ANTIPLASMODIAL AND CHEMOPREVENTIVE EFFECTS OF *PAULLINIA PINNATA* LINN. IN ETHYLENE GLYCOL MONOMETHYL ETHER - INDUCED TESTICULAR DYSFUNCTION IN RODENTS

BY

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B.Sc; M.Sc (Ibadan)

A Thesis in the Department of Biochemistry submitted to the Faculty of Basic Medical Sciences in partial fulfillment of the requirements for the award of the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

AUGUST 2017

DEDICATION

This work is dedicated to God the Father, God the Lord Jesus, God the Lord Holy Spirit, my honey (husband), my sweetheart (Anu) and my darling (Oba).

ABSTRACT

Malaria and infertility pose as threat to health where they are common globally. *Paullinia pinnata* (PP) is used in folkloric medicine to treat malaria and infertility but without scientific proof. In this study, the antiplasmodial activity of PP and its modulatory effects on ethylene glycol monomethyl ether (EGME)-induced testicular dysfunction were investigated in rodents.

Ninety-six male Wistar rats (140-190g) were treated orally for sub-chronic toxicity (SCT) and chemopreventive studies (CS) with PP authenticated at Forestry Research Institute, Nigeria (FHI No.: I06555). Leaves of PP were air-dried, pulverized and extracted with absolute methanol by cold maceration. For SCT, 36 rats were assigned into 6 groups (n=6): Normal saline, PP(50, 100, 200, 400 and 800mg/kg). On day 29, rats were sacrificed and samples collected for biochemical analysis (BA). Serum alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride were determined by spectrophotometry. In CS, 60 rats were used (6 groups, n=10) and treated with: Distilled water, 10% DMSO, EGME(200mg/kg), PP(200mg/kg), EGME+PP(100mg/kg), EGME+PP(200mg/kg). On day 22, rats were euthanized, levels of reproductive hormones and spermiogram were determined by ELISA and microscopy, respectively. Superoxide dismutase (SOD) and myeloperoxidase (MPO) were determined using spectrophotometry. For SCT and CS, histology of tissues (testes, brain, epididymes, liver, lung and kidney) was determined by microscopy. In the antiplasmodial studies, 102 Wistar mice (18-27g) were inoculated with *Plasmodium berghei* NK 65. For curative test with safe dose (CTSD), infected mice (36) were allotted into 6 groups (n=6) and treated as follows: vehicle, untreated-infected mice, chloroquine (CQ) (10mg/kg), artesunate (4mg/kg)-amodiaquine (10mg/kg) (ACT), PP(100mg/kg) and PP(200mg/kg) for 4 days and observed for 7 days post-administration. The animals were sacrificed and used for BA. Malaria aetiology profile was determined using microscopy. Data were subjected to descriptive statistics and ANOVA at $\alpha_{0.05}$.

In SCT, PP (400, 800mg/kg) significantly increased ALP (29.0, 30.0%), PP (400mg/kg) significantly increased TC (25.0%) and triglycerides (47.0%) compared to controls. Safe dose of PP was 200mg/kg. For CS, rats with [EGME+PP100mg/kg] and [EGME+PP 200mg/kg] had significantly reduced testosterone (1.2±0.2; 1.1±0.6 vs 1.6±0.1pg/mL) and increased luteinizing hormone (13.5±1.5; 14.7±0.7 vs 10.0±0.0pg/mL), decreased sperm viability (11.0±1.0; 17.5±2.5 vs 70.0±3.9%), decreased motility (3.5±1.5; 10.0±2.9 vs 67.5±5.7%) and increased abnormality (14.0±0.4; 14.6±0.1 vs 11.9±0.3%). The [EGME+PP100mg/kg] and [EGME+PP 200mg/kg] significantly reduced SOD activities in the testes (U/mg protein) (5.0±1.7; 2.4±0.2 vs 9.5±0.0) and in the brain (7.1±1.4; 4.8±0.0 vs 12.3±1.1). In contrast, [EGME+PP100mg/kg] and [EGME+PP 200mg/kg] significantly increased MPO activities in the epididymes by 91.0 and 82.0% respectively. Percentage parasitaemia for CTSD were 42.5 and 36.3% for PP at 200 and 400 mg/kg, respectively. Histology showed severe germinal erosion in the testis for CS.

Methanol leaf extract of *Paullinia pinnata* had anti-plasmodial activity but reduced chemopreventive effect on gonadal injury induced by ethylene glycol monomethyl ether.

Keywords: *Paullinia pinnata* extract, Sub-chronic toxicity, Antimalarial activity, Testicular dysfunction

Word count: 452

ACKNOWLEDGEMENTS

My inexpressible thanks goes to the Sovereign God, the Lord Jesus and the Holy Spirit for the mercy bestowed on me by which I was able to start and finish the programme.

I am indeed very grateful to my supervisor, Prof. Ebenezer Olatunde Farombi and my past teachers and supervisors at the undergraduate and postgraduate levels including Prof. G.O. Emerole, Prof. A.O. Uwaifor, Prof. L.H. Jeyakumar, Dr. Adebimpe O. Odetola, Prof. O.O. Olorunsogo, Prof. Oyeronke A. Odunola (Director, Research Management Office & Cancer research and Molecular Biology unit), late Prof. M.A. Fafunso, Prof. E.N. Maduagwu, Prof. Janet M. Makinde and Prof. R.A. Oderinde of the Department of Chemistry, University of Ibadan, Ibadan.

I appreciate the present and past Vice-Chancellors, Provosts and Deputy Provosts of the College of Medicine, Deans and Sub-Deans of the Faculty of Basic Medical Sciences, Deans and Sub-deans of the Postgraduate School, Presidents of the College of Medicine Investment and Co-operative Society and Heads of the Department of Biochemistry, University of Ibadan, Ibadan.

I am grateful to Prof. O.A. Bamiro (former Vice-Chancellor), Prof. O.G. Ademowo (Dean, Faculty of Basic Medical Sciences), Prof. M.O. Oyeyemi (Dean, Faculty of Veterinary Medicine), Prof. Chinedum P. Babalola (Dean, Faculty of Pharmacy), Prof. O.D. Olaleye (Head of Virology Department), Prof. Adeyinka G. Falusi of Institute of Advanced Medical Research and Training, Prof. Edith Ajaiyeoba of the Department of Pharmacognosy, Prof. Eme Owoaje (Head, Community Medicine), Dr. I.A. Oladosu of the Department of Chemistry, Dr. O.A. Adaramoye –Sub-Dean (Postgraduate) of the Faculty of Basic Medical Sciences and Drs. A.O. Abolaji and Gbadegesin (Postgraduate Co-ordinator) of the Department of Biochemistry, University of Ibadan amongst others.

I thank my fellow academic colleagues both at the junior and senior cadres in the Department of Biochemistry and in the University of Ibadan, and Bond Chemical Limited, Oyo State, Nigeria.

I also acknowledge the kind gesture of Prof. (Dr.) Muhammad Iqbal Choudhary for inviting me to the Hussein Ebrahim Jamal (H.E.J.) Research Institute of Chemistry at the University of Karachi, Pakistan where I utilized the Postgraduate sandwich fellowship awarded to me by the now Organization for Women in Science for the Developing Countries. I appreciate the

Federal Government of Nigeria and the Thomas-Bassir Biomedical Foundation for the grants I received.

I am grateful to the past and present non-academic, technologists and technical staff, as well as the Industrial Training, Undergraduate and Postgraduate students of the Department of Biochemistry and the Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan.

I appreciate the spiritual support of Pastor Olubi & Pastor (Dr.) Mrs. Sarah Johnson of Scripture Pasture Christian Centre (SPCC), Rt. Revd. Titus & Mrs. Mopelola Fajemirokun of All Souls' Church and Pastor Oladapo Abimbola of the Redeemed Christian Church of God (RCCG).

I also wish to express my gratitude for the contributions in various ways by my cousin- Mr. & Mrs. Tunde Fagbohunlu, my in-law- Mr. Wale & Dr. (Mrs.) Oyeduntan Adediran (Sub-Dean Sciences, Postgraduate School), Mr. A.K.B. Da-Silva of Harkods Diagnostics, Mr. E.A. Folarin of Lifelong Biomedical Diagnostics Centre and Mr. Laolu Oshundina.

I appreciate my priceless late parents and parents-in law, Mr. Matthew Taiwo & Mrs. Caroline Olayinka Adeyemo and Chief. Moses Afolabi & Mrs. Eugenia Olufunke Salami, their children and children's spouses. I am very grateful to my wonderful husband, Mr. Olatunji Oluwole Salami and my lovely children; Anuoluwagbemiro and Jesulobarotimi, as well as my late grandmother, Mrs. Comfort Adeyemo for the lessons I learnt from her.

CERTIFICATION BY SUPERVISOR

I certify that this work was carried out by Mrs. O.A. Adeyemo-Salami in the Department of Biochemistry, University of Ibadan.

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ABBREVIATIONS

ACT-	artemisinin based combination therapy
ALP-	alkaline phosphatase
ALT-	alanine aminotransferase
AST-	aspartate aminotransferase
CAT-	catalase
CQ-	chloroquine
DMSO-	dimethyl sulfoxide
DOXP-	1-deoxy-D-xylulose-5-phosphate
DSP-	daily sperm production
E-	eosinophil count
EDTA-	ethylene diamine tetra acetic acid
EGME-	ethylene glycol monomethyl ether
ESN-	epididymal sperm number
FSH-	follicle stimulating hormone
GGT-	gamma glutamyl transferase
GnRH-	gonadotropin- releasing hormone
GPx-	glutathione peroxidase
GSH-	reduced glutathione
GST-	glutathione –S-transferase
H & E-	haematoxylin and eosin
Hb-	haemoglobin concentration
L-	lymphocyte count
LDH-	lactate dehydrogenase
LH-	luteinizing hormone
LPO-	lipid peroxidation
M-	magnification
M-	monocyte count
MCH-	mean cell haemoglobin
MCHC-	mean cell haemoglobin concentration
MCV-	mean cell volume
MPO-	myeloperoxidase
N-	neutrophil count
NO-	nitric oxide
PCV-	packed cell volume

PfENR- *Plasmodium falciparum* enoyl -ACP reductase

PP- *Paullinia pinnata* leaf extract

RBC- red blood cell count

RNS- reactive nitrogen species

ROS- reactive oxygen species

SOD- superoxide dismutase

T- testosterone

T_3- triiodothyronine

T_4- thyroxine

TRH- thyrotropin- releasing hormone

TSH- thyroid- stimulating hormone

TSN- testicular sperm number

WBC- white blood cell count

CHAPTER ONE

1.1 Introduction

Mankind has been plagued with various diseases from time immemorial. As a result of this, man has exploited various means to circumvent these health challenges. The chief means has been the use of plants in his environment (Okoli *et al.,* 2007; Ogbonnia *et al.,* 2008). Medicinal plants are plants which possess in one or more of its parts, substances which have therapeutic effects or are precursors of useful synthetic drugs. The use of medicinal plants dates as far back as circa 2500 B.C. when the Chinese book on roots and grasses, "*Pen–tsao kang mu*", which was written by Emperor Shen Nung was reported. In the book, 365 drugs from dried parts of medicinal plants were recorded most of which are still being used presently and include *Theae folium, Podophyllum*, jimson weed and cinnamon bark (Petrovska, 2012).

In medical history, the great physician, Hippocrates is referred to as the father of medicine because he regarded medicine as a science (Singh, 2009). In his work "*Materia Medica*", he compiled four hundred simple remedies which he discovered from his practice and research into medicine. These remedies were herbal recipes. Some of the medicinal plants included in the recipes were sea onion, parsley, sage, opium, garlic and mandrake (Sofowora, 1993; Petrovska, 2012).

About one-quarter of the drugs in modern pharmacopoeias are from plant sources (Delahaye *et al.,* 2009; Petrovska, 2012). Efforts are being made to introduce and employ the use of herbs as alternative to, or to compliment orthodox medicine in some hospitals in urban areas (Salomonsen *et al.,* 2011). Also the knowledge of herbal treatment is being included and taught as part of the curriculum for medical training in some Institutional Teaching Hospitals (Bradshaw, 2016).

In the advent of advancement in technology, orthodox medicine is being practiced but multiple drug resistance is also posing to be a serious challenge. However, in the developing countries, the use of plants for traditional medicinal purposes is largely practiced because they are easily accessible in terms of cost and proximity (Ogbonnia *et al*., 2011). There is therefore the need to standardize these indigenous herbal preparations and assess their safety.

In tropical Africa, malaria and recently infertility are some of the health challenges that exist in the region (Guerra *et al.*, 2008; Farombi *et al.*, 2012a). Malaria is a significant public health issue in many parts of the world. Globally, malaria infects 200- 270 million people of which 1- 3 million die annually, and is responsible for infant and maternal deaths in countries where it is endemic (Becker *et al*., 2004; Iyawe *et al*., 2006; Olorunnisola and Afolayan, 2013). Initially, it results in debility thus culminating in people spending many days from their work (including farming) or school. This leads to a reduction in the money that can be earned, farm yield produced or amount of learning received in school resulting in reduced productivity (Cole and Neumayer, 2006). Presently, malaria is one of the diseases that has developed drug-resistance to conventional medicine. This has necessitated the search for alternatives which are being sought in plants.

Similarly, infertility or sterility poses as a global challenge arising, at least in part, from gonadotoxic events and effective interventions are also being sought (Boivin *et al*., 2007). There is the report that 15% of waiting couples are affected by infertility and the male factor infertility contributes to about 50% of the cases, 25% of which cause is not known (Agarwal and Prabakaran, 2005). However, continuous exposure to gonadotoxicants in the environment and workplace can contribute to or result in disruptions of the function of the reproductive system.

The mechanism(s) of action of gonadotoxicants are incompletely understood. One of the mechanisms of action of toxicants is oxidative stress which is increase or accumulation of free radical species, especially reactive oxygen species (ROS), resulting in DNA mutation and protein dysfunction that ultimately lead to cellular dysfunction of the target organ. Increased load of free radicals can also lead to cellular dysfunction. The role of antioxidants is to combat the reactive oxygen species and arrest the deleterious effect they inflict on the target thus preventing cell damage and dysfunction (Pham-Huy *et al.*, 2008). The gonadotoxic events are as a result of regular exposure to gonadotoxicants in the environment and work-place, and interventions are being sought from plants (Adaramoye *et al.*, 2010; Adedara and Farombi, 2011; Farombi *et al.*, 2012b).

1.2 Rationale

In the developing countries, tradition, poverty and inaccessibility to modern drugs and medical practice has made the use of plants to manage or cure diseases to be the norm, especially in rural areas. Similarly, there is the problem of drug resistance to conventional medicines. This has necessitated the need to search for other comparable sources thus directing the focus back to the ancient method which has been the use of plants, such as *Paullinia pinnata* Linn.

The leaves of *Paullinia pinnata* Linn. are used traditionally and taken copiously in various preparations for the treatment of diverse diseases including malaria and infertility (Burkill, 2000; Osarenmwinda *et al.*, 2009).

Everything is toxic when abused or misused and ingestion of plant products is not an exception since they also contain toxic secondary metabolites. Hence, there is a need to standardize the consumption of this plant which has been shown to contain these metabolites.

Phenolic compounds which include tannins, flavonoids and phenolic acids as well as diterpenes are of great interest because they have been shown to possess high antioxidant capacity and the leaves of *P.pinnata* have been shown to possess high phenolic content (Zamble *et al.,* 2006; Jimoh *et al.,* 2007; Tamokou *et al.,* 2013). Zamble *et al.* (2006) showed that the *P.pinnata* leaf and root extracts had the capacity to correct erectile dysfunction and attributed this to the strong antioxidant properties of the extracts *in-vitro*.

1.3 Aim

This study is therefore designed to investigate the antiplasmodial effect and the chemopreventive potential of the leaves of *Paullinia pinnata* in ethylene glycol monomethyl ether-induced testicular dysfunction model using rodents.

1.4 Objectives

The specific objectives are as follows:

(i) To determine the dose at which the methanol leaf extract of *Paullinia pinnata* can be considered safe

(ii) To investigate the suppressive, prophylactic and the curative potentials of the methanol leaf extract of *Paullinia pinnata* in mice

(iii) To investigate the anti-oxidative effect of the methanol leaf extract of *Paullinia pinnata* in the male reproductive organs of rats

(iv) To investigate the effect of the methanol leaf extract of *Paullinia pinnata* on some of the male reproductive hormones in rats

CHAPTER TWO

LITERATURE REVIEW

2.1 *Paullinia pinnata* (Linn.)

Paullinia pinnata is a woody or sub-woody climber of the family Sapindaceae. It originates from tropical America and is now common in the savanna zones of tropical Africa and Madagascar. The common names are “bread and cheese plant”, and “sweet gum”. The local names include: Yoruba; “Kakansenla” or “Ogbe-okuje”, Edo; “Aza”, Igala; “Egwubi” or “Omekpa”, Nupe; “Enu Kakanchela”, Hausa; “Goorondoorinaa”, Liberia (Basa); “Gbe-se”, Togo(AnyiAnifo); “Tolundi”, Sierra Leone (Kono); “Kamakagu” and Ghana(AdangmeKrobo); “Akplokinakpa”. (Burkill, 2000; Ikhane *et al*., 2015).

2.1.1. Medicinal uses

The belief is that the plant was introduced to Africa as a fish-poison, where it is used to serve as chewing-sticks, tying fences, hut building as well as for medicinal purposes which include the treatment of rickets, leprosy, fever debility, post-partum pain, localized pain, infectious diseases, cough, whooping cough, eye ailments and in complex treatments for jaundice and yellow fever. (Burkill, 2000).

The leaf is claimed traditionally to be a general panacea. In folklore medicine, it finds use in various forms for the treatment of colic, dysentery and diarrhoea (Akinyemi *et al.*, 2005). Though the leaf is widely used as an anti-diarrhoeatic in Africa, it is administered as a purge among the Igala tribe of Nigeria (Osarenmwinda *et al*., 2009). The leaf has also been shown to be useful gynaecologically. In various preparations, the leaf is taken to prevent miscarriage. In

Ivory Coast, Tanzania and Gabon, the leaf is used as an ocytocite to ease child birth. In Congo, it is used as a lactogene and also to help sterility. In Gabon and Ghana, it is used to ease menstrual discomfort (Burkill, 2000). The leaf is used on contusions in Ghana and Nigeria; aches, sprains, fractures, dislocations and burns in Ghana; sores in Nigeria, Mali and Ivory Coast; stiffness and lumbago in Mali; rheumatism in Nigeria; internal and external swellings in the Gambia; dermatological aberrations throughout West and Central Africa and topically for treating ulcers in Ivory Coast (Burkill, 2000; Huang *et al.*, 2007).

Other medicinal applications include the treatment of fevers in West Africa and in particular malaria in Tanzania. Likewise, it is used for the treatment of gonorrhea and paralysis in East Africa; eye treatment and leprosy in Ivory Coast; rickets in Ghana and Ivory Coast; colds in general in Nigeria; tetanus in Ghana; anti-anaemic tonic and bile stimulant in Nigeria, Congo and Senegal; snake bite in East Africa and ancylostomiasis in Tanzania. In Ghana, a leaf decoction is considered an aphrodisiac (Burkill, 2000). The leafy twigs, root, flowers, fruit and seeds are used for similar medicinal purposes as the leaves throughout Africa. Proximate analysis is reported to be 4.9 % of protein, 3.5 % of fat and 86.7 % of carbohydrate. The fruit contains a gum used by children for sticking papers together. Moreover, there are fetish claims attributed to the plant. In Ivory Coast, a wash in the decoction is believed to confer protection against evil (Burkill, 2000).

Figure 2.1. *Paullinia pinnata* Linn. Leaves

2.1.2. Biological Properties of *P.pinnata* leaves

2.1.2.1. Antibacterial capacities

Scientific investigations have been carried out on the leaves of *Paullinia pinnata*. Ikhane *et al.* (2015), using *in-vitro* and *in-vivo* assays, showed that the methanol extract had antimicrobial activity against food-borne pathogens which were *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Thus having effect on both gram-negative and gram-positive bacteria and therefore having a broad spectrum potential. This finding supports the work of Annan and Houghton (2004) who showed that aqueous decoction of leaves, stem and root of *P.pinnata* had activity against *Staphylococcus aureus* and *Bacillus subtilis*.

2.1.2.2. Pharmacological presentations

A flavotannin isolated by Bowden was shown to have cardiotonic action on isolated perfused frog heart (Broadbent, 1962). Aqueous ethanol extract of the leaves have been shown to have anti-malarial property (Maje *et al.*, 2007). Ior *et al.* (2011) showed in Wistar mice and rats that the aqueous ethanol extract of the leaves possess anti-inflammatory and analgesic properties thus supporting its use in traditional medicine for the treatment of rheumatism and arthritis, which are as a result of inflammation and pains.

2.1.2.3. Antioxidant properties

Zamble *et al.* (2006) also showed that the methanol extracts of the leaves and root had *in-vitro* scavenging activity against superoxide oxygen (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Jimoh *et al.* (2007), also confirmed the scavenging activity of the plant on 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-

sulfonic acid radicals *in-vitro*. The plant was also shown to possess moderate ferric reducing potential (Jimoh *et al.,* 2007). Using 2,2-diphenyl-1-picrylhydrazyl (DPPH) bleaching assay, Trolox equivalent antioxidant capacity (TEAC) assay and Hemoglobin ascorbate peroxidase activity inhibition (HAPX) assay, Tamokou *et al.* (2013) showed that hexane extract of the leaves possess antioxidant capacity *in-vitro*. All these indicate that *P.pinnata* is a potential source of natural antioxidants.

2.1.2.4. Anticancer and wound healing capacities

Tamokou *et al.* (2013) showed that the hexane extract inhibited the growth of the p53 wild-type malignant melanoma cell line WM35 thus suggesting that it has anti- cancer property. Annan *et al*. (2007; 2010) conducted *in-vitro* and *in-vivo* studies using human dermal fibroblast cell line 142BR and wound models in Sprague-Dawley rats. They observed that application of the methanol extract of the plant resulted in a significant increase in 142BR cell line proliferation and there was also a significant increase in tensile strength and hydroxyproline content of tissues that were healing with a decrease in epithelisation period and scar area. Moreover, the extract had cyto-protective action against hydrogen peroxide-induced damage. These findings support the use of the plant in treating wounds traditionally.

2.1.2.5. Haematological and antityphoid capacities

Adeyemo-Salami and Ewuola (2015) showed that the methanol extract of the leaves had anti-anaemic property with the ability to increase neutrophils count in normal Wistar rats. This seeks to support traditional use as an anti-anaemic tonic. Anti-typhoid activity was exhibited by leaf extracts in *Salmonella typhymurium* infected Wistar rats (Lunga *et al*., 2014a).

2.1.2.6. Phytotoxic and other biological properties

Phytotoxic activity in a dose-dependent manner was reported *in-vitro* by Salami and Fafunso (2016) against *Lemna minor* (Common duckweed), thus suggesting that it could be a good source of natural herbicides. The intermediate host of *Schistosoma mansoni* (the parasite responsible for schistosomiasis) is *Biomphalaria glabrata*. *P. pinnata* extracts was shown to kill 50 % of the snails after a twenty-four hour exposure and a day for recovery (Melendez and Capriles, 2002). Zamble *et al.* (2006) showed that *P.pinnata* extracts had the capacity to induce endothelium-dependent vaso-relaxation of bovine aortic endothelial cells through the nitric oxide pathway and inhibit endothelin-1 synthesis and therefore suggested that this could be the pharmacological mechanism responsible for the efficacy in unorthodox medicine for the treatment of impotence. Osarenmwinda *et al.* (2009) showed that the methanol extract of the leaf possesses antidiarrhoeal activity in a dose-dependent manner and inhibits gastrointestinal motility in Swiss albino mice thus corroborating or validating the folkloric use.

2.1.2.7. Biological activities of other parts of *P.pinnata*

Other investigations carried out on other parts of the plant have shown in Swiss mice that the aqueous methanol stem bark extract possesses anti-convulsant activity (Maiha *et al.*, 2009; Lunga *et al.*, 2011). The methanol bark extract showed *in-vitro* that methanol stem bark extract of *P. pinnata* possessed bactericidal activity in a dose-dependent manner against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Staphylococcus aureus, Protens mirabilis* and *Shigella flexneri*. This finding was complemented *in-vivo* (Lunga *et al*., 2011). Iful (2008) also showed that extracts from the root reversed the venom-induced increase in capillary permeability in rabbits, abnormal white blood cell, platelet and packed cell volume values to normal.

2.1.2.8. Pure compounds from *P.pinnata* and their biological capacities

Annan *et al*. (2009) also revealed that the fatty acid- azelaic acid, isolated from the methanol root extract, had anti-bacterial activity against gram negative and gram positive bacteria. The gram negative bacteria were *Pseudomonas aeruginosa* and *Escherichia coli* while the gram positive bacteria were *Staphylococcus aureus* (NCTC 4163), *Bacillus subtilis* (NCTC 10073), *Micrococeus flavus* (NCTC 9743), *Streptococcus faecalis* (NCTC 775) and resistant strains of *Staphylococcus aureus* (SA 1199B, RN 4220 and XY 212). Other fatty acids isolated from the methanol root extract had weak to moderate activity against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Annan *et al*., 2009). Annan *et al*. (2009) also showed that the methanol extract exhibited a significant radical scavenging action in the DPPH assay but the isolated fatty acids did not.

Lunga *et al.* (2014b) showed that some compounds isolated from the plant had antibacterial, anti-yeast and anti-dermatophytic activities. The compounds were (3β)-3-0-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl), oleanolic acid, (3β)-3-0-[β-D-glucopyranosyl-(1″-3′)-2′-acetamido-2′-deoxy-β-D-galactopyranosyl] oleanolic acid and (3β, 16α-hydroxy)-3-0-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl) echinocystic acid respectively.

2.1.3. Other compounds and phytochemicals from *P.pinnata*

The presence of phenolic compounds has been reported in the leaves of *P. pinnata*. A flavotannin was isolated by Bowden (Broadbent, 1962). Abourashed *et al.* (1999) isolated two flavone glycosides; diosmatin-7-0(2″-0-beta-D-apio-furanosyl-6″-acetyl-beta-D-glucopyranoside and tricetin-4'-0-methyl-7-0(2″-0-beta-D-apiofuranosyl-6″-acetyl-beta-D-glucopyranoside. Tamokou *et al.* (2013) showed that the total phenol content of the hexane extract of *P.pinnata*

leaves was significant. Jimoh *et al.* (2007) also confirmed the presence of phenolic compounds and proanthocyanidin. The presence of alkaloids, tannins, cardiac glycosides, saponins, carbohydrate, reducing sugars and anthracene have been confirmed by various researchers using phytochemical analysis (Osarenmwinda *et al*., 2009; Ior *et al.,* 2011; Adeyemo-Salami and Makinde, 2013).

The cerebroside Paullinoside A and the ceramide Paullinomide A were also isolated by Miemanan *et al.* (2006) from the leaves. β-sitosterol and daucosterol were isolated by Dongo *et al*. (2009).

Phytochemical screening of other plant parts have revealed the presence of alkaloids, tannins, saponins, triterpene, phenol and sterols in the stem bark (Lunga *et al*., 2011). In the preliminary phytochemical analysis conducted by Iful (2008), the presence of carbohydrates, saponins, steroids and tannins was observed in extracts of the root bark.

Annan *et al.* (2009) isolated and identified twelve fatty acids from methanol root extract of *P. pinnata*. These included azelaic acid, palmitic acid, oleic acid, stearic acid, eicosanoic acid, docosanoic acid and tetradecanoic acid. Dongo *et al.* (2009) isolated steroids and steroidal glycosides from the plant. Lunga *et al.* (2014b) isolated 2-0-methyl-L-chiroinositol, friedelin, (3β)-3-0-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl) oleanoic acid, (3β, 16α-hydroxy)-3-0-(2′-acetamido-2′-deoxy-β-D-glucopyranosyl) echinocystic acid and (3β)-3-0-[β-D-glucopyranosyl-(1″-3′)-2′-acetamido-2′-deoxy-β-D-galactopyranosyl] oleanolic acid from the plant. Moreover, Iful (2008) showed that the root extracts contained Zinc, Calcium, Iron and Lead.

2.2 Malaria

2.2.1 Prevalence of malaria

Malaria is an ancient disease that has been recorded since 1500 B.C. (Adinew, 2014). Malaria is one of the deadly parasitic diseases of the world. It occurs in tropical areas and is transmitted by infection with *Plasmodium* protozoa from the bite of an infected female *Anopheles* mosquito vector. In 2010, WHO estimated that there are 765 million persons at risk and 800,000 deaths annually (WHO 2010). 85% of these malaria cases and 90% of the deaths have been reported to occur in sub-Saharan Africa, especially in children younger than 5 years of age (White *et al.*, 2014).

Normally, malaria is not contagious however there are exceptions which include the following:

i) Blood transfusion

ii) Intravenous drug abuse with shared needles

iii) An infected mother to the unborn child

iv) Organ transplant

The disease condition presents strong fever, acidosis, anaemia and multiple organ failure.

Morbidity and mortality as a result of malaria infection is still on the increase despite several interventions in developing parts of the world, including Africa (Iribhogbe *et al*., 2013).

In Africa, malaria is the fourth leading cause of loss of years of productive life affecting learning in schools as well as efficiency on the farm and at the work place. Thus resulting in significant decrease in the pace of economic growth and development (Adinew, 2014).

In Nigeria, malaria is a major public health challenge where it accounts for more cases and deaths than any other country in the world. Estimates show the diagnosis of 100 million malaria cases with at least 300,000 deaths annually in Nigeria. This is more than the estimated 215,000 deaths annually in Nigeria from HIV/AIDS (reviewed in Adeyemo-Salami *et al.*, 2014).There is therefore an urgent need to seek for remedies to this scourge.

2.2.2 The *Plasmodium* species

Plasmodium species has been known to infect a wide variety of reptiles, birds and mammals including bats, rodents and ungulates. There are over fifty-two species of *Plasmodium* out of which five are known to infect humans (White *et al.*, 2014). These are:

i) *Plasmodium falciparum*- This is the most life-threatening species. It is found throughout most of the tropical and subtropical regions, especially in sub- Saharan Africa. This species does not hide in the liver.

ii) *Plasmodium vivax*- This species is found in Latin America, Asia and some parts of Africa. Infections can lead to rupture of the spleen which can be life-threatening. It hides in the liver ("hepatic phase" of the life cycle). Years after the first infection, it may cause a relapse. Special medications are used to eradicate it from the liver.

iii) *Plasmodium malariae*- This species is found worldwide although it is less common. This infection is hard to diagnose because there are usually very few parasites in the blood. It can last for many years if untreated.

iv) *Plasmodium ovale*- This species is found in western Pacific islands and Africa. Like *P. vivax*, this species can hide in the liver for years before bursting out again and resulting in symptoms which are also similar to that of *P. vivax.*

v) *Plasmodium knowlesi*- This species exists predominantly in Malaysia. It can cause organ failure or death as a result of high levels of parasites in the blood.

Other species include *P.berghei, P.yoelii, P.coatney, P.chabaudi, P.vinckei, P.inopinatum* and *P.aegyptensis.*

Plasmodium berghei is one of the four *Plasmodium* species that have been described in African murine rodents. *P. berghei* is a practical model organism in the laboratory for the experimental study of human malaria. *P.berghei* is of two main types: NK 65 and ANKA. NK 65 is of two strains: chloroquine-resistant strain and chloroquine-sensitive strain (Adeyemo- Salami *et al.*, 2014).

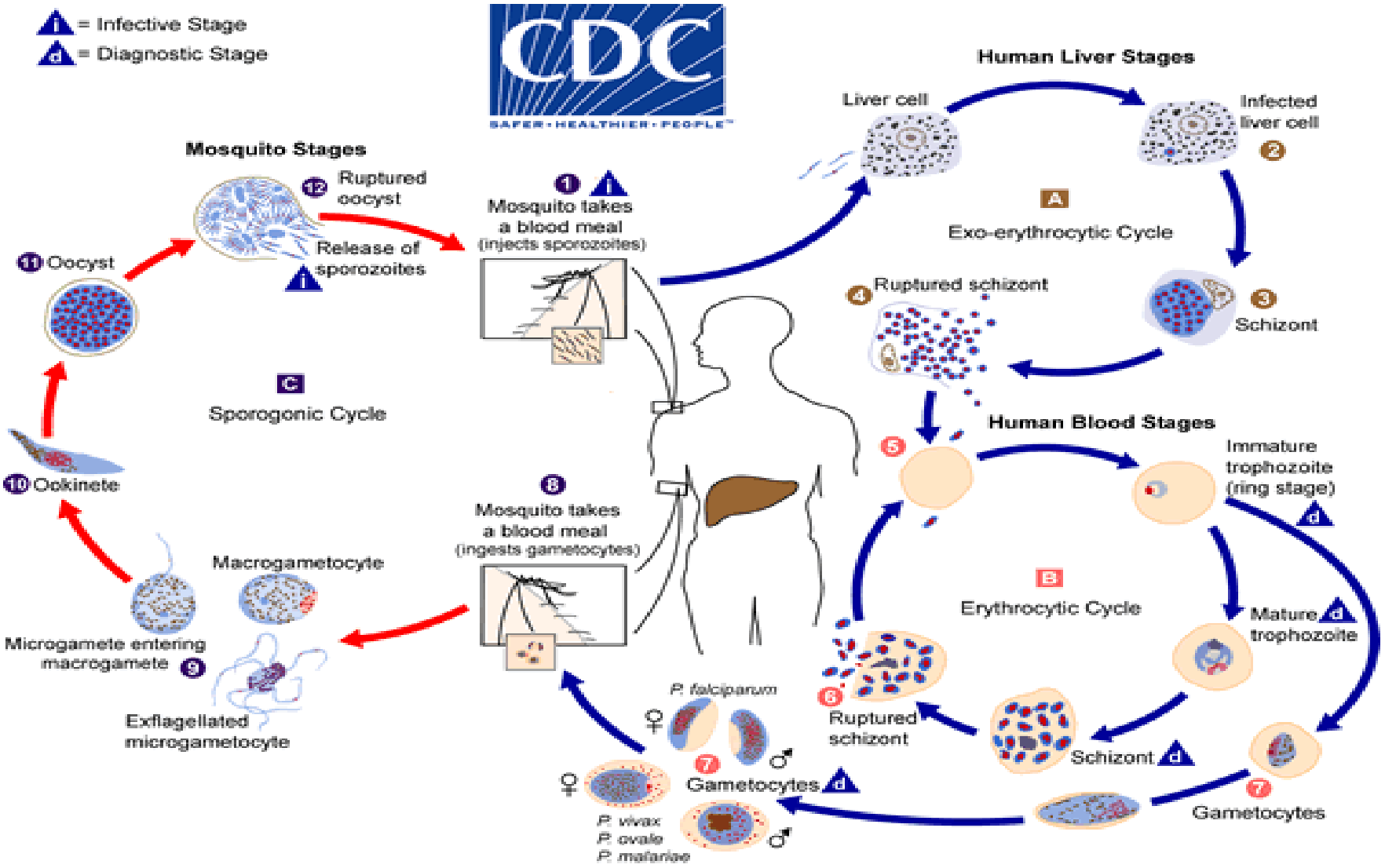

Figure 2.2 Life cycle of Malaria

Source: https://www.cdc.gov/malaria/about/biology/

2.2.3 Life cycle of malaria (Figure 2.2)

A female *Anopheles* mosquito carrying malaria-causing parasites feeds on a human and injects the parasites in the form of sporozoites into the bloodstream. The sporozoites travel to the liver and invade liver cells thus initiating the hepatic phase. Over (5-16) days*, the sporozoites grow, divide, and produce tens of thousands of haploid forms, called merozoites, per liver cell. Some malaria parasite species also produce hypnozoites in the liver that remain dormant for extended periods, causing relapses weeks or months later. The merozoites exit the liver cells and re-enter the bloodstream, beginning a cycle of invasion of red blood cells, known as asexual replication and thus initiate the erythrocyte phase. In the red blood cells, they develop into mature schizonts, which rupture and release newly formed merozoites that then re-invade other red blood cells. This cycle of invasion and cell rupture repeats every (1-3) days* and can result in thousands of parasite-infected red blood cells in the host bloodstream, leading to illness and complications of malaria that can last for months if not treated (Steps 1-6) (NIH-NIAID, 2016).

Some of the merozoite-infected blood cells leave the cycle of asexual replication. Instead of replicating, the merozoites in these cells develop into sexual forms of the parasite, called male and female gametocytes. In some malaria species, young gametocytes sequester in the bone marrow and some organs while the late stage gametocytes, circulate in the bloodstream (Step 7) (NIH- NIAID, 2016).

When a mosquito bites an infected person, it ingests the gametocytes. In the mosquito mid-gut, the infected human red blood cells burst, releasing the gametocytes, which develop further into sexual forms called gametes. Male and female gametes fuse to form

diploid zygotes, which develop into actively moving ookinetes that burrow through the mosquito mid-gut wall and form oocysts on the other side (Steps 8-10) (NIH- NIAID, 2016).

Growth and division of each oocyst produces thousands of active haploid forms called sporozoites. After (8-15) days*, the oocyst bursts, releasing sporozoites into the body cavity of the mosquito, from which they travel to and invade the mosquito salivary glands (Steps 11-12). The cycle of human infection re-starts when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the human bloodstream (Step 1) (NIH- NIAID, 2016).

*It is important to note that the time-frame depends on the malaria parasite species.

2.2.4 Antimalarial drugs and their targets

Various drugs with differing structures and therefore differing mechanisms have been isolated or discovered over time in the quest to have better acting drugs and to overcome drug resistance by the *Plasmodium* parasite (Fidock *et al.*, 2004). Presented in Table 2.1 are the currently existing antimalarial drugs and the molecule of the pathway or mechanism targeted in the *Plasmodium* parasite.

Table 2.1. Antimalarial drugs and their targets in the *Plasmodium* protozoa

Target location	Pathway/mechanism	Target molecule	Examples of therapies	
			Existing therapies	**New compounds**
Cytosol	Folate metabolism	Dihydrofolate reductase	Pyrimethamine, proguanil	Chlorproguanil
		Dihydropteroate synthase	Sulphadoxine, dapsone	
	Glycolysis	Thymidylate synthase		5-fluoroorotate
		Lactate dehydrogenase		Gossypol derivatives
		Peptide deformylase		Actinonin
	Protein synthesis	Heat-shock protein 90		Geldanamycin
	Glutathione metabolism	Glutathione reductase		Enzyme inhibitors
	Signal transduction	Protein kinases		Oxindole derivatives
	Unknown	Ca^{2+}-ATPase	Artemisinins	
Parasite membrane	Phospholipid synthesis	Choline transporter		G25
	Membrane transport	Unique channels	Quinolines	Dinucleoside dimers
		Hexose transporter		Hexose derivatives
Food vacuole	Haem polymerization	Haemozoin	Chloroquine	New quinolines
	Haemoglobin hydrolysis	Plasmepsins		Protease inhibitors
		Falcipains		Protease inhibitors
	Free-radical generation	Unknown	Artemisinins	New peroxides
Mitochondrion	Electron transport	Cytochrome *c* oxidoreductase	Atovaouone	
Apicoplast	Protein synthesis	Apicoplast ribosome	Tetracyclines, clindamycin	
	DNA synthesis	DNA gyrase	Quinolones	
	Transcription	RNA polymerase	Rifampin	
	Type II fatty acid bio-synthesis	FabH		Thiolactomycin
		Fabl/PfENR		Triclosan
	Isoprenoid synthesis	DOXP reductoisomerase		Fosmidomycin
	Protein farnesylation	Farnesyl transferase		Peptidomimetics
Extracellular	Erythrocyte invasion	Subtilisin serine proteases		Protease inhibitors

DOXP:- 1-deoxy-D-xylulose 5-phosphate; PfENR:- *Plasmodium falciparum* enoyl-ACP reductase; FabH:- β-keto acyl ACP synthase III

Source: Fidock *et al.*, 2004

2.2.5 Resistance to antimalarial drugs

Increased incidence of morbidity and mortality as a result of malaria infection has also been found to be linked to antimalarial drug resistance. Chloroquine is one of the oldest drugs used in the treatment of malaria. The mechanism of chloroquine resistance is not yet clear. However, it is well established that chloroquine, due to the weak base properties, accumulates in the vacuole of the parasite where it inhibits the process of heme polymerization and detoxification by the parasites thus resulting in their death (Loria *et al.*, 1999).

There are some genetically induced red cell factors which confer some degree of resistance against infection by malaria parasite (Cappadoro *et al*., 1998; Ademowo *et al*., 1995). These include ABO blood group antigens, glucose-6-phosphate dehydrogenase deficiency and sickle cell trait as well as chloroquine uptake in red blood cells (Ademowo *et al*., 1998).

Resistance phenotype to antifolates such as sulphadoxine, pyrimethamine and proguanil have been linked to point mutations on the dihydrofolate reductase and dihydropteroate synthase genes. Artemisinin analogs (e.g. artemether and artesunate) have proven to be effective in the treatment of malaria. In spite of this, their use as mono-therapeutic agents is limited because they have short half lives and there is occurrence of late recrudescence (WHO, 2003). This is supported by recent reports on the emergence of *Plasmodium falciparum* which are tolerant to artemisinin in the border area of Thai-Cambodia (WHO, 2010). As a result of this, artemisinin analogs are used as combination therapy with other agents having longer half lives.

Artemisinin- based combination therapy (ACT) has been shown to be effective in antimalarial treatment, contributing to the control of malaria with a decrease in the number of cases as well as in mortality (WHO, 2011). The draw -back however is that the drugs are expensive though efforts are being made to make them affordable.

2.3 Male reproductive system

2.3.1 Organs

The organs of the male reproductive system are specialized for the following functions:

- To produce, maintain and transport sperm (the male reproductive cells) and protective fluid (semen)
- To discharge sperm within the female reproductive tract
- To produce and secrete male sex hormones

The male reproductive anatomy includes internal and external structures.

a) The External Structures

Most of the male reproductive system is located outside the man's pelvis. This includes the penis, the scrotum, epididymis and the testicles.

i) **Penis**- The penis is the male organ for mating and transfer of seminal fluid for fertilization. It has three parts: the root, the body or shaft and the glans, which is the cone-shaped end of the penis (Cleveland Clinic, 2013).

- **The root:** attaches to the wall of the abdomen
- **The body:** is cylindrical in shape and consists of three internal chambers: Corpus cavernosum, spongiosum, and endothelial-lined smooth muscle sinusoids. These chambers are made up of special, sponge-like erectile tissue. This tissue contains thousands of large spaces that fill with blood when the man is sexually aroused. As the penis fills with blood, it becomes rigid and erect, which allows for

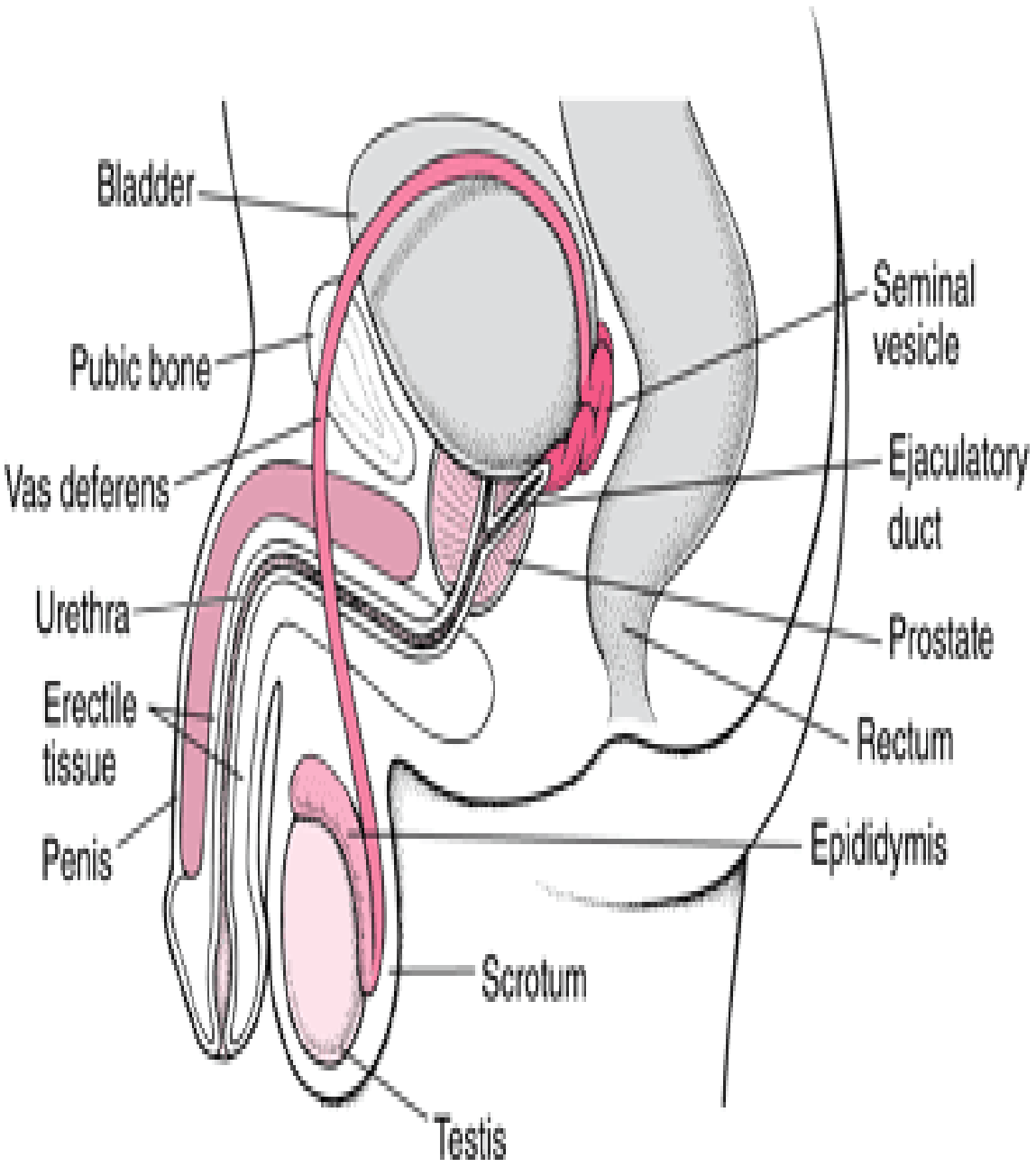

Figure 2.3. The Male reproductive organ (Source: http://www.merckmanuals.com)

penetration during copulation. The skin of the penis is loose and elastic to accommodate changes in penis size during an erection. Nitric oxide (NO) has recently been shown to be the most noncholinergic, nonadrenergic neurotransmitter responsible for penile erection. The normal growth of the penis during puberty is considered to be, in part, an androgen-dependent event and thus may be affected by endocrine disrupters or gonadotoxicants (Cleveland Clinic, 2013).

- **The glans**: also called the head of the penis, is covered with a loose layer of skin called foreskin. (This skin is sometimes removed in a procedure called circumcision). The opening of the urethra, the tube that transports semen and urine, is at the tip of the glans penis. The penis also contains a number of sensitive nerve endings.

  Semen or ejaculate, which contains sperm, is expelled (ejaculated) through the end of the penis when the man reaches sexual climax (orgasm). When the penis is erect, the flow of urine is blocked from the urethra, allowing only semen to be ejaculated at orgasm (Cleveland Clinic, 2013).

ii) **Scrotum**- The scrotum is the loose pouch-like sac of skin that hangs behind the penis. It contains the testicles (or testes), as well as many nerves and blood vessels. The scrotum has a protective function and acts as a climate control system for the testes. For normal sperm development, the testes must be at a temperature slightly cooler than the body temperature. Special muscles in the wall of the scrotum allow it to contract and relax, moving the testicles closer to the body for warmth and protection or farther away from the body to cool the temperature (OpenStax, 2016).

iii) **Testicles (testes)-**The testes are oval organs about the size of very large olives that lie in the scrotum, secured at either end by a structure called the spermatic cord. Most men have two testes. The testes are responsible for making testosterone, the primary male sex hormone, and for generating sperm. Within the testes are coiled masses of tubes called seminiferous tubules. These tubules are responsible for producing the sperm cells through a process called spermatogenesis (OpenStax, 2016).

iv) **Epididymis** - The epididymis is a long, coiled tube that rests on the backside of each testicle. It functions in the transport and storage of the sperm cells that are produced in the testes. Furthermore, the role of the epididymis is to bring the sperm to maturity, since the sperm that emerge from the testes are immature and incapable of fertilization. The mesonephric ducts in the region of the epigenital tubules become convoluted and develop to form the epididymis. Functionally, the epididymis is divided into three major regions:

a) Caput, or the head,

b) Corpus, or the body, and

c) Cauda, or the tail.

Sperm mature and acquire significant motility as they traverse the caput and corpus, and they become stored in the cauda regions. The regional expression of many different genes, proteins, and other biochemicals in the epididymis suggests a linear maturational mechanism for sperm as they traverse this organ (Cleveland Clinic, 2013).

b) **The Internal Structures**

The internal organs of the male reproductive system are the accessory organs which are vas deferens, ejaculatory ducts, urethra, seminal vesicles and prostate gland.

i) **Ejaculatory ducts-** These are formed by the fusion of the vas deferens and the seminal vesicles. The ejaculatory ducts empty into the urethra (Cleveland Clinic, 2013).

ii) **Urethra**- This is the tube that carries urine from the bladder to outside of the body. In males, it has the additional function of expelling (ejaculating) semen when the man reaches orgasm. When the penis is erect during copulation, the flow of urine is blocked from the urethra, allowing only semen to be ejaculated at orgasm (Cleveland Clinic, 2013).

iii) **Seminal vesicles** - These are sac-like pouches that attach to the vas deferens near the base of the bladder. Seminal vesicles arise as an extensive out-pocketing of the mesonephric ducts at the level of the prostate, thus sharing common embryological origins with the epididymis and vas deferens. The seminal vesicles produce a sugar-rich fluid (fructose) that provides sperm with a source of energy and helps with the sperms' motility. The fluid of the seminal vesicles makes up about 95% of semen while 5% is made up of spermatozoa (OpenStax, 2016).

iv) **Prostate gland**- This is a walnut-sized structure that is located below the urinary bladder in front of the rectum. The prostate gland contributes additional fluid to the semen. Prostate fluids also help to nourish the sperm and contains many important enzymes and polyamines, which help in lysis of the seminal coagulum and liquefaction of the ejaculate. The growth of the prostate is under control of androgens, mainly dihydrotestosterone (DHT), and the androgen-responsive elements (AREs). The AREs regulate the expression of androgen-responsive

genes. The urethra, which carries the ejaculate to be expelled during orgasm, runs through the center of the prostate gland (Cleveland Clinic, 2013).

v) **Vas Deferens -** also derived from mesonephric tubules, is a thick, muscular tube that carries sperm from the epididymis to the ejaculatory ducts in the prostate and penis. The first part of the vas deferens, originating at the cauda epididymis, is convoluted. As it approaches the prostate, adjacent to the seminal vesicles, it becomes dilated and is called the ampulla. The normal growth and development of the vas deferens during puberty is also an androgen-dependent event. During sexual arousal, contractions force the sperm into the vas deferens (OpenStax, 2016).

2.3.2 Compartments of the testis

The seminiferous tubules, the sertoli cells and the leydig cells all constitute the testis.

i) Seminiferous Tubules

About 80 % of testicular mass consists of highly coiled seminiferous tubules within which spermatogenesis takes place. The remaining 20 % consists of Leydig cells and Sertoli cells, important for establishing normal spermatogenesis. These tubules are formed by a very complex stratified epithelium containing spermatogenic cells and supporting cells called Sertoli cells. The epithelium is surrounded by a lamina propria composed of a double-layered basal lamina, three to five inner layers of myofibroblast and one or more outer layers of fibroblasts. The proliferation of the mesenchyme separates the sex cords from the underlying coelomic epithelium by the seventh week of fetal development. These sex cords become the seminiferous tubules that develop a lumen after birth. During the fourth month, sex cords become U-shaped, and their ends anastomose to form the rete testis, which provide

communication with the epididymis. The primordial sex cells are referred to as prespermatogonia, and the epithelial cells of the sex cords as Sertoli cells (Sikka and Naz, 2002).

ii) Sertoli Cells

Sertoli cells, which are also called "nurse cells," provide nourishment to the developing sperm during spermatogenesis. These cells form a continuous and complete lining within the tubular wall and establish the blood-testis barrier by virtue of tight junctions. The luminal environment is under the influence of follicle-stimulating hormone (FSH) and inhibin. Besides providing nourishment to the developing sperm cells, Sertoli cells have several other functions including:

a) Destruction of defective sperm cells

b) Secretion of fluids that help in the transport of sperm into the epididymis

c) Release of hormone inhibin that helps regulate FSH and sperm production.

The formation of a competent blood-testis barrier by differentiating Sertoli cells is essential to the establishment of normal spermatogenesis during puberty. Thus, interference by endocrine disrupters leading to spermatogenic impairment and infertility may indeed reflect changes in the function of the Sertoli cell population and not necessarily pathology in the germ cells themselves, thus creating a Sertoli-cell-only testes (OpenStax, 2016).

iii) Leydig Cells

The origin of these cells is from interstitial mesenchymal tissue during the eighth week of human embryonic development. These cells are located in the connective tissue area marked by angular interstices between the seminiferous tubules. Their main function is to produce testosterone and steroidal intermediates from cholesterol via a series of enzymatic pathways.

This steroidogenesis is under the control of luteinizing hormone (LH) from the pituitary (Sikka and Naz, 2002).

2.3.3 Male reproductive hormones

The male reproductive hormones include luteinizing hormone, follicle stimulating hormone, estradiol, testosterone and inhibin. Their sources, target organs and effects are shown in Table 2.2.

2.3.4 Hypothalamic-Pituitary-Gonadal Axis

The growth, development, and function of the mammalian male reproductive system depend upon a normal Hypothalamic-Pituitary-Gonadal (HPG) axis (Obi and Nwoha, 2014). The hypothalamus synthezises and releases, in a pulsatile fashion, the decapeptide gonadotropin-releasing hormone (GnRH) that regulates the production and release of the pituitary hormones, LH and FSH. These glycoprotein pituitary hormones regulate the gonadal function, including androgen biosynthesis and spermatogenesis. The HPG axis is under negative feedback control (long loop and short loop) mechanisms regulated by circulating steroidal hormones and inhibin. The growth and function of male reproductive organs is regulated by the action of the androgens; testosterone (T) and dihydrotestosterone (DHT), mediated via the androgen receptors (AR) (Sikka and Naz, 2002) (See Figure 2.4).

Table 2.2. Male reproductive hormones: sources, targets and effects

Hormone	**Source**	**Major Target**	**Direct or Indirect effect on target(s)**
Luteinizing hormone (LH)	Anterior pituitary	Leydig cells	Stimulate testosterone production
Follicle-stimulating hormone (FSH)	Anterior pituitary	Sertoli and/ or germ cells	i)Stimulates protein synthesis (e.g. androgen- binding protein) ii)Maturation of spermatids into spermatozoa
Testosterone	Leydig cells	i)Male accessory glands ii)Hypothalamus and pituitary	i)Maintain structure and function ii)Negative feedback control on release of FSH and LH
Estradiol	Leydig cells	Anterior pituitary	Negative feedback control on release of FSH and LH
Inhibin	Sertoli cells	Anterior pituitary	Negative feedback control on release of FSH

Source: Overstreet and Blazak, 1983

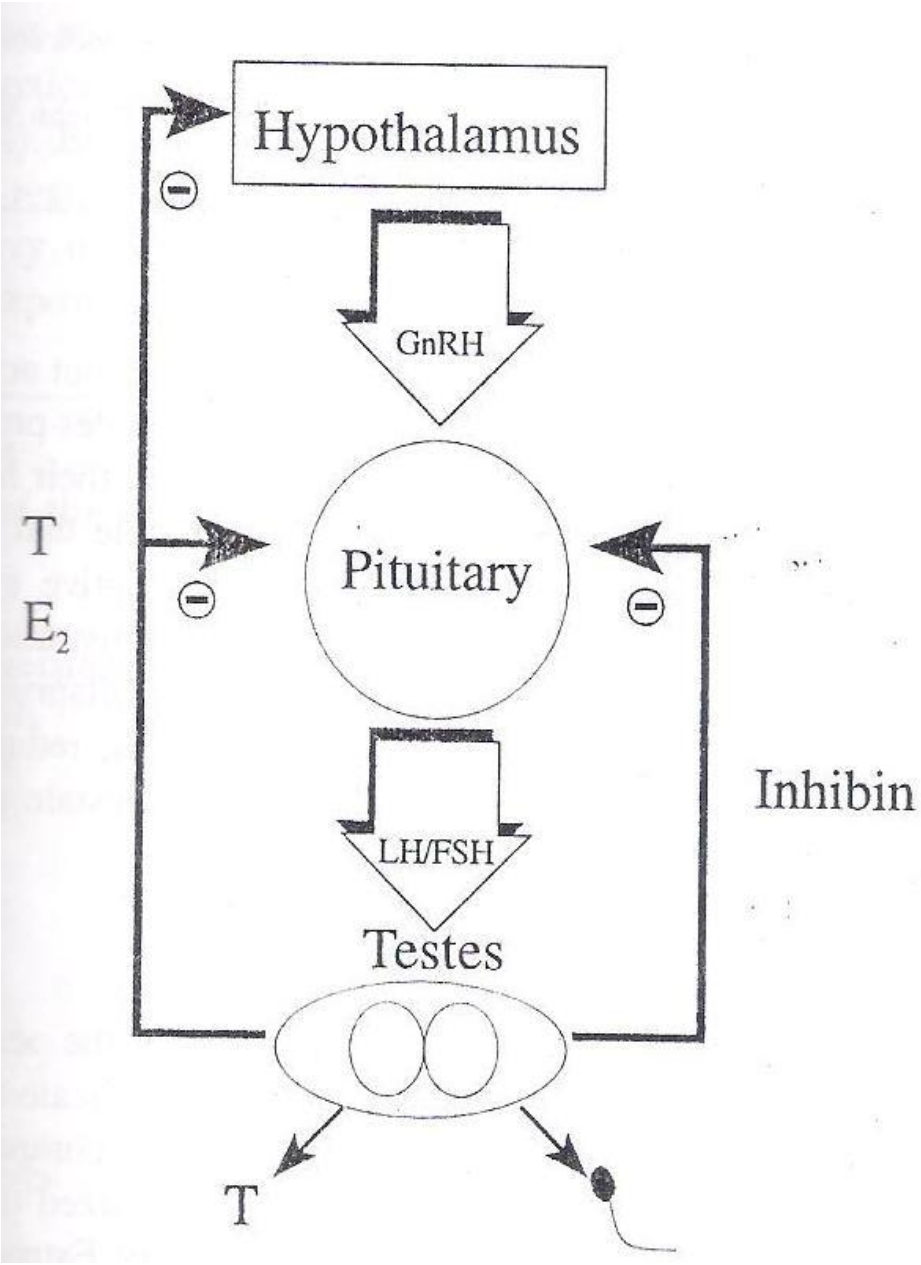

Figure 2.4. The Hypothalamic-Pituitary-Gonadal Axis

Source: Ecobichon, 2002

2.3.5 Spermatogenesis and Spermiation

The preparation for spermatogenesis begins during fetal life. After birth, stem cells are transformed to spermatogonia which lie dormant until puberty when proliferative activity resumes. These rapidly dividing, developing and maturing cells are highly susceptible to chemical insult at many stages. Spermatogenesis is a chronological process that takes about 42 days in the rodent and 72 days in man. Spermatogenesis can be divided into two separate stages (Ecobichon, 2002):

1. **Spermatocytogenesis**: during which the diploid Type A spermatogonia replicate by mitosis, each cell undergoing five mitotic divisions to form a host of Type B spermatogonia to be converted subsequently into primary spermatocytes and into haploid spermatids.
2. **Spermiogenesis**: involving the differentiation of the spermatids into spermatozoa, the metamorphosis from a rounded cell into the characteristically shaped, mature spermatozoon comprising of an acrosome covered head which contains the condensed DNA; a midpiece containing densely packed mitochondria; and a long flagellum called the tail region. When spermiogenesis is completed, the spermatozoon is released into the lumen of the seminiferous tubule. Further maturation in the epididymis results in fertility and motility being conferred on the spermatozoon and the generation of suitable fluid vehicle. This fluid is made up of secretions contributed by accessory glands (prostate), the seminiferous vesicles, and the epididymis. Gonadotoxic agents may interfere with any of these functions or act on the epididymal tissue and/or the spermatozoon during this maturation period of 12 (rat) to 21 (human) days residence time.

Spermiation is the release of mature germ cells (spermatozoa) from the seminiferous tubules. The duration of the whole process of spermatogenesis, from spermatogonial division through spermiation, is species and strain dependent (Ecobichon, 2002) (see Figure 2.5).

2.3.6 Gonadotoxicity and Gonadotoxicants

A biological, chemical or physical agent which affects physiological control processes and the normal functioning of the gonads can result in gonadotoxicity or reproductive toxicity. Such an agent is called a gonadotoxicant or an endocrine disrupter or a gonadotoxic agent. The duration of the effect may vary from transient dysfunction to permanent gonadal damage and may be mild or severe. This may be as a result of the direct chemical action of the agent or indirectly via the metabolic products formed during the reaction process. A potential gonadotoxicant can interrupt the normal function of the male reproductive system at one or more of the following points (Sikka and Naz, 2002):

i) any level of the Hypothalamic-Pituitary-Gonadal (HPG) axis

ii) directly at the gonadal level

iii) altering post-testicular events such as sperm motility or function or both.

Any disruption of these events may lead to infertility, hypogonadism and / or decreased libido/ sexual function.

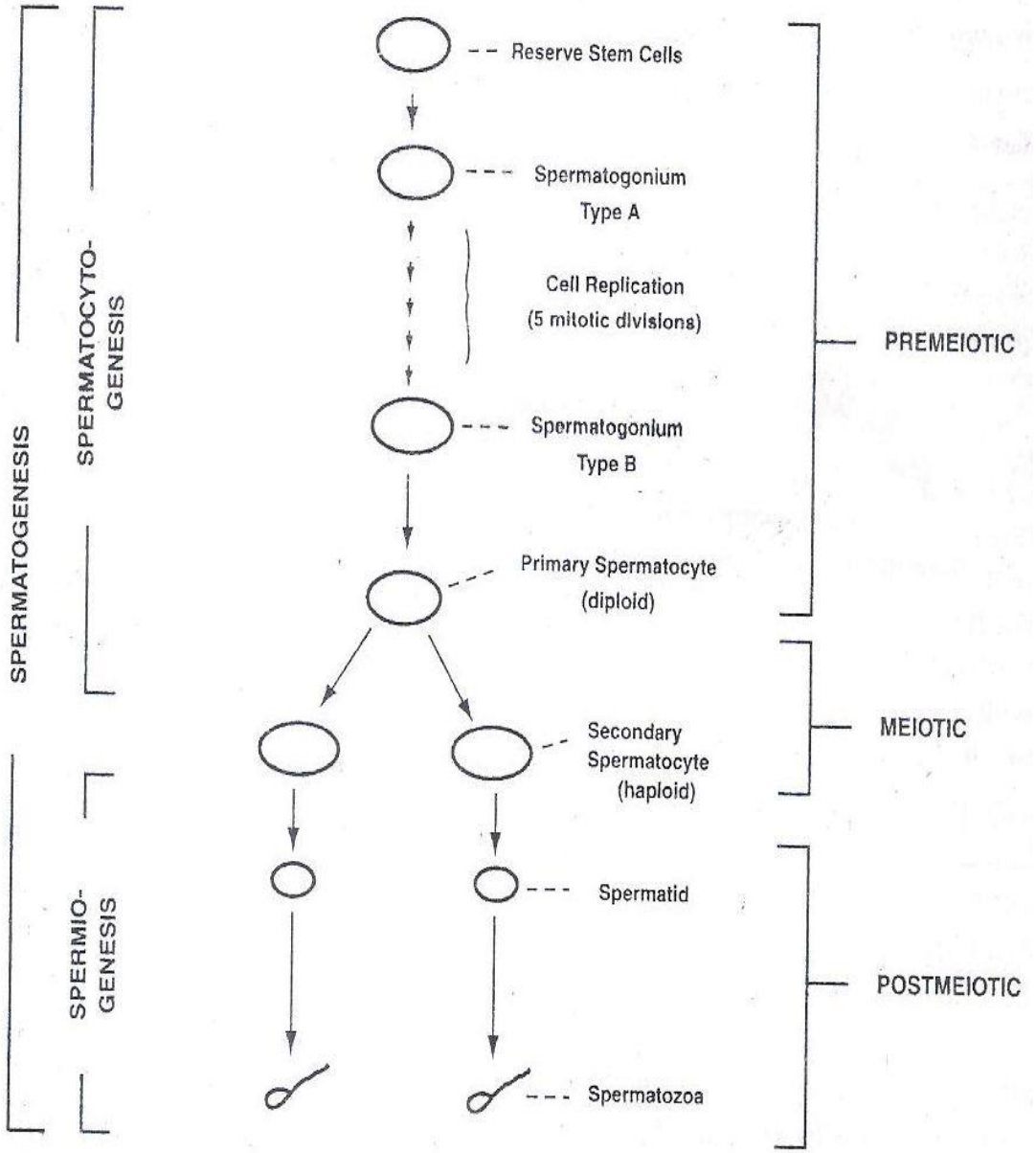

Figure 2.5. Spermatogenesis

Source: Sikka and Naz, 2002

There are three major classes of gonadotoxicants and they are as follows:

i) **Environmental/ Occupational agents**: These are wide spread in the environment. They have the ability to bioaccumulate and resist biodegradation. Examples are agricultural chemicals (e.g. *o,p*-dichlorodiphenyl-trichloroethane (DDT) a pesticide), industrial chemicals (e.g. ethylene glycol monomethyl ether a solvent) and heavy metals (e.g. lead).

ii) **Pharmacological agents**: These include radiation therapy, chemotherapeutic agents, anabolic steroids and pharmacological drugs especially at high doses.

iii) **Biological Conditions**: These include hyperthermia, chronic infection/ inflammation, aging and oxidative stress.

The mechanism(s) of action of these gonadotoxicants involve changes in hormone biosynthesis, hormone storage and/or release, hormone transport and clearance, hormone receptor recognition and binding, hormone postreceptor activation and induction of oxidative stress (Sikka and Naz, 2002).

2.3.7 Oxidative stress and the male reproductive system

Oxidative stress is a condition induced by oxygen, nitrogen-derived free radicals known as reactive nitrogen species (RNS) and oxygen- derived free radicals known as reactive oxygen species (ROS) (Lobo *et al.*, 2010; Nimse, 2015).

The presence of ROS in biological systems is as a result of normal physiological processes and cell activation (e.g. immunological responses and apoptosis) (Vernet *et al.*, 2004). Exposure to many types of environmental contaminants, aging, chronic disease state or gonadal injury can induce oxidative stress associated with an increased rate of cellular damage that results in gonadotoxicity (Sikka and Naz, 2002).

The role of spermatozoa is to fertilize the oocyte of the female reproductive system and the plasma membrane is very rich in polyunsaturated fatty acids (PUFA). This makes spermatozoa vulnerable to lipid peroxidation due to attacks from ROS which invariably leads to impairment of sperm function through oxidative stress (Vernet *et al*., 2004).

In the spermatozoa, the sources of ROS are both external and internal. External source of ROS, particularly superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) can be as a result of leukocyte contamination of the semen. This generation of ROS has been linked with sub-fertility and infertility in patients (Vernet *et al.*, 2004). Internally, two mechanisms involved in ROS have been identified in rat epididymal spermatozoa; one mechanism relies on the mitochondrial respiratory chain while the other mechanism depends on an enzymatic system related to the NADPH oxidase family found bound to the sperm plasma membrane (Vernet *et al*., 2001).

The major ROS with toxic manifestations to the male reproductive system include superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and nitric oxide ($NO^{\cdot}$) radicals. $NO^{\cdot}$ and $O_2^{\cdot-}$ combine to form highly reactive peroxynitrite radicals that induce endothelial cell injury which may lead to altered blood flow to the testis and impair testicular function. All these can contribute to hormonal imbalance, gonadal dysfunction and poor sperm motility/ function leading to infertility (Sikka and Naz, 2002).

Present within the male genital tract, seminal plasma and the spermatozoa are radical-scavenging molecules, refered to as antioxidants, which rid the system of the ROS. These radical scavenging molecules are of two types; non- enzymatic and enzymatic (Adedara and Farombi, 2010; Lobo *et al*., 2010). The non-enzymatic radical

scavenging molecules include α- tocopherol, ascorbic acid, uric acid, glutathione, pyruvate, taurine, hypotaurine and albumin. The enzymatic radical scavenging molecules include superoxide dismutase (SOD), glutathione peroxidase (GPX), indolamine dioxygenase and catalase (CAT) (Vernet *et al.*, 2004).

Oxidative stress is caused by a defect in the balance between the production of ROS and the antioxidant system. Cellular oxidative injury as a result of oxidative stress has been shown to be responsible for many pathological conditions or disease states including aging and infertility (Wang *et al*., 2003; Pham-Huy *et al*., 2008; Lobo *et al*., 2010).

2.4 Ethylene Glycol Monomethyl Ether (EGME)

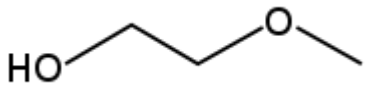

Figure 2.6. Chemical Structure of EGME

Ethylene glycol monomethyl ether (EGME) belongs to the group of solvents referred to as glycol ethers. Glycol ethers are alkyl ethers of ethylene glycol commonly used in paints and this group is made up of two classes: ethylene glycol ethers (EGEs) and propylene glycol ethers (PGEs). EGME belongs to the class of EGEs (Cordier and Multigner, 2005). EGME is also known as methyl cellosolve (commercially), 2-methoxy ethanol, monomethyl ether, methyl glycol, monomethyl glycol, monomethyl ethylene glycol ether or methyl oxitol.

The molecular formula and weight are $C_3H_8O_2$ and 76.11 respectively. The active biological oxidation product is methoxy acetic acid (MAA). EGME is a reaction product of ethylene oxide and methanol. It is highly inflammable, colorless, and moderately volatile with very good solubility properties. As a result of the

simultaneous hydrophilic and lipophilic properties, it has wide consumer and industrial applications. EGME is used as an anti-freeze additive in hydraulic fluids and jet fuel. It is also used in paints, stains, inks and surface coating, lacquers, photographic and photo lithographic processes, production of food-contact plastics, textile and leather finishing, and silk-screen printing as well as in the semi-conductor industry (Johanson, 2000; Takei *et al.*, 2010).

In humans and several other species, exposure to EGME either by inhalation, ingestion and/or dermal absorption has been reported to cause reproductive, haematopoietic and developmental toxicities with emphasis on testicular damage (Bagchi and Waxman, 2008). The evidences of haematological toxicities as a result of exposure to EGME include leucopenia; pancytopenia; marrow depression and decrease in red blood cells count, platelet count, packed cell volume, haemoglobin concentration, mean corpuscular and mean corpuscular haemoglobin (Starek *et al.*, 2010; Bendjeddou and Khelili, 2014).

The evidences of reproductive toxicities as result of exposure to EGME or the active oxidation product, MAA, include decreased sperm production as a result of increased apoptosis of primary spermatocytes (Li *et al*., 1996); gene expression changes in germ cells and Leydig cells i*n-vitro* (Bagchi *et al.,* 2010); hyper-secretion of progesterone from ovarian luteal cells both *in-vivo* and *in-vitro*; activation of caspases leading to apoptosis triggered by oxidative stress in spermatocytes (Bagchi and Waxman, 2008); prolonged estrus cycle, hypertrophy of corpora lutea evidenced by the presence of round to polygonal luteal cells with abundant vacuolated cytoplasm and ovulatory inhibition (Dodo *et al.,* 2009; Taketa *et al.,* 2011); altered androgen-dependent processes in Leydig cells *in-vitro* (Bagchi *et al.*, 2011); affects microRNAs expression

in the testes (Fukushima *et al.*, 2011); affects the antioxidant system and increase lipid peroxidation in the testes (Malik and Gupta, 2013).

Other acute and chronic toxic effects as a result of exposure to EGME have been reported in humans and animals (NIOSH/OSHA, 1981; Ohi and Wegman, 1978). These include drowsiness; weakness; irritation of throat, eyes and nose; headache and decreased mental ability. Acute toxicity studies have shown that the LD_{50}, (i.e. lethal dose, dose of 50% mortality) is 0.95 g/kg body weight in guinea pigs, 0.89 to 1.425 g/kg body weight in rabbits, 2.46 to 3.25 g/kg body weight in male rats and 3.4 g/kg body weight in female rats (NIOSH, 1991). However, MAA toxicity can be mitigated by co-administration with D- glucose, serine, acetate, glycine, certain tricarboxylic acid pathway metabolites and formate (reviewed in Bagchi and Waxman, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Reagents

Ethylene glycol monomethyl ether (EGME) and trichloro acetic acid were products of LobaChemie (Mumbai, India). Alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, total bilirubin, albumin, total cholesterol, triglyceride, urea and creatinine were kits from Randox (Antirm, U.K). Dimethyl sulfoxide (DMSO) and methanol were products of Analar (England). Immersion oil (with refractive index 1.5) was a product of PanscanXtra (U.K). Giemsa's stain, di-sodium hydrogen ortho phosphate-12-hydrate, sodium carbonate-10-hydrate, ascorbic acid, hydrogen peroxide (30 % v/v), hydrochloric acid, sulphuric acid, potassium sodium tartrate, tris-hydroxymethyl aminomethane HCl, copper (II) sulphate-5-hydrate, sulphosalicyclic acid, ammonium iron (II) sulphate, sodium carbonate anhydrous, sodium hydrogen carbonate, sodium azide, ethanol and xylenol orange were all products of Sure Chem (Suffolk, England). Tris hydrochloride, tris (hydroxyl methyl) amino methane, 2-thio barbituric acid, 1-chloro-2, 4-dinitrobenzene, epinephrine, sodium pyruvate, reduced nicotinamide adenine dinucleotide, D-sorbitol, 5,5′-Dithiobis (2-nitrobenzoic acid), gluthathione reduced, o-dianisidine, bovine serum albumin, Folin-ciocalteau were all products of Sigma-Aldrich, St. Louis (U.S.A). Potassium phosphate monobasic was a product of Fisher Scientific (New Jersey). Potassium chloride and Sodium chloride were products of MRS Scientific (Essex, U.K). Di-potassium hydrogen orthophosphate was a product of Inter Chem (U.K).

3.2 Drugs

Amodiaquine and chloroquine were products of Sigma-Aldrich (St. Louis, MO, U.S.A). Artesunate was a product of Swiss Pharma Ltd. (Lagos, Nigeria- Expiry date:- August 2019). Sulfadoxine and Pyrimethamine were products of Bond Chemicals (Oyo State, Nigeria- Expiry dates:- February 2018 and November 2017 respectively).

3.3 Plant Material

The leaves of *Paullinia pinnata* were collected by Mr. Micheal Onadeji from Odujobi village, Ido Local Government area, Oyo State, Nigeria. The plant was authenticated and deposited in the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria, and given the specimen voucher number FHI I06555.

3.4 Preparation and extraction of plant material

The leaves were air-dried, milled and extracted by cold maceration in absolute methanol initially for a period of six days. The solvent was filtered and the marc was re-soaked in absolute methanol for twenty-four hours. This was repeated three times and the recovered solvent was pooled and concentrated using a rotary evaporator (Heidolph HB, Germany) and a vacuum oven (Gallenhamp, England) at a temperature of (40-42)^{o}C. A 14 % yield of the extract was realized.

3.5 Animal experiment ethical review

The protocol for the animal studies was approved by the Animal Care and Use Research Committee of the University of Ibadan, Nigeria and the number UI-ACUREC/APP/10/2016/003 was assigned. The methods for the handling of the animals, mode of administration and the group size were in compliance with International Scientific

standard procedures for the acute toxicity study (Toskulkao *et al*., 1997 and Kennedy *et al*., 2001) and sub-chronic toxicity study (Karrow *et al*., 2001).

3.6 Experimental Animals

Fifty-four (54) six week old (18-25 g) male Wistar mice were used for the acute toxicity study. Thirty-six (36) nine week old (140-190 g) male Wistar rats were used for the sub-chronic toxicity study. Thirty-six (36) six week old (20-30 g) Wistar mice of both sexes were used for the curative study taking the safe dose of *P.pinnata* methanol leaf extract into consideration. In the anti-plasmodial activity study using the higher dose, sixty-six (66) six week old (22-25g) Wistar mice of both sexes were used for the curative test; thirty (30) six week old (18-23 g) Wistar mice of both sexes were used for the prophylaxis test and thirty (30) six week old (18-27 g) Wistar mice of both sexes were used for the suppressive test.

Fifty (50) nine week old (140-190 g) male Wistar rats were used in the sub-acute toxicity study of EGME. Sixty (60) ten week old (165-190 g) male Wistar rats were used to investigate the chemopreventive potential of *Paullinia pinnata* methanol leaf extract.

The animals were obtained from the Central Animal House, the Primate Colony of the Department of Biochemistry and the Animal House of the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan, Nigeria. The animals were left to acclimatize for a week in the Animal House of the Department of Biochemistry, University of Ibadan. They were kept in appropriate plastic laboratory animal cages and had access to mice cubes (Ladokun Feed, Ibadan, Nigeria) and water *ad libitum*. The twelve (12) hour light/dark cycle was maintained in all the *in-vivo* experiments.

3.7 Parasite and Inoculation

In the curative test taking the safe dose into consideration and anti-plasmodial study at a higher dose, chloroquine-resistant *Plasmodium berghei berghei* NK 65 obtained from Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan was used. A standard inoculum size of 1 x 10^7 of the parasite was collected from parasitized erythrocytes of a donor mouse. This was prepared by taking 0.2 ml of blood of the donor mouse in normal saline and was used to infect the experimental animals intra-peritoneally.

3.8 Experimental Design

3.8.1 Experiment I: Phytochemical screening

Phytochemical screening was conducted using the methods of Sofowora (1993) and Trease and Evans (1989). Shade- dried and pulverized leaves of *P.pinnata* (30 g) was macerated in 450 ml of redistilled methylated spirit and filtered. The process was repeated until all soluble compounds had been extracted as indicated by loss of colour of filtrate. The total extract from the leaves was evaporated to dryness *in vacuo* at 45^{o}C and further dried to constant weight at the same temperature in a hot- air oven. Portions of the extract were then used to test for the presence of alkaloids, flavonoids, saponins, tannins, anthraquinones (free and combined), terpenoids and cardiac glycosides.

3.8.1.1 Test for Alkaloids: The extract (0.5 g) was mixed with 5 mL of 1 % aqueous hydrochloric acid on a steam bath. 1 mL of the filtrate was treated with a few drops of Mayer's reagent and a second 1 mL portion similarly with Dragendorff's reagent. Turbidity or presence of precipitate was taken as a preliminary evidence for the presence of alkaloids in the extract. A confirmatory test was then carried out as follows: 1 g of the extract was mixed

with 40% calcium hydroxide solution until the extract was alkaline to litmus paper and then extracted twice with 10 mL aliquots of chloroform. The extracts were pooled and concentrated *in vacuo* to about 5 mL. The chloroform extract was then spotted on thin- layer chromatography plates. Four different solvent systems with widely different polarities were used to develop the spots. To detect the presence of alkaloids, the chromatograms were sprayed with freshly prepared Dragendorff's reagent. An orange or darker- coloured spot against a pale yellow background confirms the presence of alkaloids.

3.8.1.2 Test for Flavonoids*:* An aqueous filtrate of the extract was prepared and divided into three portions. Three methods were then used separately to test for the presence of flavonoids. First, 5 mL of dilute ammonia and 1 mL of concentrated H_2SO_4 was added to a portion of the filtrate and a yellow color which disappears on standing was observed. Secondly, to another portion of the filtrate a few drops of 1 % ammonium solution was added and again observed for yellow colouration. Thirdly, a third portion of the filtrate was heated with ethyl acetate (10 mL) for 3 minutes over a steam bath. The mixture was then filtered and 1 mL of dilute ammonia solution was added to 4 mL of the filterate, shaken and observed for a yellow colour as an indication of the presence of flavonoids.

3.8.1.3 Test for Saponins: Distilled water (5 mL) was added to 0.5 g of extract and shaken vigorously. A stable persistent froth, which remains after warming, was taken as a preliminary evidence. For confirmation, 0.5 g of the extract was boiled with 50 mL phosphate buffer (pH 7.4), cooled and filtered. 5 mL of the filtrate was passed for 3 hours through an asbestos disc (1.5 mm thick and 7 mm in diameter) which had been soaked with two to three drops of 1 % cholesterol in ether and dried. The disc was then rinsed with 0.5 mL of distilled water, dried and boiled in 20 mL of oxylol for 2 hours to break the complex that might have formed between cholesterol and any saponin in the extract. The disc was then rinsed in ether,

dried and placed on a 7 % blood nutrient agar. Complete haemolysis of red blood cells around the disc after 6 hours showed the presence of saponins in the extract.

3.8.1.4 Test for Tannins: In a boiling tube, 0.5 g of extract was boiled in 10 mL of distilled water and filtered. A few drops of 0.1 % ferric chloride was added to the filtrate and observed for blue-black, green or blue-green colour/ precipitate.

3.8.1.5 Test for Anthraquinones: Borntrager's test was employed. Briefly, 5 g of the extract was shaken with 10 mL benzene, filtered and 5 mL of 10 % ammonia solution was added to the filtrate. The mixture was then shaken and the presence of a pink, red or violet colour in the ammoniacal (lower) phase indicated the presence of free hydroxy-anthraquinones. For combined anthraquinones, 5 g of the extract was boiled with 10 mL aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5 mL of benzene. The benzene layer separated and half its volume of 10% ammonia solution was added. Similarly, a pink, red or violet colour in the ammonia phase (lower layer) indicated the presence of anthraquinone derivatives in the extract.

3.8.1.6 Salkowski Test: Chloroform (2 mL) was added to 0.5 g of the extract. Concentrated H_2SO_4 (3 mL) was then added to form a layer. Presence of terpenoids is indicated by the formation of a reddish – brown colour at the interface.

3.8.1.7 Test for Cardiac glycosides:

a. **Kedde Test:** An 8 % solution of the extract in methanol (1 mL) was mixed with 1 mL of a 2 % solution of 3,5- dinitrobenzoic acid in methanol and 1 mL of 5.7 % aqueous sodium hydroxide. The spontaneous development of a violet colour indicated the presence of cardenolides in the extract. Gradual fading of this colour to reddish-

brown and then to brownish- yellow with the precipitation of a whitish crystalline solid indicated the presence of a lactone ring in the cardenolide.

b. **Keller- Killiani Test:** The extract (0.5 g) was diluted to 5 mL in distilled water and 2 mL of glacial acetic acid containing one drop of ferric chloride solution was added. This was then underlayed with 1 mL of concentrated sulphuric acid (H_2SO_4). A brown ring forming at the interphase shows the presence of a deoxysugar which is characteristic of cardenolides. A greenish ring may appear just above the brown ring in the glacial acetic acid layer and spread throughout the layer. Also a violet ring may be seen below the brown ring.

3.8.2 Experiment II: Acute-Toxicity Study

Eight different doses of the methanol extract of the leaves were administered orally to the mice after an overnight fast. The doses were 100, 200, 400, 800, 1600, 3200, 6400 and 10,000 mg/kg body weight. The vehicle (physiological saline with Tween 80 (70:30 v/v)) was administered to the control group. The animals were then observed as follows: after Day 1, Day 7 and Day 14 (delayed toxicity) for gross behavioural (reflexes, restlessness, sleeping pattern, locomotive disability) / physiological (loss of hair, redness of eye/ blindness) changes and mortality (Toskulkao *et al.*, 1997; Kennedy *et al*., 2001).

3.8.3 Experiment III: Sub-chronic Toxicity Study

The animals were fasted for 14 hours. Five different doses of the methanol extract of the leaves were then administered orally once daily to the rats at 24 hours interval for 28 days. The doses were 50, 100, 200, 400 and 800 mg/kg body weight. The vehicle (physiological saline with Tween 80 (70:30 v/v)) was administered in the same manner to the control group. The weights of the animals were monitored weekly throughout the period of study. On day 29, after the animals were fasted initially for 12 hours, they were made inactive by cervical

dislocation. The animals were then cut open and blood was collected from the heart using a needle and 5 mL syringe into lithium heparin and ethylene diamine tetra acetic acid (EDTA) specimen bottles. The liver, lungs and kidneys from each animal were promptly removed into chilled 0.9 % NaCl solution. The organs were then blotted using Whatman No.2 filter paper before weighing with a Mettler balance. The organs were then fixed in 10 % formalin, processed, mounted on slides, stained with haematoxylin and counter-stained with eosin for histological examination. Plasma was separated from the blood in which renal and liver function indices which are the activities or levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, total bilirubin, albumin, total cholesterol, triglyceride, urea, bicarbonate and creatinine were determined using appropriate Randox bioassay kits (Karrow *et al.*, 2001). Concentrations of sodium and chloride ions were determined using the Easylite analyzer. Haematological parameters (packed cell volume, white blood cell count, red blood cell count, Heamoglobin concentration, Mean cell haemoglobin concentration, Mean cell volume, Mean cell haemoglobin, neutrophil, platelets, eosinophil, lymphocytes, monocytes) were determined using the methods of Dacie and Lewis (1984) and Baker and Silverton (1985).

3.8.4 Experiment IV: Curative test taking the safe dose into consideration

A modified Rane's method as reported by Ryley and Peters (1970) was used. Thirty-six (36) six week old Wistar mice of both sexes were infected with Chloroquine- resistant *Plasmodium berghei* NK 65 and then divided into six groups of six mice each. Parasitaemia level was determined at 72 hours after infection in blood films which were fixed in methanol, stained with 10 % Giemsa's stain and observed under immersion oil using the binocular light microscope (Olympus, Japan). The day parasitaemia was established was taken as Day 0. Group I was the negative control and received the vehicle (10% DMSO) only. Group II received normal saline. Group III received chloroquine (CQ) (10 mg/kg) only. Group IV

received artesunate (4 mg/kg) - amodiaquine (10 mg/kg) combination (ACT). Groups III and IV were the positive control groups. Groups V and VI received 100 mg/kg and 200 mg/kg doses of the methanol leaf extract of *P.pinnata* respectively. Administration was done orally once for three or four days for the standard drugs or the extract/ vehicle respectively. The body weight, percentage parasitaemia (% parasitaemia), Packed Cell Volume (PCV) and death were monitored daily throughout the period of administration and on 7^{th} day post-administration (Day 11). The percentage survival (% survival) for each of the groups and percentage chemosuppression for each treatment were calculated.

3.8.5 Experiment V: Determination of antiplasmodial activities of the methanol leaf extract of *Paullinia pinnata* at a high dose

(i) **Curative Test:** The modified Rane's method, as reported by Ryley and Peters (1970), was employed.

Sixty- six (66) six week old Wistar mice of both sexes were weight matched into six groups of eleven animals each. The animals were inoculated with chloroquine- resistant *Plasmodium berghei* NK 65 and administration was carried out for four consecutive days after the disease condition had been established.

Administration was done orally to the groups following this protocol:

Group I received the vehicle (10 % DMSO) (Control)

Group II received normal saline (Untreated Control)

Group III received chloroquine (10 mg/kg) (CQ) (Positive control 1)

Group IV received artesunate (4 mg/kg) and amodiaquine (10 mg/kg) (ACT) (Positive control 2)

Group V received *P.pinnata* extract at 200 mg/kg dose

Group VI received *P.pinnata* extract at 400 mg/kg dose

The weight of six of the animals in each group was monitored before inoculation (Day 0), throughout the period of administration (Days 1-4) and a week post- administration (Day 11, follow-up). Blood was collected from the tail vein before inoculation, throughout the period of administration and the period of follow-up. Percentage parasitaemia, packed cell volume and percentage chemosuppression were determined. Survival rate was calculated throughout the period of administration and follow-up. Five of the animals in each group were sacrificed using cervical dislocation after the period of administration. They were then dissected and the liver was excised into ice-cold 1.15 % potassium chloride, blotted with Whatman no. 2 filter paper and then weighed. 1 g of the liver samples were then homogenized, centrifuged and the supernatants were used for the antioxidant (superoxide dismutase, catalase, reduced glutathione, glutathione-S-transferase, hydrogen peroxide, lipid peroxidation, vitamin C, glutathione peroxidase) and anti-inflammatory (myeloperoxidase, nitrite) assays using standard methods described in Section 3.21. The remaining part of the liver was fixed in 10 % formalin, processed, sectioned, mounted on slides, stained with haematoxylin and counter-stained with eosin for histological examination.

(ii) Prophylaxis Test:

The method based on Peters (1967) was employed. Thirty (30) six week old Wistar mice of both sexes were weight matched into five groups of six animals each. The animals were inoculated with chloroquine- resistant *Plasmodium berghei* NK 65 (Day 4) after administration for four consecutive days (Days 1-4).

Administration was carried out orally to the groups following this protocol:

Group I received normal saline (Control)

Group II received chloroquine (10 mg/kg) (CQ) (Positive control 1)

Group III received sulphadoxine (25 mg/kg) and pyrimethamine (1.25 mg/kg) (SP) (Positive control 2)

Group IV received *P.pinnata* extract at 200 mg/kg dose

Group V received *P.pinnata* extract at 400 mg/kg dose

The weight of the animals was monitored throughout the period of administration (Days 1-4) and 72 hours post-inoculation (Day 7). Blood was collected from the tail vein before the period of administration (Day 1), on the day of inoculation (Day 4) and 72 hours post inoculation (Day 7). Percentage parasitaemia, packed cell volume and percentage chemosuppression were determined.

(iii) Suppressive Test:

The method of Peters *et al.* (2002) was employed. Thirty (30) six week old Wistar mice of both sexes were weight matched into five groups of six animals each. The animals were inoculated with chloroquine- resistant *Plasmodium berghei* NK 65 and administration started two to four hours after inoculation (Day 0) for four days consecutively (Days 0-3).

Administration was carried out orally to the groups following this protocol:

Group I received the vehicle (10 % DMSO) (Control)

Group II received normal saline (Untreated Control)

Group III received chloroquine (10 mg/kg) (CQ) (Positive control)

Group IV received *P.pinnata* extract at 200 mg/kg dose

Group V received *P.pinnata* extract at 400 mg/kg dose

The weights of the animals were monitored throughout the period of administration (Days 0 – 3) and for three days post- administration (Days 4 - 6). Blood was collected from the tail vein on the first day of administration (Day 0, which is also the day of inoculation) and for three days post- administration (Days 4 - 6). Percentage parasitaemia, packed cell volume and percentage chemosuppression were determined. Survival rate was monitored weekly from the day after inoculation (Day 1) to the 30th day (Day 30).

3.8.6 Experiment VI: Sub-acute toxicity study of Ethylene glycol monomethyl ether (EGME)

Fifty (50) nine week old (140- 190 g) male Wistar rats were randomly distributed into five groups of ten animals each.

All administrations were carried out orally daily at the same time for fourteen consecutive days and the weight was monitored weekly. The protocol for administration is as follows:

Group I received distilled water only (Control)

Group II received EGME at 100 mg/kg body weight dosage

Group III received EGME at 200 mg/kg body weight dosage

Group IV received EGME at 300 mg/kg body weight dosage

Group V received EGME at 400 mg/kg body weight dosage

On day 15, the animals were euthanized by cervical dislocation after an overnight fast. The animals were then dissected and the testes, epididymes, prostate gland and seminal vesicles

of each animal were removed into ice- cold 1.15 % potassium chloride, blotted with Whatman No.2 filter paper and weighed using a Mettler balance. The right testes and epididymes samples were used for spermatozoa analysis (testicular sperm number, daily sperm production, abnormality, motility, viability, epididymal sperm number (Zemjanis, 1970; Blazak *et al.*, 1993) and fixed in Bouin's solution, processed, sectioned, mounted on slides, stained with haematoxylin and counter-stained with eosin for histological examination. The left testes and epididymis were used for the biochemical analyses of antioxidant markers (superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, reduced glutathione, lipid peroxidation, lactate dehydrogenase, vitamin C) by employing standard methods described in Section 3.21. Blood was collected by ocular puncture before euthanasia into di- potassium ethylene diamine tetra acetic acid (K_2- EDTA) specimen bottles using heparinized capillary tubes and the haematological parameters (packed cell volume, white blood cell count, red blood cell count, heamoglobin concentration, mean cell haemoglobin concentration, mean cell volume, mean cell haemoglobin, neutrophil, platelets, eosinophil, lymphocytes, monocytes) were determined using the Mindray BC 3000 Autohaematology analyzer.

3.8.7 Experiment VII: Chemopreventive study of *Paullinia pinnata* methanol leaf extract in EGME-induced testicular dysfunction

Sixty (60) ten week old (165- 190 g) male Wistar rats were obtained from the Primate colony of the Department of Biochemistry and weight matched into six groups of ten animals each. The treatment protocol was done orally for twenty-one consecutive days as follows:

Group I was the normal rats without treatment (Control 1)

Group II received the vehicle (10 % dimethyl sulfoxide (DMSO) (Control 2)

Group III received EGME only at 200 mg/kg dose

Group IV received *Paullinia pinnata* extract only at 200 mg/kg body

Group V received EGME (200 mg/kg dose) + *P.pinnata* extract (100 mg/kg) (PP 100)

Group VI received EGME (200 mg/kg dose) + *P.pinnata* extract (200 mg/kg) (PP 200)

On the 22nd day, after an overnight fast, blood was collected by ocular puncture using heparinized capillary tubes into ethylene diamine tetra acetic acid (EDTA) specimen bottles. The animals were euthanized by cervical dislocation under light anaesthesia. The brain, seminal vesicles, epididymes, testes and prostate gland of each animal were excised and weighed. The left testes and epididymis, and the brain were homogenized and centrifuged to obtain the supernatant and used for biochemical analyses of antioxidant (superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase, reduced glutathione, lipid peroxidation, lactate dehydrogenase, vitamin C, hydrogen peroxide) and inflammatory markers (MPO, NO) by employing standard methods described in Section 3.21. The right testes and epididymes were used for spermatozoa analysis (testicular sperm number, daily sperm production, abnormality, motility, viability, epididymal volume (Zemjanis, 1970; Blazak *et al.*, 1993) and fixed in Bouin's solution, processed, sectioned, mounted on slides, stained with haematoxylin and counter-stained with eosin for histological examination. Samples of the brain were fixed in 10 % formalin then the pituitary gland and hypothalamus were sectioned and observed histologically. Plasma was separated from the blood and used to determine the level of the male reproductive hormones which are luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone, triiodothyronine (T_3) and thyroxine (T_4) using commercial enzyme immunoassay kits.

3.9 Separation of plasma

Plasma was separated by centrifuging the blood samples in the lithium heparin and EDTA

bottles at 3000 rpm for 10 mins using an MSE bench top centrifuge (England, U.K.). The plasma was removed and collected into plain bottles.

3.10 Preparation of blood smears and staining of slides for determination of parasitaemia

Blood was collected from the tail vein of the mice on two separate slides for each animal. One slide was used to make the thick film while the second slide was used to make the thin film. The slides were left to dry and the thin film was fixed with absolute methanol. The methanol was allowed to evaporate and the thin smear was then stained with 10% Giemsa stain for 30 mins. The slide was then rinsed under gently running water and then left to dry on a slide rack.

3.11 Determination of Packed cell volume (PCV)

Blood was collected from the tail vein into heparinized capillary tube, sealed with plasticine and centrifuged using a microhaematocrit centrifuge (Hawksley, England) for 6 mins. To avoid inducing anaemia in the animals a ruler was used instead of a haematocrit reader. The level of packed cell was measured, divided by the level of the whole blood and the result was multiplied by 100.

3.12 Determination of percentage parasitaemia

The stained blood smears on slides were examined under the binocular light microscope (Olympus, Japan) using the x100 objective lens under a drop of immersion oil in order to assess the activity of the drug/extract on the parasite. Percentage parasitaemia was calculated by using the following equation:

(no. of infected red blood cells/ total no. of red blood cells) x 100

3.13 Determination of percentage chemosuppression

Percentage chemosuppression was determined using the following equation:

(Average parasitaemia in control group - Average parasitaemia in treatment group/ Average parasitaemia in control group) x 100.

3.14 Determination of percentage survival

(no. of animals that survived in a day /total no. of animals in the group) x 100

3.15 Haematological Analysis

In the sub-chronic toxicity study (Experiment III), the laboratory procedure as described in the method of Baker and Silverton (1985) for haemoglobin concentration, differential leukocyte count, PCV, MCV, MCHC and MCH was employed, and the method of Dacie and Lewis (1984) was applied for the determination of RBC and WBC and are briefly described as follows:

3.15.1 Determination of packed cell volume (PCV)

The microhaematocrit method of Baker and Silverton (1985) was employed. The procedure is as follows: A plain capillary tube was used to pick anti–coagulated blood from the EDTA specimen bottle. The lower end of the tube was sealed with plasticine and was then placed on a microhaematocrit centrifuge (Hawksley England). After centrifugation for 5 min at a speed of 30,000 rpm, the PCV was determined by measuring the height of the red blood cell column with a PCV reader and this was expressed as a percentage of the height of the total blood column.

3.15.2 Determination of hemoglobin (Hb) concentration

The cyanmethaemoglobin method of Baker and Silverton (1985) was also employed. The procedure is as follows: 20 µL of blood was added to 4 mL of Drabkin's solution. The absorbance of this solution with the blood was read using a colourimeter (Gulfex, England) at a wavelength of 540 nm after mixing by inversion several times and allowed to stand at room temperature for 10 min. An ampoule of cyanmethaemoglobin standard was opened, and the absorbance was read in the same colourimeter against the reagent blank. The final Hb concentration was then calculated.

3.15.3 Determination of red blood cell count (RBC)

The procedure of Dacie and Lewis (1984) was used and is as follows: A 1 in 200 dilution of the blood sample was made in formol – citrate solution. The solution was made up by adding 10 mL of formalin (40% formaldehyde) to a 1 L solution of 32 g/L sodium citrate. 0.02 mL of each blood sample was added to 4.0 mL of diluent (formol – citrate solution), mixed thoroughly and then loaded onto an improved Neubauer counting chamber. The RBC count for the whole blood sample was then calculated.

3.15.4 Determination of white blood cell count (WBC)

The method of Dacie and Lewis (1984) was used and is as follows: 2% acetic acid - tinged with gentian violet was used as diluent. 0.02 mL of the blood sample was added to 0.38 mL of diluent to give a final dilution of 1 in 20. The diluted sample was then mixed, and a Pasteur pipette was used to load the sample on the counting chamber. The WBC for the whole blood sample was then calculated.

3.15.5 Determination of the mean cell hemoglobin concentration

This was calculated from the Hb concentration and PCV values (Baker and Silverton, 1985).

3.15.6 Determination of mean cell hemoglobin

This was calculated from the Hb concentration and the RBC count (Baker and Silverton, 1985).

3.15.7 Determination of the mean cell volume

This was calculated from the PCV and the RBC count (Baker and Silverton, 1985).

3.15.8 Differential leukocyte count

This was performed on thin blood films which were prepared on slides by the spread technique as reported by Baker and Silverton (1985). The slides were then stained with Leishmann's stain and allowed to fix for 2 min and then diluted with 1 mL of buffered distilled water (pH 6.8) (49.6 ml of 0.067 M Na_2HPO_4 (anhydrous) and 50.4 mL 0.067 M KH_2PO_4 (anhydrous) and allowed to stain for 10 min. The stain was then rinsed off with the buffered distilled water and then kept on the bench in a slant position. After drying, a drop of immersion oil was placed on the slide and the film examined with a ×100 objective lens on a binocular light microscope (Olympus, Japan). The cells were counted using the longitudinal method by counting the different types of white cells seen in one complete longitudinal strip of the film. If <100 cells are counted, a second strip was similarly enumerated. The result of each cell type was then expressed as a percentage. Nuclei of leukocytes stain purple; eosinophilic granules stain orange red; lymphocytes stain dark blue nuclei with pale blue cytoplasm; RBCs stain salmon pink; neutrophils stain lilac and cytoplasm of monocytes stain pale grey blue with Leishmann's stain.

In the sub- acute toxicity study of EGME (Experiment VI), haematological analysis was carried out using the Mindray BC 3000 Autohaematology analyzer. The packed cell volume (PCV), haemoglobin concentration (Hb), total White Blood Cell Count (WBC), Red Blood

Cell Count (RBC), platelets, differential leucocyte count (i.e. percentage values of neutrophils (N), monocytes (M), lymphocytes (L) and eosinophils (E)), blood indices and blood constants, which are the Mean Cell Haemoglobin Concentration (MCHC), Mean Cell Haemoglobin (MCH) and Mean Cell Volume (MCV), were determined.

3.16 Spermatozoa Analysis

3.16.1 Assessment of the characteristics of epididymal sperm

The method of Zemjanis (1970) was employed to evaluate the motility of the sperm. Briefly, the cauda epididymal sperm was released on a sterile clean glass slide. 2.9 % sodium citrate dehydrate solution was added to the sperm and thoroughly mixed in order to evaluate the sperm progressive motility using a light microscope within 2- 4 mins of isolation. The data were expressed as percentages. The improved Neubauer chamber haemocytometer (LABART, Munich, Germany) was used to count the sperm according to Pant and Srivastava (2003). Employing the method of Wells and Awa (1970), part of the sperm suspension on a glass slide was made into a thin smear and then stained with Wells and Awa's stain (0.6 g fast green and 0.2 g eosin dissolved in distilled water and ethanol in a 2:1 ratio) for morphologic examination and also 5% nigrosin and 1 % eosin in 3 % sodium citrate dehydrate solution for viability. A total of 400 sperm/ rat were used for examination of the morphology.

3.16.2 Testicular sperm number (TSN) and Daily sperm production (DSP) determination

The method of Blazak *et al.* (1993) was employed. Briefly, frozen right testes from the control and treatment groups were decapsulated and homogenized in ice- cold physiological saline with 0.01% Triton X- 100. An aliquot of the homogenate was transferred to a glass vial

and stored on ice. Aliquots of this stored homogenate were placed and counted twice on the improved Neubauer chamber haemocytometer at a magnification of x100 using a light microscope to determine the number of sperm heads (heads of spermatid at stage 19). Using these values, the total number of spermatids per gram of testis was determined. To calculate the DSP, the number of spermatids at stage 19 was divided by 6.1 (6.1 is the period of seminiferous cycle in which the spermatids are present in the seminiferous epithelium).

3.17 Reproductive hormone assay

3.17.1 Determination of plasma testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations

Commercial enzyme immunoassay kits (DRG Diagnostics, Germany) were used as per manufacturer's instructions to assay for the plasma concentrations of testosterone, LH and FSH. The sensitivity of the testosterone assay was 0.08 ng/mL and with negligible cross reactivity with other androgen derivatives like androstenedione, 5α-dihydrotestosterone and methyl testosterone.

3.17.2 Determination of plasma concentrations of triiodothyronine (T_3) and thyroxine (T_4)

Commercial enzyme immunoassay kits (DiaSorin, Italy) were used as per manufacturer's instructions to assay for the plasma concentrations of total T_3 and T_4. Inter assays coefficients of T_3 was 3.1- 8.9% and that of T_4 was 2.7- 3.8%. Sensitivity of the assays was 30 pg/mL for total T_4 and 700 pg/mL for total T_3. Plasma concentrations of total T_3 and T_4 were expressed as ng/mL and mg/dL respectively. All the samples were assayed on the same day to avoid the inter assay variation.

3.18 Preparation of wash buffer (1.15% Potassium chloride)

11.5 g of potassium chloride was dissolved in distilled water and made up to 1000 ml with the same and stored at 4 ^{o}C.

3.19 Preparation of homogenizing buffer (50 mM Tris- HCl, 1.15% KCl, pH 7.4)

7.80 g of Tris (hydroxymethyl) amino methane and 11.5 g of potassium chloride were dissolved in 900 mL of distilled water. The pH was adjusted to 7.4 and then made up to 1 litre with distilled water.

3.20 Preparation of supernatant

With reference to the weight of the organs, 4 volumes of the homogenizing buffer (Tris- HCl, pH 7.4) with liver, testes and brain, and 20 volumes of the homogenizing buffer with epididymes were homogenized using a Teflon homogenizer. The homogenates were then centrifuged at 10,000 g for 10 mins at 4 ^{o}C. The supernatants were then separated, stored in ice and used for the antioxidant and anti-inflammatory assays as well as the liver function test in Experiment V.

3.21 Biochemical Analysis

3.21.1 Determination of Alanine Aminotransferase:

The heparinised plasma was analysed using Glutamic- Pyruvic Transaminase Randox kits based on the method of Reitman & Frankel (1957).

Principle: Alanine aminotransferase (ALT) catalyses the transfer of the α-amino group from L-alanine to α-ketoglutarate to produce pyruvate and L-glutamate respectively. The activity of this enzyme was measured by monitoring the concentration of pyruvate hydrazone formed

during the reaction of pyruvate with 2,4-dinitrophenyl hydrazine. The absorbance of pyruvate hydrazone was read at 540 nm.

Reagents:

Content	Initial Concentration of Solution
1. Buffer Phosphate buffer L-alanine α-ketoglutarate **2. 2, 4-dinitrophenyl hydrazine**	 100 mmol/L pH 7.4 200 mmol/L 2.0 mmol/L 2.0 mmol/L

Measurement against Reagent Blank:

Pipette into Test Tube	**Reagent Blank (mL)**	**Sample (mL)**
Sample	-	0.1
Solution 1	0.5	0.5
Distilled Water	0.1	-
Mix, incubate for exactly 30 min at 37°C		
Solution 2	0.5	0.5
Mix, allow to stand for exactly 20 min at (20-25)°C		
Sodium Hydroxide	5.0	5.0

Mix, read the absorbance of sample against the reagent blank after 5 minutes.

Calculations:

The activity of ALT in the plasma was obtained from a standard reference table of ALT activity supplied with the Randox kit.

3.21.2 Determination of Alkaline Phosphatase:

Heparinised plasma samples of the rats were analysed using Alkaline Phosphatase (ALP) Randox kits based on the colorimetric optimized standard method according to the recommendations of the Deutsche Gesellschaft Klinische Chemie (1972).

Principle:

p-nitrophenyl phosphate + H_2O $\xrightarrow{ALP}$ PO_3^{2-} + *p*-nitrophenol

The absorbance of *p*-nitrophenol is read at 405 nm.

Reagents:

Content	Initial Concentration of Solution
1. **Buffer** Diethanolamine buffer $MgCl_2$ 2. **Substrate** *p*-nitrophenylphosphate	 1.0 mmol/L, pH 9.8 0.5 mmol/L 10.0 mmol/L

Procedure:

	Sample (mL)	Reagent Blank (mL)
Distilled Water	-	1.0
Sample	0.01	-
Reagent	0.50	0.50

Mix, read initial absorbance and start timer simultaneously. Read again after 1, 2 and 3 minutes.

Calculation:

ALP activity = 2760 x A 405nm/min

Where,

A = absorbance of plasma samples at 405 nm.

3.21.3 Determination of Aspartate Aminotransferase:

Heparinised plasma samples was analysed using Aspartate aminotransferase (AST) Randox kit based on the method of Reitman and Frankel (1957).

Principle: Aspartate aminotransferase catalyses the transfer of α-amino group from L-aspartate to α-ketoglutarate to produce oxaloacetate and L-glutamate respectively. The activity of the enzyme was measured by monitoring the concentration of oxaloacetate hydrazone formed upon reaction of oxaloacetate with 2, 4-dinitrophenyl hydrazine. The absorbance of oxaloacetate hydrazone was read at 540 nm.

Reagents:

Content	**Initial Concentration of Solution**
1. Buffer Phosphate buffer L-aspartate α-ketoglutarate **2. 2, 4-dinitrophenyl hydrazine**	 100 mmol/L, pH 7.4 100 mmol/L 2 mmol/L 2 mmol/L

Measurement against Reagent Blank:

Pipette into Test Tube	**Reagent Blank (mL)**	**Sample (mL)**
Sample	-	0.1
Solution 1	0.5	0.5
Distilled Water	0.1	-
Mix, incubate for exactly 30 min at 37°C		
Solution 2	0.5	0.5
Mix, allow to stand for exactly 20 min at (20-25)°C		
Sodium Hydroxide	5.0	5.0

Mix, read the absorbance of sample against the reagent blank after 5 minutes.

Calculation:

The activity of AST in the plasma was obtained from a standard reference table of AST activity supplied with the Randox kit.

3.21.4 Determination of Total Cholesterol concentration:

EDTA – plasma samples of the rats were analyzed using cholesterol Randox kits based on the methods of Richmond (1973) and Trinder (1969).

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

$$\text{Cholesterol-ester} + H_2O \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholestene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + H_2O$$

The absorbance of quinoneimine was read at 500 nm.

Reagent Composition:

Content	**Initial Concentration of Solution**
4-aminoantipyrine	0.30 mmol/L
Phenol	6.0 mmol/L
Peroxidase	0.5 U/mL
Cholesterol esterase	0.15 U/mL
Cholesterol oxidase	0.1 U/mL
Pipes Buffer	80 mmol/L; pH 6.8
Standard	5.17 mmol/L (200 mg/dL)

Procedure:

Wavelength	500 nm
Cuvette	1 cm light path
Temperature	(20-25)^{o}C
Measurement	Against reagent blank

Pipette into Cuvette			
	Reagent Blank (µL)	**Standard (µL)**	**Sample (µL)**
Distilled H_20	10	-	-
Standard	-	10	-
Sample	-	-	10
Reagent	1000	1000	1000

Mix, incubate for 10 mins at (20-25)^{o}C and measure the absorbance of the sample against the reagent blank within 60 mins.

Calculation:

Using a standard:

$$\text{Concentration of cholesterol in sample} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of Standard}$$

Where,

ΔA sample = absorbance of plasma at 500 nm

ΔA standard = absorbance of standard at 500 nm

3.21.5 Determination of Triglyceride concentration:

EDTA – plasma samples of the rats were analyzed using the Triglyceride Randox kit based on the methods of Jacobs and Van Denmark (1960), and Trinder (1969). The triglycerides are determined after enzymatic hydrolysis with lipases, using as indicator a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The absorbance of the quinoneimine formed is read at 500 nm.

Reagents

1. Buffer	
Pipes Buffer	40 mmol/L, pH 7.6
4-Chlorophenol	5.5 mmol/L
Magnesium ions	17.5 mmol/L
2. Enzyme Reagent	
4-aminophenazone	0.5 mmol/L
ATP	1.0 mmol/L
Lipases	150 U/mL
Glycerol kinase	0.4 U/mL
Glycerol-3-phosphate oxidase	1.5 U/mL
Peroxidase	0.5 U/mL
3. Standard	2.29 mmol/L (200 mg/dL)

Procedure:

Wavelength	500 nm
Cuvette	1 cm
Temperature	(20-25)^{o}C
Measurement	Against reagent blank

Pipette into Test Tubes			
	Reagent Blank (µL)	**Standard (µL)**	**Sample (µL)**
Sample	-	-	10
Standard	-	10	-
Reagent	1000	1000	1000

Mix, incubate for 10 minutes at (20-25)^{o}C and then measure the absorbance of the sample, (ΔA sample) and standard (ΔA standard) against the reagent blank within 60 minutes.

Calculation:

When using a standard:

$$\text{Triglyceride concentration} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 2.29 \text{ mmol/L}$$

$$= \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 200 \text{ mg/dL}$$

3.21.6 Determination of Creatinine concentration:

Heparinised plasma samples of the rats were analyzed using Creatinine Randox kits based on the Jaffe's Alkaline Picrate method (Henry *et al.*, 1974).

Principle:

Creatinine in alkaline medium reacts with picric acid to form a red tautomer of creatinine picrate, the intensity of which is measured at 520 nm.

Reagents:

Content	Initial Concentration of Solution
1. Creatinine Standard	177 mmol/L
2. Picric Acid	35 mmol/L
3. Sodium Hydroxide	1.6 mol/L
4. Trichloro Acetic Acid (TCA)	1.2 mol/L

Method: Deproteinization

Pipette into centrifuge tubes 1 mL of TCA and 1 mL of heparinised plasma. Mix well using a glass rod to evenly disperse the precipitate and centrifuge at 2500 rpm for 10 minutes; then pour off the supernatant.

Assay:

	Blank (mL)	Standard (mL)	Sample (mL)
Distilled Water	0.5	-	-
Standard	-	0.5	-
TCA	0.5	0.5	-
Supernatant	-	-	1.0
Reagent Mixture (1:1 mixture of reagents 2 &3)	1.0	1.0	1.0

Mix. Allow to stand for 20 minutes at 25ºC. Measure the absorbance of sample (ΔA sample) and standard (ΔA standard) against the blank.

Calculation:

$$\text{Concentration of Creatinine in plasma} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times 2 \text{ mg/dL}$$

3.21.7 Determination of Bilirubin concentration:

Principle: Bilirubin reacts with diazotized sulphanilic acid to form the red colour azobilirubin. In aqueous solution, only conjugated bilirubin (direct bilirubin) reacts and when benzoate-urea is added, all of the bilirubin (conjugated and free) react. Methyl red (2.9 mg per litre at pH 4.63 in acetate buffer) is used as a standard. The colour of this solution accurately matches the colour obtained when 0.016 mg of bilirubin is treated with the diazo reagent in a final volume of 4 mL. The absorbance is read at 520 nm (Varley *et al.*, 1980).

Reagents:

1. **Diazo Reagents:**
    - **Diazo Reagent A:** Dissolve 1.0 g of sulphanilic acid in 15 mL of concentrated hydrochloric acid and make to 1 litre with distilled water.
    - **Diazo Reagent B**: Sodium nitrite 0.5 % solution.
    - **Diazo Reagent Working Solution:** To 5.0 mL of A, add 0.15 mL of B. Prepare fresh.
2. **Diazo Blank:**
    - Hydrochloric acid 1.5 % (v/v) in distilled water.
3. **Benzoate-urea Solution:**
    - Sodium Benzoate 10.0 g
    - Urea 10.0 g

- Add distilled water to 100 mL mark and filter

4. **Methyl Red Standard:**

Stock Standard:

To 0.29 g of methyl red, add glacial acetic acid to 100 mL mark.

Working Standard:

- Stock standard 1.0 mL
- Glacial acetic acid 5.0 mL
- Sodium acetate ($CH_3COONa.3H_2O$) 14.4 g

Make up to 100 mL with distilled water. Adjust pH of this solution to 4.63.

Total Bilirubin Method:

	Test (mL)	**Blank (mL)**	**Working Standard (mL)**
Standard	-	-	0.4
Plasma	0.4	0.4	-
Diazo Reagent	0.2	-	0.2
Diazo Blank	-	0.2	-
BenzoateUrea Solution	3.4	3.4	3.4

Allow to stand at room temperature for 10 minutes and read absorbance at 520 nm wavelength, using distilled water to set at zero.

Calculation:

$$\frac{\text{Test reading}}{\text{Standard reading}} \times 0.016 \times \frac{100}{4}$$

Or

$$\frac{\text{Test reading}}{\text{Standard reading}} \times 0.4 \text{ mg/100 mL}$$

3.21.8 Determination of Total Protein:

Heparinised plasma samples of rats were analyzed using Total Protein Randox kit whose method is based on Henry *et al.* (1974), which is actually the Biuret method.

Principle: Cupric ions in an alkaline medium interact with protein peptide bonds resulting in the formation of a purplish complex, the absorbance of which is read at 550 nm.

Reagents:

Content	Concentration of Solution
1. Biuret Reagent Sodium Hydroxide Na-K-tartrate Potassium Iodide Cupric Sulphate	 100 mmol/L 16 mmol/L 15 mmol/L 6 mmol/L
2. Blank Reagent Sodium hydroxide Na-K-tartrate	 100 mmol/L 16 mmol/L
3. Standard Protein	60 g/L (6.0 g/dL)

Procedure:

Wavelength	550 nm
Cuvette	1 cm light path
Temperature	(20-25)^{o}C
Measurement	Against reagent blank

	Reagent Blank (mL)	Standard (mL)	Sample (mL)
Distilled Water	0.02	-	-
Standard	-	0.02	-
Plasma	-	-	0.02
Solution 1	1.0	1.0	1.0

Mix, incubate for 30 minutes at (20-25)^{o}C and then measure the absorbance of the sample (ΔA sample) and of the standard (ΔA standard) against the reagent blank.

$$\text{Total Protein Concentration} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of Standard}$$

3.21.9 Determination of Albumin:

Heparinised plasma samples of the rats were analyzed by the dye binding method using Bromocresol Green (BCG) (Varley *et al.*, 1980).

Principle:

The measurement of serum/plasma albumin concentrations is based on its quantitative binding to the indicator 3,3′,5,5′-tetrabromo-*m*-cresol sulphonaphthalein (bromocresol green, BCG). The albumin-BCG-complex absorbs maximally at 578 nm, the absorbance being directly proportional to the concentration of albumin in the sample.

Reagent Composition:

Content	Initial Concentration of Solution
1. BCG Concentrate Succinate Buffer Bromocresol Green Brij 35 Preservative	 75 mmol/L; pH 4.2 0.15mmol/L

2. Standard Human Serum Albumin Tris Buffer	 45 g/L (4.5 g/dL) 100 mmol/L; pH 7.3

Procedure:

Wavelength	630 nm
Cuvette	1 cm light path
Incubation Temperature	(20-25)^{o}C
Measurement	Against reagent blank

Pipette into Test tubes:

	Reagent (mL)	**Standard (mL)**	**Sample (mL)**
Distilled Water	0.01	-	-
Standard	-	0.01	-
Plasma	-	-	0.01
BCG Reagent	3.0	3.0	3.0

Mix, incubate for 5 minutes at (20-25)^{o}C. Measure the absorbance of the sample (ΔA sample) and of the standard (ΔA standard) against the reagent blank.

Calculation:

The albumin concentration in the sample may be calculated from the following formula:

$$\text{Albumin Concentration (mg/L)} = \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Concentration of standard} \times 1000$$

3.21.10 Blood Urea Determination:

Heparinised plasma samples of the rats were analyzed using the method of Varley *et al.* (1980).

Principle: When urea is heated with substances containing 2 adjacent carbonyl groups, such as diacetyl, $CH_3CO.COCH_3$, coloured compounds are formed. Diacetyl monoxime, $CH_3CO{=}NOCH_3$ is commonly used because of its greater stability. In this reaction, it decomposes to give hydroxylamine and diacetyl which then condenses with the urea.

Thiosemicarbazide and ferric ions were added to catalyse the reaction and also potentiate colour intensity.

Reagents:

1. **Acid Reagents:**

   Stock Reagent A:

   - 5 g ferric chloride hexahydrate and 20 cm^3 of distilled water.
   - 85 % phosphoric acid, 100 mL. Make up to 250 mL.

   Stock Reagent B:

   - Concentrated sulphuric acid – 200 cm^3
   - Distilled water – 800 cm^3

   Working Acid Reagent:

   - 0.5 cm^3 of Reagent A to 1 litre of Reagent B

2. **Colour Reagent:**

   Reagent A:

   - Diacetyl monoxime – 20 g/L in distilled water.

Reagent B:

- Thiosemicarbazide – 5 g/L in distilled water.

Working Colour Reagent:

- 67 cm^3 of Reagent A and 67 cm^3 of Reagent B. Make up to 1 litre with distilled water.

3. Preservative Diluent for the Urea Standard:

Dissolve 40 g of phenylmercuric acetate in about 250 cm^3 of distilled water with heating. Transfer the solution to a 1 litre flask, add 0.3 cm^3 of concentrated H_2SO_4 and make up to 1 litre with distilled water. Mix.

4. Stock Urea Standard:

- 50 g Urea /litre of preservative diluent.

Working Urea Standard: 200 mg/100 cm^3

- Dilute 10 cm^3 of stock urea standard to 250 cm^3 with preservative diluent.

Diacetyl Monoxime Method:

Stage 1:

	Standard (mL)	**Test (mL)**
Distilled Water	20.0	20.0
Sample	-	0.1
Standard	0.1	-

Stage 2:

	Blank (mL)	Standard (mL)	Test (mL)
Distilled Water	2.0	-	-
Standard from 1	-	2.0	-
Test from 1	-	-	2.0
Colour Reagent	2.0	2.0	2.0
Acid reagent	2.0	2.0	2.0

Mix thoroughly and incubate at 100ºC for 20 minutes. Cool in water and read at 520 nm.

Calculation:

$$\text{Urea mg/100 mL} = \frac{\Delta A \text{ test}}{\Delta A \text{ standard}} \times \text{Concentration of Standard}$$

Where:

ΔA test = absorbance of test at 520 nm

ΔA standard = absorbance of standard at 520 nm

3.21.11 Bicarbonate Determination:

Heparinised plasma samples from the rats were analyzed titrimetrically for bicarbonate.

Procedure:

2 mL of distilled water was added to 100 µL of plasma sample and a 0.01 N monovalent acid (e.g. HCl). Half drop of methyl red was added. The mixture was titrated with 0.01N NaOH. The end colour is yellow. The volume of 0.01 N NaOH used was read as X.

Calculation:

$$\text{YmM/L} = \frac{(1 - X)}{2} \times 100$$

X = volume of base used

Y = bicarbonate value

3.21.12 Determination of Sodium and Chloride Ions concentration:

This was carried out on the heparinised plasma samples from the rats using the Easylite Analyzer. The operation of the instrument is based on direct – ion selective electrode.

3.21.13 Protein determination

Protein concentration of homogenate supernatants of the organs was determined using the method of Lowry *et al.* (1951).

Principle: The reagent Folin- ciocalteau used in this method contains phosphomolybdic acid and tungstate. The aromatic amino acids, tyrosine and tryptophan present in protein react with Folin's reagent to produce a dark blue colour with absorbance at 650 nm.

Reagents

1. **Alkaline copper reagent**

   Reagent A: 2 g of sodium carbonate was dissolved in and made up to 100 ml with 0.1N sodium hydroxide solution.

   Reagent B: 5 mg of copper sulphate was dissolved in 1.0 mL of 4 % sodium potassium tartrate solution.

   50 mL of reagent A and 1.0 mL of reagent B were mixed fresh at the time of the assay.

2. **Sodium hydroxide (0.1N)**

   4 g of sodium hydroxide was dissolved in and made up to 1000 mL with distilled water.

3. **Sodium potassium tartrate (4%)**

   4 g of sodium potassium tartrate was dissolved in and made up to 100 mL with distilled water.

4. **Bovine serum albumin (BSA)**

   100 mg of bovine serum albumin was dissolved in and made up to 100 mL with distilled water. This solution was diluted 10 times to obtain a concentration of 0.1 mg/mL.

5. **Folin- Ciocalteau reagent (1N)**

   Commercial Folin- Ciocalteau reagent was diluted with two volumes of distilled water.

Preparation of standard BSA curve: Several dilutions of the stock solution containing 2 – 10 mg protein/mL were made. The procedure for the protein estimation is shown below. A curve of the optical densities against protein concentration was plotted.

Protocol for protein estimation according to the method of Lowry *et al.* (1951)

Test Tube No.	**1**	**2**	**3**	**4**	**5**
Stock BSA (mL)	0.2	0.4	0.6	0.8	1.0
Distilled water (mL)	0.8	0.6	0.4	0.2	-
BSA concentration (µg/mL)	20	40	60	80	100
Alkaline solution (mL)	5	5	5	5	5
Incubation for 20 minutes at room temperature					
Folin's reagent (mL)	0.5	0.5	0.5	0.5	0.5

The mixture was incubated at room temperature for 30 minutes after which the absorbance was read using a glass cuvette at 650 nm against a blank containing no BSA.

Determination of protein in the samples

The post mitochondrial fractions of the testicular and epididymal supernatants were diluted 10 times with distilled water. To 1.0 mL of the diluted supernatant, 5 ml of alkaline copper reagent was added, vortexed and allowed to stand for 20 minutes. 500 µL of Folin's phenol

reagent was added and incubated for 30 minutes at room temperature. The blue colour formed was measured at 650 nm in the spectrophotometer. A blank without the protein was used. The protein content of the samples were extrapolated from the standard curve and multiplied by 10 to get the actual amount in the supernatant.

3.21.14 Assay for the reduced glutathione level

The method of Beutler *et al.* (1963) was employed.

Principle: The reduced form of glutathione, GSH, comprises in most instances the bulk of cellular non- protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable yellow colour when 5′, 5′- dithiobis- (2- nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman reagent with the reduced glutathione, 2- nitro-5- thiobenzoic acid possesses a molar absorption at 412 nm. Reduced GSH is proportional to the absorbance at 412 nm.

This method has the following advantages over the earlier modification of the nitroprusside method:

i. The precipitation process is carried out with a single easily prepared reagent. It does not require addition of solid sodium chloride or prolonged shaking.
ii. The determination may be carried out at any temperature likely to be encountered in the laboratory.
iii. The colour formed is relatively stable.
iv. The reagent for colour development is stable for many weeks
v. The sensitivity of the method is so great that it may readily be adapted to a micro- procedure.

Reagents

1. **GSH working standard (stock)**

   40 mg GSH (Mol. Wt. 307.3 g) was dissolved in 100 mL of 0.1 M phosphate buffer, pH 7.4, and then stored in the refrigerator.

2. **Phosphate buffer (0.1 M, pH 7.4)**

   a) 7.16 g of $NaHPO_4$. $12H_2O$ (Mol. wt. 358.22) was dissolved in 200 mL of distilled water.

   b) 1.56 g of NaH_2PO_4. $2H_2O$ (Mol. wt. 156.03) was dissolved in 100 mL of distilled water.

   Finally, solutions (a) and (b) were added together and the pH adjusted to 7.4.

3. **Ellman Reagent (5′,5′ – Dithiobis- (2- nitrobenzoate)- DTNB)**

   40 mg of DTNB was dissolved in 0.1 M phosphate buffer and made up to 100 mL.

4. **Precipitating agent**

   4 % Sulphosalicylic acid (C_7H_6S. $2H_2O$; Mol. wt. 254.22) was prepared by dissolving 4 g of sulphosalicyclic acid in 100 mL of distilled water.

Procedure

Serial dilutions of the GSH working standard were prepared as shown below:

Preparation of GSH standard curve

GSH working Standard (mL)	Phosphate buffer (mL)	DTNB (mL)	Absorbance (412 nm)	GSH Conc. (µg/ mL)
0.02	0.48	4.50	0.065	8
0.05	0.45	4.50	0.177	20
0.10	0.40	4.50	0.383	40
0.20	0.30	4.50	0.695	80
0.30	0.20	4.50	1.109	120
0.40	0.10	4.50	1.514	160

Determination of GSH concentration in the samples

800 µL of the homogenate (since the assay would be done in duplicates) was deproteinated by the addition of an equal volume of 4 % sulfosalicylic acid. This was centrifuged at 4,000 x g for 5 minutes. Thereafter, 0.5 mL of the supernatant was added to 4.5 mL of Ellman reagent. A blank was prepared with 0.5 mL of the diluted precipitating reagent (1mL of distilled water: 1 mL of precipitating reagent) and 4.5 mL of Ellman reagent. Reduced glutathione (GSH) level is proportional to the absorbance at 412 nm.

3.21.15 Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined by the modified method of Misra and Fridovich (1972).

Principle: The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay of the enzyme. Superoxide anion generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adenochrome and the yield of adenochrome produced per superoxide anion introduced increased with increasing pH and also increased with increasing concentration of epinephrine. The result led to the proposal that autoxidation of epinephrine proceeds by at least two (2) distinct pathways of which only one is a free radical chain reaction involving superoxide radicals and hence can be inhibited by superoxide dismutase.

Reagents

1. **Carbonate buffer (0.05M, pH 10.2)**

   3.58 g of $Na_2CO_3.10H_2O$ and 1.05 g of $NaHCO_3$ were dissolved in 200 mL of distilled water. The pH was adjusted to 10.2 and then made up to 250 mL with distilled water.

2. **Epinephrine (0.3mM)**

0.05 g of epinephrine was dissolved in 200 mL of distilled water and 0.5 mL conc. HCl was added. Always prepare fresh when needed.

Procedure

10 µL of sample was added to 2.5 mL of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer (i.e. allow the reading to be stable in the spectrophotometer before adding the epinephrine) and the reaction started by adding 300 µL of freshly prepared 0.3 mM epinephrine to the mixture which was quickly mixed by inversion. The reference glass cuvette contained 2.5 mL buffer, 300 µL of epinephrine and 10 µL of water. The increase in absorbance at 480 nm was monitored at 0 and at every 30 seconds for 150 seconds (2.5 mins).

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_0}{2.5}$$

Where A_0 = absorbance at 0 second

A_3 = absorbance at 150 seconds

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

1 unit of SOD activity was given as the amount of SOD necessary to cause 50 % inhibition of the oxidation of epinephrine.

3.21.16 Determination of catalase activity

Catalase (CAT) activity was determined according to the method of Claiborne (1985).

Principle: Catalase catalyzes the hydrolysis of hydrogen peroxide into oxygen and water. The rate of decrease in the absorbance of hydrogen peroxide at 240 nm is therefore an indication of the activity of the enzyme.

Reagents

1. **0.05 M Phosphate buffer, pH 7.4**

   174.19 g of K_2HPO_4 and 0.163 g of KH_2PO_4 were dissolved and made up to 100 mL with distilled water. The pH was adjusted to 7.4.

2. **19 mM H_2O_2**

   215 µL of 30% H_2O_2 was mixed in 100 mL of phosphate buffer (pH 7.4)

Procedure

50 µL of sample was added to 2.95 mL of the H_2O_2/ phosphate buffer mixture in a quartz cuvette and mixed by inversion. The absorbance was read at 240 nm in 0 and 1 minute interval for 5 minutes.

Calculation:

$$\text{Catalase activity} = \frac{(\text{Change in absorbance/ min}) \times \text{vol. of mixture}}{0.0394 \times \text{vol. of sample} \times \text{mg protein}}$$

Where 0.0394 = millimolar extinction coefficient of H_2O_2 at 240 nm

Volume of mixture = 3 mL

Volume of sample = 50 µL

Change in absorbance/ min = $(Abs_0 – Abs_5)/ 5$

Unit = mmoles H_2O_2 consumed/ min/ mg protein.

3.21.17 Estimation of glutathione –S- transferase activity

Glutathione -S-transferase (GST) activity in supernatants of homogenates of the organs was determined according to the method of Habig *et al.* (1974).

Principle: The principle is based on the fact that all known GSTs demonstrate a relatively high activity with 1-chloro-2,4- dinitrobenzene as the second substrate. Consequently, the conventional assay for GST activity utilizes 1-chloro-2,4- dinitrobenzene as substrate. Conjugation of this substrate with reduced glutathione, led to a shift in its maximum absorption to a longer wavelength. The increase in absorption at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction.

Reagents

1. **20 mM 1- Chloro-2,4- dinitrobenzene (CDNB)**

   3.37 mg of CDNB was dissolved in 1 mL of ethanol

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

   30.73 mg of GSH was dissolved in 1 mL of 0.1 M phosphate buffer (pH 6.5)

3. **0.1 M Phosphate buffer, pH 6.5**

   4.96 g of dipotassium hydrogen phosphate (K_2HPO_4; Mol. wt. 174.18) and 9.73 g of potassium dihydrogen phosphate (KH_2PO_4; Mol. wt. 136.09) were dissolved in 1000 mL distilled water and the pH was adjusted to 6.5.

Procedure

Glutathione-S-Transferase assay medium

Reagent	**Blank**	**Test**
Reduced glutathione (GSH)	30 µL	30 µL
CDNB	150 µL	150 µL
Phosphate buffer, pH 6.5	2.8 mL	2.8 mL
Distilled water	30 µL	-
Sample	-	30 µL

The medium for the estimation was prepared as shown above. The reaction was allowed to run with the absorbance being read against the blank at 0 second and at 60 seconds interval for 3 minutes at 340 nm. The temperature was maintained at approximately 31^{o}C.

Calculation:

ΔABS, Change in absorbance = Abs_{180}- Abs_{0}

Where: Abs_{0} – Absorbance at 0 second

Abs_{180} – Absorbance at 180 seconds (3 minutes)

GST activity = ΔABS x 3.47/ mg protein

= µmole/ min/ mg protein

3.21.18 Assay for lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was determined according to the method of Vassault (1983).

Principle: The reaction velocity is determined by a decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit of the enzyme causes the oxidation of one micromole of NADH per minute at 25^{o}C and pH 7.3.

Reagents

1. **0.2 M Tris HCl (pH 7.3)**

   3.94 g of Tris HCl was dissolved in 125 mL of distilled water and the pH adjusted to 7.3.

2. **30 mM Sodium pyruvate**

0.033 g of sodium pyruvate was dissolved in 10 mL of Tris HCl buffer.

3. **6.6 mM NADH**

0.047 g of NADH was dissolved in 10 mL of Tris HCl buffer

Procedure

Reagents	**Blank**	**Test**
Tris HCl	2.8 mL	2.8 mL
NADH	100 µL	100 µL
Sodium pyruvate	100 µL	100 µL
Distilled water	100 µL	-
Sample	-	100 µL

The mixture was incubated in the spectrophotometer for 3 minutes to achieve equilibration and establish a blank rate, if any. For the epididymis homogenates, the samples were not diluted since the volume of homogenising buffer used to prepare the sample was twenty times the weight of the epididymis. For the testes homogenates, 1 in 5 dilution of the homogenates was made. 100 µL of diluted (where necessary) sample was added and change in absorbance at 340 nm was read at 0 second and at 60 seconds intervals for 3 minutes. 100 µL distilled water was used for the blank.

Calculation

ΔABS, Change in absorbance = Abs_{180}- Abs_0

Where: Abs_0 – Absorbance at 0 second

Abs_{180} – Absorbance at 180 seconds (3 minutes)

LDH (unit/ mg protein) = ΔABS/ 6.22 x mg protein

3.21.19 Assay for glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined by the method of Rotruck *et al*. (1973) with some modifications, which is based on the reaction between 5′- 5′- dithiobis- (2-nitrobenzoic acid) and glutathione remaining after the action of GPx.

Reagents

1. **10 mM Sodium azide (NaN_3)**

   0.03 g of sodium azide was dissolved in 50 mL of distilled water.

2. **4 mM Reduced glutathione (GSH)**

   0.01 g of reduced GSH was dissolved in 100 mL of phosphate buffer.

3. **2.5 mM Hydrogen peroxide (H_2O_2)**

   28 µL of H_2O_2 was dissolved in 100 mL of distilled water

4. **10 % Trichloroacetic acid (TCA)**

   2 g of TCA was dissolved in 20 mL of distilled water.

5. **0.3 M Di- potassium hydrogen orthophosphate (K_2HPO_4)**

   5.23 g of K_2HPO_4 was dissolved in 100 mL of distilled water.

6. **5′- 5′- dithiobis- (2-nitrobenzoic acid) (DTNB)**

   0.04 g of DTNB was dissolved in 100 mL of phosphate buffer.

7. **Phosphate buffer, pH 7.4**

   0.992 g of K_2HPO_4 and 1.946 g of KH_2PO_4 were dissolved in 200 mL of distilled water and the pH adjusted to 7.4.

Protocol for GPx assay medium

	Blank (µL)	Test (µL)
Phosphate buffer	500	500
NaN_3	100	100
GSH	200	200
H_2O_2	100	100
Sample	-	500
Distilled water	500	-

The whole reaction mixture was incubated at 37ºC for 3 minutes after which 500 µL of TCA was added and thereafter centrifuged at 3000 rpm for 5 minutes. To 1 mL of each of the supernatants, 2 mL of K_2HPO_4 and 1 mL of DTNB was added and the absorbance was read at 412 nm against a blank. Glutathione peroxidase activity was determined by plotting the standard curve and the concentration of the remaining GSH was extrapolated from the curve.

GSH consumed = initial GSH concentration (254.34) – GSH remaining

Glutathione peroxidase activity = GSH consumed/ mg protein

3.21.20 Determination of hydrogen peroxide generation

Hydrogen peroxide (H_2O_2) generated in the organs was evaluated using the method of Wolff (1994).

Principle: Hydroperoxides oxidize ferrous to ferric ion selectively in dilute acid and the resultant ferric ions can be determined using ferric – sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange (o- cresolsulfonaphthalein 3′, 3″- bis (methyl-

imino) diacetic acid, sodium salt) binds ferric ion with high selectivity to produce a coloured (blue- purple) complex with an extinction coefficient of 1.5 x 10^4 M^{-1} cm^{-1} at 560 nm, the absorbance maximum. In the presence of sorbitol the yield of ferric ion per mole hydroperoxide increases enormously relative to a system containing only ferrous ions and xylenol orange. Therefore the apparent extinction coefficient obtained for H_2O_2 in an optimized reaction mixture comprising of 100 µM xylenol orange, 250 µM ammonium ferrous sulfate and 100 mM sorbitol in 25 mM H_2SO_4 is 2.24 ± 0.067 x 10^5 $M^{-1}cm^{-1}$ at 560 nm (the absorbance maximum).

Reagents

1. **100 µM H_2O_2**

   57 µL of 30 % H_2O_2 was made up to 100 mL with distilled water. 1 mL of the resulting solution was taken and made up to 50 mL with distilled water.

2. **100 µM xylenol orange tetrasodium salt (760.58 g/mol)**

   7.61 mg of xylenol orange tetrasodium salt was dissolved in 10 mL of distilled water

3. **100 mM sorbitol**

   0.182 g of sorbitol was dissolved and made up to 10 mL with distilled water.

4. **25 mM H_2SO_4**

   136 µL of conc. H_2SO_4 was made up to 50 mL distilled water.

5. **250 µM ammonium ferrous sulfate (392.14 g/mol)**

   9.8 mg of ammonium ferrous sulfate was dissolved in 50 mL of 25 mM H_2SO_4.

6. **Fox 1 reagent**

10 mL of xylenol orange + 10 mL of sorbitol + 50 mL of ammonium ferrous sulfate, made up to 100 mL with distilled water.

Procedure

The procedure for the determination of the standard curve for hydrogen peroxide within the range of 0 - 5 µM is shown below:

Procedure for hydrogen peroxide standard curve

	Blank	**1**	**2**	**3**	**4**	**5**
H_2O_2 solution (µL)	-	20	40	60	80	100
Distilled water (µL)	100	80	60	40	20	-
Fox 1 reagent (mL)	1.9	1.9	1.9	1.9	1.9	1.9
H_2O_2 concentration (µM)	0	1	2	3	4	5
Vortex, incubate for 30 minutes at room temperature and centrifuge for 5 minutes at 3000 x g						

Absorbance was then read at 560 nm.

Procedure for determination of hydrogen peroxide generation in the samples

100 µL of test sample was added to 1.9 mL of Fox 1 reagent. The mixture was vortexed, incubated for 30 minutes at room temperature and then centrifuged at 3000 x g for 5 minutes to remove any flocculated material. The absorbance is read at 560 nm against the hydrogen peroxide standard curve.

3.21.21 Determination of ascorbic acid concentration

Ascorbic acid (Vitamin C) concentration in the supernatants of the homogenates was determined according to the method of Jakota and Dani (1982).

Principle: Ascorbic acid present in biological samples react with Folin reagent, an oxidizing agent to give a blue colour which has maximum spectrophotometrical absorption at 760 nm. Complete absence of the interference of even those substances which interfere in protein estimation by Folin reagent may be due to either dilution of the Folin reagent or to acidic pH. The dissociation of phosphate from molybdate leads to disappearance of the yellow colour of phosphomolybdate which decreases the reactivity of Folin reagent. Only strong reductants like ascorbic acid can react with Folin reagent under these conditions and any interference by other possible substances is eliminated.

Reagents

1. **10 % Trichloroacetic acid (TCA)**

   10 g of TCA was dissolved in distilled water and made up to 100 mL.

2. **Folin- ciocalteau reagent**

   Commercially prepared Folin reagent of 2.0 M was diluted 10- fold with double distilled water.

3. **Ascorbic acid standard solution (stock)**

   0.1 g of ascorbic acid was dissolved in distilled water and made up to one litre to give a final concentration of 100 µg ascorbic acid/ ml.

Preparation of ascorbic acid standard curve

A standard curve was prepared by taking varying concentrations of standard solutions of ascorbic acid in water. To this, 0.8 mL of 10 % TCA was added and vigorously shaken, incubated in ice bath for 5 minutes and centrifuged at 3000 x g for 5 minutes. Supernatant of the same range were withdrawn and diluted to 2.0 mL using double distilled water. 0.2 mL of

diluted Folin reagent was added and the tubes were vigorously shaken and incubated in ice bath for 10 minutes. The absorbance of the blue colour developed was measured at 760 nm.

Procedure for ascorbic acid (vitamin C) standard curve

Test- Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Ascorbic acid (mL)	-	0.05	0.1	0.2	0.3	0.4	0.5
TCA (mL)	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Vortex, incubate in ice for 5 minutes and centrifuge at 3000 x g for 5 minutes							
Supernatant (mL)	-	0.05	0.1	0.2	0.3	0.4	0.5
Distilled water (mL)	2.0	1.95	1.9	1.8	1.7	1.6	1.5
Folin reagent (mL)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vortex, incubate in ice for 10 mins. Read absorbance at 760 nm							
Ascorbic acid conc. (µg/ mL)	-	5	10	20	30	40	50
Absorbance (760 nm)	-	0.003	0.005	0.015	0.028	0.047	0.070

Estimation of ascorbic acid level in test samples

The same procedure employed for the standard curve was used in the determination of ascorbic acid concentration in the test samples. 500 µL of the supernatants of the homogenates was used in place of the ascorbic acid standard.

3.21.22 Nitrite determination

Principle: Nitrite (NO) mediates the nitrosative modification of sulphanilic acid which then reacts with N-naphthyl ethylenediamine dihydrochloride forming an orange coloured product with maximum absorbance at 496 nm (Green *et al.*, 1982).

Procedure

The amounts of nitrite in tissue homogenates were measured following the Griess reaction by incubating a 100 µL of sample with 100 µl of Griess reagent [0.1 % N-(1-naphthyl) ethylene diamine dihydrochloride: 1 % sulfanilamide in 5 % phosphoric acid; 1:1] at room temperature for 20 min. The absorbance at 550 nm (OD 550) was measured with a spectrophotometer. Nitrite concentration was calculated by comparison with the OD 550 of a standard solution of known sodium nitrite concentrations.

Standard Curve of Nitric oxide concentration

NITRITE CONC (mM nitrite)	ABSORBANCE (at 550 nm)
0	0
0.2	0.077
0.4	0.112
0.6	0.166
0.8	0.225
1	0.215

3.21.23 Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined by the modification of the method described by Trush *et al.* (1994).

Principle: MPO is a lysosomal enzyme present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. However, monocytes contain only one-third of the MPO found in PMNs. MPO utilizes H_2O_2 produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate

radicals for antibacterial activity. This enzyme is unique however in that it can oxidize chloride ions to produce a strong non-radical oxidant, hypochlorous acid (HOCl). HOCl is the most powerful bactericide produced by neutrophils. Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury (Klebanoff *et al.*, 2013).

In this method, MPO activity was measured spectrophotometrically using *O*-dianisidine (Sigma-Aldrich) and hydrogen peroxide. In the presence of H_2O_2, as oxidizing agent, MPO catalyses the oxidation of *O*-dianisidine yielding a brown coloured product oxidized *O*-dianisidine, with a maximum absorbance at 470 nm, according to the following overall reaction:

$2\ H_2O_2$ + *O*-dianisidine $\longrightarrow$ oxidized dianisidine + $4\ H_2O$

Procedure

200 µL of *O*-dianisidine (16.7 mg in 100 mL phosphate buffer) and 50 µL of dilute H_2O_2 [4 µL of H_2O_2 + 96 µL of distilled H_2O] was added to 7 µL of sample homogenate in duplicate. One unit of MPO is defined as that giving an increase in absorbance of 0.001 per min and specific activity is given as IU/ mg protein.

Calculation:

$$\text{MPO activity} = \frac{(A_{180} - A_0) \times 1000}{\text{Change in absorbance} \times \text{mg protein}}$$

Where A_0- absorbance at 0 second

A_{180}- absorbance at 180 seconds

3.21.24 Estimation of lipid peroxidation

Lipid peroxidation (LPO) was determined by measuring the levels of malondialdehyde produced during lipid peroxidation according to the method described by Varshney and Kale (1990).

Principle: This method is based on the reaction between 2- thiobarbituric acid (TBA) and malondialdehyde: an end product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532 nm and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of free MDA produced.

Reagents

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

   9 g of TCA was dissolved in distilled water and made up to 30 mL

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

   This was prepared by dissolving 0.225 g of TBA in 0.1M HCl and made up to 30 mL with same.

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

   1.12 g of KCl and 2.36 g of Tris base were dissolved in 100 mL of distilled water and the pH was then adjusted to 7.4.

Procedure

400 µL of the sample was mixed with 1.6 mL of Tris- KCl buffer to which 500 µL of 30 % TCA was added. Then 500 µL of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80^{o}C. This was then cooled in ice and centrifuged at 3000 g for 5 minutes. The clear supernatant was collected and absorbance measured against a reference blank of

distilled water at 532 nm. Lipid peroxidation expressed as MDA formed/ mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

$$\text{LPO (MDA formed/ mg protein)} = \frac{\text{Absorbance x volume of mixture}}{E_{532nm} \text{ x volume of sample x mg protein}}$$

Where : volume of mixture = 3 mL

volume of sample = 400 µL

$E_{532nm,}$ molar extinction coefficient $=1.56 \times 10^5\ M^{-1}\ cm^{-1}$

3.21.25 Determination of Gamma-glutamyl transferase activity

Principle: L-γ- glutamyl-3- carboxy-4-nitroanilide, in the presence of glycylglycine, is converted by gamma- glutamyl transferase (GGT) in the sample to 5-amino-2-nitrobenzoate which can be measured at 405 nm. A modified method of Theodorsen and Stromme (1976).

Reagent Composition

Content	**Concentration**
1. Buffer/ Glycylglycine	
Tris buffer	100 mmol/L, pH 8.25
Glycylglycine	100 mmol/L
2. Substrate	
L-γ- glutamyl-3- carboxy-4-nitroanilide	2.9 mmol/L

Procedure

Pipette into cuvette	
Sample	0.10 mL
Reagent	1.00 mL

The initial absorbance was read after mixing and the timer started immediately. Readings after 1, 2 and 3 mins were taken.

Calculation

GGT activity (U/L) = 1158 x ΔA 405 nm/min

3.22 HISTOPATHOLOGICAL STUDIES

The samples were fixed in either 10 % formalin or Bouin's solution as indicated in the experiments. Sectioning technique was employed to process and mount the organ sections on microscope slides. Briefly, the fixed tissues were dehydrated by passing the tissue through different grades of alcohol, starting from 30% alcohol to absolute alcohol. Using xylene, the tissues were de-alcoholized. They were embedded in paraffin and then blocked using moulds. The blocks were trimmed and sectioned using a microtome. The sections were dewaxed in xylene and hydrated through different percentage grades of alcohol. They were then stained in British haematoxylin. Using water, excess stain was washed off and the sections were differentiated in 70 % acid alcohol. The sections were counter stained in alcoholic eosin and dehydrated through increasing percentage grades of alcohol. The sections were then cleared in xylene and mounted in Canada balsam on slides. The cover slips were fixed and the slides

were labelled. The mounted slides were examined using a light microscope and photographed using a digital camera (Laboratoryinfo.com, 2017).

3.23 STATISTICAL ANALYSIS

All data, as appropriate, were expressed as mean ± standard error of mean (S.E.M). Statistical analyses were carried out using one- way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be significant and post- hoc tests were carried out using the least significant difference (LSD). The statistical package used was the International Business Machines (IBM) brand of the Statistical Package of the Social Science (SPSS) Version 23.

CHAPTER FOUR

EXPERIMENTS AND RESULTS

EXPERIMENT I: DETERMINATION OF PHYTOCHEMICAL CONSTITUENTS OF THE METHANOL LEAF EXTRACT OF *PAULLINIA PINNATA*

Introduction

Different parts of plants have been used to treat and manage various ailments traditionally.

Secondary metabolites such as alkaloids, flavonoids, saponins and tannins have been shown to be responsible for the medicinal properties of plants.

The aim of this experiment is to investigate the phytochemical content of the leaves of *P. pinnata.*

Procedure

The methods of Sofowora (1993) and Trease and Evans (1989) were used to conduct the phytochemical screening of the plant. This is briefly reported in Section 3.8.1.

Result

The phytochemical screening shows the presence of alkaloids, flavonoids, tannins, terpenoids and cardiac glycosides in the leaves of *P.pinnata*. The absence of saponins and anthraquinones (free and combined) was also observed (Table 4.1).

Conclusion

The leaves of *P.pinnata* have the potential for medicinal purposes because of the presence of the phytochemicals.

Table 4.1. Secondary metabolite constituents of the leaves of *P. pinnata*

TEST	RESULT
Alkaloids	
a. 1% Hydrochloric acid	+ve
b. Thin layer chromatography	+ve
Flavonoids	
a. Ammonia/H_2SO_4	+ve
b. Aluminium solution	+ve
c. Ethyl acetate/ammonia	+ve
Saponins	
Frothing	-ve
Tannins	
Ferric Chloride	+ve
Anthraquinones	
a. Free	-ve
b. Combined	-ve
Terpenoids	
Salkowski	+ve
Cardiac glycosides	
a. Kedde	+ve
b. Keller – Killiani	+ve

Key: +ve - present; -ve – absent

EXPERIMENT II: ACUTE TOXICITY STUDY OF THE METHANOL LEAF EXTRACT OF *P.PINNATA*

Introduction

The essence of acute toxicity study is to observe the biological activity of a chemical and to have a lead into the mechanism of action. These observations give information on the hazard and the risk involved in the handling and use of chemicals (Walum, 1998).

The LD_{50} value, which is the statistically derived dose at which when administered 50 % of the treated animals die within a given period, was determined. Various researchers have conducted acute toxicity study in order to be able to estimate the safe dose for a chemical.

Secondary metabolites of plants which include saponins, tannins, cardiac glycosides and flavonoids are all chemical compounds. Therefore, there is a need to determine the quantity that can be tolerable to a biological system.

Usually, laboratory mice or rats are used for acute toxicity study. The aim of this experiment therefore is to determine the LD_{50} value of the methanol leaf extract of *P.pinnata* using Wistar mice.

Procedure

Fifty- four (54) male Wistar mice whose weight ranged from 18- 25g were obtained from the Central Animal House, College of Medicine, University of Ibadan, and were kept in the Animal House, Department of Biochemistry of the same Institution where they were placed in standard laboratory cages and left to acclimatize. They were given mice cubes (Ladokun Feed, Nigeria) and water *ad libitum*. They were weight matched into nine groups of six animals each and the 12 hour light/ dark cycle was maintained. After an overnight fast, eight different doses; 100, 200, 400, 800, 1600, 3200, 6400 and 10,000 mg/kg body weight were

administered to the different groups while the vehicle (physiological saline with Tween 80 (70:30 v/v) was administered to the control group. The animals were then observed for gross behavioural (reflexes, restlessness, sleeping pattern, locomotive disability)/ physiological (loss of hair, redness of eye/ blindness) changes and mortality after Day 1, Day 7 and Day 14 (delayed toxicity).

Result

The treated animals did not show any gross behavioural (reflexes, restlessness, sleeping pattern, locomotive disability) or physiological (loss of hair, redness of eye or blindness) changes. Moreover, mortality was not observed even at a dose of 10,000 mg/kg body weight (Table 4.2).

Conclusion

This finding suggests that the leaves of *P.pinnata* may be well tolerated by the metabolic system of the mice even when taken in large amounts.

Table 4.2. Influence of the methanol leaf extract of *P. pinnata* in the acute toxicity study

	Parameters						
Dose	**Reflexes**	**Restlessness**	**Sleeping pattern**	**Locomotive disability**	**Loss of hair**	**Redness of eyes/ blindness**	**Mortality**
Control	No change	Not observed	No change	Not observed	Not observed	Not observed	Not observed
100	"	"	"	"	"	"	"
200	"	"	"	"	"	"	"
400	"	"	"	"	"	"	"
800	"	"	"	"	"	"	"
1,600	"	"	"	"	"	"	"
3,200	"	"	"	"	"	"	"
6,400	"	"	"	"	"	"	"
10,000	"	"	"	"	"	"	"

Note: n = 6

EXPERIMENT III: SUB- CHRONIC TOXICITY STUDY

Introduction

Plant parts are taken in different types of preparations for the treatment and management of various disease conditions traditionally without any proper consideration for possible evidences of toxicity as a result of the antinutrients and phytochemicals present in the plants.

Paullinia pinnata leaves are taken orally in various preparations without appropriate regulations hence the need for standardization. The aim of this experiment is to determine a safe dose for the oral administration or ingestion of *Paullinia pinnata* leaves.

Procedure

Thirty-six (36) male Wistar rats whose body weight ranged from 140- 190 g were obtained from the Central Animal House, College of Medicine, University of Ibadan and kept in standard laboratory cages at the Animal House of the Department of Biochemistry of the same Institution to acclimatize. They were given mice cubes (Ladokun Feed, Nigeria) and water *ad libitum*. They were weight matched into six different groups of six animals each and the 12 hour light/ dark cycle was maintained. After an overnight fast, five different doses; 50, 100, 200, 400 and 800 mg/kg body weight were administered once orally to the rats for 28 consecutive days at 24 hour intervals. The vehicle (physiological saline and Tween 80 (70:30 v/v) was administered in the same manner to the control group. The weight of the animals was monitored at 7- day intervals throughout the period of the study. On day 29, after an overnight fast, the animals were euthanized by cervical dislocation. The animals were then dissected and blood was collected from the heart with the aid of a needle and 5 mL syringe into lithium heparin and ethylene diamine tetra acetic acid (EDTA) specimen bottles. Three organs; liver, lungs and kidneys were promptly removed from each animal into ice-cold 0.9

% sodium chloride solution. The organs were then blotted using Whatman No. 2 filter paper, weighed with a Mettler balance and fixed in 10 % formalin for histology. Plasma was separated from the anti-coagulated blood in the specimen bottles as described in Section 3.9. EDTA- plasma samples were used to carry out the haematological analysis and the lipid profile while the heparinized plasma was used for the liver function (total bilirubin, total protein, albumin, alkaline phosphatase (ALP), aspartate amino transferase (AST), alanine amino transferase (ALT)) and renal function (creatinine, urea, sodium ion, chloride ion, bicarbonate) tests.

Results

The weight of the animals showed that the rate of percentage weight gained weekly decreased in a dose- dependent manner in the treated groups compared to the control (Fig. 4.1). The

group treated with 800 mg/kg body weight dose showed the least rate of percentage weight gain over the period of study (23 %, Fig. 4.1). Apart from the insignificant decreased weight gained, no change in morphology or gross behavioural changes was observed and there was no incidence of mortality by the termination of the study.

The relative weights of the vital organs; kidneys, lungs and liver, in the treatment groups do not vary significantly from that of the control. Although there was a dose- dependent increase (Table 4.3).

The plasma levels of total proteins, total bilirubin and albumin were not significantly elevated in the treatment groups compared to the control (Table 4.4).

Plasma cholesterol and triglyceride levels in the group treated with a dose of 400 mg/kg body weight (80.00 ± 4.97 and 99.75 ± 8.61 mg/dL respectively) increased significantly ($p < 0.05$) compared to the control and the other treatment groups (Table 4.5).

Table 4.6 showed that the plasma alanine aminotransferase (ALT) activity was not significantly affected by the various doses of treatment and the increased activity at 400 mg/kg body weight dose (128.25 ± 30.16 IU/L) was not significant. Plasma aspartate aminotransferase (AST) activity in the group treated with the dose of 400 mg/kg body weight (430.00 ± 15.82 IU/L) varied significantly ($p < 0.05$) from that of the control and the other treatment groups (Table 4.6). Also, the alkaline phosphatase (ALP) activity in the groups treated with 400 and 800 mg/kg body weight doses (646.75 ± 32.95 and 649.15 ± 71.27 IU/L respectively) varied significantly ($p < 0.05$) from that of the control (499.50 ± 26.39 IU/L) and the other treatment groups (Table 4.6).

The plasma creatinine levels of the treatment groups do not vary significantly from that of the control (Table 4.7). This was also observed in the concentrations of urea, sodium ion, chloride ion and bicarbonate in the plasma. However, there was an increase in the urea concentration at the 800 mg/kg body weight dose (61.66 ± 13.40 mg/dl) which was not significant (Table 4.7).

The results for the haematological analysis are presented in Tables 4.8 and 4.9. Table 4.8 shows that the packed cell volume (PCV) increased significantly $(p < 0.05)$ at 50, 100 and 400 mg/kg body weight doses while there was a significant decrease at the 200 mg/kg body weight dose compared to the control. The increase in PCV observed at the 800 mg/kg dose was not significant. The haemoglobin concentration (Hb) increased in all the treatment groups compared with the control, with an increase at the 200 mg/kg dose not being significant ($p < 0.05$). The values for the red blood cell count (RBC) increased significantly ($p < 0.05$) in all the treatment groups with the exception of the 200 mg/kg dose, which showed a significant decrease compare to the control. With the exception of the 100 and 800 mg/kg doses which showed a significant ($p < 0.05$) decrease in the white blood cell count (WBC), all the other doses showed a significant ($p < 0.05$) increase compared with the

control and the highest increase was at 400 mg/kg body weight. Table 4.8 also shows that the mean cell haemoglobin concentration (MCHC) decreased significantly ($p < 0.05$) in all the treatment groups compared to the control. The mean cell volume (MCV) increased in a dose-dependent manner from 100 to 400 mg/kg body weight doses and was significant ($p < 0.05$) in the groups treated with 200 and 400 mg/kg doses. The mean cell haemoglobin (MCH) increased at 100 and 200 mg/kg doses but was significant ($p < 0.05$) at the 200 mg/kg dose and increased significantly at the 800 mg/kg dose compared to the control. Table 4.9 revealed that in the groups treated with 50, 200 and 800 mg/kg doses, there were significant ($p < 0.05$) increases in the neutrophils compared to the control while the group treated with 400 mg/kg dose showed a significant decrease. The increase at the 100 mg/kg dose was not significant. The lymphocytes showed a significant ($p < 0.05$) increase at 100 and 400 mg/kg doses while the eosinophils decreased significantly ($p < 0.05$) in all the treatment groups compared to the control. The values for the monocytes decreased significantly in the groups treated with the 100, 200 and 400 mg/kg body weight doses compared to the control while it increased significantly in the group treated with the 800 mg/kg body weight dose.

For the histopathology examination, periportal lymphocytic infiltration was observed in the liver of one of the rats each treated with 400 and 800 mg/kg body weight doses while the control group had no lesions (Fig. 4.2). In the lungs, the control group had no lesions while the groups treated with 400 and 800 mg/kg body weight doses showed marked diffuse hyperemia and thick blood vessel associated lymphocytic aggregates. In addition, pulmonary emphysema was observed in the group treated with 800 mg/kg body weight dose.

Conclusion

A tolerable dose for the oral administration of the methanol leaf extract of *Paullinia pinnata* (Linn.) would be 200 mg/kg body weight.

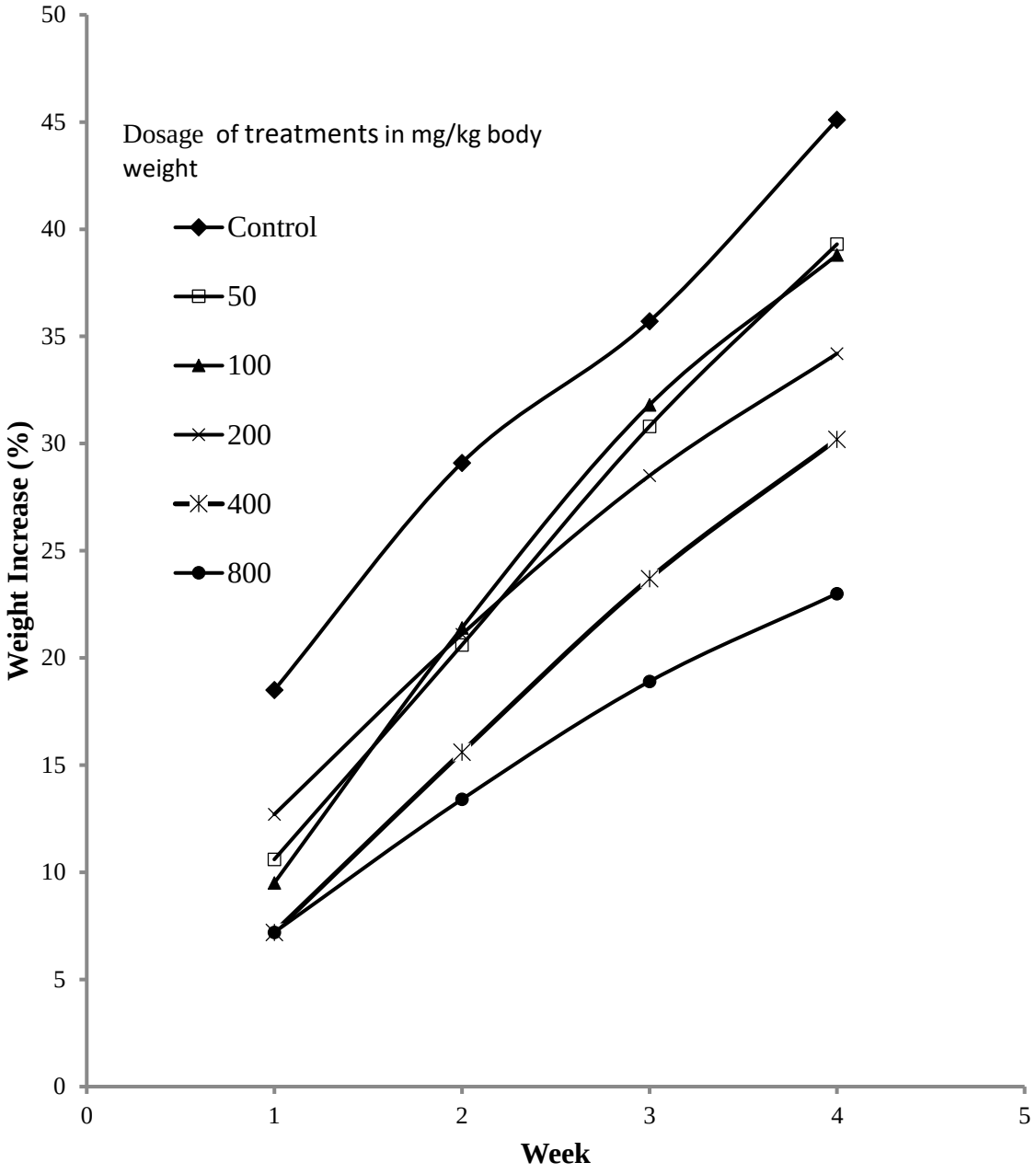

Figure 4.1. Percentage increase in weight of treated Wistar rats and control group over a period of four weeks

Table 4.3. Percentage weight of organs in the *P.pinnata* treated and control Wistar rats

Dose (mg/kg)	Lungs	Liver	Kidneys
Control	0.58 ± 0.11	3.67 ± 0.30	0.64 ± 0.03
50	0.60 ± 0.02	3.50 ± 0.08	0.61 ± 0.04
100	0.62 ± 0.03	3.52 ± 0.28	0.66 ± 0.02
200	0.69 ± 0.05	3.99 ± 0.34	0.70 ± 0.06
400	0.76 ± 0.04	4.11 ± 0.05	0.78 ± 0.03
800	0.83 ± 0.07	4.07 ± 0.07	0.75 ± 0.03

Note: n = 6

Table 4.4. Effect of *P.pinnata* on plasma concentrations of bilirubin, total proteins and albumin in Wistar rats

Dose (mg/kg)	Total Bilirubin (mg/dL)	Total Protein (mg/dL)	Albumin (mg/dL)
Control	0.08 ± 0.05	6.78 ± 0.25	2.45 ± 0.03
50	0.05 ± 0.03	6.13 ± 0.29	2.43 ± 0.10
100	0.10 ± 0.04	6.68 ± 0.17	2.60 ± 0.04
200	0.20 ± 0.04	5.73 ± 0.17	2.30 ± 0.13
400	0.13 ± 0.03	6.73 ± 0.36	2.58 ± 0.11
800	0.10 ± 0.06	6.13 ± 0.42	2.15 ± 0.18

Note: n = 6

Table 4.5. Effect of *P.pinnata* on plasma lipid profile of Wistar rats

Dose (mg/kg)	Cholesterol (mg/dL)	Triglyceride (mg/dL)
Control	60.25 ± 1.65	67.75 ± 8.87
50	55.00 ± 6.78	69.50 ± 15.47
100	71.75 ± 4.37	72.75 ± 9.13
200	68.00 ± 6.67	76.75 ± 11.63
400	80.00 ± 4.97*	99.75 ± 8.61*
800	66.00 ± 4.00	69.00 ± 10.85

Note: n = 6

*- significant at $p < 0.05$

Table 4.6. Effect of *P.pinnata* on the activities of plasma liver enzymes in Wistar rats

Dose (mg/kg)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)
Control	499.50 ± 26.39	202.50 ± 7.54	85.75 ± 6.74
50	512.00 ± 49.09	300.25 ± 88.35	93.75 ± 11.63
100	587.50 ± 16.26	252.00 ± 11.97	90.75 ± 3.09
200	440.75 ± 15.82	324.75 ± 71.02	91.50 ± 6.12
400	646.75 ± 32.95*	430.00 ± 15.82*	128.25 ± 30.16
800	649.15 ± 71.27*	222.50 ± 13.03	78.50 ± 8.17

Note: n = 6

*- significant at $p < 0.05$

Table 4.7. Effect of *P.pinnata* on renal function indices in Wistar rats

Dose (mg/kg)	Creatinine (mg/dL)	Urea (mg/dL)	Sodium ion (mg/dL)	Chloride ion (mg/dL)	Bicarbonate (mg/dL)
Control	0.53± 0.03	44.17± 3.99	128.08± 1.54	97.80± 1.03	16.75± 0.63
50	0.50± 0.00	40.17± 3.36	127.20± 6.48	97.75± 4.15	18.00± 0.91
100	0.48± 0.03	46.82± 3.78	128.50± 3.17	100.28± 1.77	16.75± 1.31
200	0.43± 0.03	44.51± 3.80	118.51± 5.01	92.55± 5.19	15.50± 0.96
400	0.53± 0.06	45.28± 4.98	127.53± 2.58	100.28± 1.03	16.75± 0.25
800	0.45± 0.06	61.66± 13.40	126.93± 1.59	101.68± 0.45	17.00± 1.47

Note: n = 6

Table 4.8. Influence of varying doses of *P.pinnata* on haematological parameters in rats

Dose (mg/kg)	PCV (%)	Hb (g/ 100 mL)	RBC (x 10^{12}/L)	WBC (/mm^3)	MCHC (g/ 100mL)	MCV (fL)	MCH (pg)
Control	52.67 ± 1.08	16.34 ±0.33	5.43 ± 0.09	7.57 ± 1.34	32.68± 0.05	96.57± 0.76	31.67± 0.65
50	55.17 ± 4.22*	17.50 ± 1.34	5.83 ± 0.27*	9.53 ± 1.48*	31.33± 0.03*	96.57± 4.41	31.38± 1.49
100	60.67 ± 2.04*	18.36 ± 0.63*	5.64 ± 0.20*	6.84 ± 0.59*	31.35± 0.03*	97.10± 3.25	32.17± 1.83
200	50.92 ± 5.66	16.39 ± 1.14	5.33 ± 0.31*	8.43 ± 1.33*	31.31± 0.10*	99.50± 5.32*	33.38± 1.49*
400	61.63 ± 2.66*	18.63 ± 0.81*	6.05 ± 0.24*	10.26 ± 1.37*	31.33± 0.05*	100.29± 1.13*	31.49± 0.48
800	53.40 ± 2.10*	16.75 ± 0.63*	5.60 ± 0.81*	6.31 ± 0.27*	31.42± 0.07*	95.63± 1.11	28.67± 0.05*

Note: n = 6; * - significant at $p < 0.05$; PCV – Packed cell volume; Hb – Haemoglobin concentration; RBC – Red blood cell count; WBC – White blood cell count; MCHC – Mean cell haemoglobin concentration; MCV – Mean cell volume; MCH – Mean cell haemoglobin

Table 4.9. Influence of varying doses of *P.pinnata* on differential leukocyte count

Dose (mg/kg)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	Monocytes (%)
Control	16.25±2.90	76.66±2.98	3.28±0.48	1.48±0.29
50	19.56±1.93*	75.46±2.78	2.69±0.48*	1.46±0.50
100	16.76±1.89	80.33±1.47*	2.26±0.25*	1.10±0.41*
200	19.27±2.99*	76.04±3.59	2.52±0.50*	0.76±0.48*
400	15.22±2.63*	81.54±2.81*	2.00±0.00*	1.32±0.75*
800	19.83±2.94*	75.75±3.66	2.47±0.50*	1.72±0.25*

Note: n = 6; *- significant at $p < 0.05$

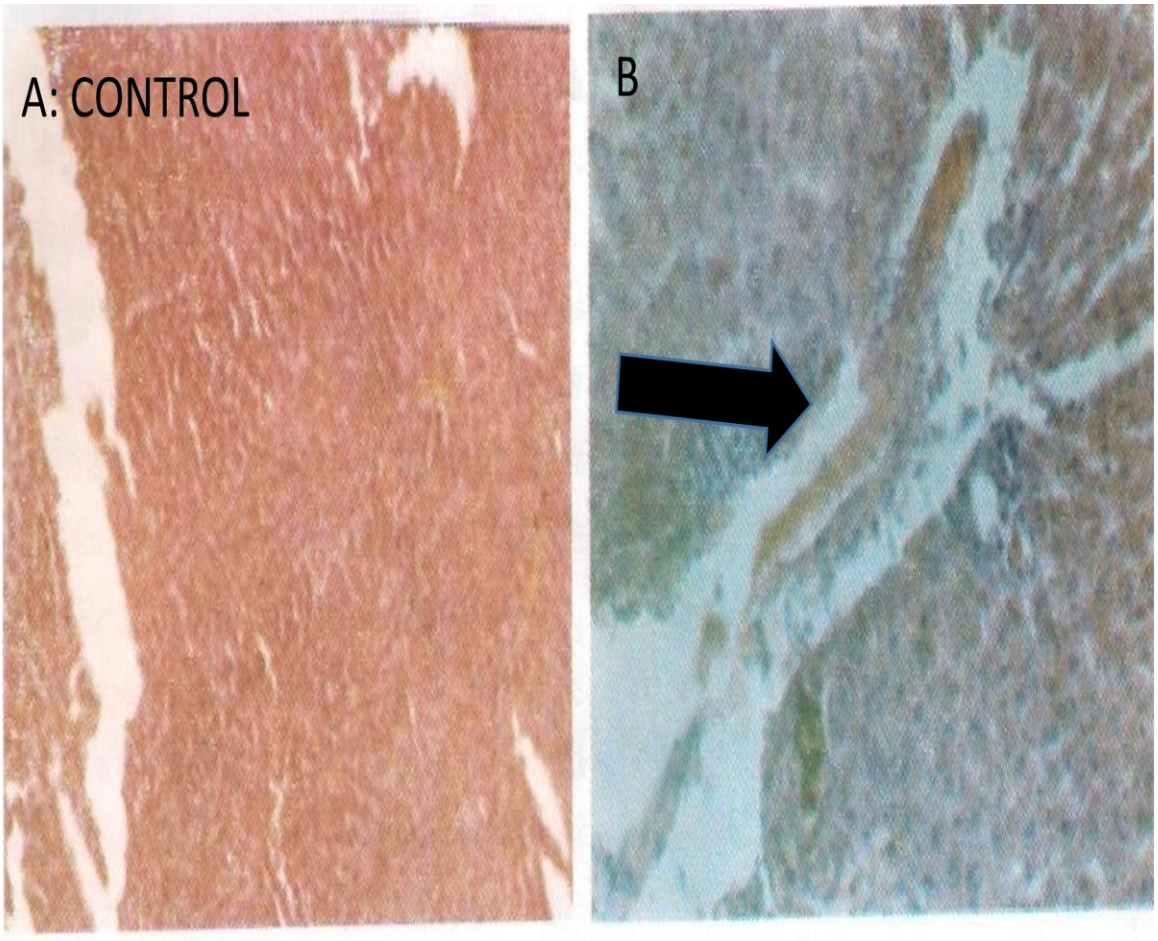

Figure 4.2. Photomicrographs of the liver samples for the sub-chronic toxicity study

A: No lesion; B: Periportal lymphocytic infiltration in one of the rats of each of the 400 and 800 mg/kg body weight doses. H & E, M x 250.

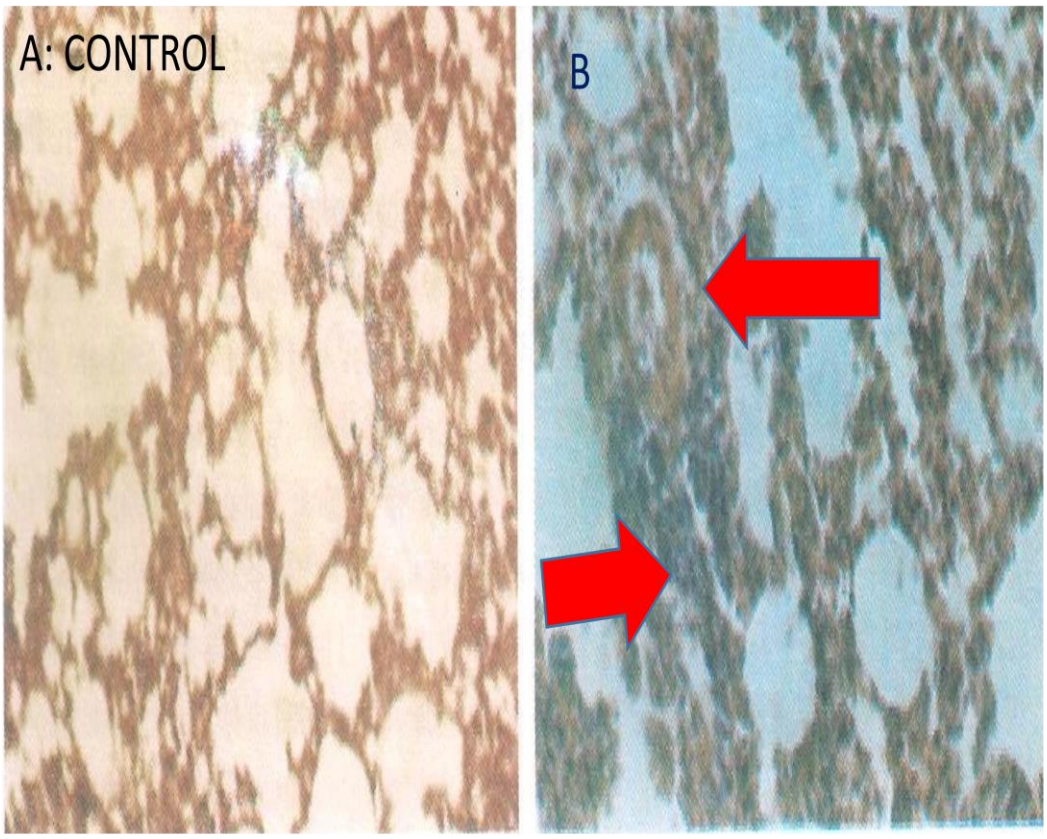

Figure 4.3. Photomicrographs I of the lung samples for the sub-chronic toxicity study
A: No lesion; B: Marked diffuse hyperemia and thick blood vessel associated lymphocytic aggregates in the lungs of the groups treated with 400 and 800 mg/kg body weight doses. H & E, M. x 250.

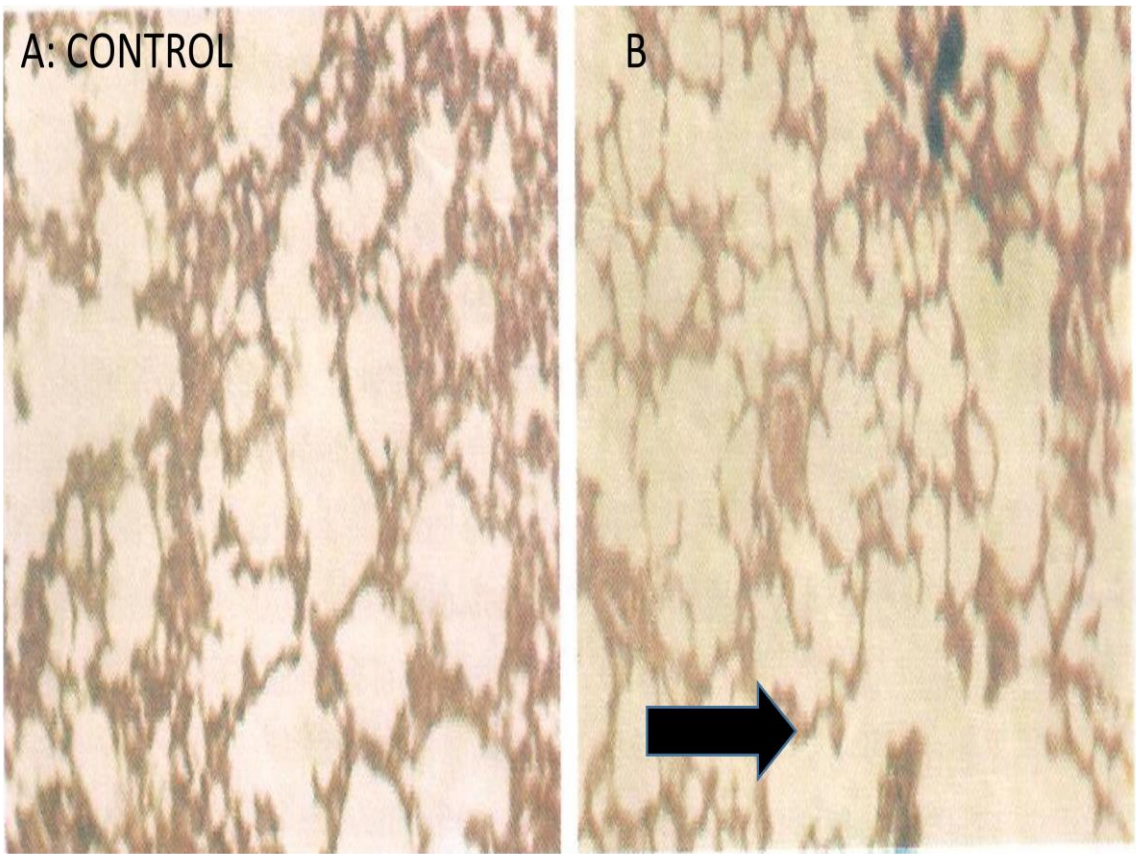

Figure 4.4. Photomicrographs II of the lung samples for the sub-chronic toxicity study

A: No lesion; B: Pulmonary emphysema observed only in the group treated with the 800 mg/kg body weight dose. H &E, M x 250.

EXPERIMENT IV: CURATIVE TEST TAKING INTO CONSIDERATION THE SAFE DOSE

Introduction

Folkloric medicine reports that *Paullinia pinnata* leaves are taken as decoction, concoction, infusion as well as in other preparations for the treatment of malaria.

The aim of this experiment is to establish the antiplasmodial activity of the methanol extract of the leaves using the curative test and taking into consideration the safe dose which has been determined in Experiment III to be 200 mg/kg body weight.

Procedure

Thirty- six (36) Wistar mice of both sexes weighing between 20- 30 g were obtained from the Animal House of the Institute for Advanced Medical Research and Training (IAMRAT), College of Medicine, University of Ibadan. The animals were kept there in appropriate standard laboratory cages and were fed mice cubes (Ladokun Feed, Nigeria) and water *ad libitum*. The animals were weight matched into six groups of six animals each and the treatment protocol as reported in Section 3.8.4 was followed. The body weight, percentage parasitaemia (% parasitaemia), Packed Cell Volume (PCV) and death were monitored daily throughout the period of administration and on 7^{th} day post-administration (Day 11). The percentage survival (% survival) for each of the groups and percentage chemo- suppression for each treatment were calculated. The mode of determination of these parameters are stated in Sections 3.11 – 3.14.

Results

A gradual loss of weight, which was not significant ($p < 0.05$), was observed in the groups treated with the extract by day 11 while the ACT group showed a gradual increase in weight.

The untreated and control groups also presented a non-statistically significant weight loss on day 11 (Table 4.10). A reduction in PCV was observed in the control and untreated groups (Figure 4.5). CQ and the ACT groups showed a rise in the PCV from the 2nd day of administration while the group treated with 100 and 200 mg/kg body weight doses of the extract did not show a significant increase on day 4. With the exception of the ACT group, all the other groups showed a decrease in the PCV in the 11th day.

The percentage parasitaemia reduced gradually in the groups treated with CQ and ACT with a total removal of the parasites by the 4th day of administration. The groups treated with 100 and 200 mg/kg doses of the extract did not show a significant decrease in parasitaemia on day 4 and on day 11 there was an increase in the level of parasitaemia (Figure 4.6).

Table 4.11 shows that none of the animals died during the period of administration. However, the survival rate of the animals post administration is in the following order: ACT > 100 mg/kg dose of extract > CQ and 200 mg/kg dose of the extract > control and untreated groups.

In Table 4.12, *Paullinia pinnata* had no chemosuppressive activity at both doses on day 4. By day 11, the percentage chemosuppressive activities were 13% and 26% at the 100 and 200 mg/kg doses of the extract respectively.

Conclusion

The methanol leaf extract of *Paullinia pinnata* has weak curative activity when taking into consideration the safe dose.

Table 4.10. Effect of *P.pinnata* leaf extract on the body weight of *P. berghei* infected Wistar mice

	Body Weight (g)					
Day	**Control**	**Untreated**	**CQ**	**ACT**	**100 mg/kg**	**200 mg/kg**
0	24.05 ± 1.23	26.48 ± 1.28	25.97 ± 0.59	25.55 ± 1.44	23.70 ± 0.42	25.07 ± 0.76
1	24.32 ± 1.01	27.13 ± 1.39	25.17 ± 0.81	26.44 ± 1.52	22.22 ± 0.54	25.32 ± 1.21
2	23.60 ± 2.17	26.65 ± 1.39	24.75 ± 1.17	26.52 ± 1.57	21.25 ± 0.69	24.68 ± 1.25
3	23.12 ± 2.24	26.03 ± 1.56	24.00 ± 1.49	26.82 ± 1.77	20.57 ± 0.91	24.43 ± 1.21
4	22.78 ± 0.85	26.68 ± 0.78	25.08 ± 2.66	27.84 ± 1.85	19.90 ± 1.28	23.80 ± 1.10
11	14.90 ± 3.20	22.50 ± 3.50	24.83 ± 3.59	28.82 ± 1.60	14.90 ± 1.24	16.68 ± 0.46

Note: n = 6

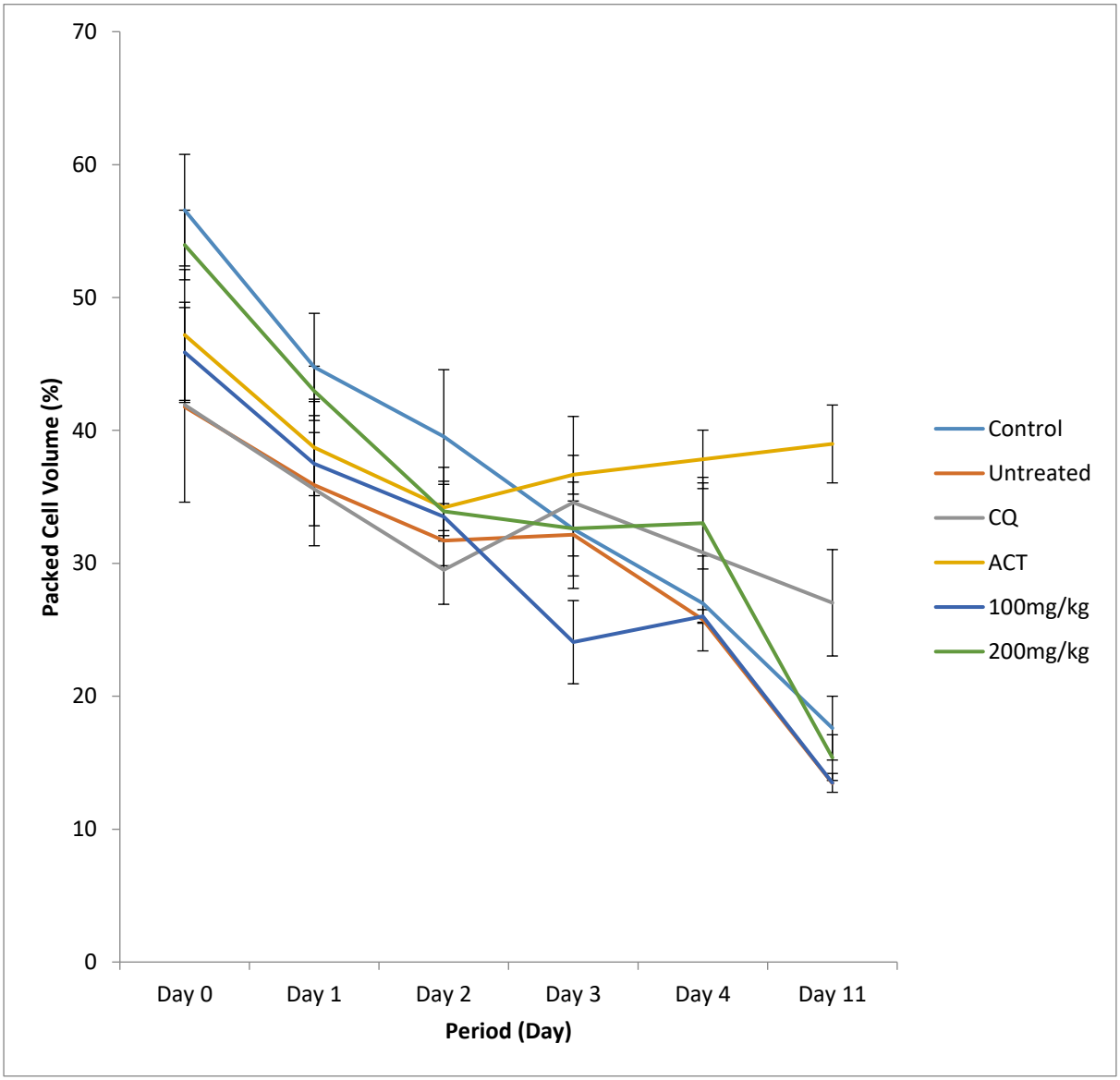

Figure 4.5. Effect of *P.pinnata* on the Packed Cell Volume over the period of study

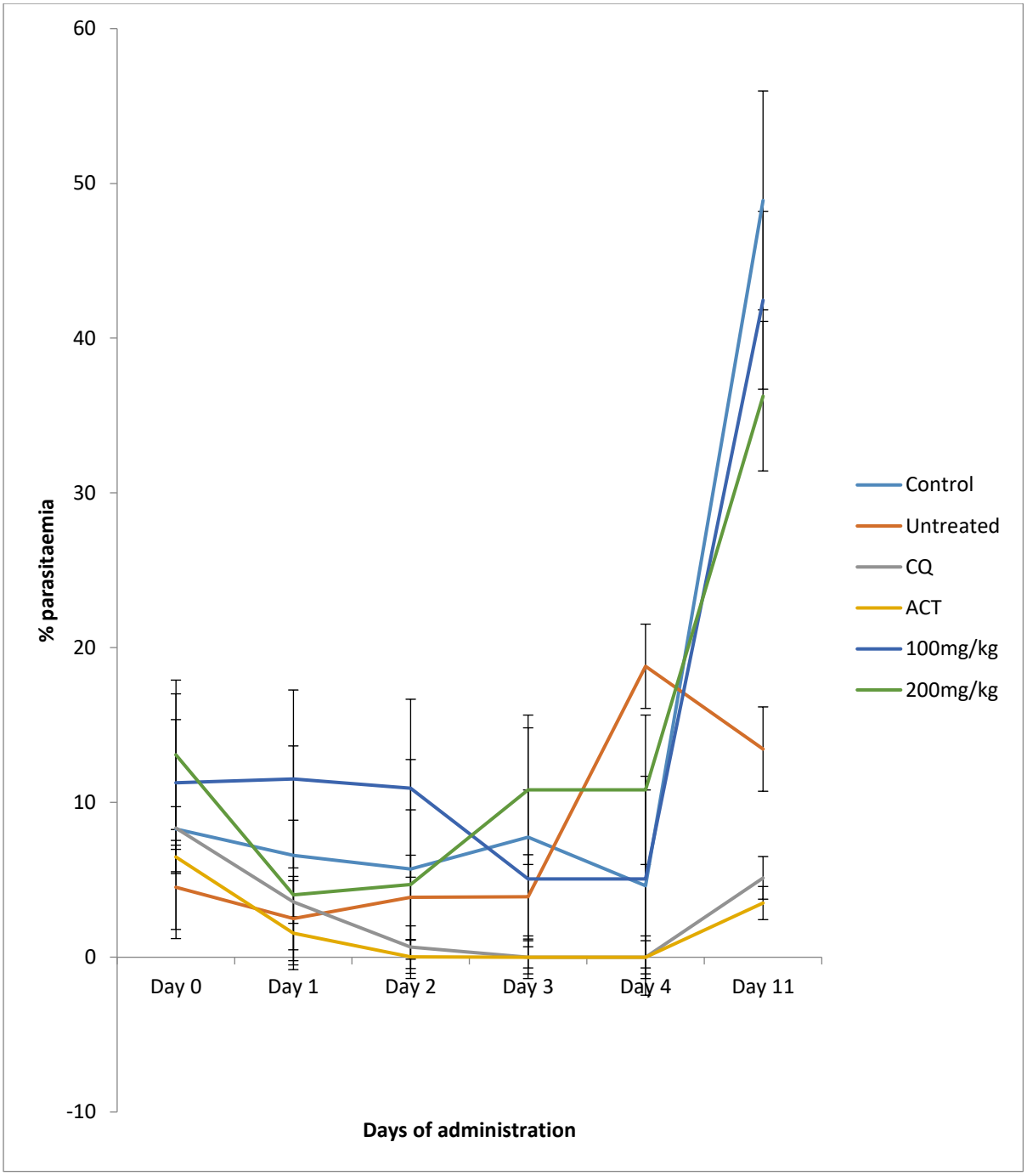

Figure 4.6. Effect of *P.pinnata* on percentage parasitaemia over the period of study

Table 4.11. Percentage survival of the *P.pinnata* leaf extract treated *P.berghei* infected Wistar mice

Day	Control	Untreated	CQ	ACT	100 mg/kg	200 mg/kg
1	100	100	100	100	100	100
2	100	100	100	100	100	100
3	100	100	100	100	100	100
4	100	100	100	100	100	100
11	40	40	66.67	100	80	66.67

Note: n = 6

Table 4.12. Percentage chemosuppression of *P. pinnata* extract compared to the standard drugs

Day	CQ	ACT	100 mg/kg	200 mg/kg
1	53	75	0	39
2	90	99	0	18
3	100	100	34	0
4	100	100	0	0
11	90	93	13	26

Note: n = 6

EXPERIMENT V: DETERMINATION OF ANTIPLASMODIAL ACTIVITES OF THE METHANOL LEAF EXTRACT OF *PAULLINIA PINNATA*

Introduction

Paullinia pinnata leaves are used in folkloric medicine for the treatment of malaria (Maje *et al*., 2007). However, taking into consideration the safe dose we see that it has weak curative property for malaria infection.

Malaria infection confers oxidative stress on both the parasite and the host as a result of increased levels of reactive oxygen species produced during degradation of haemoglobin in the parasite and by activated neutrophils in the host. The combined effect of these processes results in the symptoms and complications observed when the infection is presented (Becker *et al.*, 2004; Iyawe *et al.*, 2006). The liver is the primary target of the parasite after infection of the host.

The aim of this experiment therefore is to investigate the antiplasmodial activity of *P.pinnata* methanol leaf extract at a higher dose and to observe the effect on oxidative damage in the liver as a result of the infection.

Procedure

The protocol for the experiment is reported in Section 3.8.5. The modified Rane's method, as reported by Ryley and Peters (1970), was employed for the curative test and the assessment of the antioxidant and anti-inflammatory parameters with histological examination of the liver was carried out. The method based on Peters (1967) was employed for the prophylaxis test and the method of Peters *et al.* (2002) was employed for the suppressive test.

Results

In the curative test, Table 4.13 shows that there was no significant change in weight of the animals in the control group over the period of the study. The weights of the untreated, 200 and 400 mg/kg doses of extract groups increased till the 1st day of administration after which there was a steady decrease which was significant ($p < 0.05$) by day 11 (7 day post administration) compared to day 0 (before inoculation) within the groups. The weights of animals in the CQ and ACT groups increased steadily throughout the period of study. The CQ, ACT and 400 mg/kg dose of extract groups showed significant ($p < 0.05$) decreases in the percentage parasitaemia over the period of study with the 400 mg/kg dose of extract group showing an insignificant decrease on day 11 (7 day post administration) compared to day 0 (before inoculation) within the groups (Table 4.14). The control, untreated and 200 mg/kg dose of extract showed a steady increase in percentage parasitaemia with the increase being significant ($p < 0.05$) on day 4 (4th day of administration) and day 11(7 day post administration) compared to day 0 within the groups (Table 4.14). In Table 4.15, the values of the packed cell volume decreased significantly ($p < 0.05$) from day 2 to day 11 (7 day post administration) in all the groups but the decrease in the CQ and ACT groups on day 11 (7 day post administration) was not significant ($p < 0.05$) compared to day 0 (before inoculation) within the groups. Table 4.16 shows that the percentage chemosuppression for the CQ, ACT and 400 mg/kg dose of extract on day 4 of administration was 100%, 100% and 85.67% respectively while that of 200 mg/kg dose of extract was 0%. Table 4.17 reveals that the percentage survival in the groups by day 11 (7 day post administration) is ACT > CQ = Control = 400 mg/kg dose of extract > 200 mg/kg dose of extract > Untreated.

The antioxidant assay for the curative test in Table 4.18 reveals that the activities of catalase, glutathione-S-transferase and glutathione peroxidase as well as the reduced glutathione concentration were not affected in the untreated and treatment groups. Lipid peroxidation was

insignificantly elevated in the untreated and 200 mg/kg dose of extract groups. The activity of superoxide dismutase was significantly ($p < 0.05$) elevated in the ACT and chloroquine groups compared to the untreated group. Hydrogen peroxide generated was significantly ($p < 0.05$) reduced in the ACT and 400 mg/kg dose of extract groups compared to the untreated group. Vitamin C concentration reduced significantly ($p < 0.05$) in the ACT, 200 mg/kg and 400 mg/kg doses of the extract groups compared to the untreated group, and it was elevated significantly ($p < 0.05$) in the chloroquine group. Table 4.19 shows that the concentration of nitric acid is not affected in the untreated and treatment groups while the activity of myeloperoxidase is significantly ($p < 0.05$) reduced in the treatment groups compared to the untreated group. The activities of alanine aminotransferase and gamma-glutamyl transferase are significantly reduced in the chloroquine, ACT and 400 mg/kg body weight dose of extract groups compared to the untreated group (Table 4.19). For the histopathology of the curative test, Figure 4.7 shows that the untreated group showed mild to moderate Kupffer cell hyperplasia. This was also observed in the group treated with 200 mg/kg dose of the extract. In the chloroquine treated group, only one of the animals showed severe portal and central venous congestion while the others had no lesions. The ACT group had no lesions except one animal that showed mild periportal cellular infiltration and there were no visible lesions in the group treated with the 400 mg/kg dose of the extract (Figure 4.7).

For the prophylaxis test, Table 4.20 shows that there was no significant change in weight in the untreated and treatment groups over the period of study but the chloroquine treated group showed significant increase ($p < 0.05$) on day 7 when compared to day 1. Table 4.21 reveals that the percentage parasitaemia decreased significantly ($p < 0.05$) in all the treatment groups compared with the untreated group. Table 4.22 shows that the values for the packed cell volume decreased in the untreated, CQ and SP groups over the period of study with the decrease in the SP group being significant ($p < 0.05$) when comparing day 1 with day 4, day

4 with day 7 and day 1 with day 7. The packed cell volume increased insignificantly ($p < 0.05$) in the group treated with 200 mg/kg doses of extract over the period of the test (Table 4.22). It can be seen in Table 4.23 that the percentage chemosuppression for the CQ, SP, 200 and 400 mg/kg doses of the extract are 53.99 %, 100 %, 57.06 % and 49.09 % respectively.

For the suppressive test, Table 4.24 reveals that the weight of the animals decreased in the control, untreated, 200 and 400 mg/kg body weight doses of the extract over the period of study, while the CQ group showed insignificant ($p < 0.05$) increase in weight compared to day 0 (before inoculation and 1st day of administration) within the group. Table 4.25 shows that the percentage parasitaemia increased in the control, untreated and 400 mg/kg body weight dose of extract groups from day 4 to day 6, while the 200 mg/kg of extract group showed decrease when comparing with day 0 within the groups. There was no change in the CQ group. Table 4.26 reveals that the value of packed cell volume decreased significantly ($p < 0.05$) in the control, untreated and the treatment groups compared with day 0 within the groups.

Table 4.27 shows that the percentage chemosuppression in CQ, 200 and 400 mg/kg body weight doses of the extract groups by day 6 were 100%, 66.67% and 0% respectively. The survival rate by day 22 is in the order CQ > Control = 200 mg/kg dose of extract > Untreated = 400 mg/kg dose of extract (Table 4.28).

Conclusion

The data at the higher dose demonstrates that *P.pinnata* methanol leaf extract had mildly moderate prophylactic potential, low suppressive activity and curative potential which was reflected in the oxidative status, anti-inflammatory result and function of the liver of the animals, while the safe dose showed prophylactic and suppressive capacities with weak curative activity.

Table 4.13. Effect of *P.pinnata* on the body weight of the mice in the curative test

	Body Weight (g)					
	Day					
	0	**1**	**2**	**3**	**4**	**11**
10% DMSO	24.26 ± 1.23	24.13 ± 1.23	24.10 ± 1.47	23.94 ± 1.55	22.90 ± 1.57	15.05 ± 2.52
Untreated	24.80 ± 0.54	25.06 ± 0.77	25.20 ± 0.74	25.06 ± 0.83	24.46 ± 0.85	20.38 ± 1.33*
CQ	23.00 ± 1.88	23.26 ± 1.24	23.54 ± 1.26	23.70 ± 0.98	23.53 ± 1.01	23.40 ± 0.50
ACT	24.67 ± 0.37	25.05 ± 0.54	25.08 ± 0.52	24.97 ± 0.70	25.08 ± 0.67	25.54 ± 0.70
200 mg/kg	22.98 ± 0.86	23.37 ± 1.06	23.63 ± 1.01	23.60 ± 1.06	22.88 ± 1.04	19.22 ± 0.57*
400 mg/kg	25.93 ± 0.52	26.03 ± 0.74	25.98 ± 0.74	26.05 ± 0.94	25.70 ± 0.94	21.90 ± 1.82

Note: n = 6; * - significant at $p < 0.05$ when comparing Day 11 with Day 0 within the group

Table 4.14. Effect of *P.pinnata* on percentage parasitaemia in the curative test

	Parasitaemia (%)					
	Day					
	0	**1**	**2**	**3**	**4**	**11**
10% DMSO	1.65 ± 0.15	2.03 ± 0.72	2.20 ± 0.80	4.53 ± 1.07	6.00 ± 1.83	19.68 ± 3.57e
Untreated	3.25 ± 0.52	2.80 ± 0.70	4.10 ± 0.85	4.35 ± 0.76	7.00 ± 0.00d	15.90 ± 1.70e
CQ	2.53 ± 0.24	0.12 ± 0.06a	0.00 ± 0.00b	0.00 ± 0.00c	0.00 ± 0.00d	0.40 ± 0.40e
ACT	3.53 ± 0.63	0.72 ± 0.40a	0.00 ± 0.00b	0.00 ± 0.00c	0.00 ± 0.00d	0.88 ± 0.57e
200 mg/kg	2.43 ± 0.28	2.08 ± 0.16	1.76 ± 0.20	4.63 ± 0.80	6.16 ± 0.72d	26.17±2.92e
400 mg/kg	2.73 ± 0.33	1.30 ± 0.10a	1.27 ± 0.09b	0.66 ± 0.13c	0.86 ± 0.18d	2.10 ± 0.30

Note: n = 6; a, b, c, d, e – significant at $p < 0.05$ when Day 1,2,3,4 and 11 are compared with Day 0 respectively within the group

Table 4.15. Effect of *P.pinnata* on Packed cell volume in the curative test

	Packed cell volume (%)					
	Day					
Group	**0**	**1**	**2**	**3**	**4**	**11**
10% DMSO	48.07 ± 1.07	47.82 ± 1.08	36.58 ± 2.10b	33.87 ± 1.37c	34.05 ± 4.01d	21.63 ± 1.99e
Untreated	48.67 ± 1.47	44.78 ± 4.21	37.24 ± 2.80b	35.73 ± 4.90c	31.64 ± 3.25d	25.15 ± 3.36e
CQ	52.88 ± 3.04	49.25 ± 1.38	36.40 ± 1.49b	36.11 ± 0.69c	37.87 ± 1.33d	48.71 ± 2.18
ACT	49.30 ± 2.31	45.02 ± 1.07	37.40 ± 1.31b	39.43 ± 1.09c	42.05 ± 1.32d	43.34 ± 2.39
200 mg/kg	50.41 ± 1.52	44.75 ± 1.93a	37.92 ± 1.24b	35.50 ± 1.61c	33.49 ± 0.88d	30.50 ± 0.07e
400 mg/kg	50.72 ± 2.50	45.43 ± 1.41	36.84 ± 2.62b	38.87 ± 2.50c	38.53 ± 2.19d	33.46 ± 2.26e

Note: n = 6; a, b, c, d, e – significant at $p < 0.05$ when Day 1,2,3,4 and 11 are compared with Day 0 respectively within the group

Table 4.16. Percentage chemosuppression of *P.pinnata* in the curative test

	Chemosuppression (%)			
Day	**CQ**	**ACT**	**200 mg/kg**	**400 mg/kg**
1	94.09	64.53	0	35.96
2	100	100	20	42.27
3	100	100	0	85.43
4	100	100	0	85.67
11	97.97	95.53	0	89.33

Note: n = 6

Table 4.17. Survival rate of the animals in the curative test of *P.pinnata*

	Survival (%)					
Day	**10% DMSO**	**Untreated**	**CQ**	**ACT**	**200 mg/kg**	**400 mg/kg**
1	100	100	100	100	100	100
2	100	100	100	100	100	100
3	100	100	100	100	100	100
4	100	100	100	100	100	100
11	80	60	80	100	70	80

Note: n = 6

Table 4.18. Influence of *P.pinnata* on the antioxidant parameters in the curative test

Group	CAT	SOD	GST	VIT. C	GSH	GPx	LPO	H_2O_2
Untreated	3.75± 0.13	11.83± 0.54	22.75± 1.83	109.50± 5.50	3.06± 0.28	0.83± 0.05	34.55± 2.55	8.79± 1.56
ACT	3.51± 0.77	25.81± 1.86*	24.80± 4.07	82.67± 0.33*	2.39± 0.17	0.87± 0.13	13.90± 2.10	4.98± 0.30*
CQ	2.79± 0.52	25.00± 1.04*	27.58± 3.33	133.75± 6.22*	3.83± 0.50	0.88± 0.02	16.40± 1.65	6.74± 0.12
200 mg/kg	3.64± 0.41	9.14± 0.54	24.61± 0.73	82.67± 2.73*	2.56± 0.28	0.71± 0.10	22.95± 11.75	7.61± 0.30
400 mg/kg	3.72± 0.44	15.32± 2.42	20.72± 1.36	84.00± 0.00*	2.50± 0.72	0.69± 0.05	16.51± 7.50	4.40± 0.38*

Note: n = 5; *- significant at $p < 0.05$; KEY: Parameter (unit):- CAT- Catalase (mmoles H_2O_2 consumed/ min/ mg protein), SOD- Superoxide dismutase (unit/ mg protein), GST- Glutathione S- Transferase (IU), VIT. C- Ascorbic acid (µg/mL), GSH- reduced glutathione (µg/mL), GPx- Glutathione peroxidase (µg/mL/mg protein), LPO- Lipid peroxidation (malondialdehyde formed/ mg protein), H_2O_2- Hydrogen peroxide generation (nM)

Table 4.19. Influence of *P.pinnata* on inflammation and liver function markers in the curative test

Group	NO	MPO	ALT	GGT
Untreated	72.86± 4.83	0.36± 0.02	158.00± 0.26	11.00± 2.90
ACT	63.05± 2.02	0.15± 0.02*	154.76± 1.35*	3.48± 2.32*
CQ	55.45± 7.71	0.20± 0.04*	157.52± 0.82	5.02± 0.77*
200 mg/kg	70.66± 3.34	0.21± 0.04*	156.96± 0.75	11.19± 0.77
400 mg/kg	61.43± 8.79	0.14± 0.01*	153.75± 0.24*	0.10± 0.00*

Note: n = 5; *- significant at $p < 0.05$; KEY:Parameter (unit):- NO- Nitric oxide (mM), ALT- Alanine aminotransferase (U/I), MPO- myeloperoxidase (IU/ mg protein), GGT- L-gamma glutamyl transferase (U/I)

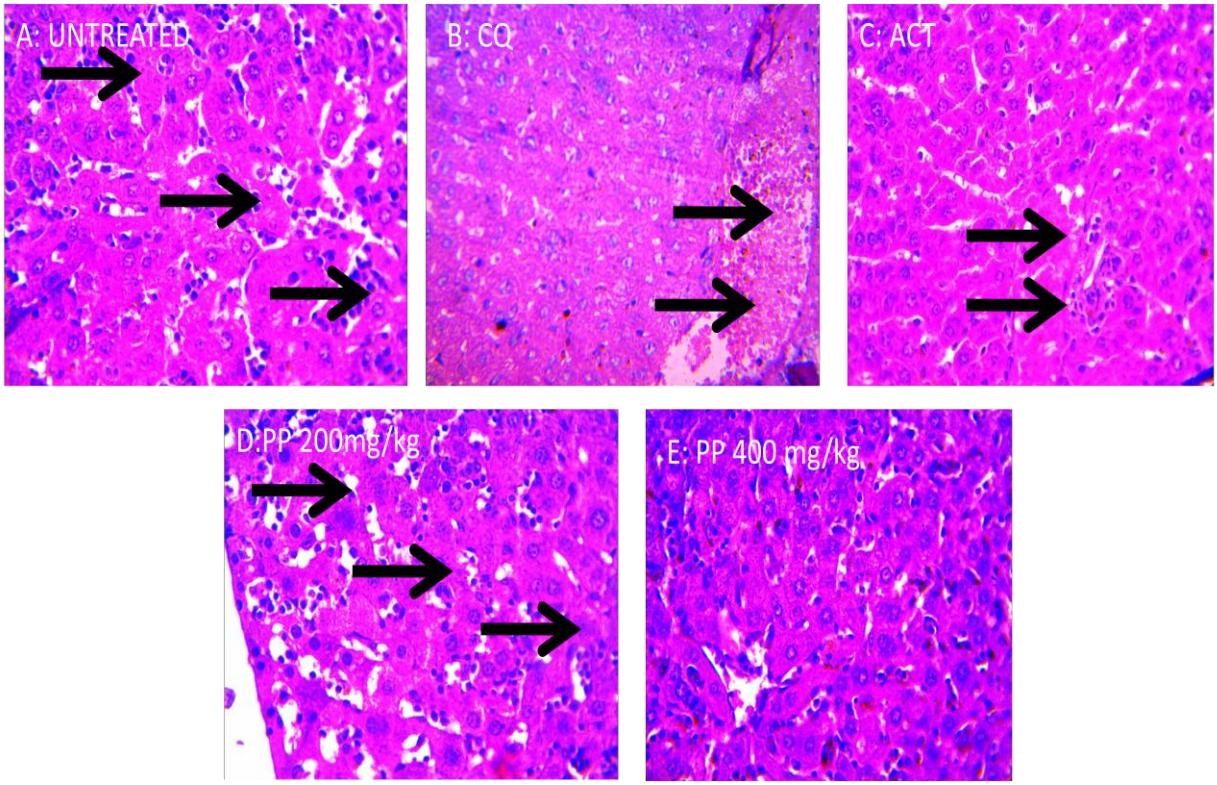

Figure 4.7. Photomicrographs of the liver in the curative study of *P.pinnata* leaf extract .H & E M x 400

A: Mild to moderate Kupffer cell hyperplasia. B:Severe portal and central venous congestion in one of the animals while the others showed no lesions . C: Mild periportal cellular infiltration in one of the animals while the others showed no lesions. D: Mild to moderate Kupffer cell hyperplasia. E: No visible lesions

Table 4.20. Effect of *P.pinnata* on the body weight of animals in the prophylaxis test

	Body weight (g)				
	Day				
Group	**1**	**2**	**3**	**4**	**7**
Untreated	22.00± 0.93	22.78± 0.93	23.22± 0.81	22.90± 0.76	22.90± 1.01
CQ	18.50± 0.11	18.85± 0.45	18.40± 0.48	18.75± 0.47	19.78± 0.50*
SP	20.00± 0.10	19.94± 0.57	19.56± 0.62	19.86± 0.54	19.38± 0.57
200 mg/kg	21.32± 0.77	21.53± 0.67	21.44± 0.34	21.20± 0.35	21.57± 0.73
400 mg/kg	21.08± 0.61	21.62± 0.44	21.93± 0.59	21.65± 0.65	22.10± 0.62

Note: n = 6; *- significant at $p < 0.05$ when comparing Day 1 with Day 7 within the group

Table 4.21. Effect of *P.pinnata* on percentage parasitaemia in the prophylaxis test

Group	Parasitaemia (%)
Untreated	1.63± 0.27
CQ	0.75± 0.12*
SP	0.00± 0.00*
200 mg/kg	0.70± 0.18*
400 mg/kg	0.83± 0.15*

Note: n = 6; *- significant at $p < 0.05$

Table 4.22. Effect of *P.pinnata* on Packed cell volume in the prophylaxis test

Group	Packed cell volume (%)		
	Day		
	1	4	7
Untreated	50.00± 0.30	51.40± 1.57	48.89± 1.11
CQ	49.69± 1.34	47.51± 1.80	45.99± 1.72
SP	57.75± 0.91	51.47± 0.72a	46.25± 1.50b,c
200 mg/kg	49.66± 1.55	53.19± 2.82	51.74± 1.06
400 mg/kg	49.61± 0.98	49.76± 0.79	47.96± 0.94

Note: n = 6; a- significant at $p < 0.05$ when Day 4 and Day 1 are compared within the group; b- significant at $p < 0.05$ when Day 7 and Day 4 are compared within the group; c- significant at $p < 0.05$ when Day 7 and Day 1 are compared within the group

Table 4.23. Percentage Chemosuppression of *P.pinnata* in prophylaxis test

Group	Chemosuppression (%)
CQ	53.99
SP	100
200 mg/kg	57.06
400 mg/kg	49.08

Note: n = 6

Table 4.24. Effect of *P.pinnata* on the body weight of animals in the suppressive test

	Body Weight (g)			
	Day			
Group	**0**	**4**	**5**	**6**
10% DMSO	17.74± 0.98	17.37± 0.34	17.20± 0.40	17.20± 0.40
Untreated	29.03± 0.62	28.75± 0.32	28.57± 0.75	28.10± 0.26
CQ	25.38± 1.17	25.96± 1.08	26.08± 1.08	25.63± 1.19
200 mg/kg	25.18± 0.79	24.72± 0.98	24.06± 0.93	22.03± 0.64
400 mg/kg	25.40± 1.52	25.55± 1.40	25.10± 1.88	24.05± 1.97

Note: n = 6

Table 4.25. Effect of *P.pinnata* on Percentage Parasitaemia in the suppressive test

	Parasitaemia (%)			
	Day			
Group	**0**	**4**	**5**	**6**
10% DMSO	0.00± 0.00	2.97± 0.28a	3.38± 0.85b	3.60± 0.38c
Untreated	0.00± 0.00	1.58± 0.21a	2.40± 0.06b	2.62± 0.12c
CQ	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
200 mg/kg	0.00± 0.00	1.88± 0.32a	1.53± 0.30b	1.20± 0.23c
400 mg/kg	0.00± 0.00	2.63± 0.84a	3.83± 0.88b	5.23± 0.36c

Note: n = 6; a, b, c- significant at $p < 0.05$ when Day 4, 5 and 6 are compared with Day 0 respectively within the group

Table 4.26. Effect of *P.pinnata* on Packed cell volume in the suppressive test

	Packed cell volume (%)			
	Day			
Group	**0**	**4**	**5**	**6**
10% DMSO	49.41± 1.85	45.92± 1.88	40.92± 2.36b	38.14± 3.63c
Untreated	45.63± 2.65	45.98± 0.97	40.95± 2.38	41.46± 1.58
CQ	50.65± 2.28	47.01± 1.08	46.46± 1.77	41.20± 1.47c
200 mg/kg	52.52± 2.15	47.92± 3.48	42.45± 2.30b	41.87± 0.69c
400 mg/kg	52.40± 1.59	43.89± 1.89a	42.68± 3.86b	40.91± 3.59c

Note: n = 6; a, b, c- significant at $p < 0.05$ when Day 4, 5 and 6 are compared with Day 0 respectively within the group

Table 4.27. Percentage Chemosuppression of *P.pinnata* in the suppressive test

	Chemosuppression (%)		
Day	**CQ**	**200 mg/kg**	**400 mg/kg**
4	100	36.70	11.45
5	100	54.73	0
6	100	66.67	0

Note: n = 6

Table 4.28. Survival rate of the animals in the suppressive test

	Survival (%)				
Day	**10%DMSO**	**Untreated**	**CQ**	**200 mg/kg**	**400 mg/kg**
1	100	100	100	100	100
8	100	100	83.3	60	100
15	80	50	83.3	40	40
22	20	0	50	20	0
30	0	0	33.3	0	0

Note: n = 6

EXPERIMENT VI: SUB-ACUTE TOXICITY STUDY OF ETHYLENE GLYCOL MONOMETHYL ETHER

Introduction

In humans and various species, EGME has been shown to cause developmental and reproductive toxicities.

Welsch (2005) showed that pregnant mice treated with EGME at 100- 500 mg/kg body weight doses for 1-3 days presented disruption at two developmental phases: digit differentiation in all the limbs and neurogenesis at the stage of the closure of the anterior neural tube.

Foster *et al.* (1984) revealed using male rats that the treatment of a single dose of EGME at 100 mg/kg/day results in degeneration of pachytene spermatocytes within 24 hours and treatment at the dose of 500 mg/kg/day for 2 or more days resulted in significant decreases in relative testicular weight. Continuation of the dosing resulted in progressive depletion of early spermatid population and spermatocytes and Ku *et al*. (1995) showed that this was via apoptotic mechanism.

There was an observed increase in the occurrence of oligospermia and azospermia as well as low sperm counts per ejaculate in seven- three shipyard painters exposed to 2.6 mg/m^3 EGME (Welch *et al.*, 1988).

The aim of this experiment is to investigate the toxicity of EGME in the male reproductive organ of Wistar rats in order to determine the tolerable dose.

Procedure

Fifty (50) nine weeks old male Wistar rats weighing 140- 190 g were obtained from the Primate Colony of the Department of Biochemistry, University of Ibadan and randomly distributed into five groups of ten animals each. They were kept in appropriate laboratory cages and given mice cubes (Ladokun Feed, Nigeria) and

water *ad libitum* in the Animal house of the same Department where they were acclimatized for a week. The 12 hour light/dark cycle was maintained.

All administrations were carried out orally daily at the same time for fourteen consecutive days and the weight was monitored weekly. The protocol for administration is as follows:

Group I received distilled water only (Control)

Group II received EGME at 100 mg/kg body weight dosage

Group III received EGME at 200 mg/kg body weight dosage

Group IV received EGME at 300 mg/kg body weight dosage

Group V received EGME at 400 mg/kg body weight dosage

On day 15, the animals were euthanized by cervical dislocation after an overnight fast. The animals were then dissected and the testes, epididymes, prostate gland and seminal vesicles were removed into ice- cold 1.15% potassium chloride, blotted with Whatman No.2 filter paper and weighed using a Mettler balance. The right testes and epididymes samples were used for spermatozoa analysis and fixed in Bouin's solution, processed, sectioned, mounted on slides and stained for histological examination. The left testes and epididymis were used for the biochemical analyses of antioxidant markers. Blood was collected by ocular puncture before euthanasia into di-potassium ethylene diamine tetra acetic acid (K_2- EDTA) specimen bottles using heparinized capillary tubes and the haematological parameters were determined using the Mindray BC 3000 Autohaematology analyzer.

Results

Figure 4.8 shows the percentage weight gained weekly over the fourteen day treatment period. The groups of the control, 100, 200 and 300 mg/kg doses showed an increase in weight weekly over the period of study while the 400 mg/kg dose group decreased from 12% to 10% between day 7 and day 14.

Table 4.29 reveals that the weight of the testis decreased significantly ($p < 0.05$) in the 200, 300 and 400 mg/kg dosage groups compared to the control. This is also presented in the relative weight. The weights and relative weights of the epididymis, seminal vesicle and prostate gland decreased insignificantly ($p < 0.05$) in the dosage groups compared to the control (Table 4.29).

The haematological analysis (Table 4.30) shows that the haemoglobin concentration, white blood cell count, platelet count, neutrophils, red blood cell count, mean cell haemoglobin and mean cell haemoglobin concentration decreased significantly ($p < 0.05$), especially at 300 and 400 mg/kg doses compared to the control. The eosinophils, monocytes and packed cell volume were not significantly affected whereas there were significant increases in lymphocytes at the 300 mg/kg dose and in the mean cell volume compared to the control.

The spermatozoa analysis (Table 4.31) shows that the testicular spermatozoa number, epididymal spermatozoa number, sperm motility and sperm viability decreased significantly ($p < 0.05$), especially at the 200, 300 and 400 mg/kg doses compared to the control. The percentage sperm abnormalities increased significantly ($p < 0.05$) in the 200, 300 and 400 mg/kg doses (Table 4.31) while the daily spermatozoa production decreased insignificantly in a dose-dependent manner.

Table 4.32 reveals the effect of EGME on testicular antioxidant parameters. The activities of catalase, glutathione-S-transferase and glutathione peroxidase increased significantly ($p > 0.05$) at the 200, 300 and 400 mg/kg doses compared to the control. The activities of superoxide dismutase and lactate dehydrogenase decreased significantly ($p < 0.05$) and lipid peroxidation increased significantly ($p > 0.05$) in the 200, 300 and 400 mg/kg dosage groups compared to the control. The levels of vitamin C and reduced glutathione were not affected (Table 4.32).

The antioxidant analysis in the epididymis (Table 4.33) showed that there were increases in lipid peroxidation; in the activities of catalase, superoxide dismutase, glutathione-S-transferase and glutathione peroxidase; and in the levels of vitamin C and reduced glutathione compared to the control. These increases were significant ($p < 0.05$) at the 300 and 400 mg/kg doses. There was significant ($p < 0.05$) decrease in the activity of lactate dehydrogenase in all doses compared to the control. Figure 4.9 shows the histopathology of the testis in the control and treatment groups. The control group (A) had no lesions and the 100 mg/kg group (B) showed oedema in the interstitium. The 200 mg/kg dose group (C) showed that some of the sections of the seminiferous tubules have a greatly reduced germinal epithelial height, late maturing stages were absent and there were few cellular clumps in the lumina. The 300 mg/kg dose group (D) showed mild congestion of the interstitial vessels while the 400 mg/kg dose group (E) showed severe epithelial erosion. Figure 4.10 shows the histopathology of the epididymis in the control and treatment groups. No lesions were observed in the control group (A) and the treatment groups, but the 400 mg/kg dose group (B) showed severe diffuse germinal cell erosion.

Figure 4.11 showed the survival rate of the animals over the period of the study. There was 90% survival in the 200 mg/kg dose group, 80% survival in the 300 mg/kg dose group and 70% survival in the 400 mg/kg dose group.

Conclusion

It is evident in this experiment that Ethylene glycol monomethyl ether effects its gonadotoxic potential by inducing oxidative stress and a tolerable dose would be 200 mg/kg body weight.

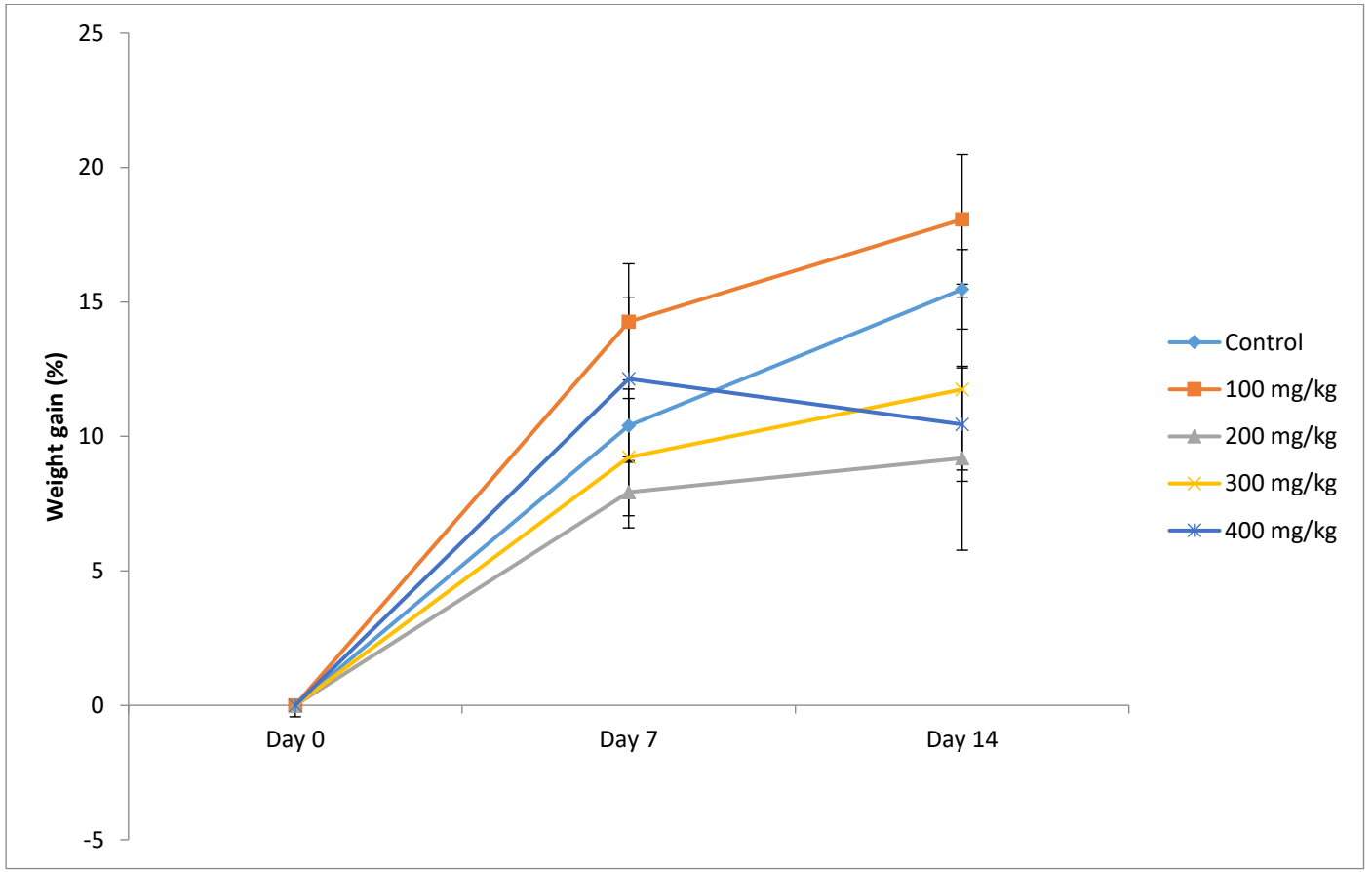

Figure 4.8. Percentage weight gain of male Wistar rats over fourteen day treatment of EGME

Table 4.29. Effect of EGME on weight and relative weight of organs in male Wistar rats after a fourteen day treatment

	Weight of organs (g)				Relative weight of organs (%)			
Dose (mg/kg)	**Testes**	**Epididymis**	**Seminal Vesicle**	**Prostate Gland**	**Testes**	**Epididymis**	**Seminal Vesicle**	**Prostate Gland**
Control	2.10± 0.06	0.16± 0.01	0.75± 0.10	0.28± 0.03	1.07± 0.02	0.08± 0.01	0.38± 0.05	0.14± 0.01
100	1.94± 0.11	0.16± 0.01	0.66± 0.05	0.26± 0.02	1.02± 0.05*	0.09± 0.00	0.35± 0.02	0.14± 0.01
200	1.28± 0.06*	0.16± 0.00	0.66± 0.13	0.23± 0.03	0.69± 0.03*	0.08± 0.01	0.35± 0.07	0.12± 0.02
300	1.13± 0.04*	0.14± 0.01	0.49± 0.12	0.21± 0.04	0.63± 0.02*	0.08± 0.00	0.27± 0.05	0.10± 0.02
400	1.12± 0.05*	0.12± 0.01	0.35± 0.03	0.22± 0.04	0.65± 0.07*	0.07± 0.01	0.19± 0.02	0.12± 0.02

Note: n = 6; *- significant at $p < 0.05$

Table 4.30. Effect of EGME on Haematological parameters in male Wistar rats

Dose (mg/kg)	PCV (%)	Hb (g/ 100mL)	WBC Total (x 10^3)	N (%)	L (%)	M (%)	E (%)	Platelets (x 10^3)	MCV (fl)	MCH (pg)	MCHC (g/ 100mL)	RBC (x 10^{12}/ L)
Control	41.80±	12.77±	10,857.14±	47.60±	44.90±	7.11±	1.10±	509.50±	61.50±	19.10±	30.40±	6.68±
	1.62	0.49	939.32	4.04	4.14	0.72	0.46	59.42	1.45	0.41	0.34	0.25
100	43.80±	12.89±	6,800.00±	46.80±	48.20±	7.50±	1.30±	446.70±	61.10±	18.00±	29.30±	7.16±
	1.69	0.52	371.29*	3.37	2.30	0.50	0.54	51.20	1.36	0.26	0.47	0.26
200	41.78±	12.24±	6,950.00±	41.11±	49.56±	7.89±	1.22±	352.56±	63.22±	18.11±	29.22±	6.66±
	1.52	0.46	1,119.90*	2.35	2.11	0.82	0.49	35.53	0.95	0.56	0.72	0.23
300	38.25±	10.70±	3,616.67±	31.43±	58.63±	8.00±	1.50±	351.67±	63.88±	18.00±	28.13±	5.99±
	0.88	0.27	388.52*	2.94*	3.83*	0.73	0.38	59.21*	1.30	0.27	0.40*	0.17*
400	35.86±	9.57±	3,716.67±	37.50±	52.83±	8.00±	1.71±	266.50±	68.43±	16.43±	27.14±	5.27±
	2.60	0.80*	387.66*	3.10	3.50	0.44	0.36	31.88*	1.99*	1.43*	0.80*	0.41*

Note: n = 6; *- significant at $p < 0.05$; PCV- Packed Cell Volume; Hb- Haemoglobin concentration; WBC- White Blood Cell count; N- Neutrophils, L- Lymphocytes; M- Monocytes; E- Eosinophils; MCV- Mean Cell Volume; MCH- Mean Cell Haemoglobin; MCHC- Mean Cell Haemoglobin Concentration; RBC- Red Blood Cell count

Table 4.31 Effect of fourteen day treatment of EGME on the spermiogram in Wistar rats

Parameter	Control	100 mg/kg	200 mg/kg	300 mg/kg	400 mg/kg
Daily Spermatozoa Production	19.31±	15.06±	15.05±	16.54±	14.80±
(DSP)	0.61	1.68	0.96	2.16	1.14
Testicular Spermatozoa Number (TSN)	46.00±	33.33±	26.67±	24.67±	21.00±
	3.61	3.53	3.28*	2.40*	1.73*
Epididymal Spermatozoa Number (ESN)	132.33±	123.00±	107.57±	102.57±	107.14±
	3.62	3.14	6.93*	5.98*	5.75*
Motility (%)	92.22± 0.88	80.00± 2.11*	71.25± 2.95*	70.00± 3.78*	65.00± 3.42*
Viability (%)	97.33± 0.44	96.50± 0.50	93.78± 1.72	95.25± 1.56	86.57± 7.94*
Abnormalities (%)	10.60± 0.46	11.53± 0.27	12.28± 0.60*	12.05± 0.59*	12.52± 0.40*

Note: n = 6; *- significant at $p < 0.05$; KEY: Parameter (unit):- DSP (x 10^6 cells/ gm testis), TSN (x 10^6/ gm testis), ESN (x 10^6/ ml)

Table 4.32. Effect of EGME on testicular antioxidant parameters in Wistar rats

Dose (mg/kg)	LPO	CAT	SOD	GST	VIT.C	GSH	GPx	LDH
Control	4.22± 0.13	49.33± 1.02	8.83± 0.61	6.39± 0.25	34.50± 2.01	4.84± 0.12	238.01± 7.51	23.05± 1.00
100	4.50± 0.12	49.32± 1.82	3.44± 0.57*	7.13± 0.51	41.83± 3.17	4.50± 0.08	260.32± 14.25	16.27± 1.43*
200	6.86± 0.30*	79.96± 3.23*	3.74± 0.75*	9.97± 0.60*	41.00± 3.71	4.49± 0.25	395.00± 18.69*	14.06± 1.54*
300	6.18± 0.39*	105.79±6.57*	4.69± 0.70*	11.83± 0.28*	40.25± 3.12	4.85± 0.16	439.77± 15.21*	8.24± 0.17*
400	7.14± 0.60*	101.75± 14.43*	2.85± 0.16*	12.38± 0.92*	35.67± 1.20	4.57± 0.10	464.22± 22.65*	6.24± 0.45*

Note: n = 6; *- significant at $p < 0.05$; KEY: Parameter (unit):- LPO- Lipid peroxidation (µM malondialdehyde formed/ mg protein), CAT- Catalase (mmoles H_2O_2 consumed/min/mg protein), SOD- Superoxide dismutase (unit/mg protein), GST- Glutathione-S-Transferase (IU), VIT. C- Ascorbic acid (µg/mL), GSH- reduced glutathione (µM/ g tissue), GPx- Glutathione peroxidase (µg/mL/mg protein), LDH- Lactate dehydrogenase (unit/ mg protein)

Table 4.33. Effect of EGME on epididymal antioxidant parameters in Wistar rats

Dose (mg/kg)	LPO	CAT	SOD	GST	VIT.C	GSH	GPx	LDH
Control	17.62± 0.17	127.48± 12.14	16.67± 2.38	9.18± 0.75	3.50± 0.50	12.64± 1.26	3.05± 0.20	30.92± 3.75
100	17.79± 1.68	120.32± 13.20	19.05± 2.38	14.24± 1.99*	4.67± 0.67	12.84± 0.77	3.15± 0.09	2.66± 0.12*
200	17.40± 1.28	135.97± 15.08	21.43± 5.05	13.09± 1.05	3.50± 0.22	10.80± 1.17	3.16± 0.10	3.37± 0.56*
300	24.60± 3.21*	140.27±18.89	28.57± 7.14	20.36± 2.84*	7.20± 0.58*	15.89± 1.24	3.68± 0.26*	2.99± 0.92*
400	24.42± 2.18*	135.06± 7.14	30.95± 2.38*	21.27± 3.44*	6.00± 0.41*	16.25± 1.37*	4.02± 0.28*	0.97± 0.10*

Note: n = 6; *- significant at $p < 0.05$; KEY: Parameter (unit):- LPO- Lipid peroxidation (µM malondialdehyde formed/ mg protein), CAT- Catalase (mmoles H_2O_2 consumed/min/mg protein), SOD- Superoxide dismutase (unit/mg protein), GST- Glutathione-S-Transferase (IU), VIT. C- Ascorbic acid (µg/mL), GSH- reduced glutathione (µM/ g tissue), GPx- Glutathione peroxidase (µg/mL/mg protein), LDH- Lactate dehydrogenase (unit/ mg protein)

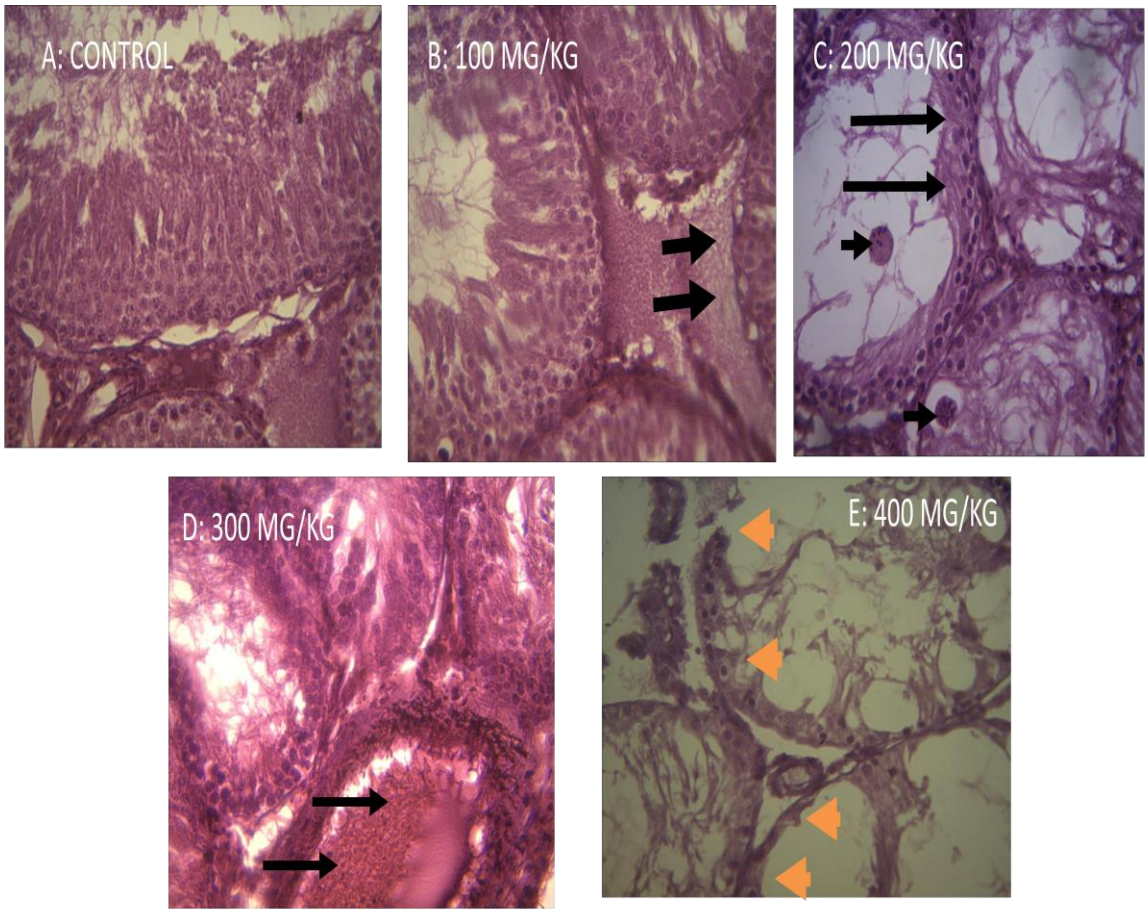

Figure 4.9. Photomicrographs of Testis in the control and treatment groups after 14 day treatment of EGME H&E, M X 400.

A: No lesions; **B**: Mild quantity of pink staining (oedema) fluid in the interstitium; **C**: Some sections of the seminiferous tubules have a greatly reduced germinal epithelial height (*long arrows*). Late maturing stages appear absent and there are few cellular clumps in the lumina (*short arrows*); **D**: Mild congestion of the interstitial vessels; **E**: Severe epithelial erosion.

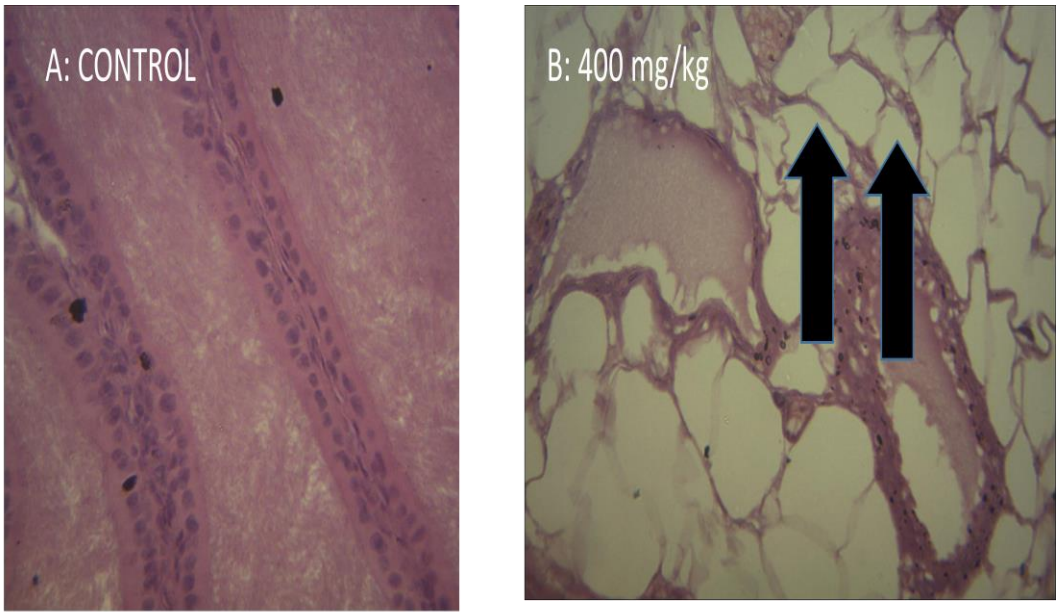

Figure 4.10. Photomicrographs of Epididymis in the control and 400 mg/kg dose group after 14 day treatment of EGME. H&E, M X 400.
A: No lesion; **B**: Severe diffuse germinal cell erosion

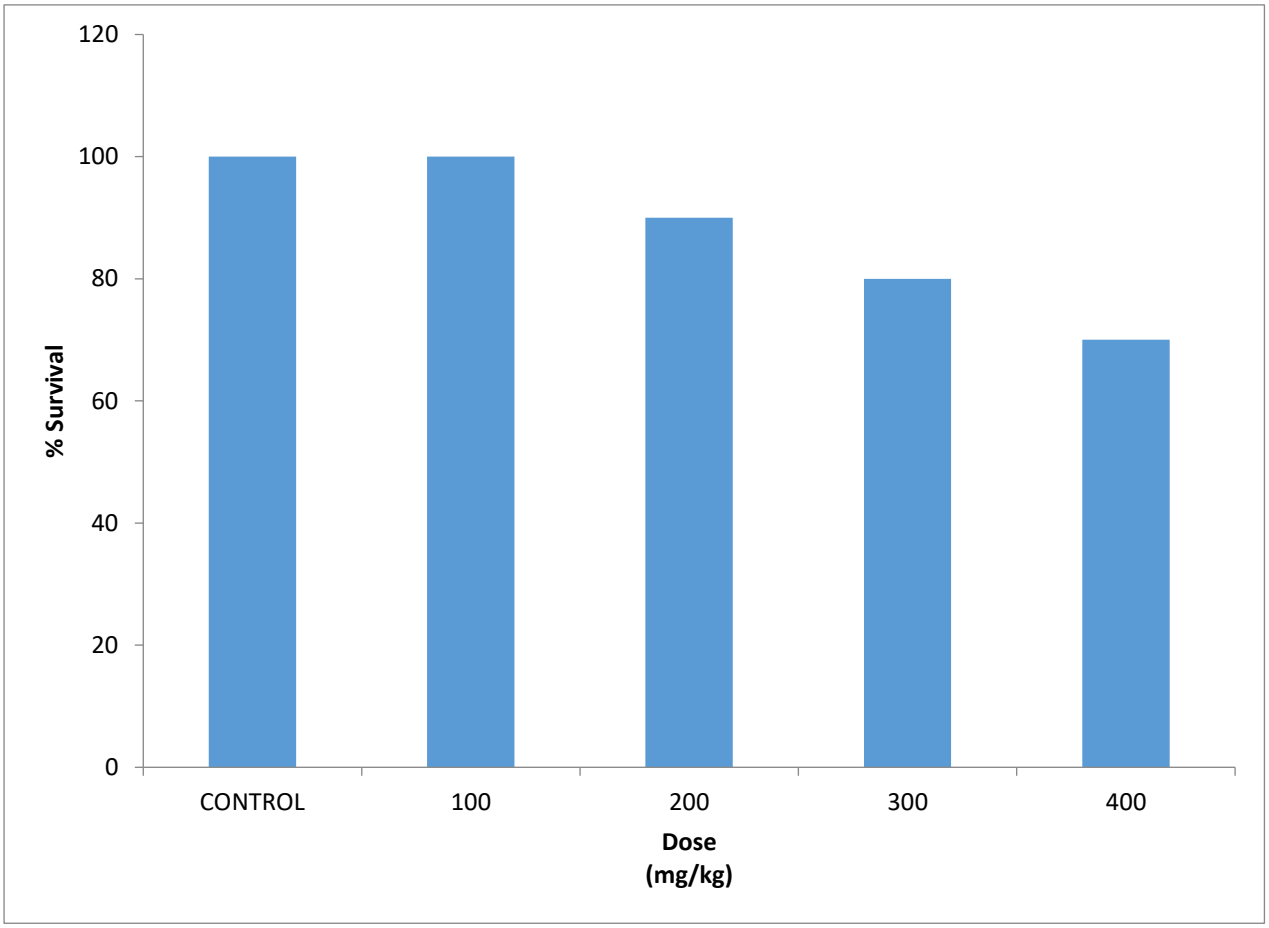

Figure 4.11. Survival rate in the control and treatment groups of Ethylene glycol monomethyl ether

EXPERIMENT VII: CHEMOPREVENTIVE STUDY OF *PAULLINIA PINNATA* METHANOL LEAF EXTRACT IN EGME-INDUCED TESTICULAR DYSFUNCTION

Introduction

Zamble *et al.* (2006) showed that *P.pinnata* extract, which has been shown to be rich in polyphenols, has the ability to induce endothelium- dependent vaso-relaxation of bovine aortic endothelial cells through the nitric oxide pathway and inhibit endothelin-1 synthesis thus suggesting that *P.pinnata* can help impotence.

Moreover, there is the folkloric claim that the leaf is used to help sterility or infertility (Burkill, 2000).

The extract has been shown to have antioxidant properties (Zamble *et al.*, 2006; Jimoh *et al.*, 2007; Tamokou *et al.*, 2013) and Sikka and Naz (2002) have explained that infertility can be as a result of oxidative stress amongst other factors.

Ethylene glycol monomethyl ether (EGME) has been shown to elicit its gonadotoxic potential by altering the oxidative stress status in the male reproductive system (Malik and Gupta, 2013).

The aim of this experiment therefore, using EGME as the reproductive dysfunction model, is to investigate the ability of the methanol leaf extract of *P.pinnata* to prevent oxidative stress, thus improving infertility.

Procedure

Sixty (60) ten week old (165- 190 g) male Wistar rats were obtained from the Primate colony of the Department of Biochemistry, University of Ibadan and divided randomly into six

groups of ten animals each. The animals were kept in appropriate laboratory cages in the Animal house of the same Department to acclimatize for a week and had access to mice cubes (Ladokun Feed, Nigeria) and water *ad libitum*. The 12:12 light/dark cycle was maintained. The treatment protocol was done orally for twenty-one consecutive days as follows:

Group I was the normal rats without treatment (Control 1)

Group II received the vehicle (10% dimethyl sulfoxide (DMSO) (Control 2)

Group III received EGME only at 200 mg/kg dose

Group IV received *Paullinia pinnata* extract only at 200 mg/kg body

Group V received EGME (200 mg/kg dose) + *P.pinnata* extract (100 mg/kg) (PP 100)

Group VI received EGME (200 mg/kg dose) + *P.pinnata* extract (200 mg/kg) (PP 200)

On the 22nd day, after an overnight fast, blood was collected by ocular puncture using heparinized capillary tubes into ethylene diamine tetra acetic acid (EDTA) specimen bottles and plasma was separated. The animals were euthanized by cervical dislocation under light anaesthesia. The brain, seminal vesicles, epididymes, testes and prostate glands were excised and weighed. The left testes and epididymis, and the brain were homogenized and centrifuged to obtain the supernatant and used for biochemical analyses of antioxidant and inflammatory markers. The right testes and epididymes were used for spermatozoa analysis and fixed in Bouin's solution, processed, sectioned, mounted on slides and stained for histological examination. The pituitary gland and the hypothalamus of the brain were also observed histologically. Plasma was separated from the blood and used to determine the level of the male reproductive hormones which are luteinizing hormone, follicle stimulating hormone, testosterone, triiodothyronine and thyroxine using commercial enzyme

immunoassay kits.

Results

Figure 4.12 shows the percentage change in weight in the control and treatment groups. The groups that received co-administrattion of EGME with the extract showed weight loss ranging from 0.29% - 0.59% and 7.02% - 7.82% between day 8 to day 16 and day 16 to day 21 respectively, while the other groups showed increases in weight.

Table 4.34 shows that the weights of the testes and epididymis decreased significantly ($p < 0.05$) compared to the control in the EGME only group and in the co-administered groups. The weight of the seminal vesicle and the prostate gland was also decreased compared to the control, but the decrease was significant ($p < 0.05$) in the EGME + PP (200 mg/kg) group. The relative weight of the testis (i.e. weight of testes per 100 g of body weight) decreased significantly ($p < 0.05$) compared to the control in the EGME only and the EGME + PP co-administered groups.

The reproductive hormone profile is presented in Table 4.35. The plasma levels of luteinizing hormone, follicle stimulating hormone, triiodothyronine and thyroxine were significantly ($p < 0.05$) increased compared to the control in the EGME + PP co-administered groups while the plasma testosterone level is significantly ($p < 0.05$) reduced.

The spermatozoa analysis (Table 4.36) shows that spermatozoa motility, spermatozoa viability (live:dead ratio) and testicular spermatozoa number were significantly ($p < 0.05$) reduced compared to the control in the EGME only and EGME + PP co-administered groups. The total sperm abnormality and daily sperm production is significantly ($p < 0.05$) increased compared to the control in the EGME only and EGME + PP co-administered groups.

The antioxidant profile in the epididymis (Table 4.37) shows that there are decreases in the activities of superoxide dismutase, catalase, glutathione-S-transferase and glutathione peroxidase in the EGME only and EGME + PP co-administered groups. The levels of reduced glutathione and vitamin C was also reduced significantly ($p < 0.05$) in these groups and lipid peroxidation was not significantly affected.

The antioxidant profile in the testis (Table 4.38) shows that the activities of glutathione-S-transferase, catalase and glutathione peroxidase are increased while the activity of super-oxide dismutase and the level of vitamin C are decreased significantly ($p < 0.05$) in the EGME only and EGME + PP co-administration groups compared to the control. Lipid peroxidation is significantly ($p < 0.05$) elevated in the EGME only group and insignificantly ($p < 0.05$) elevated in the EGME+ PP co-administered groups compared to the control. Hydrogen peroxide generation increases significantly ($p < 0.05$) in the EGME only and EGME + PP co-administered groups compared to the control. The activity of lactate dehydrogenase is increased, though not significantly in the EGME only and EGME-PP co-administered groups. Reduced glutathione is significantly ($p < 0.05$) increased in the EGME only and EGME+PP co-administered groups compared to the control.

The antioxidant profile in the brain (Table 4.39) shows that the activity of superoxide dismutase is significantly ($p < 0.05$) reduced in the EGME + PP co-administered groups compared to the control. Also the glutathione-S-transferase activity is increased significantly ($p < 0.05$) in the EGME only and the EGME + PP (100 mg/kg) groups compared to the control, and the activity of glutathione peroxidase is increased significantly ($p < 0.05$) in the EGME + PP (200 mg/kg) group compared to the control. The level of vitamin C is significantly ($p < 0.05$) reduced in the EGME+PP co-administered groups compared to the control.

Table 4.40 shows the anti-inflammatory profile in the epididymis, testis and brain. In the epididymis, nitric oxide concentration is not affected but the activity of myeloperoxidase is significantly ($p < 0.05$) increased in the EGME only and EGME + PP co-administered groups compared to the control. In the testis, there are significant ($p < 0.05$) elevations in the activity of myeloperoxidase and nitic oxide concentration in the EGME only and EGME + PP co-administered groups compared to the control. In the brain, there is no obvious effect on the myeloperoxidase activity and the nitric oxide concentration.

Figure 4.13 shows the histopathology of the testis. The control, 10 % DMSO and *P. pinnata* only groups showed no lesions (A). The EGME only group showed severe germinal erosion and severely congested interstitium (B). The EGME + PP co-administered groups showed severe germinal erosion (C & D).

Figure 4.14 shows the histopathology of the hypothalamus. Again the control, 10 % DMSO and *P. pinnata* only groups showed no lesions (A). The EGME only group showed moderate to severe diffuse spongiosis of the parenchyma and the submeningeal portion was mildly haemorrhagic (B). The EGME + PP (100 mg/kg) group showed moderate spongiosis of the parenchyma (E) and the EGME + PP (200 mg/kg) showed severe spongiosis of the same (F). The pituitary and the epididymis showed no lesions in the control and treatment groups.

The histological examination of the pituitary gland is presented in Figure 4.15. There was no lesion in the control (A) and all the treatment groups (B- F).

Figure 4.16 presents the survival rate and it shows that there was 100 % survival in the control, 10 % DMSO and *P. pinnata* only groups. The EGME only group showed 90 % survival while the EGME + PP (100 mg/kg) and EGME + PP (200 mg/kg) groups had 70 % and 50 % survival respectively.

Conclusion

Paullinia pinnata methanol leaf extract does not have chemopreventive effect against gonadotoxicity by Ethylene glycol monomethyl ether.

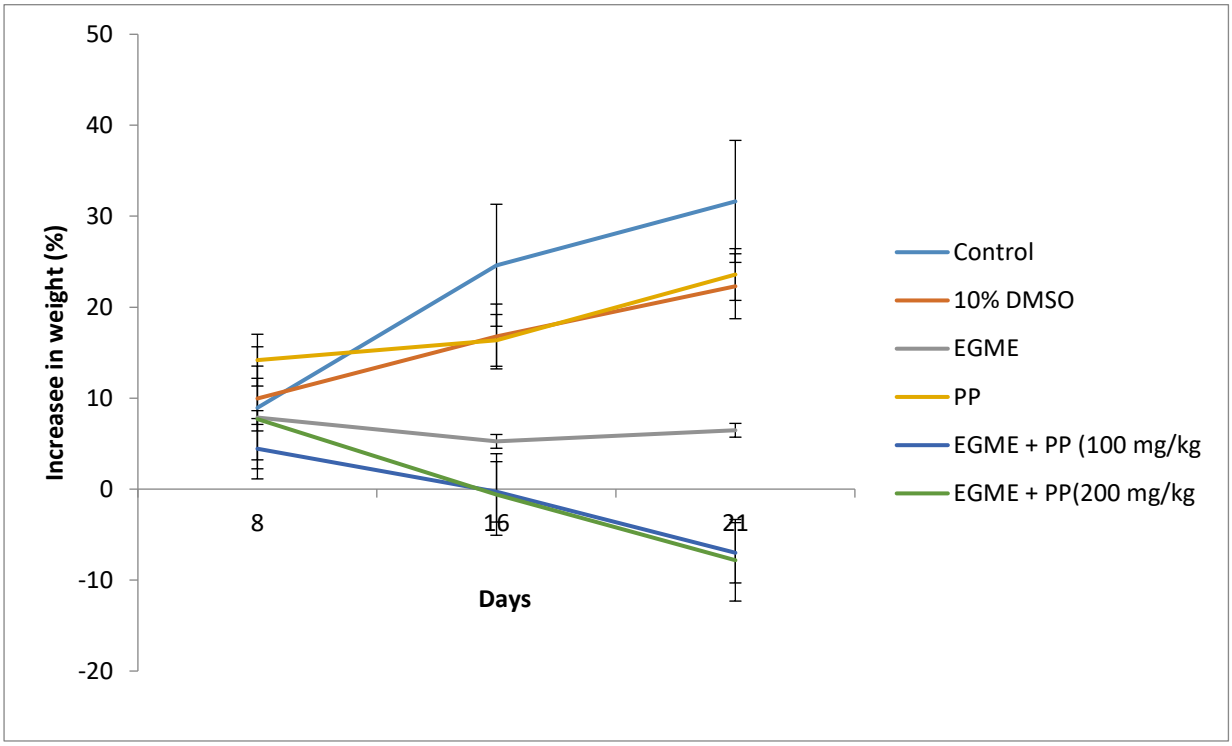

Figure 4.12. Percentage change in weight in the control and treatment groups

Table 4.34. Weight and relative weight of organs after 21 day treatment of EGME and *Paullinia pinnata* methanol leaf extract

Group	Weight of organs (g)					Relative weight of organs (%)				
	Testes	**Epididymis**	**Seminal Vesicle**	**Prostate Gland**	**Brain**	**Testes**	**Epididymis**	**Seminal Vesicle**	**Prostate Gland**	**Brain**
Control	2.46± 0.11	0.57± 0.02	0.94± 0.07	0.25± 0.03	1.77± 0.03	1.16± 0.06	0.25± 0.02	0.42± 0.03	0.11± 0.01	0.79± 0.04
10 % DMSO	2.24± 0.06	0.52± 0.03	0.92± 0.05	0.28± 0.03	1.76± 0.03	1.11± 0.04	0.26± 0.02	0.44± 0.02	0.14± 0.02	0.82± 0.01
EGME	0.96± 0.05*	0.47± 0.03*	0.94± 0.06	0.20± 0.03	1.73± 0.01	0.49± 0.02*	0.22± 0.02	0.46± 0.05	0.14± 0.02	0.83± 0.02
PP	2.36± 0.11	0.54± 0.03	1.01± 0.05	0.33± 0.02	1.75± 0.03	1.09± 0.05	0.24± 0.02	0.47± 0.03	0.14± 0.01	0.81± 0.03
EGME + PP(100)	0.95± 0.06*	0.41± 0.03*	0.84± 0.11	0.20± 0.03	1.78± 0.03	0.55± 0.05*	0.23± 0.02	0.43± 0.04	0.12± 0.02	1.05± 0.06*
EGME + PP(200)	0.76± 0.06*	0.34± 0.03*	0.71± 0.07*	0.15± 0.02*	1.71± 0.04	0.48± 0.05*	0.22± 0.02	0.38± 0.09	0.10± 0.02	1.07± 0.05*

Note: n = 10; *- significant at $p < 0.05$

Table 4.35. Reproductive hormone profile after 21 day treatment of Ethylene glycol monomethyl ether and *Paullinia pinnata* methanol leaf extract

Group	LH	FSH	TESTOSTERONE	T_3	T_4
Control	10.00± 0.00	8.50± 0.50	1.57± 0.12	1.37± 0.07	68.33± 1.76
10 % DMSO	11.67± 0.88	6.00± 2.65	1.50± 0.06	1.47± 0.07	75.50± 2.50
EGME	12.00± 1.00	9.50± 1.50	1.50± 0.10	1.50± 0.00	74.00± 4.00
PP	11.50± 0.50	9.67± 0.33	1.63± 0.09	1.50± 0.20	80.00± 1.00*
EGME + PP(100)	13.50± 1.50*	11.00± 2.00	1.15± 0.15*	1.77± 0.09*	87.67± 4.91*
EGME + PP(200)	14.67± 0.67*	11.67± 0.67	1.13± 0.07*	1.40± 0.10	87.00± 2.08*

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- LH- Luteinizing Hormone (pg/mL), FSH- Follicle Stimulating Hormone (pg/mL), Testosterone (pg/mL), T_3- Triiodothyronine (pg/mL), T_4- Thyroxine (pg/mL)

Table 4.36. Spermatozoa analysis after 21 day treatment of Ethylene glycol monomethyl ether and *Paullinia pinnata* methanol leaf extract

Parameters	Control	10 % DMSO	EGME	PP	EGME + PP(100)	EGME + PP(200)
Abnormality (%)	11.94±	11.62±	13.01±	12.79±	14.03±	14.59±
	0.25	0.22	0.31*	0.52	0.37*	0.14*
Motility (%)	67.50±	72.50±	12.50±	80.00±	3.50±	10.00±
	5.74	4.96	2.50*	7.79	1.50*	2.89*
Viabilty (%)	70.00±	70.00±	11.75±	82.86±	11.00±	17.50±
	3.87	5.88	1.18*	2.40*	1.00*	2.50*
Testicular Spermatozoa Number (TSN)	116.83±	98.14±	86.83±	105.00±	88.50±	93.00±
	9.03	6.14	5.53*	7.42	0.50	5.00
Daily Sperm Production (DSP)	49.40±	40.90±	58.67±	45.73±	67.24±	101.00±
	0.22	1.53	1.29	4.75	3.72*	4.91*

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- TSN (x 10^6/ gm testis), DSP (x 10^6 cells/ gm testis)

Table 4.37. Antioxidant profile in the epididymis after 21 day treatment of EGME and *Paullinia pinnata* methanol leaf extract

Group	SOD	CAT	GSH	LPO	GST	H_2O_2	VIT. C	GPx
Control	9.63± 1.48	17.52± 1.23	2.04± 0.35	6.80± 0.81	0.17± 0.02	0.59± 0.04	14.00± 1.05	322.81± 24.51
10 % DMSO	7.56± 0.88	18.34± 1.37	1.47± 0.21	5.81± 0.48	0.13± 0.00	0.57± 0.03	11.00± 3.51	298.80± 7.02
EGME	2.22± 0.00	11.79± 1.40*	0.72± 0.13*	7.23± 0.14	0.23± 0.04	0.67± 0.12	6.12± 0.70*	281.86± 22.57
PP	13.33± 1.11	16.77± 1.47	1.48± 0.04	6.73± 0.75	0.18± 0.03	0.54± 0.05	13.40± 1.17	309.49± 20.99
EGME + PP(100)	6.67± 0.00	15.86± 1.43	1.54± 0.24	7.35± 1.46	0.20± 0.01	0.58± 0.03	3.60± 1.43*	295.11± 5.05
EGME + PP(200)	3.70± 1.48	13.67± 0.27	1.23± 0.12*	6.83± 0.74	0.18± 0.04	0.62± 0.05	3.00± 2.00*	273.60± 24.80*

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- SOD- Superoxide dismutase (unit/ mg protein), CAT- Catalase (µmoles H_2O_2 consumed/min/mg protein), GSH- reduced glutathione (µM/ g tissue), LPO- Lipid peroxidation (µM malondialdehyde formed/ mg protein), GST- Glutathione-S-Transferase (IU), H_2O_2- Hydrogen peroxide generation (µM), VIT. C- Ascorbic acid (µg/mL), GPx- Glutathione peroxidase (mg/mL/mg protein)

Table 4.38. Antioxidant profile in the testis after 21 day treatment of EGME and *Paullinia pinnata* methanol leaf extract

Group	GSH	SOD	CAT	LPO	GST	H_2O_2	VIT. C	GPx	LDH
Control	1.91± 0.08	9.52± 0.00	9.81± 0.82	2.06± 0.08	0.85± 0.02	0.59± 0.02	30.00± 3.81	43.86± 1.45	4.64± 1.58
10% DMSO	2.14± 0.05	9.52± 0.00	9.05± 1.12	2.04± 0.11	0.77± 0.02	0.62± 0.06	26.00± 1.53	40.67± 1.43	4.55± 1.10
EGME	2.32± 0.18*	7.14± 2.38	15.20± 1.33*	3.13± 0.11	1.27± 0.04*	0.87± 0.04*	23.67± 4.67*	48.07± 0.83	4.88± 1.44
PP	1.77± 0.10	11.11± 1.59	10.34± 0.66	1.99± 0.30	0.81± 0.06	0.76± 0.10	25.00± 2.20	46.07± 0.96	4.23± 1.02
EGME + PP(100)	2.00± 0.14	5.00± 1.67*	14.71± 0.71*	2.56± 0.37	1.28± 0.07*	1.11± 0.09*	20.25± 3.09*	57.08± 3.08*	4.76± 0.14
EGME + PP(200)	2.43± 0.00*	2.38± 0.24*	17.33± 1.23*	2.58± 0.39	1.38± 0.05*	1.03± 0.09*	17.00± 5.00*	55.13± 3.64*	7.80± 0.72

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- GSH- reduced glutathione (µM/ g tissue), SOD- Superoxide dismutase (unit/ mg protein), CAT- Catalase (µmoles H_2O_2 consumed/min/mg protein), LPO- Lipid peroxidation (µM malondialdehyde formed/ mg protein), GST- Glutathione-S-Transferase (IU), H_2O_2- Hydrogen peroxide generation (µM), VIT. C- Ascorbic acid (µg/mL), GPx- Glutathione peroxidase (mg/mL/mg protein), LDH- Lactate dehydrogenase (unit/ mg protein)

Table 4.39. Antioxidant profile in the brain after 21 day treatment of EGME and *Paullinia pinnata* methanol leaf extract

Group	SOD	CAT	GSH	LPO	GST	H_2O_2	VIT. C	GPx
Control	12.25± 1.11	3.89± 0.78	5.18± 0.74	1.61± 0.13	0.13± 0.01	0.53± 0.03	7.50± 0.56	40.44± 1.05
10 % DMSO	11.11± 0.00	5.08± 0.42	4.90± 0.80	1.61± 0.09	0.12± 0.01	0.56± 0.05	8.00± 0.71	42.41± 1.62
EGME	11.11± 0.00	3.68± 0.41	4.40± 0.35	1.44± 0.08	0.17± 0.01*	0.51± 0.06	6.33± 0.33	43.65± 1.13
PP	12.70± 1.59	4.51± 1.28	6.36± 0.50	1.63± 0.32	0.14± 0.01	0.54± 0.08	8.67± 0.67	39.72± 1.95
EGME + PP(100)	7.14± 1.37*	3.80± 0.65	6.93± 0.50	1.57± 0.09	0.15± 0.02*	0.54± 0.08	5.50± 0.65*	39.71± 0.91
EGME + PP(200)	4.76± 0.00*	2.46± 0.51	4.36± 0.07	1.25± 0.10	0.14± 0.01	0.54± 0.12	3.00± 0.00*	46.98± 0.78*

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- SOD- Superoxide dismutase (unit/ mg protein), CAT- Catalase (µmoles H_2O_2 consumed/min/mg protein), GSH- reduced glutathione (µM/ g tissue), LPO- Lipid peroxidation (µM malondialdehyde formed/ mg protein), GST- Glutathione-S-Transferase (IU), H_2O_2- Hydrogen peroxide generation (µM), VIT. C- Ascorbic acid (µg/mL), GPx- Glutathione peroxidase (mg/mL/mg protein)

Table 4.40. Anti-inflammatory profile in the epididymis, testis and brain after 21 day treatment of Ethylene glycol monomethyl ether and *Paullinia pinnata* methanol leaf extract

	Epididymis		Testis		Brain	
Group	**MPO**	**NO**	**MPO**	**NO**	**MPO**	**NO**
Control	8.97± 1.15	1.53± 0.21	13.83± 1.03	0.77± 0.08	12.31± 0.27	0.98± 0.10
10 % DMSO	8.36± 0.54	1.51± 0.17	13.61± 1.44	0.69± 0.09	12.72± 1.29	1.06± 0.05
EGME	19.42± 1.98*	1.35± 0.23	18.32± 0.44*	1.40± 0.10*	13.33± 3.13	1.20± 0.08
PP	12.00± 2.08	1.27± 0.69	14.53± 1.63	0.92± 0.06	15.67± 1.54	1.05± 0.09
EGME + PP(100)	17.14± 1.91*	1.01± 0.05	19.04± 1.77*	2.16± 0.18*	13.67± 1.85	1.15± 0.03
EGME + PP(200)	16.34± 1.24*	1.22± 0.07	17.27± 1.12	1.67± 0.24*	14.00± 1.41	0.91± 0.02

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- MPO- myeloperoxidase (IU/ mg protein), NO- Nitric oxide (mM)

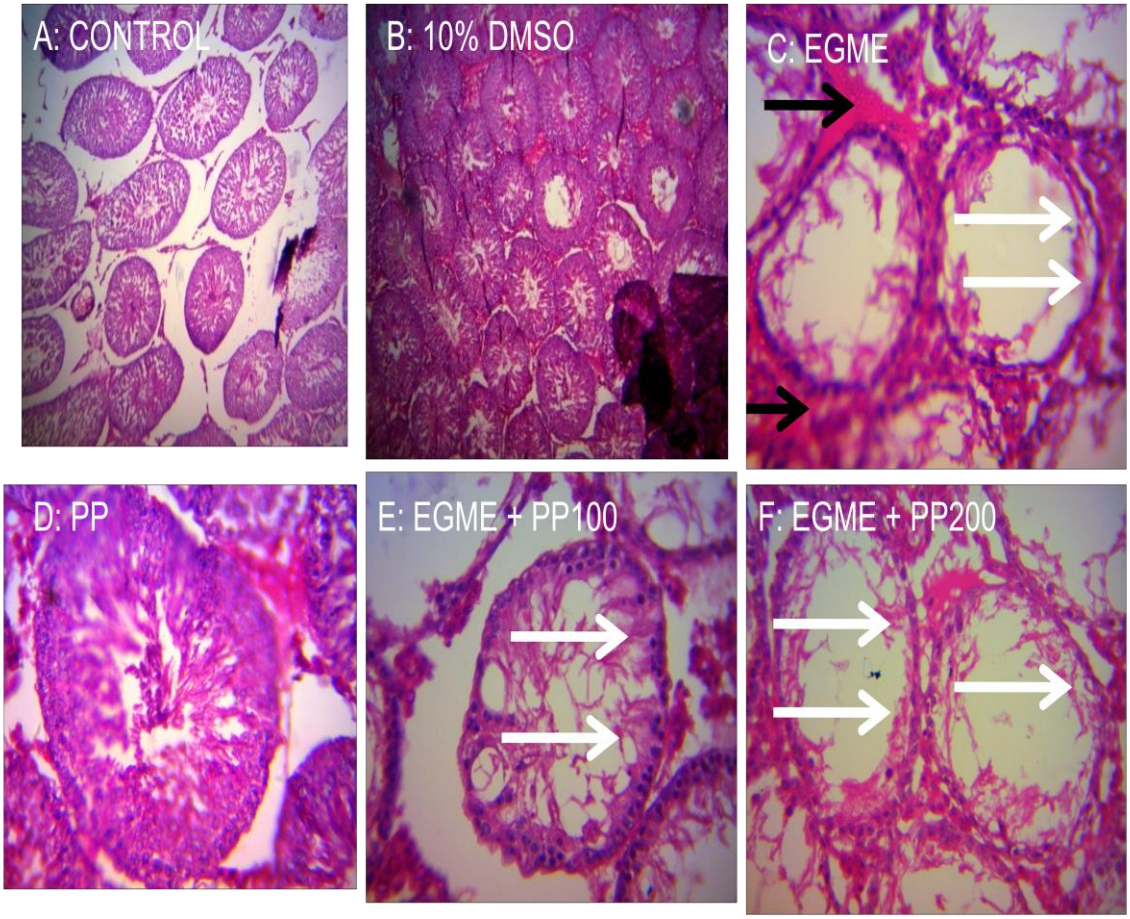

Figure 4.13. Photomicrographs of the Testis in the study of the chemopreventive role of *P.pinnata*. H & E, M x 400

A: No lesion; B: No lesion; C: Severe germinal erosion (white arrows) and severely congested interstitium (black arrows); D: No lesion; E: Severe germinal erosion; F: Severe germinal erosion

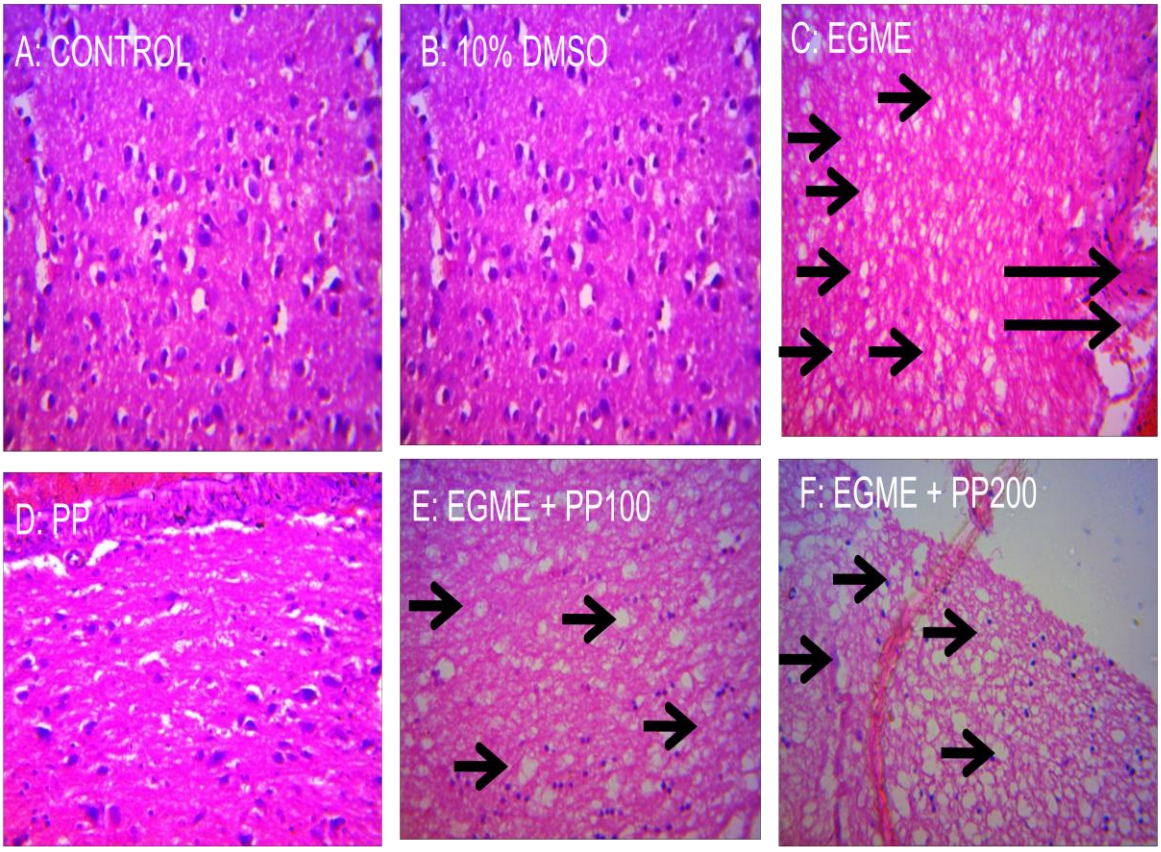

Figure 4.14. Photomicrographs of the Hypothalamus in the study of the chemopreventive role of *P.pinnata*.
H. & E., M x400
A: No lesion; B: No lesion; C: Moderate to severe diffuse spongiosis of the parenchyma (short arrow) and submeningeal portion is mildly haemorrhagic (long arrows); D: No lesion; E: Moderate spongiosis of the parenchyma; F: Severe spongiosis of the parenchyma

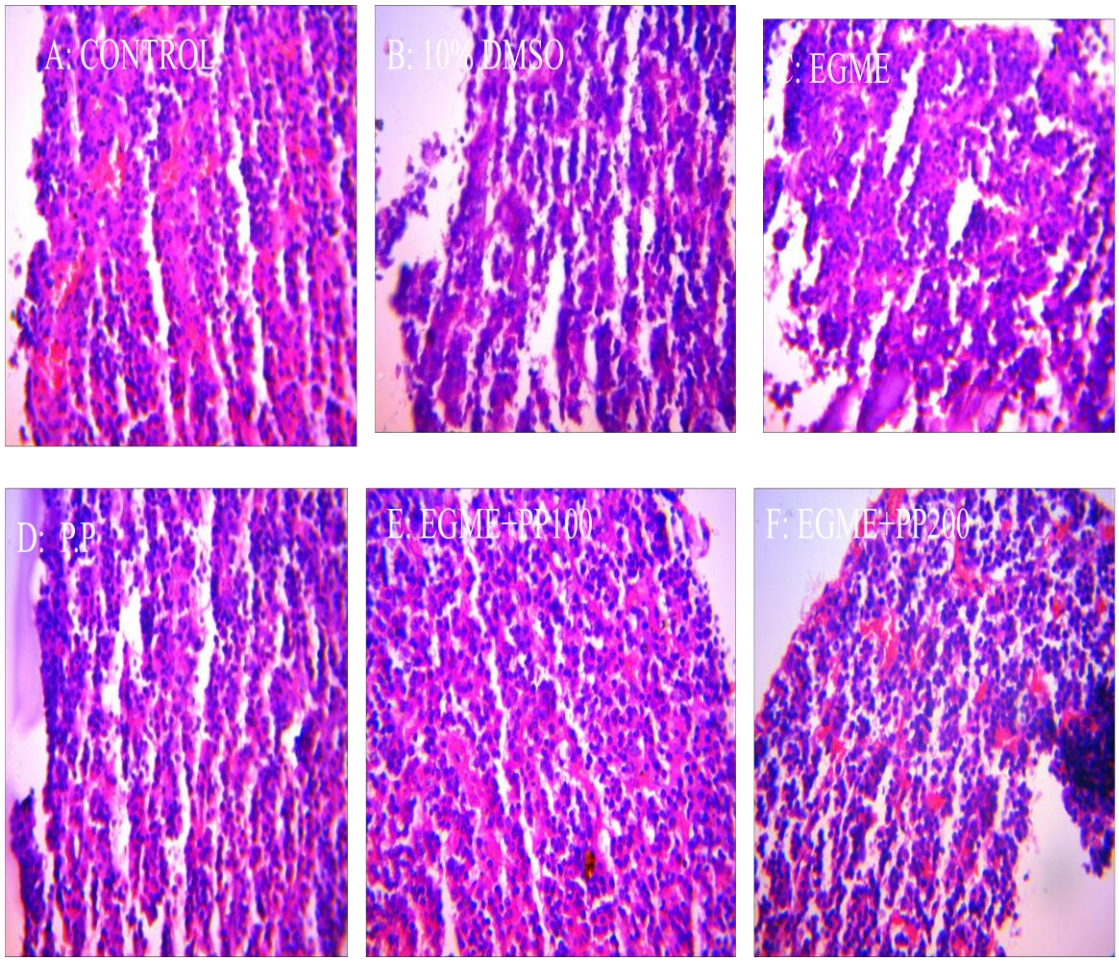

Figure 4.15. Photomicrographs of the Pituitary gland in the study of the chemopreventive role of *P.pinnata*. H.& E., M x 400
A-F: No Lesion

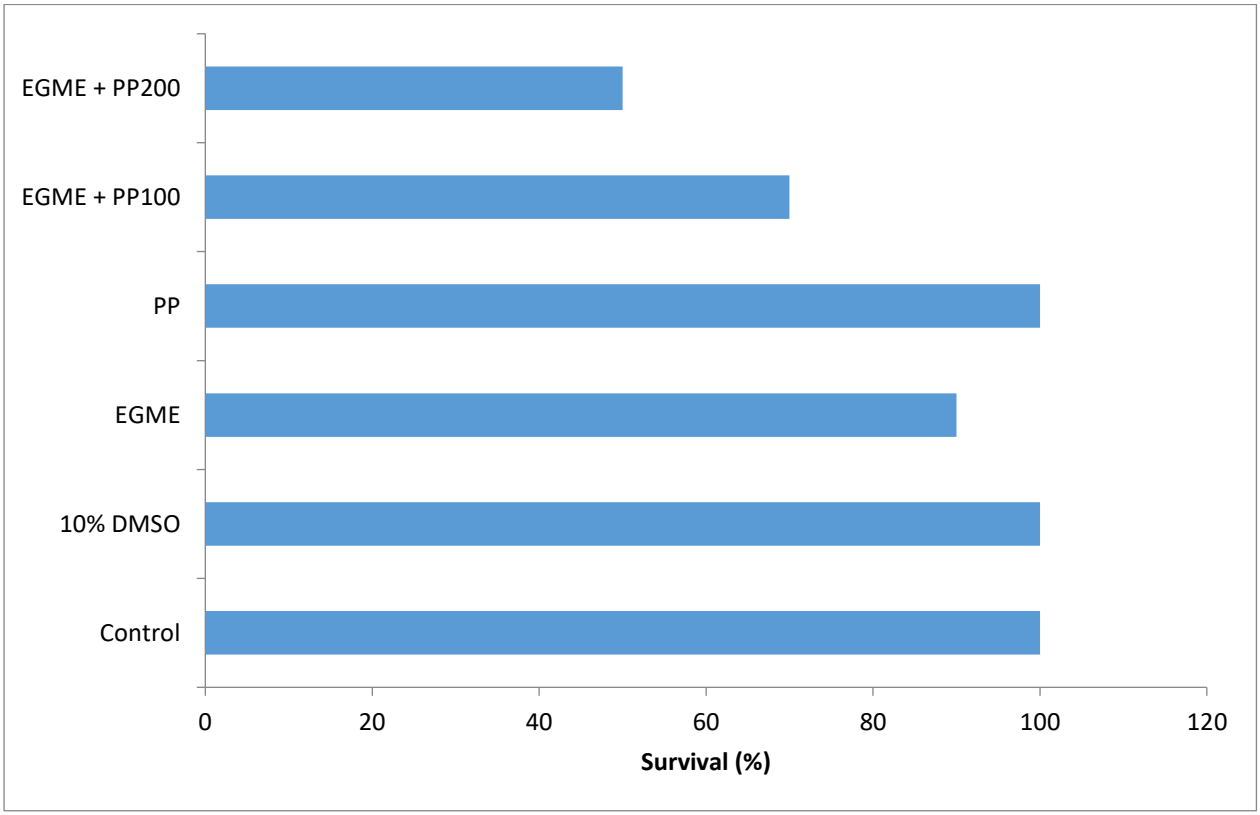

Figure 4.16. Survival rate in the chemoprevention study of *P.pinnata*

CHAPTER FIVE

DISCUSSION

The bioactivities of medicinal plants are as a result of their active phytochemical constituents which elicit physiological and biochemical actions on the human body (Ekpo *et al*., 2013). The phytochemical screening confirms the presence of alkaloids using 1% hydrochloric acid and thin layer chromatography; flavonoids when tested with ammonia/H_2SO_4, aluminium solution and ethyl acetate/ ammonia; tannins using the ferric chloride test; terpenoids using the Salkowski test, and cardiac glycosides using the kedde and keller-killiani tests in the leaf extract of *P.pinnata*. This supports the findings of various researchers including Jimoh *et al.* (2007), Osarenmwinda *et al.* (2009) and Ior *et al.* (2011). Anthraquinones, both free and combined, and saponins were absent.

The leaves of *P.pinnata* are being used and taken copiously in various forms in the treatment of various ailments and diseases. Investigations need to be carried out to establish the toxic level especially when it is known to also be used for lethal purposes. The acute – toxicity study showed that the treatment did not cause any physiological (loss of hair, redness of eyes or blindness) or gross behavioral (reflexes, restlessness, sleeping pattern, locomotive disability) changes, and it did not result in mortality even at a dose of 10,000 mg/kg body weight. This implies that the leaves of *P.pinnata* may be tolerable to the metabolic system of the mice even when taken in large amounts.

For the sub – chronic toxicity study, the percentage weight of the vital organs; kidneys, lungs and liver, did not vary significantly from that of the control rats although a dose- dependent increase was observed in all the organs except the kidneys. This suggests that the methanol extract of the leaves may not have any deleterious effect on these organs and therefore on the metabolic system of the animals even at a dose of 800 mg/kg body weight.

Plasma concentrations of total bilirubin, total protein and albumin did not vary significantly in the treatment groups. This suggests that the liver may not be affected. However, plasma cholesterol and triglyceride levels in the group treated with a dose of 400 mg/kg body weight increased significantly compared to that of the control. This suggests impairment in the function of the liver at this dose. Studies have shown that the impairment of liver catabolic processes for chylomicron and Very Low Density Lipoprotein (VLDL) remnants result in an increased concentration of both cholesterol and triglycerides in the plasma (Cooper, 1990). This could be the reason for the increase in cholesterol and triglyceride concentrations in the plasma of the group treated with a dose of 400 mg/kg body weight. This may also be as a result of damage or inhibition of the activity of the chylomicron remnant- and VLDL remnant – receptor sites, either directly or indirectly in the liver and in particular, the hepatocytes. Statistically significant elevations of cholesterol and triglyceride in the plasma were not observed at the 800 mg/kg body weight dose (Table 4.5). This may be as a result of unavailability of the compounds in the extract at this dose to carry out more damage in the liver.

Increased plasma activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are indicators of hepatocellular necrosis, especially ALT (Stolz and Kaplowitz, 1990; Plaa and Charbonneau, 2007). Since the activity of ALT did not vary significantly in the treatment groups, it implies that the liver is not affected by the treatment doses. However, activity of alkaline phosphatase (ALP) in the plasma was significantly increased at the 400 and 800 mg/kg body weight doses. ALP is an indicator of cholestasis in the liver (Stolz and Kaplowitz, 1990) although it is not exclusive to the liver. Increased activity of AST is also known to be due to damage done to other organs apart from the liver (Plaa and Charbonneau, 2007). Therefore, increased activity of this enzyme at the 400 mg/kg body weight dose implies that some other organ(s) and probably the liver were affected at this dose. The

observed differences in the activities of these enzymes at the 800 mg/kg body weight dosage, which is twice the 400 mg/kg body weight dose, could be that the concentration of the AST and ALT released into circulation have their active sites being partially masked or inhibited by the compounds present in the leaf extract. This may result in the observed insignificant decreased activity of ALT and AST in the plasma at this dose.

The bio-markers for renal function are creatinine, urea, bicarbonate, sodium ions and chloride ions (Laragh and Pickering, 1991; Smith and Dunn, 1991; Nduka, 1999). The results show that these were not affected by the various dosages of treatment (Table 4.7). This further shows that the methanol extract of the leaves of *P.pinnata* did not have any obvious effect on renal function.

Results of serum/plasma enzyme tests are to be complimented by histological studies (Plaa and Charbonneau, 2007). The histological findings complimented the results of the tests of the plasma enzymes and the interpretation. There was a dose-dependent occurrence of the degree of lesions in the treatment groups with the group treated with 800 mg/kg body weight showing the highest occurrence of lesions as a result of toxicity in the lungs but not the liver. This further supports the finding that AST is present in a wide variety of tissues apart from the liver (Plaa and Charbonneau, 2007). Therefore the lesions, which are an indication of toxicity in the lungs, may be responsible for the significant increase in the activity of AST. Increased activity of ALP at the 400 mg/kg body weight and 800 mg/kg body weight doses are reflected in the observed lesions in the liver at these dosages. No lesions and therefore no toxic effects were observed in the sections of the kidneys among the treatment groups and the control.

For the haematological analysis, the results of the treated groups were compared with observed physiological ranges in normal animals (as reported by Mitruka *et al.*, 1981) and

control. Packed cell volume (PCV), which is also known as the haematocrit, is a measure of the volume of the red blood cells (erythrocytes) in the blood. In the rats treated with 50, 100 and 400 mg/kg body weight doses, the PCV values showed that blood volume was adequate and even higher than that of the control. The values of the PCV observed in the control and the treatment groups were above the physiological range for normal rats (42.50 – 49.40 %), (Mitruka *et al.*, 1981).

Similiarly, Haemoglobin (Hb) concentrations was also higher in the treated rats than in the control group which may be an indication of adequate blood formation and pigmentation (Cole, 1986). This implies that the oxygen carrying capacity of the blood was higher in the treated rats than in the control. This may be attributed to the extract having the ability to enhance erythropoiesis. Moreover, the groups treated with 50, 100 and 400 mg/kg had higher levels of Hb concentration than the upper limit of the physiological range for normal rats (12.00 – 17.50 g/100ml), (Mitruka *et al.*, 1981). This is similar to the results of Obeagu *et al.* (2014) and Egba *et al.* (2013) using the leaf extract of *Telfairia occidentalis* and the observations of Kadham (2008) in rabbits using the aqueous extract of *Haloxylan salicornicum*. The treated rats recorded higher values for erythrocytes than the control with the exception of those treated with 200 mg/kg dose. This compares well with the results of Dharmarathna *et al*. (2013) which showed that the leaf extract of *Carica papaya* causes increase in red blood cell count (RBC) in a murine model without causing any form of toxicity. Increase in the production of RBCs may be an indication of increased bone marrow function and sufficient erythropoiesis (Cole, 1986) induced by the extract. It seems that the plant extract may contain erythropoietic property that enhances erythropoiesis as also observed in the aqueous extract of the fruit of *Solanum torvum* which increased both the numbers of RBCs and Hb concentration at 37.5 – 150 mg/kg doses in phenyl hydrazine-induced anaemic rats (Koffuor *et al.,* 2011). The results of the PCV, Hb concentration and

RBC count as shown by the animal model would suggest that the plant extract had the potential of reversing anemia since they showed increase in blood formation.

The Mean Corpuscular Volume (MCV) is a measure of the average RBC volume and the Mean Corpuscular Haemoglobin (MCH) is the average mass of Hb per RBC in a sample of blood. These values increased, especially at 200 mg/kg body weight dose. This may be an indication of increase in reticulocytes (immature RBCs). This may be due to increased erythropoiesis caused by the extract. The values of MCV and MCH for the control and treated groups were higher than the physiological range in normal rats (57.00 – 65.00 fL and 14.60 – 21.30 pg respectively) (Mitruka *et al.*, 1981). This may imply that the reticulocytes are macrocytic as expected.

Mean Cell Haemoglobin Concentration or Mean Corpuscular Haemoglobin Concentration (MCHC) is a measure of the concentration of haemoglobin in a given volume of packed RBCs. The values of MCHC for the treated groups showed that the RBCs were hypochromic because they were lower than the lower limit of the normal physiological range (32.00 – 38.50 g/100mL), (Mitruka *et al.*, 1981) and the control.

The white blood cells (WBCs or leukocytes), neutrophils, lymphocytes, monocytes and eosinophils constitute the defense mechanism of the body system. The WBC count of the group treated with the 50, 400 mg/kg body weight doses exceeded the normal physiological range (5.00 – 8.96 mm^3) (Mitruka *et al.*, 1981). Increase in the leukocyte production is always initiated either by infection, injury or toxic substance in the body (Cole, 1986). In this case, it could be due to anti-nutrients such as tannins that have been shown to be inherent in the plant extract (Adeyemo-Salami and Makinde, 2013). Anti-nutrients are natural or synthetic compounds that interfere with the absorption of nutrients. The percentage values for neutrophils, lymphocytes and monocytes in the control and treatment groups were all within

the physiological range (9.00 – 34.00 %), (65.00 – 84.50 %) and (0.00 – 5.00 %) respectively (Mitruka *et al.*, 1981). However, there were increases in the values for the neutrophils at the 50, 100, 200 and 800 mg/kg doses compared to the control. This result is comparable with that of *Momordica charantia* that increased the adhesion of neutrophils to nylon fibers at 900 mg/kg (Meera and Nagarjuna, 2009). Furthermore, it is similar to the observations of Vinothapooshan and Sundar (2011). This indicated that the extract could be useful as chemotherapy for neutrophils. The apparent reduction in eosinophils in the treated rats may be to the advantage of the animals. This is an indication that the animals were not adversely affected in such a way that could elicit the response of eosinophils that are detoxifiers in the respiratory and gastrointestinal tracts (Cole, 1986).

From the results, the maximum tolerable dose of the extract is 200 mg/kg body weight.

Malaria causes the excessive destruction of red blood cells during the parasite's life cycle resulting in anaemia. Anaemia is a haematological disorder caused by a decrease in the production of red blood cells, a bone marrow failure or by an increased destruction of red cells. It is a predominant symptom of malaria in which there is decreased erythropoiesis due to the malarial infection. There is the parasitization of red cells by the malaria parasite which leads to shortened survival or death of erythrocytes (Menendez *et al.*, 2000; Almeida and Mehta, 2000). As a result of this destruction, PCV and hemoglobin values are reduced when measured in this disease condition. For the curative test taking into consideration the safe dose, the PCV for the untreated group decreased throughout the period of study. The groups treated with chloroquine and ACT showed a gradual decrease until Day 2 after which the PCV began to rise steadily but dropped after Day 3 in the chloroquine treated group while the group treated with ACT continued to show a steady rise. The groups treated with 100 and 200 mg/kg doses of the extract did not show a significant increase in the PCV on Day 4 while there was a decrease by the 11^{th} day. This shows that the extract was not effective at the 100

and 200 mg/kg doses to arrest the destruction of the red blood cells by the parasites after the period of administration.

Percentage parasitaemia is a measure of the level of parasites in the blood. Figure 4.6 shows that the extract at 100 and 200 mg/kg doses had mild effects in reducing the percentage parasitaemia by Day 4 and by Day 11, there was an increase in the level of parasitaemia. This implies that the extract does not have the ability to eliminate the parasites and therefore can not effectively ameliorate the disease condition. As expected, by day 4, artesunate – amodiaquine combination (ACT) and chloroquine removed the parasites from the blood but recrudescence occurred by day 11 thus showing the curative potential of this drugs. The survival rate of the animals in the control groups compared with that of the treatment groups showed that the extract is not toxic to the animals at the doses administered and the death observed are likely due to the effect of the parasite.

The percentage chemosuppressive activity of the extract within the safe dose was higher in day 11 at 200 mg/kg dose than at the 100 mg/kg dose, but it was not significant.

Malaria precipitates loss of appetite which eventually leads to weight loss (Kabiru *et al.*, 2012). In the curative test at the higher dose, the control, untreated and 200 mg/kg doses of the extract groups showed weight losses over the treatment period while the chloroquine, ACT and 400 mg/kg doses of extract groups showed weight gain, especially during the first three days of treatment. This shows that the effect of malaria is evident in the control, untreated and 200 mg/kg dose of extract groups and it is mitigated during the period of administration in the chloroquine, ACT and 400 mg/kg dose of extract groups. In the prophylaxis test, there was no significant change in the weight of the untreated group and the treatment groups. This is expected because the symptoms of malaria become evident 72 hours (3 days) after infection. In the suppressive test, the gradual weight loss in all the groups

buttresses the presence of the infection and also supports the observed percentage chemosuppression which shows that the leaf extract of *P.pinnata* is moderately suppressive at the dose of 200 mg/kg body weight.

Parasitaemia is the quantitative estimation of parasites in the blood and is an index of the severity of the disease condition. In the curative test at the higher dose, the percentage parasitaemia increased throughout the period of administration and on day 11 (7 day post administration) in the control, untreated and 200 mg/kg dose of extract groups resulting in the 0 % chemosuppression in days 3, 4 and 11 in the 200 mg/kg dose of extract. At the 400 mg/kg dose of extract, the percentage parasitaemia reduced over the period of administration (days 1 – 4) and this reflects in the percentage chemosuppression which was 85.67 % by day 4, and was sustained until day 11 (7 day post administration). In the prophylaxis test, the percentage parasitaemia was reduced in the treatment groups compared to the untreated group showing the prophylactic activity of the leaves extract, especially at the 200 mg/kg dose, and this activity was shown to be comparable with chloroquine but not as effective as the standard prophylactic therapy (sulphadoxine/pyrimethamine). This suggests therefore that the leaf extract of *P. pinnata* has moderate prophylactic activity as presented in the percentage chemosuppression. In the suppressive test, the percentage parasitaemia increased in the control, untreated and 400 mg/kg dose of extract from days 4 to 6 (1^{st} day – 3^{rd} day post administration respectively) while there was no increase in the 200 mg/kg dose of extract. This reflects the suppressive activity of the extract at the dose of 200 mg/kg body weight.

Anaemia is a very important pathological feature of malaria infection (Haldar and Mohandas, 2009). It is as a result of hemolysis of the red blood cells due to oxidative stress arising from imbalance between plasma oxidants (generated by the parasite and the host and the antioxidant system of the host (Isah and Ibrahim, 2014). For the curative at the higher dose, reduced packed cell volume which is an indicator of anaemia was observed in the control,

untreated and 200 mg/kg dose of extract groups. The observed decrease in the 400 mg/kg dose of the extract may be because the animals have not recovered from the infection. For the prophylaxis test, reduced packed cell volume was observed in the untreated, chloroquine, sulfadoxine/ pyrimethamine and 400 mg/kg dose of extract groups indicating anaemia, but in the group of 200 mg/kg dose of extract, the packed cell volume increased thus confirming the prophylactic potential of the extract at this dose and is reflected in the percentage chemosuppression (57.06 %). This may be that the extract, at the 400 mg/kg dose (which is twice 200 mg/kg dose) undergoes a kind of autolysis since there is no target, so that when the parasite is introduced, it is not active enough to prevent the development of the parasite. The reduction in the packed cell volume in the chloroquine and sulfadoxine/ pyrimethamine groups supported the findings of Chikezie *et al.* (2010), who showed that these drugs caused an initial destabilization of red blood cells. For the suppressive test, reduced packed cell volume was observed in all the treatment groups as well as the untreated and the control groups thus indicating anaemia. In the chloroquine group, this may be due to the fact that chloroquine has been reported to cause heamolytic anaemia (Tchandema and Lutgen, 2013), while in the 200 mg/kg dose, the percentage chemosuppression is 66.67 % by day 6 reflecting that at this dose the extract has suppressive activity. Again at 400 mg/kg dose, there is sign of autolysis because the chemosuppression is 0 (zero) by day 6.

For the curative test at the higher dose, the leaf extract of *P. pinnata* had curative potential, mildly moderate prophylaxis capacity and no suppressive activity at the 400 mg/kg dose while the 200 mg/kg had suppressive and prophylatic capacities with no curative potential. We propose that this may be because the extract undergoes some kind of autolysis at the 400 mg/kg dose since the target (parasites) are not available in the system for there to be suppressive and prophylactic activities at this dose.

The survival rate for the curative and suppressive tests at the higher dose complimented the results.

Different complex mechanisms have been shown to be responsible for the pathogenesis of malaria infection and one that has been found to be common to various aspects of the disease pathology is oxidative stress (reviewed in Isah and Ibrahim, 2014). The elevated superoxide dismutase activity in the ACT and chloroquine groups is as a result of the activity being reduced in the untreated group because of the free radicals generated as a result of the high metabolic rate of the rapidly growing and multiplying parasite. Similarly, this is responsible for the reduced activity of the enzyme in the *P.pinnata* treated groups. However, the activity of the enzyme in the 400 mg/kg dose of extract is slightly elevated compared to the untreated and 200 mg/kg dose of extract group. This suggests that the extract is mitigating the effect of the free radicals released as a result of the activity of the parasites. This is supported by the observed decrease in hydrogen peroxide generated in the 400 mg/kg dose of extract group and the ACT group. Furthermore, lipid peroxidation is increased, though not significantly, in the untreated and 200 mg/kg dose of extract groups while it is insignificantly reduced in the chloroquine, ACT and 400 mg/kg dose of extract. Thus showing the effect of the treatments on the disease condition. The decreased level of vitamin C in the ACT and *P.pinnata* treatment groups may be due to artemisinin reacting with heme moieties released by the parasites and forming cytotoxic radicals as well as the presence of secondary metabolites in the extract (Becker *et al.*, 2004), thus resulting in decreased vitamin C levels in these groups. Chloroquine functions by preventing the sequestration of free heme by the parasites and not generation of free radicals (Becker *et al.*, 2004) hence the elevated vitamin C level. This is similar to the report of Olorunnisola and Afolayan (2013) and shows the presence of oxidative stress in the untreated and 200 mg/kg dose of extract groups as a result of the disease condition as reflected in the percentage parasitaemia.

Polymorphonuclear (PMN) leukocytes facilitate the phase of inflammation at which vascular responses result in tissue injury (Wiessmann *et al.*, 1978) and myeloperoxidase (MPO) is a lysosomal enzyme present in the azurophilic granule of polymorphonuclear leukocytes which is unique to neutrophils and monocytes. Myeloperoxidase activity is therefore an indication of inflammation. Polymorphonuclear neutrophil leukocytes, tumour necrosis factor – alpha and proinflammatory cytokines have been shown to be involved in the pathogenesis of malaria (Clark *et al.*, 2006; Elias *et al.*, 2012) thus suggesting that inflammation is one of the pathophysiological manifestations of malaria infection. The activity of myeloperoxidase is increased only in the untreated group but reduced significantly in the treatment groups compared to the untreated group thus presenting one of manifestations of malaria infection in the untreated group.

Nitric oxide (NO) is known to play several roles, and has been shown to be involved in the pathogenesis of inflammation when there is impaired or over-production (Sharma *et al.*, 2007). This has been associated with malaria infection and several disease conditions (Polat *et al.*, 2002; Sharma *et al.*, 2007). The level of nitric oxide was elevated, though not significantly in the untreated and 200 mg/kg dose of extract groups (72.86 ± 4.83 mM and 70.66 ± 3.34 mM respectively) while the observed level in the chloroquine group (55.48 ± 7.71 mM) was the lowest, though not significantly. This supports the anti-inflammatory property of chloroquine as reviewed by Mishra *et al.* (2013) and Fang *et al.* (2013).

Alanine aminotransferase (ALT) and γ- glutamyl transferase (GGT) are enzymes found in the liver and elevated activities are an indication of liver damage or injury (Plaa and Charbonneau, 2007). The elevated activity of these enzymes in the untreated and 200 mg/kg dose of extract groups is an indication of liver damage and this is supported by the histopathology result. The elevated ALT activity in the chloroquine group may be as a result

of the animal that showed severe portal and central venous congestion but no lesion was observed in the other animals of the group thus making the observation spurious. Mild to moderate Kupffer cell hyperplasia was observed in the untreated and 200 mg/kg dose of extract groups (Figure 4.7). This resonates with reported histopathological features observed as a result of malaria infection (Nobes *et al.*, 2002; Baheti *et al.*, 2003; Rupani and Amarapurkar, 2009; Viriyavejakul *et al.*, 2014). No lesion was observed at the 400 mg/kg dose of extract thus supporting the curative effect of the extract.

The decrease in weight in the 400 mg/kg dose of EGME is a sign of toxicity. The decrease in weight and relative weight of testis in the treatment groups especially 200, 300 and 400 mg/kg doses of EGME is similar to or supported by the observations of Foster *et al*.(1994); Watanabe *et al*. (2000); Welsch (2005) and Malik and Gupta (2013) in rats and humans.

Significantly decreased Hb, WBC, platelet count, MCH, MCHC and RBC are signs of haematological aberrations and is an indication that the function of the bone marrow is being disrupted (Aster and Bunn, 2017). These findings are consistent with the report of Shih *et al*. (2000) in humans exposed to EGME at the workplace. The gradual increase in MCV in the treatment groups, which was significant at the 400 mg/kg dose despite significant decrease in the red blood cell count, suggests myelodysplastic syndromes (a heterogeneous group of bone marrow disorders where the marrow does not produce sufficient healthy blood cells) (Russell and Wilson, 2017). The white blood cells is composed of neutrophils, monocytes, lymphocytes, basophils and eosinophils which makeup the defense system of the body (Adeyemo-Salami and Ewuola, 2015). The elevated level of the lymphocytes at the 300 mg/kg dose was not significant enough to ameliorate the effect of the significant decrease in neutrophils on the white blood cell count.

The daily spermatozoa production decreased insignificantly in all the treatment groups compared to the control. This is consistent with the findings of Mebus *et al*. (1989) also in rats. The testes are the sites for spermatogenesis and androgen production. The gradual significant decrease in testicular spermatozoa number shows a disturbance in spermatogenesis. The epididymis is the site for sperm maturation as they traverse the different regions of the epididymis from the testis. Significant decreased epididymal spermatozoa number is therefore expected since the testicular spermatozoa number is decreased. The epididymis is also the site where sperm acquires significant motility. Significant decrease in sperm motility in the treatment groups suggests that the integrity of internal milieu of the epididymis is compromised.

The spermatozoa viability decreased gradually in a dose-dependent manner in the treatment groups and was significant at the 400 mg/kg dose of EGME. This implies that there is a larger number of dead spermatozoa compared to live spermatozoa with increased dosage of EGME. Thus showing that EGME has adverse effect on the spermatozoa. Moreover, the percentage abnormalities which increased significantly in the treatment groups showed that there was increased presence of deformed sperms in the treatment groups.

Oxidative stress is a condition where the enzymatic and/or non-enzymatic scavengers of reactive oxygen species (ROS) are overwhelmed by the level of ROS in the system. Catalase, glutathione-S-transferase, superoxide dismutase, glutathione peroxidase are enzymatic ROS scavengers while reduced glutathione and vitamin C are non-enzymatic ROS scavengers. In the testes, the activities of catalase, glutathione-S-transferase and glutathione peroxidase were increased significantly in the treatment groups especially at 200, 300 and 400 mg/kg doses of EGME. This is a sign of adaptive response. However the activity of superoxide dismutase was significantly reduced in the treatment groups thus confirming oxidative stress. Lactate deydrogenase is an enzyme of the glycolytic pathway and is used to identify the location, and

severity of tissue damage. The significant dose-dependent decreased activity of this enzyme in the treatment groups may be as a result of its inhibition by the metabolite of EGME. These observations are at variance with the findings of Malik and Gupta (2013) who administered EGME dermally to rats for 28 days at a dose of 2g/kg body weight. This may be because the route of administration was different as well as the duration was longer and the dose higher.

Lipid peroxidation, which is an indication of oxidative degradation of lipids by free radicals, was significantly elevated in a dose-dependent manner in the testes of the treatment groups. This shows damage to the membrane of the cells of the testis. The histopathology compliments the results showing varying degrees of lesions in the treatment groups with the 400 mg/kg dose of EGME group reflecting severe epithelial erosion.

In the epididymis, the activities of glutathione peroxidase, reduced glutathione, catalase, superoxide dismutase, glutathione-S-transferase and vitamin C level were elevated and were significant at the 400 mg/kg dose of EGME. Again this shows adaptive response. Lipid peroxidation was also increased significantly at the 300 and 400 mg/kg dose while the activity of lactate dehydrogenase was decreased significantly in a dose-dependent manner in the treatment groups. This again suggests that lactate dehydrogenase may be inhibited by the metabolite of EGME. The histopathology showed that there was severe diffuse germinal cell erosion only at the 400 mg/kg dose of EGME thus buttressing the elevated lipid peroxidation.

All these findings support the results of the spermatozoa analysis. The toxicity of EGME is further made obvious by the survival rate which shows that EGME was very potent at 400 mg/kg dose with a survival of 70 %.

The observed weight loss in the EGME-PP co-administered groups is a sign of toxicity. As expected, there was significant decrease in the weight of testes in the EGME only group but this was also observed in the EGME- PP co-administered groups and reflected in the relative

weight of the organ. Also, there was significant decrease in the weight of epididymes in the EGME only group and the EGME- PP co-administered group. In addition, the significant increase in the relative weight of the brain for the EGME- PP co-administered groups is as a result of the significant loss in body weight of the animals. All these manifestations are signs of toxicity. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are glycoprotein hormones released from the pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus. LH and FSH regulate gonadal function, including spermatogenesis and androgen biosynthesis (Sikka and Naz, 2002; O'Shaughnessy *et al*, 2010). Elevated plasma levels of LH and FSH in the EGME- PP co-administered groups, with that of LH being statistically significant, is an indication of the impairment in the function of the hypothalamus which stimulates the pituitary through GnRH. Decreased plasma level of testosterone in the EGME-PP co-administered groups is an indication of improper function of the leydig cells of the testes because the leydig cells are the site of androgen production (Sikka and Naz, 2002).

Thyroxine (T_4) is the prohormone of triiodothyronine (T_3) and both are tyrosine-based hormones produced by the thyroid gland (Baldo, 2016). Their primary role is the regulation of metabolism. The pituitary, in response to stimulation by thyrotropin-releasing hormone (TRH) from the hypothalamus, releases thyroid-stimulating hormone (TSH) which stimulates the thyroid to secrete T_4 and then T_3. Positive feedback to the hypothalamus and pituitary by T_4 and T_3 results in the stimulation or suppression of these organs depending on the plasma levels (Beckman Coulter, 2013). Significantly elevated plasma levels of T_3 and T_4 in the EGME-PP co-administered groups is an indication of over-stimulation of the thyroid gland by the pituitary in response to the TRH from the hypothalamus and may therefore suggest impairment in hypothalamus function. Thus resulting in hyperthyroidism, which may contribute to weight loss, which is one of the symptoms (Bowen, 2010).

The daily sperm production is significantly increased in the EGME- PP co-administered groups thus indicating over-stimulation of the seminiferous tubules. The significant decrease in motility and viability of the sperms with significant increases in the percentage total sperm abnormality in the EGME only and the EGME-PP co-administered groups point to the existence of aberrations in the sperms of the animals in these groups and shows that the function of the sertoli cells of the testes, which is to remove defective sperm cells, is impaired. This implies that co-administration of EGME with *P.pinnata* affects the proper function of the sertoli cells and this can result in infertility. Since the sperms of the EGME-PP co-administered groups showed decreased viability and motility with increased abnormality, the ability of the sperms in these groups to fertilize the ovum of a female is minimized and therefore infertility is likely to be observed.

Oxidative stress is a condition induced by nitrogen-derived free radicals, oxygen and oxygen-derived free radicals known as reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Lobo *et al.*, 2010). The presence of ROS in biological systems is as a result of normal physiological processes and cell activation (e.g. immunological responses and apoptosis) (Pham-Huy *et al.*, 2008). Also, exposure to many types of environmental contaminants, aging, chronic disease state or gonadal injury can induce oxidative stress associated with an increased rate of cellular damage that results in gonadotoxicity (Sikka and Naz, 2002). Enzymatic and non-enzymatic antioxidants in the gonads exist to combat these ROS (Adedara and Farombi, 2010). In the EGME only and EGME-PP co- administered groups, the activities of superoxide dismutase, catalase, glutathione peroxidase and levels of reduced glutathione and vitamin C were largely insignificantly reduced, and this had resulted in no observable lipid peroxidation which is reflected in no visible lesions in the epididymis. This shows that the oxidative stress status in the epididymis was not appreciably affected.

In the testes, activity of superoxide dismutase and vitamin C level were significantly reduced while that of catalase, glutathione-S-transferase, glutathione peroxidase, reduced glutathione and hydrogen peroxide were significantly increased in the EGME only and EGME-PP co-administered groups. This resulted in insignificant increases in lipid peroxidation thus suggesting minimal deleterious effect of the reactive oxygen species in these groups. The activity of lactate dehydrogenase (an indicator of tissue damage) increased insignificantly in the EGME only and EGME-PP co-administered groups. This suggests that there is minimal damage done to the testes but the histological examination shows that there are lesions in the EGME only and EGME-PP co-administered groups. This indicates that lactate dehydrogenase is probably being inhibited in these groups and since EGME is common to the groups, it may be that the metabolite(s) of EGME is/are responsible.

In the brain, there were no significant changes in the activity of catalase and levels of lipid peroxidation, reduced glutathione and hydrogen peroxide generation. However, there were significant decreases in the activity of superoxide dismutase and vitamin C level with significant increases in the activities of glutathione-S-transferase and glutathione peroxidase in the EGME-PP co-administered groups. This suggests that co-administration of EGME with *P.pinnata* facilitates the crossing of the blood –brain barrier.

Regarding the anti-inflammatory profile, the significantly elevated activity of myeloperoxidase in the epididymis of the EGME only and EGME-PP co-administration groups supports the observed decreased activity of superoxide dismutase. This is because when neutrophils are functioning, they generate free radicals, especially superoxide radical, and this in turn is scavenged by superoxide dismutase forming hydrogen peroxide which is inturn cleaved by catalase and other peroxidases. When catalase is overwhelmed by the level of hydrogen peroxide, the hydrogen peroxide feed–back inhibits superoxide dismutase thus resulting in the decreased activity (Rahman, 2007; Lobo *et al*., 2010). In the testes, the

elevated activity of myeloperoxidase and level of nitric oxide again supports the observed decreased activity of superoxide dismutase.

The pituitary gland and hypothalamus are parts of the brain that control the male reproductive system. Histopathology of the pituitary shows that there were no lesions in the control and treatment groups but the hypothalamus showed moderate to severe diffuse spongiosis of the parenchyma with the submeningeal portion being mildly haemorrhagic in the EGME only group while the EGME-PP co-administration groups showed moderate and severe spongiosis of the parenchyma. This corroborates the results of the analysis of the reproductive hormones and shows that the hypothalamus, and therefore its function, is affected in the EGME only and EGME-PP co-administered groups and this is reflected in the function of the pituitary gland.

The survival rate showed that co- administration of the methanol leaf extract of *Paullinia pinnata* with EGME exacerbated the effect of EGME. This is because there was 50 % mortality in the group administered with EGME + 200 mg/kg of PP and the group that received EGME + 100 mg/kg of PP had 30 % mortality but the EGME only group had a mortality of 10 %.

CHAPTER SIX

SUMMARY AND CONCLUSION

6.1 Summary

1. The methanol leaf extract of *Paullinia pinnata* reveals the presence of alkaloids, flavonoids, tannins, terpenoids and cardiac glycosides.
2. A tolerable dose, for the oral administration of the methanol extract of the leaves of *Paullinia pinnata* (Linn.), would be 200 mg/kg body weight.
3. The methanol leaf extract of *Paullinia pinnata* showed that it has potential to be used to treat anaemia and therefore may improve bone marrow failure as well as serve as a therapeutic agent to increase neutrophil count, thus corroborating the traditional use of the leaves as an anti-anaemic tonic.
4. The methanol leaf extract of *Paullinia pinnata* possesses a weak curative effect on malaria when working within the safe dose.
5. The methanol leaf extract of *Paullinia pinnata* has curative capacity at a dose that is double the safe dose but possesses prophylactic and suppressive activities at the safe dose.
6. The methanol leaf extract of *Paullinia pinnata* does not have chemopreventive potential against Ethylene glycol monomethyl ether induced male reproductive dysfunction in Wistar rats.

6.2 Conclusion

The study supports the folkloric use of *Paullinia pinnata* leaves in the treatment of malaria. However, caution should be exercised when administering for curative

purpose. In addition, the leaves do not possess chemopreventive capacity against insult on the male gonads by Ethylene glycol monomethyl ether.

6.3 Recommendations

1. Anti- anaemic potential of the methanol leaf extract can be investigated in anaemic experimental models.
2. Caution should be exercised when taking or ingesting *Paullinia pinnata* leaves or the preparations with drugs containing glycol ethers as excipients. These drugs include the following:
    - Chlordiazepoxide Hydrochloride (Teva Pharmaceuticals, USA)
    - Piroxicam (Teva Pharmaceuticals, USA)
    - Tetracycline Hydrochloride (Watson Pharmaceuticals, Inc.)
    - Trimethobenzamide Hydrochloride (Gavis Pharmaceuticals)
    - Vitamin D ergocalciferol (Paddock Laboratories, Inc.)
3. Persons exposed regularly to Ethylene glycol monomethyl ether, especially at the work place, should avoid ingesting or taking preparations from the leaves of *Paullinia pinnata*.

6.4 Contributions to knowledge

1. The study shows for the first time that *Paullinia pinnata* leaves have anti-anaemic potential. There were elevated levels of packed cell volume, haemoglobin concentration and red blood cell count.
2. The study shows for the first time that *Paullinia pinnata* leaves have the potential to enhance neutrophil count since elevated levels was observed, and therefore may be used to fight infections and treat leukemias
3. The antiplasmodial studies show that administration of the extract of *Paullinia pinnata* leaves has varying suppressive, prophylactic and curative potentials depending on the level of the extract.
4. The study showed for the first time that treatment with the extract of *Paullinia pinnata* leaves removed lesions from *Plasmodium berghei berghei* infected liver in the curative test.
5. The study showed for the first time that ethylene glycol monomethyl ether elicits its toxic effect on the epididymes by inducing oxidative stress.
6. Co-administration of the extract of *Paullinia pinnata* leaves and ethylene glycol monomethyl ether adversely affected plasma levels of testosterone, thyroxine, triiodothyronine as well as morphology and daily production of sperms.
7. The study further supports the observation that administration of herbs with chemicals (especially in form of drugs) may have adverse effect on the subject because co-administration of the extract of *Paullinia pinnata* leaves with ethylene glycol monomethyl ether resulted in mortality in some of the animals.

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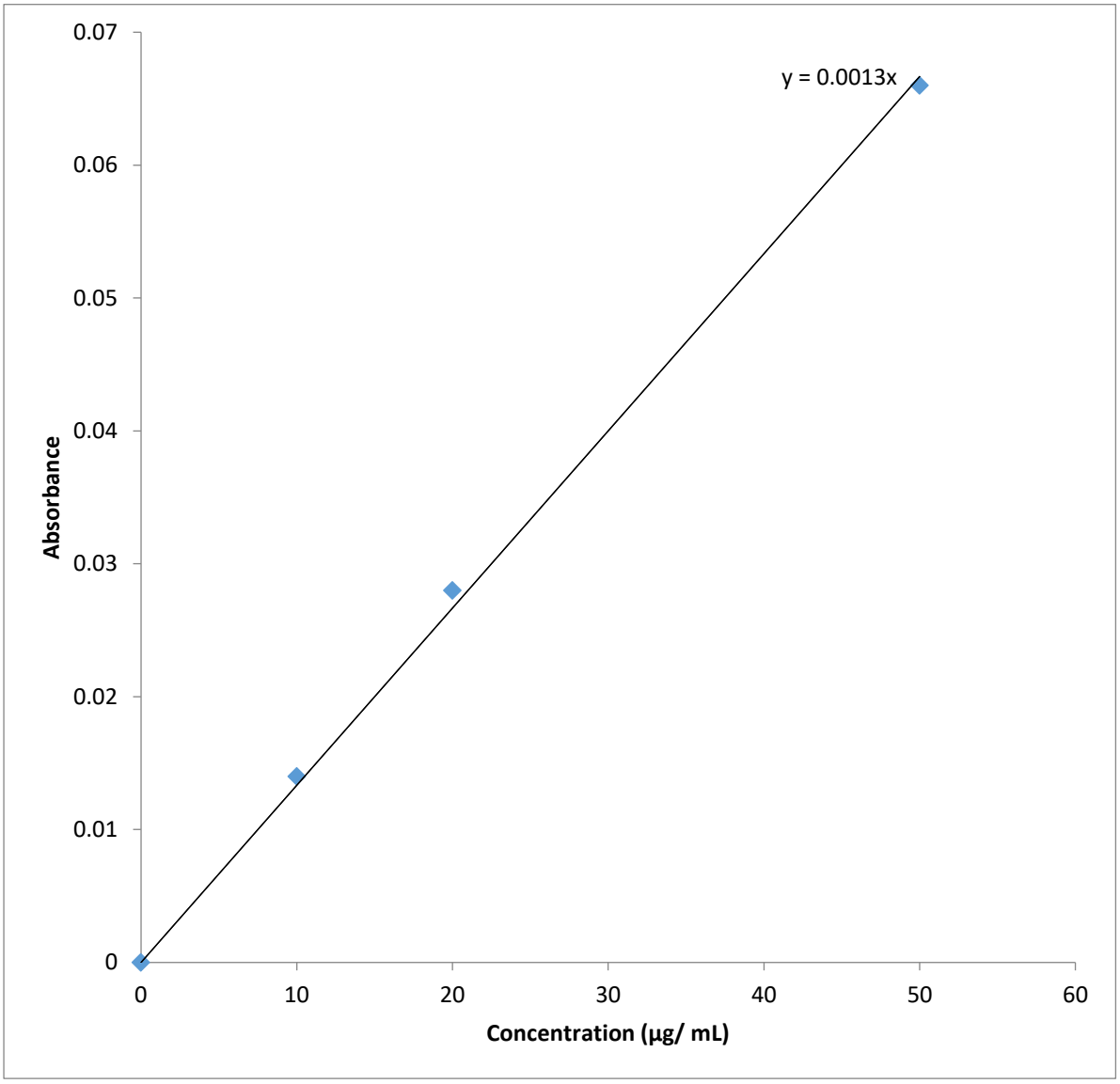
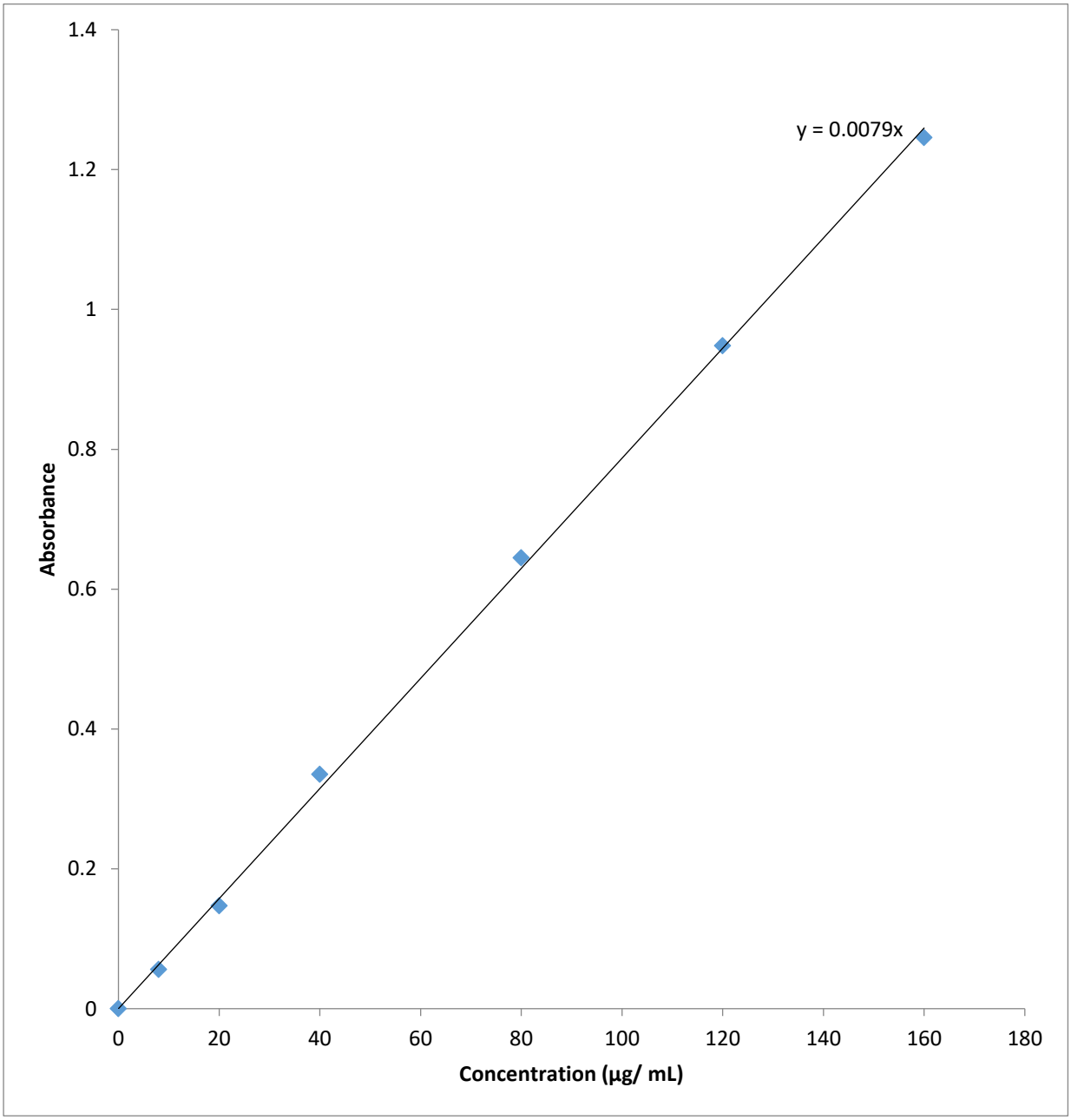
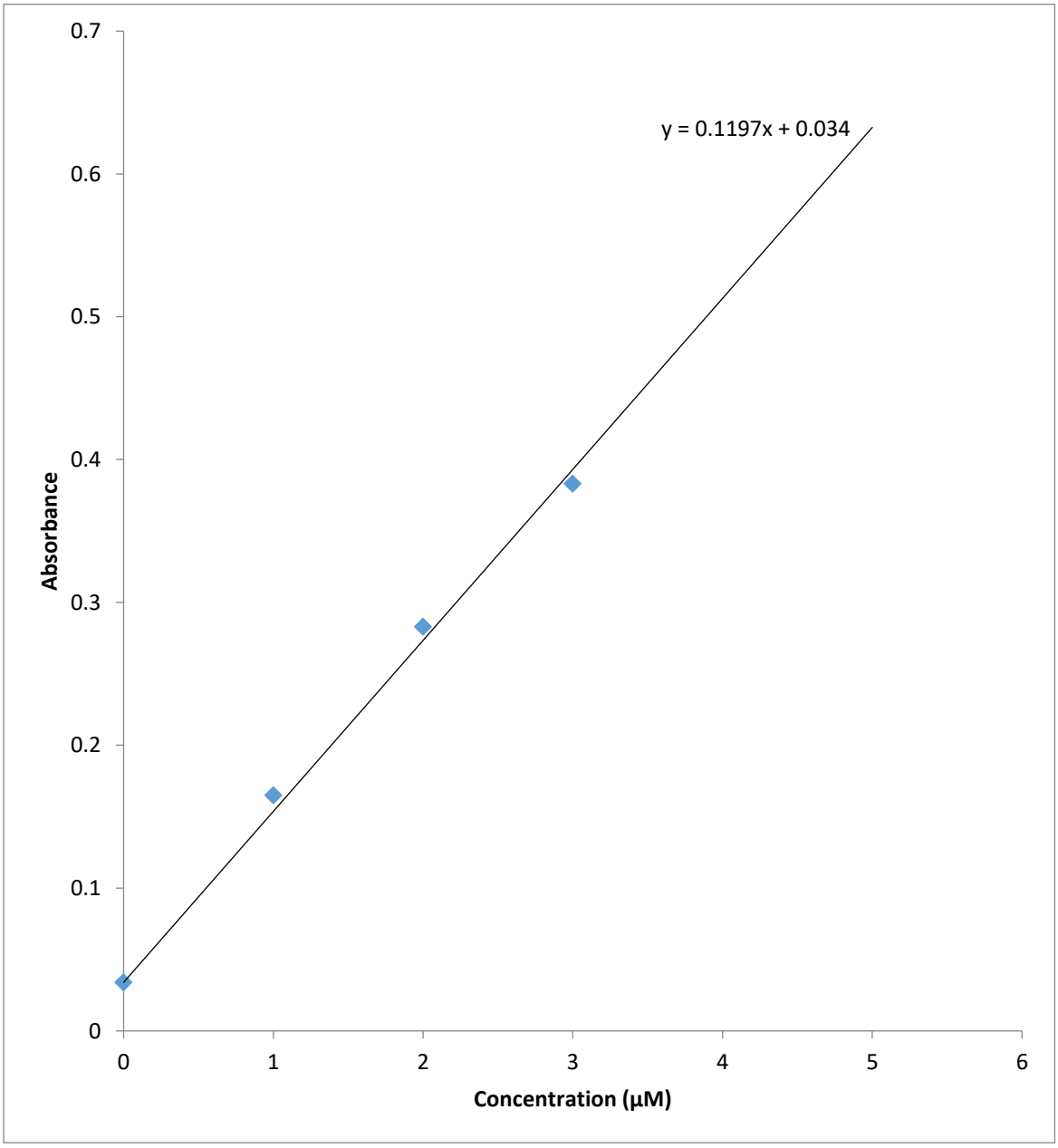
APPENDICES



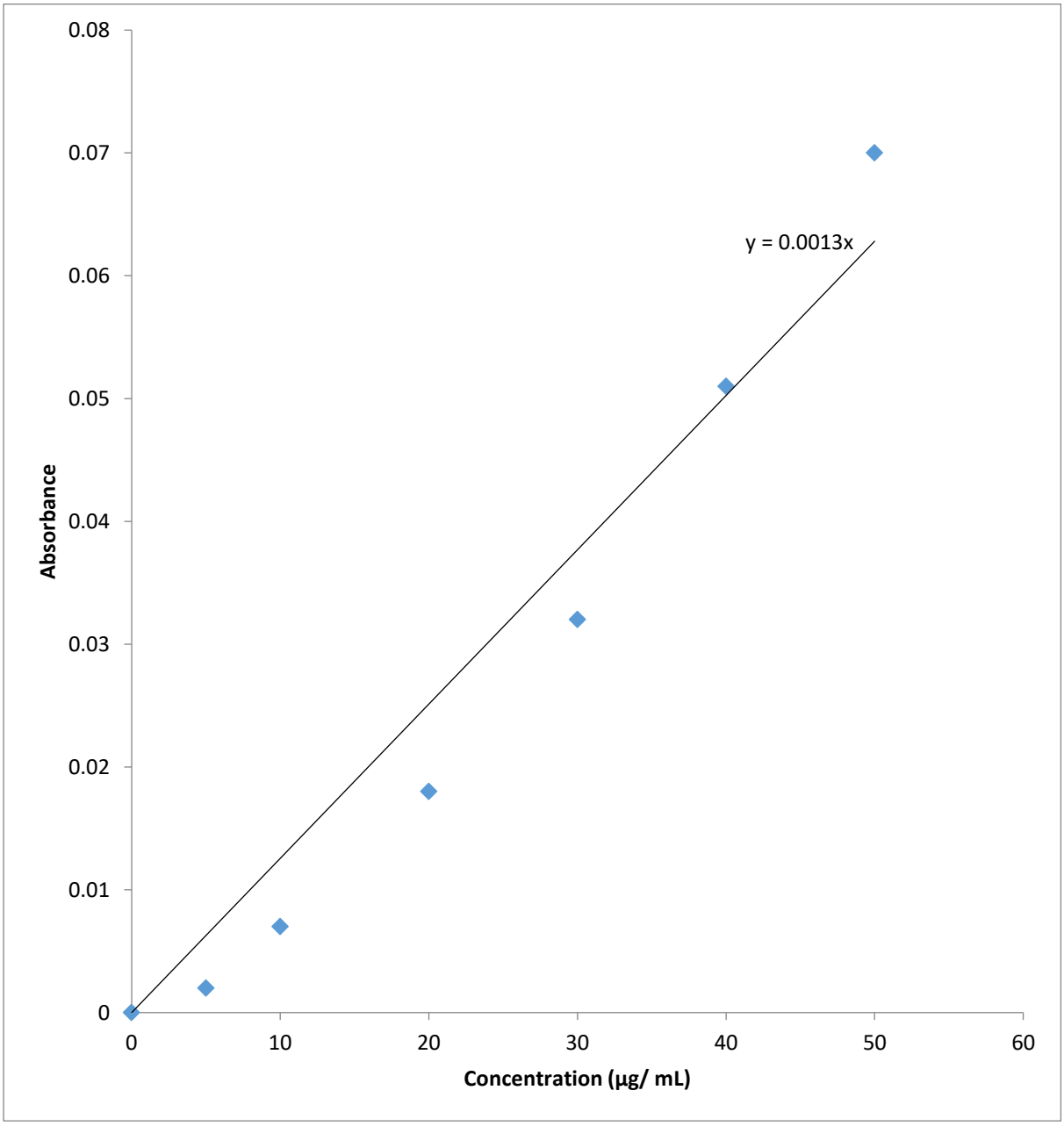
I. Standard curve for protein determination



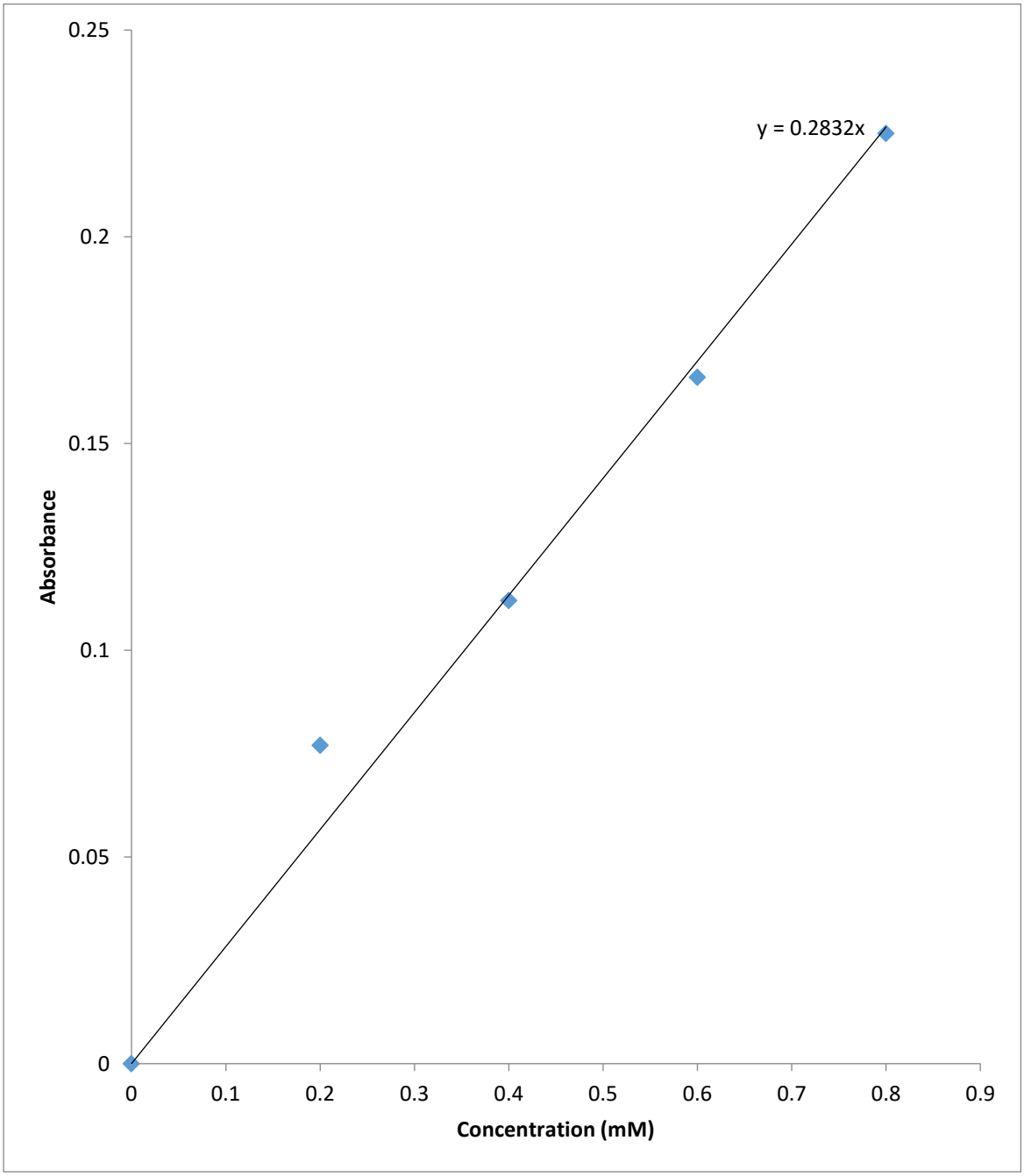
II. Standard curve for reduced glutathione



III. Standard curve for hydrogen peroxide concentration



IV. Standard curve for ascorbic acid concentration



V. Standard curve of nitrite concentration

(ii) **Published Papers**

1. **Adeyemo- Salami O.A.** and Makinde J.M. 2013. Acute and sub-acute toxicity studies of the methanol extract of the leaves of *Paullinia pinnata* (Linn.) in Wistar albino mice and rats. *African Journal of Medicine and medical sciences* 42(1):81-90. PMID: 23909098.
2. **Adeyemo-Salami O.A.**, Farombi E.O. and Ademowo O.G. 2014. An investigation into the antimalarial effect of methanolic extract of *Paullinia pinnata* leaves in *Plasmodium berghei* infected mice and course of infection. *African Journal of Medicine and medical sciences* 43 Suppl.: 93-100. PMID: 26688604 PMCID: PMC4682908.
3. **Adeyemo-Salami O.A.** and Ewuola E.O. 2015. Hematological effects of repeated graded doses of the methanol extract of *Paullinia pinnata* (Linn.) leaves in Wistar albino rats. *Pharmacognosy Research* 7(Suppl. 1): S34-S38. PMID: 26109785.
4. **Adeyemo-Salami O.A.** and Farombi E.O. 2018. Sub-acute toxicity study of ethylene glycol monomethyl ether on the antioxidant defense system of the testes and epididymes of Wistar rats. *Nigerian Journal of Physiological Sciences* 33(2): 195- 200. PMID: 30837775.
5. **Adeyemo-Salami O.A.** and Farombi E.O. 2019. Exacerbative effect of *Paullinia pinnata* methanol leaves extract on ethylene glycol monomethyl ether-induced testicular dysfunction in male Wistar rats. *Archives of Basic and Applied Medicine* 7(1): 47-56.
6. **Adeyemo-Salami O.A.,** Ademowo O.G. and Farombi E.O. 2020. Antioxidant and antiplasmodial activities of methanol leaf extract of *Paullinia pinnata*. *Journal of Herbs, Spices and Medicinal Plants* 26 (3): 315-328. DOI:10.1080/ 10496475.2020.1740905.
7. **Adeyemo-Salami O. A.** 2020. The medicinal properties of *Paullinia pinnata* Linn. leaves. *International Journal of Phytomedicine* 12 (2). https:// arjournals. org/ index. php /ijpm/issue/view/119