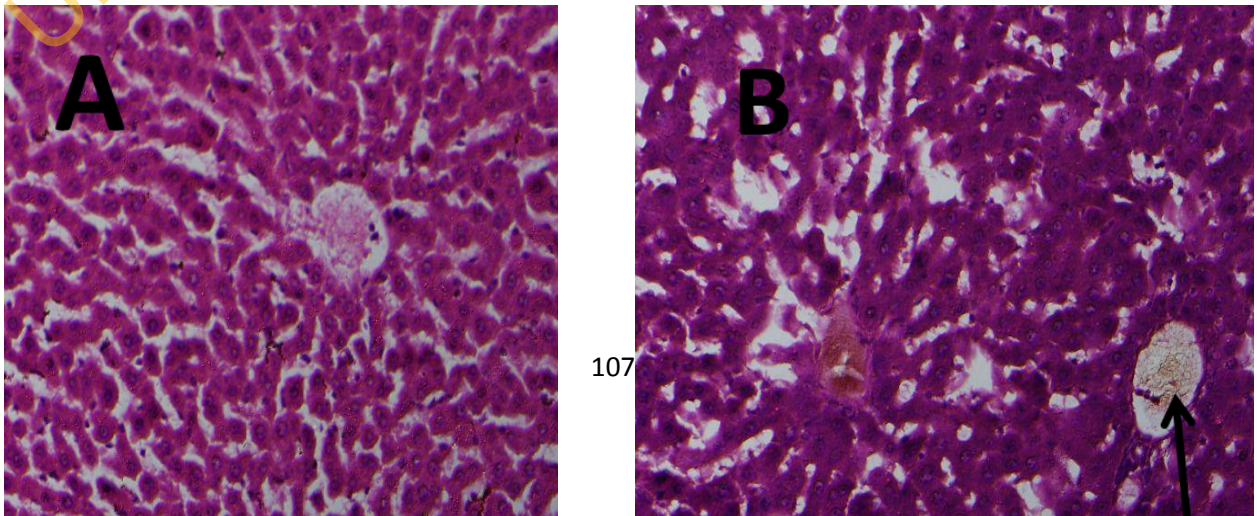


Plate 1: Light microscopic appearance of the kidney. Control kidney **(A):** Glomerular histo-architecture appears normal without any visible lesion. PRP-treated (25 mg/kg bw) kidney **(B):** congestion of cortical blood vessels. PRP-treated (150 mg/kg bw) kidney**(C):** focal hemorrhages between the tubules of the cortex. PRP-treated (200 mg/kg bw) kidney **(D):** focal hemorrhages and severe tubular necrosis. Original magnification X100. Propanil (PRP).



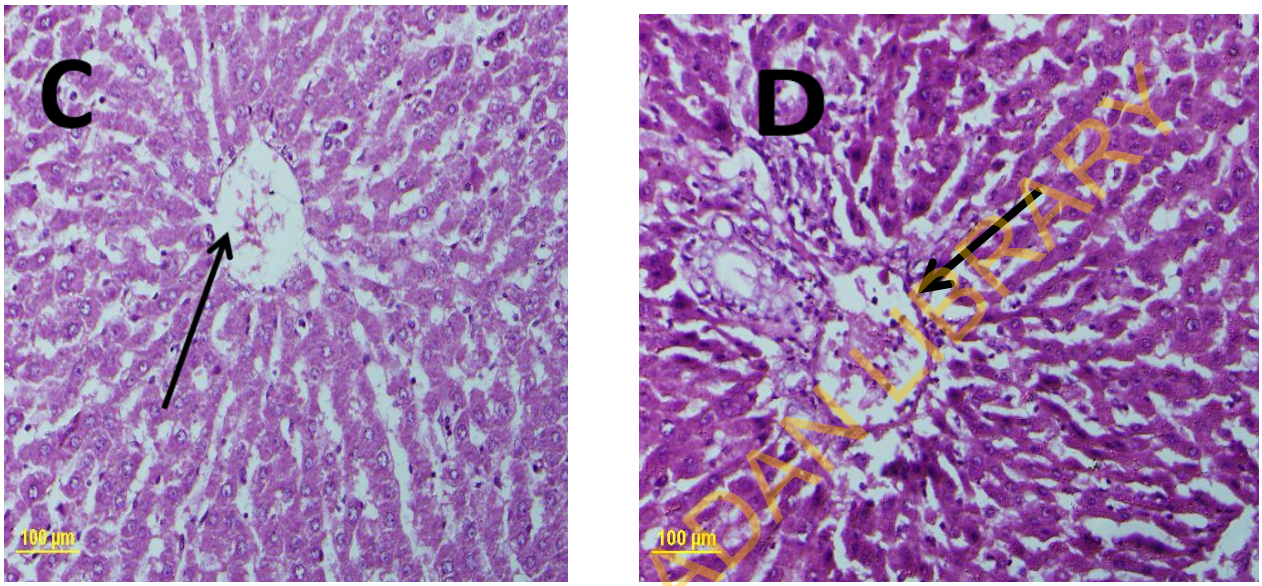


Plate 2: Light microscopic appearance of the liver. Control liver **(A):** Histoarchitecture appears normal without any visible lesion. PRP-treated (25 mg/kg bw) liver **(B):** portal congestion. PRP-treated (150 mg/kg bw) liver **(C):** mononuclear cellular infiltration and periportal fibroplasias. PRP-treated (200 mg/kg bw) liver **(D):** showing severe mononuclear cellular infiltration. Original magnification X100. Propanil (PRP).

Conclusion

The study demonstrated that PRP exposure produced damages on two key organs, the kidney and liver, by alteration of the anti-oxidant systems and lipid peroxidation. Additionally, PRP adversely affected clinical parameters in the kidney and liver. The primary alterations observed for liver tissue damage were hydropic degeneration of the hepatocytes, portal inflammation and fibrosis of the liver. Pathologies induced in kidney included necrosis of the proximal tubular epithelium, and focal hemorrhages in the interstitial areas of the cortex.

4.2.0 EXPERIMENT II: EFFECT OF *Pterocarpus mildbraedii* EXTRACT ON PROPANIL-INDUCED LIVER TOXICITY IN RATS

INTRODUCTION

Reactive oxygen and nitrogen species are produced by normal cellular metabolism with beneficial effects such as cytotoxicity against bacteria and other pathogens. Enzymes, such as nicotinamide adenine dinucleotide phosphate oxidases, nitric oxide synthases and myeloperoxidases whose functions are to produce ROS/RNS, also exist. Since these free radicals may also damage normal tissue, the balance between antioxidants and pro-oxidants is critical for normal function. An imbalance favoring pro-oxidants is defined as oxidative stress. Oxidative stress is proposed to be critical in various diseases including liver diseases (Muriel,

2009). Propanil, 3, 4 dichloropropionanilide, is a post-emergent herbicide used extensively for broad spectrum weed control in the production of rice and wheat. Multiple applications of propanil during a growing season are used to control weeds (Dahchour, *et al.*, 1986). It is now established to have adverse effects on the liver via its induction of oxidative stress amongst other mechanisms (Cakici and Akat, 2013**;** Otuechere *et al*., 2012; Santillo *et al*., 1995). Our preliminary study showed that exposure to PRP induced oxidative damage in the kidney and liver of rats.

The role of dietary phenolic compounds in the prevention of several chronic diseases has been the subject of intense research interest. Epidemiological studies have shown that the consumption of vegetables and fruits can protect humans against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species. Many plants including fruits and vegetables are recognized as sources of natural antioxidants that can protect against oxidative stress and thus play an important role in the chemoprevention of diseases that have their aetiology and pathophysiology in reactive oxygen species (Hamza *et al*., 2013). *Pterocarpus mildbraedii* (Harms), a medium sized tree found in the Eastern and Southern Nigeria, produces leaves which are used for soup making in Nigeria. The leaves, found to contain alkaloids, saponins, tannins, phenols and flavonoids, have been reported to protect against degenerative diseases and diabetes species. In the present study, we examined the protective effects of *Pterocarpus mildbraedii* extract against the toxicity of PRP in the liver of rats.

PROCEDURE

40 male albino rats, obtained from the Primate colony of the Faculty of Vertinary Medicine, University of Ibadan, were kept in wire mesh cages under controlled light cycle (12h/12 dark) and fed with commercial rat chow *ad libitum* and liberally supplied with water. The rats were distributed into eight groups of five animals each. Group A was the control and received normal saline. Group B received 100 mg/kg *Pterocarpus mildbreadii* extract (PME) while Groups C and D: received 200 mg/kg and 400 mg/kg PME respectively. Group E received Propanil (200 mg/kg); Group F: Propanil (200 mg/kg) + PME 100 mg/kg; Group G: Propanil (200 mg/kg) + PME 200 mg/kg; Group H: Propanil (200 mg/kg) + PME 400 mg/kg. Treatments were by oral intubation and lasted for 7 days. At the end of treatment, samples were collected for biochemical analyses as described in section 3.4.2 (page 48). The 7 day

treatment period and 200 mg/kg dose of PRP was based on findings that these conditions caused changes in antioxidant status in the liver and alterations in liver function indices (Santillo *et al.*, 1995; Otuechere *et al.*, 2012). Serum biochemical markers were carried out to determine the concentrations of alanine and aspartate aminotransferases, alkaline phosphatase, lactate dehydrogenase, gamma glutamyl transferase, bilirubin, cholesterol, triglycerides, urea and creatinine using diagnostic kits from Randox Laboratories Limited, Crumlin, United Kingdom while estimation of tissue total protein, superoxide dismutase, catalase, glutathione s-transferase, glutathione and lipid peroxidation were quantified as described on pages 53-74.. Histological observations of the liver were examined under a light microscope (section 3.9, page 74).

RESULTS

Organ and relative organ weights

The absolute and relative weights of organs of control animals and those treated with PRP alone and PRP plus various doses of PME are shown in Table 2A. There were no significant difference in absolute organ and relative weights when compared with control rats.

Table 2A: The absolute weights (g) and relative organ weights (g/100g body weight) of rats treated with Propanil (PRP) and *Pterocarpus mildbraedii* (PME) Extract

Treatment Groups	Absolute liver weight(g)	Relative liver weight/100g body weight
Control	6.56±0.49	3.96±0.29
PMEI	6.50±0.16	3.89±0.09
PMEII	6.94±0.44	4.10±0.26

PMEIII	7.24±0.63	4.04±0.35
PRP	8.47±0.53	4.59±0.29
PRP+PMEI	7.38±0.34	4.34±0.20
PRP+PMEII	8.30±0.59	4.79±0.34
PRP+PMEIII	8.34±0.46	4.53±0.25

Data expressed as mean ± SEM for five rats per group. Values are not significantly different when compared with the control ($p>0.05$). PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; PRP=200 mg/kg.

Liver Function Clinical Parameters

The effect of propanil treatment on liver function test is illustrated in Figures 2A to 2F. Propanil produced a significant increase in serum Aspartate aminotransferase (AST) activity ($p<0.05$), Lactate dehydrogenase (LDH) activity ($p<0.01$) and Bilirubin ($p<0.01$) concentrations and a moderate increase in serum Alanine aminotransferase, Alkaline phosphatase and γ-Glutamyl Transferase activities when compared with the control rats. Pretreatment with PME significantly improved hepatic function in the propanil-treated rats. Propanil -induced elevations in LDH decreased by 92%, 83% and bilirubin by 63%, 50% following treatment with 100 (PME I) and 200 mg/kg (PME II) PME respectively. The larger

dose (400 mg/kg) of the extract, however, did not seem to offer better protection against propanil toxicity when compared with that of the lower doses.

Antioxidant system

Following exposure to PRP, the activity of SOD increased compared with control animals. The extract dose dependently prevented PRP- induced increase in SOD activity of rats. In addition, PRP treatment resulted in a moderate decrease in catalase activity and GSH levels. PME treatment prevented the alterations in the activities of these enzymes and maintained their normalcy in PRP-treated rats. Propanil treatment increased activity of GST by 50% in the rats. PME when administered at 100 and 200 mg/kg/day doses restored the GST activity relative to control (Figure 2I). PRP treatment resulted in a significant increase in MDA levels, however treatment with PME at doses of 100 and 200 mg/kg reversed the increase in MDA levels back to normal (Figures 2G to 2K).

Histology and Gross Morphology

Control animals and groups treated with PME extract at the doses of 100 mg/kg and 200 mg/kg showed normal hepatic histoarchitecture. The livers of animals treated with PRP were degenerated as characterized by severe periportal infiltration and necrosis. Furthermore, exposure to PRP altered the gross histological appearance of the liver including increased liver weight and the presence of white pustular nodules on the surface of the liver. The morphologic characteristics of the liver were preserved and comparable to those in the control groups in animals treated with PRP plus PME (100 mg/kg and 200 mg/kg) (Plates 3 and 4).

Conclusion

The present study suggested that exposure of rats to propanil elicited marked alterations in cellular antioxidant and biochemical parameters in the liver. The dichloromethane: methanol leaf extract of *Pterocarpus mildbraedii* protected the liver from the detrimental effects of propanil through its effect on the antioxidative defense system and its ability to attenuate lipid peroxidation.

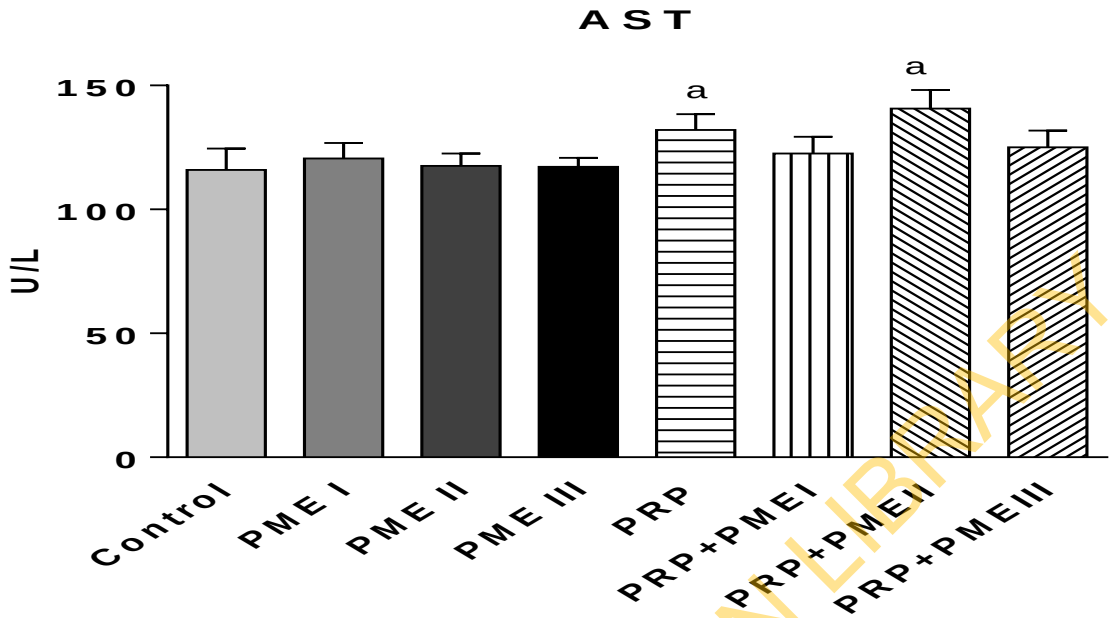


Figure 2A: Effect of *Pterocarpus Mildbraedii* extract (PME) on serum aspartate aminotransferase (AST) activity in PRP-exposed rats. Each bar represents mean± SEM of 5 animals. [a] Values differ significantly from control ($P<0.05$). PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

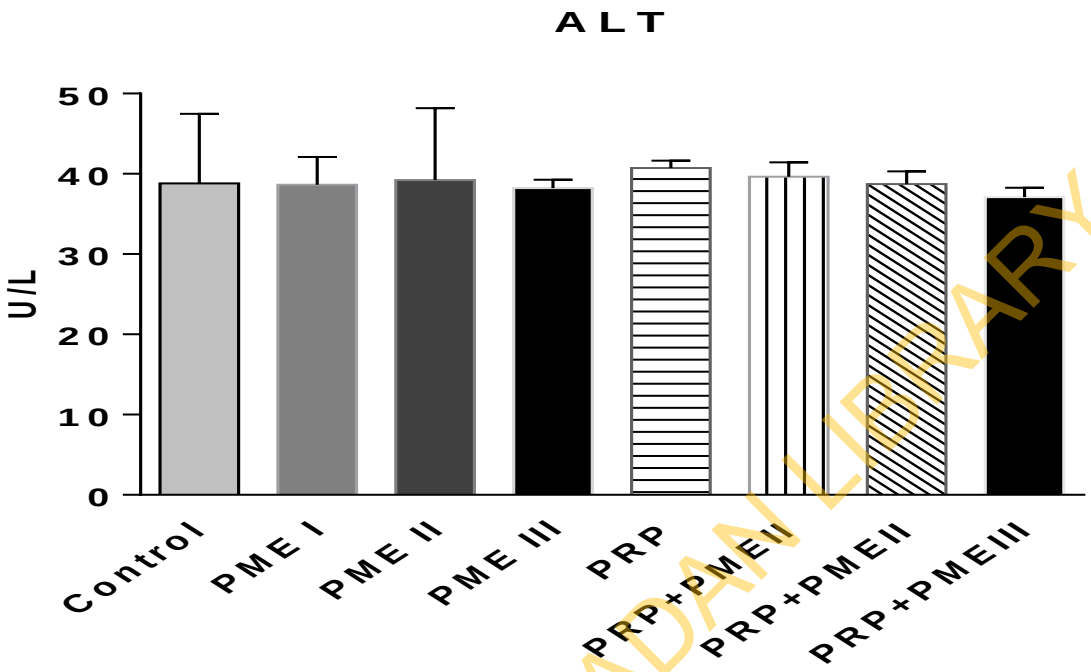


Figure 2B: Effect of *Pterocarpus Mildbraedii* extract (PME) on serum alanine aminotransferase (ALT) activity in PRP-exposed rats. Each bar represents mean± SEM of 5 animals. PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

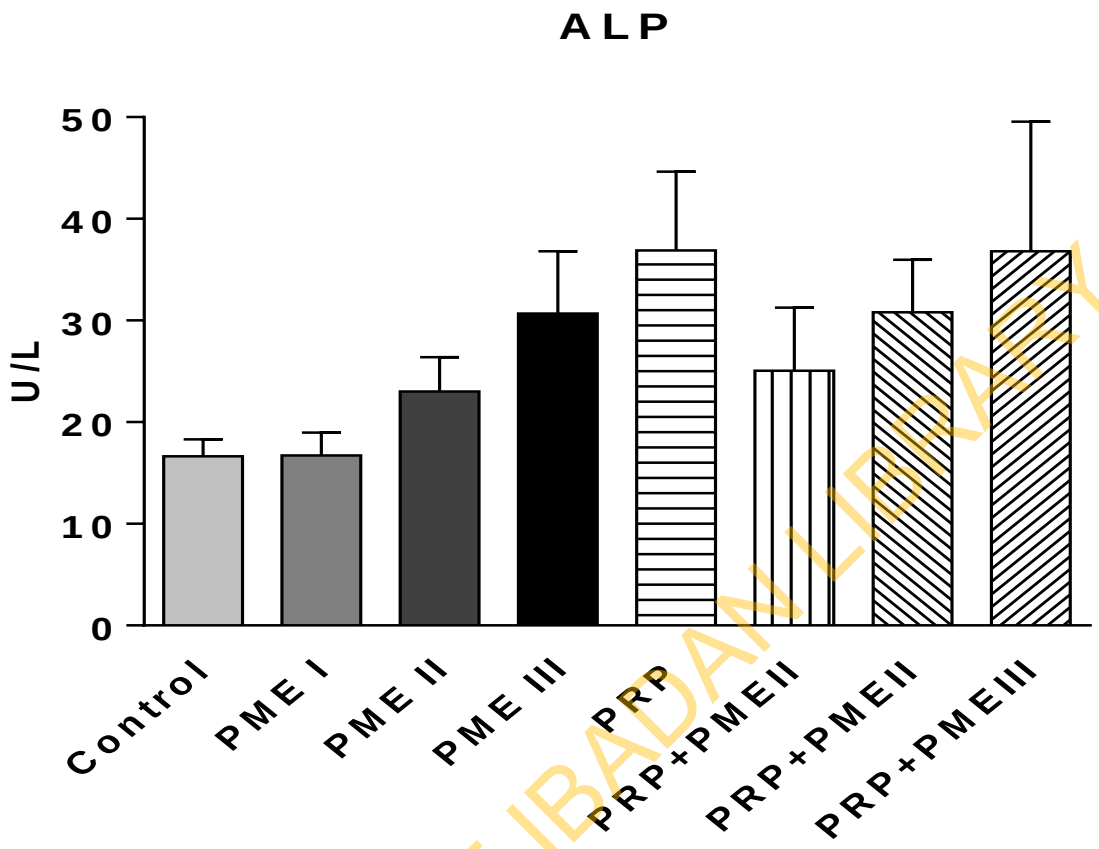


Figure 2C: Effect of *Pterocarpus mildbraedii* (PME) extract on serum Alkaline Phosphatase (ALP) activity in PRP-exposed rats. Each bar represents mean± SEM of 5 animals. PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

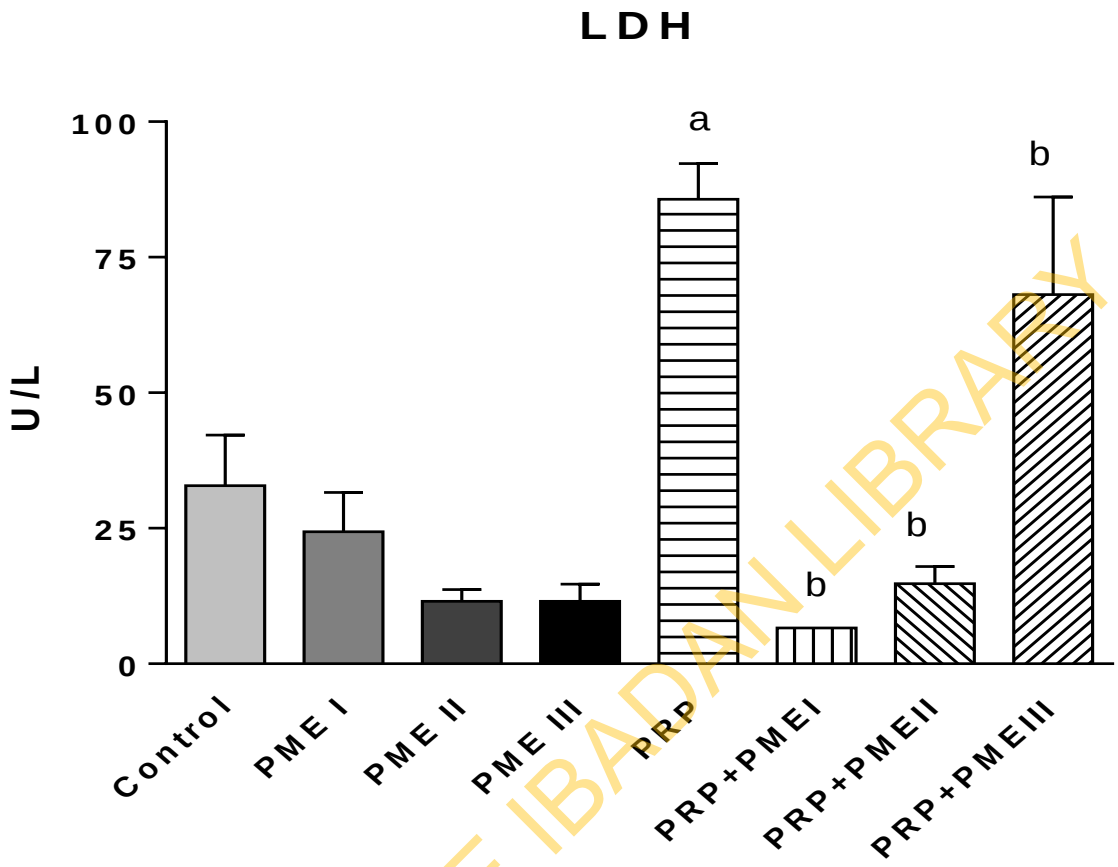


Figure 2D: Effect of *Pterocarpus mildbraedii* (PME) extract on serum Lactate Dehydrogenase (LDH) activity in PRP-exposed rats. Each bar represents mean± SEM of 5 animals. [a] Values differ significantly from control (P<0.01).[b] Values differ significantly from PRP (P < 0.001). PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

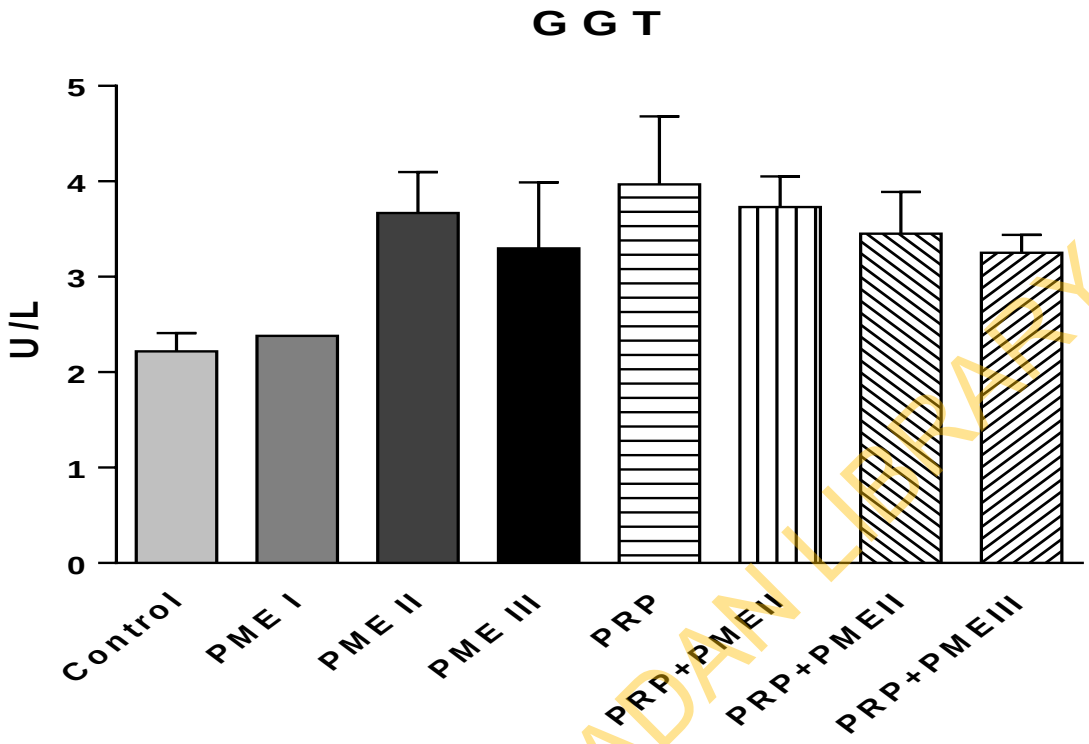


Figure 2E: Effect of *Pterocarpus mildbraedii* (PME) extract on serum γ-Glutamyl Transferase (GGT) activity in PRP-exposed rats. Each bar represents mean± SEM of 5 animals. PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

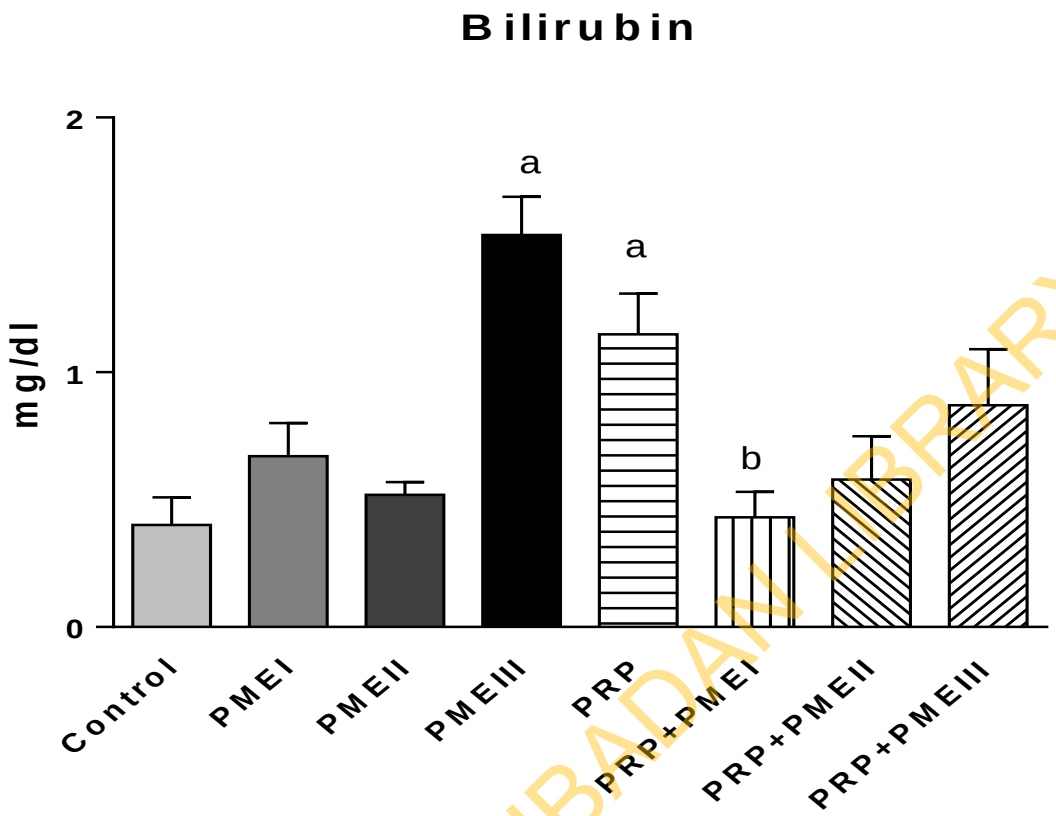


Figure 2F: Effect of *Pterocarpus mildbraedii* (PME) extract on Bilirubin level in PRP-exposed rats. Each bar represents mean± SEM of 5 animals. [a] Values differ significantly from control (P<0.001). [b] Values differ significantly from PRP (P<0.05). PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

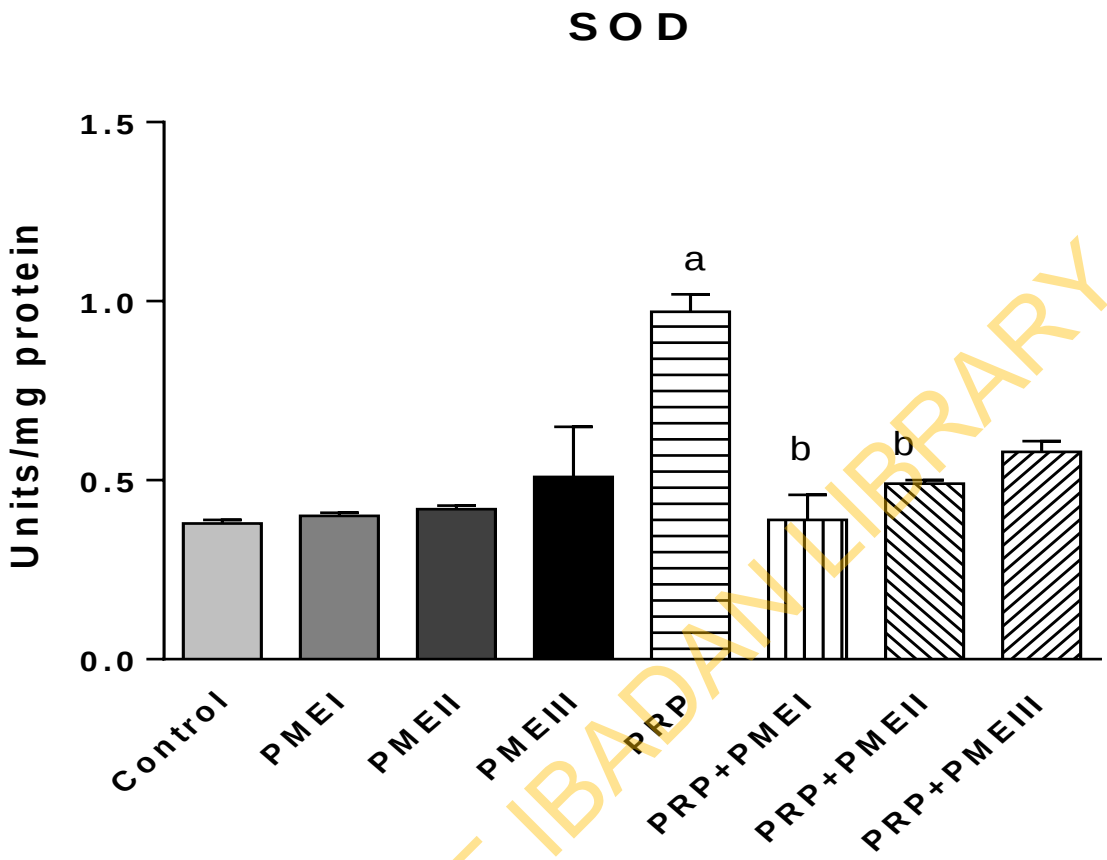


Figure 2G: Effect of *Pterocarpus mildbraedii* (PME) extract on Superoxide Dismutase (SOD) activity in liver of PRP-exposed rats. Each bar represents mean± SEM of 5 animals. [a] Values differ significantly from control (P<0.001).[b] Values differ significantly from PRP (P<0.05).PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

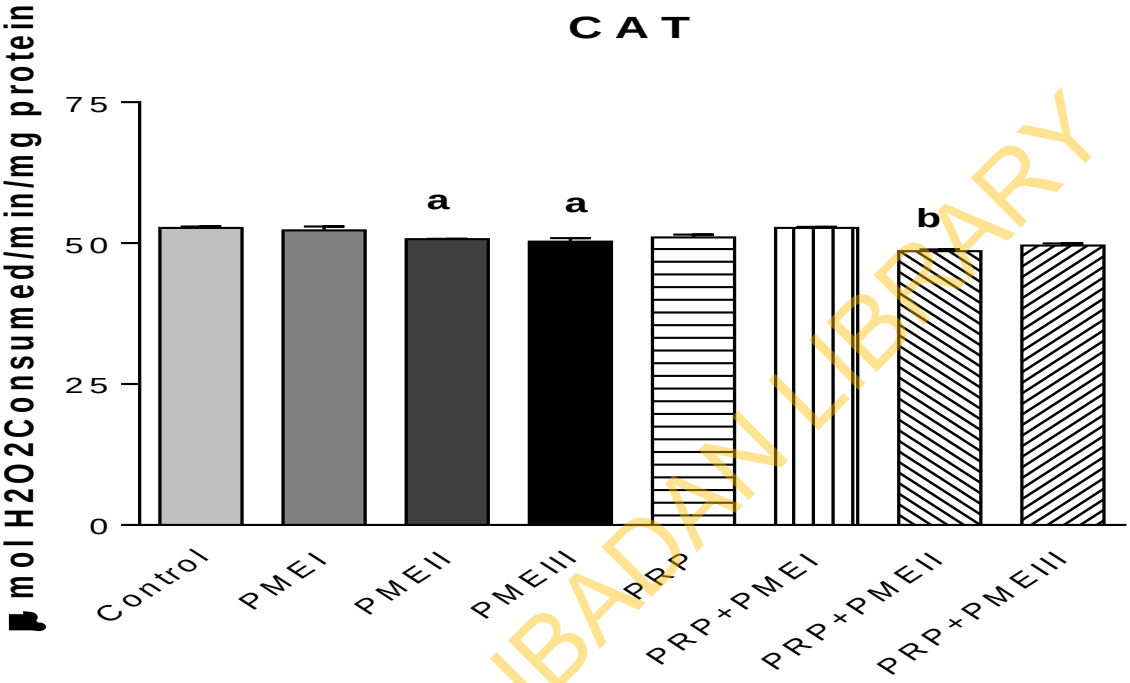


Figure 2H: Effect of *Pterocarpus mildbraedii* (PME) extract on Catalase (CAT) activity in liver of PRP-exposed rats. Each bar represents mean± SEM of 5 animals. [a] Values differ significantly from control($P<0.001$).[b] Values differ significantly from PRP ($P<0.05$).PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP)=200 mg/kg.

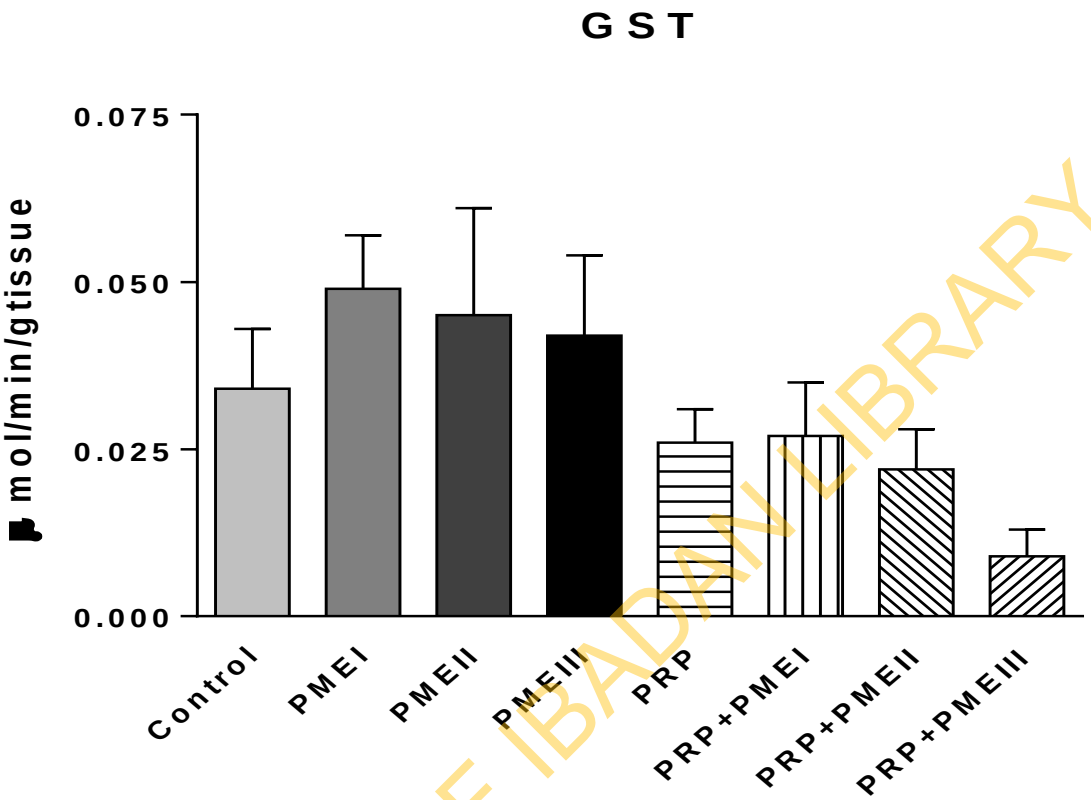


Figure 2I: Effect of *Pterocarpus mildbraedii* (PME) extract on Glutathione S-Transferase (GST) activity in liver of PRP-exposed rats. Each bar represents mean± SEM of 5 animals. PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

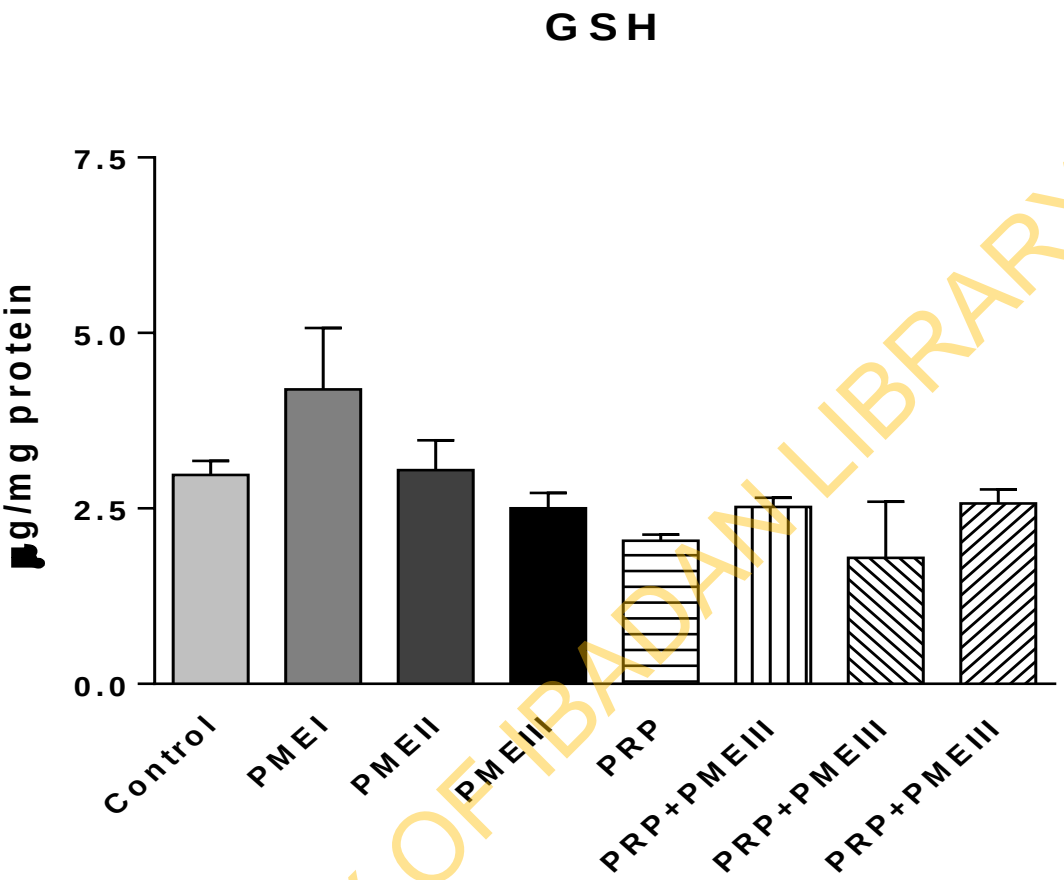


Figure 2J: Effect of *Pterocarpus mildbraedii* (PME) extract on Glutathione (GSH) levels in liver of PRP-exposed rats. Each bar represents mean± SEM of 5 animals. PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

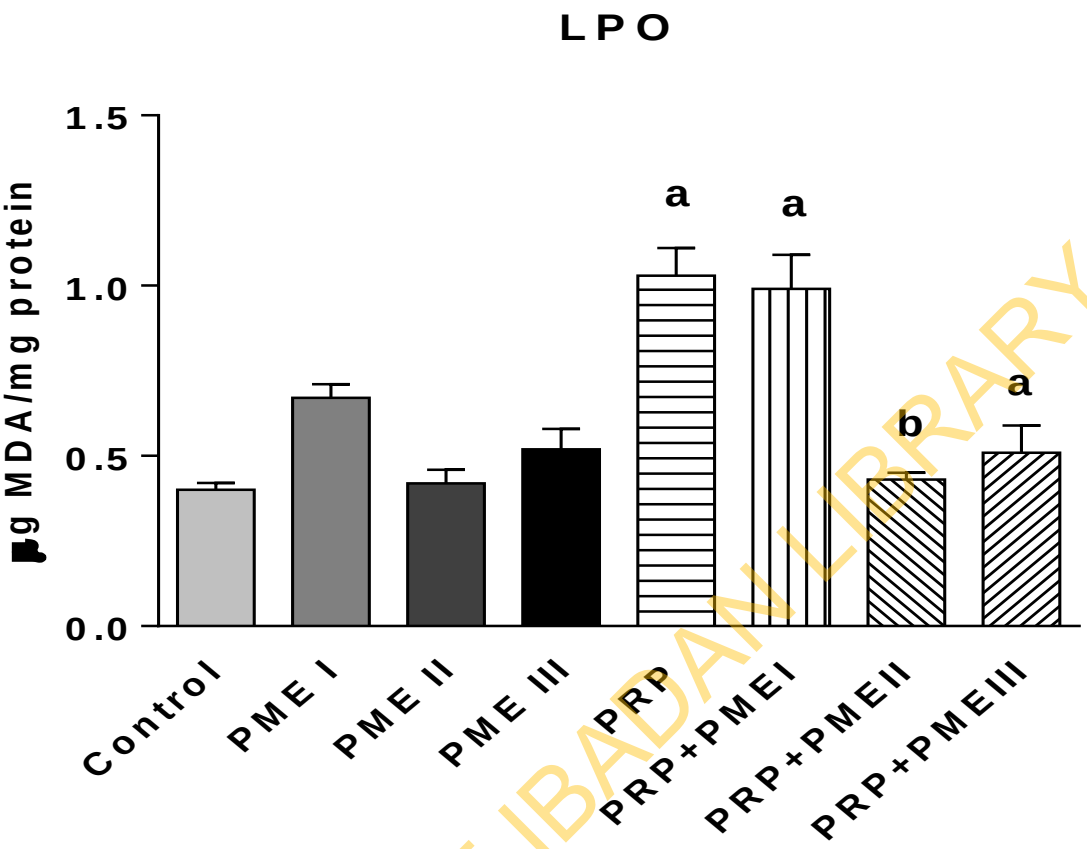


Figure 2K: Effect of *Pterocarpus mildbraedii* (PME) extract on Malondialdehyde (MDA) level in liver of PRP -exposed rats. Each bar represents mean± SEM of 5 animals. [a] Values differ significantly from control (P<0.001).[b] Values differ significantly from PRP (P<0.001). PMEI=100 mg/kg; PMEII=200 mg/kg; PMEIII=400 mg/kg; Propanil (PRP) =200 mg/kg.

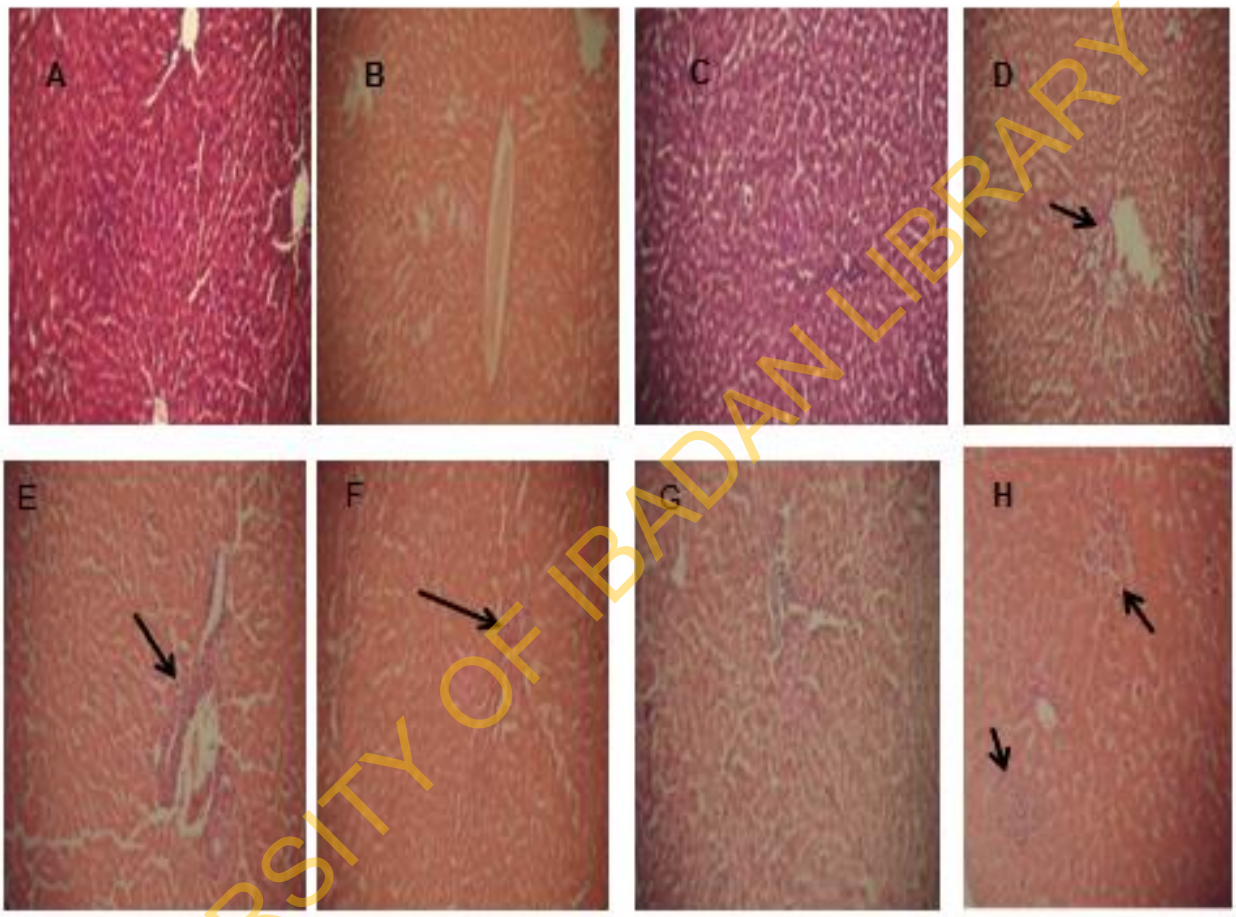


Plate 3: Representative photomicrographs of control and treated liver. (A) Control; (B) PME I (100 mg/kg) and (C) PMEII (200 mg/kg) with normal architecture and showing no visible lesion.(D) PME III (400 mg/kg) showing mild periportal cellular infiltration. (E) PRP (200 mg/kg) showing severe periportal cellular infiltration. (F) PRP + PMEI (100 mg/kg) and (G) PRP+PMEII (200 mg/kg) showing moderate periportal cellular infiltration. (H) PRP +PMEIII (400 mg/kg) showing diffuse hydropic degeneration of hepatocytes. H&E stain at X 400. *Pterocarpus mildbraedii* (PME) extract; Propanil (PRP).

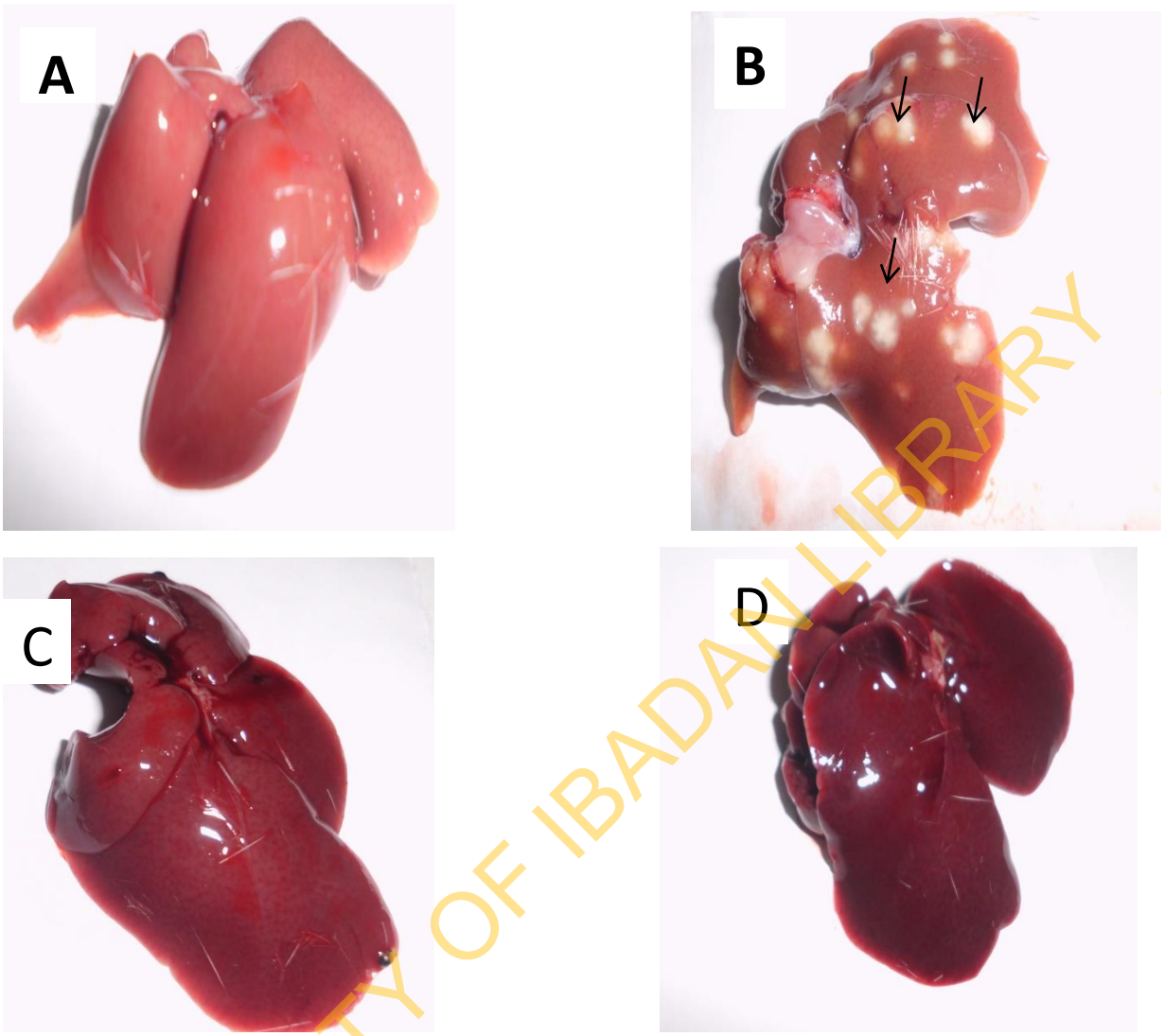


Plate 4: Representative gross morphology of control and treated liver. (A) Control with normal looking morphology; (B) PRP (200 mg/kg) showing gross alterations and appearance of white nodules on the surface (arrow) of liver. (C) PME (200 mg/kg) showing a normal looking appearance; (D) PME (200 mg/kg) + PRP (200 mg/kg) with normal architecture, but vascularized appearance and showing no visible lesion. *Pterocarpus mildbraedii* (PME) extract; Propanil (PRP).

4.3.0 EXPERIMENT III: EFFECT OF *Pterocarpus mildbraedii* EXTRACT ON PROPANIL-INDUCED INFLAMMATION IN RATS

Introduction

Inflammation is a defense response of living tissues to injurious stimulus. The inflammatory response involves the activation of several immune cells such as monocytes/macrophages which secrete a series of pro-inflammatory mediators such as enzymes, cytokines, chemokines as well as signaling proteins at the site of infected tissues and cells. Dysregulation of the inflammatory immune responses can lead to a variety of diseases, such as cancer, atherosclerosis, rheumatoid arthritis, neurological diseases, diabetes, pulmonary disorders, and allergies (Choudhari et al., 2013).

Cyclooxygenase-2 is an enzyme involved in inflammatory processes and a rate limiting enzyme in prostaglandin biosynthesis from arachidonic acid (Farombi *et al*., 2009). Inducible nitric oxide synthase (iNOS) is another inducible enzyme that causes the overproduction of nitric oxide during inflammation and tumor development (Chung *et al*., 2007). Nitric oxide has been implicated in initiation, promotional stage of neoplasmic transformation and in tumor progression by regulating angiogenesis. Therefore, suppression of the induction and activity of COX-2 and/or iNOS has been considered a new paradigm in chemoprevention in several organs (Chung *et al*., 2007). NF-κB signaling, a regulator of cell survival, immunity and inflammation, is one of the more important pathways that is activated during liver injury and inflammation and has been studied quite extensively in mouse models of liver carcinogenesis. Furthermore, NF-κB is recognized to be an important redox-sensitive transcriptional factor that regulates transcription of genes encoding inflammatory cytokines, adhesion molecules, and chemokines. STAT 3 is another transcriptional factor involved in immune responses, inflammation and tumorigenesis, and was found to be critical for compensatory liver regeneration (He and Karin, 2011). One of the most relevant aspects in the regulation of chemical apoptosis is the signaling by mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases which mediate intracellular signal transduction in response to different physiological stimuli and stressing conditions. Three major MAPKs have been identified, namely c-Jun NH2- terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) 1/2. It is normally considered that JNK and/or p38 activation is associated to apoptosis induction, and that ERK activation favors proliferation and promotes cell survival. However, the roles of these kinases either pro-apoptotic or survivalist depend on the cell model and experimental conditions (Kim and Chung, 2008; Brozovic and Osmak, 2007; Roux and Blenis, 2004). The present study is designed to investigate the possible effects of PRP on the levels of

inflammation-related proteins and upstream kinases in adult rat liver. A further aim of this study was to identify the molecular targets for PME.

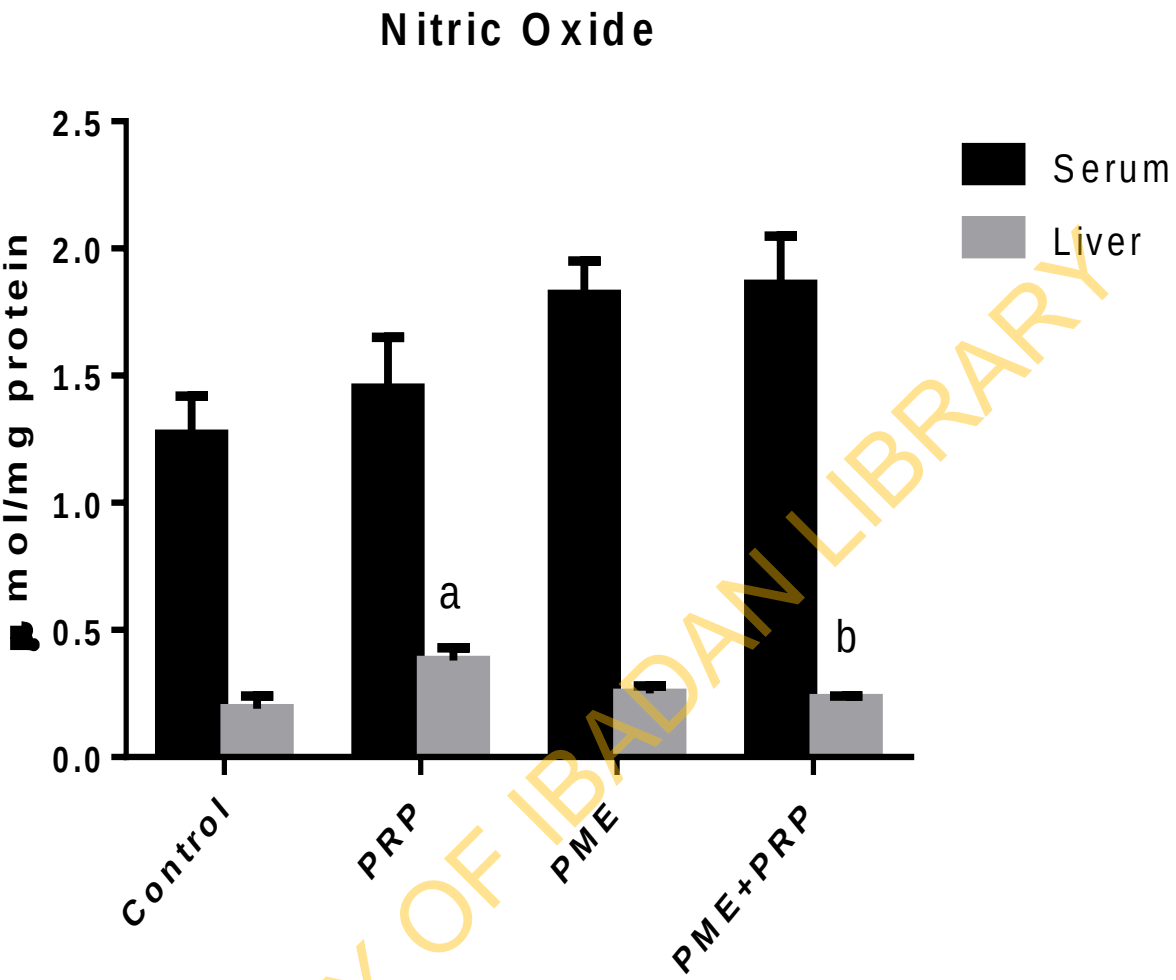
PROCEDURE

The experiment was set up as described in section 3.4.3, page 47. Liver lysates were processed for ELISA and biochemical analyses as follows: Liver tissues weighing 100 mg were homogenized in 1 mL lysis buffer; 20 mM Tris pH=7.5, 150 mM Nacl, I mM ethylene diamine tetraacetate, 1 mM ethylene glycol bis (2 amino ethyl) N, N, N', N' tetraacetic acid, 1% Triton X-100, 2.5 mM pyrophosphate, 1mM ß-glycerophosphate, 1 mM sodium vanadium oxide, 1µg/mL leupeptin. Samples were then centrifuged at 10,000 *g* for 15 minutes at 4^{o}C and supernatants collected and stored at -20^{o}C (page 47), and liver tissue was processed for immunohistochemistry (section 3.10.0, page 74) as described in the materials and method section of Chapter three.

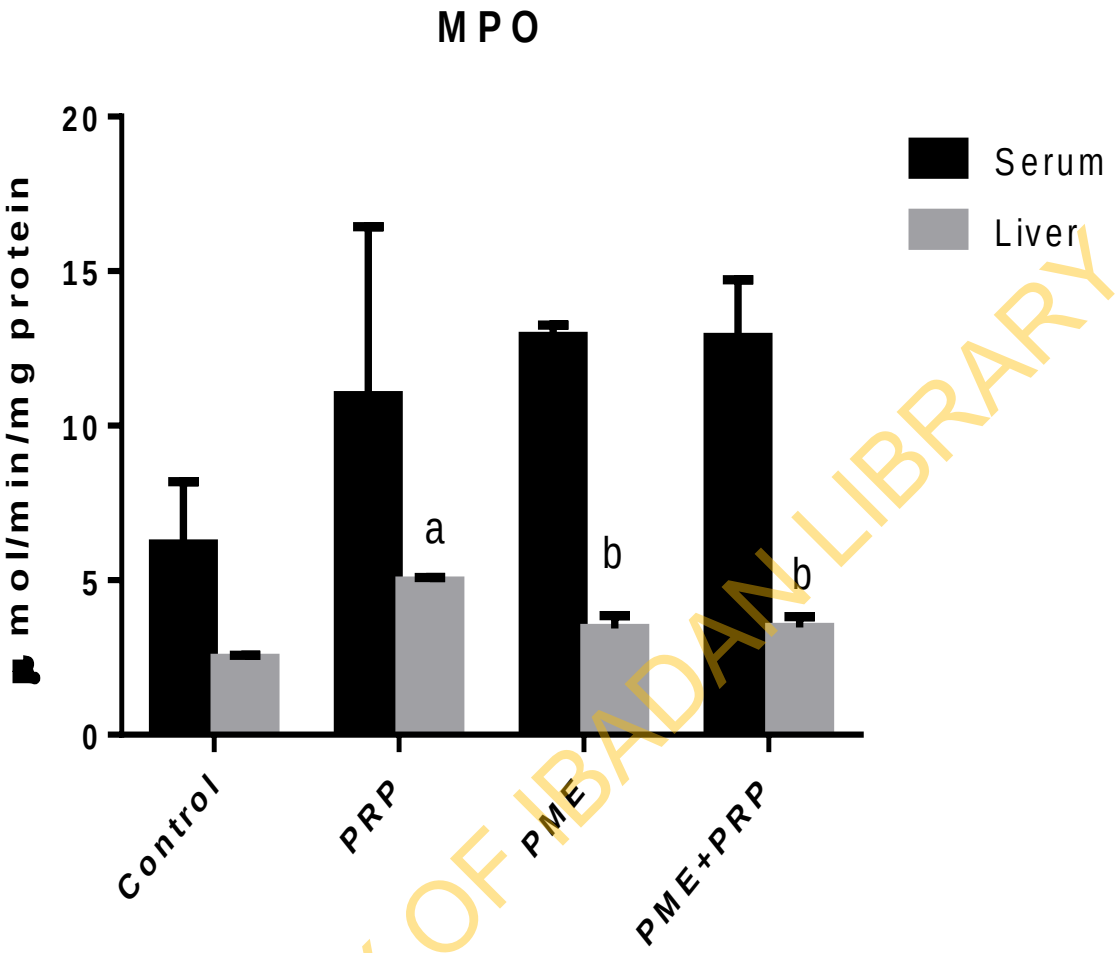
RESULTS

Serum and organ markers of inflammation

The effect of *Pterocarpus mildbraedii* (PME) extracts on levels of Nitric Oxide (NO) and Myeloperoxidase (MPO) in serum and liver of PRP exposed rats is shown in Figures 3A and 3B. There were no significant changes in the serum NO and MPO levels across all treatment groups. However, PRP elicited a significant increase in liver NO levels when compared with controls, and this increase was mitigated upon administration of PRP. This trend was also observed in the liver MPO activity, where PME restored the PRP-induced elevation to near normal levels.



Figures 3A: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of Nitric Oxide (NO) in serum and liver of Propanil (PRP) exposed rats. The data are expressed as mean± SEM; n=5. [a]Values differ significantly from control ($p<0.01$). [b]Values differ significantly from PRP ($p<0.05$).



Figures 3B: Effect of *Pterocarpus mildbraedii* (PME) extract on Myeloperoxidase (MPO) activity in serum and liver of Propanil (PRP) exposed rats. The data are expressed as mean± SEM; n=5. [a]Values differ significantly from control($p<0.01$). [b]Values differ significantly from PRP ($p<0.05$).

Effect of PME on PRP-induced levels of NF-κB, phospho-NF-κB p65 and I-κB

Following exposure, the livers of treated rats were lysed and cell lysates assayed at absorbance 450 nm for the determination of protein levels of NF-κB and phospho-NF-κB p65(ser 536), using a Pathscan Inflammation Multi-Target Sandwich ELISA from Cell Signaling Technology. Compared to control, cytosolic NF-κB p65 levels were depleted following treatment with PRP. However there was an increase in phospho NF-κB p65 levels in the PRP-treated group. Co-treatment of PME + PRP resulted in a significant reduction in these alterations (Figures 3C and 3D). Furthermore, administration of PME significantly reduced the PRP-induced increase in I-κB levels (Figure 3E). Immunohistochemical staining also showed an increased immune- positive cells in the PRP-treated groups when compared with control group as well as the amelioration afforded by PME co-treatment (Plate 7).

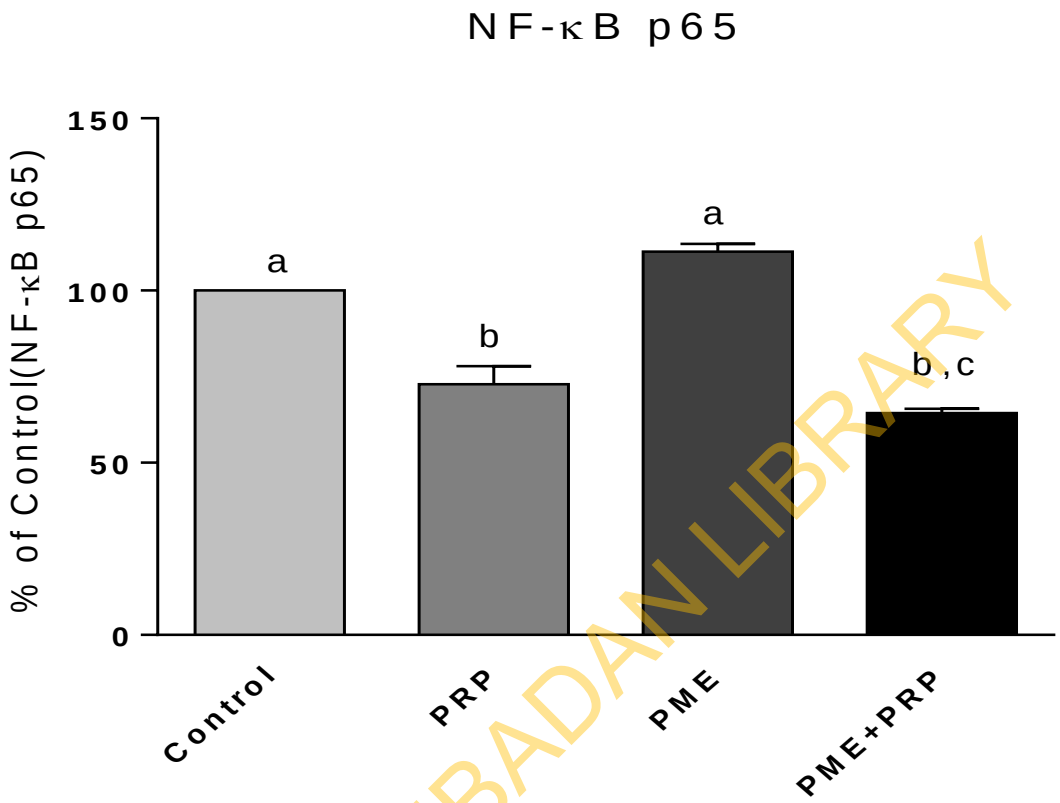


Figure 3C: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of Nuclear factor kappa B (NF-κB p65) in Propanil (PRP) - exposed rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.

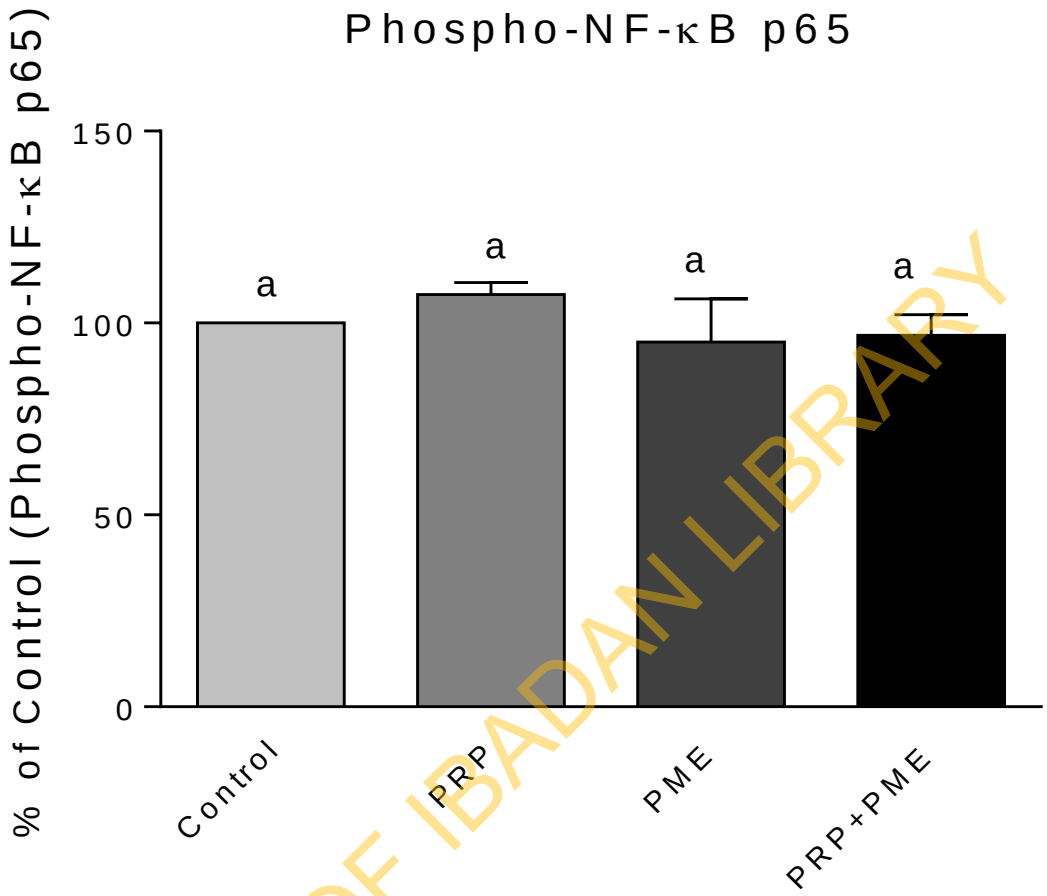


Figure 3D: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylated Nuclear factor kappa B (Phospho-NF-κB p65 (S536) in Propanil (PRP) - exposed rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.

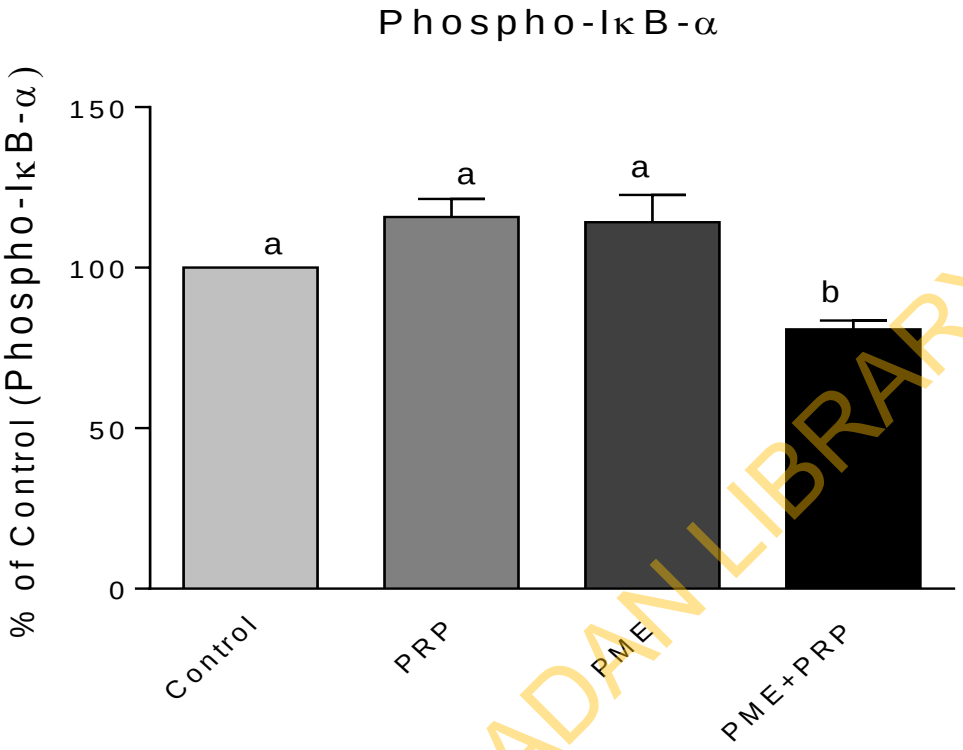


Figure 3E: Effect of *Pterocarpus mildbraedii* (PME) extract on levels Phospho- IκB-α in Propanil (PRP) - exposed rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.

Immunohistochemical staining of COX-2 and INOS

On immunohistochemical evaluation, plates 5 and 6 shows the effects of the treatment of PME on the expression of COX-2 and iNOS, respectively, in the liver of PRP-treated rats. The expressions of the COX-2 and iNOS in rats exposed to PRP alone was more intense compared with control. There were minimal or no expression of these proteins in the control group. There was an observed decrease in the number of COX-2 and iNOS immune positive cells in the PRP group pre-treated with PME.

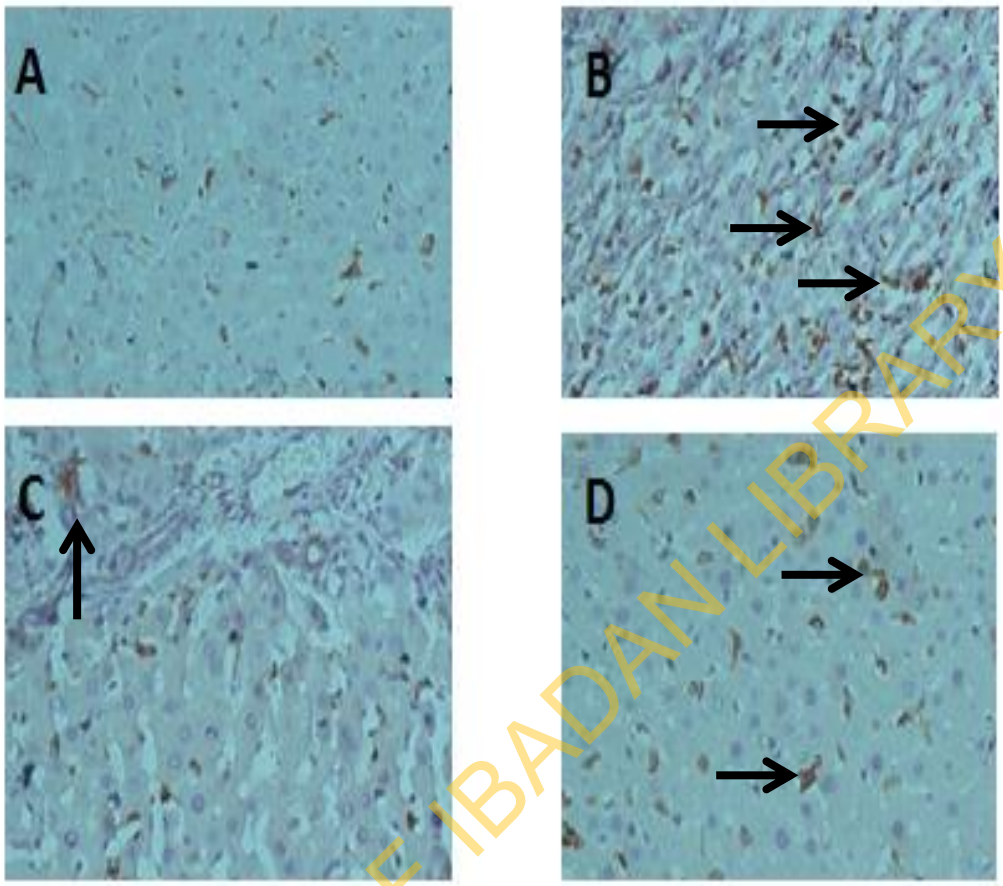


Plate 5: Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on Cyclooxyegense-2 (COX-2) expression in the liver of Propanil (PRP) -treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. More intense expression of COX-2 was observed in B when compared with other groups. Original magnification X 400.

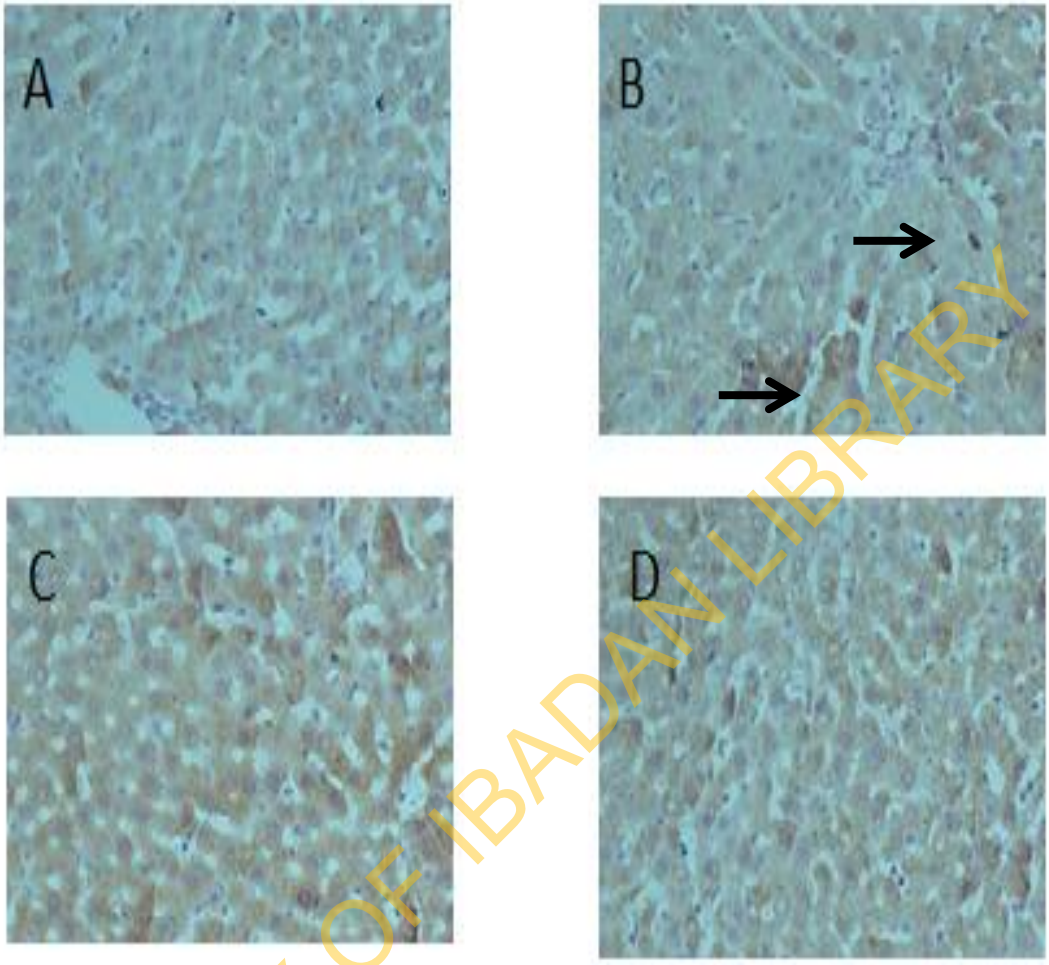


Plate 6: Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on inducible nitric oxide synthase (iNOS) expression in the liver of Propanil (PRP) - treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. Abnormal expression of iNOS was observed in B when compared with other groups. Original magnification X 400.

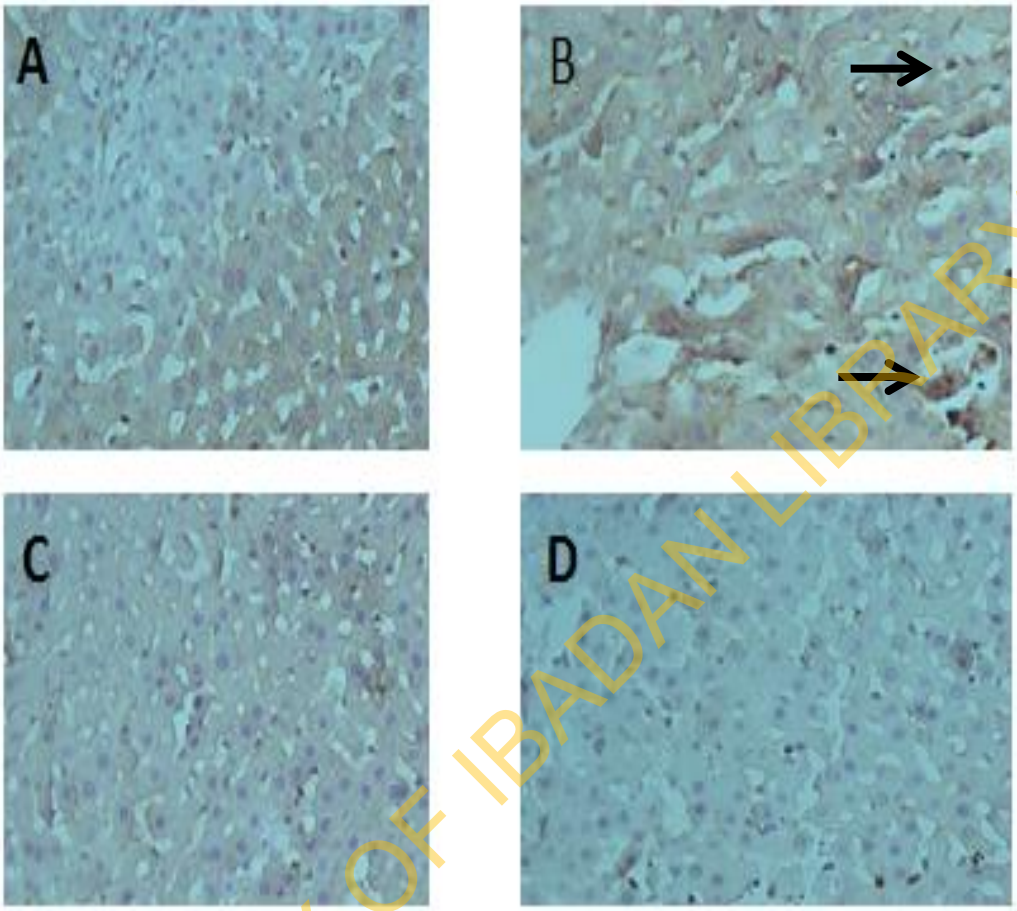


Plate 7: Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on Nuclear factor kappa B (NF-*κ*B) expression in the liver of Propanil (PRP) -treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. Abnormal expression of NF-*κ*B was observed in B when compared with other groups. Original magnification X 400.

Effect of PME on PRP-induced expression of phospho- p38MAPK, phospho- SAPK/JNK and phospho- Stat3

Following exposure of rats to PRP and PME treatment, the livers from animals were lysed in lysis buffer and assayed at absorbance of 450 nm for the determination of protein levels of phospho- p38MAPK (Thr 180/Tyr 182), phospho-SAPK/JNK (Thr 183/Tyr 185) and phospho--Stat 3 (Tyr 705) using a Pathscan Inflammation Multi-Target Sandwich ELISA from Cell Signaling Technology. There was a significant decrease ($p < 0.05$) in the phospho- p38MAPK, phospho- SAPK/JNK and phospho-Stat 3 levels in PME-treated rats compared with the PRP group (Figures 3F to 3H).

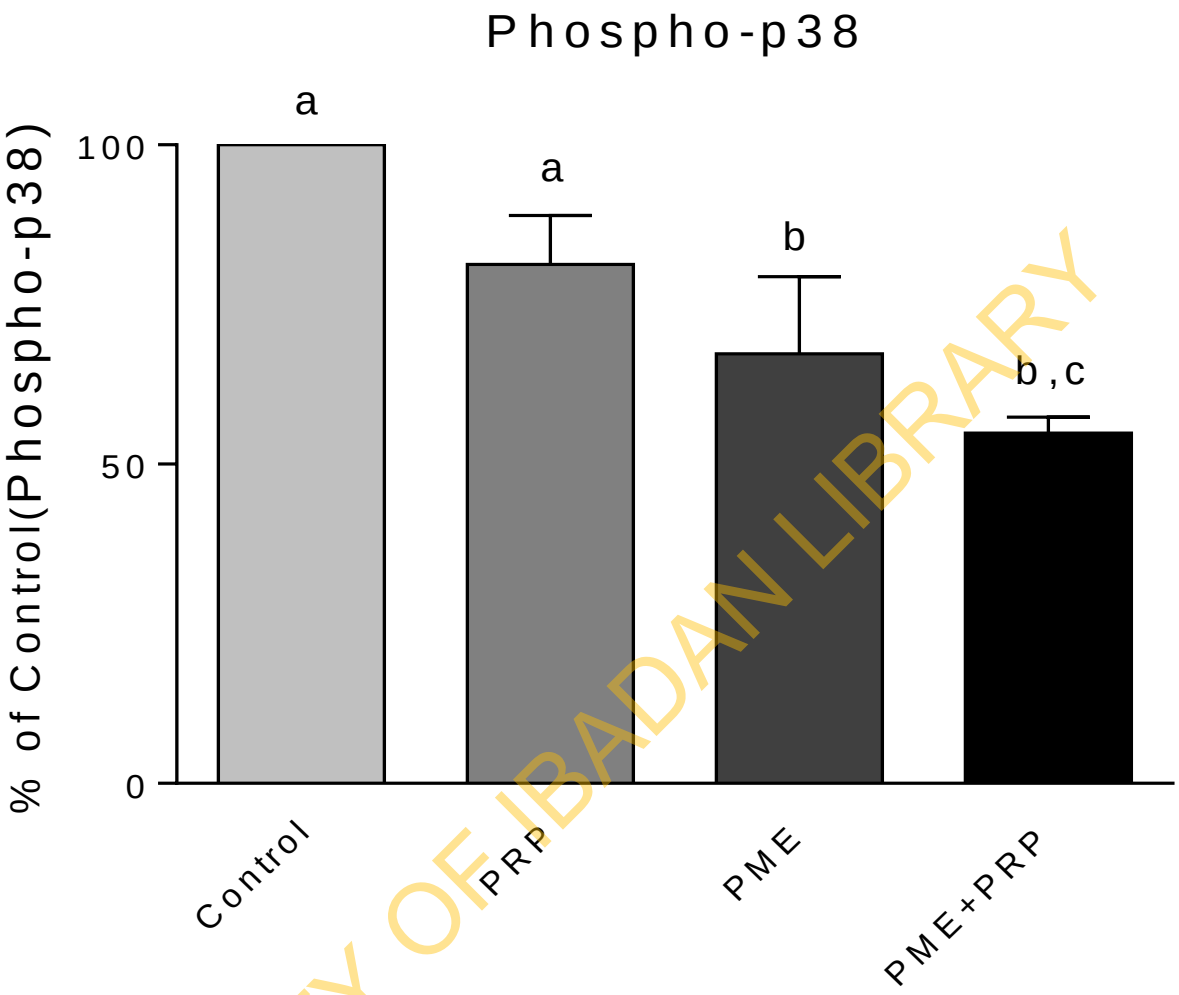


Figure 3F: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylated p38 mitogen-activated protein kinase (Phospho-p38) in Propanil (PRP) exposed rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. [a,b,c] Mean values with unlike letters are significantly different.

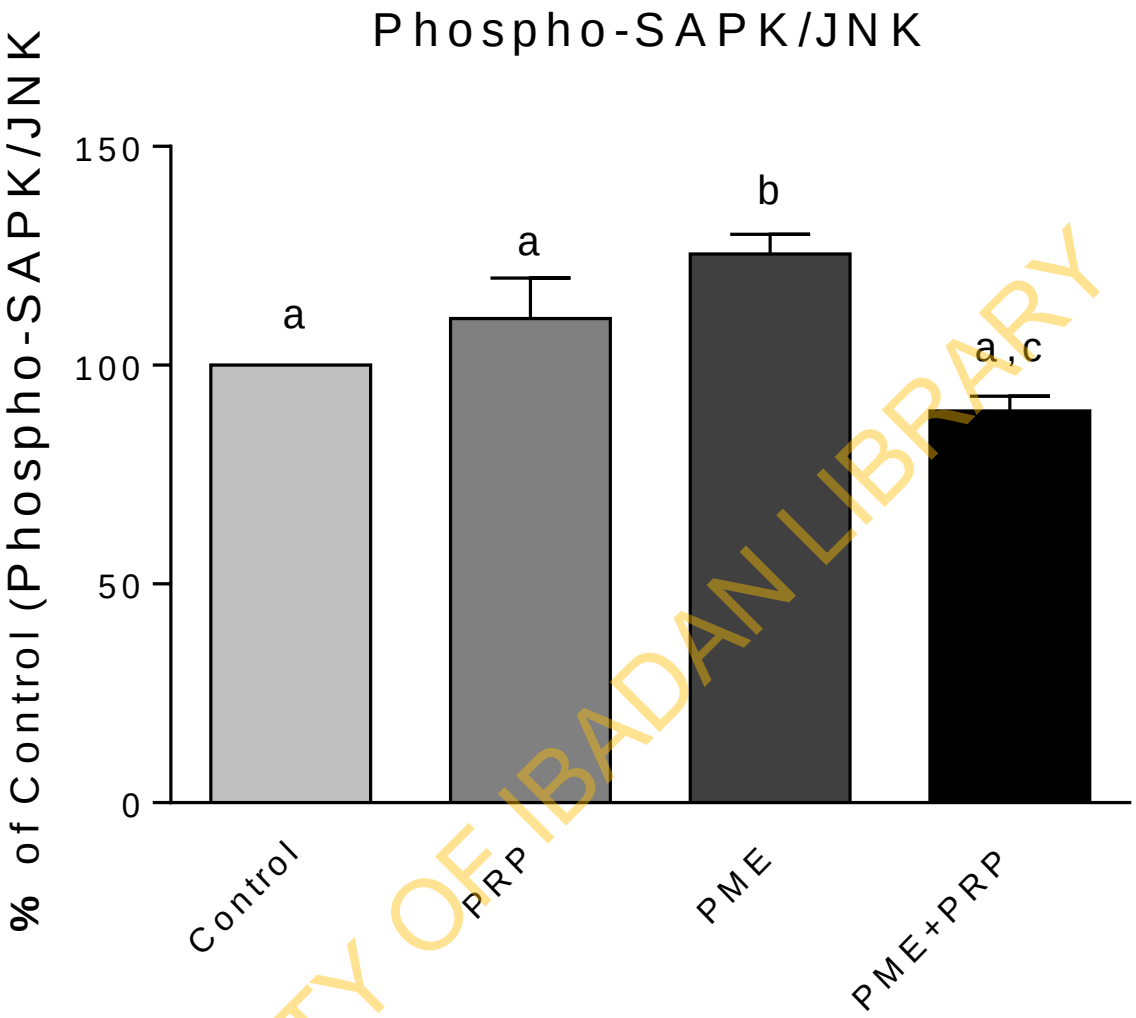


Figure 3G: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylated stress activated protein kinase/ C Jun NH_2-terminal kinase (Phospho-SAPK/JNK) in Propanil (PRP) exposed rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. [a,b,c] Mean values with unlike letters are significantly different.

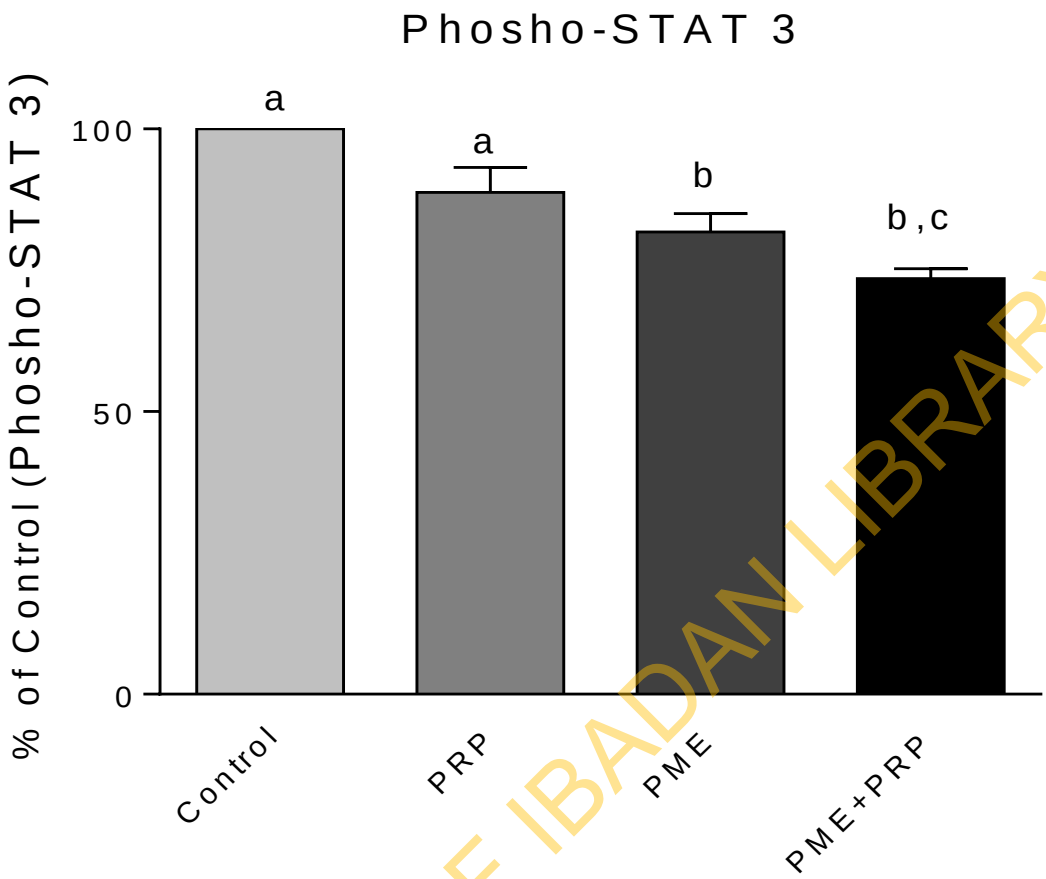


Figure 3H: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylated signal transducer and activator of transcription 3 (Phospho-Stat 3) in Propanil (PRP) exposed rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. [a,b,c] Mean values with unlike letters are significantly different.

Conclusion

In conclusion, data from this study demonstrated the ability of PME to inhibit COX-2 and iNOS expression through down regulation of NF-κB and deregulaton in the activities of the upstream kinases. This could be a mechanism to explain the hepatoprotective effect of PME on propanil-induced hepatotoxicity.

4.4.0 EXPERIMENT IV: EFFECT OF *Pterocarpus mildbraedii* EXTRACT ON PROPANIL-INDUCED APOPTOSIS IN RATS

Introduction

Statistics reveal that over 98% of sprayed insecticides and 95% of applied herbicides reach a destination other than their targeted species, including air, water, bottom sediments, food and non-target living systems, eventually producing devastating effects on non- target organisms, including humans (Agrawi and Sharma, 2010). The ability to trigger apoptosis, through alterations in redox homeostasis generated by a decrease of antioxidant defenses and accumulation of reactive oxygen species, is a possible mechanism of toxicity of several pesticides (Elsharkawy *et al.,* 2013; Franco *et al.,* 2009).

Apoptosis is a cellular process of self-destruction with a distinctive morphological appearance characterized by organized nuclear and cellular fragmentation. It is known to play a role in a wide variety of liver diseases, such as immune, viral, malignant, drug-induced, alcohol- induced and ischaemic disease, copper storage disease, and cholestatic liver disease (Gores and Rust, 2000; Kaplowitz, 2000). Apoptosis is critically important for the survival of multicellular organisms by getting rid of damaged or infected cells that may interfere with normal function. The extrinsic and intrinsic pathways represent the two major well-studied apoptotic processes. The extrinsic pathway is mediated by a sub-group of Tumor Necrosis Factor receptors (TNFR) superfamily that includes TNFR, Fas and TRAIL. Activation of these death receptors leads to the recruitment and activation of initiator caspases such as caspases 8 and 10. The process involves the formation and activation of complexes such as the death inducing signaling complex (DISC). This leads to the activation of caspase 3, known as the effector caspase. The active caspase 3 is responsible for the cleavage of a number of so-called death substrates that lead to the well-known characteristic hallmarks of an apoptotic cell including DNA fragmentation, nuclear fragmentation, membrane blebbing and other morphological and biochemical changes (Labbe and Saleh, 2008; Vicencio *et al.*, 2008; Sprick and Walczak, 2004). Alternatively, intrinsic apoptosis is initiated by the stress-mediated release of cytochrome c from the mitochondria resulting in the formation of the apoptosome, which then activates the initiator caspase, caspase 9, which leads to the activation of the executioner or effector caspase 3 thereby culminating in an apoptotic response(Galluzzi *et al.,* 2010; Brunelle and Letai, 2009).

The present study is designed to investigate the possible effects of PRP on the levels of apoptosis-related proteins in adult rat liver and also to explore the effect of PME on cell signal transduction pathways that lead to the induction of apoptosis in PRP-treated rats.

PROCEDURE

The experiment was set up as described in section 3.4.4, page 48 (Materials and methods). PME was dissolved in normal saline, while PRP was dissolved in olive oil. Both were orally administered to rats at a dose of 200 mg/kg body weight/ day for 7 days. Each group consisted of 6 animals. After treatment with PRP for 7 days, animals were sacrificed by cervical dislocation. Caspase 3, Caspase 9, Bax, Bcl-2 expressions were measured using immunohistochemistry, while Tumour suppressor p53, Bcl-2 antagonist of cell death (Bad), NF-κB, inhibitor of total nuclear factor-kappa B α (I-κB α), stress activated protein kinase/ C Jun NH_2-terminal kinase (SAPK/JNK), p38 mitogen-activated protein kinase (p38) and signal transducer and activator of transcription 3 (STAT 3) were assessed using ELISA kits procured from Cell Signaling Techology, USA, as described on pages 74-75. Liver tissue was also prepared for the Terminal de-oxy nucleotidyl dUTP nick end labeling (TUNEL) assay(page 75).

RESULTS

Liver: Body weight ratio

Compared to control animals (0.030±0.003), exposure to PME (0.031±0.002), PRP (0.041±0.002) and PME+PRP(0.036±0.003) increased liver weight to body weight ratio by ~3%, 36% ($p<0.05$), and 20%, respectively. Intervention with PME preserved the Liver/Body Weight ratio, but this was not statistically significant (Table 4A).

Table 4A: The liver weight to body weight ratio of rats treated with PRP and *Pterocarpus mildbraedii* Extract (PME)

Treatment	Body weight(g)	Liver weight(g)	Liver : Body weight	Change in L: B ratio (%)
Control	147.8±8.05	4.47±0.46	0.030±0.003	0
PRP	132.7±6.52	5.37±0.21	0.041±0.002*	36
PME	154.3±9.67	4.70±0.29	0.031±0.002	3
PRP+PME	161.1±8.65	5.82±0.41	0.036±0.003	20

Data expressed as mean ± SEM for six rats per group. * Significantly different when compared with the control ($p<0.05$). L: B= Liver: Body weight ratio.

Effect of PME on PRP-induced expression of p53, phospho-53, Bad, and phospho- Bad

Enzyme linked immunosorbent assay (ELISA) technique was adopted to investigate the effects of PRP on apoptotic-related proteins. Following exposure, the livers of treated rats

were lysed and cell lysates assayed at absorbance 450 nm for the determination of protein levels of p53, phospho-p53, Bad, and phospho-Bad using a Pathscan Inflammation Multi-Target Sandwich ELISA kit from Cell Signaling Technology. There were significant decreases ($p < 0.001$) in the Total p53 and phospho-p53 levels in PRP-treated rats compared with controls. Co-treatment with PME significantly ($p<0.01$) reversed these decreases in the expression of phospho- p53 when compared with the control groups (Figures 4A and 4B). Furthermore, treatment with PRP down-regulated the expression of the pro-apototic protein Bad. However, there were no significant changes ($p > 0.05$) in the levels of phospho-Bad in the livers of rats across all treatment groups (Figures 4C to 4D).

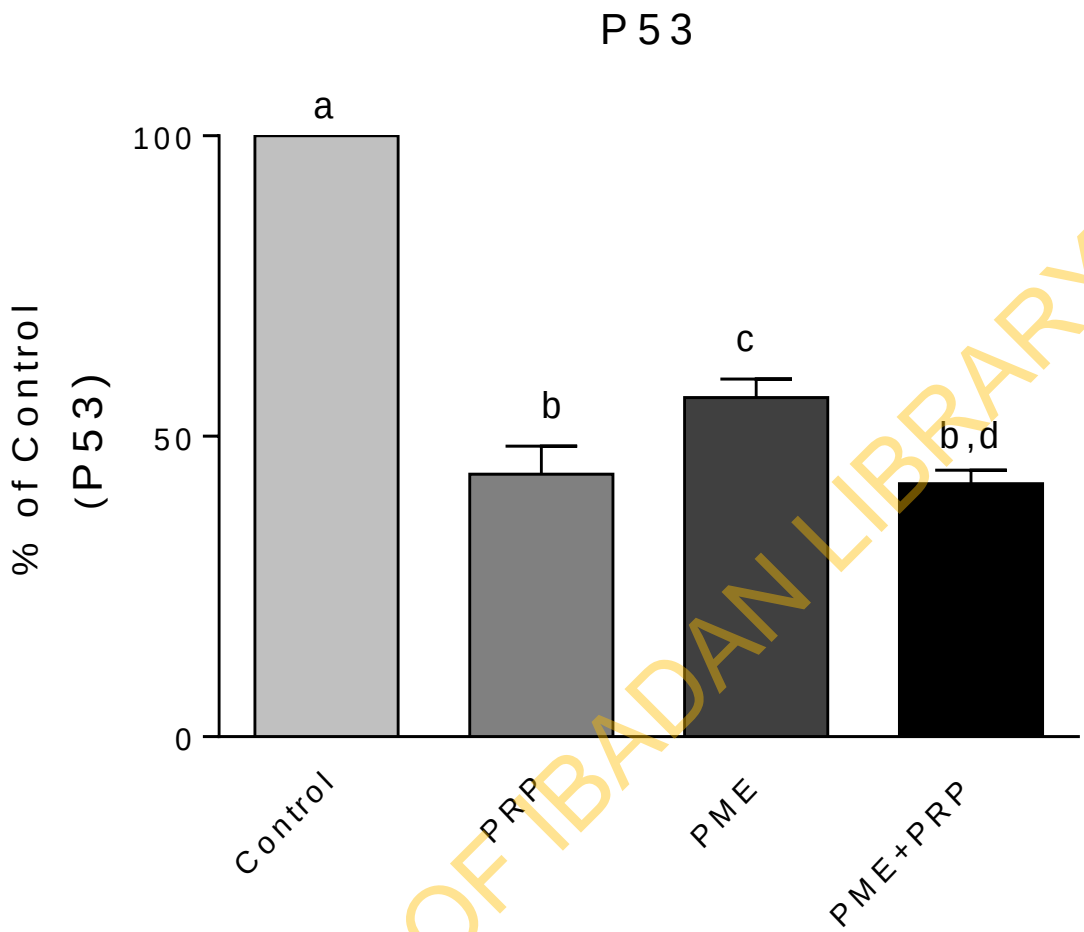


Figure 4A: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of Tumour suppressor p53 (p53) in Propanil (PRP) - treated rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.

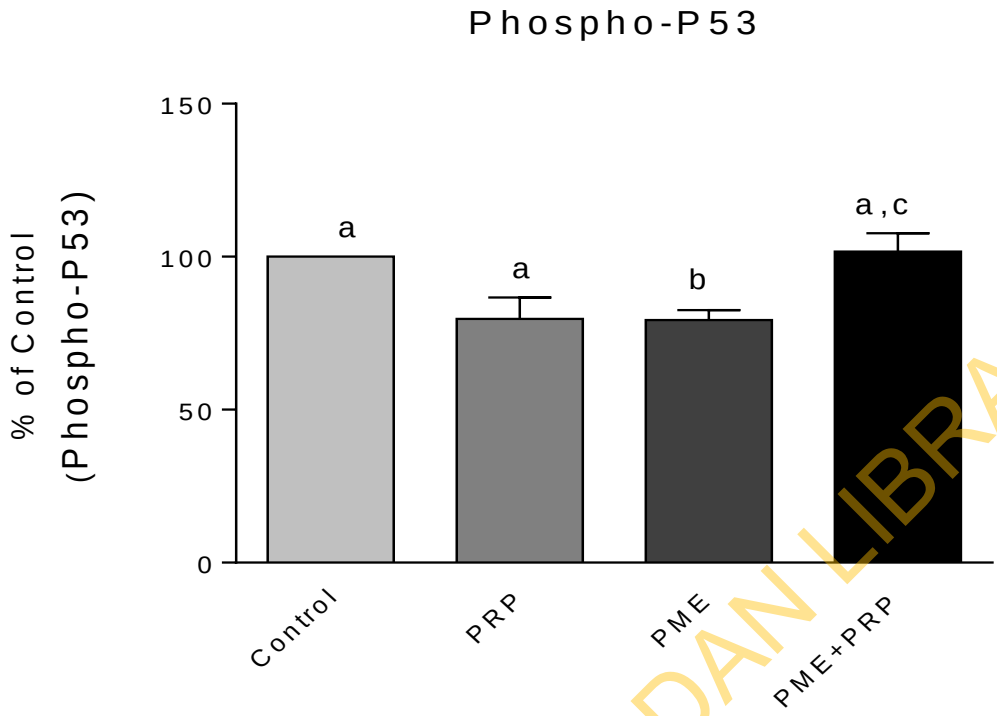
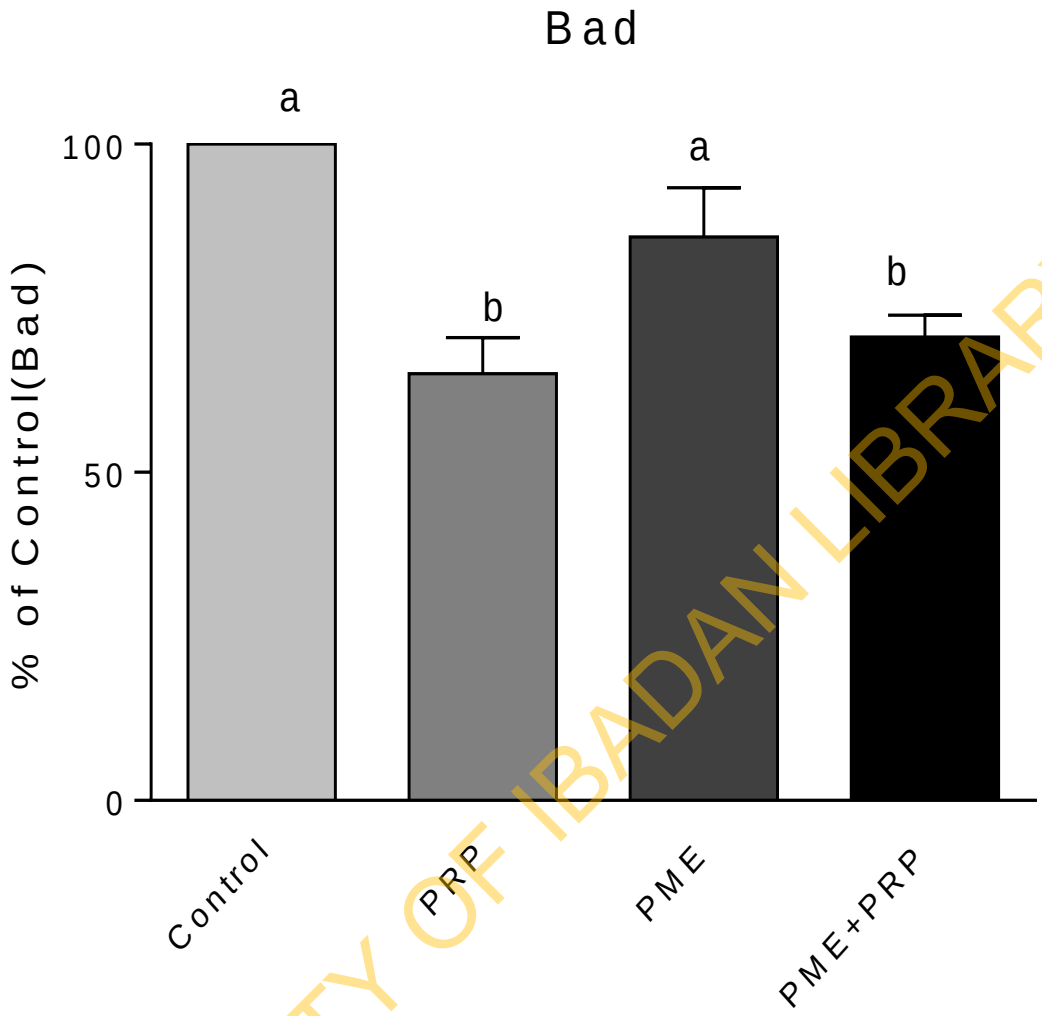


Figure 4B: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylated Tumour suppressor p53 (Phospho p53) in Propanil (PRP) - treated rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.



Figures 4C: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of Bcl-2 antagonist of cell death (Bad) in Propanil (PRP) - treated rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.

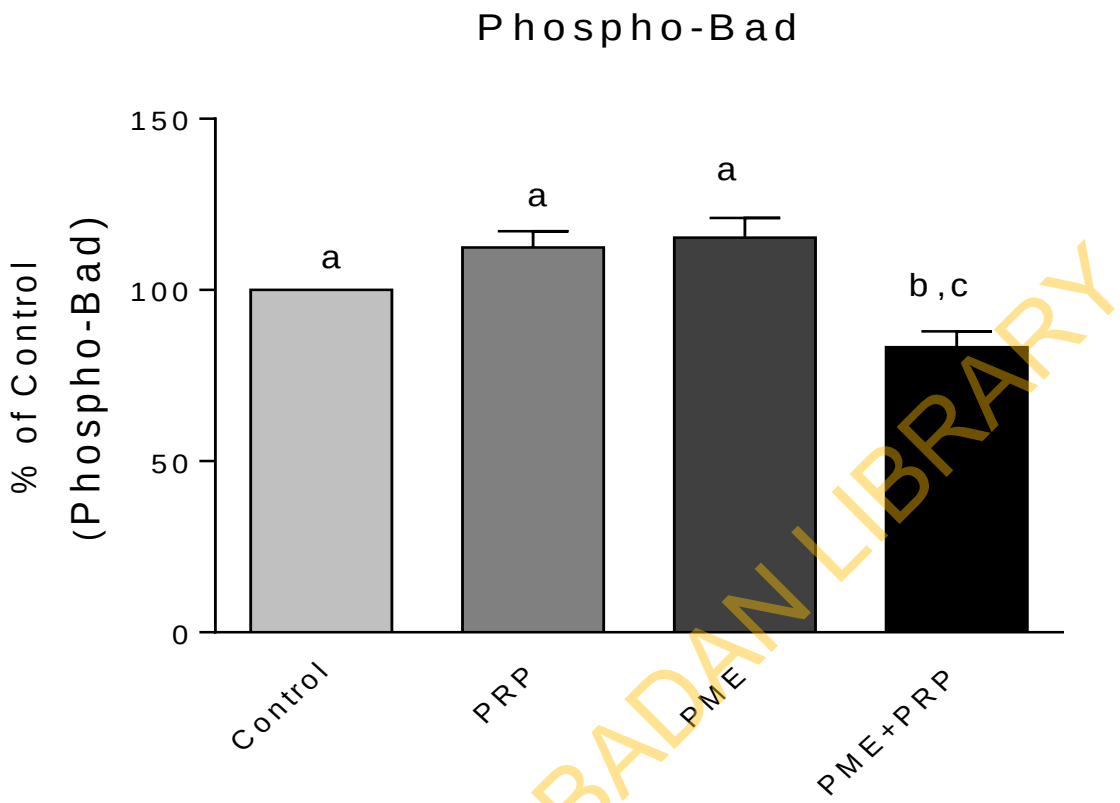


Figure 4D: Effect of *Pterocarpus mildbraedii* (PME) extract on levels of phosphorylated Bcl-2 antagonist of cell death (Phospho- Bad) in Propanil (PRP) - treated rats. Results are expressed as percentage of relative levels of phosphoproteins and are normalised to the control (100%). Values are means with their standard errors of technical duplicates. a,b,c Mean values with unlike letters are significantly different.

Immunohistochemical staining of Caspases 9 and 3, and Bax and Bcl-2

On immunohistochemical evaluation, Figures F, G, H and I show the effects of the treatment of PME on the expression of caspase-9, caspase-3, Bax and Bcl2, respectively, in the liver of PRP-treated rats. The expressions of the caspases and Bax in rats exposed to PRP alone was more intense compared with control. There were minimal or no expression of these apoptotic proteins in the control and PME+ PRP treatment groups, with the exception of an observed increase in the number of caspase-9 immune positive cells in the PRP group pre-treated with PME, when compared with the control.

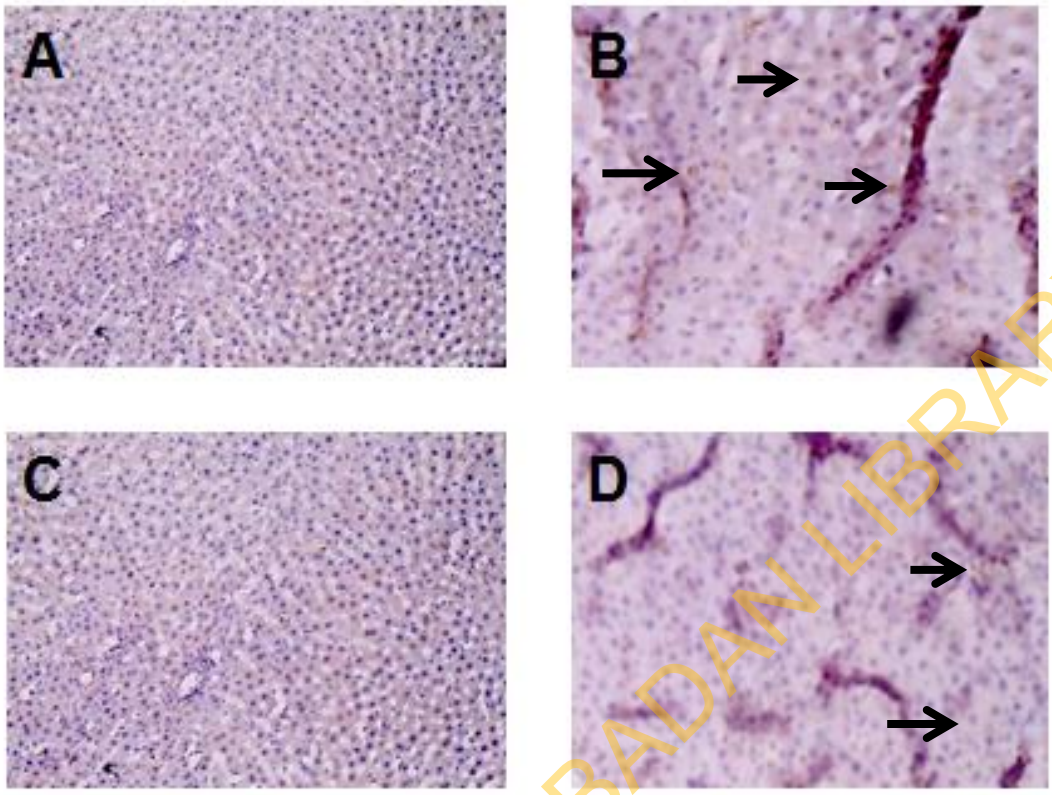


Plate 8: Immunohistochemical staining showing the effects of PME on caspase 9 expression in the liver of PRP-treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. Arrows in panels identify immunopositive cells.

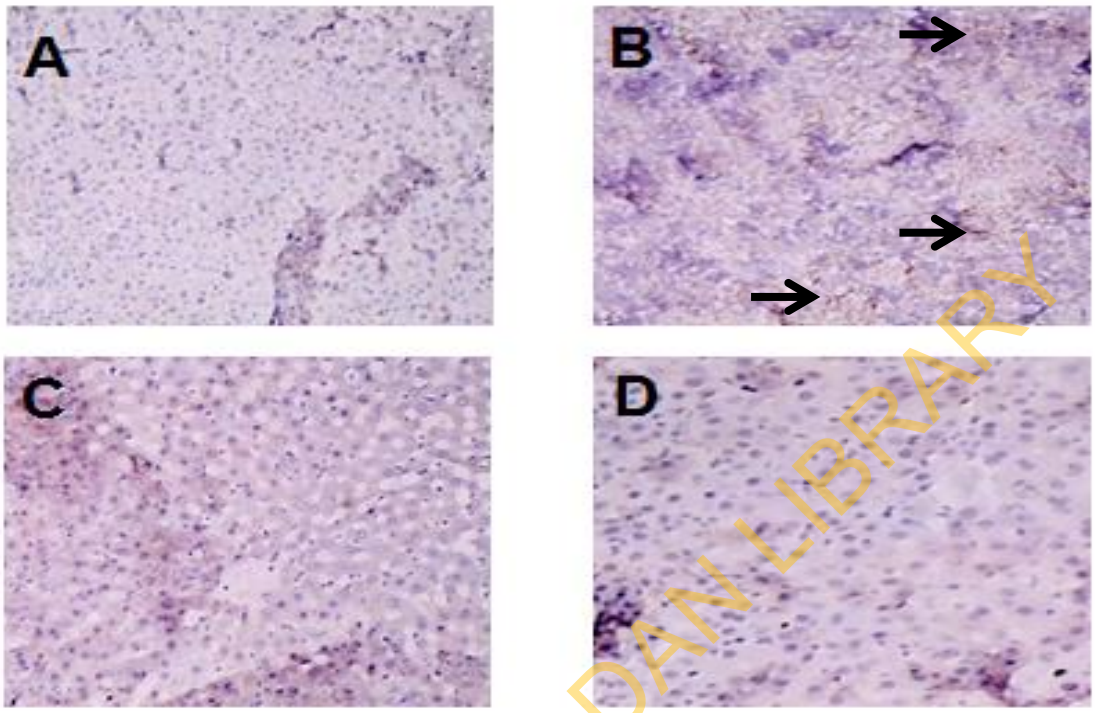


Plate 9: Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on caspase 3 expressions in the liver of Propanil (PRP)-treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. Arrows in panels identify immunopositive cells.

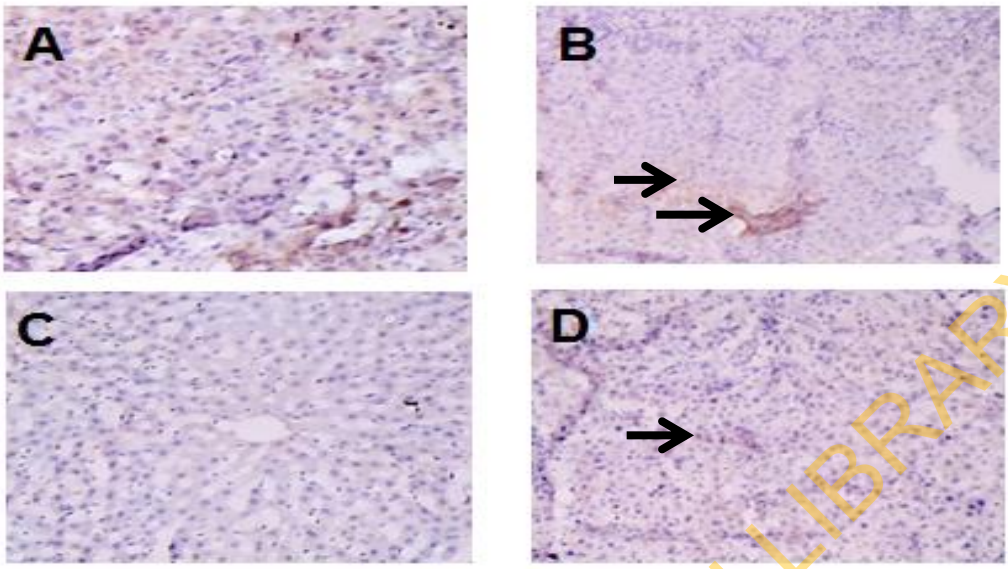


Plate 10: Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on Bcl-2 - associated X protein (Bax) expressions in the liver of Propanil (PRP) -treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. Arrows in panels identify immunopositive cells.

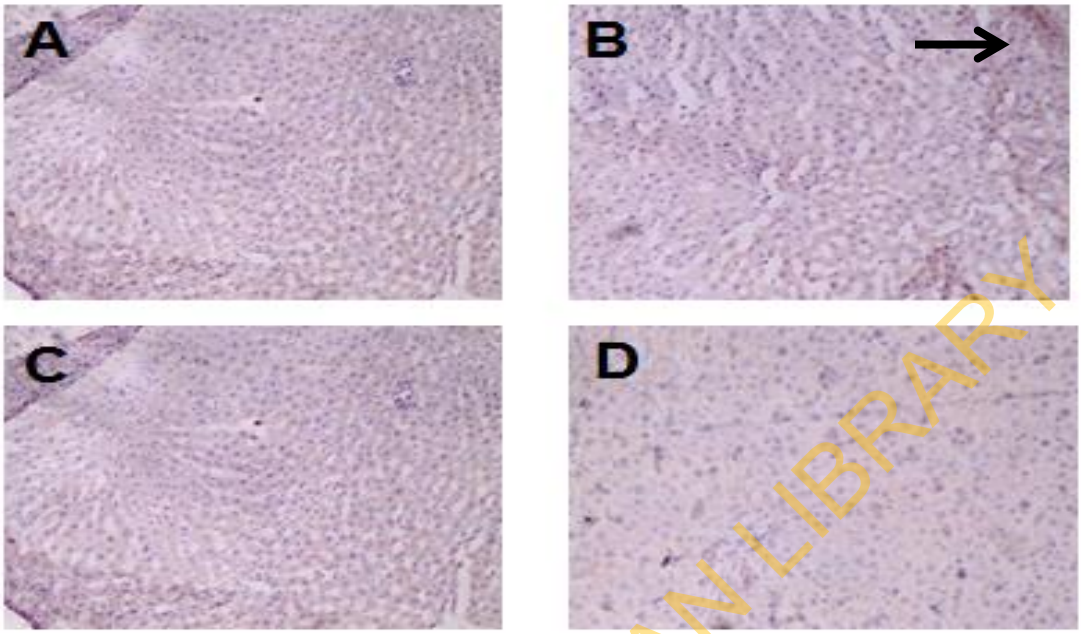


Plate 11: Immunohistochemical staining showing the effects of *Pterocarpus mildbraedii* (PME) extract on Bcl-2 expression in the liver of Propanil (PRP) -treated rats. (A) Control (B) PRP-induced group (C) PME group; and (D) PME+PRP. Arrow heads in panels identify immunopositive cells.

Level of apoptosis in the liver

Assessment of TUNEL-positive nuclei for all the treatment groups is represented in Plate 12. The number of TUNEL-positive nuclei in the control group was negligible. Administration of PRP resulted in a marked increase in liver apoptosis compared to control group. Co-infection with PME elicited a significant decrease in the programmed cell death in the PRP-treated liver compared to animals exposed to PRP alone.

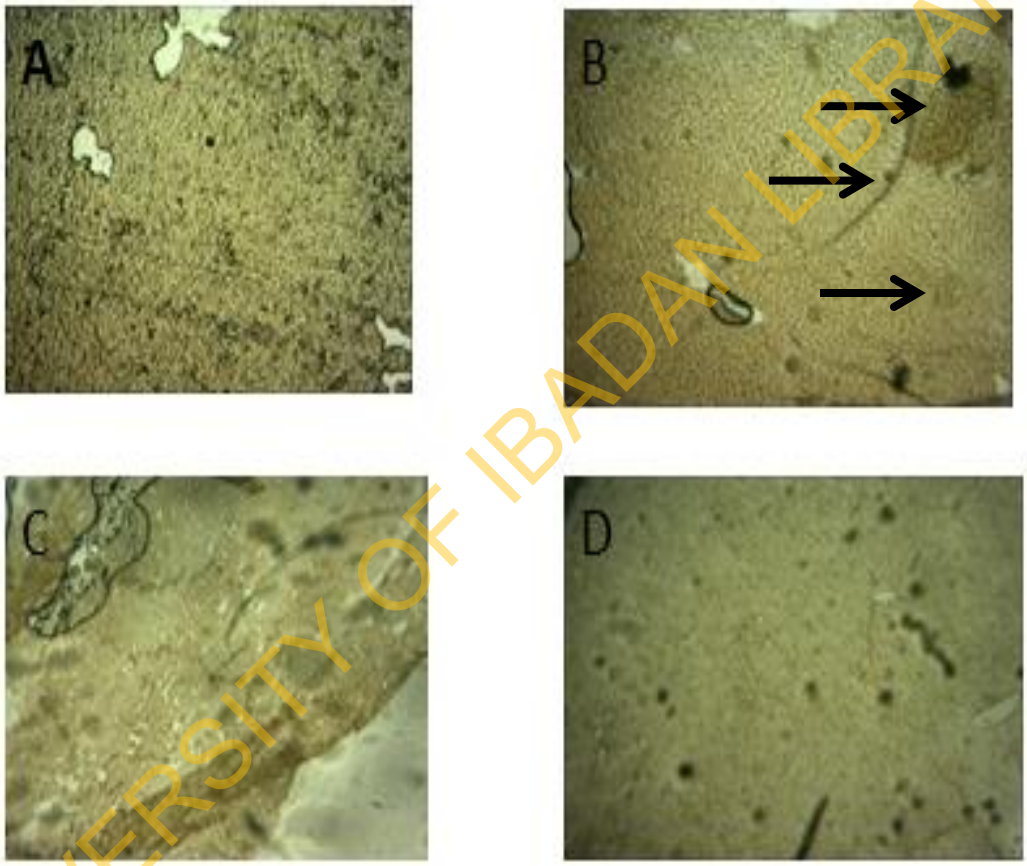


Plate 12: Liver histopathology guide showing the effect of *Pterocarpus mildbraedii* (PME) extract on Propanil (PRP) -induced apoptosis in rats. Arrows identify TUNEL-positive nuclei. Increased number of TUNEL-positive nuclei are seen in liver sections of PRP-treated rats. Original magnification X 40

Conclusion

The present investigation suggests that PRP exposure induced alterations in apoptotic- and inflammatory-related proteins in the hepatic system of rats by decreasing the expression of some transcription factors and increasing the expression of caspase 9, caspase 3, and Bax. PME intervention was able to ameliorate most of the observed alterations.

4.5.0 EXPERIMENT V: PRELIMINARY PHYTOCHEMICAL SCREENING AND CHARACTERISATION OF *Pterocarpus mildbraedii* LEAVES

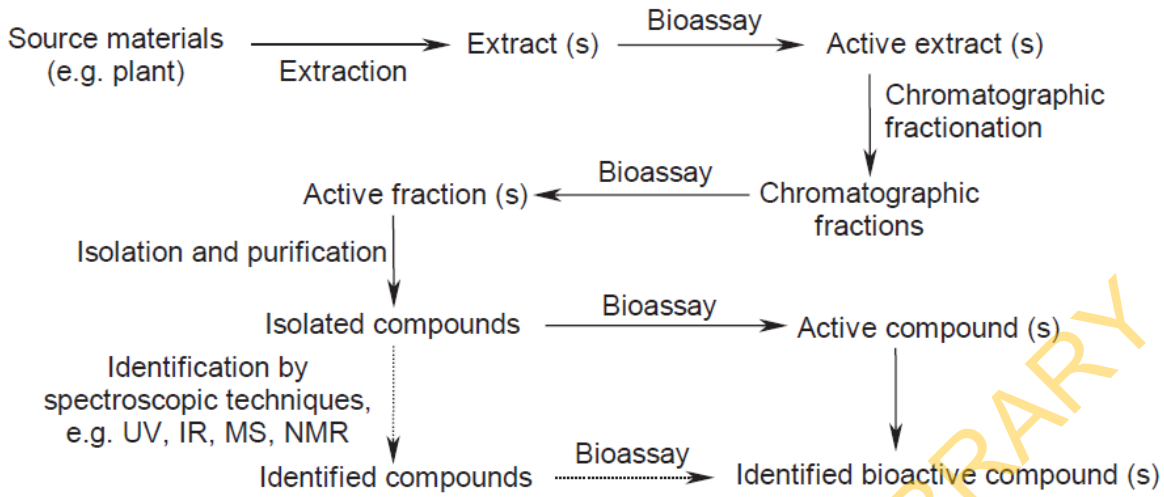
Introduction

In the traditional method of drug discovery from natural products, drug targets are exposed to crude extracts, and in the case of a hit, i.e. substantial evidence of activity, the extract is fractionated and the active compound is isolated and identified. Every step of fractionation and isolation is usually guided by bioassays, and the process is called bioassay-guided isolation. Sometimes, a straightforward natural product isolation route, irrespective of bioactivity, is also applied, which results in the isolation of a number of natural compounds (small compound library) suitable for undergoing any bioactivity screening. However, the process can be slow, inefficient and labour- intensive (Satyajit and Lutfun 2007). Scheme 4.1 presents an overview of a bioassay-guided traditional natural product drug discovery process.

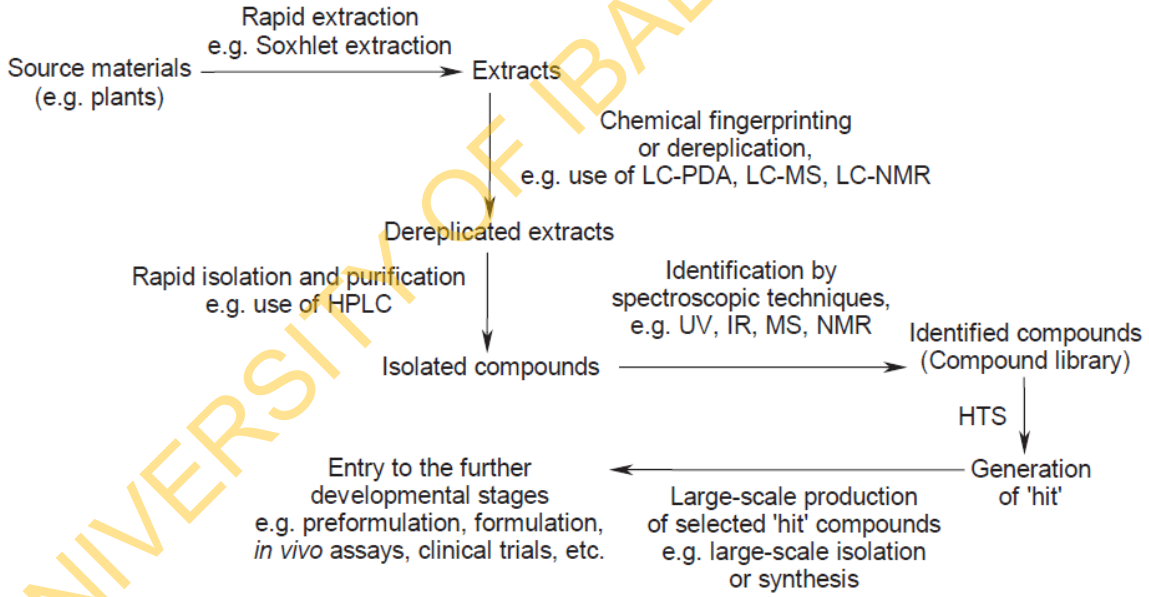
On the other hand, modern drug discovery involves High Throughput Screening (HTS), in which, several molecules can be screened using several assays within a short time, by applying full automation and robotics. In order to incorporate natural products in the modern HTS programmes, a natural product library (a collection of dereplicated natural products) needs to be built. Dereplication is the process by which one can eliminate recurrence or re-isolation of same or similar compounds from various extracts. A number of hyphenated techniques are used for dereplication, e.g. liquid chromatography–photo-diode-array detector (Satyajit and Lutfun 2007).

The screening of natural products from plant sources remain unabated and advances in isolation techniques have resulted in a variety of chromatographic techniques applicable to the isolation and purification of natural products. Thus, the present experiment was undertaken to identify some of the known compounds and/or possibly isolate new compounds.

A



B



Figures 5A and B: Traditional Drug Discovery Method and Modern Drug Discovery Method (Satyajit and Lutfun 2007).

PROCEDURE

Phytochemical tests were carried out on plant extracts to screen for the presence or absence of secondary metabolites as described in section 3.5.0, pages 49 and 50**.** The antioxidant and the 1, 1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging activity (RSA) activities, and the Total phenolic and flavonoid content of PME were investigated as described on pages 51-52. Column Chromatography, extraction and methanolysis of fixed oil, and spectroscopic measurements (Infrared and Gas Chromatography-Mass Spectrometry) procedures were detailed on pages 52.

RESULTS

Phytochemical Screening conducted on *P. mildbraedii* extracts revealed the presence of steroids, flavonoids, phenols, tannins, cardiac glycosides, quinones and saponins (Table 5A). Presence of phenolic compounds and flavonoids are possible indication of antioxidant property of the extracts. PME exhibited maximum RSA of 55% on DPPH radical indicating a significant free radical scavenging activity (Figures 5A and 5B).

Table 5A: Phytochemical components of crude *Pterocarpus mildbraedii* extracts

Phytochemical Compounds	Aqueous	Methanol	Chloroform	Gallic acid Equivalent(mg/g)
Steroids	+	+	+	ND
Flavonoids	+	+	+	ND
Phenols	+	+	_	ND
Tannins	+	+	-	ND
Cardiac glycosides	+	+	-	ND
Saponin	+	+	-	ND
Quinones	+	+	-	ND
Total flavonoids	ND	+	ND	0.082 ± 0.003
Total phenol	ND	+	ND	0.215 ± 0.005

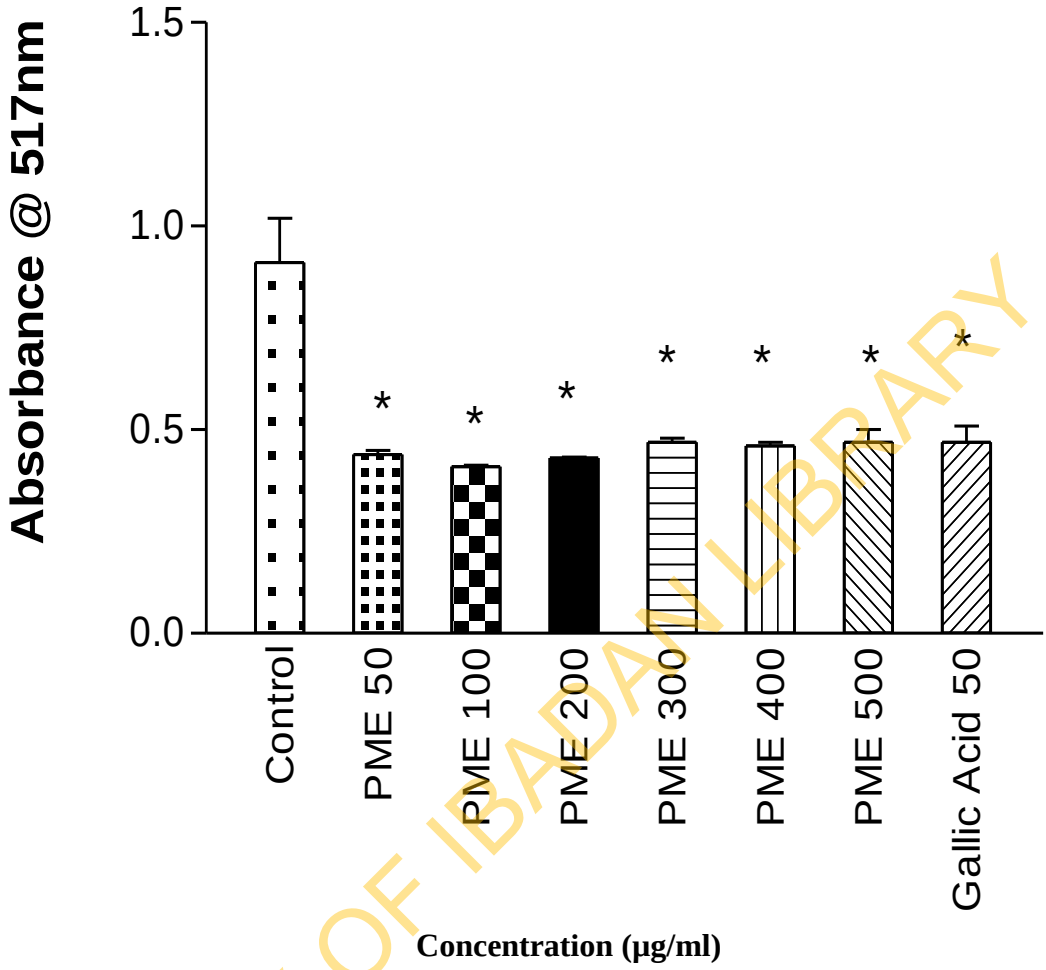


Figure 5C: DPPH· Radical Scavenging Activity (RSA) of PME; $p<0.01$ when compared with control

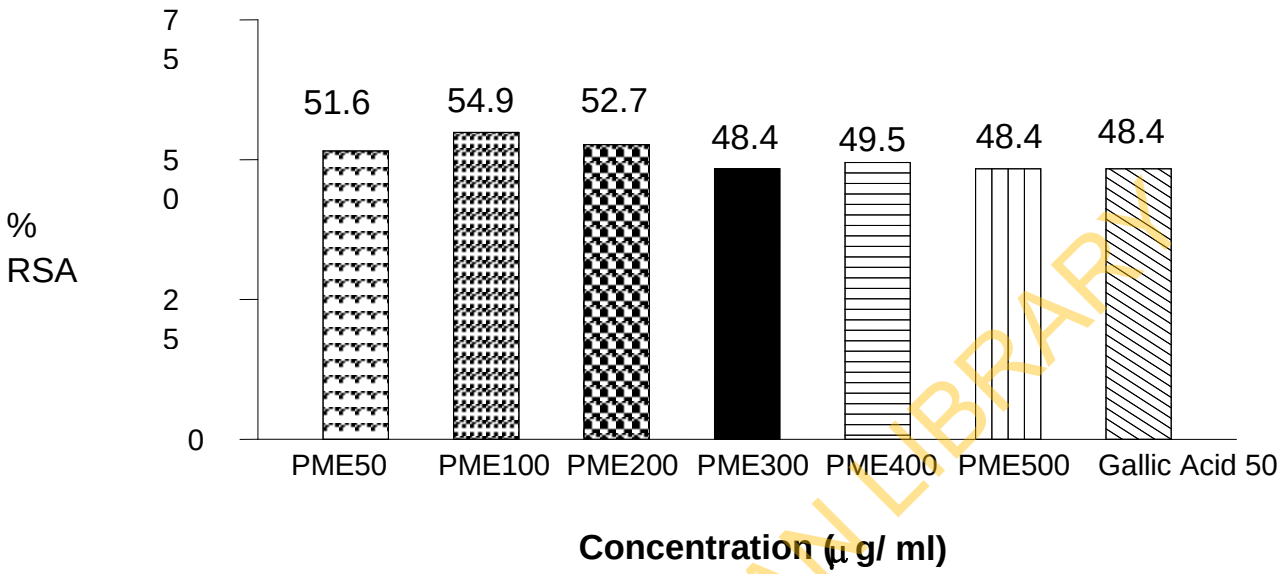


Figure 5D: Percentage DPPH· Radical Scavenging Activity (RSA) of PME

General column chromatographic fractionation

The concentrated PME was fractionated in silica gel open column using n-hexane and methanol in increasing order of polarity to give forty three (43) fractions of about 5 to 10 mL each. Fractions with similar TLC profile were combined and concentrated using the rotary evaporator. Fraction 1 afforded a compound coded PMEA, while fractions 2 to 3 afforded a compound coded PMEB. Combined fractions 4 to 19 were pulled together and chromatographed on a preparative silica gel TLC plates eluted with a mixture of dichloromethane and methanol. The major sport afforded compound PMEC with R_f value of 0.7. Fraction 35 afforded another compound coded PMEF. Combined fractions 20 to 34 and 36 to 43 were not worked up. PMEA and PMEB were subjected to further spectroscopic analyses.

Pulverized Leaves (236g)

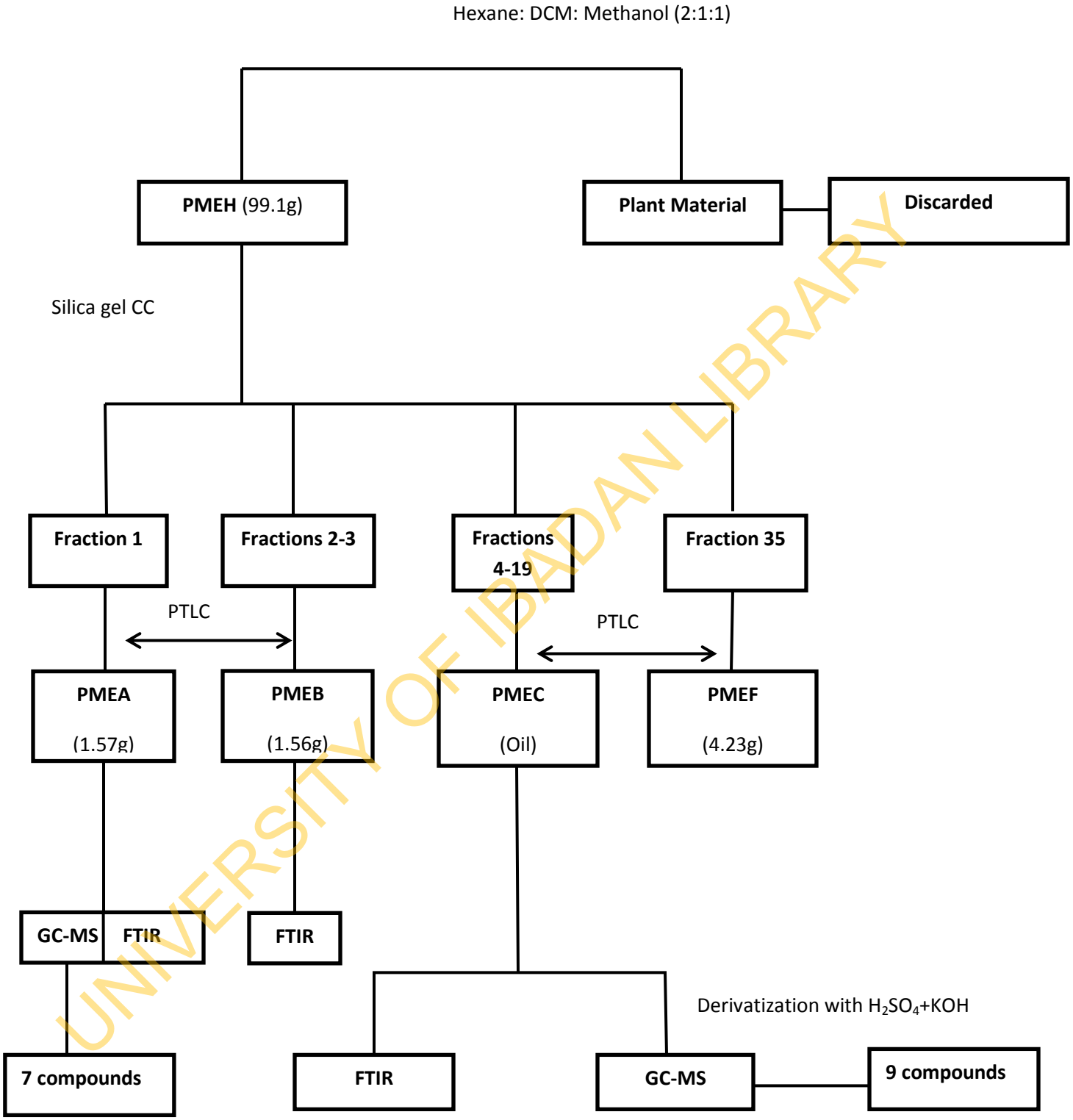


Figure 5E: Fractionation/Isolation Scheme for P. *mildbraedii* extract

Table 5B: Chemical Composition of PMEA

Hydrocarbon	RT (min)	% Yield
C27	18.908	5.9
C28	19.598	1.5
C29	20.325	69.1
C30	20.925	1.5
C31	21.624	10.0
C32	22.413	0.3
C33	23.372	0.9
Unidentified		10.9
identified		89.1
Total		**100.0**

FTIR SPECTRA OF PMEA, PMEB and PMEC

The FTIR spectrum of PMEA indicated the presence of O-H stretching vibration of alcohol at 3450 cm^{-1}, aliphatic C-H stretching vibrations at 2918 and 2848 cm^{-1}, and C=C stretch of alkene at 1634 cm^{-1} which is an indication of the presence of polar unsaturated aliphatic compounds. On the other hand, the FTIR spectrum of PMEB indicated the presence of O-H stretching vibration of alcohol at 3444 cm^{-1}, aliphatic C-H stretching vibrations at 2918 and 2848 cm^{-1}, and C=C stretch of alkene at 1635 cm^{-1}. Aromatic C-H stretching is conspicuously absent thereby suggesting that the sub-fraction is a mixture of polar unsaturated aliphatic compounds. FTIR spectrum of PMEC showed a strong OH stretching vibration at 3446cm^{-1} and C-H of aliphatic hydrocarbon at 2924 and 2854 cm^{-1}. It also showed the stretching vibration of C=C of alkene at 1622 and 1714 cm^{-1} suggesting that presence of polar unsaturated aliphatic compounds (Spectra shown in the appendix).

Fatty acid methyl esters (FAMEs) from the leaves of *P.mildbraedii*

The GC analysis of fatty acid methyl esters obtained from the leaves allowed the identification of nine FAMEs (Table 5B). The major constituent of the leaves were C16:0 and C18:0, at 22.65% and 13.05% respectively. The chain lengths of the FAMEs were between C-12 and C-20. The total polyunsaturated fatty acid was 8.34%, while the saturated fatty acid and mono unsaturated fatty acid were 39.81% and 7.60%, respectively.

Table 5C: Fatty acid composition of *Pterocarpus mildbraedii* leaves

Peak #	Compounds	RT (min)	% Yield
1	Dodecanoic acid(12:0)	2.1	0.89
2	Tetradecanoic acid (14:0)	3.0	0.73
3	Hexadecanoic acid (16:0)	4.5	22.65
4	Palmitoleic acid (16:1)	5.0	0.94
5	Octadecanoic acid (18:0)	6.0	13.04
6	Oleic acid (18:1 n9)	6.5	6.66
7	Linoleic acid (18:2 n6)	7.4	1.49
8	α-linolenic acid (18:3:3)	8.0	6.85
9	Eicosanoic acid (20:0)	7.7	2.50
10	Others	-	44.25

CHAPTER FIVE

5.0 DISCUSSION

The herbicide, propanil (3, 4 dicholoropropionalide) is one of the widely used agricultural pesticides in the world in the control of weeds in rice and paddy farms. Though the World Health Organization recognized propanil as slightly hazardous in terms of human risk (WHO, 2004a), there are growing concerns that the widespread use of the herbicide on rice and wheat crops means that individuals in agriculture are particularly at risk of high level exposure. The incidence or prevalence of organ injury arising from the use of chemicals continues to soar, posing serious challenges to therapeutic outcomes in clinical practice. Unfortunately, several synthetic therapeutic agents used in clinical practice have been reported to produce functional impairment and toxic injuries to the liver and kidney. The reason for this is not unconnected with the fact that the these two organs are involved in biotransformation and excretion roles in the body and as a result are exposed to large amount of parent and active metabolites of these chemicals. It has therefore become important that deliberate efforts are directed towards protecting these organs from injuries and damage. This study primarily attempted to elucidate the effect of propanil on clinical biochemical indices and antioxidant system in the kidney and liver of rats exposed to graded doses of the xenobiotic.

It has been reported that increase or decrease in either absolute or relative weight of an organ after administration of a chemical or drug is an indication of the toxic effect of that chemical (Orisakwe *et al*., 2003). In the present study, there was a dose-dependent increase in the liver weight in all the groups, however, the absolute kidney weight were decreased across the treatment groups. There were no significant effects of the relative weights of the liver and kidney in all the groups (Table 1A). The present result is consistent with the study of Van Birgelen *et al* (1999) who reported an increase in liver weight of rats and mice exposed to 3, 3, 4,4 tetrachloroazobenzene, a contaminant generated during the synthesis of 3, 4 dichloroanilide-derived pesticides. In their report, it was indicated that the male rodents were exposed at dose levels of 0.1,1,3,10 and 30 mg/kg, 5 days in a week, for 13weeks.

Furthermore, the liver integrity of control and propanil-intoxicated animals was assessed by measuring biochemical endpoints such as the levels of serum transaminases, alkaline phosphatase, lactate dehydrogenase, gamma glutamyltransferase, bilirubin, and cholesterol (Table 1B). Aminotransferases are localized in periportal hepatocytes where they involved in amino acid metabolism; transamination reactions and their activities in blood was elevated

sequel to cellular injury. The increases in AST observed in the propanil treated rats indicate hepatic damage, which may be associated with altered membrane permeability (Adedara *et al.*, 2014).

Lactate dehydrogenase is widely distributed throughout the body; cellular damage causes an elevation of the total serum LDH. When disease or injury affects tissues containing LDH, the cells release LDH into the bloodstream, where it is identified in higher than normal level. Propanil administration elicited a significantly increased LDH activity in the rat serum and this may be attributed to hepatocellular necrosis as a result of leakage of the enzyme into the blood stream (Wang and Zhai, 1988). Bilirubin is a yellow pigment produced when heme is catabolized. In this study, we observed an elevation in serum bilirubin levels in the propanil-treated rats compared with the control rats. This consequent hyper-bilirubinemia may result from ineffective erythropoiesis, impaired ability of the liver to excrete normal amounts of bilirubin, or obstruction of excretory ducts of the liver (Olaleye *et al.*, 2010). An increase in cholesterol as a result of pesticide exposure, as observed in our study, may indicate loss of membrane integrity. This is in accordance with previous reports that the accumulation of pesticide in the liver was associated with the disturbance of lipid metabolism and elevation of serum cholesterol (Lari *et al.*, 2014, Ubachukwu *et al.*, 2012). However, the present result is inconsistent with Santillo *et al.* (1995) who reported a significant decrease in the serum cholesterol levels of rats exposed to propanil. The differences in these data may be due to disparity in treatment dose, mode of administration and/or species differences.

Kidney damage is associated with decline in renal function which could lead to renal failure. The decrease in renal function evidenced by significant increase in serum levels of urea and creatinine in rats treated with propanil was clearly demonstrated in the present investigation. While an increase in serum urea may indicate decrease in reabsorption at the renal epithelium, an increase in serum creatinine reflects impairment in the kidneys, particularly for glomerular filtration rate (Adedara *et al.*, 2012, Ekor *et al.*, 2006).

The histopathological report revealed that oral exposure to propanil at environmentally relevant concentrations produced remarkable dose-dependent damaging effect to both liver and kidney of the treated rats, hence supporting the observed biochemical observations. In this study, we observed that propanil-treatment led to infiltration of mononuclear cells, focal hepatocellular necrosis, and widespread vacuolar degeneration. These changes could be

consequences of an increase in free radical species formation in the hepatocytes. In addition, propanil- treated kidney showed focal haemorrhages between the tubules of the cortex and renal tubular necrosis. The susceptibility of the tubular epithelium to toxicants has been attributed to the intense filtration of substances from the blood, their transport, and the high energy requirement of these functions (Stacchiotti *et al.*, 2003).

Furthermore, the effect of propanil on the hepatic and renal antioxidant status was studied as a way of detailing the mechanism of action of the toxicant. SOD, CAT and GST provide the cellular defence against the intermediates of dioxygen reduction (superoxide radical, hydrogen peroxide and hydroxyl radical). SOD converts superoxide radicals into hydrogen peroxide, which in turn has to be removed by CAT. On the other hand, GSTs are group of multifunctional proteins encoded by a multigene family. They perform functions ranging from catalyzing the detoxification of electrophilic species via a spontaneous enzyme catalyzed conjugation reaction to protect the cells against peroxidative damage (Brandsch *et al.*, 2010). In the present study, the activities of hepatic and renal SOD and GST were markedly increased in rats treated with propanil. The dose-dependent induction of these antioxidant enzymes may indicate an adaptive response to counter the damaging effect of oxidative stress possibly generated during propanil metabolism (Adedara *et al.*, 2014). With exposure to propanil, CAT activity was not affected in the liver but decreased significantly in the kidney. It is plausible that this decrease in CAT in the kidney would allow more of the H_20_2 to be converted to toxic hydroxyl radicals, which might contribute to severe oxidative damage to the cellular membrane of the tissue. GSH level remained unchanged, and this is in accordance with similar reports by other workers (Abarikwu *et al.*, 2014, 2012). Reactive oxygen species attack cellular components containing polyunsaturated fatty acid residues to produce peroxyl radicals which undergo a cyclization reaction to form endoperoxides and MDA. The dose-dependent increases in the hepatic and renal MDA levels observed in this study clearly indicate a state of stress in the tissues possibly induced by propanil or its metabolites (Table 1C).

The liver is the most sensitive and main target organ of pesticide toxicity and damage, and plays an essential role in metabolism and detoxification of pesticides. Due to these functions, hepatotoxicity continues to be among the main threats to public health, and they remain problems throughout the world. The goal of reducing or protecting against pesticide-

induced hepatotoxicity has attracted much effort and attention over the last one decade (Mossa *et al.*, 2015; Beydilli *et al.*, 2015; Sushma and Devasena, 2010; Khan, 2006). Environmental exposure to pesticides may affect the human health by increasing the incidence of certain disorders at the level of the general population. The toxic effects of organic compounds are clearly mediated by reactive oxygen species (ROS) which can react with biological molecules and initiate oxidative damage including protein oxidation, reduced glutathione depletion and lipid peroxidation. LPO, variations in levels of GSH and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) have been proposed as indicators of pollutant mediated oxidative stress (Mitra *et al.*, 2012; Yuanxiang *et al.*, 2011). Administration of free radical scavengers like N-acetyl cysteine, silibinin and vitamin E has provided some benefit in ameliorating this toxicity (Beydilli *et al*., 2015; Lasram *et al.*, 2014, Araoud *et al.*, 2014).

In this study, the hepatoprotective effect of PME against propanil-induced toxicity in rats was established. Secondary metabolites in green leafy vegetables include alkaloid, steroids, saponins, flavonoids, tannins and they are known for their biological activities including anticancer, antioxidant and antitumour activities (Soria *et al.*, 2015; Kawabata et al., 2015). It was on this basis that PME was screened for possible chemopreventive action against propanil hepatotoxicity. It has been reported that increase or decrease in either absolute or relative weight of an organ after administration of a chemical is an indication of the toxic effect of that chemical. Administration of propanil significantly increased the relative weight of liver when compared with controls. This is contrary to an earlier report that atrazine administration resulted in a decrease in these parameters (Sharma *et al.*, 2005). Treatment with PME at the various doses did not significantly reverse the increased weight of the liver.

In the present study propanil elicited a 14.8% increase in AST activities, and this was significantly different from the control. Furthermore, propanil caused a 5%, 122% and 78% increase in ALT, ALP and GGT activities (Figures 2A to 2F). Elevation of AST and ALT has been associated with hepatocellular injury while ALP and GGT are important in assessing obstructive liver injury. Though these increases were not significant, possibly due to the short term duration of the exposure, there is a clear indication that at this treatment dose they are mildly hepatotoxic. Patterns of mild liver enzyme elevations include hepatocellular injury pattern (elevated ALT or AST), cholestatic pattern (elevated alkaline phosphatase or GGT, or

both), and mixed pattern (elevation of ALT, AST, and alkaline phosphatase). The administration of PME had no deleterious effects on serum enzyme activities. At a dose of 100 mg/kg and 200 mg/kg, the extract showed promise of a modulating effect.

Laperche (2003) announced that hepatocytes, which are known for their production of bilirubin, in case of liver cell damage ultimately leads to raised levels of this substance in the sera. Bilirubin is a yellow pigment produced when heme is catabolized. In this study, we observed significant increases in serum bilirubin levels in all doses of propanil-treated rats when compared with the controls. It is possible that this hyperbilirubinemia maybe as a result of impaired ability to excrete normal amounts of bilirubin or obstruction of the excretory ducts of the liver (Olaleye *et al.,* 2010). Propanil-induced elevations in bilirubin decreased by 63% and 50% following treatment with 100 mg/kg (PME I) and 200 mg/kg (PME II) PME respectively. The larger dose (400 mg/kg) of the extract, however, did not seem to offer better protection against propanil toxicity when compared with that of the lower doses. LDH is present in the cells of almost all body tissues and in the case of damage; it is released into the blood. In this study, propanil induced a dose-dependent increase in serum LDH activity and this may be attributed to hepatocellular necrosis as a result of the leakage of the enzyme into the blood stream (Kaptanoglu *et al.,* 2008). The treatment of propanil-exposed rats with PME at the 100 mg/kg and 200 mg/kg doses significantly reduced the increased observed increase in serum LDH activity and this is an indication of the protective effect of this extract.

Literature report suggests that free radical mediated oxidative stress is responsible for the toxicity of pesticides (Abdollahi *et al.,* 2004). The peroxidation of lipid membranes is an indication of an alteration in balance between the generation of free radicals and the potential of the antioxidant defense system to detoxify reactive products (Mena *et al.,* 2009). Therefore, the relationship between propanil - oxidative stress and hepatotoxicity was further established in this present study. Propanil-induced toxicity was associated with increased activities of SOD and GST, and non-significant depletion of CAT activity in the liver (Figures 2G to 2K). This trend was also observed in a similar study. Dwivedi and Flora (2015) reported that a combined exposure of rats to arsenic (25 ppm in drinking water) and dichlorvos (2.5 mg/ kg, orally) for 56 days increased the levels thiobarbituric acid reactive substances (TBARS), and SOD activity while the catalase activity remained unchanged. Consistent with this study, propanil treatment also produced a significant increase in the level of MDA in the hepatic tissues. The

increase in the concentration of MDA is an indicator of propanil-induced LPO leading to tissue injury. Oral administration of PME at 200 mg/kg and 400 mg/kg led to a significant reduction in the oxidative stress parameter. Modulation of lipid peroxidation is a good indicator of the protective effect of an extract that possesses antioxidant activities. The mechanism of this protection could be the inhibition of reactive oxygen species generation by suppressing cytochrome P450 bioactivation of propanil to reactive metabolites such as 3, 4 dichloroaniline which has been implicated as the reactive metabolite of propanil (Kamalakannan *et al.*, 2005). GSH is the intracellular non-protein sulfhydryl compound that act as both nucleophile and an effective reductant by interacting with various electrophilic and oxidized compounds and its depletion results in increased defenselessness of cell to oxidative stress. The consequence of decrease in cellular GSH levels is an increase of free radical intermediates leading to oxidative stress and potent cellular damage. The reduced form of GSH becomes readily oxidized to oxidized glutathione (GSSG) on interacting with free radicals (Bose and Agarwal, 2007). After 7 days administration, the levels of CAT and GST were not significantly altered across all treatment groups, which may suggest that these molecules may not be a major source of oxidative stress in the rat liver under short–term propanil exposure. Histopathological examination of liver sections from normal rats showed normal parenchyma architecture while the histoarchitecture of rats treated with propanil presented with severe periportal infiltrations and periportal fibroplasias in the liver. Control animals and groups treated with PME extract at the doses of 100 mg/kg and 200 mg/kg showed normal hepatic histoarchitecture (Figures 2L). Furthermore, exposure to PRP altered the gross histological appearance of the liver including increased liver weight and the presence of white pustular nodules on the surface of the liver. The morphologic characteristics of the liver were preserved and comparable to those in the control groups in animals treated with PRP plus PME (100 mg/kg and 200 mg/kg) (Figure 2M). However, the livers of rats treated with PMEI and PME II showed no visible lesion unlike those treated at the higher dose of 400 mg/kg which showed mild cellular infiltration.The present study suggested that exposure of rats to propanil elicited marked alterations in cellular antioxidant and biochemical parameters in the liver. Findings of this investigation reveal that oxidative stress plays a major role in the development of propanil-induced hepatic dysfunction and that the dichloromethane: methanol leaf extract of

Pterocarpus mildbraedii protected the liver from the detrimental effects of propanil through its effect on the antioxidative defense system.

In this study, possible role of Inflammation in propanil-induced hepatic damage and the possible protective effects of *Pterocarpus mildbraedii* were established. Inflammation is a defense response of living tissues to injurious stimulus. The inflammatory response involves the activation of several immune cells such as monocytes/macrophages which secrete a series of pro-inflammatory mediators such as enzymes, cytokines, chemokines as well as signaling proteins at the site of infected tissues and cells. Dysregulation of the inflammatory immune responses can lead to a variety of diseases, including liver disorders (Choudhari *et al.*, 2013). Under normal physiological conditions, NO produces an anti-inflammatory effect. However, it is considered as a proinflammatory mediator that induces inflammation due to overproduction by inducible nitric oxide synthase (iNOS) in abnormal situations (Cross and Wilson, 2003). The elevated concentration of NO in PRP-exposed rats observed in the current study could lead to NO reaction with superoxide anion to form more poisonous nitrite anion and impair the liver. Similar increases in NO concentration in rodent models of chemically-induced liver injury have been reported (Shehata *et al*., 2015; Freitag *et al*., 2015). Evidence from the present study seems to suggest that the extract is capable of decreasing excessive $NO^{\cdot}$ generation. The effect of the extract in lowering serum nitrate/nitrite concentration (index of NO. production) in this study was significant ($p<0.05$) at 200 mg/kg doses when compared with the rats treated with propanil only.

Furthermore results from this study show that propanil - induced liver injury can cause polymorphonuclear leukocyte infiltration and accumulation as indicated by the significant increase in myeloperoxidase (MPO) activity in the propanil - treated rats. This observation which supports inflammatory mechanism in propanil hepatototoxicity corroborates similar findings from recent studies (Beydilli *et al.*, 2015; Freitag *et al*., 2015). The ability of PME to decrease the hepatic activity of MPO in the propanil - treated rats suggests its protective effect is also related to its ability to reduce leukocyte infiltration and subsequently down-regulate some components of the inflammatory mechanisms that contribute to this injury. The decrease in hepatic activity of MPO produced by PME in this study may enhance $NO^{\cdot}$ bioactivity and reduce oxidative stress associated with the increased MPO activity in the propanil - treated rats.

Infact, it will not be unreasonable to speculate that inhibition of either NF-κB production or its activity is involved in the protection afforded by PME against the hepatic dysfunction and structural damage observed in this study. NF-kB is a redox-sensitive transcriptional factor that is activated by oxidative stress. The suppression of NF-κB –induced expression in propanil-treated rats by PME could be as a result of its antioxidant properties. This is in agreement with the report that antioxidants, such as N-acetylcysteine, suppress hepatic NF-kB activation and the production of proinflammatory cytokines (Li et al, 2002).

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases which mediate intracellular signal transduction in response to different physiological stimuli and stressing conditions. Three major MAPKs have been identified, namely c-Jun NH2- terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) 1/2. It is normally considered that JNK and/or p38 activation is associated to apoptosis induction, and that ERK activation favors proliferation and promotes cell survival but this rule is subjected to multiple exceptions (Kim and Chung, 2008; Abarikwu, 2014). These kinases could play pro-apoptotic or survival roles depending on experimental models conditions (Brozovic and Osmak, 2007). Furthermore, inhibitors of MAPK pathways exhibit anti-inflammatory effects. Our study showed that PME suppressed propanil-induced phosphorylation of MAPK related proteins suggesting that their anti-infalmmatory actions may be derived through the regulation of MAPK pathways. Our present results showing that PME decreases p38- MAPK phosphorylation, agrees with earlier reports (Song *et al.,* 2012; Ki *et al.,* 2013; Maurya *et al.,* 2014). In addition, the PME- mediated decrease in phosphorylation of STAT-3 (Tyr705) as observed in our report seemed to parallel p38 MAPK down-regulation because a previous work has demonstrated that p38 MAPK can act as an upstream activator of STAT-3 (Blanc *et al.,* 2010). These results suggest a model for PME chemoprevention in which PME depresses JNK and p38 activities, causing a decrease in Stat-3 phosphorylation.

NF-κB is a central transcription factor of inflammation because it regulates pro-inflammatory genes including cytokines and chemokines. Reversible phosphorylation and dephosphorylation of proteins catalyzed by protein kinases and protein phosphatases respectively, is one of the most important and widespread molecular regulatory mechanisms. It controls almost all aspects of cellular physiology (Zhang. and Pelech, 2012). In our study, the cytosolic p65 subunit of NF-κB was decreased after PRP administration suggesting its

activation and translocation, and this is confirmed by the increased level of P-NF-κB. After PRP exposure, there was also an increased IκB- α phosphorylation. Co-exposure with PME suppressed PRP-induced NF-κB activation and IκB- α phosphorylation .Consequentially we propose that PME might inhibit the expression of pro-inflammatory mediators via the regulation of NF-κB activation. These modulations of inflammation transcription factors may occur by a mechanism involving direct or indirect effect of PME on proteins associated with inflammation and apoptosis. In addition, we observed increased immune-histochemical expressions in NF-κB, iNOS and COX-2 in the liver of PRP-treated rats. Since iNOS is induced in response to inflammation, this confirms our observations with NO. COX-2 has been involved in inflammation. So the over-expression of COX-2 in the liver is evidence of PRP-induced inflammation. However, treatment with PME reduced these adverse effects.

Apoptosis is a cellular process of self-destruction with a distinctive morphological appearance characterized by organized nuclear and cellular fragmentation. It is known to play a role in a wide variety of liver diseases, such as immune, viral, malignant, drug-induced, alcohol-induced and ischaemic disease, copper storage disease, and cholestatic liver disease(Gores and Rust, 2000; Kaplowitz, 2000). To explore the potential mechanisms of PRP-induced liver apoptosis and the mechanisms underlying the possible anti-apoptotic effect of PME, the expressions of apoptosis related proteins involving the mitochondrial pathways were investigated (Figures 4F and 4G). The intrinsic apoptosis pathway is triggered by intracellular signals such as cellular and DNA damage. Key events in this pathway include the depolarization of mitochondrial membrane potential, mitochondrial outer membrane permeabilisation, release of mitochondrial cytochrome c into the cytosol, which results in activation of initiator caspase-9. Mitochondrial activation is controlled by the family of Bcl-2 proteins, which consists of anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1), pro-apoptotic multidomain proteins (Bax and Bak) and proapoptotic BH3-only proteins (e.g. Bad, Puma and Noxa). According to the intrinsic and extrinsic pathways, caspase cascades appear as central in apoptosis regulation. Initiator caspases cleave and activate downstream effector caspases, which themselves target a number of death substrates to set apoptosis into work (Elmore, 2007; MacFarlane and Williams, 2004). Summarily, the free cytosolic cytochrome c rapidly assembles a multi-protein complex involving Apaf-1 and pro-caspase-9 leading to caspase-9 activation. Caspase-3 which is considered to be a major execution protease can be triggered by

active caspase-9. The cleavage of caspase-3 from its pro-form to its active form has been shown to be critical for its role in apoptosis.

P53 tumor suppressor protein is a transcription factor that regulates the transcription rate of several genes involved in the regulation of cell cycle, DNA repair and apoptosis (Wang *et al*., 2005). In the current study, PRP was associated with the down-regulation of p53, phospho p-53 and Bad (Figures 4A, 4B and 4C). Phosphorylation, induced by multiple stress-activated kinases, has been proposed to be essential for p53 stabilization, interaction with transcriptional co-activators, and activation of p53 target genes. Our study suggests that propanil-activated phosphorylation may not be essential for p53 activation, which corroborates another report that phosphorylation may not be required for execution of biological functions of p53 (Thompson *et al*., 2004). Our study is further re-enforced by a report that arsenic could induce apoptosis by a p53 independent pathway (Huang et al, 1999). The decreased expression of p53 further contributed to the down-regulation of the p53-dependent protein Bad (Figure 4C). Although the apoptotic activity of Bad is largely determined by its phosphorylational status, upon apoptotic stimuli, Bad is rapidly dephosphorylated and targets to the mitochondria where it induces cell death. It has been demonstrated that p53 can form a complex with dephosphorylated Bad and this p53/Bad interaction appears to be independent of the phosphorylation of Bad at Ser-112(Jiang *et al.*, 2007). Our results indicated the above trend.

Immunohistochemical (IHC) staining is currently the gold standard for the quantitative measurement of protein biomarkers (Dunstan *et al.*, 2011). Several studies have demonstrated a significant correlation between results of IHC protein quantification and quantification using established methods including enzyme-linked immunosorbent assay and western blot (Harford-Wright *et al.*, 2014; Sysel *et al*., 2013; Thornton and Vick, 2012). IHC staining uses specific antibodies to differentially stain for the antigens of choice, and relies on the principle that greater antigen content leads to increased precipitation of immune complexes coupled to a chromophore such as 3, 3'diaminobenzidine. Computer-assisted analysis of digitally imaged IHC-stained tissues has been introduced as a means of overcoming the shortfalls associated with manual counts (Lejeune *et al.*, 2008; Sysel *et al*., 2013). In this study, we deployed immunohistochemical staining techniques to examine the protective role of PME on PRP-induced alterations in apoptotic markers caspases 9 and 3, Bax and Bcl-2 (Figures 4F-4I).

The mechanisms of apoptosis could be explained by the intrinsic and extrinsic pathways. The intrinsic apoptotic pathway, also known as mitochondrial pathway, is initiated by intracellular signals that target on the mitochondrial membrane potential and in turn result in the opening of mitochondrial permeability transition pore. Subsequently, some pro-apoptotic molecules such as cytochrome c are released from mitochondria to successively activate caspase-9 and caspase-3. Once caspase-3 is activated, the execution pathway is initiated which will lead to nuclear DNA fragmentation, degradation of nuclear and cytoskeletal proteins, and finally the formation of apoptotic bodies (Green and Kroemer, 2004). The extrinsic apoptotic pathway, also known as death receptor pathway, is initiated by the death signal which is transmitted from cell surface to the intracellular signaling pathways by the death receptors. And then caspase-8 and caspase-3 are successively activated to execute cell apoptosis (Elymore, 2007).

Consequences from the toxin-induced excessive oxidative stress, depletion of antioxidant enzymes and induction of membrane lipid peroxidation may prompt the extrinsic or intrinsic apoptotic pathways (Kaplowitz, 2002; Zhang *et al*., 2003). These pathways eventually lead to the activation of caspases pathway for apoptosis that ends up with caspase-3 activation, thereby initiating cellular apoptosis (Guicciardi and Gores, 2005; Riordan and Williams, 2003). In the present study, we detected an increased caspase-9 activity in the liver of PRP-treated rats. Furthermore, in the same line with the previous studies, our results revealed significantly increased active caspase-3 content in liver tissue samples collected from propanil-treated rats in comparison with controls. The significant elevation of caspases-9 and 3 contents was significantly abrogated close to normal in sections obtained from animals treated with a combination of PME and propanil, indicating that PME could inhibit apoptosis in hepatocytes which might support and at the same time explain our histological examination results and liver function results. Hence, PME could act upstream of caspase-3 to block caspase-3 dependent cell death.

The control and regulation of apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins. The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic or anti-apoptotic. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, and some of the pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. These proteins have special

significance because they can determine if the cell commits to apoptosis or aborts the process (Elymore, 2007). It has been reported that Bcl-2 is released from its binding to Bax and loses its function in the regulation of anti-apoptosis processes and that activated Bax protein, by forming oligomeric pores that constitute the apoptosis-induced mitochondrial channels in the outer membrane, allow the release of cytochrome c into the cytosol to bind to pro-caspase 9 and Apaf-1 and thereby forming the apoptosome (Rousset *et al.,* 2012; Dejean *et al.,* 2010). In the present study, staining for Bax in liver tissues was significantly increased in rats given propanil (Figure 4H). Our findings are similar to several reports from other groups with respect to the mechanisms of apoptosis. For example, Ayan *et al*. (2012) demonstrated that Bax activities increased in livers of rats exposed to the organic solvent, toluene. It was also observed by Abarikwu *et al.* (2015) that an elevated expression of cell death effector Bax may contribute to apoptosis in SH-SY55 cells exposed to the pesticide, Atrazine. Since Bax is a protein involved in the mitochondrial pathway, it can be said that PRP leads to mitochondrial damage and thus activates the intrinsic pathway. As expected, Bcl-2 levels were down-regulated by PRP in the present study (Figure 4I). Therefore, this alteration in the expression of Bax and Bcl-2 leads to high Bax/Bcl-2 ratio which is an important factor in determining the cell's vulnerability to apoptosis. We also observed that PME mediated significant restoration in the PRP induced alterations in the expression of Bax and Bcl-2 in liver cells.

An increase in apoptosis was verified using the TUNEL method, which is an advanced method used to detect cellular damage by binding to broken DNA ends. TUNEL assay analysis of liver cells is presented in Figure 4J. The number of TUNEL-positive nuclei among control group was negligible. Administration of PRP resulted in a significantly higher frequency of apoptotic cells compared to those of control. It is worthy of note that PME prevented PRP induced hepatic apoptosis via downregulation of proteins involved in the mitochondrial pathways. Taken together, the attenuation of these apoptotic markers by pretreatment with PME indicates the chemopreventive role of these antioxidants in PRP-induced hepatic toxicity.

The present study provides evidence about the antioxidant and free-radical scavenging properties of *Pteropcarpus mildbraedii*. The phytochemical analysis conducted on the *Pterocarpus mildbraedii* extracts revealed the presence of tannins, flavonoids, steroids, phenols, cardiac glycoside, quinones and saponins (Table 5.1). These phytochemicals, also known as plant secondary metabolites, are known to support biological activities of medicinal

plants. According to Cia (2003), plants are endowed with free radical scavenging molecules, such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites, which are rich in antioxidant activity. Saponins are valuable principles found in various herbal medicines with pharmaceutical, cosmetical and nutraceutical merits. They have been reported to exhibit remarkable hepatoprotective activity (Dong *et al.*, 2015; Kim *et al.*, 2009). Thus the presence of these constituents in *Pterocarpus mildbraedii* should support the use of this plant against liver injury. Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defense mechanism (Parr and Bolwell, 2000). In recent years, flavonoids have attracted a tremendous interest as possible therapeutics against free radical mediated diseases (Middleton *et al.*, 2000). Scavenging of free radicals has been suggested to play a considerable part in the antioxidant activity of these compounds. The antioxidant effect of *Pterocarpus mildbraedii* has been reported (Omale *et al*., 2011; Odukoya *et al.*, 2007). Results from previous experiments in this study have demonstrated the ability of the dichomethane: methanol extract of *Pterocarpus mildbraedii* (PME) to protect against propanil-induced hepatoxicity and apoptotic cell death. The protective action by this extract was suggested to be largely dependent on the observed antioxidant activity and in addition, anti-apoptotic property. It was also suggested that these actions in this experimental model of hepatotoxicity is related to the total phenolic content of PME. To validate these speculations and provide explanations for the effects observed *in vivo*, the antioxidant and free radical scavenging activity of the extract was evaluated *in vitro*.

Phenolic compounds have been reported to function as antioxidants by virtue of their ability to donate hydrogen to stabilize reactive and unstable free radicals (Rice-Evans *et al.*, 1996). Indeed, results from this experiment indicate that phenolic compounds may make a major contribution to the antioxidant capacity of the PME. The presence of these phenolic compounds in this plant contributed to their antioxidative properties and thus the usefulness of these plants in herbal medicament. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) has been used extensively as a free radical to evaluate reducing substances. PME in this study exerted significant scavenging effect on $DPPH^{\cdot}$ radical at all concentrations (50 and 500 µg). This is depicted by the significant decrease in absorbance values ($p<0.05$) of reaction mixtures

containing PME at these concentrations when compared with the control. DPPH· solution freshly prepared in methanol exhibits a deep purple colour with maximum absorption at 517 nm. This purple colour generally bleaches, in the presence of an antioxidant molecule which can quench DPPH·, with the formation of 1, 1-diphenyl-2-hydrazine resulting in a decrease in absorbance (Yamaguchi *et al.,* 1998).This scavenging activity against DPPH· radical was also comparable to those of the standard, Gallic acid. This ability of PME to scavenge DPPH radical indicates that the plant was potently active. This suggests that the plant extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for the radical's reactivity. In addition, the ability of the FAMEs to scavenge the DPPH radical is an indication that the plant could be useful in slowing down the progress of oxidative stress (Atolani *et al.,* 2015).

There is a current shift towards evaluating the chemical composition and nutritive value of tropical plants, many of which are medicinal and the relevance of fatty acids (FAs) in human diet and health cannot be overemphasized. FAs have been reported to possess a myriad of activities including anticancer (Robinson *et al.,* 2002), anti-inflammatory (Calder, 2005) and antioxidant (Atolani *et al.,* 2012). The GC-MS profile of the fatty acid composition of *Pterocarpus mildbraedii* leaves is shown in Table 5B. A total of nine fatty acids were identified from the total ion chromatogram of the GC. The ratio of the total unsaturated to saturated fatty acids is approximately 1:3. Monounsaturated accounted for about 12.6 % while polyunsaturated accounted for about 13.9 % of total fatty acids. The dominant fatty acid was Hexadecanoic acid (C18:2) in a concentration of 22.65 %. Other major fatty acids included octadecanoic acid (13.04 %), oleic acid , an omega-9 monounsaturated fatty acid (6.66%), linolenic acid (5.89 %), Ceric acid 6.85%) and eicosanoic acid (4.56%). It has been argued that though diets inordinately high in any component, including saturated fat, are likely to be deleterious, but finite quantities of a variety of saturated fatty acids may provide distinct benefits to various metabolic processes and overall health (Feinman, 2010). The three-fold content of saturated fatty acids could also be responsible for the hepatoprotective potential of PME. This observation is strengthened by a reports stating that rats fed a saturated fat diet were protected from experimentally induced alcoholic and acetaminophen hepatotoxicity. According to the authors, as dietary saturated fat content increased, all measures of hepatic pathology and oxidative stress were progressively reduced (Ronis *et al*., 2004; Hwang *et al*.,

2011). Another classical research investigated the potential of dietary saturated fatty acids to reverse alcoholic liver injury despite continued administration of alcohol. The authors observed that the rats exposed to saturated fat showed marked histological improvement with decreased levels of endotoxin and lipid peroxidation, absence of NF-κB activation, and reduced expression of TNF-α and COX-2 (Nanji et al 2001). Taken together, the therapeutic effect of PME could also be attributed to the high content of saturated fatty acids leading to reduced lipid peroxidation, COX-2 and iNOS levels, which in turn resulted in decreased activation of NF-κB, C and reduced levels of P38 and Stat-3.

5.1.0 CONCLUSION

The relationship between propanil-induced hepatotoxicity and oxidative stress was further established in this study. The dichloromethane: methanolic extracts of *Pterocarpus mildbraedii* (PME) attenuated the hepatotoxicity and acute hepatic dysfunction induced by propanil in rats by reinforcing the antioxidant defense system *in vivo*. Propanil-induced hepatic toxicity was associated with the alterations in the expression of key proteins involved in apoptotic and inflammatory pathways.Specifically, PRP-induced increase in the expressions of NF-κB, COX-2, iNOS and other apoptotic-related proteins may likely contribute to its hepatotoxic effects. The immunohistochemical studies and TUNEL analysis suggested that the toxicity of PRP seems to be mediated by DNA damage.

PME at a relatively low dose probably has a specific stabilizing effect on the cell membrane and other vital cellular macromolecules and can thus protect against PRP- induced hepatic damage and periportal necrosis. PME exhibited significant free radical scavenging and antioxidant activities, *in vitro*. Phytochemistry revealed the presence of antioxidant phenolics, flavonoids and saponins. In addition, the determination of the chemical composition of PME revealed the presence of unsaturated fatty acids, which has been reported in literature, to be anti-inflammatory. PME via its antioxidant, free radical scavenging activities and anti-inflammatory properties offered protection against PRP- mediated hepatotoxicity. Therefore,

PME may represent therapeutic tools in clinical conditions characterized by exposure to pesticides.

CONTRIBUTIONS TO KNOWLEDGE

The findings of the present study show that:

1. Free radicals and altered antioxidant defense system are involved in the pathophysiology of PRP- induced hepatotoxicity.
2. PME protected against PRP-induced hepatic damage via up-regulation of antioxidant status and enzymes in rats.
3. Caspases 3 and 9 and Bax expressions play key roles in the pathogenesis of PRP-induced oxidative injury in rat liver.
4. PME prevented induction of apoptosis and DNA damage in PRP-exposed rats via down-regulation of Caspase 3, caspase 9 and Bax expressions.
5. PME ameliorated the PRP-induced elevations in some tissue markers of inflammation viz; Nitric Oxide and Myeloperoxidase.
6. Our studies also revealed that PME suppressed PRP-induced phosphorylation of MAPK related proteins.
7. PME prevented inflammation in PRP-exposed rats via down-regulation of NFκB, COX-2 and iNOS expressions.

8. *P. mildbraedii* leaves are rich in saturated fatty acids and these could afford hepato-protection by decreasing levels of lipid peroxidation and inflammatory proteins

RECOMMENDATIONS

1. The consistency in our results from all experiments conducted strengthens the claim that the pesticide propanil induces oxidative stress, inflammation and apoptosis in the liver. However, no single assay could conclusively detect and measure inflammation and apoptosis in the cells. Therefore, it is important to examine multiple parameters using multi-pronged techniques before drawing conclusions.
2. The antioxidant activity of the *Pterocarpus mildbraedii* also indicated that the leaves could be a viable natural renewable source of antioxidant compounds.
3. This study lends credence to a growing body of literature that saturated fats could have a hepatoprotective effect. This could be harnessed for chemo-therapeutic purposes.

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APPENDIX

CALCULATIONS

Creatinine, Urea and Protein Concentrations

Concentration = O.D. of test X Concentration of standard **(**mg/100mL) O.D. of standard

Superoxide Dismutase (SOD) activity

Increase in absorbance per minute = A3 – A0/2.5

Where A0=absorbance after seconds and A3=absorbance 150 seconds

$$\% \text{ inhibition} = \frac{\text{increase in absorbance for substrate}}{\text{increase in absorbance of blank}} \times 100$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 minute.

Glutathione-S-transferase activity

The extinction coefficient of CDNB = $9.6mm^{-1}$ Cm^{-1}

$$\text{GSH-S-transferase activity} = \frac{OD/min}{9.6} \times \frac{1}{0.03mL \text{ mg protein}}$$

= µmole/min/mg protein.

Malondialdehyde (MDA) level

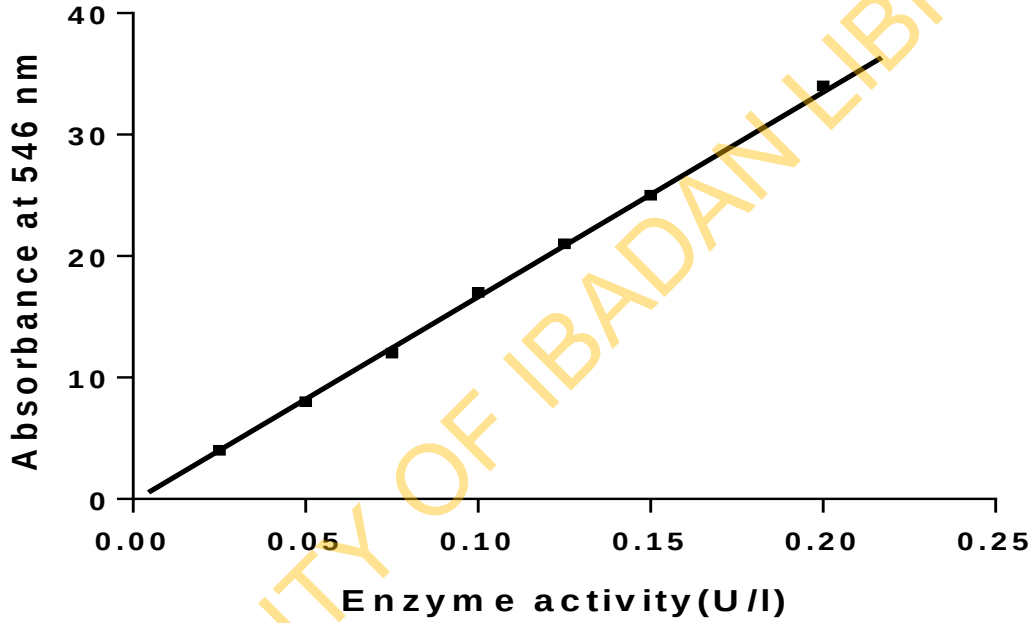
$$\text{MDA (units/mg/protein)} = \frac{\text{Absorbance x volume of mixture}}{\text{E532nm x volume of sample x mg protein}}$$

1,1-Dipheny-2-picrylhydrazyl (DPPH.) Radical Scavenging assay

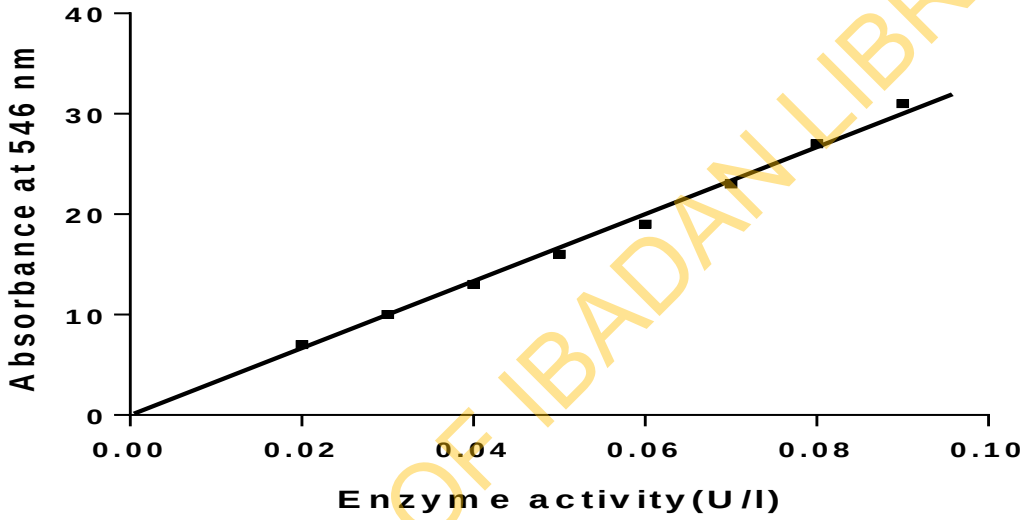
% RSA = 100 x (1-AE/AD)

Where AE is the absorbance of the solution when extract has been added at a particular level, and AD is the absorbance of the $DPPH^{\cdot}$ solution without extract.

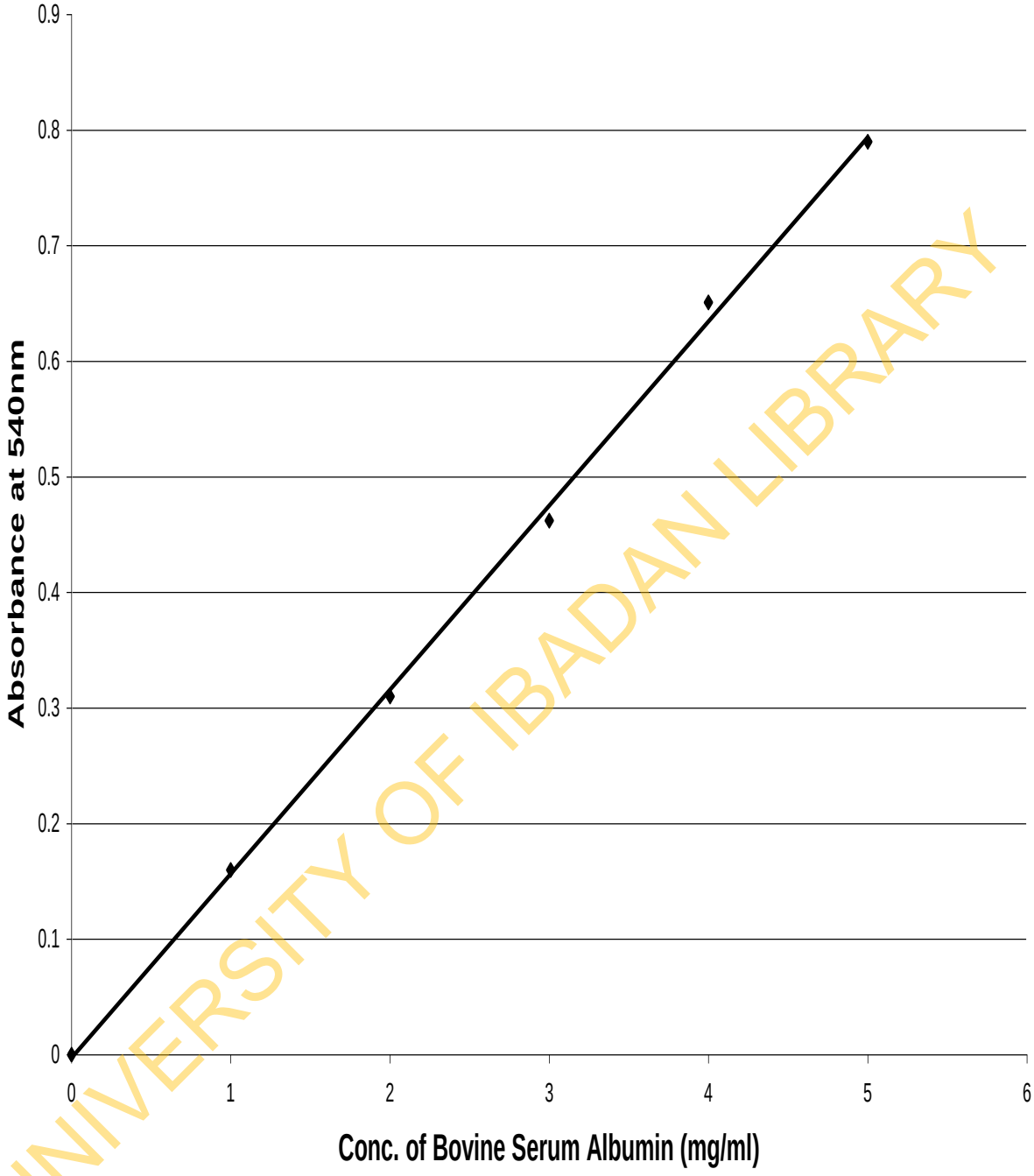
Standard curve for ALT determination



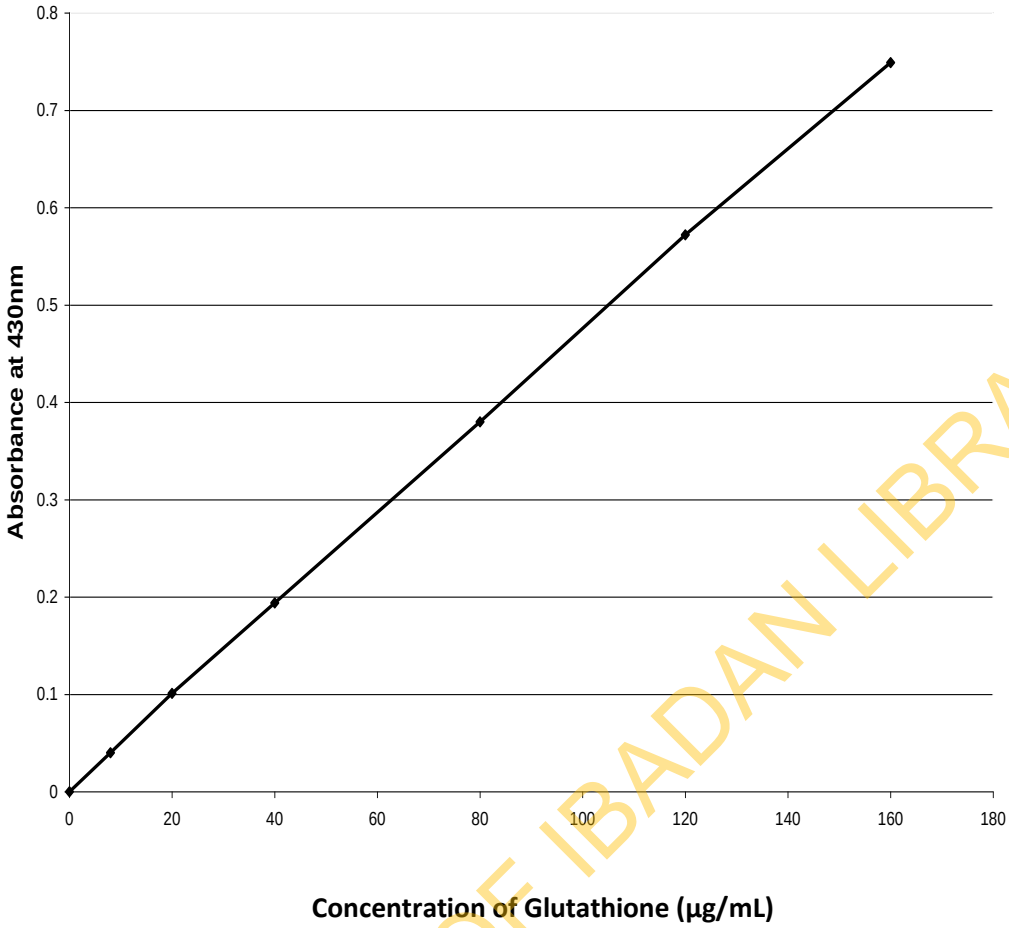
Standard curve for AST determination



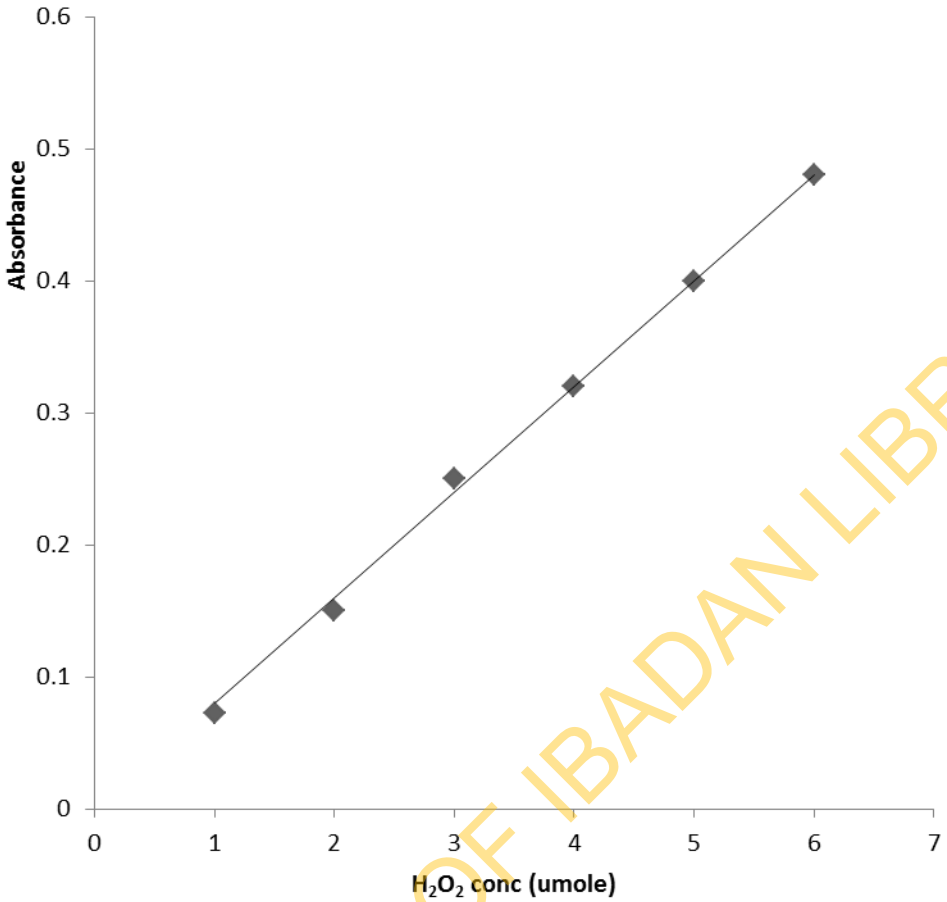
Standard curve for Protein level estimation



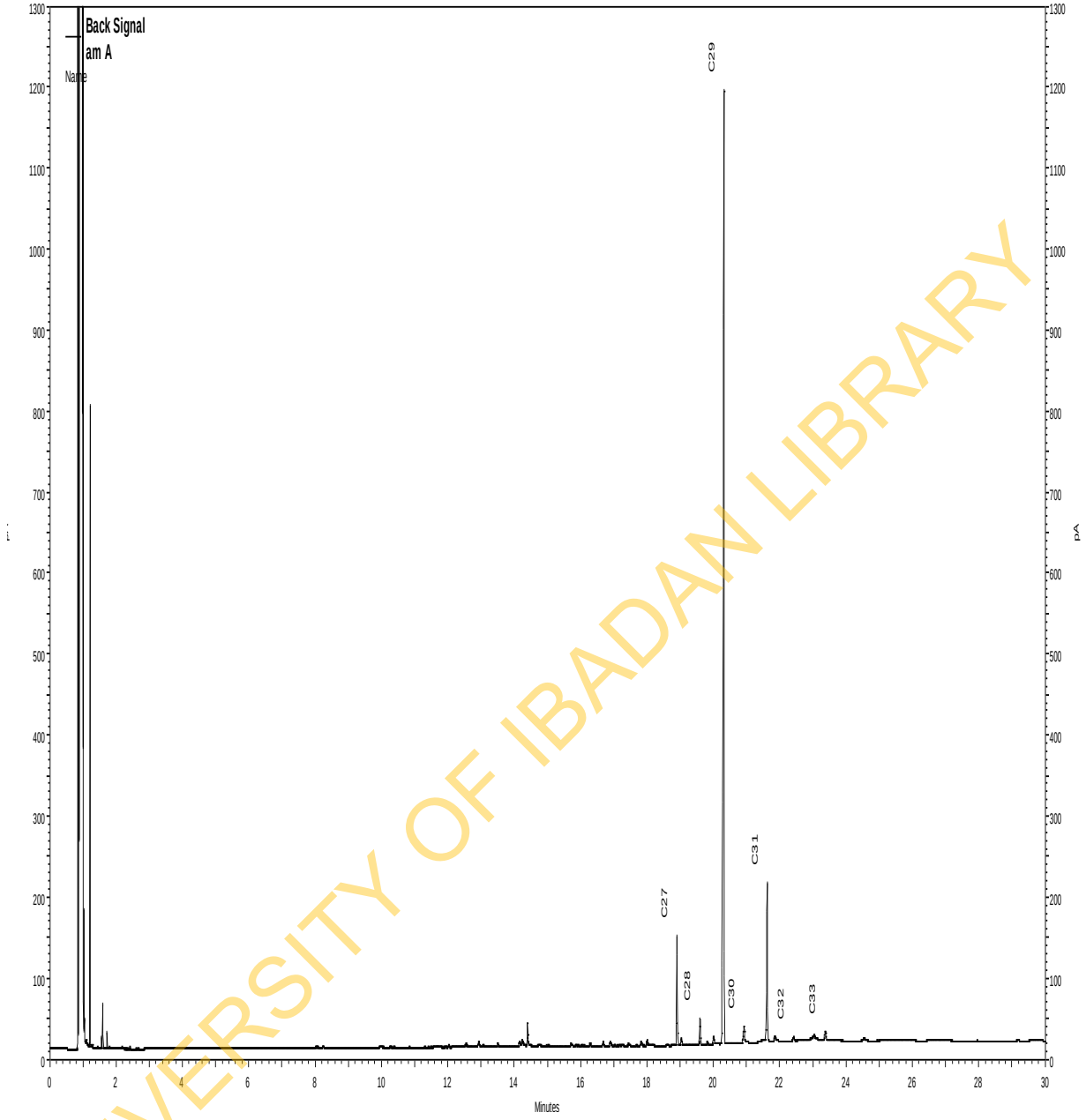
Standard curve for Glutathione estimation



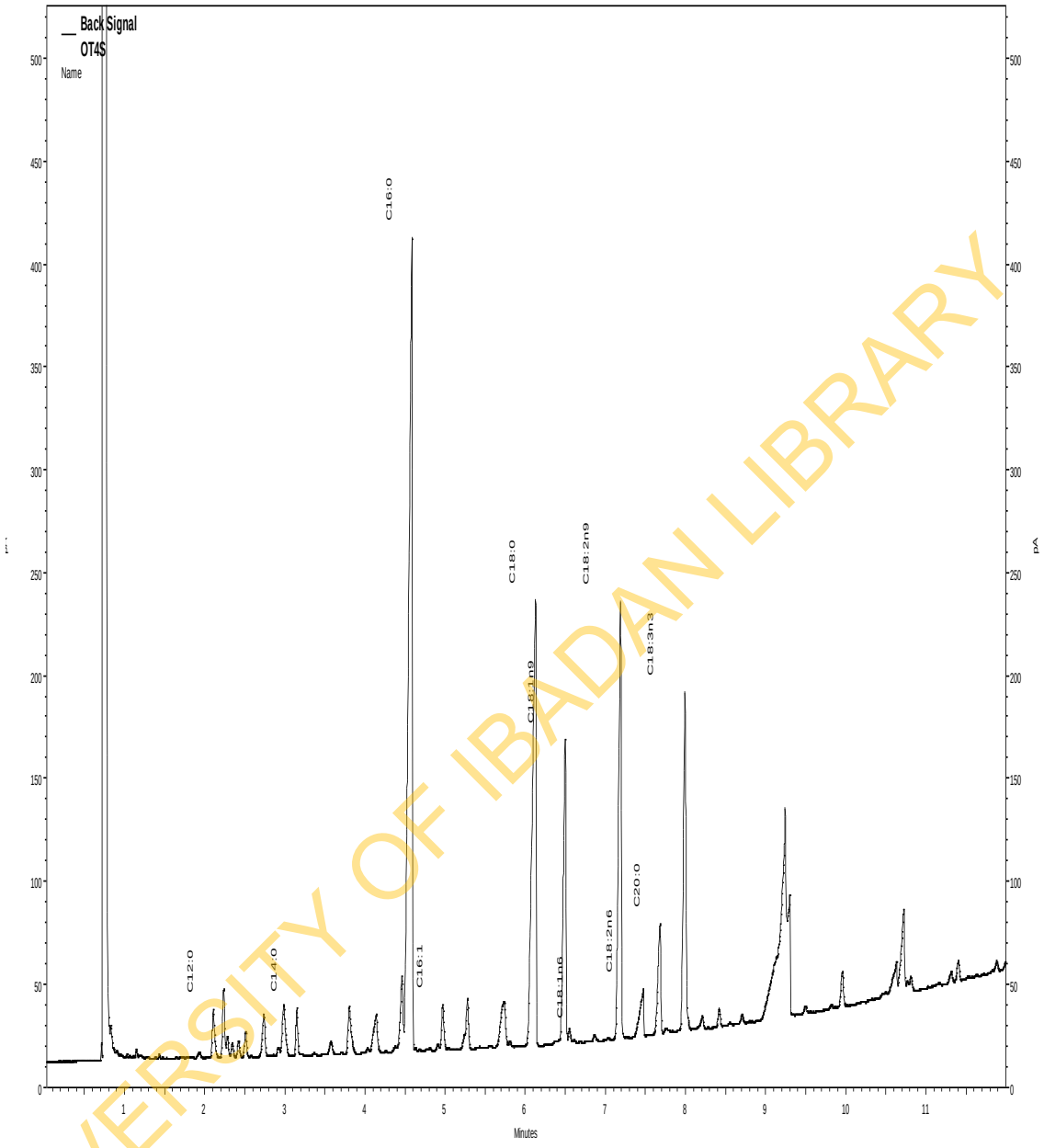
Standard curve for catalase



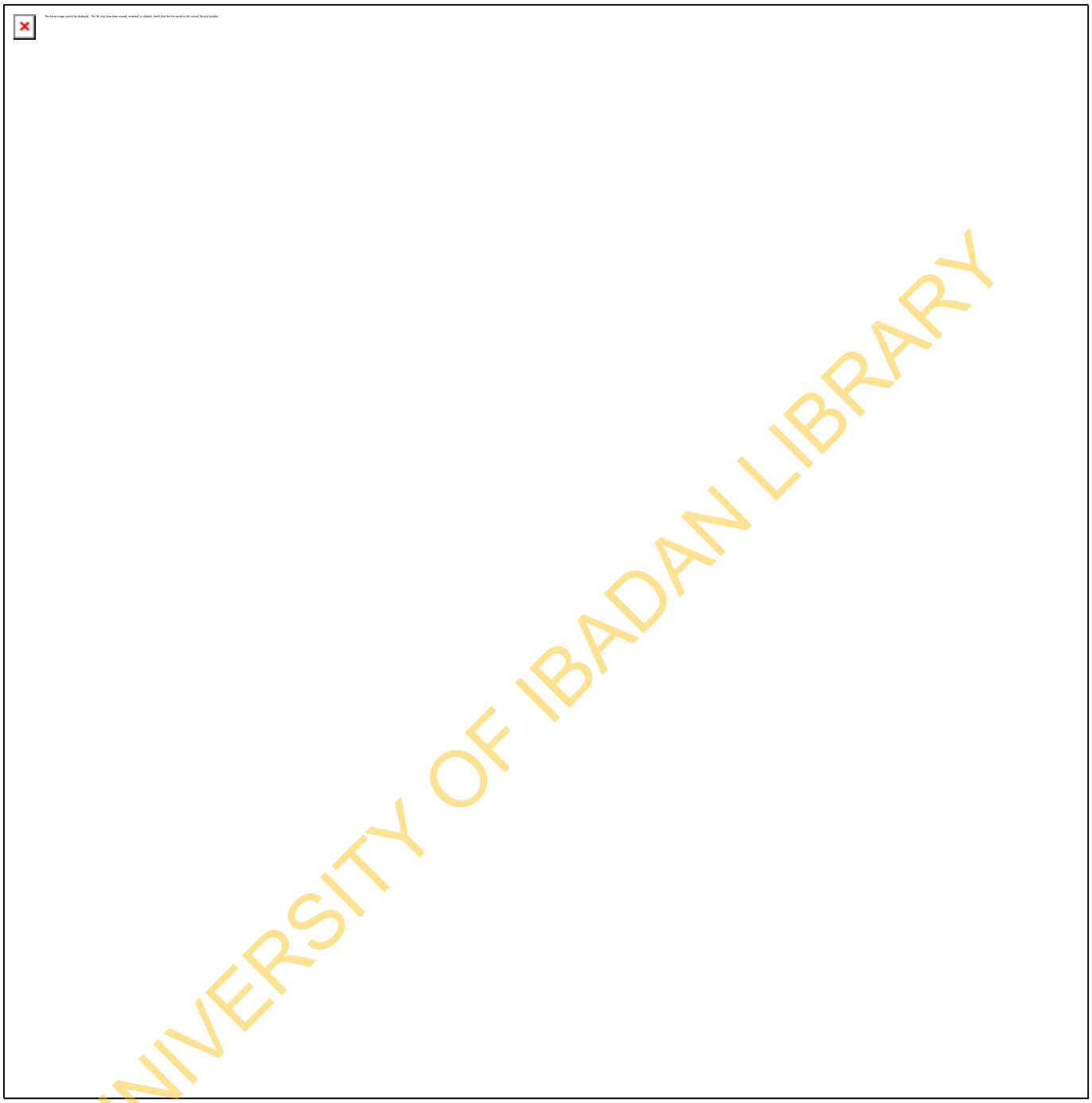
Chromatogram of GC analysis of PMEA



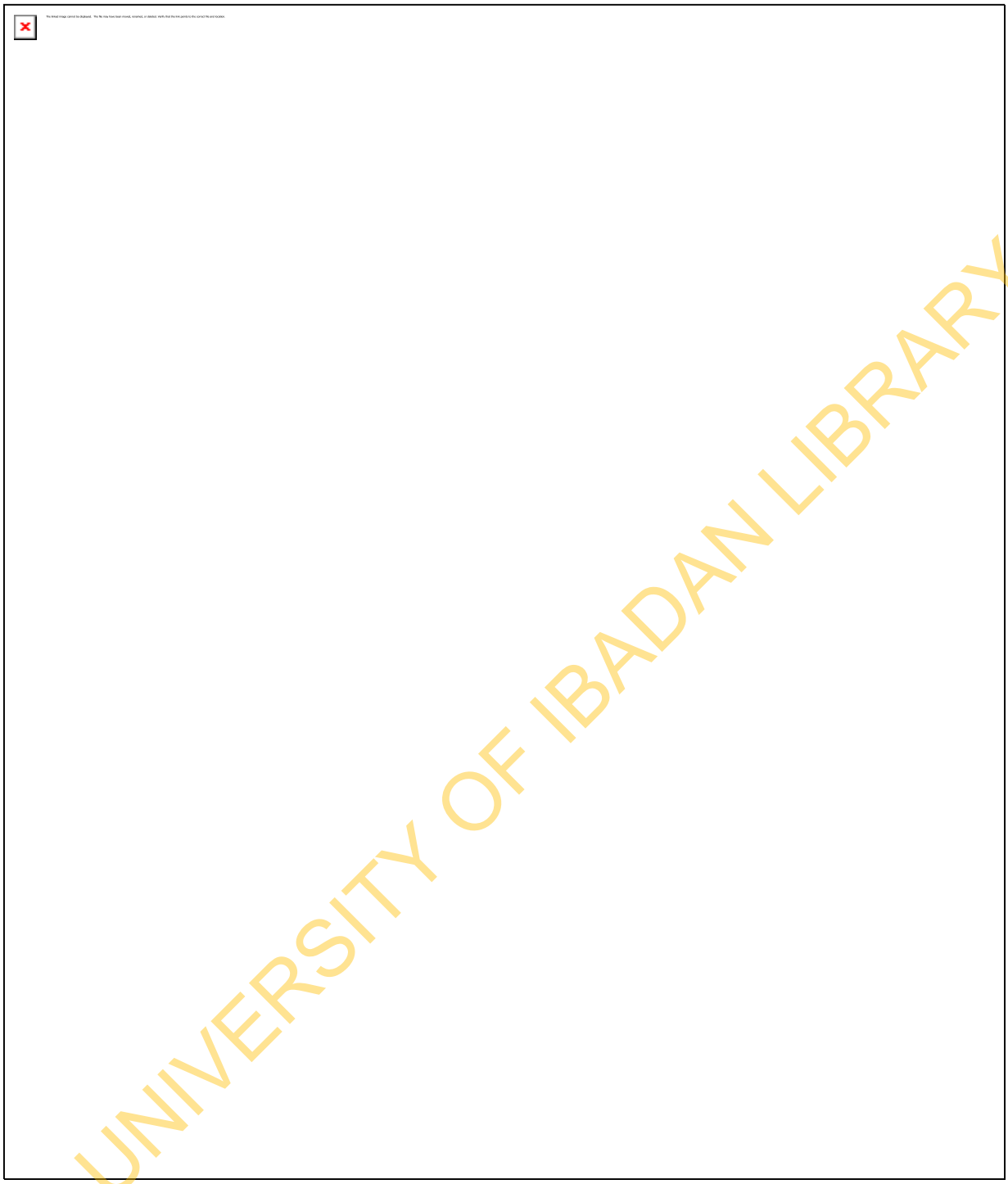
Chromatogram of GC analysis of Fatty acid methyl esters of *Pterocarpus mildbraedii*



FTIR Spectrum of Fraction PMEA



FTIR Spectrum of Fraction PMEB



FTIR Spectrum of Fraction PMEC