THE EFFECT OF AQUEOUS LEAF EXTRACT OF *Psidium guajava* (GUAVA) ON ALCOHOL INDUCED LIVER DAMAGE IN ADULT WISTAR RATS.

BY

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B.Sc. ANATOMY (ABU - 2006)
(MSc/MED/7926/2009-2010)

DEPARTMENT OF HUMAN ANATOMY,
FACULTY OF MEDICINE,
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

NOVEMBER, 2014.

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DEPARTMENT OF HUMAN ANATOMY,
FACULTY OF MEDICINE,
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA.

NOVEMBER, 2014.

DECLARATION

I, AFUWAI Vera Kasang hereby declare that this thesis titled "The study of the effect of aqueous leaf extract of *Psidium guajava* (guava) on alcoholinduced liver damage in adult Wistar rats" was performed by me in the Department of Human Anatomy, under the supervision of Prof. S. S.Adebisi (BSc., MSc., Ph.D) and Dr. A. A. Buraimoh (BSc., MSc., Ph.D). The information derived from the literature has been duly acknowledged in the text and a list of the references is provided. No part of this work has been presented for another degree or diploma at any other institution.

---------- ----------

(Signature) **(Date)**

CERTIFICATION

This project report/thesis/dissertation titled: The effect of aqueous leaf extract of *Psidium guajava* (guava) on alcoholinduced liver damage in adult Wistar rats meets the regulation governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty, the giver of everything, to my daughter, and my family.

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ABSTRACT

Plants are an important source of medicine, and the plant *Psidium guajava* has been used for the treatment and management of different aliments including liver diseases. This study was designed to investigate the effect of aqueous leaf extract of *Psidium guajava* on alcohol induced liver damage. 50adult Wistar rats were used for the experiment.The experimental animals were randomly divided into ten groups, each group comprised of 5 rats. The experiment was divided into 2 protocols; protocols A and B. Protocol A was for hepatoprotective study, comprising 7 groups while protocol B was for curative study comprising 3 groups. Absolute ethanol was used to induce liver damage in the Wistar rats. In the hepatoprotective study, group 1 rats were administered distilled water (5mg/kg b/w) orally daily for 7 days. Group-2 rats were administered ethanol 6000mg/kg b/w orally as a single application on the day 7. Group-3 rats were administered 100mg/kg b/w of silymarin daily for 7days plus 6000mg/kg b/w of ethanol on day 7 orally. Group-4 rats were administered 800mg/kg b/w of aqueous extract of *Psidium guajava* orally for 7 days. Group-5 rats were administered 300mg/kg b/w of aqueous extract of *Psidium guajava* orally for 7 days plus 6000mg/kg b/w single dose of ethanol on day 7. Group-6 rats were administered 500mg/kg b/w of aqueous extract of *Psidium guajava*orally for 7days plus 6000mg/kg b/w single dose of ethanol on day 7. Group-7 rats were administered 800mg/kg b/w of aqueous extract of *Psidium guajava* orally for 7 days plus 6000mg/kg b/w of single dose of ethanol on day 7. In the curative study, group-8 rats were administered 6000mg/kg b/w single dose ethanol on day 1. Group-9 rats were administered 6000mg/kg b/w single dose ethanol on day 1 plus 500mg/kg b/w aqueous extract of *Psidium guajava* for 3 days. Group-3 rats were administered 6000mg/kg b/w single dose ethanol on day 1 plus

800mg/kg b/w aqueous extract of *Psidium guajava* for 3 days. In the hepatoprotective study, the liver function test analysis, Alanine Amino transaminase, Aspartate Amino transaminase and Alkaline Phosphatase (ALT, AST and ALP), AST showed statistically significant ($p<0.001$) increase in groups 3, 4 and 5. The oxidative stress markers, Catalase, Superoxide Dismutase and Lipid Peroxidation (CAT, SOD and LOP), CAT showed significant ($p<0.001$) increase in group 4, SOD showed significant ($p<0.01$) decreased in groups 2 and 5. Haematological indices showed significant increase in Red Blood Cell count (RBC) and Haemoglobin count (HGB) in group 2, significant ($p<0.001$) increase in group RBC and platelet count in group 4, significant ($p<0.001$) increase in RBC, HGB and platelet count in groups 6 and 7. Neutrophil count significantly ($p<0.001$) increased in groups 2, 4, 5 and 7. Lymphocyte count significantly ($p<0.001$) decreased in groups 2, 5 and 7. In the curative study, ALP was significantly ($p<0.005$) increased in group 10, WBC was significantly increased in groups 9 and 10, and RBC was significantly ($p<0.01$) increased in group in group 10. The histological study revealed almost normal hepatic architecture in all the groups except in Groups 2 and 4 (in the hepatoprotective study) and group 8 (in the curative study) which showed necrosis, mild oedema and congestion. Glycogen deposit was decreased in all the treatment groups in both protocols. The administration of aqueous extract of *Psidium guajava* significantly protected and restored tissue damage caused by ethanol and it can also be used as a blood booster.

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LIST OF ABBREVIATIONS

Abbreviation	Translation
ALT	Alanine amino Transaminase
ADH	Alcohol Dehydrogenase
ALD	Alcohol Liver Disease
ALDH	Aldehyde Dehydrogenase
ALP	Alkaline Phosphatase
AST	Aspartate amino Transaminase
BASO	Basophil
CAT	Catalase
CYP2E1	Cytochrome P450-2E1
GPT	Glutamate pyruvate transaminase
g	gram
g/L	gram/liter
HCl	Hydrochloric Acid
<	Less than
LPO	Lipid Peroxidation
LYMP	Lymphocytes

µmol/mg	micromole/milligram
MEOS	Microsomal Enzyme Oxidizing System
mg	Milligram
ml	Mililiter
MONO	Monocytes
nm	Nanomole
nmol/mg	nanomole/milligram
NEUT	Neutrophil
%	Percentage
PAS	Periodic Acid Schiff
PLT	Platelet
PGE	*Psidium guajava* extract
RBC	Red Blood Cell
SOD	Superoxide Dismutase
TBA	Thiobabuturic Acid
TBARS	Thiobabuturic Acid Reactive Substance
TCA	Trichioro acetic Acid

WBC	White Blood Cell

CHAPTER ONE

INTRODUCTION

1.1BACKGROUND OF THE STUDY

Plant products have been important sources of medicine for thousands of years. The World Health Organisation (WHO) estimated that 80% of people still rely on traditional remedies such as herb for their medicines (Tripathi and Tripathi, 2003). Plants are also sources of many modern medicines. It is estimated that approximately a quarter of processed drugs contain plant extracts or active ingredient obtained from or modeled on plant substances (Tripathi and Tripathi, 2003).

The plant,*Psidium guajava* is from the family myrtaceae. It is a small tree of about 8m high and often cultivated throughout the tropics with the leaves and the fruit being the most used part of the plant. The long history of*Psidium guajava*(guava) for medicinal purposes led to the documentation of its properties/actions which includes antioxidant effect which is beneficial to the heart as a cardiprotective agent (Jimenej-Escrig, 2002). Its analgesic, anti-bacterial, anti-diarrhoea,anti-hypoglycemic, cytotoxic, anti-malarial, anti-ulcerous and antioxidant properties have been well documented (Caceres *et al.,* 1993,Lutterodt*et al.*,1992, Malcolm *et al.,* 1969, Maruyama *et al.,* 1985, Gessler*et al.*,1994, Villareal*et al.,* 1992, Qian*et al.*,2004).

Guava has been reportedly used as a cardio depressant, CNS depressant, cough suppressant, hypotensive agent, sedative and a vasoconstrictor. Other properties/action

documented by traditional users include: anti-anxiety, anticonvulsant, blood cleanser and a menstrual stimulant.The fruit is still enjoyed as a sweet treat and for medicinal uses by indigenous people throughout the rain forest zone up till today (Gill, 1992).

Chemical analysis of guava plant extract have revealed the presence of anti-microbial compounds (Arima, 2002), tannins, phenol,triterpenes, flavonoids, guajivolic acid, guajavanoic acid, linolenic acid, guavacoumaric acid, galaturonic acid, asphaltic acid, benzaldehyde, essential oils, saponins, carofenoid,cectin, fiber, fatty acids and a high content of vitamins C and A in its fruit (Suntornonk,2002).

The LD_{50} of *Psidium guajava* leaf extract is found to be above 5000mg/kg b/w (Jaiarj*et al.*, 1999).

The common names of this plant include *guaba*in Yoruba, *guwaiva*in Hausa, *gova*in Igbo and *ugwaba*in Efik (Gbile, 1984;Okujagu*et al.*, 2005).

Liver is an organ in the upper abdomen that aids in digestion and removes waste products and worn-out cells from the blood. It is a vital organ present in vertebrate and some other animals, which has a wide range of functions including detoxification and protein synthesis. The liver is our greatest chemical factory, builds complex molecules from simple substances absorbed from the digestive tract, neutralizes toxins, manufactures bile which aids fat digestion and removes toxins through the bowels (Maton*et al.*, 1993). But the ability of the liver to perform these functions is often compromised by numerous substances we are exposed to on a daily basis; these substances include certain medicinal agents which when taken in over doses and sometimes when introduced within therapeutic ranges injures the organ (Gaglianо*et al.*, 2007).

Alcohol-related disorders are one of the challenging health problems with far reaching medical, social and economic consequences (Devipriya*et al*., 2007). The World Health Organization reported thatwith about two billion alcohol consumers worldwide, 76.3 million people have diagnosable alcohol use disorders (WHO, 2004). Globally, alcohol causes 3.2% deaths for all causes (8 million deaths annually) and accounts for 4% of disease burden (WHO, 2007). Ethanol (C_2H_5OH) has been used extensively to study liver injury induced by free radicals in an animal model system.

Ethanol treated rats are widely used to study its toxicity (Adebisi, 2002,2003,2004) including liver damage where it was reported that ethanol induced not only necrosis but also apoptosis in rat liver (Hiroshi *et al*., 2000; Zili*et al*., 2005).Ethanol is removed from the body mainly by metabolism in the liver. Approximately 90% of the alcohol is metabolized in the liver, while the gastrointestinal tract, lungs, and kidneys play only a minor role (Lieber, 1994). Studies have also emphasized the role of bacteria in the colon that are capable of ethanol metabolism (Salaspuro, 1996).

The LD_{50} of absolute ethanol in rats is 10,300mg/kg (Robert, 2004).

In the liver, there are three metabolic pathways involved in the clearance of ethanol. The major pathway, cytosolic alcohol dehydrogenase (ADH), catalyses reversible oxidation of ethanol to acetaldehyde. In normal conditions, it accounts for about 80-90% of ethanol oxidation. The pathway through cytochrome P450-2E1 (CYP2E1) is normally of minor quantitative importance but is induced by chronic alcohol consumption. The role of

peroxisomal ethanol metabolism mediated by catalase is less important under physiologic conditions in humans except in the fasted state (Handler and Thurman 1990). Chronic alcohol consumption by rats has been shown to result in increased H_2O_2 production in percentral regions of the liver and increased catalase activity (Misra*et al.*, 1992). Ethanol oxidation may produce pathogenic effects as a result of altered redox state, toxic products made by the induced CYP2E1, and the direct cellular toxicity of acetaldehyde.

In the absence of reliable liver protective drugs in allopathic medical practices, herbs play a role in the management of various liver disorders especially in developing countries. However, we do not have satisfactory remedy for serious liver disease; most of the herbal drugs speed up the natural healing process of liver, so the search for effective hepatoprotective drug continues.

1.2 STATEMENT OF THE PROBLEM

In recent times, hepatotoxicity and liver related diseases are becoming prevalent especially in developing countries, such as, Nigeria. Some of these diseases result from the kind of diet and life style of the individuals.

Over the years, *Psidium guajava* leaves have been reportedly used traditionally for the management of different ailments including those of liver diseases,therefore, there should be a foundation to the use of *Psidium guajava* leaves for hepatoprotection.

1.3 JUSTIFICATION FOR THE STUDY

Popular and indigenous medical systems are mostly the immediate intervention for most of the people that lack access to formal health resources. Since plants are integral elements of these medical systems, it is important to understand indigenous therapeutic objectives in using plant medicine.

The improvement of health in developing nations requires an understanding of indigenous and popular concepts of liver related diseases, including its etiology and treatment.

1.4 SIGNIFICANCE OF THE STUDY

Results of the study can be exploited in the design of hepatoprotective drugs which are natural, easily accessible, cheaper, andmay have minimal side effect, thus serving as a better alternative to the other known hepatoprotective drugs e.g.,silymarin erythromycin, alpha methyldopa (Shah, 2010).

1.5AIM OF THE STUDY

To evaluate the effect of aqueous guava leaf extract on ethanol-induced liver damage in adult Wistar rats.

1.6OBJECTIVES OF THE STUDY

The objectives of the study are to:

i. investigate the effect that aqueous leaf extract of guava could have on alcohol induced liver damage using histological techniques.
ii. determinehepatic enzyme activity using biochemical techniques.

iii. investigate the effect of aqueous guava leaf extract on some haematological indices.

1.7 STUDY HYPOTHESES

i. Aqueous extract of guava leaf has no effect on alcohol induced liver damage.

ii. There is no hepatic enzyme activity using biochemical techniques.

CHAPTER TWO

LITERATURE REVIEW

2.1 GUAVA TREE

The long history of guava for medicinal purposes led to the documentation of its properties/actions which includes antioxidant effect which is beneficial to the heart as a cardio protective agent (Jimenej-Escrig, 2002), its analgesic, anti-bacterial, anti-dysenteric, anti-malarial and anti-ulcerous properties has also been Documented. Guava has also been used as a cardio depressant, CNS depressant, cough suppressant, hypotensive agent, sedative and a vasoconstrictor. Other properties/action documented by traditional users include: anti-anxiety, anticonvulsant, blood cleanser and a menstrual stimulant. The fruit is still enjoyed as a sweet treat and for medicinal uses by indigenous peoples throughout the rain forest up till today(Gill, 1992).

2.2 CHEMICAL COMPOSITION OF *Psidium guajava*LEAF

The chemical analysis of guava plant extract have revealed the presence of anti-microbial compounds (Arima, 2002), tannins, phenol triterpenes, flavonoids, guajivolic acid, guajavanoicacid,linolenic acid, linoleic acid, guavacoumaric acid, galaturonic acid, asphaltic acid, benzaldehyde, essential oils, saponins, carofenoid, cectin, fibre ,fatty acids and a high content of vitamins C and A in its fruit (Suntornonk*et al* 2002). The leaves are rich in flavonoids, (quercetin) attributing for most of its therapeutic activity.

2.3 MAIN ACTION

The long history of guava use has led moderndayresearchers to study guava extracts.
Its traditional use against diarrhea, gastroenteritis and other digestive complaints has been validated in numerous clinicalstudies.

Anti-diabetic activity: The ethanolic stem bark extract exhibited statisticallysignificant hypoglycaemic activity in alloxan-inducedhyperglycaemic rats but was devoid of significanthypoglycaemic effect in normal and normal glucose loadedrats (OGTT). In both acute and sub-acute tests, the water extract, at an oral dose of 250 mg/kg, showed statistically significant hypoglycemic activity (Mukhtar*et al.*, 2004).

Antimicrobial activity: *Psidiumguajava*aqueous bark and methanolic extracts werefound to possess anti-bacterial activity. Four antibacterialcompounds were isolated from leaves of guava wereidentified. The minimum inhibition concentration of morin-3-Oalpha-L-lyxopyranoside and morin-3-O-alpha-Larabopyranosidewas 200 microg/ml for each against*Salmonella enteritidis*, and 250 microg/ml and 300 microg/mlagainst *Bacillus cereus*, respectively. The hot water extractand the methanol extract of*Psidiumguajava*showed high activity against *Arthriniumsacchari*and *Chaetomiumfunicola*strains (Abdelrahim*et al.*, 2002; Arima andDanno, 2002; Sato *et al.*, 2000).

Antidiarrhoeaactivity: *Psidiumguajava*leaf aqueous extract (PGE) (50-400 mg/kgp.o.) produced dose-dependent and significant protection ofrats and mice against castor oil-

induced diarrhoea, inhibitedintestinal transit, and delayed gastric emptying. Like atropine(1 mg/kg, p.o.), PGE produced dose-dependent andsignificant antimotility effect, and caused dose-relatedinhibition of castor oil-induced enteropooling in the animals.Like loperamide (10 mg/kg, p.o.), PGE dose-dependently andsignificantly delayed the onset of castor oil-induced diarrhoea,decreased the frequency of defaecation, and reduced theseverity of diarrhoea in the rodents(Ojewole*et al.,* 2008).

Treatment of plaque: The active flavonoid compound, quercetin-3-O-alpha-larabinopyranoside(guaijaverin) isolated from *Psidiumguajava*demonstrated high potential antiplaque agent by inhibiting the growth of the Streptococcus mutans (Prabu*et al.,* 2006).

Spermatoprotective activity: The extracts of the leaves of *Psidiumguajava*Linn. possessbeneficial effects on sperm production and quality, and maythus improve the sperm parameters of infertile males witholigospermia and nonobstructiveazoospermia(Akinola*et al.,* 2007).

Spasmolytic effect: The spasmolytic activity of the *Psidiumguajava*leaf remedy ismainly due to the aglyconequercetin, present in the leaf andin the extract mainly in the form of five flavonols, and whoseeffect is produced when these products are hydrolyzed bygastrointestinal fluid (Lozoya*et al.,* 1994).

Treatment of infantile rotaviral enteritis: *Psidiumguajava*showed good curative effect on infantilerotaviral enteritis (Wei *et al.,* 2000).

Anti- cancer activity: Aqueous extract of *Psidiumguajava*L. budding leaves hasbeen shown to possess anti-cancer activity in a cell line model. Treatment with *Psidiumguajava*L. budding leaves(1.5 mg/mouse/day) significantly diminished both the prostatespecific antigen (PSA) serum levels and tumor size in axenograft mouse tumor model. Guava leaf essential oil hasbeen shown to possess cytotoxic effect on Human cervicalcancer cell lines (Chen *et al.,* 2010**;**Joseph *et al.,* 2010).

Analgesic & anti-inflammatory activity: The aqueous extract of *P. guajava*leaves possessesanalgesic and anti-inflammatory properties. The hexane, ethylacetate and methanol extracts of *Psidiumguajava* leaves (20,100,500 and 1250 mg/kg) exhibited mostly dose-dependentantinociceptive effects in chemical and thermaltests of analgesia (Ojewole, 2006;Shaheen*et al.,* 2000).

Anti-proliferative activity: Guava leaf extract has anti-proliferative activity caused byinhibition of the catalytic activity of Prostaglandinendoperoxide-H synthase (PGHS) isoforms (Kawakami *et al.,* 2009).

Contractile effect: Aqueous leaves extract of *Psidiumguajava*significantly anddose-dependently (0.25-2 mg/ml) contracted aorta rings. Theeffect of *P. guajava*was to a large extent mediated byactivation of alpha-adrenoceptor and to a lesser extent byacting via calcium ion channel (Olatunji-Bello *et al.*,2007).

Hypotensive: *P. guajava*leaf aqueous extract (PGE, 50-800 mg/kg i.v)produced dose-dependent, significant reductions in systemicarterial blood pressures and heart rates of hypertensive, Dahlsalt-sensitive rats. The extract causes hypotension in themammalian experimental animal model used via cholinergicmechanisms (Ojewole, 2005).

Malaria: The leaves are used as an ingredient in the preparation of ever "teas". They are also used as a part of the pot herb usedin steam treatment for malaria. The stem bark extractcontained anthraquinones, flavonoids, seccoirridoids andterpenoids and were found to be effective for the treatmentand/or prophylaxis of malaria (Nundkumar andOjewole, 2002).

Oral care: In southern Nigeria the twigs are used as chew sticks and thepresence of bioactive compounds comprising of saponins,tannins, flavonoids, alkaloids are responsible for theireffectiveness. Chewing sticks when used without toothpasteare very efficient, effective, and reliable for cleaning teeth. Theteeth of chewing sticks users are usually strong, clean, fresh,and devoid of dental plaques carries (Okwu and Ekeke,2003).

Antipyretic: The methanol extract of the leaves of *Psidiumguajava*exhibited an antipyretic effect (Olajide*et al.*, 1999).

2.4 OTHER ACTIONS

CNS Activity: The leaves of the guava tree in decoction are used forspasms, epiand even for cerebral affections (Ticzon and Romeo,1997).

Conjunctivitis: Flowers are also used as a poultice for conjunctivitis(Ayensu, 1978)

Vaginal Disorders: The leaves of the guava tree in decoction are recommended for uterine haemorrhage. The same decoction is used as awash for vaginal and uterine problems, and especially wherean astringent remedy is needed (Ticzon and Romeo,1997).

Immunomodulatory activity: Extracts derived from *Psidiumguajava*revealedimmunomodulatory activities (Kailehet *al.*, 2007).

Antimutagenic activity: The water extract of *P.guajava*was effective in inactivating themutagenicity of direct-acting mutagens (Grover and Bala, 1993)

Inotropic effect: The extract from P. guajava leaves depress myocardialinotropism(Garcia *et al.*, 2003).

Treatment of acne: *Psidiumguajava*leaf extracts are used in treatment of acne(Qadan*et al.*, 2005).

2.5 CLINICAL USES

Antioxidant activity: The extracts from distilled water, 65% ethanol and 95% ethanol respectively showed effects on scavenging hydroxyl radicals and inhibiting lipid peroxidation in the dose-dependent manner, had 50% effective concentration (EC50) on scavenging hydroxyl radicals of 0.63, 0.47 and 0.58g/L, had EC50 on inhibiting lipid peroxidation of 0.20, 0.035, 0.18g/L (Wang *et al.*, 2007).

Treatment of cough: The water extract of the plant at doses of 2 and 5 g/kg, p.o.decreased the frequency of cough induced by capsaicin aerosol by 35 and 54%, respectively, as compared to the control, within 10 min after injection of the extract, ($P < 0.01$) (Chulasiri*et al.*, 1986).

Hepatoprotective activity: the aqueous extract of *psidiumguajava leaves* showed good hepatoprotective activity when administered ta doses of 250 and 500mg/kg orally and the effect observed was dose dependent. The lower dose (250mg//kg p.o) did not show hepatoprotective effect in chronic hepatic damage(Chanchal*et al.*, 2005).

2.6 ALCOHOL (ETHANOL)

Alcohol is a well knownhepatotoxin, and alcoholic hepatitisis a serious health problem (Lieber 1994). The mechanisms responsible for ethanol-induced hepatotoxicity have been the subject of investigation for many years. Unfortunately, answers remain elusive. One approach to this problem is to use a clinically relevant model in which an ethanol containing, corn oil-based, high-fat diet is given continuously to rats, as described originally by Tsukamoto *et al.* (1984). Unlike other models that only produce steanosis, this model also exhibits inflammation and necrosis that is similar to that observed in humans. Many studies have shown that ethanol administration *invivo* is associated with the formation of free radicals and oxidative stress(Lieber 1994,Moncada*et al.*, 1994, Albano*et al.*,1996, Misra*et al.*, 1992). Lipid peroxidation increases (French*et al.*, 1993) and hepatic antioxidants decrease (Morimoto*et al.*, 1994) after ethanol administration.

The pathophysiology of alcohol liver disease(ALD) is quitecomplex, encompassing factors related to genetics, gender, ethnicity, consumption patterns and co-morbid conditions. From such a composite interplay, several clinical manifestations ensue, ranging from a benign condition, such as steatosis, to deadly diseases, such cirrhosis and hepatocellular carcinoma. The mechanisms leading to alcohol-induced liver damage are also complex and not completely clarified. Altered metabolic pathways, energy metabolism impairment, immune-mediated events and oxidative stress all cooperate, with a relatively prevailing importance in different settings. It is generally accepted thatethanol induces an altered redox state associated withfree radical generation, resulting in lipid peroxidation, cell-membrane damage and depletion of mitochondrial antioxidants such as reduced glutathione (GSH). Aldehydes generated by ethanol oxidizing pathways are involved in several toxic effects of alcohol by forming protein adducts which are responsible for activation of specific intracellular signalling pathways modulating collagen synthesis and inflammatory response. This later effect is also coordinated by Kupffer cells, activated by portal vein endotoxin to release cytokines and chemokines. These changes are, in part, dependent on genetic factors including polymorphism of genes belonging to ethanol-metabolizing enzymes and to inflammatory/ immune response and, in part, are modulated by the presence of co-morbid conditions (Gramenzi*et al.,* 2006).

In the liver, there are three metabolic pathways involved in the clearance of ethanol. The major pathway, cytosolic alcohol dehydrogenase (ADH), catalyses reversible oxidation of ethanol to acetaldehyde. In normal conditions, it accounts for about 80-90% of ethanol oxidation. The pathway through cytochrome P450-2E1 (CYP2E1) is normally of minor quantitative importance but under situations of sustained exposure to ethanol it can be

induced several-fold. The role of peroxisomal ethanol metabolism mediated by catalase is less important under physiologic conditions in humans. Ethanol oxidation may produce pathogenetic effects in several ways as a result of an altered redox state, toxic products made by the induced CYP2E1, and the direct cellular toxicity of acetaldehyde.

2.7 ALCOHOL METABOLISM

An understanding of alcohol metabolism provides the basis for understanding alcohol-induced liver damage. Most of the alcohol that people drink is metabolized in the liver. The major pathway for alcohol metabolism involves the enzyme alcohol dehydrogenase (ADH). Thisenzyme converts alcohol to acetaldehyde through a chemical process called oxidation. As alcohol is broken down in the liver, a number of potentially dangerous by-products are generated. Acetaldehyde is highly toxic to thebody, even in low concentrations (Jacquelyn *et al.,* 1997). Normally,however, the enzyme aldehyde dehydrogenase (ALDH) rapidly oxidizes acetaldehyde to acetate. Most of the acetate travels through the bloodstream to other parts of the body, where it can enter other metabolic cycles (Lieber 1994) that produce energy or useful molecules. The usual biological role of both ADH and ALDH is to metabolize vitamin A (retinol). The microsomal enzyme oxidizing system (MEOS) is an alternate pathway for alcohol metabolism in the liver. Microsomal enzymes belong to a family of proteins called cytochromes. Some cytochromes, located in a cellular substructure called the endoplasmic reticulum detoxify harmful substances that enter the body. The MEOS oxidizes alcohol to acetaldehyde by means of a cytochrome called cytochrome P450 2E1, or CYP2E1, which is found in the endoplasmic reticulum of livercells. Normally functioning at a low level,

CYP2E1 is stimulated to a higher level by the presence of alcohol. Thus, the MEOS becomes increasingly important as alcohol consumption becomes heavier and more chronic (Adachi*et al.*, 1995).Excessive alcohol consumption is also associatedwith the enzymatic induction of CYP2E1 pathway of alcohol metabolism. The recruitment of this pathway may indirectly contribute to ALD development by excess production of superoxide radicals via the interaction of CYP2E1 with cytochrome reductase, which leads to electron leaks in the respiratory chain and reactive oxygen species (ROS) production (Polavarapu*et al.*, 1998, Lieber, 1999). The species produced in this cascade can interact with iron (Fenton reaction) generating even more potent hydroxyl, ferryl and perferrylradicals which perpetuate liver damage (Rouault 2003,Purohit*et al.*, 2003).

2.8 THE LIVER

The liver is the largest organ in the body and it accounts for approximately $1/4^{th}$ of adult body weight. It lies in the right and left upper quadrant of the abdomen, inferior to the diaphragm (Moore and Dalley, 1999).The liver is a reddish brown organ located immediately below the diaphragm, filling most of the right hypochondrial and epigastric regions and weighing about 1.4kg (Kenneth, 1999). The liver is responsible for various functions which include; filtration and storage of blood, metabolism of carbohydrate, protein, fat hormones, and foreign chemicals, formation of bile, storage of vitamins and iron and the formation of coagulation factors (Guyton and Hall 2000).

2.9 GROSS ANATOMY OF THE LIVER

The liver is wedge shaped and is covered by a network of connective tissues called the Glisson's capsule which comprises of the serous and fibrous coats. The liver has four surfaces which are superior, inferior, anterior, and posterior. The liver has four lobes which are the right lobe, left lobe, quadrate lobe and caudate lobe and these lobes are separated by ligaments of the liver. The liver is connected to the diaphragm and abdominal walls by five ligaments through the membranous falciform ligament, coronary ligament, right and left triangular ligament and the fibrous round ligament. The large right lobe and small left lobe are visible anteriorly. They are separated from each other by the falciform ligament, which is a sheet of mesentery that suspends the liver from the diaphragm and the anterior abdominal wall. The round ligament (ligamentumteres), which is a ruminant of the umbilical cord to the liver of foetus is also visible anteriorly. At the interior aspect, the quadrate lobe is next to the gallbladder and a tail-like caudate lobe is posterior to it. The gallbladder adheres to the depression on the interior surface of the liver, between the right and quadrate lobes. The posterior aspect of the liver has a deep groove which accommodates the inferior vana cava. Most of the liver is covered by a serosa but it is defective from the bare area of its superior surface, where the liver is attached to the diaphragm (Susan *et al.,* 2004; Henry *et al*, 1995).

2.10 MICROSCOPIC STRUCTURE OF THE LIVER

The liver parenchyma consist mainly of cuboidal cells, called hepatocytes which are arranged in innumerable cylinders, called hepatic lobules. Each lobule is about 1mm in diameter and 2mm in length and has a central vein passing through its core. The

hepatocytes form thin epithelial plates which fan out from the central vein. These plates are separated from each other by blood-filled channels (discontinuous capillaries), called hepatic sinusoids. The hepatocytes are in direct contact with the blood from which they continually extract and add substances. The sinusoids are lined by the phagocytic Kupffercells and typical endothelial cells. The endothelial lining of the sinusoids have extremely large pores and lying between these and the hepatic cells are narrow tissue spaces called space of disse, inter lobular septa (Moore and Dalley, 1999).

2.11 DEVELOPMENTAL STAGES OF THE LIVER

The liver develops from an endodermal bud that arises from the ventral aspect of the gut, at the junction between the foregut and the midgut. This bud grows into the ventral mesogastrium and passes through it into the septum tranversum. It enlarges and soon shows a division into a large cranial part called the pars hepatica, and a smaller caudal portion called the pars cystica. The pars hepatica divides into the right and left parts, which forms one lobe of liver. The left and right divisions of the pars hepatica enlarge and extend into the septum transversum, the cell arising from them are broken up into interlacing colomns called hepatic trabeculae. The umbilical and vitelline veins which lie in the septum transversum are broken up to form the sinusoids of the liver. The sinusoid are also formed from the mesenchyme of the septum transversum. The endodermal cell of the hepatic bud gives rise to the parenchyma of the liver and the bile capillaries. The mesoderm of the septum transversum forms the capsule and fibrous tissue of the liver (Singh, 2003).

2.12 VASCULAR SUPPLY OF THE LIVER

The liver is supplied by the hepatic artery and portal vein and it transmits a large volume of blood when the mesenteric arteries are dilated. The blood drains into the inferior vena cava principally through the right and left hepatic veins, and also through some smaller veins (Susan *et al.*, 2004).

The nerves are derived from the coeliac plexus and the gastric branches of the vagus nerve. Some sensory fiber in the phrenic nerve appears to reach the biliary tract (Susan *et al.*, 2004).The lymphatic vessels emerge either through the portal hepatic to the nodes in the portal and lesser omentum, or through the bare area of the pancreaticosplenic nodes through the diaphragm to the nodes in the thorax (Susan *et al.*, 2004).

CHAPTER THREE

MATERIALS AND METHOD

3.1 CHEMICALS

Absolute ethanol was used for this experiment, the epthanol was colourless, contained in an amber coloured bottle

withcas number 28304. The ethanol was obtained from BDH Chemical Limited, Poole, England. The ethanol weighing 0.87mg was dissolved in 1ml.

The silymarine (contained 70 mg / tablet) used for the experiment was obtained from Jerrusave Pharmaceutical, Sabon Tasha Kaduna, manufactured by Alexandria Co. Pharmaceutical, GamilaBohreid Road Awayed-Alexandria, Egypt.

3.2 PLANT MATERIAL

3.2.1 COLLECTION AND IDENTIFICATION OF THE PLANT

The guava leaveswereobtained from Botanical Garden, Department of Biological Sciences, Faculty of Sciences, Ahmadu Bello University, Zaria. The plant was identified and authenticated at the Department of Biological Sciences, Herbarium, Ahmadu Bello University, Zaria by Mallam Musa with a voucher number 3253.

3.2.2 SAMPLE PREPARATION

The leaf was air dried at room temperature for 3 days. The sample was grounded using pestle and motar and filtered using sieving net to obtain fine powder.

3.2.3 AQUEOUS EXTRACTION

Aqueous extraction of guava leaf was prepared according to the procedure of Taju*et al.,* (2011).The leaf was shade dried at room temperature for 3 days. The leaves were grounded using mortar and pestle. For each 100g of crushed leaves 300ml of distilled water was added. The crushed leaves were then boiled in a water bath for 1 h. The boiled leaf extract was then filtered through a muslin cloth. The aqueous extract obtained was evaporated to get a powdery mass that yielded (4.0% w/w). The powder obtained was then subjected to phytochemical analysis to determine the chemical constituents present in the extract. The powdery extract of PGJ leaves was diluted in water to obtain a concentration of 300mg, 500mg and 800mg/ml for oral administration.

3.3PRELIMINARY PHYTOCHEMICAL SCREENING OF THE PLANT

3.3.1 Test for Carbohydrates

3.3.1.1 Molisch's test

Few drops of Molisch reagent was added to a little quantity of the extract in a test tube and a small quantity of concentrated sulphuric acid was allowed to run down the side of the test tube. A purple to violet colour formed at the interphase indicates the presence of carbohydrate (Trease and Evans, 1983).

3.3.1.2 Fehling's test

Fehling's test was conducted by adding 2ml of the extract, 5ml of a mixture of Fehling solution A and B in the ratio of 1:1 and the mixture was allowed to boil for 5minutes. A

brick-red precipitate indicates the presence of free reducing sugar (Trease and Evans, 1983).

3.3.2 Test for Glycosides

Glycosides test was carried out by adding 5ml of concentrated sulphuric acid to the extract and boiled for 15 minutes. This was the cooled and neutralized with 20% KOH and was divided into two portions. Another part of the extract was dissolved in distilled water, this was used as a control to indicate the absence of acid hydrolysis (Trease and Evans, 1983).

3.3.2.1 Fehling's solution test

Fehling's solution A and B was added to a small portion of the extract and boiled for a few minutes. A brick-red precipitate indicates the presence of glycoside as a result of the hydrolysis of glycoside (Trease and Evans, 1983).

3.3.2.2 Ferric Chloride Test

Three drops of ferric chloride solution was added to a small portion of the extract. Green to black precipitate indicates the presence of phenol aglycone as a result of hydrolysis of glycoside (Trease and Evans, 1983).

3.3.3 Test for Antraquinones Derivatives

3.4.3.1 Test For Antraquinones (Borntrager's Test)

A small portion of the extract was shaken with 10ml of benzene and filtered. 5ml of 10% of ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet colour indicates the presence of free antraquinones (Trease and Evans, 1983).

3.3.3.2 Test For Combined Anthracene (MordifiedBorntrageer's Test)

The extract was boiled with 5ml of 10% hydrochloric acid for 3 minutes. This will hydrolyze the glycosides to yield aglycone which are soluble in hot water only. The solution was filtered hot, the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was filtered off and shaken gently with half volume of 10% ammonia solution. A rose-pink or a cherry red colour indicates the presence of combined anthracene (Trease and Evans, 1983).

3.3.4 Test for Cardiac Glycosides

3.3.4.1 Kella-Killiani Test

The extract was dissolved in glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45 degree, 1ml of concentrated sulphuric acid was added down the side. The appearance of purple ring colour at the interphase indicates the presence of cardiac glycosides (Trease and Evans, 1983).

3.3.4.2 Legal Test

Small quantity of the extract was evaporated to dryness in an evaporating dish. The residue was dissolved in a few drops of pyridine in the fumed cupboard. Drops of 2% sodium nitroprusside and a drop of sodium hydroxide was added. The production of a deep red colour indicates the presence of cardenolides (Trease and Evans, 1983).

3.3.4.3 Kadde Test

Kadde test was performed by adding 1ml of 2% 3,5-dinitrobenzoic acid in 5% alcohol to the extract. The solution was made alkaline with 5% sodium hydroxide. The appearance of purple-blue colour indicates the presence of cardenolides in the ring (Trease and Evans, 1983).

3.3.4.4 Salkowsk Test

About 0.5g of extract was dissolved in 2ml of chloroform and few drops of concentrated hydrogen sulphate were added to form a lower layer. A reddish-brown colour at the interphase indicates the presence of a steroidal ring (Trease and Evans, 1983).

3.3.5 Test for Saponins

3.4.5.1 Frothing Test

Small quantity of the extract was dissolved in 10ml of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb formed for more than 30 minutes indicate saponin (Trease and Evans, 1983).

3.3.5.2 Haemolysis Test

Haemolysis test was carried out by adding 2ml of 1.5% aqueous sodium chloride solution into two test tubes. To one test tube, 2ml of the extract was added while same volume of distilled water was added to the other test tube, then 3 drops of human blood was added to each test tube. The content was observed for 30 minutes. Haemolysis in the test tube containing the extract indicates the presence of saponins (Trease and Evans, 1983).

3.3.6 Test for Steroid and Triterpenes (Lieberman-Burchards Test)

Equal volume of acetic anhydride was added to the extract. 1ml of concentrated sulphuric acid was added down the side of the tube. The colour change was observed immediately and later. Red, pink or purple colour indicates the presence of triterpenes while blue or blue-green indicate steroids (Trease and Evans, 1983).

3.3.7 Test for Flavonoids

3.3.7.1Shinoda Test

Shinoda test was carried out by dissolving 0.5g of the extract in 1-2ml of 50% methanol in tile heat. Metallic magnesium and 4-5 drops of concentrated HCl were added. A red or orange colour indicates the presence of flavanoicaglycones (Trease and Evans, 1983).

3.3.7.2 Sodium Hydroxide Test

Few drops of aqueousNaOHwas added to 5ml of the extract. A yellow coloration observed indicates the presence of flavonoid (Trease and Evans, 1983).

3.3.8 Test for Tannins

3.3.8.1Lead sub-acetate test

Three drops of lead sub-acetate solution was added to a solution of the extract. A coloured precipitate indicates tannins (Trease and Evans, 1983).

3.3.8.2 Ferric chloride test

About 0.5g of the extract was dissolved in 10ml of distilled water, and then filtered. Few drops of ferric chloride solution was added to the filtrate. Formation of a blue-black precipitate indicates hydrolysable tannins and a green precipitate indicates the presence of condensed tannins (Trease and Evans, 1983).

3.3.8.3 Bromine water test

Three drops of bromine water was added to a solution of the extract (in a fumed cupboard). A buff coloured precipitate indicates the presence of condensed tannins (Trease and Evans, 1983).

3.4.9 Test for Alkaloids

3.3.9.1Meyer's test

Few drops of Meyer's reagent was added to the sample of the extract in a test tube. A cream precipitate indicates the presence of alkaloids (Trease and Evans, 1983).

3.3.9.2 Dragendoff's Test

Few drops of Dragendoff's reagent was added to the extract. A rose red precipitate indicates the presence of alkaloids (Trease and Evans, 1983).

3.3.9.3 Wagner's Test

Drops of Wagner's reagent was added to a small amount of the extract. A whitish precipitate observed indicates the presence of alkaloids (Trease and Evans, 1983).

3.3.9.4 Picric Test

Few drops of 1% picric acid solution was added to the extract. A yellow coloured solution observed indicates the presence of alkaloids (Trease and Evans, 1983).

3.3.9.5 Tannic Acid Test

Few drops of tannic acid was added to a sample of the extract. A black precipitate observed indicates the presence of alkaloids. (Sofowora, 1982).

3.4 EXPERIMENTAL ANIMALS

A total number of 50 young male and female Wistar rats were obtained from the Department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria. The animals were housed in plastic cages in the animal house of the Department of Human Anatomy, Ahmadu Bello University, Zaria. The animals were between the ages of 7-10 weeks and weighed between 180 to 200g.

The animals were kept and maintained on standard laboratory condition of room temperature, humidity and under twelve hours dark and light cycle. The animals were fed with standard pellet diet and clean water.

The animals were allowed to acclimatize to the new condition for two weeks before the commencement of the experiment. The cages were cleaned daily.

3.5 EXPERIMENTAL PROTOCOL

The experiment was divided into two protocols, A and B, with protocol A having seven groups and protocol B having three groups. The 50 animals were randomly selected and divided into five rats per group and each rat in every group was marked for identification purpose.

3.5.1 Protocol A (Hepatoprotective Study)

Group 1 rats were administered distilled water (5 ml / kg b/w)orally once daily for seven days. Group 2 rats were administered absolute ethanol 6000 mg / kg body weight orally as a single dose on day seven. Group 3 rats were administered 100 mg / kg body weight Silymarin orally once a day for seven days and a single dose of 6000 mg/kg body weight of absolute ethanol on day seven, 1 hour 30 minutes after administration of the silymarin. Group 4 rats were administered 800mg /kg body weight extract orally once a day for seven days. Group 5 rats were administered 300mg /kg body weight extract once a day for seven days and a single dose of 6000 mg/kg body weight of absolute ethanol on day seven, 1 hour 30 minutes after administration of the extract. Group 6 rats were administered 500mg /kg body weight extract once a day for seven days and a single dose of 6000 mg/kg body

weight of absolute ethanol on day seven, 1 hour 30 minutes after administration of the extract. Group 7 rats were administered 800mg /kg body weight extract once a day for seven days and a single dose of 6000 mg/kg body weight of absolute ethanol on day seven, 1 hour 30 minutes after administration of the extract.

3.5.2 Protocol B (Curative Study)

Group 8 rats were administered absolute ethanol 6000 mg / kg body weight orally as a single dose on day one. Group 9 rats were administered a single dose of 6000 mg/kg body weight of absolute ethanol on day one, and 500mg /kg body weight extract 1 hour 30 minutes after administration of ethanol, and then once a day for three days. Group 10 rats were administered a single dose of 6000 mg/kg body weight of absolute ethanol on day one, and 800mg /kg body weight extract 1 hour 30 minutes after administration of ethanol, and then once a day for three days.

3.6 SACRIFICE OF ANIMALS

On day 7, all the animals were humanely sacrificed. Blood was collected from the jugular vein of the animals under deep anesthesia with chloroform for haematological indices and biochemical analysis. The liver was removed for histological examination.

3.7 HAEMATOLOGICAL INDICES

3.7.1 Evaluation of Haematological Parameters

Haematological parameters including differential white blood cell count were measured and calculated by fully-automatedhaematology analyzers. The obtained results are precise (error of the measurement is lower than 1 %) and are automatically compared with standard values for individual parameters.

3.7.2 Materials

Haematology analyzer Mindray BC-5500, test tube with ethylenediaminetetraacetic acid (EDTA-2) as anticoagulant agent, sterile injection needle and 5 ml syringe for single use, rubber strangulator, cottonwool, disinfection solution. Haematology analyzer Mindray BC-5500 measures and calculates more than 20 haematologic parameters characterizing.

3.7.3 Red Blood Cells (RBC)

RBCcount and Haemoglobin content (HGB).

3.7.4 White Blood Cells (WBC)

WBCcount: Absolute and relative counts of WBC populations: lymphocytes,monocytes, neutrophils, eosinopils and basophils.

3.7.5 Platelets (PLT)

PLTcount.Mean platelet volume (MPV), Platelet volume distribution width (PDW), Plateletcrit (PCT).

For the measurement of the above mentioned parameters, laser scatter technology combined with chemical dye method and flow cytometry were used.



Figure 3.1 Haematological Auto-Analyzer Machine (GuangzdouMecan Trading Co., Ltd. China).

The analyzer enables the possibility to choose one of 2 modes of measurement. The analyzer can analyze small volumes of blood: as pre-diluted sample (minimum volume 40µl) or full blood (minimum volume 120 µl) sucked directly from the test tube, up to 80 samples per hour.

In addition to results in numerical form, 2 histograms (for RBC and PLT) and 2 scattergrams for differential WBC count (1 scattergram for neutrophil, monocytes, lymphocytesand eosinophiland 1 independent scattergram for basophil) are displayed on the large touch screen.

3.7. 6 Method

5 ml of venous blood was taken into a sterile syringe. The test-tube containing EDTA-2 was filled in with the blood precisely up to mark. The test-tube was repetitively turned over and the content mixed with the anticoagulant agent covering the internal walls of the test-tube. The haematological parameters were measured using automatic analyzer within 1-2 hours after the blood was taking.

3.8 BIOCHEMICAL ANALYSES

The blood collected in plane centrifuge tube was centrifuged at 10,000rmp for 10 minutes, and the serum collected was used for enzyme assay for the estimation of non-enzymatic and enzymatic antioxidant.

3.8.1 Determination of Catalase Activity

Catalase activity was determined using the method described by Sinha (1972).

3.8.1.1Principle

The method is based on the reduction of dichromate in acetic acid to chromic acetate, when heated in the presence of hydrogen peroxide with the formation of perchromic acid as unstable intermediate.

3.8.1.2 Reagents

5% potassium heptaoxochromate (VI) $K2Cr_2O_7$; 5g of$K2Cr_2O_7$ was dissolved in little quantity of distilled water and made up to 100ml.

0.2M H_2O_2; 0.6ml of H_2O_2 was dissolved in little quantity of distilled water and made up to 100ml. it was stored at 4°C.

0.01M phosphate buffer; 1.2g of NaH_2PO_4 and 1.41g of $Na_2HPO_4$was dissolved in distilled water and made up to 1000ml mark in a volumetric flask. The buffer was adjusted to pH 10.2.

Dichromic/acetic acid solution: 5% potassium heptaoxochromate (VI), $K2Cr_2O_7$ was mixed with glacial acetic acid in the ratio 1:3, and was stored in a brown bottle at room temperature.

3.8.1.3 Procedure

The procedure involves; 0.9ml of distilled water was added to 0.1 ml of microsome and mixed thoroughly. 2.5ml of phosphate buffer was put in a small conical flask; 0.5ml of microsome was added, and 2.0ml of H_2O_2 was added, and the stop watch was started. The reaction mixture was allowed to thoroughly mix and the reaction was stopped after every

60 seconds for three minutes with Dichromate/acetic acid solution. It was heated in water bath for 10 minutes at 80°C. Absorbance was read at 570nm. The standard cure was obtained using absorbance obtained at various H_2O_2.

3.8.1.4 Calculations

The quantity of H_2O_2 consumed was obtained from the graph of the catalase standard curve, which determined the catalase activity.

3.8.2 Determination of Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined by a method described by Fridovich (1989).

3.8.2.1 Principle

The ability of superoxide dismutase (SOD) to inhibit auto oxidation of adrenaline at pH 10.2 forms the bases of this assay.

3.8.2.1 Reagents

0.5M phosphate buffer; 6.97g of diphosphate) and 1.36g of KH_2PO_4 was dissolved in distilled water and made up to 1000ml mark in a volumetric flask. The buffer was adjusted to pH 7.8.

0.05M carbonate buffer; 14.3g of Na_2CO_3 and 4.2g of $NaHCO_3$ was dissolved in distilled water and made up to 1000ml mark in a volumetric flask. The buffer was adjusted to pH 10.2.

0.3mM adrenaline; 0.01g of adrenaline was dissolved in 17ml of distilled water. The solution was prepared fresh.

3.8.2.3 Procedure

The procedure involve; 0.1ml of microsome was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.20ml of the diluted microsome was added to 2.5ml of 0.05M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3Mm adrenaline. The reference mixture contains 2.5ml of 0.05M carbonate buffer, 0.3ml of 0.3mM of adrenaline and 0.20ml of distilled water. Absorbance was measured over 30 s to 150 sat 480nm.

3.8.2.4 Calculation

Increase in absorbance per minute = $(A_5 –)/2.5$

% inhibition = 100 – increase in absorbance for substrate x 100

Increase in absorbance of blank

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adenochrome in 1 minute.

A_5= Initial reading, A_1= final reading, 2.5 = standard value.

3.8.3 Assessment of Lipid Peroxidation

Lipid peroxidation as evidenced by the formation of TBARS was measured by the modified method of Niehaus and Samuelson (1968) and described by Akanji*et al.* (2009).

3.9.3.1 Reagents

15% trichloroacetic acid (TCA) solution

0.37% thiobarbituric acid (TBA) solution

3.8.3.2 Procedure

To 150 of serum and tissue homogenate, (0.25M sucrose solution) were treated with 2ml of 1:1:1 TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 0.25M HCl and 15% TBA) and placed in water bath for 1 hour at 90°C. The mixture was cooled and centrifuged at 3000rpm for 5 minutes at 4°C. The absorbance of the pink supernatant 2.0ml was measured against a reference blank using spectrophotometer at 535nm. Values were expressed as Mn /1 g tissue.

3.9 HISTOPATHOLOGICAL EXAMINATION

Animals from each of the groups were anaesthetized with chloroform until they were unconscious. The abdomens were opened by a midline incision and the liver of the rats in each group was excised and the tissues were placed in formol saline.

3.10 TISSUE PREPARATION FOR MICROSCOPY

Tissue preparation for histological studies was carried out using the following materials; 10% formol saline, absolute alcohol, 95% alcohol, 70% alcohol, xylene, paraffin wax, egg albumen, oven, microtome, glass slides and microscope.

3.10.1 Methods

Tissue preparations were made using routine and special techniques as outlined by Bradbury (1977); Culling (1993); Drury and Wallington (1980) and Gurr (1992).

3.10.2 Fixation

The excised liver tissues were trimmed and immediately transferred to 10% formol saline contained in a transparent container. This was done separately for the tissues from the different study groups and labeled appropriately.

3.10.3 Tissue Processing

The tissue processing techniques were both manual and automatic. The manual processing technique used was as outlined by Bradbury (1977); Culling (1993); Drury and Wallington (1980) and Gurr (1992).Some of the tissues were processed using the Histokinetic Bench Model Automatic Tissue Processor (Leica).

3.10.4 Sectioning and Mounting

Tissues were embedded in paraffin wax and sections were cut using the rotary microtome. The sizes of the tissue sections were between 5-10 microns. The cut sections were floated on hot water bath and they were picked on clean albumenized glass slides for staining.

3.10.5 Staining

Different staining techniques were employed, both routine staining and special staining techniques. The routine staining technique used was the hematoxilin and eosin (H and E)

method.The special staining technique used was the periodic acid schiff(PAS) method for glycogen deposits. These methods were carried out as outlined by Bradbury (1977); Culling (1993); Drury and Wallington (1980) and Gurr (1992).

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3.11 STATISTICAL ANALYSES

One way analysis of variance (ANOVA) was used to analyze the data and data were presented as mean $\pm$ standard deviation. For establishing significant differences between groups, the Tukey*post hoc*test was used. Values were considered statistically significant if P value is less than 0.05 ($p< 0.05$). Statistical analyses were carried out using Sigma Stat 2.0 for Window (Soft Stat, San Rafael, CA).

CHAPTER FOUR

RESULTS

4.1 PLANT PHYTOCHEMICAL SCREENING

The phytochemical screening results of *Psidium guajava* leaf showed the presence of carbohydrates (monosaccarides and free reducing sugar), flavonoids, tannins, alkaloids, glycosides, saponins and cardiac glycosides. While some chemical constituents like anthraquinones, unsaturated steroids and triterpenes were absent. The details are shown in Table 4.1

Table 4.1: Results of phytochemical screening of the aqueous extract of *Psidium guajava* leaf

Constituents	Test	Observation	Inference
Carbohydrates	Molisch test	Purple colouration at interface	+ ve
Monosaccharide	Barfoed's test	Red precipitate	+ ve
Free reducing sugar	Fehlings test	Brick red precipitate	+ ve
Flavonoid	NaOH test	Yellow colouration formed	+ ve
Tannins	Lead sub-acetate test	Brown precipitate formed	+ ve
	Ferric chloride test	Green black precipitate formed	+ ve
	Bromine water test	Brown precipitate formed	+ ve
Alkaloids	Mayer's test	Slight white precipitate	+ ve
Glycosides	Fehling's test	Brick red precipitate	+ ve
	$FcCl_3$ test	Red green precipitate formed	+ ve
Saponins	Frothing test	Froth occurred for more than 15 min	+ ve
	Hemolysis test	Lysis of blood occurred in test tube containing extract	- ve
Unsaturated steroids and triterpenes	Lieberman Burchard test Salkwoski's test	No change No change	-ve - ve
Anthraquinones	Bontrager's test	No colour change	- ve
	Mordified test	No colour change	-ve
Cardiac glycosides	Kella-killiani test	Purple ring interface formed	+ ve
	Kadde's test	Purple blue colour formed	+ ve
	Legal test	No change	- ve

4.2 GENERAL OBSERVATIONS

Following the administration of 6000mg/kg body weight of ethanol, 300mg/ kg body weight, 500mg/ kg body weight, and 800mg/ kg body weight of aqueous leaf extract of *Psidium guajava*to the rats, the following observations were made. There was increase in activity, increased sensitivity to touch and sound. There was decreased body weight in all the groups except in groups 1, 3, and 5 which gained weight. These changes were gradually replaced by decreased activity, decreased sensitivity to both touch and sound.

4.3RAT SURVIVAL AND BODY WEIGHT

During the treatment of the animals, there was one mortality recorded in the group that received only ethanol.

At the end of the experiment, the mean body weights of the rats showed no significant difference between groups but there was decrease in the body weight in all the experimental groups except in groups 3, and 5 where there was increase in body weight as seen in the control group. The group that was administered ethanol alone lost more weight as shown in Table 4.2.

There was significant decrease in organ-body weight ratio in groups 2, 4 and 7 when compared to the control group, $p< 0.05$ as shown in Fig 4.1.

4.4 HEPATOPROTECTIVE STUDY

Table 4.2 Effect of aqueous leaf extract of *Psidium guajava* on body weight in ethanol induced liver damage.

Grps	Dosage	Body weight (g)		
		Initial weight	**Final weight**	**Diff. in weight**
Grp 1	Distilled water	178.80 ± 34.77	195.60 ± 35.79	16.80 ± 4.55
Grp 2	6000mg/kg ethanol	187.20 ± 26.81	167.40 ± 24.92	23.00± 32.94
Grp 3	100 mg/kg silymarine + 6000mg/kg ethanol	178.60 ± 17.70	190.40 ± 22.12	14.33± 12.53
Grp 4	800mg/kg extract	179.80 ± 30.29	172.80 ± 28.84	17.80± 14.76
Grp 5	300mg/kg extract + 6000mg/kg ethanol	193.20 ± 77.77	176.00 ± 72.94	19.67± 15.66
Grp 6	500mg/kg extract + 6000mg/kg ethanol	184.20 ± 22.12	181.20 ± 42.21	35.33± 36.36
Grp 7	800mg/kg extract + 6000mg/kg ethanol	166.60 ± 29.28	171.60 ± 45.46	23.80± 19.41

g = gram, diff. in weight = difference in weight, n = 5.

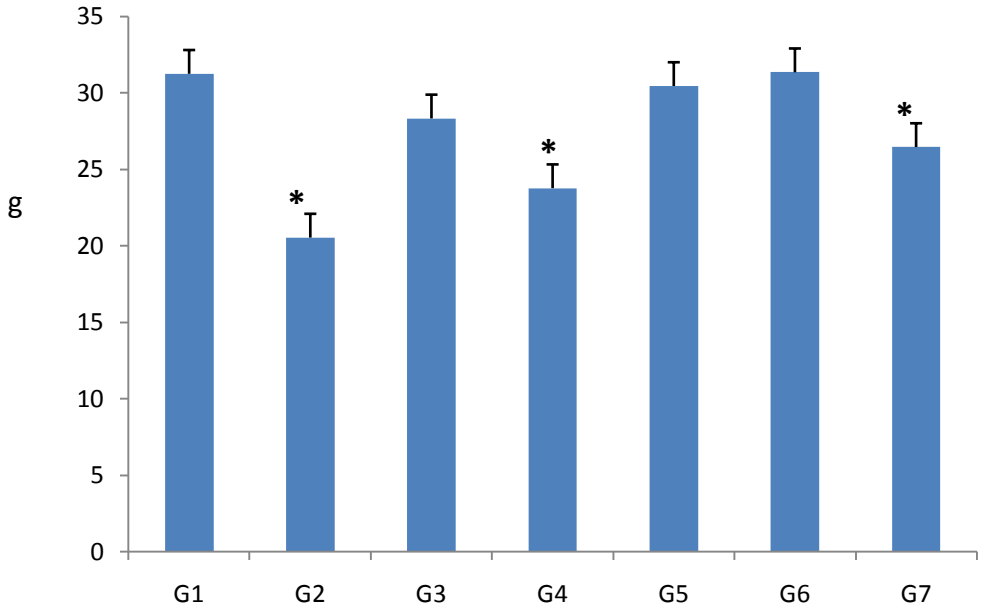


Fig 4.1: Effect of aqueous leaf extract *Psidium guajava* on organ-body weight ratio in ethanol induced liver damage.

G = group, g = grams, n = 5,*p<0.05

4.4.1 Biochemical Parameters

The results of the liver enzyme analysi; aspartate amino transaminase (AST), alanine amino transaminase (ALT) and alkaline phosphatase (ALP)) following ethanol and aqueous leaf extract of *Psidium guajava* administration showed increase and decrease in some parameters as shown in Table 4.3.

The mean aspartate amino transaminase (AST) values shows significant increase in groups 3, 4 and 5, when compared with the control group, $p < 0.001$.

The mean alanine amino transaminase (ALT), and alkaline phosphatase (ALP) showed no significant difference between the groups but there was increase in ALT in groups 2 and 4 when compared with the control group.

The result of the biochemical analysis of lipid peroxidation (LPO), catalase (CAT) and superoxide dismutase (SOD)) following ethanol and aqueous leaf extract of *Psidium guajava* administration showed increase and decrease in CAT and SOD as shown in Table 4.4. The mean CAT value of the liver showed significant increase in group 4 when compared with the control group, $p<0.001$.

The mean SOD value of the liver showed significant decrease in groups 2 and 5 when compared with the control group, $p< 0.01$.

The mean lipid peroxidation values of the liver showed no significant difference between the treatment groups and the control group.

Table 4.3 Effect of aqueous leaf extract of *Psidium guajava* on liver enzymes (AST, ALT and ALP) in ethanol induced liver damage.

GRP	DOSAGE	AST(IU/l)	ALT(IU/l)	ALP(IU/l)
GP 1	Distilled water	24.26 ± 2.71	7.36 ± 1.53	25.16 ± 7.55
GP 2	6000mg/kg ethanol	35.54 ± 2.85	10.42 ± 4.98	13.14 ± 4.67
GP 3	100mg/ kg silymarine + 6000mg/kg ethanol	42.94 ±12.58*	9.12 ± 1.81	26.18 ± 5.06
GP 4	800mg/kg extract	46.55 ± 6.33*	10.23 ± 1.34	45.10 ± 17.87
GP 5	300mg/kg extract + 6000mg/kg ethanol	35.64± 5.11	9.34± 1.76	74.04±104.65
GP 6	500mg/kg extract + 6000mg/kg ethanol	27.26 ± 2.08	8.88 ± 0.47	32.54 ± 5.16
GP 7	800mg/kg extract + 6000mg/kg ethanol	38.12±6.68*	9.90± 1.44	14.26±6.04

AST = aspartate amino transaminase, ALT = alanine amino transaminase, ALP = alkaline phosphatase, n = 5, P < 0.001. mean with one super script *

Table 4.4 Effect of aqueous leaf extract of *Psidium guajava* on biochemical parameters (CAT, SOD and LPO) in ethanol induced liver damage.

GRP	DOSAGE	CAT (µmol/mg protein)	SOD (µmol/mg protein)	LPO (nmol/mg protein)
GP 1	Distilled water	1.67 ± 0.89	31.13 ± 0.00	1.19 ± 0.84
GP 2	6000mg/kg ethanol	1.23 ± 0.16	16.85 ± 6.49*	2.38 ± 0.51
GP 3	100mg/ kg silymarine + 6000mg/kg ethanol	1.87 ± 1.01	22.23 ± 8.43	0.93 ± 0.92
GP 4	800mg/kg extract	4.59 ± 0.66**	21.89 ± 5.35	1.68 ± 1.25
GP 5	300mg/kg extract + 6000mg/kg ethanol	1.60± 0.07	21.81± 4.95	1.24± 0.56
GP 6	500mg/kg extract +6000mg/kg ethanol	1.75 ± 0.44	22.12 ± 2.88	1.05 ± 0.92
GP 7	800mg/kg extract + 6000mg/kg ethanol	1.79± 0.31	18.45± 4.79*	1.38± 0.67

CAT = catalase, SOD = superoxide dismutase, LPO = lipid peroxidation, n = 5, $P < 0.01$. mean with one super script * and $p<0.001$, mean with two super script **

4.4.2 Haematological Parameters

The results of the haematological analysis following ethanol and aqueous leaf extract of *Psidium guajava* administration showed increase and decrease in some parameters as shown in Table 4.5.

The mean red blood cell (RBCs) showed significant increase in groups 2, 4, 5 and 7 when compared with the control group, $p<0.001$.

The mean white blood cell (WBCs) showed no significant difference between the treatment groups and the control group.

The mean haemoglobin concentration (HGB) showed significant increase in groups 2, 5 and 7 when compared with the control group, $p < 0.001$.

The mean platelets count (PLT) showed significant increase in groups 4, 5 and 7 when compared with the control group, $p < 0.001$.

Table 4.5 Effect of aqueous leaf extract of *Psidium guajava* on some haematological parameters in ethanol induced liver damage.

GRP	DOSAGE	WBC ($X10^{9/L}$)	RBC ($X10^{12/L}$)	HGB (g/L)	PLT ($X10^{9/L}$)
GP 1	Distilled water	11.06 ± 1.09	6.99 ± 0.59	12.90 ± 0.57	6.98 ± 0.47
GP 2	6000mg/kg ethanol	16.68 ± 7.19	9.86 ± 0.48 *	16.04 ± 1.00 *	7.98 ± 1.85
GP 3	100mg/ kg silymarin+ 6000mg/kg ethanol	13.36 ± 5.85	8.16 ± 0.26	14.12 ± 0.38	6.02 ± 1.60
GP 4	800mg/kg extract	7.13 ± 0.95	8.65 ± 0.88 *	13.58 ± 0.74	8.97 ± 0.30*
GP 5	300 mg/kg extract + 6000mg/kg ethanol	8.36±4.28	9.39± 0.47*	18.00± 0.59*	11.28± 0.53*
GP 6	500 mg/kg extract + 6000mg/kg ethanol	11.74 ± 4.38	7.93 ± 0.45	13.86 ± 0.37	6.10 ± 0.52
GP 7	800 mg/kg extract + 6000mg/kg ethanol	9.52± 4.66	8.31± 0.89*	15.76± 0.62*	8.63± 0.58*

WBC = white blood cell count, RBC = red blood cell count, HGB = haemoglobin content, PLT = platelet count, n = 5, $P < 0.001$. mean with one super script *

4.4.3 Differential White Blood Cell Count

The result of the differential white blood cell (WBC) count following ethanol and aqueous leaf extract of *Psidium guajava* administration showed increase and decrease in some parameters as shown in Table 4.6. The mean neutrophil values showed significant increase in groups 2, 4, 5, and 7 when compared with the control group, $p< 0.001$.

The mean lymphocyte values showed statistically significant decrease in groups 2, 5 and 7 when compared with the control group, $p< 0.001$.

However, the basophil, monocytes, and eosinophil values showed no statistical significant between the treatment groups and the control group.

Table 4.6 Effect of aqueous leaf extract of *Psidium guajava* on differential white blood cell count in ethanol induced liver damage.

GRP	DOSAGE	LYMPHOCYTE %	MONOCYTE %	NEUTROPHIL %
GP 1	Distilled water	89.00 ± 2.72	0.00 ± 0.00	9.76 ± 3.09
GP 2	6000mg/kg ethanol	47.40 ± 4.26*	0.00 ± 0.00	51.50 ± 5.60*
GP 3	100mg/ kg silymarine + 6000mg/kg ethanol	70.48 ± 10.64	0.00 ± 0.00	35.94 ± 12.33
GP 4	800mg/kg extract	65.10 ± 0.74	0.00 ± 0.00	40.08 ± 4.58*
GP 5	300mg/kg extract + 6000mg/kg ethanol	62.36± 10.17*	0.00 ± 0.00	38.42 ± 10.64*
GP 6	500 mg/kg extract + 6000mg/kg ethanol	79.70 ± 11.92	0.00 ± 0.00	20.08 ± 11.86
GP 7	800 mg/kg extract + 6000mg/kg ethanol	63.72± 2.51*	0.00 ± 0.00	38.72 ± 3.84*

pn = 5, P < 0.001. mean with one super script *

4.5 Therapeutic Study

At the end of the experiment, the mean body weights of the rats showed no significant difference between groups but there was decrease in the body weight in all the experimental groups. The group that was administered ethanol alone lost more weight when compared to the other groups as shown in Table 4.7.

There was significant decrease in organ-body weight ratio groups 8, and 9 (ethanol control and 6000mg/kg b/w ethanol + 500mg/kg b/w extract) when compared to the normal control group, $p < 0.05$ as shown in Fig 4.2.

Table 4.7 Effect of alcohol and aqueous leaf extract of *Psidium guajava* on body weight in ethanol induced liver damage.

		Body weight(g)		
Grp	**Dosage**	**Initial**	**Final**	**Diff. in weight**
Control	Distilled water	178.80 ± 34.77	195.60 ±35.79	16.80 ± 4.55
Grp8	6000 mg/kg ethanol	199.20 ± 47.46	180.00± 53. 61	43.00± 45.83
Grp9	500mg/kg extract + 6000mg/kg ethanol	162.40 ± 35.13	156.40 ± 30.48	32.80± 37.53
Grp10	800mg/kg extract + 6000mg/kg ethanol	169. 40 ± 30.11	167.25 ± 33.35	37.00± 23.19

g = gram, diff. in weight = difference in weight, n = 5.

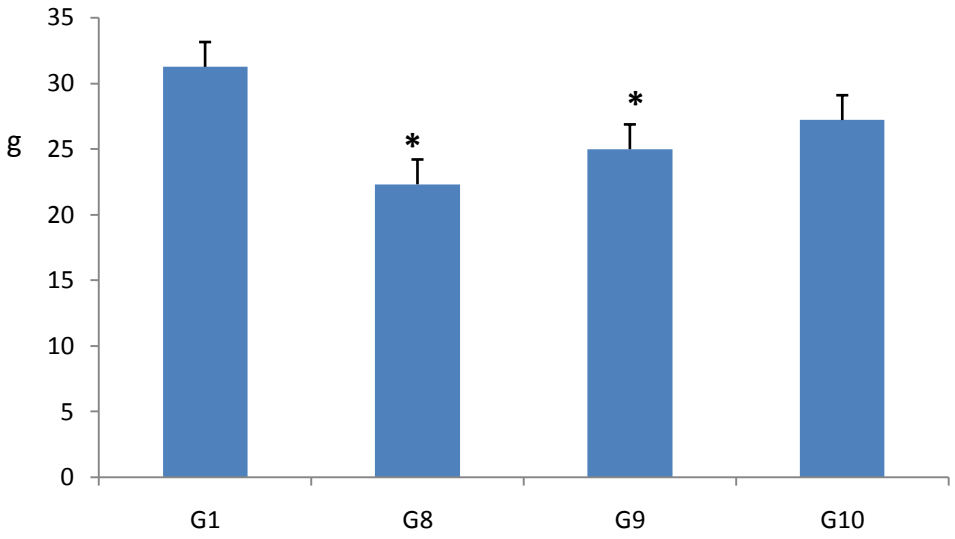


Fig 4.2: Effect of aqueous leaf extract *Psidium guajava* on organ-body weight ratio in ethanol induced liver damage.
G = group, g = grams, P< 0.05

4.5.1 Biochemical Parameters

The mean alkaline phosphatase (ALP) values showed significant increase in group 3 when compared with the ethanol control group, $p< 0.005$.

The mean alanine amino transaminase (ALT), showed significant increase in both the ethanol control group (group 8) and the extract treatment groups (groups 9 and 10), $p< 0.005$.

Aspartate amino transaminase (AST) showed no significant difference between the treatment groups and the control group as shown in Table 4.8.

The mean SOD showed significant decrease in the ethanol control group (group 8) when compared with the control group as shown in Table 4.9.

The mean CAT and lipid peroxidation values of the liver showed no significant difference between the treatment groups and the control group as shown in Table 4.9.

Table 4.8 Effect of aqueous leaf extract of *Psidium guajava* on liver enzymes (AST, ALT and ALP) in ethanol induced liver damage.

GRP	DOSAGE	AST(IU/l)	ALT(IU/l)	ALP(IU/l)
Control	Distilled water	24.26 ± 2.71	7.36 ± 1.53	25.16 ± 7.55
GP 8	6000mg/kg ethanol	31.53 ± 4.96	17.35 ± 8.26*	22.65 ± 9.28
GP 9	500mg/kg extract + 6000mg/kg ethanol	37.46 ± 7.19	13.18 ± 3.99*	26.28 ± 8.62
GP 10	800mg/kg extract + 6000mg/kg ethanol	29.00 ± 4.47	13.85 ± 2.10*	46.38 ± 6.47*

AST = aspartate amino transaminase, ALT = alanine amino transaminase, ALP = alkaline phosphatase, n = 5,P < 0.005. mean with one super script *

Table 4.9 Effect of aqueous leaf extract of *Psidium guajava* on biochemical parameters (CAT, SOD and LPO) in ethanol induced liver damage.

GRP	DOSAGE	CAT (µmol/mg protein)	SOD (µmol/mg protein)	LPO (nmol/mg protein)
Control	Distilled water	1.67 ±0.89	31.13 ± 0.00	1.19 ± 0.84
GP 8	6000mg/kg ethanol	1.57 ± 0.38	18 .40 ± 12.99*	2.37 ± 0.82
GP 9	500mg/kg extract + 6000mg/kg ethanol	1.68 ± 0.31	20.93 ± 6.71	1.76 ± 1.47
GP 10	800mg/kg extract + 6000mg/kg ethanol	1.87 ± 0.58	25.56 ± 10.29	1.64 ± 1.13

CAT = catalase, SOD = superoxide dismutase, LPO = lipid peroxidation, n = 5, $P < 0.01$ mean with one super script *

4.5.2 Haematological Parameters

The results of the haematological parameters analysis following ethanol and guava leaf extract administration showed increase and decrease in some parameters as shown in Table 4.10. The mean red blood cell (RBCs) showed significant increase in group 10 when compared with the ethanol control group, $p < 0.01$.

The mean white blood cell (WBCs) showed significantdecrease in groups 8 when compared with the control group and extract treated groups, $p < 0.01$.

The mean haemoglobin concentration (HGB) and platelets count showed no significant difference between the treatment groups and the ethanol control group.

Table 4.10 Effect of aqueous leaf extract of *Psidium guajava* on some haematological parameters in ethanol induced liver damage.

GRP	DOSAGE	WBC ($X10^{9/L}$)	RBC ($X10^{12/L}$)	HGB (g/L)
Control	Distilled water	11.06 ± 1.09	6.99 ± 0.59	12.90 ± 0.57
GP 8	6000mg/kg ethanol	8.48 ± 2.19*	6.60 ± 0.38	11.08 ± 0.76
GP 9	500mg/kg extract + 6000mg/kg ethanol	13.08 ± 2.84	6.15 ± 0.41	11.32 ± 0.66
GP 10	800mg/kg extract + 6000mg/kg ethanol	14.00 ± 0.43	7.03 ± 0.27*	12.30 ± 1.62

WBC = white blood cell count, RBC = red blood cell count, HGB = haemoglobin content, PLT = platelet count, n = 5, $P < 0.01$. mean with one super script *

4.5.3 Differential White Blood Cell Count

The results of the mean differential white blood cell (WBC) count,lymphocytes, basophil and monocytes values following ethanol and aqueous guava leaf extract administration showed no statistical significant between the treatment groups and the ethanol control group as shown in Table 4.11. The mean neutrophil count showed significant increase in the treatment groups (groups 8, 9 and 10) when compared to the control group, $p< 0.05$ as shown in Table 4.11.

Table 4.11 Effect of aqueous leaf extract of *Psidium guajava* on differential white blood cell count in ethanol induced liver damage.

GRP	DOSAGE	LYMP %	MONO % ($X10^{9/L}$)	NEUT %	BASO % ($X10^{9/L}$)
Control	Distilled water	89.00 ± 2.72	0.00 ± 0.00	9.76 ± 3.09	0.00 ± 0.00
GP 8	6000mg/kg ethanol	80.93 ± 4.17	0.00 ± 0.00	17.70 ± 2.61*	0.00 ± 0.00
GP 9	500mg/kg extract + 6000mg/kg ethanol	83.32 ± 4.40	0.00 ± 0.00	17.38 ± 2.14*	0.00 ± 0.00
GP 10	800mg/kg extract + 6000mg/kg ethanol	84.53 ± 6.78	0.00 ± 0.00	36.83 ± 42.15*	0.00 ± 0.00

LYPH = lymphocyes, MONO = monocytes, NEUT = neutrophil, BASO = basophil, n = 5, $p < 0.05$ mean with one super script.

4.6 HISTOPATHOLOGICAL FINDINGS

4.6.1 Hepatoprotective Study

Liver sections from control rats showed normal lobular architecture and normal hepatic cells with a well-preserved cytoplasm, nuclei and nucleoli were defined (Plate I). Whereas rats treated with ethanol showed degenerative activity in the form of binucleation, loss of nuclei, mild necrosis and congestion (Plate II). No significant morphological changes were noted in liver of animals given silymarin, as compared to that of animals in the control group (Plate III). Treatment with different doses of *Psidiumguajava* showed almost normal hepatocytes with centrally located nuclei, almost normal hepatic cords, normal sinusoids, and normal central vein of the liver of rats in groups 5, 6 and 7(Plates V, VI, and VII) while group 4 rats showed marked necrosis (Plate IV).

There was decreased glycogen deposit in the liver of all the treatment groups (Plate XII, XIII, XIV, XVXVI and XVII) but there was normal glycogen deposit seen in the liver of the control group (Plate XI).

4.6.2 Therapeutic Study

The histological findings showed mild oedema, necrosis and congestion in group 8 rats (Plate VIII). The histological examination of the liver in group 9 and 10 rats showed almost normal hepatocytes with centrally located nuclei, almost normal hepatic cords, normal sinusoids, and normal central vein (Plate IX and X). There was decreased glycogen deposit in the liver of all the treatment groups (Plates XVIII, XIX and XX).

Histological Findings

Hepatoprotective Study

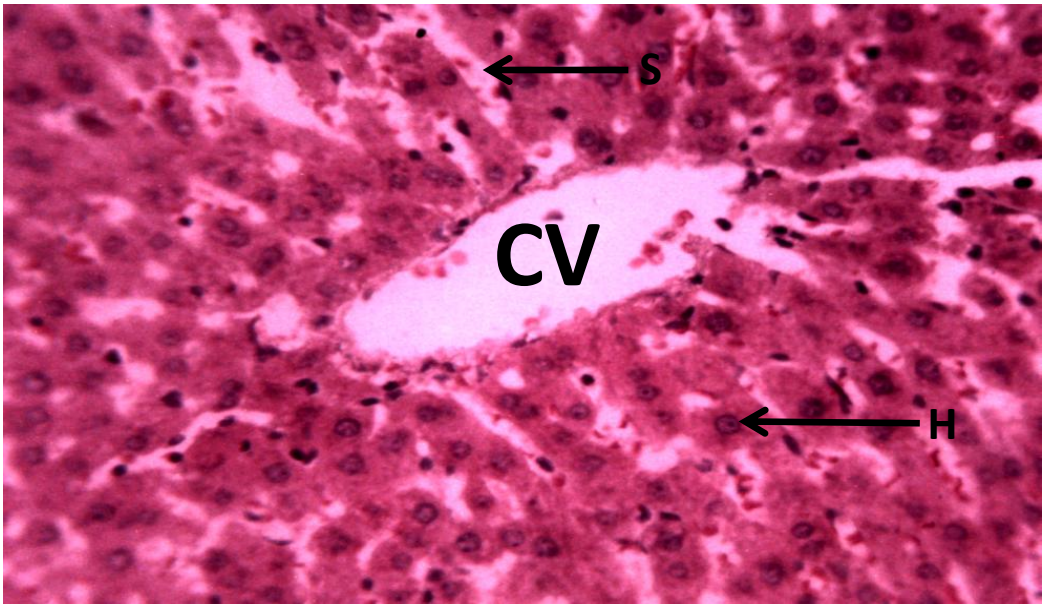


Plate I: Micrograph of liver from group 1 (control) rats showing normal central vein (CV), sinusoid (S) and hepatocytes (H), H and E (x 250)

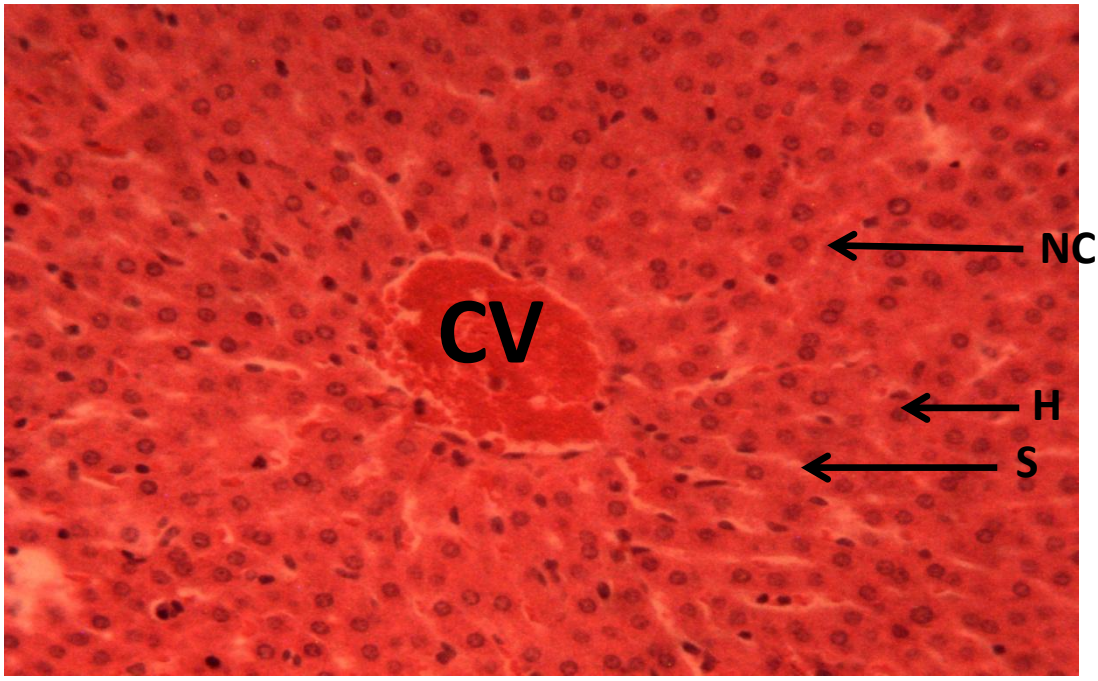


Plate II: Micrograph of liver from group 2 (ethanol control) rats showing central vein (CV), sinusoid (S), hepatocytes (H) and necrotic cell (NC), H and E (x 250)

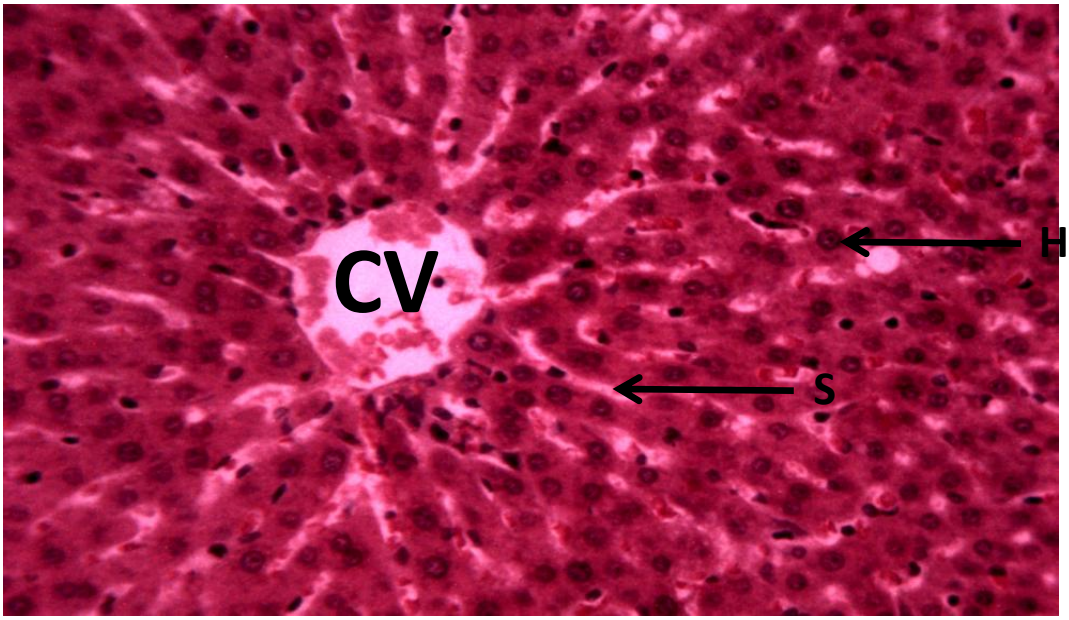
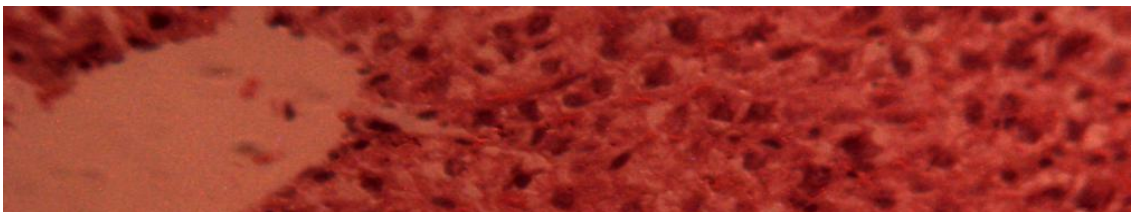


Plate III: Micrograph of liver from group 3 (silymarin + 6000mg/kg body weight ethanol) rats showing normal central vein (CV), sinusoid (S), hepatocytes (H), H and E (x 250)



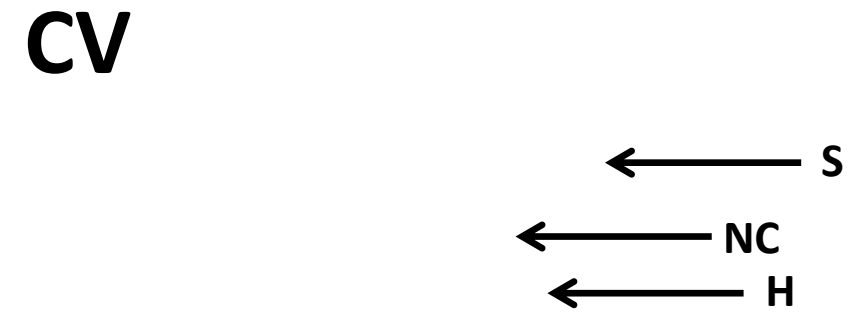
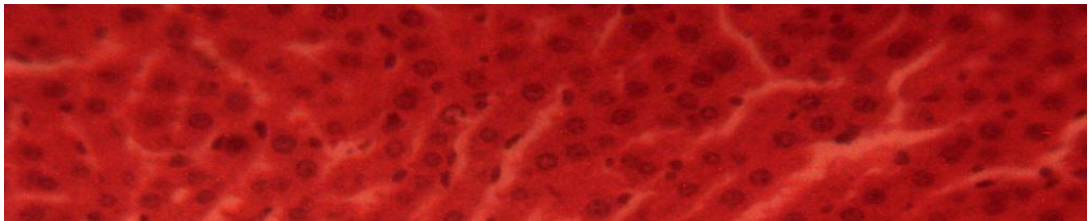


Plate IV: Micrograph of liver from group 4 (800mg/kg body weight extract) rats showing central vein (CV), sinusoid (S) and necrotic cell (NC), H and E (x 250)



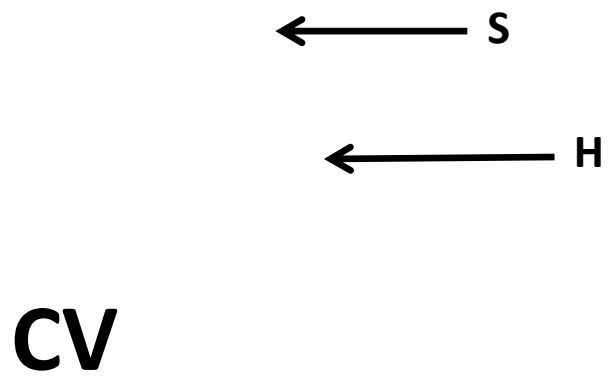


Plate V: Micrograph of liver from group 5 (300mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing normal central vein (CV), sinusoid (S) and hepatocytes (H), H and E (x 250)

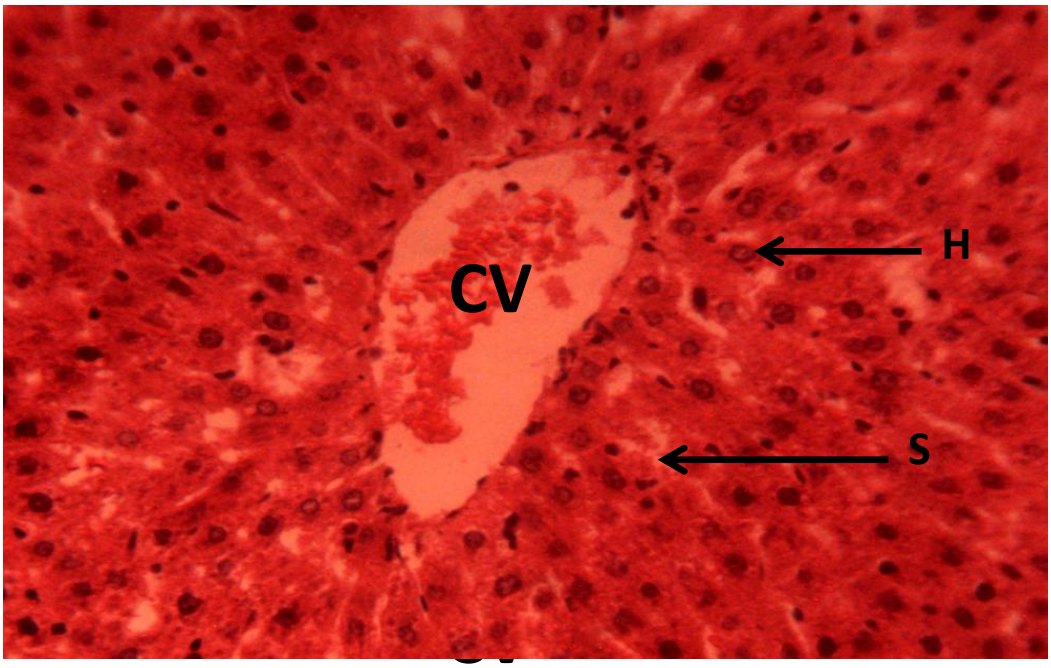


Plate VI: Micrograph of liver from group 6 (500mg/kg extract body weight + 6000mg/kg body weight ethanol) rats showing central vein (CV) sinusoid (S) and hepatocytes (H), H and E (x 250

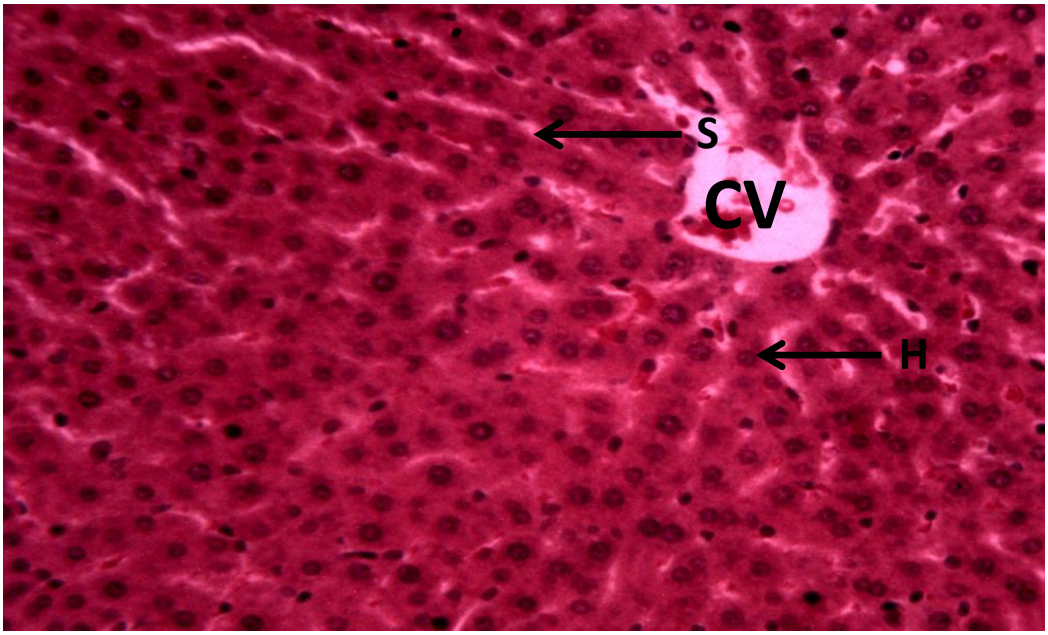


Plate VII: Micrograph of liver from group 7 (800mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing normal central vein (CV), sinusoid (S), and hepatocytes (H) , H and E (x 250)

Therapeutic Study

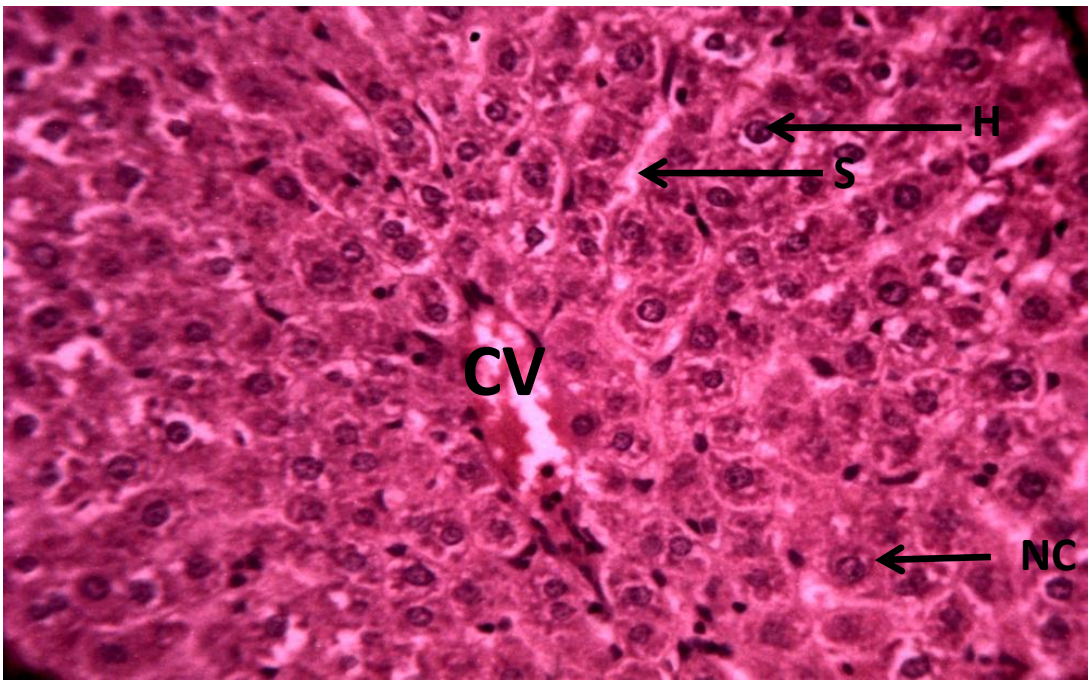
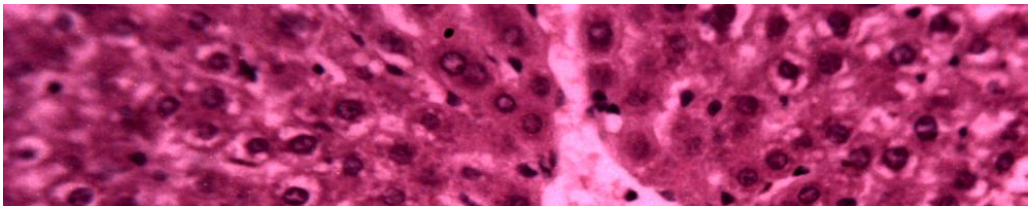


Plate VIII: Micrograph of the liver of group 8 rats treated with 6000mg/kg body weight ethanol showing the central vein (CV), sinusoid (S) and hepatocytes (H) and necrotic cell (NC), H and E (x 250)



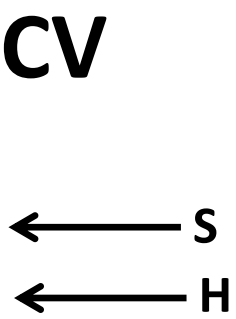


Plate IX: Micrograph of liver from group 9 (500mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing normal central vein (CV), sinusoid (S) and hepatocytes (H), H and E (x 250)

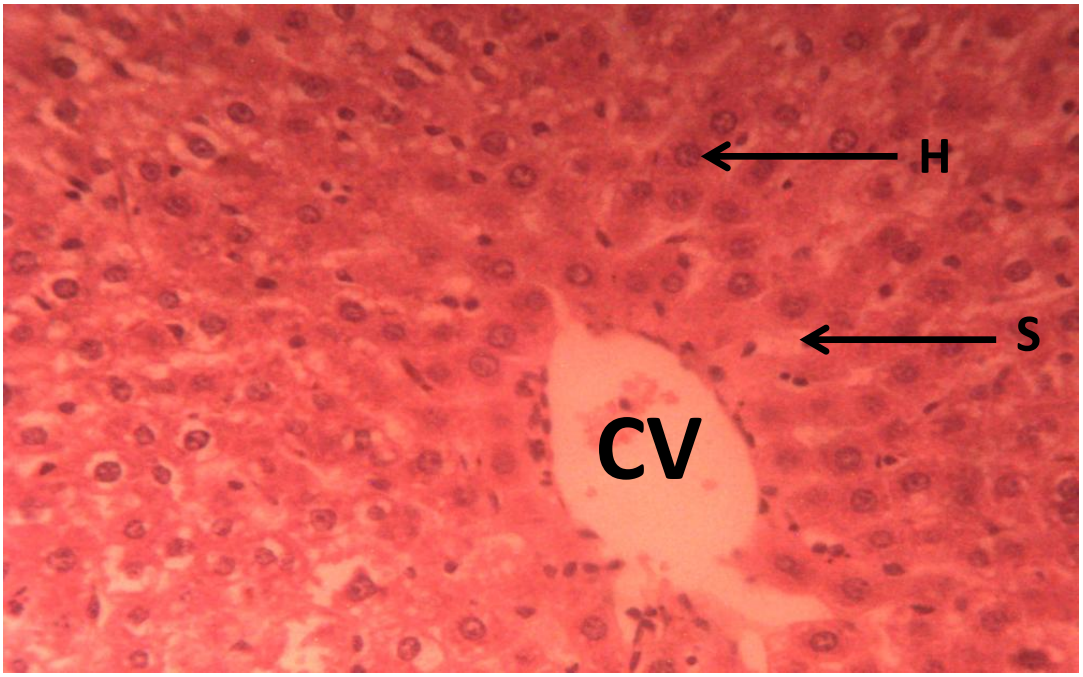


Plate X: Micrograph of liver from group 10 (800mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing normal sinusoid (S) and hepatocytes (H), H and E (x 250)

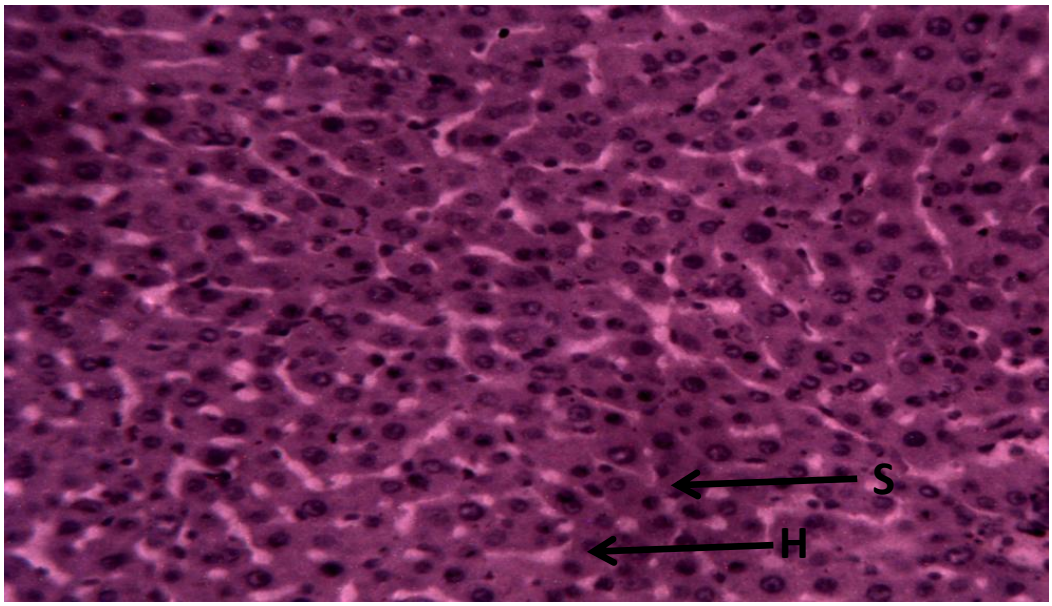


Plate XI: Micrograph of liver from group 1 (control) rats showing hepatocyte (H), and sinusoid(S), PAS (x 250)

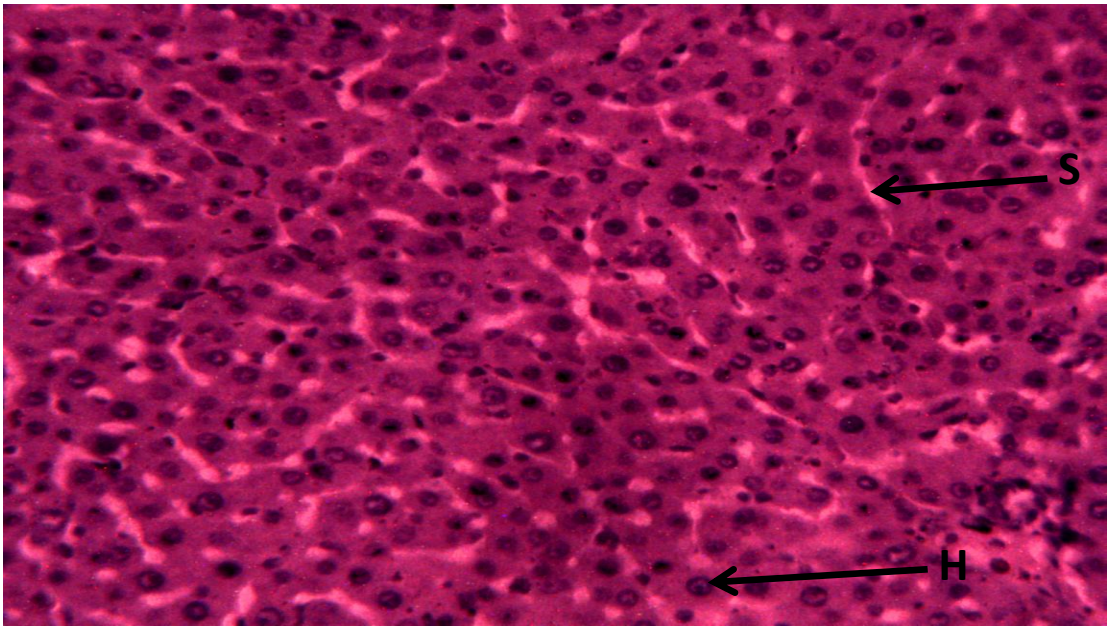


Plate XII: Micrograph of liver from group 2 (6000mg/kg body weight ethanol control) rats showing hepatocyte (H) and sinusoid (S), PAS (x 250)

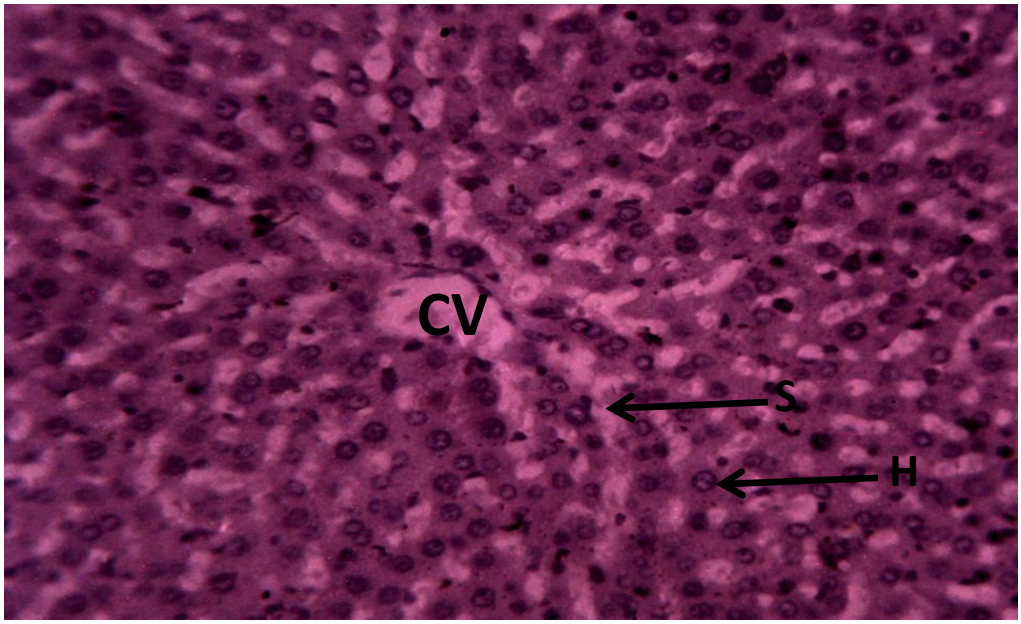


Plate XIII: Micrograph of liver from group 3 (silymarin + 6000mg/kg body weight ethanol) rats showing central vein (CV), hepatocyte (H)and sinusoid (S), PAS (x 250)

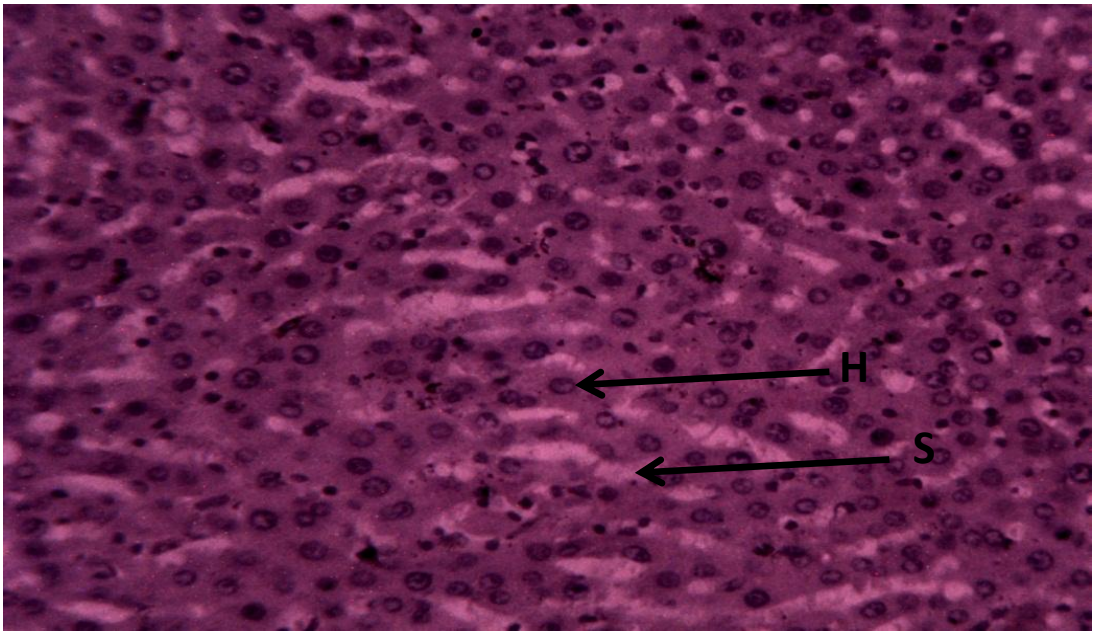


Plate XIV: Micrograph of liver from group 4 (800mg/kg body weight extract) rats showing hepatocyte (H)and sinusoid (S), PAS (x 250)

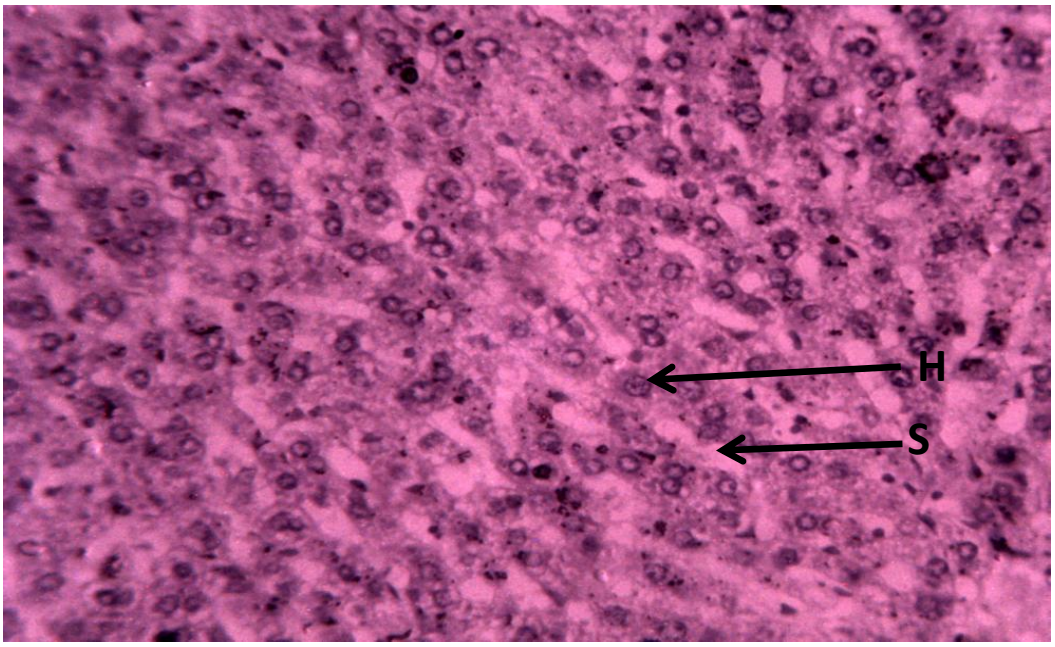


Plate XV: Micrograph of liver from group 7 (800mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing hepatocyte (H)and sinusoid (S), PAS (x 250)

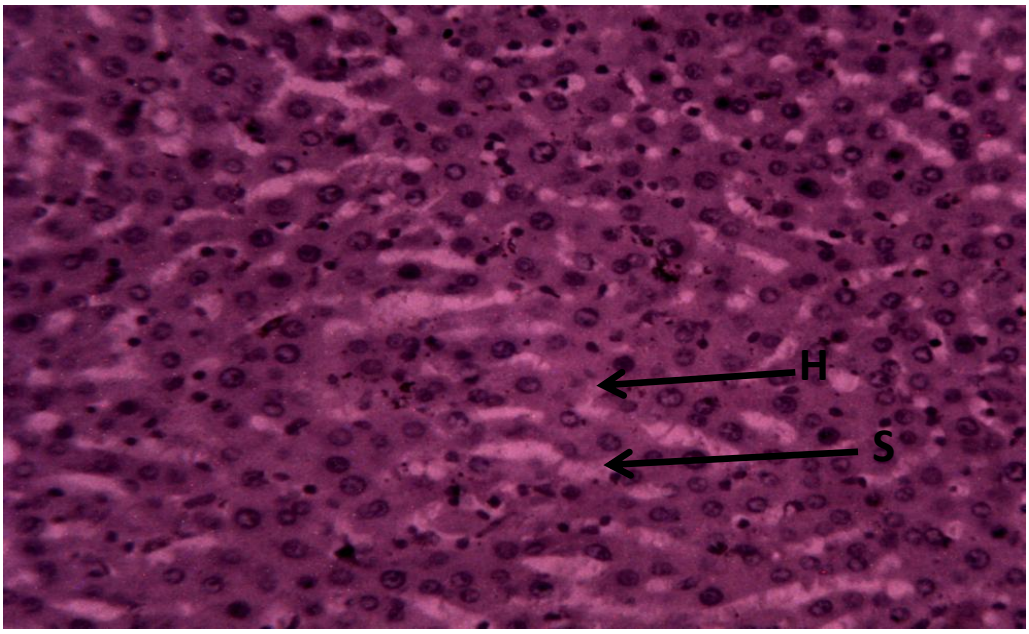


Plate XVI: Micrograph of liver from group 6 (500mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing hepatocyte (H)and sinusoid (S), PAS (x 250)

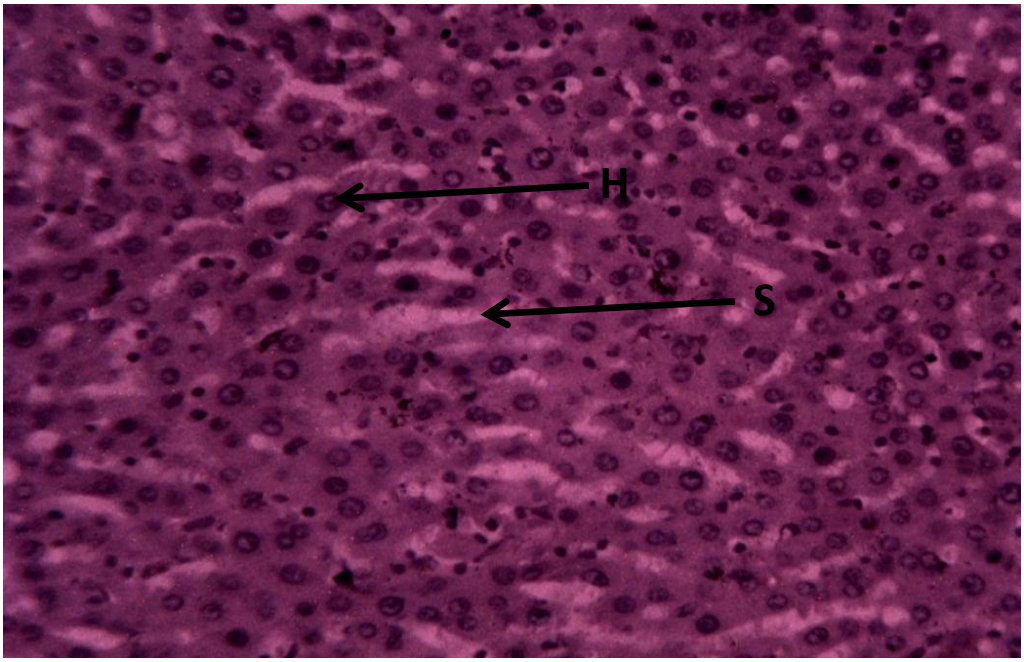


Plate XVII: Micrograph of liver from group 7 (300mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing hepatocyte (H)and sinusoid (S), PAS (x 250)

Therapeutic Study

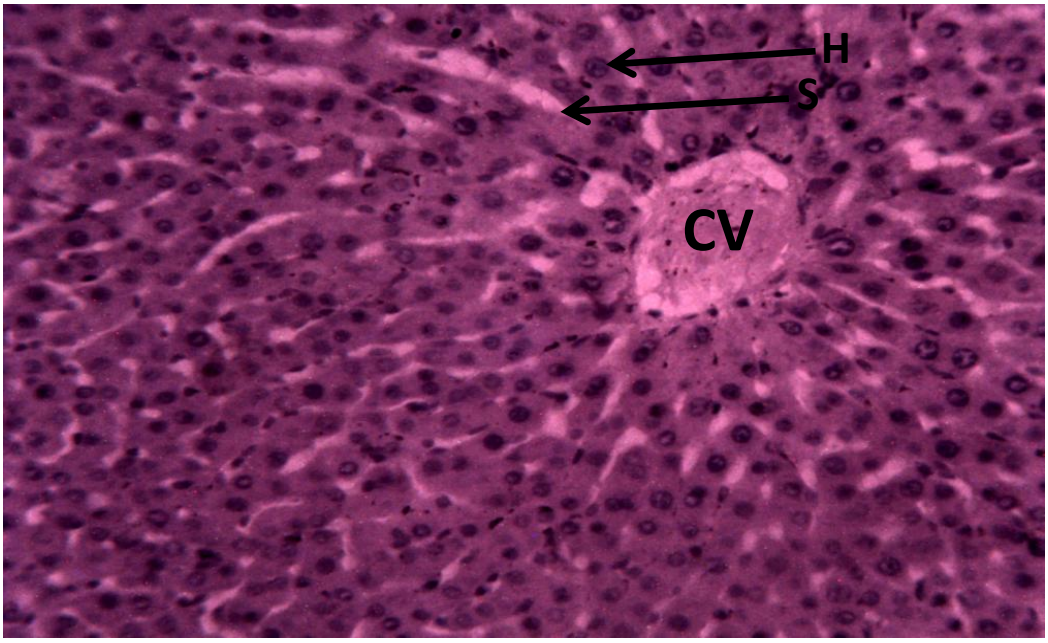


Plate XVIII: Micrograph of liver from group 8 (6000mg/kg body weight ethanol control) rats showing central vein (CV), hepatocyte (H)and sinusoid (S), PAS
(x 250)

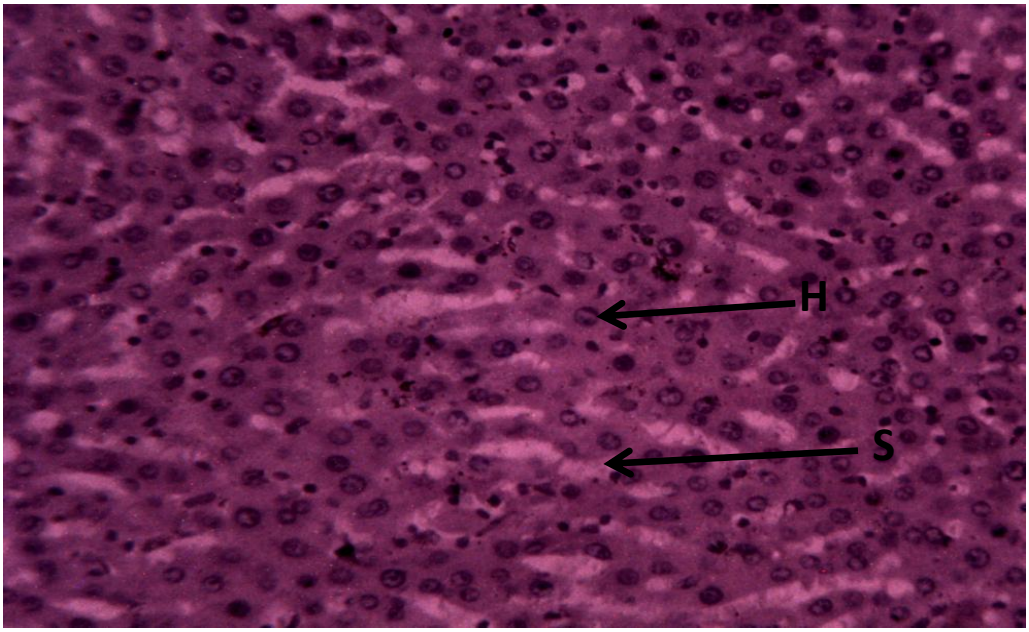


Plate XIX: Micrograph of liver from group 9 (500mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing central vein (CV), hepatocyte (H), and sinusoid (S), PAS (x 250)

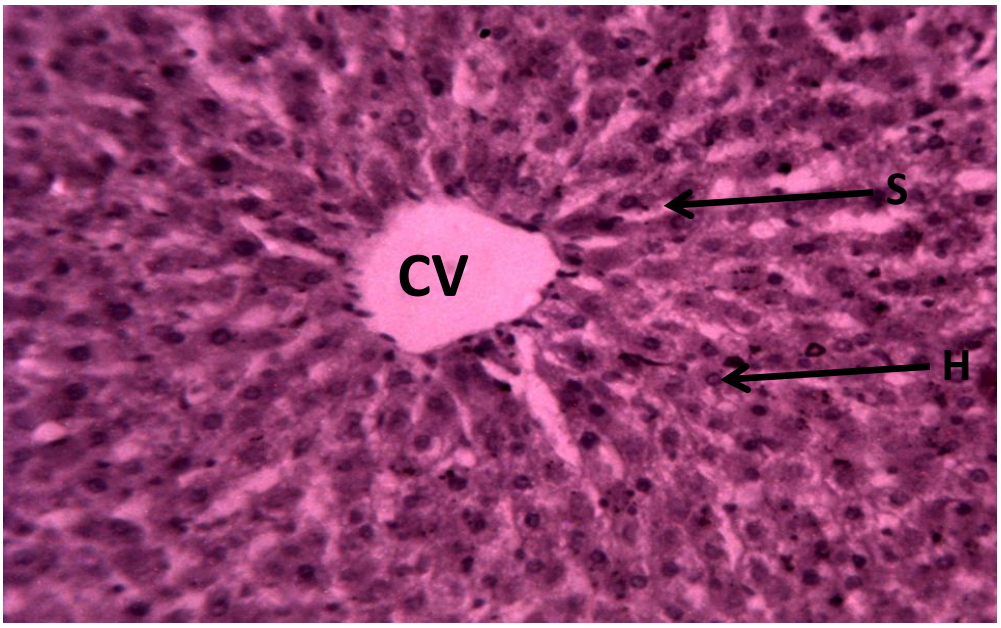


Plate XX: Micrograph of liver from group 10 (800mg/kg body weight extract + 6000mg/kg body weight ethanol) rats showing central vein (CV), hepatocyte (H)and sinusoid (S), PAS (x 250)

CHAPTER FIVE

DISCUSSION

The liver is a major target organ for toxicity of xenobiotics and drugs, because most of the orally ingested chemicals and drugs first go to the liver where they are metabolized, producing various metabolites, including toxic intermediates. Various pharmacological or chemical substances are known to cause hepatic injuries such as alcohol, acetaminophen, CCl4 and dimethylnitrosamine. Excessive dose exposure to these hepatotoxins may induce acute liver injury characterized by abnormality of hepatic function and degeneration, necrosis or apoptosis of hepatocytes (Higuchi *et al.,* 2003). With the increasing ingestion of drugs or exogenous chemicals, the possibilities of liver injury will undoubtedly increase (Lee, 2003). At present, drug or chemically-induced liver injury has become a major clinical problem hence, much attention should be paid to the mechanisms involving drug or chemically-induced liver injury. In addition, the search for effective therapeutic methods for the treatment of drug or chemically-induced liver injury is also very important (James *et al.,* 2003).

5.1 MORPHOLOGICAL OBSERVATION

Chronic ethanol consumption can exert deleterious effects on the structures and functions of all parts of the Gastro intestinal tract (GIT) via oxidative stress (Al-Attar, 2011). This may interfere with digestion and absorption of essential nutrients in the GIT (Lieber, 2003). Poor nutritional status, as a consequence of alcohol-induced primary and secondary

malnutrition, has been reported to contribute to loss in body weight and the development of other alcohol related pathologies (Pushpakiran*et al.,* 2004).

In this study, Silymarin standard treated rats (group 3) exhibited an increase in body weight as in the normal control group rats. The guava leaf treated group 7 rats (800mg / kg body weight extract + 6000mg/kg body weight ethanol) showed increase in body weight throughout the experiment. Rats of groups 2, 4, 5 and 6 (ethanol control and extract + ethanol treated) exhibited decease in body weight at the end of the administration period as shown in Table 4.2.

The loss of body weight observed in the groups administered ethanol and ethanol together with *Psidium guajava* extract when compared with the rats in the control group that were administered only distilled water might be attributed to the loss of appetite, malnourishment and nausea (Adebisi, 2004).

The rats in groups 8, 9, and 10(ethanol control, 6000mg/kg ethanol + 500mg/kg extract and 6000mg/kg ethanol + 800mg/kg extract) in the curative study also showed decreased body weight.

In the hepatoprotective study, the body-organ weight ratio was significantly decreased in the ethanol control treated group, 800mg/kg b/w extract alone and 800mg/kg b/w extract + ethanol. The Silymarin + ethanol, 300mg/kg b/w extract + ethanol and 500mg/kg b/w extract + ethanol had a ratio that is close to the ratio in the control group which indicates that the low and medium doses of the extract has better protective and restorative ability than the high dose which may potentiate more hepatotoxicity.

5.2 SERUM BIOCHEMICAL PARAMETERS

Marker enzymes assay plays a significant role in diagnosis of disease, investigation and assessment of drugs of plant extracts for safety and toxicity (Adeoyo and Oyedepo, 2004). Hepatocellular necrosis or membrane damage leads to very high levels of serum AST and ALT released from the liver into circulation.The increased serum levels of the transaminases refects the cytosolic release of liver marker enzymes into serum, which results from the necrotic and degenerative response of hepatocytes (Chawla, 1999). The magnitude of hepatic damage is usually assessed by measuring the level of the release of cytosolic transaminases (AST, ALT) in circulation (Perez Gutierrez and Solis 2009). Among the two, ALT (GPT) is a better index of liver injury, since ALT catalyzes the conversion of alanine to pyruvate and glutamate, and is released in a similar manner, thus liver ALT represents 90% of total enzyme present in the body (Achliya*et al.*, 2003).

The increased levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cellular membrane in liver (Drotman and Lawhorn, 1978). ALP activities on the other hand are related to functioning of hepatocytes, its increase in serum is due to increased synthesis in the presence of increased biliary pressure (Moss and Butterworth 1974). In the present study, groups 5 and 6 showed that treatment with PGJ leaf (300 mg/kg, and 500mg/kg bw) suppressed the elevated serum levels of AST, towards the value of the control group , this clearly indicates that the plant extract can stabilize the plasma membrane as well as help in healing of the hepatic tissue damage, which corresponds to the work of Taju*et al.,* (2011). However, AST was significantly elevated in groups 3, 4 and 7 (silymarine + ethanol, 800mg/kg extract and 800mg/kg extract + ethanol) which indicate that the extract high dose further potentiate hepatotoxicity instead of

protecting and maintaining the integrity of the hepatocytes and this corresponds with the work of Sambo*et al.,* (2009).

The therapeutic study showed no significant elevation of AST in the ethanol control and extract treated groups when compared to the control group but it was decreased in the high dose extract group (800mg/kg b/w + ethanol) in the curative study.

There was no significant elevation of serum ALT in the ethanol control and extract treated groups in the protective study (protocol A) but it was more elevated in the ethanol only (group 2) and extract control (group 4) which indicates tissue (hepatocytes) damage.In the therapeutic study (protocol B), there was significant increase in serum ALT level in the ethanol control and extract treated groups when compared to the control group thus this indicates that there was hepatocytes damage due to ethanol toxicity.

Serum ALP and total bilirubin levels are also related to the status and function of hepatic cells. Increase in serum ALP is due to increased synthesis, in the presence of increasing biliary pressure (Williamson*et al.,*1996). The study revealed no significant elevation of ALP in the ethanol control and extract treatment groups in the protective study.

In the curative study, serum ALP level was significantly elevated in the 800mg/kg body weight extract + ethanol group when compared to the ethanol control and 500mg/kg body weight extract + ethanol groups. The site specific oxidative damage of some of the susceptible amino acids of proteins is regarded as the major cause of metabolic dysfunction during pathogenesis (Gressner 1991).

5.3 OXIDATIVE STRESS MARKERS

Oxidative stress is increasingly suspected to contribute to the initiation and progression of many diseases, including those caused by alcohol exposure (Gavin, 2008). Both acute and chronic consumption of alcohol can increase production of reactive oxygen species (ROS) and enhance peroxidation of lipids, proteins and DNA, as has been demonstrated in a variety of systems, cells, and species, including humans (Defeng and Arthur, 2009). Living tissues are endowed with innate antioxidant defense mechanisms, such as the presence of the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx). A reduction in the activities of these enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes (Sheela*et al.,* 1995). Antioxidant enzymes such as SOD and CAT are easily inactivated by lipid peroxides or reactive oxygen species, which results in decreased activities of these enzymes in acetaminophen toxicity. It is most abundant in the liver and is responsible for the catalytic decomposition of H_2O_2 to oxygen and water (Reiter *et al.,* 2000). SOD is an extremely effective antioxidant enzyme, and is responsible for catalytic dismutation of highly reactive and potentially toxic superoxide radicals to H_2O_2(Krishnakantha *et al.,* 1993).The effects of chronic alcohol exposure on the cellular content or activity of SODs are controversial, with reports of increases, no changes or decreases, depending on the model, diet, amount and time of alcohol feeding (Defeng and Arthur,2009). The present study revealed normal catalase (CAT) level in the blood of the experimental animals and control group except in group 4 (800mg/kg extract control) which showed significant increase in CAT level and this may be due to the fact that this group was not administered any hepatotoxic agent which will lead to the release of free

radicals which deplete CAT. CAT protects protecting tissues from extreme toxic hydroxylation by decomposing hydrogen peroxide (H_2O_2) (Chance *et al.,* 1952).

SOD was significantly deceased in the ethanol control (group 2) and 800mg/kg body weight extract + ethanol (group 7) when compared with the normal control group. The result suggest that the significant decrease of hepatic SOD activities observed in rats treated with ethanol and 800mg/kg extract + ethanol may be largely due to increased free radical production caused by administration of ethanol(Krishnakantha *et al.,* 1993). In rats treated with *Psidium guajava* extract, however, the activities of these antioxidant enzymes wereelevated than in the rats exposed to ethanol alone. *Psidium guajava* is similarly rich in phytochemical and exhibits antioxidant capacity against oxidative stress (Qian *et al.,* 2004).

Lipid peroxidation has been postulated to be the destructive process in liver injury due to CCl4 administration (Rao *et al.,* 1993). The study showed no significant increase in LPO activity among the control and experimental groups but it was elevated in the ethanol control group (group 2) which indicates the destructive process of liver injury due to ethanol administration and it was lower in the Silymarin + ethanol group (group 3).

The therapeutic study revealed significant decrease in SOD in the ethanol control group when compare with the normal control group and the extract treated groups, this indicates that the extract contains some antioxidants which mop up the free radicals release due to ethanol toxicity .There was no significant difference in CAT and LPO level in the ethanol control and 500mg/kg, 800mg/kg body weight extract + ethanol groups when compared to

the control group but CATwas decreased in the ethanol control group while LPO was elevated in the ethanol control group which indicates tissue damage via lipid peroxidation.

5.4 HAEMATOLOGICAL PARAMETERS

Evaluation of haematological indices plays a vital role in assessing the toxicity of any exogenous compound in the system. Since blood accounts for the greatest percentage of the total body fluid, it remains the commonest route of transport of food, drugs and foreign substances (Saka*et al.,* 2011). Assessment of haematological parameters can not only be used to determine the extent of deleterious effect of extracts on the blood of an animal, but it can also be used to explain blood relating functions of a plant extract or its products (Yakubu*et al.,* 2007). In assessing haematoxicity, increase or decrease in blood variables indicates excess or suppression of blood cell production respectively or an imbalance between blood cell production and destruction (Saka*et al.,* 2011). Such analysis is relevant to risk evaluation as the changes in the haematological system have higher predictive value for human toxicity, when the data are translated from animal studies (Olson *et al.,* 2000).Stimulation of haematopoietic growth factor and erythropoietin systems has been reported to enhance rapid synthesis of blood cells (Murray, 2000).

Similar alterations in lipid as well as haematological profiles were reported in various other plant extracts such as *Bulbinenatalensis*(Yakabuand Afloyayan, 2009), *Bougainvillea spectabilies leaves*(Adebayo *et al*., 2005) and *Fadogiaagrestis*stem (Yakabu*etal.,* 2007). Platelets play a major role in the development as well as in the stability of atherosclerotic

plaques and as a consequence, anti-platelet agents have been used clinically in patients at risk for myocardial ischemia, unstable angina and acute myocardial infarction (Albers, 1995; George, 2000). Therefore the low (300 mg/kg body weight) and high doses (800 mg/kg body weight)of the *P. guajava* extracts significantly increased the platelet count which in turn might increase the risk of cardiovascular diseases. The haemoglobin content (HGB), and RBC showed significant increase in ethanol control, 300mg/kg body weight + ethanol and 800mg/kg body weight + ethanol groups, while RBC significantly increased in extract control, this indicates that the low and high doses of the extract stimulate erythropoietin release in the kidney which is the humoralregulators of RBC production(Degruchy, 1976; Polenakovicand Sikole, 1996).This work agrees with the work of Aruna*et al.*, (2013).There was no significant difference in WBC between the control and treatment groups in the protective study.

The results obtained in the curative study shows significant decrease in values of WBC in the ethanol control group when compared to the normal control groupand this is due to the haematotoxic effect of ethanol on haematological indices.

The differential white blood cell count showed a significant increase in the neutrophil level in the extract treated animals in group 4, ethanol treated rats in group 2, and ethanol and extract treated rats in groups 5 and 7. This increase may be due to the immunomodulatory activity of guava leaf extract which is involved in the stimulation of bone marrow to synthesize and release large number of neutrophil into the blood stream. The ethanol treated rats in group 2, and ethanol and extract treated rats in groups 5 and 7 showed a significant decrease in lymphocyte level.

The curative study showed significant increase in mean neutrophil level in the ethanol control group and the extract treated groups when compared to the normal control group. Careiro and Lister (1992) reported that during acute phase of inflammation, more neutrophil were produced while lymphocytes and monocytes predominate in chronic phase.

5.5 THE LIVER

The initial processing of the ethanol in the liver includes several biochemical changes that modulate the process of liver damage. Some of the metabolites for example acetaldehyde formed are hepatotoxic. Therefore, the liver plays a primary role in the metabolism of ethanol and is the most significantly affected organ following the experimental exposure of ethanol to the animals.

Many indigenous plants are claimed to have a stimulant and restorative effect on the liver (Kirtikar and Basu, 1933).

In the hepatoprotective study, the histological finding shows normal hepatic architecture of the liver in Plates I, while III, V, VI and VII showed mild necrosis. The histological examination of the liver in group 4 revealed marked necrosis, while group 2 (ethanol control) showed regenerative activity in the form of nuclei disintegration, loss of nucleus, necrosis and congestion. This study suggest that *Psidium guajava* extract has protective potentials through its high antioxidant content especially flavonoid which is an antioxidant that protect the liver tissue against liver injury induced by ethanol. This study agrees with the work of Taju*et al.,* (2011) on the hepatoprotective effect of psidium guajava extract.

The administration of the high dose (800mg/kg body weight) of extract alone showed hepatotoxicity to the liver thus the high extract dose may contain some hepatotoxic components which potentiate tissue damage when administered alone. This work agrees with the work of Sambo*et al.*, (2009).

The curative study revealed almost normal hepatic architecture in Plate IX and X. The effect is dose dependent as 800mg/kg showed a better healing effect than the 500mg/kg. The liver of plate 8 showed necrosis, mild oedema and congestion which are due to the administration of ethanol which is a known hepatotoxic agent. This suggest that the extract has some restorative and regenerative potential which enhanced the regeneration of the damaged hepatocytes.

It has also been shown that the inability to synthesize protein that has been destroyed by the direct toxigenic action of ethanol may be responsible for the initiation of necrogenesis present in the test group (Wood, 1965; Wepler and Wildhirt, 1972).

Chronic ethanol consumption results in a dramatic decrease in liver glycogen concentration which could be related to either a depressed rate of glycogen synthesis or an increased rate of glycogen breakdown (Van *et al.,* 2001). Several flavonoid have been shown to have potential to inhibit glycogen synthesis by inhibition of glycogen phosphorylase (Atsushi *et al.,* 2008).The effect of antioxidants on hepatic glycogen phosphorylase are not well understood. Both inhibitory and stimulatory effect on hepatic glycogen phosphorylase activity by – catechin and + catechin as a flavonoid have been reported (Nyfeler*et al.,* 1983). The glycogen storage in the liver in all the test groups for both the hepatoprotective and the curative study showed decreased glycogen deposit and this is due to the effect of

ethanol and the *psidium guajava* extract administration which deplete glycogen deposit in the liver.

CHAPTER SIX

SUMMARY, CONCLUSION, AND RECOMMENDATION

6.1 Summary and Conclusion

The hepatoprotective role of guava (*Psidium guajava)* leaf extract on ethanol induced liver damage was conducted using adult male and female Wistar rats. Significant toxicity was seen in the liver hepatocytes with congestion, and necrosis in group 2 (ethanol control) animals (Plate II) and necrosis in group 4 (800mg/kg body weight extract) rats (Plate IV) in the hepatoprotective study. The curative study showed marked oedema and necrosis in group 8 (ethanol control) rats (plate VIII). Aqueous extract leaf of *Psidium guajava*exhibited hepatoprotective effect in group 5, 6 and 7 (300mg/kg extract + 6000mg/kg ethanol, 500mg/kg extract + 6000mg/kg ethanol and 800mg/kg extract + 6000mg/kg ethanol) (Plates V, VI and VII) and some degree of therapeutic/restorative effect on the hepatocytes of groups 9 and 10 (6000mg/kg ethanol + 500mg/kg extract and 6000mg/kg ethanol + 800mg/kg extract) animals (plates IX and X).

The difference in biochemical parameters (LPO, CAT and SOD) in the rats after ethanol administration showed significant decreased in groups 2 (ethanol control) and 7 (800mg/kg

extract + 6000mg/kg ethanol) rats, SOD when compared with the control group (group 1). AST was significantly increased in groups 3, 4, and 7(100mg/kg silymarin, 800mg/kg extract and 800mg/kg extract + 6000mg/kg ethanol) while ALP was significantly increased in group 10 (6000mg/kg ethanol + 800mg/kg extract) in the curative study.

RBC, HGB and platelets count were significantly increased in groups 5 and 7 (300mg/kg extract + 6000mg/kg ethanol and 800mg/kg extract + 6000mg/kg ethanol), RBC and platelets were significantly increased in groups 4 (800mg/kg extract) while RBC and HGB were significantly increased in group 2 (ethanol control). In the curative study, RBC and WBC were significantly increased in group 10 (6000mg/kg ethanol + 800mg/kg extract) while WBC was significantly high in group 9 (6000mg/kg ethanol + 500mg/kg extract).

The differential white blood cell count showed significant increase in the mean neutrophil and significant decrease in mean lymphocyte values in groups 2, 5 and 7 (6000mg/kg ethanol, 300mg/kg extract + 6000mg/kg ethanol and 800mg/kg extract + 6000mg/kg ethanol) and also significant increase in neutrophil in group 4 (800mg/kg extract).

In conclusion, the administration of aqueous guava (*Psidium guajava*) leaf extract significantly reduced the biochemical and histological alterations to near normal level in the experimental animals while protecting the liver in pretreatment rats and showed some degree of restoration of the liver tissue from ethanol induced liver damage in the therapeutic study. Thus, aqueous leaf extract of *Psidium guajava*(guava)positively modulated antioxidant activity by reducing and detoxifying the free radicals tissue injuries induced by ethanol.

6.2 RECOMMENDATION

Ethanol is toxic to the body organs if consumed in large quantity and without caution. Thus the following recommendations are suggested:-

i. Chronic study with simultaneous administration of ethanol plus extract should be carried out.

ii. Fractional distillation of the crude extract should be carried out and the active ingredients used to determine its hepatoprotective activities.

iii. The full extent of the antioxidant activities of guava leaf should be determined by the use of tissue culture techniques.

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APPENDIX I

Effect of alcohol and aqueous leaf extract *psidium guajava* on organ (liver) weight of control and experimental animals.

Hepatoprotective study

GROUP/TREATMENT	**LIVER WEIGHT**
Group 1 Distilled water	6.26 ± 0.55
Group 2 6000mg/kg ethanol	8.15 ± 0.53
Group 3 100mg/ kg silymarine + 6000mg/kg ethanol	6.72 ± 0.31
Group 4 800mg/kg extract	7.27 ± 0.71
Group 5 800mg/kg extract + 6000mg/kg ethanol	6.29 ± 0.58

Group 6 500mg/kg extract + 6000mg/kg ethanol	5.78 ± 0.27
Group 7 300mg/kg extract + 6000mg/kg ethanol	7.63 ± 0.25

APPENDIX II

Effect of alcohol and aqueous leaf extract *psidium guajava* on organ (liver) weight of control and experimental animals.

Therapeutic study

GROUP/TREATMENT	**LIVER WEIGHT**
Group 1 6000mg/kg ethanol	8.07 ± 0.77
Group 2 500mg/kg extract + 6000mg/kg ethanol	6.50 ± 0.89
Group 3 800mg/kg extract + 6000mg/kg ethanol	6.19 ± 0.55

APPENDIX III

TISSUE PREPARATION METHOD

1. Fixation in 10% formalin for 48 hours

2. Washing in running tap water for 3 minutes

3. Dehydration in grades of alcohol

   i. 30% alcohol for 4 hours

   ii. 50% alcohol for 4 hours

   iii.70% alcohol for 4 hours

   iv.1 change in 95% alcohol for 4 hours

   v.1 change in 95% alcohol for 4 hours

   vi.1 change in 95% alcohol for 4 hours

vii.1 change in 95% alcohol for 4 hours

4. Clearing

i. Xylene I, for 2 hours

ii. Xylene II for 2 hours

iii. Xylene III for 2 hours

5. Infiltration

i. Xylene + paraffin wax for 2-4 hours

ii. Pure paraffin wax for 2 hours

6. Embedding in paraffin wax

7. Sectioning

APPENDIX IV

HAEMATOXYLIN AND EOSIN STAINING

PREPARATION OF STAINING SOLUTION

A.HAEMATOXYLIN

i. Haematoxylin	1g
ii. Absolute alcohol	10ml
iii. Ammonium or potassium alum	20g
iv. Mercury oxide	0.5g

Haematoxylin was dissolved in alcohol and alum, which was then dissolved in water. The mixture was then boiled and the mercuric oxide was added, the solution was allowed to turn dark purple, then it was cooled rapidly.

B. EOSIN

i. Eosin 5g

ii. Crystal of thymol

iii. Distilled water 100ml

About 5g of eosin was dissolved in 100ml of distilled water. To prepare growth of mould in the stock solution, a crystal of thymol was added to obtain 1% staining solution. The 20ml of stock solution was diluted with distilled water by making it up to 100ml.

C. STAINING

1. Rehydration

2. Dewax I for 3 minutes

3. Dewax II for 3 minutes

4. Absolute alcohol for 1 minute

5. 95% alcohol for 1 minute

6. 70% alcohol for 2 minutes

7. 50% alcohol for 2 minutes

8. 30% alcohol for 2 minutes

9. Haematoxylin for 5 minutes

10. Distilled water

11. 35% alcohol for 1 minute

12. Acid alcohol for 30 seconds (for differentiation into nucleus and cytoplasm)

13. Distilled water for 1 minute

14. Alkaline alcohol for 2 minutes (bluing of the nucleus)

15. Water for 1 minute

16. Mount in Canada balsam of D.P.X

APPENDIX V

P.A.S STAINING TECHNIQUE

A. Gomori's silver methenamine solution

Add 5ml of 5% silver nitrate to 100ml of 3% methenamine (hexamethylenetetramine) to prepare stock solution. To use, add 3ml of 5% borax to 25ml of stock silver and 25ml of distilled water.

B. Differentiator

This is a mixture of 0.5% sulphuric acid in 0.2% ferric sulphate

C. Method

1. Bring section to water

2. Oxidize in 5% chromic acid for 1 hour

3. Wash in running tap water for 1-2 minutes

4. Treat with 2% sodium bisulphate for 1 minute to remove chromic acid

5. Wash in running tap water for 5 minutes

6. Place in methenamine silver solution at 60°C for 1-3 hours

7. Wash well in distilled water

8. Toned in 0.2% gold chloride solution for 2 minutes, then rinsed in distilled water

9. Place in 3% sodium thiosulphate for 2 minutes, washed in running tap water for 2-3 minutes

10. Sections may be counter stained if desired with haematoxylin or 0.2% light green in 0.2% acetic acid.

11. Dehydrate, clear, and mount in Canada balsam or D.P.X

D. Result

P.A.S positive structure	magenta
Background	pink