TOXICOLOGICAL STUDIES OF *Piliostigma reticulatum* (Kalgo) METHANOLIC LEAF EXTRACT IN RATS

BY

MOHAMMED SANI MAMMANI

(SPS/17/MBC/00078)

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JULY, 2021.

DECLARATION

I hereby declare that this work is the product of my own research efforts, undertaken under the supervision of Professor A. M. Wudil and has not been presented and will not be presented elsewhere for the award of any degree or certificate. All sources have been duly acknowledged.

_____ _____

Mohammed Sani Mammani **Date**
(SPS/17/MBC/00078)

CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent preparation of this report by Mohammed Sani Mammani (SPS/17/MBC/00078) were carried out under my supervision.

_____ _____

Professor A. M. Wudil **DATE**
Supervisor

APPROVAL PAGE

This is to certify that this dissertation has been examined and approved for the award of the degree of Master of Science in Biochemistry.

Name and signature **Date**

_____ _____
External Examiner

_____ _____
Internal Examiner

_____ _____
Supervisor

_____ _____
Representative of Board of PG Studies

_____ _____
HOD/Chief Examiner

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DEDICATION

I dedicate this work to my beloved parents, Mr & Mrs. Mammani Sani who struggled to see me through in the pursuit of my M.Sc. program. May Allah reward them abundantly, Ameen,

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ABSTRACT

Plants have been used for several decades for the treatment of various diseases, but their harmful effects are common clinical occurrence. This study was designed to investigate the effects of methanolic leaves extract of *P. reticulatum* on liver and kidney function indices as well as oxidative stress markers in rats. Initially, the qualitative phytochemical screening of *P. reticulata* revealed that the methanolic leave extract of the plant contains seven (7) out of the nine phytochemicals tested: alkaloids, tannins, terpenoids, flavonoid, phenols, saponins and glycosides, while coumarins and phlobatannis were absent. Similarly, the result of the quantitative phytochemical analysis showed that the extract has high concentration of phenols followed by alkaloids, tannins, terpenoids, flavanoids, saponins and the least were the glycosides. Acute oral toxicity test showed that the extract was non toxic with an LD_{50} greater than 5000mg/kg body weight. Results from the Sub-chronic toxicity study showed that the extracts did not significantly ($p>0.05$) affect serum Na^+, Cl^- HCO_3^- and Creatinine levels when compared to control, however, there was significant differences ($p<0.05$) in serum K^+, and Urea levels in groups treated for 4 weeks when compared to the control. There was no significant difference ($p>0.05$) in the activities of AST, ALT, ALP and concentrations of albumin, total protein, and conjugated bilirubin after 2 and 4 weeks of administration, while total bilirubin level increased significantly ($p<0.05$) compared to the control group. Also, while MDA level in treated rats was not significantly different from control, CAT and SOD levels increases significantly ($p<0.05$) with increasing extract concentration after 2 and 4 weeks, compared to control group. In conclusion, the results demonstrate that *P. reticulatum* leaf possess antioxidant activity probably due to its high polyphenolic content. It also has no effect on liver and kidneys function indices, and this may be safe for treatment of diseases.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Man has been using medicinal preparations derived from plants and other sources like animals and minerals from time immemorial, the practice being as old as man himself. These medicinal plants pass through several developmental stages from their crude form to drugs like tablets, capsules, injections, ointments, creams, etc. There are records of the use of plants in traditional medicines by the ancient civilization like the Chinese, Greek, Indians and the Babylonians are still in existence (Snowdon and Cliver, 1996). Ancient texts of Indian and Chinese origin contain exhaustive depictions of the use of a variety of plant-derived medications (Ahmad *et al*., 2006). The Chinese Emperor, Shen Nung, published a book on herbs in 370BC. Also an Apothecary by the name Theophrastus born in Athens in the present day Greece around 370BC also published a compendium on the uses of close to 500 medicinal plants. To this day, plants remain the main source of medicines for a large proportion of the world's population, particularly in the third world countries (Barnes *et al*., 2008).

Piliostigma reticulatum is one of the medicinal plants of the Ivorian rich flora used traditionally for the treatment of several diseases including as colic, hemorrhoids, diarrhea, dysentery, inflammation, infections, neuralgia, smallpox, malaria and rheumatism. In addition, the leaves and barks of *P. reticulatum* are used in food for the preparation of local alimentary paste, while the barks are used to stimulate digestion (Yelemou *et al*, 2007 and Dosso *et al*, 2012). *Piliostigma reticulatum* is a tree of 8 to 10 m high, rarely straight, sometimes bushy, with a rounded, bushy top. Its bark is deeply fissured and cracked, sometimes ferruginous gray with a pink fibrous slash turning brown. The leaves are alternate, leathery, couplets and hairless

beneath. They are heavily lobed with rounded lobes or corner. The fruit is a woody pod, flat, hairless, sometimes twisted and cracked (Arbonnier, 2002). For the traditional treatment of diarrhoea, alcohol macerated of *Piliostigma reticulatum* stem bark is used in beverage for two or three days (Dosso *et al*., 2012). Pharmacological studies have shown that the leaves of *Piliostigma reticulatum* possess antibacterial, sedative and anticonvulsant properties (Bum *et al.,* 2009). The leaf extract from the plant was found to exhibit anti-microbial activity against some bacteria and fungi such as *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418), *Bacillus subtilis* (NCTC 8236), *Proteus vulgaris* (NCTC 4175), *Aspergillus niger* (ATCC 10578), and *Candida albic ans* (NCTC 10231) (Bum *et al.,* 2009). Vibriocidal action of *P. reticulatum* among other medicinal plants was studied and proved to be effective in killing *Vibrio* sp. In Nuba mountains in Sudan, the fresh bark of the plant is used for fresh wound dressing as it coagulates blood and is believed to enhance healing of the wound by keeping it clean. In the old days before the introduction of primary healthcare and midwifery facilities, the inner soft bark of *P. reticulatum* was widely used after delivery to cover the episiotomy wound (Irvine, 1961). In Tanzania and Zimbabwe, a cough remedy prepared from the root bark of *p.reticulata* has been shown to exhibit significant anti-inflammatory and analgesic activity. Certain compounds isolated from its leaves have been reported to elicit anti-inflammatory and antibacterial activities (Akinpelu and Obuotor, 2000).

Recently, there is a growing interest in substances with antioxidant and antimicrobial properties that are supplied to human as food components or as specific pharmaceutics. The role of free radicals is becoming increasingly recognized in the pathogenesis of many human diseases, including inflammation, cancer, diabetes, renal failure, atherosclerosis and hypertension (Aderogba *et al*., 2005). Antioxidants retard oxidation and arrest the adverse effect of free

radicals.They are sometimes added to meat and poultry products to prevent or slow oxidative rancidity of fats that cause browning and deterioration. However, antioxidant compounds from natural sources are receiving consideration due to the side effect of the synthetic ones.

Generally, plants reported to exhibit lipid lowering activity are rich in flavonoids and tannins which play significant roles in the mobilization and metabolism of lipids. Preliminary phytochemical studies on *Piliostigma reticulata* reveals high levels of flavonoids, tannins, and alkaloids (Akindahunsi and Salawu, 2005). The plant is also reported to contain nutritionally important vitamins (such as C, E, and beta-carotene) and minerals (such as Calcium, Magnesium, Zinc, and Potassium) all of which contribute to its high-antioxidant properties.

A major challenge to herbal medicine and development of drugs from herbal medicine is the validation of their folkloric claims to provide scientific basis for the conservation of tropical medicinal resources, the deployment of the beneficial ones and the development of potential bioactive constituents. Hence, the present study evaluates the safety and toxicity of *P. reticulatum* methanolic leaf extract in organ such as liver and kidney of experimental rats.

1.2 Statement of Research Problem

Medicinal plants such as *Piliostigma reticulata* also known as “Kalgo” in Hausa are commonly used as traditional medicine, being natural, are considered as harmless (Ekor, 2014). Despite this positive perception of herbal treatments, their safety has most often not been evaluated per modern standards (Pelkonen *et al*., 2014), and cases of contamination, toxicity or poisoning are regularly detected (Liu *et al*., 2014), and the situation appears even worse for herbs used in developing countries, notably in African traditional medicine (Poivre *et al.,* 2017). Despite

frequent and regular use, there is paucity of information with regard to its effect on liver and kidney function indices.

1.3 Justification to the Study

According to World Health Organization (WHO**,** 2017) plants extracts or their active constituents are used as folk medicine in traditional therapies of 80% of the world population. Due to the frequent use of these herbs, it is imperative to check their effects on organs such as liver and kidneys.

1.4 Aim and Objectives of the Study

Aim of the Study

The aim of this research is to determine the effects of methanolic leaf extract of *P. reticulatum* on liver and kidney function indices in rats.

Objectives of the Study

The specific objectives of this study are:

1. To determine the phytochemical composition of the methanolic leaf extract of the plant.
2. To carry out acute and sub-chronic toxicity studies of the extract by administering different doses of the extract to rats for 2 and 4 weeks, and determinng their effect on liver and kidney function indices as well as oxidative stress markers before and after administration

CHAPTER TWO

LITERATURE REVIEW

2.1 *Piliostigma reticulata* (BUKHAN 72)

2.1.1 Botanical Classification

The botanical classification of *P. reticulata* is as follows:

Kingdom: Plantae

Division: Angiosperms

Class: Eudicots

Order: Fabales

Family: Fabaceae

Subfamily: Caesalpinioideae

Genus: *Piliostigma*

Species: *reticulata*

BUKHAN 72:Bayero University Kano, Herbarium Accession Number 72

Figure 2.1: The leaves and fruits of *Piliostigma reticulata*

2.2 Ethno-medicinal (Traditional) uses of *Piliostigma reticulata*

Piliostigma and other species in the genus have been reported to have a wide range of uses to mankind, ranging from food for man and animals, and also a wide range of medicinal uses (Ibewuike *et al*., 1996), which includes the treatment of loose stool in teething children, wound dressing, ulcer treatment, worms infestation, arrest bleeding, inflammations, bacterial infections, gonorrhoea, stomach ache, headache, etc (Ozolua *et al*., 2009). The roots and twigs have are used locally in the treatment of dysentery, fever, respiratory ailments, snake bites, hookworm and skin infections, and the leaf extracts are used for the treatment of malaria (Kwaji *et al*., 2010). The plant is used in rope making, dye making, tanning of leather, as household utensils, for roofing, fence and bridge building, and farm implements, they are also used as erosion control measures, and as stakes to support plants of weak stems or creepers like yams, because they are deep rooted.

2.3 Phytochemistry of *Piliostigma reticulata* and other species in the genus

Phytochemical investigations reported on *Piliostigma thonningii*, shows that the plant contains a wide range of compounds, ranging from alkalloids, anthraquinones, flavonoids, glycosides, saponins, sterols and tannins (Bello *et al*., 2013).Compounds isolated from the plant include Piliostigmin (XXI), 16α - hydroxy - (-) - kauran-18-oic acid (XXII), (Ibewuike, *et al*., 1996), 6, 8 – di – C - methyl quercetin 3 - methyl ether (XXIII), 6 – C - methyl quercetin 3, 7- dimethyl ether (XXIV) and 6, 8 – di – C - methyl quercetin3, 7 – dimethyl ether (XXV), have been shown to possess antibacterial and anti – inflammatory activities for the first time. Flavonoids isolated from the plant include quercetin, quercitrin, 6 –C - methyl quercetin 3,3,3 – trimethyl ether

(XXVI) and 6, 8 – di – C - methyl kaempferol 3, 7- dimethyl ether (XXVII) (Ibewuike, et al.,1996).

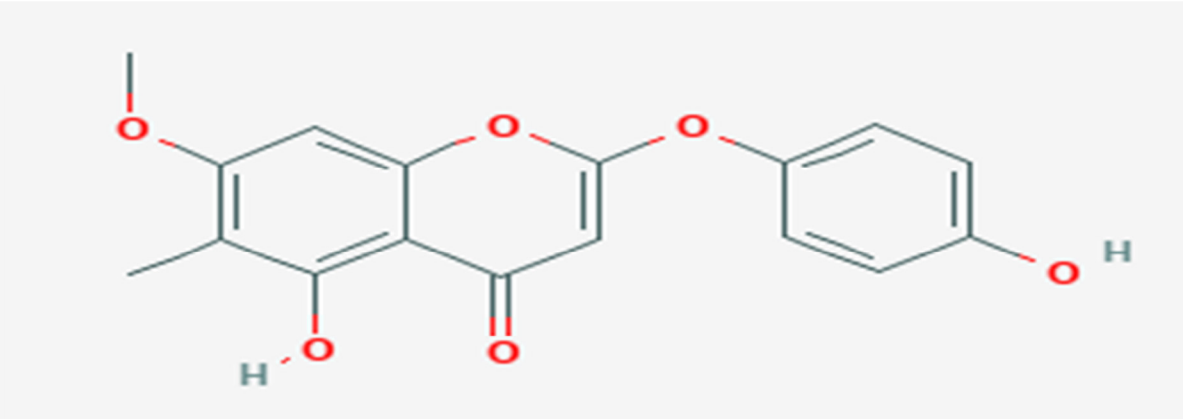

Figure 2.2: Structure of Piliostigmin (Ibewuike, et al.,1996).

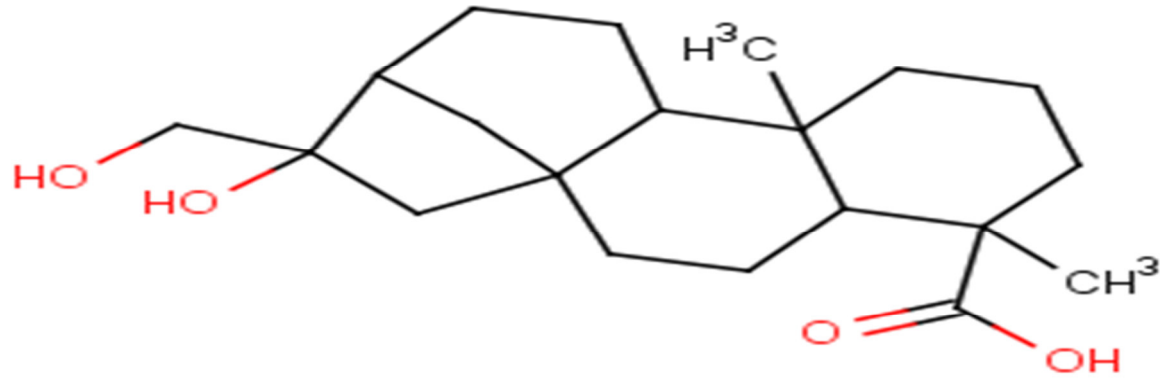

Figure 2.3: Structure of 16α - hydroxy - (-) - kauran-18-oic acid (XXII) (Ibewuike, et al.,1996).

Although flavonoids are well known anti - inflammatory agents, the anti-inflammatory activity of the relatively rare C - methyl flavonoids was reported for the first time by Ibewuike *et al.*(1996). A comparative study carried out on the only co–generic specie of *Piliostigma*

reticulatum showed similarities both in chemical constituents and biological activity and can be conveniently substituted for each other (Ogundaini, 2005).

Methanolic root extracts have shown *in vivo* anti-diarrhoeal and anti-ulcerogenic properties in rats. Aqueous extracts of the bark showed *in-vivo* anti-inflammatory and analgesic effects in mice and rats. Ethanolic and aqueous bark extracts showed antibacterial activity. Leaf extracts showed anti-inflammatory activity in the carrageenan-induced rat paw oedema model and antimicrobial activity, especially against Gram positive bacteria (Jada *et al.,*2015). Piliostigmol and various flavonoids isolated from the leaf also showed anti-inflammatory and antimicrobial activity (Yeo *et al.,*2011). Methanol extracts from leaves have proved to be trypanocidal against *Trypanosoma brucei* and *Trypanosoma congolense* (Chibale.,2005). Leaf decoctions showed in-vivo anticonvulsant and sedative activity in mice (Konate *et al.,*2012). Butanol and ethyl acetate fractions of the leaf extract exhibited strong antioxidant activity (Nwaehujor *et al.,* 2015). Quercetin and quercetin glycosides isolated from the ethyl acetate fraction also showed antioxidant activity (Nwaehujor *et al.,* 2015).

2.4 General Mechanisms of Action of Medicinal Plants

Plants produce a wide diversity of secondary metabolites which serve not only as defense against herbivores, other plants and microbes, but also as signal compounds. In general, some exhibit a wide array of biological and pharmacological properties. Because of this, some plants or products isolated from them have been and are still used to treat infections, health disorders or diseases, as they often interact with proteins, biomembranes or nucleic acids which are the main targets in cells. Whereas some of the compounds, can be optimized on a few molecular targets, e.g alkaloids on receptors of neurotransmitters, others, such as phenolics and terpenoids are less

specific and attack a multitude of proteins by building hydrogen, hydrophobic and ionic bonds, thus modulating their 3D structures and in consequence their bioactivities, which is the main modes of action for the major groups of common plant secondary metabolites. The multi-target activities of many compounds explains the medical application of complex extracts from medicinal plants for several diseases. Herbal medicine is not a placebo medicine but a rational medicine, and for several of them clinical trials have shown efficacy (Frenkel *et al*., 2014).

2.5 Uses of *P. reticulata*

2.5.1 Uses of some Parts of *P. reticulata* Tree

The Tree: Provides poles, and the wood is used for making tool handles, household utensils, stools, masks and other small articles. It is also used as tinder, as fuel-wood and for making charcoal, in central Burkina Faso, the plant is considered to contribute to soil improvement and farmers use it for mulching. In addition *Piliostigma reticulatum* provides shade during agricultural activities, e.g. in nurseries (Dambatta et al., 2011).

The Bark: Is widely used on wounds, cuts, ulcers and sores as an astringent, haemostatic, antiseptic and cicatrisant. Bark decoctions are used for washing wounds and as a mouth wash, and for the treatment of fever, colds, stomach-ache, indigestion and diarrhoea. A decoction of the bark boiled in milk or bouillon is drunk against gonorrhoea. Bark infusions are taken against toothache and vomiting(Mustapha *et al.*,2013) . The bark is either chewed or macerated and taken against cough. A maceration of the inner bark is used as a wash against ringworm, attached to swollen body parts against inflammation (Yeo *et al.,*2011). In Niger republic, the aerial parts are used as tonic for lactating women and also against fever in children (Ighodaro *et al*. 2012). Boiled young shoots are chewed by children with toothache (Aiyeloja *et al.,* 2006).

The Leaves: Are used for the treatment of fevers, as a tranquillizer, and for the treatment of a range of ailments including colds, bronchitis, headache, rheumatism, ophthalmia, toothache, mumps, syphilis, vertigo and epilepsy (Aiyeloja *et al.,* 2006). Leaf preparations are considered haemostatic, antiseptic and cicatrisant, and so applied on wounds, ulcers and sores. Ground fresh leaves are applied for inflammation, young leaves are eaten raw against nausea and boiled leaves are rubbed in against lumbago (Mustapha *et al.*,2013). In northern Senegal, a decoction of the leaves in a vapour-bath is used against conjunctivitis. Leaf decoctions are taken by women in labour to ease delivery, and are used in baths as a sedative and against epilepsy, dysentery, haemorrhoids, malaria and hernia (Alfred, 2013). In Nigeria leaf decoctions are used to foment fractures and to get rid of guinea worm. A leaf decoction is rubbed into scarifications for the treatment of leg pain, while leaf infusions are used in drinks or baths as a sedative and anti-rachitic for new-born children, and to stimulate their appetite (Egharevba *et al*. 2010). Macerations of young leaves and flower buds are given against rickets in babies, kwashiorkor and anorexia. The plant leaves are also used to wrap foods (Vodouhe *et al.*, 2010).

The Fruit and Seed: The young fruits are eaten as a vegetable, and the seeds as a condiment or as food in times of scarcity. The pounded and boiled fruits are made into drinks, perfume is made from the seeds. In Nigeria the seeds are sometimes chewed as a substitute of kola nuts or to stain the lips red (Jiofack et al., 2009), or eaten by cattle, sheep, goats and camels, or burnt for smoking beehives to make these attractive to bees, and as fuel or used for strengthening uncured clay pots.

Also, it is used as a laxative and for the treatment of wounds, sores, ringworm, headache, encephalitis, bronchitis, cough, liver problems and indigestion. In Burkina Faso, crushed or powdered fruits are applied on the skin for the treatment of wounds and skin problems, and burnt

and crushed fruits against cough. In Senegal, the powdered fruit in water is taken by draught or used topically in case of snake bites. In traditional veterinary medicine, the powdered root is put into drinking-water for the treatment of diarrhoea in cattle. In Nigeria, powdered seeds are added to brewery waste and given to animals against trypanosomiasis (Vodouhe *et al.,* 2010).

The Roots: Yield red dye, The roots are used for the treatment of gonorrhoea, hookworm, ascites and dropsy. Root infusions are taken against diarrhoea and uterine pain. Root decoctions are used in preparations against liver and gall complaints, and are drunk as an antidote for plant poison. Root decoctions or macerations are taken or used in vapour baths against cough, diarrhoea, constipation, stomach-ache and muscular pain. The sap of ground roots is applied on swellings in dislocations, and taken against painful pregnancy and nausea (Noumi et al., 2015).

2.5.2 Livestock Feed

Although there is limited information on the use of *P. reticulatum* seed as ingredients in livestock feed formulation, Akin-Osanaiye (2009), conducted a research on the nutritional potentials of *P. reticulatum* with view of exploiting the seeds as an alternative plant protein source for human and livestock feed formulation, He reported that methods of the different ways of feed stuff processing may significantly affect the concentration and availability of minerals, vitamins and other essential nutrients in feedstuffs formulated using *P. reticulatum.*

2.5.3 Antimicrobial Activity

There were common beliefs that certain plants contained potential healing features which characterize them today as antimicrobial principles (Laly and Nyazema, 2011). Historically, plants have provided good sources of anti-infective agents in the fight against microbial

infections (Gull *et al*., 2011; Shih-Lu *et al*., 2011), but, increased resistance to antibiotics has given rise to the search for new antimicrobial agents, focusing on plant derived active principles (Sousa *et al.*, 2016). Antimicrobial activities of *P. reticulata* extracts on the growth of bacteria depends on the parts used, solvent of extraction and concentration of the extracts applied, the presence of chemical components of the studied plant parts, the inhibitory zone and concentrations at which values were effective on the tested organism. This could also be attributed to the different growth rate of tested organisms, nutritional requirements, temperature and inoculum size (Kalimuthu, 2013).There are reports on the antimicrobial activities of extracts from different parts of *P*. reticulata plant, however their efficacies depend on the extraction procedure and assay (Kalimuthu, 2013). This report is in line with the observation of (Alfred, 2013), who reported the antimicrobial activities of six ethnomedicinal plants used in the treatment of infectious diseases, with *P. reticulata* shown to be active against *E. coli*, *P. aeroginosa*, *S. typhi* and *S. aureus* with significant diameter of inhibition in all the tested organisms ($P < 0.05$) (Jada *et al.,*2015).

The activity of the methanolic extract of the plants against *Staphylococcus* species has been attributed to the presence of constituents like terpenoids, tannins, saponins, phenolic compounds, and flavonoids (Selvamohan *et al*., 2012).

2.5.4 Anti-diarrhoeal Activity

Diarrhoea is one of the leading cause of mortality in developing countries, accounting for more than 5-8 million deaths each year in infants and small children under 5 years old (Mohd *et al.,* 2004; Mujumdar *et al*., 2005). Salawu *et al.* (2007) demonstrated that the methanolic leaf extract of *P. reticulatum* inhibited diarrhoea in rats, as shown by the significant reduction in the number

of diarrhoeal and total faeces. The leaves inhibited diarrhea by 36.12, 49.47 and 60.85% and reduction of gastrointestinal tract by 38.78, 60.99 and 64.15%. The study reported that the bark and the roots of P. reticulatum inhibited diarrhea by 53.5 and 72.1% and a reduced gastrointestinal tract by 9.38 and 21.74% respectively, suggesting that the roots of *P. reticulatum* may contain high concentration of antidiarrheal components compared to the stem bark. Di Carlo *et al*.(2005) reported an inhibition of castor oil-induced entero pooling as shown by the decrease in the volume of intestinal content after treatment of rats with *P. reticulatum*. They concluded that the anti-diarrhoeal action of the ethanol extract of *P. reticulatum* could be mediated in part by a mechanism involving a decrease of gastrointestinal secretion and motility. The phytochemical screening of the stem bark of *P. reticulatum* showed tannins and flavonoids as the major constituents of the extract (Di Carlo *et al*., 2005).

2.5.5 Anti-ulcerogenic Activity

P. reticulatum has been reported to contain tannins, saponins and sterols as phytochemical constituents (Salawu *et al*., 2007). Tannins are known to affect the integrity of mucosa membrane. Tannins being astringent may precipitate micro-proteins in the site of ulcer, thus preventing absorption of toxic substances by forming a protective layer and resisting the mucous layer against the attack of proteolytic enzymes (Bigoniya *et al*., 2006). Saponins protect stomach mucosa from acid by selectively inhibiting prostaglandin F2 (PGF2), which causes vasoconstriction of mucosal blood vessels. According to the presence of these phytochemical components in *P. reticulatum*, may have contributed to its ulcerogenic protective effect by maintaining an efficient gastric mucosa microvascular supply. Their research validated the folkloric use of *P. reticulatum* root in the therapy of gastric ulcer disease, as the extract was

shown to be gastric cytoprotective and anti-secretory effects of the extract (Bigoniya *et al*., 2006).

2.5.6 Antioxidant Activity

Several plants or parts of plants are used for several reasons. Recent studies have shown a relationship between the use or consumption of products rich in polyphenols and prevention of many diseases related to oxidative stress such as cancer, coronary heart disease, inflammation, thrombosis and other (Latte and Kolodziej, 2004). This is linked in part by their ability to inhibit free radicals and thus the oxidation of some compounds such as LDL-cholesterol (Auddy *et al.,* 2003; Aderogba *et al*., 2006). The methanolic extract of *P. reticulatum* demonstrated a high antioxidant activity with good ability of scavenging DPPH free radicals which was correlated with the high quantity of phenolic contents (Karou *et al*., 2005), and the IC_{50} value was lower than quercetin and gallic acid employed as standard agent. The IC_{50} value of aqueous extract of the plant was larger, with low amount of phenolic compounds suggesting that it may be a weak antioxidant. The low total phenolic content of the aqueous extract was attributed to the low solubility of these compounds in water (Oliveira *et al*., 2008), while the high antioxidant effect of the methanolic extract of the plant was attributed to the high amount of phenolic compounds. This could explain the fairly frequent use of *P. reticulatum* bark extracts in Senegalese traditional medicine.

2.6 The Liver

The liver is a large glandular organ located in the abdominal region in the human body that is responsible for an array of functions that help support metabolism, immunity, digestion, detoxification, vitamin storage among other functions. It comprises around 2% of an adult's

body weight. The liver is a unique organ due to its dual blood supply from the portal vein (approximately 75%) and the hepatic artery (approximately 25%) (Figure 2.4) (Hoekstra et al., 2013).

It is lined by endothelial cells that contain the kupffer cells, which are typical macrophages (Si-Tayeb *et al.,* 2010). The space of Disse also contains Kupffer cells (macrophages) and Ito cells (stellate cells). The Kupffer cells sit in the space to filter out unnecessary or pathologic material from the circulation. The Ito cells serve as storage for fat, and vitamins. In the right setting, they can also serve as myofibroblasts and aid in the regeneration of the liver cells (Si-Tayeb *et al.,* 2010).

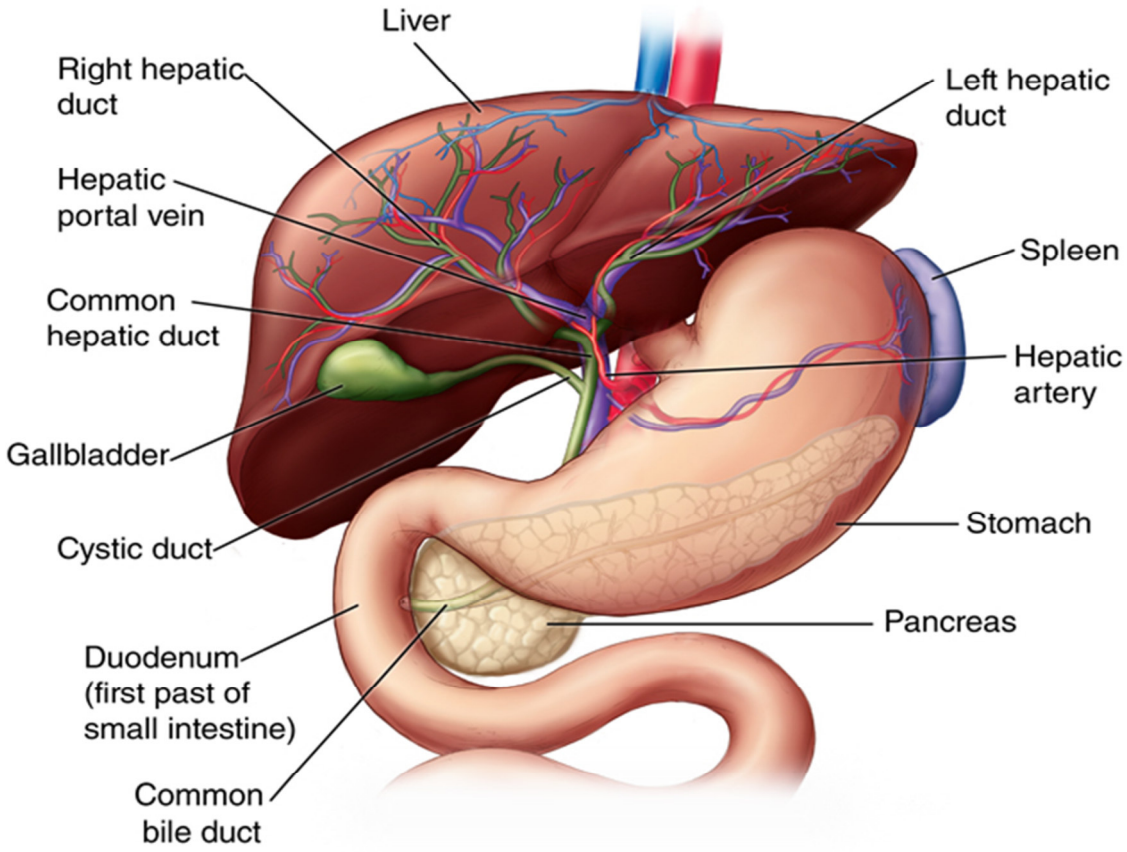

Figure 2.4: Diagram showing the human liver

It is involved in metabolism and detoxification of most substances through biotransformation, to transform xenobiotics mainly by converting them from a lipophilic form to a hydrophilic form through 2 reactions: phase I and phase II. These reactions mainly take place in the smooth endoplasmic reticulum of hepatocytes. Phase I reactions create a more hydrophilic solute via oxidation, reduction, and hydrolysis using primarily the cytochrome P450 (CYP450) family of enzymes. The product of phase I has an oxygen species that reacts better with enzymes involved with phase II reactions. Phase II reactions conjugate the metabolites created in phase I to make them more hydrophilic for secretion into blood or bile (Su-W *et al.,*2019), factors such as age, gender, drug-drug interactions, diabetes, pregnancy, liver or kidney disease, inflammation, or genes, etc, affect drug metabolism (Almazroo *et al.,* 2017).

In humans, the majority of drugs administered are eliminated by a combination of hepatic metabolism and renal excretion (Almazroo *et al.,* 2017). Since the liver is susceptible to injuries particularly in situations of toxicity, it would therefore be worth while to examine the effects of *Piliostigma reticulata* methanolic leaves extract on the liver of rats.

2.7 The Kidneys

Human kidneys have a specific range of thickness, length and breadth. The thickness varies from approximately 2.5 cm to 3.0 cm; the width varies from 5.0 cm to 7.5 cm and the length varies from 11 cm to 12 cm. The kidney is surrounded by a tough fibrous capsule, in normal condition this cover can be easily removable (Standring, 2006), (Figure 2.5). The two kidneys constitute one of the basic urine excretory systems inside human body which can help in excretion of nitrogenous waste of basic protein metabolism from the blood. By removing all

these waste materials, excess nutrients and harmful stuffs from our body, kidneys help to maintain the electrolyte and water balance of the body (Blaine et al., 2014).

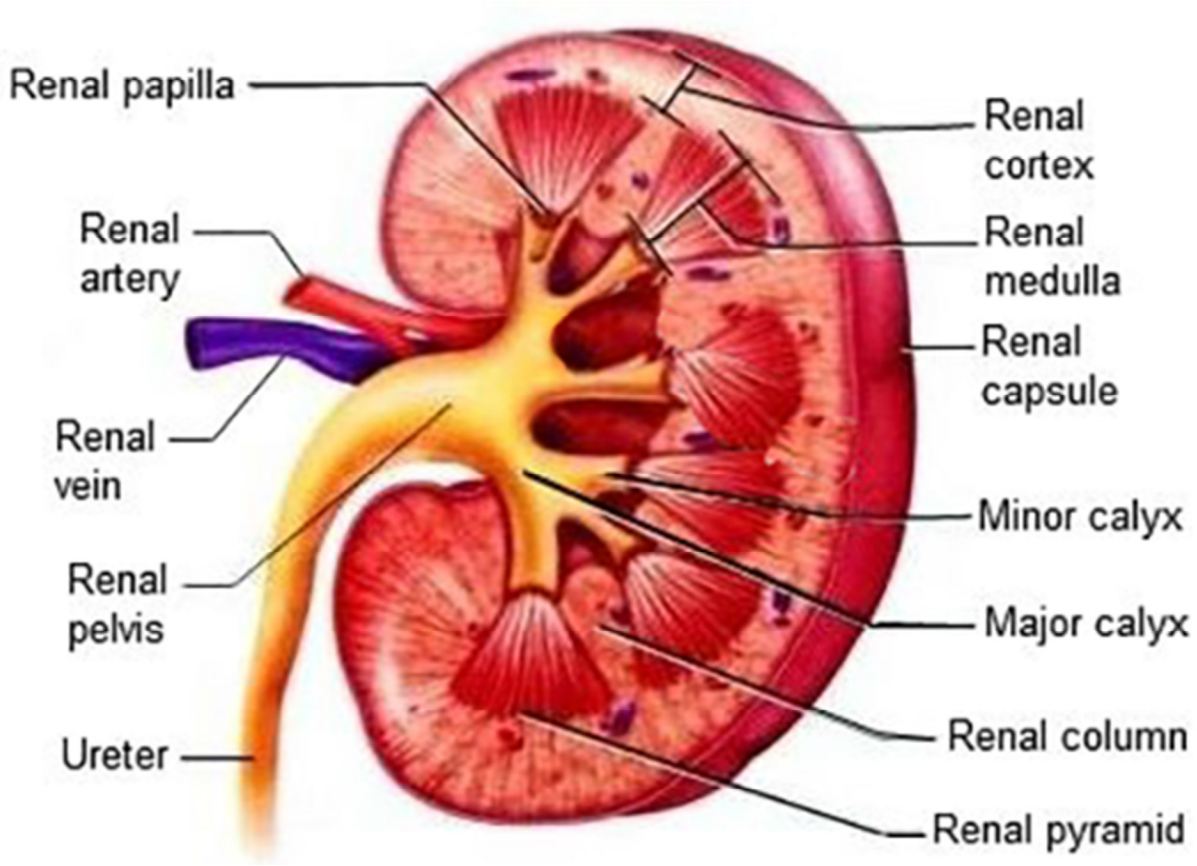

Figure 2.5: Diagram showing the human Kidney

Inside the kidney, filtration and reabsorption of blood is done. During the filtration blood enters by means of the afferent arteriole and directed to flows towards the glomerulus of kidney. Inside the glomerulus both the filterable and non-filterable blood is present. The filterable blood contain some components like nutrients, water, salts such as ions and nitrogenous wastes, these were taken in plasma form called glomerular filtrate, while the non filterable components of blood which include elements such as plasma proteins, blood cells and platelets, are by passed by the process of filtration and through the way of efferent arteriole, which are exited from the

glomerulus (Pollak *et al.,* 2014). The reabsorption occurred when the filtrate passes through the tubules of the nephron which is known as the basic functional unit of the kidney (Arkill *et al.,* 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials Used

P. reticulata leaves, methanol, 500ml beaker, test tube, test tube rag, measuring cylinder (1000mls), water bath, distill water, pen, Notebook, masking tape, pestle and mortar, rotary evaporator, 2ml syringe, weighing balance, battery cages, plain and EDTA containers, pipette, spectrophotometer, micropipette, razor blade, Refrigerator, Hand gloves, Stop watch and sterile bottles

3.2 Collection of Plant Material

Fresh leaves of *P. reticulata* were collected from New Campus of Bayero University, Kano, Nigeria in September, 2019. It was taxonomically identified and authenticated at the Department of Biological Sciences, where a voucher specimen Number BUKHAN 72 was deposited by Dr Yusuf Nuhu. The fresh leaves were thoroughly washed and shade dried in the laboratory for 7 days, pulverized with a pestle and mortar, and kept in air tight containers.

3.3 Experimental Animals

Thirty six (36) apparently healthy *albino* rats of both sex weighing between 80-180 g were used for this study. The rats were obtained from the Animal House of the Department of Biological sciences, Bayero University, Kano, Nigeria. They were maintained under standard laboratory conditions with free access to water and standard rodent feed *ad libitum* (Growers mash, and Vita feed, Nigeria). They were kept to acclimatize for two weeks, before commencement of the experiment in accordance with the principles of laboratory animal care (NIH, 1985).

3.2.0 METHODS

3.2.1 Preparation of Extract

Three hundred grams (300g) of the powdered leaves was macerated in 1.5L methanol in a flask, the content of the flask was shaken and the top covered with aluminium foil and kept at room temperature (25^{o}C) for 72 hours. The extract was then obtained by filtration using Whatman No.1 filter paper and concentrated using rotary evaporator.

Administration of Extract

A 60mg/ml stock concentration of methanol extract was prepared, and dose of extract administered to each rat was calculated thus:

$$\text{Volume to be administered (ml)} = \frac{\text{Weight of rats (kg) X Dose (mg/kg)}}{\text{Concentration of extract 60mg/ml}}$$

3.2.2 Preliminary Phytochemical Screening

Phytochemical screening of *P. reticulata* methanolic leaves extract was carried out using standard methods :

Test for alkaloids

A 0.5g of the extract was dissolved in 5ml of 1% aqueous hydrochloric acid on a water bath and filtered. The filtrate was divided into three. To the first portion few drops of freshly prepared Dragendorff reagent was added and observed for formation of orange to brownish precipitate. To the second portion 1 drop of Mayer reagent was added and observed for formation of white to yellowish or cream colour precipitate. To the third portion 1 drop of Wagner reagent was added

to give a brown or reddish or reddish- brown precipitate. The presence of precipitate in most or all of the above reagents indicates the presence of alkaloids (Evans *et al* 1989,Silva *et al*., 1998).

Tests for Anthraquinones

Exactly 0.5g of the extract was shaken with 10 ml of benzene for 5minutes, the content was filtered and 5ml of 10% ammonia solution was added to the filtrate, the mixture was shaken. Presence of a pink, red or violet colour in the ammonia layer (lower phase) indicates the presence of free anthraquinones.

Test for Carbohydrates/Reducing Sugar

Exactly 5ml of a mixture (1:1) of Fehling solution A and Fehling solution B was added to 2ml of the extract dissolved in water and the mixture boiled on water bath for 5 minutes. A brick – red precipitate indicates the presence of reducing sugars (Trease and Evans, 1996).

Tests for flavonoids

Exactly 5ml of dilute Ammonia solution was added to a portion of the filtrate (obtained using filter paper) of the plant extract followed by addition of concentrated H_2S0_4. A yellow coloration observed indicated the presence of flavonoids. The yellow coloration disappeared on standing (Sofowara, 1993).

Test for Glycosides

Five 5ml of extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated Sulphoric acid, a brown ring was formed at the interface, indicating a deoxysugar characteristic of cardenolides, while in the

acetic acid layer, a greenish ring was formed gradually throughout the thin layer (Sofowara, 1993).

Test for Saponins (Frothing test).

About 0.5g of the extract was shaken with 5ml of water in a test tube for 30 seconds. A persistent froth for 15 minutes on water bath indicated the presence of saponins (Sofowora, 1993, Silva *et al*., 1998).

Tests for Steroids and Terpenoids

To five 5ml of the plant extract, 2ml chloroform and 3ml concentrated H_2S0_4 was carefully added and a layer was formed. The formation of a reddish brown coloration at the interface indicated positive result for the presence of steroids and terpenoids (Sofowara, 1993).

Test for Tannins

Exactly 0.5g of the dried powder of the plant extract was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green coloration (Earl and Waren, 1962).

Test for Coumarins

Exactly 3 ml of 10% NaOH was added to 2 ml of methanolic extract. The formation of yellow colour indicated the presence of coumarins.

3.4 Quantitative Determination of Phytochemicals

Quantitative determination of Phytochemicals was carried out using standard methods of El-Olemy *et al* (1994), as shown below:

Determination of Alkaloids

Alkaloid content was determined by the method of Harborne (1973). Exactly 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hrs. This was filtered and the extract concentrated on a water bath, to one-quarter of the original volume, concentrated Ammonium hydroxide was added drop wise to the extract until the precipitation was completed. The whole solution was allowed to settle and the precipitate collected, washed with dilute Ammonium hydroxide, and filtered. The residue which is the alkaloid was dried and weighed (Harborne (1973).

Determination of Flavonoids

Principle:

The method depends on the UV absorption of free flavonoids. Most flavonoids show maximum absorbance at 370nm after the hydrolysis of their glycosides. At that wave length, most other extractives do not interfere in the essay (Chang *et al*., 2002).

Procedure: The extract 5ml was transferred into a conical flask and then hydrolysed by heating on a water bath with 10% H_2S0_4 10ml, the original volume was reduced to half after heating and the mixture was cooled on ice for 15 minutes where the flavonoids were precipitated. The cooled solution was then filtered and the residue dissolved by adding warm 95% ethanol 50ml and made

up to 100ml with 95% ethanol. Aliquot 5ml was pipette into 25ml volumetric flasks and diluted to volume with 50% ethanol. The absorbance of the resulting solution was measured at 370nm against 50% ethanol as blank.

Determination of Total Phenols

The fat free sample was boiled with 50 ml of ether for 15 minutes to extract the phenolic component. About 5 ml of the extract was then pipetted into a 50 ml flask, and 10 ml of distilled water. 2 ml of Ammonium hydroxide solution and 5 ml of concentrated amyl alcohol added. The samples left to react for 30 min for colour development. Absorbance was measured at 505 nm (MacDonald *et al*., 2001).

Determination of Tannins.

Principle:

The method is based on quantitative consumption of tannins and pseudo tannins by iodine in alkaline medium, a character which is attributed to their phenolic nature (both true and pseudo tannins). True tannins, in contrast to pseudo-tannins can be removed from the extract precipitation with gelatin, permitting the determination of each group of constituents alone. Excess iodine was determined by titration, after being rendered acidic with Sodium Thiosulphate standard solution (Peri and Pompei 1971).

Procedure:

5ml Plant extract was transferred to a stopped conical flask and 0.1N iodine 25ml and 4% NaOH added. This was mixed and kept in the dark for 15 minutes. The mixture was diluted with water

and acidified with 4% H_2SO_4 10ml, before titrating with 0.IN Sodium Thiosulphate solution using starch solution as indicator. The volume of 0.1N iodine used corresponds to the sum of tannins and pseudo tannins. Another 25ml of the extract was mixed with gelatin solution (15ml made up to 100ml in measuring flask and filtered), and 20ml of the mixture mixed with 0.1N iodine (25ml) and 4% NaOH (10ml) and kept in the dark for one minute. The mixture was then diluted with water (10ml) and titrated with 0.1N Sodium Thiosulphate used as indicator. The volume of 0.1N iodine used corresponds only to pseudo tannins content (B). Additionally, a blank experiment was carried out using distilled water. 1ml of N10 $N_{A2}S_203$= lcm^3 of N/10 1_2 solution = 0.0290g of tannin.

$$\% \text{ of total tannins} = \frac{(\text{Blank} - \text{Exp A}) \times 0.029 \times 100}{5(\text{Volume taken})}$$

$$\% \text{ pesudotannins} = \frac{(\text{Blank} - \text{Exp B}) \times 0.029 \times 100}{5(\text{Volume taken}}$$

% True tannin = (A-B) = g% W/V

Determination of Saponins.

Principles:

The method of isolation of saponins depends on the fact that saponins are soluble in water or boiling dilute alcohol and are precipitated upon cooling by adding acetone to their hydro alcoholic solution (Obadoni and Ochuko 2001).

Procedure:

The plant extract (50ml) was placed in a 500ml flask and 50% alcohol (300ml) was added, boiled under reflux for 30 minutes and filtered while hot through a coarse filter paper.

Charcoal (2g) was added to the filtrate, boiled and filtered again while hot. The filtrate was cooled and an equal volume of acetone was added to completely precipitate the saponins. The precipitated saponins were collected by decantation and dissolved in small amount of boiling 95% alcohol and filtered while hot. The filtrate was cooled to room temperature to separate the saponins in the relatively pure form. The clean supernatant fluid was decanted and the saponins suspended in alcohol (20ml) and filtered. The filter paper was then transferred into a desiccator containing anhydrous Calcium chloride, left to dry and weighed.

Determination of Steroids.

1ml of test extract was transferred into 10ml volumetric flasks. Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by Potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70±20^{o}C for 30 minutes with occasional shaking and diluted with distilled water. The absorbance was measured at 780 nm against the reagent blank (Jayaraman, 1980).

Total Carbohydrate Determination.

For estimating the polysaccharide content, 1ml of extract solution was added to 1ml of 5% phenol, before adding 5ml of concentrated Sulphuric acid, mixed and left for 10 minutes. The absorbance was measured at 488nm against blank, then compared with standard solution of

glucose. Blank was prepared by adding 1ml of distilled water to 1ml of 5% phenol followed by 5ml of concentrated Sulphuric acid (Hedge and Hofreiter, 1962).

Determinationof Cardiac Glycosides.

Principles:

Cardiac glycosides develop an orange-red color with Baljet's reagent (picric acid in alkaline medium). The intensity of the colour produced is proportional to the concentration of the glycosides at 495nm (Geetha and Geetha.,2014).

Procedure:

Plant extract (8ml) was transferred to a 100ml volumetric flask and H_2O (60ml) and 12.5% lead acetate (8ml) was added, mixed then filtered. The filtrate (50ml) was transferred into another 100ml flask and 4.7% Na_2HPO_4 (8ml) was added to precipitate excess Pb^{++} ions. This was mixed and made up to volume with water. The mixture was filtered twice through some filter paper to remove excess lead phosphate. Purified filtrate (10ml) was transferred into clean Erlyn-Meyer flask and Baljet reagents (10ml) was added. A blank titration was carried out using distilled water (10ml) and Baljet reagent (10ml). ,which were allowed to stand for one hour for complete colour development. The colour intensity was measured colometrically at 495nm.

$$\% \text{ of total glycosides} = \frac{\text{A x 100g\%}}{17}$$

Determination of Lethal Dose (LD_{50})

The lethal dose of the leaves extract of *p. reticulatum* was determined using the Lorkes method (1983). In the first phase, nine rats were divided randomly into three groups of three rats each.

They were orally given 10,100 and 1000 mg/kg body weight of the extract, respectively, and observed for 24 hours for signs of toxicity and mortality. An absence of mortality in first phase led to the choice of higher doses 1600,2900 and 5000 mg/kg, at second phase and also observed for possible signs of delayed toxicity.

3.5 Sub-chronic Toxicity Study

The study of subchronic toxicity was conducted according to the OECD 407 guidelines. Twenty-four rats were divided into four groups of six rats each, comprising of three test groups and one control group. Three doses: 200, 300 and 600 mg / kg body weight were selected which are slight modification of the work of Dosso *et al*. (2012).The doses were administered to groups 1, 2 and 3 respectively, while control group were given distilled water only. The rats were individually marked and dosed orally and treated for 2 and 4 weeks to check for toxic effects on the liver and kidney function indices.

Experimental Design:

The aspect of this research was divided into two phases and involved a total of twenty-four (24) *albino* rats.

Phase One; This phase focused on the effect of the extract in rats for a period of 2 weeks. The rats were weighed and grouped randomly into three groups (1-3) of animals (n=3) each.

Control Group: Comprising six (6) rats. The rats, were given normal feeds and distilled water only.

- **Group One**; Rats in this group were administered 200mg/kg body weight of extract.

- **Group Two**; Rats in this group were administered 300mg/kg body weight of extract.
- **Group Three**; Rats in this group were administered 600mg/kg body weight of extract.

Phase two; This phase focused on the effect of the extract in rats for a period of 4 weeks. The rats were weighed and grouped randomly into three groups (1-3) of animals (n=3) each.

- **Group One**; Rats in this group were administered 200mg/kg body weight of extract.
- **Group Two**; Rats in this group were administered 300mg/kg body weight of extract.
- **Group Three**; Rats in this group were administered 600mg/kg body weight of extract.

3.6 Sample Collection

On the last day of the experiment, rats in all groups were deprieved of food from 10 p.m. to 7:00 am in the morning, but allowed free access to water (OECD, 2008), and blood collected in a plain collection tubes. This was centrifuged at 3000 revolutions / minute for 5 minutes to obtained the serum used for the determination of biochemical parameters.

3.7 Liver Function Indices

3.7.1 Determination of Serum Alkaline phosphatase (ALP) activity

Principle

Phenol is released by enzymatic hydrolysis from Phenylphosphate under defined condition of time, temperature and pH and estimated calorimetrically. The incubation of serum with Phenylphosphate buffered at pH 10 for 15 minutes at 37℃, produces the hydrolytic product,

phenol which condenses with 4-aminoantipyrene and then oxidized with alkaline solution to give a yellow colour that is measured colorimetrically at 450nm.

$$\text{P-nitro phenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{Phosphate} + \text{P-nitrophenol}$$

ALP: Alkaline Phosphatase

Procedure:

Into a test tube, 500µL buffer substance (p-nitrophenylphosphate, diethanolamine buffer and $MgCl_2$) and 10µL of the serum sample was pipetted, mixed, and absorbance read after 1, 2 and 3 minutes (Rec 1972).

Calculation:

ALP activity was calculated using formulae:

U/I =2760 x absorbance at 405nm/min

3.7.2 Determination of Serum Aspartate aminotransferase (AST) activity.

Principle:

AST activity was measured by monitoring the concentration of oxaloacetic hydrazine formed with 2,4-dinitrophenylhydrazine colorimetrically at 546nm.

$$\alpha\text{-Ketoglutarate} + \text{L-aspartate} \xrightarrow{\text{GOT}} \text{L-glutamate} + \text{oxaloacetate}$$

GOT: Glutamate Oxaloacetic transaminase

Procedure:

Two test tubes were labelled "reagent blank" (RB) and "test" (T), and then 500µL of R1 buffer (Phosphate buffer, L-aspartate and a-Ketoglutarate) was pipetted followed by addition of 100µl serum into the test tube (T) and 100µL into the reagent blank tube (RB). The test tubes was mixed and incubated for exactly 30 minutes at 37℃. Exactly 500µL of 2(2,4-dinitrophenylhydrazine) was added to each of the test tubes and allowed to stand for 20 minutes at 25℃, followed by incubation with 5.0ml of 0.4N NaOH for 5 minutes. The absorbance of the test was read against sample blank at 546nm (Reitman and Frankel 1957).

Calculation:

Activity of the AST was obtained from the calibration plot Table (Appendix II).

3.7.3 Determination of Serum Alanine Aminotransferase (ALT) activity.

Principle:

Alanine aminotransferase was measured by monitoring the concentration of pyruvate hydrazine formed with 2,4-dinitrophenylhydrazine.

$$\alpha\text{-Ketoglutarate} + \text{L-aspartate} \xrightarrow{\text{GPT}} \text{L-glutamate} + \text{pyruvate}$$

GPT: Glutamate Pyruvate transaminase

Procedure:

Two test tubes were labelled "reagent blank" (RB) and "test" (T), and 500µL of solution RI buffer (Phosphate buffer, L-alanine and α-ketoglutarate) was pipetted followed by addition of

100µL serum into the test tube (T) and 100µL of distilled water into the reagent blank tube(RB). The test tubes were mixed and incubated for 30 minutes at 37℃. About 500µL of solution R2 (2, 4-dinitrophenylhydrazine) was added to each of the test tubes and allowed to stand for 20 minutes at 25℃, followed by incubation with 5.0ml of 0.4N NaOH for 5 minutes. The absorbance of the test was read against sample blank at 546nm (Reitman and Frankel, 1957).

Calculation:

Activity of the ALT was obtained from the calibration plot (Appendix II).

3.7.4. Determination of Serum Total Protein Concentration.

Principle:

In alkaline medium copper reacts with the peptide bonds of proteins to form the characteristic pink to purple biuret complex. Sodium Potassium tartarate prevents Copper hydroxide precipitation, and potassium iodide prevents the autoreduction of Copper.

$$\text{Protein} + Cu^{2+} \xrightarrow{\text{Alkaline pH}} \text{Cu – protein complex}$$

The color intensity is directly proportional to the protein concentration. It is determined by measuring the increase in the absorbance at 546nm.

Procedure:

Four test tubes were labelled reagent blank, standard, sample and sample blank. To reagent blank, distilled water (0.02ml) and biuret reagent (1.0ml) were added. To the standard, standard solution (0.02ml) and biuret reagent (1.0ml) were added. To the sample, Serum (0.02ml) and Biuret reagent (1.0ml) were added. The tubes were mixed and incubated for 30 minutes at room

temperature. The absorbance of standard and sample was read against sample blank at 530nm (Tietz, 1995).

Calculation:

$$\text{Total protein concentration} = \frac{Absorbance\ of\ the\ sample\ x\ Standard\ concentration}{\text{Absorbance of standard}}$$

3.7.5 Determination of Serum Albumin concentration.

Principle

The measurement of serum albumin is based on its quantitative binding to the indicator 3,3,5,5'-tetrabromo-cresol sulphonaphthalene (bromocresol green, BCG). The albumin-BCG-complex absorbs maximally at 578mm, the absorbance being proportional to the concentration of albumin in the sample.

Procedure:

Three test tubes were labelled test (T) standard (S) and reagent blank (B). About 3000µl of R1 (Bromocresol green concentrate) was pipetted into all the tubes, followed by 10µl of serum, standard and distilled water to T, S and B, respectively. The tubes were mixed and incubated for 5 minutes at 25℃, reagent blank was used to zero the colorimeter. The absorbance of test and standard were measured against the reagent blank at 578nm (Grant 1987).

Calculation:

$$\text{Albumin concentration} = \frac{Absorbance\ of\ the\ test\ x\ 4.68 g/dl}{\text{Absorbance of standard}}$$

3.7.6 Determination of Serum Total and Direct Bilirubin concentration.

Principle:

This was determined using the colorimetric method as described by Jendrassik and Grof (1938). Direct (conjugated) bilirubin reacts with diazotized Sulphanilic acid in alkaline medium to form a blue coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin-bound bilirubin, by the reaction with diazotized sulphanilic acid.

Procedure:

Total Bilirubin

To two test tubes labelled test (T) and blank (B), 200µL of reagent 1 (Sulphanilic acid) was added, followed by 50µL of reagent 2 (titrate) to test tube labelled T. Reagent 3 (Caffeine, 1000µl) was added to both tubes followed by 200µL of serum to the test tube labelled T. The tubes were mixed and incubated for 10mins. at 25°C. Finally, 1000µL of reagent 4(titrate) was added to both tubes, mixed and incubated for 10 mins. at 25°C.The absorbance of the sample was measured against the sample blank at 578nm (Sherlock, 1951).

Direct Bilirubin

To two tubes labeled test (T) and blank (B), 200uL of reagent 1 (Sulphanilic acid) was added followed by 50µL of reagent 2 (titrate) to test tube labeled T. About (2000µL) of 0.9% NaC1 was added to both tubes followed by 200uL of serum to the test tube labeled T. The tubes were mixed and incubated for 10mins. at 25°C, and absorbance of test was measured against blank at 546nm (Sherlock, 1951).

Calculation:

a. Total bilirubin (µmol/1) = 185 x Absorbance at 578nm

b. Direct bilirubin (µmol/1) = 246 x Absorbance at 578nm.

3.8 Antioxidant enzymes assay: All enzymes were assayed using the Megazymes Diagnostic test kit according to the manufacturer's instructions.(Sigma Chemical CO., USA)

3.8.1 Catalase (CAT) Assay.

Principle:

Catalase activity in a sample is determined by measuring the decrease in H_2O_2 concentration observed following incubation of the analyte sample with an H_2O_2 standard solution. In order to determine catalase activity using the Megazyme Catalase Assay Kit, two separate reactions were completed. In reaction A, the catalase sample of interest is incubated with a known concentration (~ 65 mM in assay) of H_2O_2. The reaction is stopped by the addition of 15 mM sodium azide which strongly inhibits Catalase.

Reaction A:

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

For reaction B, the exact concentration of H_2O_2 remaining is measured using an enzyme-linked colourimetric detection method employing 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DHBS), 4-aminoantipyrine (AAP) and peroxidase. The resulting quinoneimine dye is measured at 520 nm. A 'blank' reaction is performed in parallel to provide the colourimetric response for the initial H_2O_2 concentration. The rate of H_2O_2 decomposition (i.e. Catalase activity) can then

be determined.

Reaction B:

$2\ H_2O_2 + DHBS + AAP \xrightarrow{Peroxidase} Quinoneimine\ dye + 4H_2O$

Procedure:

To a cleaned cuvette, 30µL of CAT working reagent was added, and then 35µL of the serum sample was added to the cuvette and mixed for 5 seconds, covered with plate and incubated for 5minutes at room temperature on a shaker. First absorbance was then measured at 240nm (A_1) immediately after inserting the cuvette in the spectrophotometer and then after 1minute, second absorbance was measured (A_2), (Hadwan 2016).

The change in the absorbance was calculated, thus :

Change in absorbance (ΔA) = (A_1-A_2), and

CAT Activity = µM of Sample /20 min. x Sample dilution = nmol/min/ml.

Note:

When necessary, samples should be diluted with sample buffer to bring the enzymatic activity in place and initiate the reaction by adding 20 µL diluted H_2O_2 to wells been used.

3.8.2 Superoxide Dismutase (SOD) Assay.

Principle:

Xanthine Oxidase generates superoxide in the presence of oxygen, which converts a colourless substrate in the detection reagent into a yellow colored product. The coloured product is read at 450nm. Increasing levels of SOD in a samples causes a decrease in superoxide concentration and a reduction in yellow product. The activity of SOD in the sample is calculated after making suitable dilution correction, using softwares available in most plate readers. The results are expressed in terms of units of SOD activity per mL.

Procedure:

The plate layout sheet on the back page was used to aid in proper sample and calibrator identification. The calibrator were carefully pipetted, introduced into the wells and the sample added slowly down the side of the well by reverse pipetting to avoid bubbles.

10 µL of samples was pippetted into duplicate wells in the plate, and 10 µL of assay buffer into duplicate wells as blank, 50µL of the substrate preparation was then added to each well. Samples with significant yellow coloration were pre-read, 25µL of Xanthine Oxidase Preparation was added to each well, incubated at room temperature for 20 minutes before taking absorbance at 450 nm (Veskoukis et al., 2018).

Calculation:

SOD activity (inhibition rate %) = $(\Delta A_B - \Delta A_T) / \Delta A_B \times 100$

Where

ΔA_B: Change in absorbance of blank

ΔA_T: Change in absorbance of sample (Test)

If the color of the sample solution is strong, measure blank at each dilution of the sample.

3.8.3 Malondialdehyde (MDA) Assay.

Principle:

MDA assay is based on the reaction of MDA with Thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm. Butylated hydroxytoluene (BHT) and EDTA are added to the sample and reaction mixture to minimize oxidation of lipids that contribute artificially during sample processing and the TBA reaction. The temperature of the reaction mixture is reduced to minimize the decomposition of lipid hydroperoxides. Because much of the MDA is protein bound, mostly as a Schiff base, the pH of the reaction is optimized to facilitate hydrolysis of the MDA (Gerard-Monnier, 1997). Additionally, the reaction mixture is subjected to derivative spectrophotometric analysis that resolves the problem of the variable and nonlinear baseline observed, and the absorbance was measured at 532 nm in various biological samples.

Procedure:

Wells for diluted standard, blank and sample were labelled. About 50µL each of dilutions of standard, blank and samples were added into the appropriate wells, before adding 50µL of detection Reagent A to each well immediately. The plate was shaked gently using a microplate shaker, while covered with a plate sealer and Incubated for 1 hour at 37^{o}C. Detection of

Reagent A appear cloudy, this was warmed to room temperature and mixed gently until solution appears uniformed. The solution was then aspirated and washed with 350µL of 1X Wash solution and left to stand for 1-2 minutes. This was repeated 3 times. After the last wash, any remaining wash buffer was removed by aspirating, inverting the plate and blotted against absorbent paper. 100µL of detection Reagent B working solution was added to each well and incubated for 30 minutes at 37^{o}C after covering it with the Plate sealed. And then, run the microplate reader and measured at 450nm immediately (Zhaoul *et al.*, 2019).

3.9 Kidney Function Indices

Kidney function analysis was carried out on serum to determine urea, creatinine, electrolytes such as chloride, sodium, potassium and bicarbonate using Randox diagnostic and TECO kits according to the manufacturers' instructions.

3.9.1 Determination of Serum Urea Level.

Principle:

Urea in serum is hydrolyzed to Ammonia in the presence of Urease. The Ammonia is then measured photometrically by Berthelot's reaction.

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

NH_3 + hypochlorite + phenol → indophenols (blue compound).

Procedure:

To three test tubes labelled test (T), standard (S) and blank (B), 1000 µL of reagent 1 (Sodium nitroprusside and Urease) was added, followed by 10 µL of serum, standard and distilled water

to T, S and B, respectively. The tubes were mixed and incubated at 37°C for 10 mins., before adding 2500 µL of reagent 2 (diluted phenol 1:6) and reagent 3 (diluted sodium hypo chloride: 22cm^3 in 750cm^3 of distilled water) to all the tubes. They were mixed immediately and incubated at 37^oC for 15 min. The absorbance of the test and standard were measured against the reagent at 546nm (Weatherburn, 1967).

Calculation:

$$\text{Urea concentration} = \frac{\text{Absorbance of the test x 80.35mg/dl}}{\text{Absorbance of standard}}$$

3.9.2 Determination of Serum Creatinine Concentration.

Principle:

Creatinine in alkaline solution reacts with picric acid to form a coloured complex, the amount of which is directly proportional to the creatinine concentration.

Procedure:

To three test tubes labelled test (T), standard (S) and blank (B), 1000µL working reagent (equal volume of picric acid and sodium hydroxide) was added followed by 100µL of serum, standard and distilled water to T,S and B, respectively. The tubes were mixed and incubated for 30 seconds and the absorbance, A_1 of the standard and test were measured. After 2 minutes, absorbance, A_2 of the standard and sample were measured again at 492nm (Bartels and Bohmer, 1972).

Calculation:

$$\text{Creatinine concentration} = \frac{\text{Absorbance of the test x 2.04mg/dl}}{\text{Absorbance of standard}}$$

Electrolytes

Electrolytes are the most commonly requested biochemistry tests. They provide essential information on renal function, principally in excretion and homoeostasis. They include: Sodium (Na^+), potassium (K^+), Chloride (Cl^-) and bicarbonate (HCO_3).

3.9.3 Determination of Serum Sodium Concentration.

Principle:

This method is based on modification of the method described by Maruna and Trinder (1958) in which sodium is precipitated as the triple salt, Sodium Magnesium Uranyl acetate, with the excess Uranium reacting with ferrocyanide to produce a chromophore whose absorbance varies inversely as the concentration of Sodium in the test specimen.

Procedure:

Three test tubes labelled blank (B), test (T) and standard (S) were arranged in a test tube rack and 100ml of the reagent added to the three test tubes. About 10ml of the sample and 10ml of the standard was added to the tube. They were mixed and allowed to stand at room temperature for 5 minutes and absorbance of sample and standard were read against blank at 680nm (Maruna 1958).

Calculation:

$$\text{Sodium concentration (Na)} = \frac{\text{Absorbance of the test}}{\text{Absorbance of standard}} \text{ x concentration of standard}$$

3.9.4 Determination of Serum Potassium Concentration.

Principle:

The amount of potassium is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity of which is proportional to potassium concentration in the range of 2-7 mEq/L.

Procedure:

To three test tubes labelled test (T), standard (S) and blank (B), Potassium reagent (Sodium tetraphenylboron/1000µL) was added, followed by 10µL of serum, standard and distilled water to T, S and B, respectively. The tubes were mixed and incubated for 3 minutes at room temperature. The absorbance of the test and standard were measured against reagent blank at 500nm (Henry, 1974).

Calculation:

$$\text{Potassium concentration } (K^{+}) = \frac{\text{Absorbance of the test x 4mEq/L}}{\text{Absorbance of standard}}$$

3.9.5 Determination of Serum Chloride concentration.

Principle:

Chloride ions form a soluble, non-ionized compound, with Mercuric ions and will displace thiocyanate irons from non-ionized Mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a coloured complex that absorbs light at 480nm. The intensity of the colour produced is directly proportional to the chloride concentration.

$Hg(SCN)_2 + 2C1^- \rightarrow HgCl_2 + 2SCN^-$

$3SCN^- + Fe^3 \rightarrow 4\ Fe(SCN)_3$ red complex

Procedure:

To three test tubes labelled test (T), standard (S) and blank (B), 1500µL of Chloride reagent (Mercuric nitrate, Mercuric thiocyanate, Mercuric chloride and ferric nitrate) was added. This was followed by addition of 10µL serum, chloride calibrator (sodium chloride) and distilled water to the test tubes labelled T, S and B, respectively. The tubes were mixed and incubated for 5 minutes at room temperature. The absorbance of the test and standard were measured against the reagent blank at 480nm (White, 1970).

Calculation:

$$\text{Chloride concentration } (Cl^-) = \frac{\text{Absorbance of the test x 100mEq/L}}{\text{Absorbance of standard}}$$

3.9.6 Determination of Serum Bicarbonate Concentration.

Principle:

Phosphoenol Pyruvate Carboxylase (PEPC) catalyzes the reaction between Phosphoenol Pyruvate and Carbon dioxide (bicarbonate) to form Oxaloacetate and Phosphate ion. Oxaloacetate is reduced to Malate with simultaneous oxidation of an equimolar amount of reduced nicotinamide adenine dinucleotide (NADH) to NAD; the reaction is catalyzed by Malate dehydrogenase (MDH). This results in a decrease in absorbance at 340nm that is directly proportional to CO_2 concentration in the sample.

$$\text{Phosphoenol Pyruvate} + HCO_3^- \xrightarrow{\text{PEPC}} \text{Oxaloacetate} + H_2PO_4$$

$$\text{Oxalate} + \text{NADH} \longrightarrow \text{Malate} + \text{NAD}$$

PEPC:Phosphoenol Pyruvate Carboxylase., MDH: Malate dehydrogenase

Procedure:

To the three test tubes labelled T (test), S (standard) and B (blank), 1000µL of Carbon dioxide reagent, PEP, Magnesium sulphate, NADH, MDH (porcine), PEPC(microbial), Sodium oxalate, buffer, PH 7.0, non-reactive filler and stabilizers with sodium azide, 5µL of serum, standard (Sodium bicarbonate in an aqueous solution) and distilled water were added. The tubes were mixed and incubated for 5 minutes at room temperature. The absorbances of the test and standard were measured against the reagent blank at 340nm (Forrester *et al.*, 1976).

Calculation:

Bicarbonate concentration (HCO_3^-) = $\frac{\text{Absorbance of the test x 30Mmol/l}}{\text{Absorbance of standard}}$

3.10 STATISTICAL ANALYSIS

All data were analyzed using SPSS 20. Data are represented as mean±SD of three different experiments. Comparison between the groups were performed using one way analysis of variance (ANOVA) followed by GraphPad multiple comparison. Values with $p < 0.05$ were considered statistically significant.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Phytochemical screening of *Piliostigma reticulata* methanolic leaf extract

The result of the present study on the qualitative phytochemical screening of *P. reticulata* revealed that the methanolic leave extract of the plant contains seven (7) out of the nine phytochemicals tested: alkaloids, tannins, terpenoids, flavonoid, phenols, saponins and glycosides were present while coumarins and phlobatannis were absent. Similarly, the result of the quantitative phytochemical revealed that the extract has highest concentration of phenols followed by alkaloids, tannins, terpenoid, flavanoids, saponins and the least phytochemicals were glycosides (Table 4.1)

Table 4.1: Phytochemical analysis of *Piliostigma reticulata* methanolic leaf extract

Phytochemicals	Qualitative	Concentration (mg/100gm)
Alkaloids	+	1.43±0.47
Phlobatanins	-	ND
Flavonoids	+	0.71±0.23
Tannins	+	1.35±0.07
Saponins	+	0.23±0.13
Terpenoids	+	1.23±0.181
Glycosides	+	0.18±0.014
Phenols	+	2.96±0.521
Coumarins		ND

Keys: – absent, + present, ND: Not detected.

4.2 Acute Oral Toxicity Study of *Piliostigma reticulata* methanolic leaf extract

The results of the acute oral toxicity study is shown in (Table 4.2). The results revealed that there was no change in behaviour, body weight and no mortality after the rats in groups 1, 2 and 3 were orally administered *Piliostigma reticulata* methanolic leaf extract at concentrations of 10, 100 and 1000 mg/kg body weight respectively. Similarly, there was no recorded mortality in phase II of the experiment where rats in groups 1, 2 and 3 were given the extract at 1600, 2900 and 5000 mg/kg body weight.

Table 4.2: Acute Oral Toxicity (LD_{50}) of *Piliostigma reticulata* methanolic leaf extract

Groups	No. of Rats	Dose (mg/kg)	Mortality ratio
Phase I			
1	3	10	$0/3$
2	3	100	$0/3$
3	3	1000	$0/3$
Phase II			
1	1	1600	$0/1$
2	1	2900	$0/1$
3	1	5000	$0/1$

4.3 Sub-chronic Toxicity Study of *Piliostigma reticulata* methanolic leaf Extract

Table 4.3 shows the effect of oral administration of *Piliostigma reticulata* methanolic leaf extract on kidney function indices. From the result, administration of 200, 300 and 600mg/kg methanolic leaf extracts of *Piliostigma reticulata* for two weeks did not significantly ($p>0.05$) affect serum concentrations of Na^+, K^+, HCO_3 and Creatinine when compared to the control group.

However, for rats administered with 200, 300 and 600mg/kg methanolic leaf extracts of *Piliostigma reticulata* for four weeks, there was significant increase ($p<0.05$) in serum concentrations of K^+, and Urea when compared to the control group.

In addition, there were no significant differences ($p>0.05$) in HCO_3 and Cl^- levels of rats administered with 200, 300 and 600mg/kg of extracts for four weeks when compared to the control group.

Table 4.3: Effect of oral administration of *Piliostigma reticulata* methanolic leaf extract on kidney function indices

Parameters	Na^+ (mmol/L)	K^+ (mmol/L)	Creatinine (mg/dL)	HCO_3^- (mmol/L)	Cl^- (mmol/L)	Urea (mmol/L)
Control	137.00 ±3.22[a]	4.28 ±0.35[a]	4.70 ±2.27[a]	23.97 ±0.98[a]	74.07 ±2.62[a]	5.83 ±0.07[a]
Group I (200mg/kg PRE + 2wks)	137.36 ±2.81[a]	4.51 ±0.16[a]	4.84 ±0.07[a]	23.90 ±0.18[a]	76.48 ±3.81[a]	5.98 ±0.22[a]
Group II (300 mg/kg PRE + 2wks)	136.59 ±4.99[a]	4.40 ±0.55[a]	4.81 ±0.18[a]	24.31 ±1.38[a]	78.38 ±4.51[a]	6.02 ±0.22[ac]
Group III (600 mg/kg PRE + 2wks)	137.64 ±1.18[a]	4.65 ±0.15[a]	4.85 ±0.08[a]	23.78 ±0.21[a]	78.94 ±1.43[a]	6.24 ±0.16[a]
Group IV (200 mg/kg PRE + 4wks)	141.80 ±0.57[a]	4.69 ±0.16[a]	5.01 ±0.10[a]	25.72 ±0.86[a]	79.30 ±0.49[a]	6.38 ±0.28[a]
Group V (300 mg/kg PRE + 4wks)	139.09 ±2.31[a]	5.14 ±0.11[b]	4.98 ±0.13[a]	25.43 ±0.46[a]	83.29 ±7.98[a]	6.76 ±0.32[b]
Group VI (600 mg/kg PRE + 4wks)	142.71 ±2.79[a]	5.21 ±0.19[b]	5.15 ±0.10[a]	25.90 ±0.58[a]	82.38 ±2.48[a]	6.88 ±0.62[bc]

Results are expressed as Mean±SD, n=3. Values in the same column with different superscript letters are significantly different at ($p < 0.05$) when compared to control.

PRE: *Piliostigma reticulata* extract; Sodium: Na^+, Potassium: K, Bicarbonate: HCO_3^-, Chloride: Cl^-.

Table 4.4 shows the effect of oral administration of *Piliostigma reticulata* methanolic leaf extract on liver enzyme function, albumin, total protein, total bilirubin and conjugated bilirubin. From the results, administration of 200, 300 and 600mg/kg methanolic leaf extracts of *Piliostigma reticulata* for two weeks have mean serum activities of AST, ALT, ALP and concentration of albumin, total protein, and conjugated (direct) bilirubin, not significantly different ($p>0.05$) from the control group, while total bilirubin of rats given 200mg/kg of the extract was significantly ($p>0.05$) lower than control and all other groups (Table 4.4).

The same was observed in rats administered 200, 300 and 600mg/kg of the extract for four weeks, as there were no significant differences ($p>0.05$) in mean serum activities of AST, ALT, ALP and concentrations of albumin, total protein and conjugated (direct) bilirubin compared to control, while total bilirubin level increased significantly ($p<0.05$) compared to control group.

Table 4.4: Effect of oral administration of *Piliostigma reticulata* methanolic leaf extract on Liver function indices

Parameter	AST(IU/L)	ALT(IU/L)	ALP (IU/L)	Albumin (g/dl)	Total Protein (g/dl)	Total Bilirubin (µmol/L)	Conj. Bilirubin (µmol/L)
Control	23.17 ±0.34[a]	20.37 ±0.96[a]	64.79 ±1.18[a]	34.73 ±0.86[a]	7.41 ±0.40[a]	11.59 ±0.17[a]	8.91 ±0.13[a]
Group I (200 mg/kg PRE + 2wks)	22.95 ±0.60[a]	21.21 ±0.50[a]	65.64 ±4.05[a]	34.57 ±0.79[a]	7.47 ±0.46[a]	11.47 ±0.30[a]	8.83 ±0.23[a]
Group II (300 mg/kg PRE + 2wks)	23.01 ±0.94[a]	20.83 ±1.21[a]	64.57 ±3.85[a]	35.08 ±2.50[a]	7.35 ±0.10[a]	11.51 ±0.47[a]	8.85 ±0.36[a]
Group III (600 mg/kg PRE + 2wks)	23.39 ±0.54[a]	20.11 ±1.54[a]	63.44 ±6.57[a]	34.10 ±0.21[a]	7.10 ±0.47[a]	11.70 ±0.27[a]	9.00 ±0.21[a]
Group IV (200 mg/kg PRE + 4wks)	24.21 ±0.31[a]	22.14 ±2.10[a]	67.87 ±3.79[a]	35.43 ±0.84[a]	7.43 ±0.50[a]	13.08 ±0.83[b]	9.78 ±0.29[a]
Group V (300 mg/kg PRE + 4wks)	24.51 ±0.52[a]	22.24 ±1.41[a]	72.55 ±1.51[a]	36.08 ±0.31[a]	7.44 ±0.52[a]	13.61 ±0.78[b]	10.47 ±0.60[b]
Group VI (600 mg/kg PRE + 4wks)	24.46 ±0.49[a]	22.57 ±3.13[a]	73.01 ±1.51[a]	36.38 ±0.72[a]	7.51 ±0.44[a]	13.16 ±0.33[b]	10.12 ±0.25[b]

Results are expressed as Mean±SD, n=3. Values in the same column with different superscript letters are significantly different at ($p < 0.05$) when compared to control.

PRE: *Piliostigma reticulata* extract; Alanine aminotransferase (ALT); Alkaline phosphatase (ALP); Aspartate aminotransferase (AST).

Table 4.5 shows results of the antioxidant activities of *Piliostigma reticulata* methanolic leaf extract on malondialdehyde (MDA), Catalase (CAT) and Superoxide dismutase (SOD), after oral administration of *Piliostigma reticulata* methanolic leaf extract. From the result, there were no significant differences ($p>0.05$) in MDA, CAT and SOD of rats given 200g, 300, and 600mg/kg methanolic leaf extracts of *Piliostigma reticulata* for two weeks compared to the control group.

Also, when rats where given the extract at 200, 300 and 600mg/kg for four weeks, no significant difference ($p>0.05$) in MDA concentration was observed, but there was significant increase in serum concentration of SOD and CAT compared to the control group. The increase in SOD and CAT was found to be time and dose dependent.

Table 4.5: Effect of *Piliostigma reticulata* methanolic leaf extract on lipid peroxidation and some antioxidants enzymes

Groups	MDA (nmol/L)	CAT (U/mL)	SOD(U/mL)
Control	3.76±0.48[a]	8.06±0.18[a]	7.36±0.22[a]
200mg/kg PRE +2wks	4.07±0.19[a]	8.14±0.36[a]	7.28±0.19[a]
300mg/kg PRE+2wks	3.88±0.29[a]	8.14±0.17[a]	7.22±0.30[a]
600mg/kg PRE+2wks	4.14±0.14[a]	8.16±0.33[a]	7.23±0.24[a]
200mg/kg PRE +4wks	4.13±0.20[a]	8.70±0.79[a]	8.22±0.19[b]
300mg/kg PRE +4wks	4.34±0.12[a]	9.72±0.08[b]	8.80±0.47[b]
600mg/kg PRE +4wks	4.53±0.40[a]	9.87±0.55[ab]	8.81±0.39[b]

Results are expressed as Mean±SD, n=3. Values in the same column with different superscript letters are significantly different at ($p < 0.05$) when compared to control.
PRE: *Piliostigma reticulata* extract; SOD: Superoxide dismutase; CAT: Catalase; MDA: Malondialdehyde.

4.2 DISCUSSION

This research was carried out to evaluate the phytochemical and toxicological effects of methanolic leaf extract of *Piliostigma reticulata* in albino rats.

The qualitative phytochemical screening of the methanolic leaf extract of *Piliostigma reticulata* revealed the presence of seven (7) phytochemicals out of the nine (9) tested: alkaloids tannins, phenols, saponins, cardiac glycosides, flavonoids and terpenoids were present while phlabotannis and coumarins were absent. Quantitative investigation of the phytochemical showed high concentration of phenols, followed by alkaloids, tannins, terpenoid, flavanoids, saponins and the least were the cardiac glycosides (Table 4.1). Abdallah *et. al.,* (2016) in a study to determine the biological activities of methanolic leaf extract of *Piliostigma reticulata* found that the methanolic leaf extract of the plant contains bioactive compounds such as saponins, tannins, alkaloids, phenolic compounds and terpenoids, while flavonoids and anthraquinones were not detected. This may be attributed to the extraction procedure as various methods of extraction may result in different products (Abdallah *et. al.,* 2016).

The absence of death after oral administration of 5000mg/kg body weight methanolic leaf extract (table 4.2) showed that the leaf extract of *P. reticulata* has no discernible acute toxicity, and the LD_{50} of *P. reticulata* leaf extract is greater than 5000mg/kg. This conforms to the study by Suriyavadhana and Pakutharivu (2011) who evaluated the acute toxicity of ethanol extract of *P.reticulata* and observed no sign of toxicity during the experimental period, no significant variation in the body weights between the control and the treated group was observed after 28 days of treatment, and mortality was not recorded. An LD_{50} greater than 5000mg/kg is an indication that the extract may be safe for human consumption, validating its use by herbalists who believed that *Piliostigma reticulata* leaf extract is not harmful. Signs of toxicity are the most

important indicators of drug or clinical related toxicity or morbidity in all types of toxicity studies.

The results of this study demonstrates that the oral consumption of *Piliostigma reticulata* methanolic extract did not produce any sign of toxicity or death in the rats after 24 hours of administration, at up to 5000 mg/kg.

Alkaloids, tannins and saponins are known to have antimicrobial activity and other physiological activities (Sofowora, 1993). Some alkaloids such as those that inhibit enzymatic activities and affects glucagon, thyroid stimulating hormone, are carcinogenic and toxic to the body system (Cragg and Newman, 2013). The non-toxic alkaloids are those that are used as anagelsic, antispasmodic and bactericidal agents. Saponins can be used as detergents, foaming and emulsifying agents and can help reduce inflammation of upper respiratory passage (Li et al., 2015). Also saponin has the ability to coagulate and precipitate red blood cells (Mahalakshmi *et al*., 2015). According to reports, steroids have antibacterial properties while Glycosides help to lower blood pressure (Mahalakshmi *et al*., 2015). Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many phenolic compounds including flavonoids, tannins and phenolic acid, exhibit a strong antioxidant activity (Rohman *et al*, 2010). Phenolic compounds present in most plants have been shown to possess some biological activities such as anti-aging, anti-carcinogen, anti-inflammation, anti-diabetic, anti-apoptosis, anti-atherosclerosis, cardiovascular protection and improvement of endothelial functions as well as inhibition of angiogenesis and cell proliferation activities (Charles *et al*., 2018). Flavonoids are synthesized by plants in response to microbial infection, and have been shown to be good antimicrobial, anticancer and antioxidant substances, due to their ability to

form complexes with extracellular and soluble proteins, and with bacterial cell wall (Mahalakshmi *et al*., 2015).

These phytochemicals represent major groups of plant constituents that work predominantly as powerful antioxidants or scavenger of free radicals. They play beneficial roles in human health and cure or prevent ailments such as inflammatory disorders, cardiovascular diseases, cancer and diabetes which occur due to the deregulation of free radicals generation in the cells (Chouhan and Singh, 2011). A study by Alhassan *et al.* (2012), attributed the hepatocurative effect of *Calitrophis procera* on CCl_4-induced liver toxicity in rabbits with some phytochemicals saponins and terpenoids, two phytochemicals found in *P. reticulata*. In addition to saponins and terpenoids, *P. reticulata* also contains cardiac glycosides, phenols, flavonoids and tannins. Thus this shows that *P. reticulata* may possess hepatocurative effect providing additional evidence of its inherent pharmacological potentials. Our result also agrees with findings by Dahiru and Obidoa (2008) that *P. reticulata* has curative effect on ethanol induce.

Subchronic toxicity study was performed in order to evaluate the toxic effects of *P. reticulata* on liver and kidney function, thus parameters such as, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, Albumin, total protein, total and conjugate bilirumin, creatinine, chloride, bicarbonate, Potassium, Sodium and Urea were monitored. It is known that the liver and kidneys play significant roles in various metabolic processes. The liver plays an important role in xenobiotic metabolism while the kidneys are the main organs involved in drug elimination, thus both organs are exposed to the toxic effects of exogenous compounds (Bidhe and Ghosh, 2004).

Liver serum enzymes like AST, ALT and ALP are well-known enzymes that serves as indicators of liver function (Rahman *et al*, 2001) and as biomarkers predicting possible toxicity (Mdhluli, 2003). The transaminases, AST and ALT leak into circulation when hepatocytes or their cell membranes are damaged. ALT is a more specific marker of hepatocellular injury because it occurs exclusively in the liver, whereas AST occurs to some extent also in heart, skeletal muscle, kidney, brain, pancreas and blood cells (Kew, 2000; Belguet, 2010). Moreover, serum ALP activity increases in case of damage to the hepatic cells and obstruction of the bile ducts (Akhtar *et al*., 2012; Dahamna, 1987). From our results (Table 4.4), there were no signicant differences ($P>0.05$) in ALT, AST and ALP activities in rats treated with *P. reticulata* compared to control groups, suggesting that the subchronic administration of *Piliostigma reticulata* extract did not alter the hepatocyte integrity.

Other liver function parameters measured such as albumin was not significantly different in treated groups compared to the control group. Albumin is the protein with the highest concentration in plasma. It transports many small molecules in the blood, such as bilirubin, Calcium, progesterone, and drugs. However, because it is produce by the liver, decreased serum albumin may be as result of liver disease. It can also be due to kidney disease, as the kidneys allow albumin to escape into the urine. Decreased albumin may also be explained by malnutrition or a low protein diet (Russo *et al*., 2007). From our results, sub-chronic administration of *P.reticulata* did not compromised the conjugating ability of the liver as conjugated bilirubin level, was not significantly ($p>0.05$) different from control, while total bilirubin was significantly higher ($p<0.05$) in rats treated for 4 weeks. The synthetic ability of the liver was also maintained judging from total protein and albumin concentration.

Also, there were no significant increases in serum Urea and creatinine concentration, in treated rats compared to rats in the control groups. Indeed, Urea and creatinine are considered important markers of kidney function (Mukinda and Eagles, 2010; Gnanamani *et al*., 2008). Creatinine is a nitrogenous end product of metabolism removed from the blood by the kidneys. It is the most commonly used clinical serum biomarkers of renal damage (Hayes, 2008). A rise in creatinine concentration is occurs only if there is marked damage to functional nephrons (Lameire *et al*., 2005). Thus, the results obtained in this study suggest that *Piliostigma reticulata* methanolic leaf extract did not alter renal function.

The liver and kidney are most vulnerable to injury by free radicals resulting in serious health problems (Sreenivasamurthy *et al*., 2012). This is due to their involvement in metabolism, detoxification, storage and excretion of xenobiotics and their metabolites, making them important target organs for xenobiotic induced injuries (Tayeb *et al*., 2010). ROS and RNS deteriorate many biological molecules like fatty acid, lipids, proteins and DNA, and become a major cause of heart diseases, diabetes, cancer, inflammations and weak immune system (Gulcin *et al*., 2007; Jayakumar *et al*., 2009;).The body has a complex defence strategy to minimize the damaging effects of various oxidants, central to this are the non-enzymatic and enzymatic antioxidants. These include reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which acts in concert to protect the organism from oxidative damage (Magdi *et al*., 2011). Thus, in the present work, the effect of the extract on oxidative stress parameters MDA, SOD and CAT in the liver and kidneys was evaluated.

Lipid peroxidation is an auto-catalytic, free-radical mediated, destructive process, which allows polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Linden *et al*., 2008). Reactive oxygen species degrade polyunsaturated lipids, forming

malondialdehyde (He et al., 2018). This compound is a reactive aldehyde, and is one of the many reactive electrophilic species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end-products. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del Rio *et al.* 2005).

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues. It decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals (Young and Woodside,2001). Catalase activity varies greatly from tissue to tissue, with the highest activity found in the liver and kidneys, whereas the lowest activity is seen in connective tissues (Valko et al., 2007). Inhibition of this enzyme may enhance sensitivity to free radical-induced cellular damage. Therefore reduction in the activity of CAT may lead to deleterious effects as a result of superoxide and hydrogen peroxide assimilation (Jonsson et al., 2009).Thus

increased ($p<0.05$) levels of antioxidant enzymes: SOD and Catalase observed after 4 weeks oral administration of *p. reticulata* at higher doses, may stimulate increased antioxidant activity and reduce hepatic lipid peroxidation. Our result, agrees with the findings by Olukiran *et al.* (2014), that saponins (phytochemical also found in *P.reticulata*) from the defatted seeds of *Camellia oleifera* improves antioxidant level *in vivo*, which may be attributed to the presence of Tigloyl group and triterpenes in saponins which help to increase antioxidant ability (Ye *et al.,* 2013).

Also, a study by Khan *et al.* (2015) reported that treatment with *P. reticulata* also showed a significant ($p < 0.01$) improvement of serum antioxidant status in tested animals fed high cholesterol diet. Also, insignificant increase ($p>0.05$) in MDA concentration observed in a dose and time-dependent manner agrees with findings by He *et al.* (2018), which demonstrated that

elevated MDA level in groups of rats feed with Hyper feed diet, when administered with *M. charantia* leave extract reduces hepatic MDA in the rats, similarly, Khodayar *et al.*(2014) demonstrated that using atorvastatin reduces MDA in rats, which is an additional point in support of the extract antioxidant potentials.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

The present study was carried out to evaluate the toxicological effects of methanolic leaf extract of *Piliostigma reticulata*. The extract was administered to the rats at 200, 300 and 600mg/kg body weight for 2 and 4 weeks respectively, and its effects on liver and kidney function as well as antioxidant parameters were investigated *in vivo* using *Albino* rats**.** Initial qualitative phytochemical screening of leaf extract shows the presence of seven (7) out of the nine phytochemicals tested: alkaloids, tannins, terpenoids, flavonoid, phenols, saponins and glycosides while coumarins and phlobatannis were absent. The quantitative analysis revealed that the extract has highest concentration of phenols followed by alkaloids, tannins, terpenoids, flavanoids, saponins and the least were the glycosides (Table 4.1).

The acute toxicity study of *Piliostigma reticulata* extract at 10, 100, 1000, 1600, 2900 and 5000 mg/kg body weight showed no mortality (Table 4.2). The sub-chronic toxicity study of the extract on biochemical parameters of liver function showed that the extract was not toxic to the liver as there were no significant ($p>0.05$) differences on serum levels of AST, ALT, ALP, Albumin, Total protein and conjugated bilirubin when compared to control groups (Table 4.4).. Similarly, treatment with the extract did not significantly ($p>0.05$) affect kidney function, as there were no significant ($p>0.05$) increases in kidney function indices measured except in K^+ and Urea levels of rats treated for 4 weeks compared to control groups (Table 4.3).

Administration of the extract led to a dose and time-dependent increase in antioxidant enzymes (Table 4.5).

5.2 Conclusion

The result of this current study showed that *Piliostigma reticulata* methanolic leaf extract contains alkaloids, tannins, terpenoids, flavonoids, phenols, saponins and glycosides. The toxicity study revealed that methanolic leaf extract from *Piliostigma reticulata* did not cause any mortality or other toxicity signs. Acute toxicity study suggested that methanolic extract of the plant is safe up to the dose of 5000mg/kg body weight when administered orally. Therefore, according to the classification of Hodge and Sterner, the methanolic leaf extract of the plant could be considered practically non-toxic. In sub-chronic toxicity study, the methanolic extract did not affect biochemical parameters as the serum levels of most parameters studied were not different from control rats, while antioxidant enzymes increased. This demonstrates that *Piliostigma reticulata* methanolic leaf extract has no adverse effect on liver and kidney function in rats.

5.3 Recommendations

The following are recommendations made based on the result findings;

1. Further studies aimed at isolation and characterization of the bioactive compounds present in the plant should be conducted.

2. A subsequent study aimed to evaluate the anti-inflammatory, antimicrobial and antidiabetic activity of the different solvent extract should be conducted.

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APPENDICE I

Statistical Analysis

Kidney Function Test (KFT)

One Single Factor ANOVA

Parameters	Variation	Sum of Squares	Df	Mean Square	*F*	*Sig.*
Cl^-	Between Groups	.013	6	.002	1.876	.156
	Within Groups	.016	14	.001		
	Total	.029	20			
Creatinine	Between Groups	.001	6	.000	.089	.996
	Within Groups	.015	14	.001		
	Total	.015	20			
Potassium	Between Groups	.003	6	.001	4.969	.006
	Within Groups	.002	14	.000		
	Total	.005	20			
Sodium	Between Groups	.006	6	.001	2.165	.110
	Within Groups	.006	14	.000		
	Total	.012	20			
Urea	Between Groups	.029	6	.005	4.844	.007
	Within Groups	.014	14	.001		
	Total	.043	20			
Bicarbonate	Between Groups	15.562	6	2.594	4.304	.011
	Within Groups	8.437	14	.603		
	Total	23.999	20			

Liver Function Test (LFT)

One Single Factor ANOVA

Parameters	Variation	Sum of Squares	Df	Mean Square	*F*	*Sig.*
Albumin	Between Groups	12.171	6	2.029	1.577	.226
	Within Groups	18.006	14	1.286		
	Total	30.177	20			
Total Bilirubin	Between Groups	15.728	6	2.621	10.142	.000
	Within Groups	3.619	14	.258		
	Total	19.346	20			
Conjugate Bilirubin	Between Groups	8.489	6	1.415	13.207	.000
	Within Groups	1.500	14	.107		
	Total	9.989	20			
Total Protein	Between Groups	.329	6	.055	.295	.929
	Within Groups	2.600	14	.186		
	Total	2.929	20			
ALP	Between Groups	275.683	6	45.947	3.395	.028
	Within Groups	189.467	14	13.533		
	Total	465.150	20			
AST	Between Groups	8.717	6	1.453	4.519	.009
	Within Groups	4.500	14	.321		
	Total	13.217	20			
ALT	Between Groups	17.077	6	2.846	.940	.497
	Within Groups	42.389	14	3.028		
	Total	59.465	20			

Antioxidants

One Single Factor ANOVA

Parameters	Variation	Sum of Squares	Df	Mean Square	*F*	*Sig.*
SOD	Between Groups	9.934	6	1.656	18.049	.000
	Within Groups	1.284	14	.092		
	Total	11.218	20			
CAT	Between Groups	11.187	6	1.865	10.507	.000
	Within Groups	2.485	14	.177		
	Total	13.672	20			
MDA	Between Groups	1.218	6	.203	2.450	.078
	Within Groups	1.160	14	.083		
	Total	2.379	20			

APPENDIX II

PREPARATION OF REAGENTS

AST assay kit (Randox), which consist of the following solutions were purchased Randox

SOLUTION I: This contain phosphate buffer (100mmol/L, pH 7.4) L-aspartate (100mmol/L) and Alpha-ketoglutarate (20mmol/L).

SOLUTION II: This contain 2,4-dintrophenyl hydrazine (2mmnol/L).

Commercially prepared ALT kit (Randox) which consist of the following solutions

SOLUTION I: this contains phosphate buffer (100mmol/L, pH7.4), L-alanine (100mmol/L) and alpha-ketoglutarate (2mmol/L).

SOLUTION I: containing 2,3dinirophenyl hydrazine (2mmol/L).

ALP Assay kit was purchased from Randox: It contains two reagents;

Reagent 1a: Contain diethanolamine buffer (91mmol/L) pH9.8) and $MgCl_2$ (0.5mmol/L),

Reagent 1b: Contain p-nitrophenyl phosphate (910mmol)

Bilirubin assav kit was purchased from randox which contains the following reagents;

Reagent 1: Containing sulphanilic acid (30mmol/L)

Reagent 2: Contain sodium nitrate (50mmol/L)

Reagent 3: Contain caffeine (100mmol/L)

Total protein kit Reagent composition

Contents	**Concentration in the test**
Biuret reagent	
Sodium hydroxide	200mmol/1
Na-K tartrate 32mmol/1	32mmol/1
Potassium iodide	15mmol/1
Cupric sulfate	6mmol/1
Blank reagent	
Sodium hydroxide	200mmol/1
Na-K tartrate 32mmol/1	32mmol/1
Protein standard	5.5g/dl(58.48g/l)

Sodium hydroxide (0.4N): 16 of NaOh was dissolved with distilled water in IL volumetric flask and the solution was made up to IL with water.

Urea reagents: It consists of the following reagent:

Acid reagent (stock A); Ferric chloride (5g) was dissolved in 20m of H_2O. This solution was then transferred to a graduated cylinder. 85% phosphoric acid

(100ml) was then added slowly with swirling. The volume was made up to 50ml with distilled water.

Acid reagent (stock B): Conc, sulphuric acid (300m) was added to 700ml of distilled water in a 2L volumetric flask slowly with swirling

Mixed acid reagent (working reagent): Solution A (0.5m) was added to solution (IL).

Colour reagent (stock A): Diacetylmonoxime (20) was dissolved in distilled water (100ml), which was then filtered.

Colour reagent (stock B); thiosemicarbazide (5g) was dissolve in distilled water (1000ml).

Working colour reagent: solution A (67ml) was mixed with solution B (97m)) and it was then made up to 1L distilled water

Preservative, diluents for standards: phenylmercuric acetate (40mg) was dissolved in 25l of water. The solution was transferred to a measuring cylinder. Conic, Sulphuric acid (0.3) was then added and the volume was made to IL, with distilled water.

Table 1: The activity of AST in the serum was obtained from the table below:

Absorbance	**U/1**
0.020	7
0.030	10
0.040	13
0.050	16
0.060	19
0.070	23
0.080	27
0.090	31
0.0100	38
0.0110	41
0.0120	47
0.0130	52
0.0140	59
0.0150	67
0.0160	76
0.0170	89

Table 2: The activity of ALT in the serum was obtained from the table below:

Absorbance	**U/1**
0.025	4
0.050	8
0.075	12
0.100	17
0.125	21
0.150	25
0.175	29
0.200	34
0.225	39
0.250	43
0.275	48
0.300	52
0.325	57
0.350	62
0.375	67
0.400	72
0.425	77
0.450	83
0.475	88
0.500	94