Ameliorative Potential of Methanol Extract of *Tithonia diversifolia* (HEMSL.) Leaf on Lead Acetate-Induced Reproductive Toxicity in Male Wistar Rats

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MATRICULATION NUMBER: 90465

A THESIS IN THE DEPARTMENT OF PHYSIOLOGY
SUBMITTED TO THE FACULTY OF BASIC MEDICAL SCIENCES COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (Ph.D)

OF THE

UNIVERSITY OF IBADAN

MAY, 2019

ABSTRACT

Prolonged exposure to lead induces oxidative stress which is implicated in male infertility. *Tithonia diversifolia* Leaf (TDL) has been reported in folkloric medicine to possess anti-inflammatory properties. There is dearth of information on the effects of TDL on lead-induced male reproductive dysfunctions. Therefore, the effects of Methanol Extract of TDL (METDL) on Lead Acetate (PbA)-induced reproductive toxicity were investigated in male Wistar rats.`

Tithonia diversifolia leaves were collected from Botanical Garden, University of Ibadan, and identified at Forestry Research Institute of Nigeria, Ibadan (FHI No.: 110652). The leaves were air-dried, pulverised, macerated in methanol, and the filtrate was concentrated using rotary evaporator. Identification of the phytochemical constituents present in METDL was done by Gas Chromatography Mass Spectrometry (GC-MS). Forty male Wistar rats (120-150 g) were assigned into eight groups (n=5) and treated thus: Group 1 (Distilled Water, 5 mL/kg), Group 2 (15 mg/kg PbA), Group 3 (50 mg/kg METDL), Group 4 (15 mg/kg PbA + 50 mg/kg METDL), Group 5 (100 mg/kg METDL), Group 6 (15 mg/kg PbA + 100 mg/kg METDL), Group 7 (50 mg/kg phytol) and Group 8 (15 mg/kg PbA + 50 mg/kg phytol). Twenty-four hours after the last administration, blood samples were collected *via* cardiac puncture from the animals under anaesthesia (50 mg/kg sodium thiopental, i.p.). Serum Gonadotropin-Releasing Hormone (GnRH), Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), testosterone, nitrotyrosine, Interleukin-1β (IL-1β) and Tumour Necrosis Factor Alpha (TNF-α) were measured using ELISA technique. Testis and hypothalamus were harvested for spectrophotometric analysis of Malondialdehyde (MDA), Superoxide Dismutase (SOD), catalase and reduced glutathione. Histological analysis of parafine-embedded testis and hypothalamus was done *via* Hemotoxylin and eosin staining technique. Semen analysis was done using Computer Aided Sperm Analyser (CASA). Data were analysed using Students' *t*-test and ANOVA at $\alpha_{0.05.}$

Phytol (3,7,11,15-tetrametylhexadec-2-en-1-ol) had the highest percentage (14.7%) of all the compounds that have antioxidant properties identified in METDL. Sperm motility, livability and counts were significantly increased in Groups 4 (78.8±1.8%; 66.0±3.3%; 383.2±2.1x10^6 cell/mL) and 8 (75.0±3.2%; 66.0±4.0%; 78.0±3.7 x10^6 cell/mL) compared to Group 2 (46.4±1.1%; 42.0±3.7%; 34.8±1.3 x10^6 cell/mL). Testicular SOD (31.2±1.5 vs. 17.3±0.9 Unit/mg tissue) and catalase (2.5±0.2 vs. 0.9±0.2 Unit/mg tissue) were significantly increased in Group 4 compared to Group 2. Testicular and hypothalamic MDA were significantly decreased in Group 8 (0.4±0.0 µM/mg protein; 0.2±0.0 µM/mg protein) compared to Group 2 (0.5±0.0 µM/mg protein; 0.5±0.0 µM/mg protein). Serum TNF-α, IL-1β and nitrotyrosine levels were significantly decreased in Group 8 (63.0±2.0 pg/mL; 433.8±1.8 pg/mL; 3.5±0.2 ng/mL) compared to Group 2 (95.5±1.9 pg/mL; 683.8±2.3 pg/mL; 7.5±0.2 ng/mL). Significant increase was observed in testosterone, LH, FSH and GnRH in Groups 4 (0.2±3.2x10^3 ng/mL; 4.2±1.2 mIU/mL; 0.7±0.1 mIU/mL; 1260.0±5.4 pg/mL) and 8 (0.1±3.5x10^3 ng/mL; 5.1±1.1 mIU/mL; 0.8±0.1 mIU/mL; 1319.0±3.6 pg/mL) compared to Group 2 (0.1±3.8x10^3 ng/mL; 1.7±0.7 mIU/mL; 0.4±0.0 mIU/mL; 1056.0±3.9 pg/mL), respectively. Histology of the testes and epididymides showed cyto-architectural distortion only in Group 2.

Tithonia diversifolia alleviated lead acetate-induced reproductive toxicity *via* the mitigation of oxidative stress. The methanol extract also prevented inflammatory response to lead acetate toxicity.

Keywords: *Tithonia diversifolia,* Lead Acetate-induced reproductive toxicity, markers of oxidative stress

Word count: 497

DEDICATION

This research work is dedicated to:

Allah, the lord of the worlds

My parents

My family

ACKNOWLEDGEMENT

All praises and thanks are due to Allah whom out of His infinite mercy I am able to go this far in life. You alone I will continue to worship and from you alone I will always seek aid.

My utmost appreciation goes to my amiable Supervisor, and Head of Department of Physiology, Prof. Y. Raji, for his fatherly guidance, support and constructive criticism which have immensely contributed to the success of this research work. I thank you so much sir, for believing in me despite all odds. I pray Allah's blessing will never depart from you.

I am very grateful to all academic staff of the Department of Physiology: Prof. A.R.A Alada, Prof. A. A. Fasanmade, Prof. S.B Olaleye, Prof. F.S Oluwole, Prof. E.O. Adewoye, Dr G.F Ibironke, Dr O.O. Akande, Dr A.S. Onasanwo, Dr A.G. Adeleye, Dr O.A. Aiku, Dr J.T. Lasisi, Dr G.O. Isehunwa, Dr O.A. Ige, Dr A.T. Salami, Dr O.T. Kunle- Alabi, Dr A.O. Odukanmi, Dr O.O. Akindele, Dr. S.T. Shittu, and the support of all the Administrative and Technical staff of the Department.

I thank all members of the Reproductive Physiology and Developmental Programming Unit, Department of Physiology, College of Medicine, University of Ibadan.Your contributions and constructive criticism during unit meetings made this work a better one.

Special thanks go to the entire members of Staff of the Department of Physiology, UNIOSUN for their support especially, Dr O.O. Obembe (Ag HOD), Dr I. P. Oyeyipo, Dr O.O. Oladokun, Dr T.O. Usman, Dr O.S. Osuntokun, and Mr. D. H. Adeyemi. God bless you all.

I appreciate the support of the following individuals during the course of this work: Dr. A. Attah (Department of Pharmacognocy, University of Ibadan); Mr O. Adebowale (Mr Kits); Mr.S.P. Otegbade (Department of Histopathology, UCH, Ibadan); Dr S. Akinsomisoye (Department of Physiology, OAU, Ile-Ife); Dr W. Oyeyemi (Department of Physiology, Igbinedion University, Okada); Dr. G.O. Atere (Department of Biochemistry, UNIOSUN); Dr O.E. Adeleye (FUNNAB); Dr and Mrs. L.A. Baiyewu (Ibadan). May God Almighty always be there for all of you.

To my wonderful parent, Alhaji and Mrs Fasasi Adedokun. I say thank you so much for your moral and spiritual support. I pray you live long to reap more, the fruit of your labour.

To my family, I say thank you to Mariam Adedokun and Oluwatoyin Adedokun, for your support and patience. My children, I love you so much. God bless you.

To my students: Ranti, Musa, Chioma, Bolaji, Quadri and Tomiwa, I say thank you.

CERTIFICATION

I certify that this work titled: **Ameliorative Potential of Methanol Extract of *Tithonia diversifolia* (HEMSL.) Leaf on Lead Acetate-Induced Reproductive Toxicity in Male Wistar Rat** was carried out by Kabiru Isola **ADEDOKUN** in the Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Nigeria.

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ABBREVIATIONS

ROS - Reactive Oxygen Species

SOD -Superoxide dismutase

GPx -Glutathione peroxidase

WHO-World Health Organisation

TCA- tricyclic antidepressant

GMP - guanosine monophosphate

NO –Nitric oxide

TD–Methanol extract of *Tithonia diversifolia* leaf

BTB- blood testis barrier

T - testosterone

FSH- Follicle-Stimulating Hormone

LH - Luteinising Hormone

GnRH - gonadotropin-releasing hormone

ABP - androgen-binding protein

DHT - dihydrotestosterone

ARs - androgen receptors

DNA- deoxyribonucleic acid

OS - Oxidative stress

GC-MS - Gas chromatography-mass spectrometry analysis

RNS - Reactive Nitrogen Species,

H_2O_2 - hydrogen peroxide

TAC -total antioxidant capacity

ArO* - aryloxy radical

ORAC - oxygen radical absorbance capacity

AAPH - 2,2'-azobis (2 amidinopropane) hydrochloride

ABTS - 2,2'- Azinobis-(3-ethylbenzothiazoline 6 Sulfonic acids)

TEAC- Trolox equivalent antioxidant capacity

GSH - glutathione

OECD - Organization of Economic Cooperation and Development

ALT - Alanine aminotransferase

AST - aspartate aminotranferase

ALP- alkaline phosphatise

NIST-National Institute of Standards and Technology

LD_{50} - Median lethal dose

EDTA- ethylenediaminetetraacetic acid

ANOVA- analysis of variance

SEM- standard error of mean

CAT- catalase

MDA- malondialdehyde

TD- Tinthonia diversifolia

CHAPTER ONE

1.1 Introduction

Heavy metal contamination is a globally recognized environmental issue, threatening human life and resulting in catastrophic health hazards (Anwarzeb *et al.,* 2015). The rate at which metals gain entrance into the environment through anthropogenic activities is on the increase in the last decade ranging from coal driven power plants to waste incinerators (Arif *et al.,* 2015). Human beings are at risk of being exposed to such metals. The most prevalent heavy metal contaminant is lead (Di Maio, 2001). It was estimated that, lead poisoning resulted in 853,000 deaths globally, and "contributed to16.8 million disability-adjusted life years due to its long-term effects on health (WHO, 2016).

For decades, interest on how environmental and occupational exposures to toxicants contribute to decreasing sperm concentration and human male infertility is reported to be on the increase (Becker and Berhane, 1997). Lead was one of the first industrial metals to be demonstrated as detrimental to fertility (Benoff *et al.,* 2000). Environmental lead toxicity is a persistent world problem in public health, and children are more prone to lead than adults (Ahmed and Siddiqui, 2007). Lead appears in homes in many forms as lead piping, lead-containing solders, paints, ceramic glazes, pewter and base metal utensils and fixtures. Cream powder, lipstick and hair color equally have lead. Agricultural soil contamination may be responsible for lead found in many herbal medicines and cigarettes (Benoff *et al.,* 2003).

Lead is a male reproductive toxicant (Winder, 1989) and the primary mechanism of the toxic action of lead appears to be through disruption of the hypothalamic control of pituitary hormone secretion and in turn, spermatogenesis (Sokol, 1987). In men, especially in

professional workers, lead exposure results in infertility and sterility (Cullen *et al.,* 1984). Testicular atrophy, cellular degeneration, reductions in FSH, LH, testosterone, seminiferous tubule diameter and sperm count, depending on the dose and time of exposure (Nadia *et al.,* 2013). The study on lead's effects on testis of rats showed that lead alters the organ and there was testicular necrosis and atrophy in rodents (McGivern *et al.,* 1991). Nadia *et al.,* (2013) observed that lead acetate may be considered to have an environmental genotoxic and cytotoxic effects in male rats which may contribute to a reduction of fertility. It has equally been shown that maternal exposure of Wistar rats to lead acetate during neonatal period has a dose-related effect on postnatal development of testis in offspring (Mehran *et al.,* 2011).

Heavy metal poisoning is treated with the administration of chelators (Blann and Ahmed, 2014). Chelating agents are chemical compounds (e.g calcium disodium ethylenediamine tetraacetate, CaNa2 EDTA) that convert heavy metals to chemically inert forms that can be excreted without further interaction with the body. Chelates are not without side effects and can also remove beneficial metals from the body. Vitamin and mineral supplements are sometimes co-administered for this reason.

Plants have always been used as a common source of medicine, both for traditional remedies and in industrialized products (Newman and Cragg, 2012). *Tithonia diversifolia* is an annual plant from the compositae (Asteraceae) family. It is commonly known as Mexican Sunflower, tree marigold, Shrub sunflower or Japanese sunflower (Oyewole *et al.,* 2008). It is called 'Ewe Awolowo or Sepeleba' in Yoruba land. For centuries, different parts of this plant have been used in different countries around the world as a traditional medicine for the treatment of various diseases including pulmonary infections, diarrhea, inflammation, bacteria and parasitic infections as well as malaria (Gu *et al.,* 2002; Ogunfolakan *et al.,* 2010). In addition,

infusion from its leaves has been used for subdue swelling, dissolving lumps and treating enteritis and gastritis in local folk medicine (Tona *et al.,* 1998). It has anti-inflammatory and analgesic activities (Owoyele *et al.,* 2004). It is used in the treatment of bile, kidney, urinary and venereal diseases, testicular inflammation, frigidity, sterility, heavy menstruation, rheumatism and arthritis, upper respiratory tract infections, ranging from cough to tuberculosis, intestinal worms and schistosomiasis, cancer chemopreventive activity (Jian-Qiao *et al.,* 2002); and antimicrobial activity (Ogundare, 2007).

Ajayi *et al.,* (2009) showed that inclusion of Wild sunflower leaf meal mixture diet supported optimum caudal epididymal sperm characteristics and testicular morphometrics in rabbits at 10% level. Administration of crude extract of *Tithonia diversifolia* at 50 mg/kg improved reproductive profile (Ajayi and Jegede, 2012). *Tithonia diversifolia* is capable of scavenging reactive oxygen species (ROS) and reduce oxidative stress (Di Giacomo *et al.,* 2015; Mayara *et al.,* 2016).

1.2 Statement of Problem

Lead is a well-known environmental toxicant that is a major threat to public health (Akkus and Ozdenerol, 2014). Occupational exposure may include crystal ware, glazed pottery, in some instances home-made wine (Janin *et al.,* 1985). Exposure comes from the use of fossil fuels, leaded gasoline, some types of industrial facilities, lead-based paint, ceramics, pipes and plumbing materials, solders, gasoline, batteries, ammunition, and cosmetics. The mechanism of action of Lead is in part via generation of ROS with antifertility effects on male reproductive functions (Anjum and Reddy, 2015). Lead equally act via disruption of hypothalamic pituitary testicular axis and direct toxic effect on sperm (Nadia *et al.,* 2013).

1.3 Justification for the Study

Tithonia diversifolia has been reported to have potent antioxidants and capable of scavenging reactive oxygen species (ROS) thereby reducing oxidative stress (Mayara *et al.,* 2016). *Tithonia diversifolia* leaf is used in folkloric medicine for treatment of testicular inflammation and sterility (Jian-Qiao *et al.,* 2002). However, there is paucity of information on the activities of *T. diversifolia* leaf and its active compound on male reproductive functions.

1.4 Aim of the Study

To evaluate the effects of methanol extract of *Tithonia diversifolia* leaf and its active compound on lead-induced reproductive toxicity in male Wistar rats.

1.5 Specific Objectives:

1. To identify the phytochemical constituents present in methanol extract of *Tithonia diversifolia* leaf.
2. To examine the toxicity of methanol extract of *Tithonia diversifolia* leaf in male Wistar rats
3. To evaluate the effects of methanol extract of *Tithonia diversifolia* leaf and its active compound on reproductive functions in lead acetate-induced reproductive toxicity in male Wistar rats.
4. To assess the effects of methanol extract of *Tithonia diversifolia* leaf and its active compound on oxidative stress and inflammatory biomarkers in lead acetate-induced reproductive toxicity in male Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Tithonia diversifolia* plant

The plant for the study in this research is an annual plant, from Compositae (Asteracea) family, known as Shrub Sunflower or Japanese Sunflower (in English), Mexican Sunflower and Tree Marigold. The Yorubas from Southwest Nigeria, in Africa call it 'Sepeleba' or 'Ewe Awolowo' acoording to Oyewole and collegues in 2008. In old times, it was a plant introduced and used in West Africa for ornamental purpose but has eventually become a weed, while is commonly found on road sides and waste lands (Akinbodun and Agyakwa, 1998)

2.1.1 Plant Description

Tithonia diversifolia is synonymous to *Mirasolia diversifolia* Hemsley. It belongs to a family of Asteracea (Queensland, New South Wales, the ACT, Victoria Tasmania, Western Australia and Northern Territory). Compostae (South Australia). Its common names includes: Giant Mexican sunflower, Japanese sunflower, Mexican sunflower, Shrub sunflower, Tree marigold.

It is a native to Eastern Mexico and Central America (i.e Beliza, Costa Rica, El Savador, Guatamala, Honduras, Nicaragua and Panoma. Despite its common name, it is not native to Japan. *T. diversifolia* is widely naturalised in the Coastal district of Queensland and Northern South wales. It is also present on Christmas Island and widely naturalised throughout the tropical and subtropical regions of the world including South-eastern-USA (i.e Florida and Texas) and several Pacific Islands (i.e the Cook Island, Galapagoa Island, Fiji, French Polynesia, New Calendon, Niue, Palav, Western Samoa, Tonga and Hawaii (Tongma *et al.*, 1998).

Plate 2.1. *Tithonia diversifolia* (Mexican sunflower) (Orwa *et al.*, 2009).

T. diversifolia is a common weed of roadsides, railway lines, Greek banks, disturbed sites, waste area, embarkments, hill sides and neglected Sub-urban sites in tropical, sub-tropical and warmer temperature regions. *T. diversifolia* is a robust and short lived (i.e annual) or long lived (i.e perennial plant with upright stem forming shrubby bushes usually growing 2-3 meters tall, but occasionally reached 5 meters. Its leaves have three to five pointed lobes and scallope margins. Its large yellow daisy-like flower heads are about 10 cm across and have yellow centres. Its seeds (6-8 mm long) are lopped with 4 rings of scale and two awns.

The stems are slightly ridged (i.e striate) and hairy (i.e pubescent) when young. The alternately arranged leaves are borne on stalks (i.e petiole), 2-10 cm long. The leaves blade (6-33 cm long and 5-22 cm wide) have tapered (i.e uneate) bases and 3-7 pointed lobes with scalloped (i.e crenale) or tooth (i.e serate) margin. These leaves are finely hairy (i.e puberulent) and greyish-green in colour (Lalith, 2009).

The large flower-head (i.e capitula) are borne in small groups and the ends of the leafy branches on stalks (i.e peduncle) 7-30 cm long. These flower-heads (5-15 cm wide) look like sun flowers, but have yellow centres (3-5 cm across). They have 7-15 bright yellow petals (i.e ray florets) that are 4-7 cm long and 9-16 mm wide each with three small teeth at their tips (Orwa *et al.,* 2009).

There are also numerous (80-120) tiny yellow flowers (i.e tubular florets in the centre of the flower-heads and they are surrounded by several rows of green brats (i.e an involucres). Flowering occurs during spring but mainly during autum and early winter (i.e April to June).

The ‘seeds’ (i.e achenes are 4-8 mm long and topped with a ring (i.e pappus) of scales and two awns (about 5 mm long). These ‘seeds’ are covered in close-lying hair (i.e they are appressed pussecent); blackish in clour and are somewhat four angled.

This species reproduce by seed. The seeds may be dispersed by animals, water and in clothing. They also spread dumped garden waste and contaminated agricultural produce. *T. diversifolia* retains its seeds until the plant dries in the dry season when the seed are spread by the various modes of dispersal (Jama *et al.,* 2009).

The plants are also present in many Asian countries, and are very dominant in regions, where they are grown (Tongma *et al.,* 1998). The increased rate of propagation and allelopathy might be the major influence of the weed's dominance in any environment, where the weeds are found (Dutte *et al.,* 1993).

2.1.2 Similar Spicies of *Tithonia diversifolia*

Japanese sunflower (*Tithonia diversifolia*) is similar to Mexican sunflower (*Tithonia rotundifolia*), Wild sunflower (*Helianthus annuus*) and Crown beard (*Verbesinaen celioides*).

These species can be distinguished by following differences:

Japanese Sunflower (*Tithonia diversifolia*) has leaves with 3-7 pointed lobes. Its flower-heads have bright yellow centres and yellow petals i.e ray florents 4-7 cm long. Mexican Sunflower (*Tithonia rotundifolia*) has leaves without lobes or with rounded lobes. Its flower-heads have bright yellow centres and orange or reddish petals (i.e ray florets) 2-3.5 cm long. Wild Sunflower (*Heliantus annuus*) has leaves without lobes. Its flower-heads have orange-brown to dark brown centres and yellow petals (i.e ray florets) 2-3 cm long. Crown beard (*Verbesina encelioides*) has leaves without lobes. Its flower-heads have bright yellow centres and yellow petals (i.e ray florets) 1-2 cm long. (Bio security Queensland Edition fact sheet).

2.1.3 Medicinal and Agricultural Uses of *Tithonia diversifolia*

Different parts of the plants have been useful in different countries over the years. *T. diversifolia* is therapeutic and hence has been used for a variety of diseases ranging from pathogenic to pathophysiological conditions.

Gu and colleagues (2002), reported the use of the plant in various diseases like diahrroea, inflammation, pulmonary diseases, microbic and parasitic infections like malaria. The broad spectrum antibacterial effects on human pathogens potential of *T. Diversifolia* have been discussed by Ogunfolahan and others (2010). The leafy part of the plant has been useful for dissolving body swelling and lump, and used in treating enteritis and gastritis in local folk medicine (Tona *et al.,* 1998).

The antimalaria prospect of crude extract of *T. diversifolia* has been explored extensively in Nigeria and the possibility of limited deleterious effects has been proposed although the pharmacological and toxicological evidence of these have been scantly documented (Elufioye and Agbedahunsi, 2004). In vitro studies have shown that the activities of three different strains of *Plasmodium falciparum* have been impeded by the ether extract from the aeria part of the plant. Additionally, antiplasmodia activity of *Plasmodium berghei* has also been documented in vivo (Elufioye and Agbedahunsi, 2004). The aqueous and methanol extract of *Tithonia diversifolia* leaf also contain antimalaria substances with properties that showed possibility of both preventing and curing malaria. The Japanese used *T. diversifolia* to induce fever to fight food poisoning because of its characteristic bitter taste.

As much as the plant has been useful medicinally; the agricultural benefit cannot be overemphasized. *T. diversifolia* has been used by poor African farmers as green fertilizers. Other agricultural uses of the plant include provision as poultry meal, fuel wood, prevention of soil erosion and building materials (Olabode *et al.,* 2007).

As fertilizers, it can be either spread on the soil surface or buried in the soil (Liasu and Achakazi, 2007). *T. diversifolia* as fertilizers has power to increase the yields of crops (Jana *et al.,* 2000). The plant is made up of 1.76% of Nitrogen, 0.82% phosphorus and 3.92% potassium. These combinations of essential nutrients for increase plant yield make it possible for *T. diversifolia* used for fertilizers to restore high amount of Nitrogen into the soil (Nziguheba *et al.,* 2002). Lastly the nitrogen, phosphorus and potassium proportions showed better combination than cattle manure, which has limited composition and in poultry and swine manure with higher phosphorous (Olabode *et al.,* 2007).

2.2. Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol)

2.2.1 Chemistry and source of Phytol

The diterpene phytol (3,7,11,15-tetrametilhexadec-2-en-1-ol) is a member of branched-chain unsaturated alcohols whose common characteristic structural elements are one hydroxyl group

per molecule and a twenty-one double bond carbon atoms ($C_{20}H_{40}O$), molecular weight 296.54 mol/L. It is colorless with a boiling point of 202 ^{o}C, flash point >200 ^{o}C and liquid at room temperature, with a density of 0.8533 g/cm3 and refractive index 1.460–1.466. It is optically active in as much as it contains three asymmetric carbon atoms. The aqueous solubility of phytol is about 0.00327 mg/L (McGinty *et al.,* 2010). This compound is particularly interesting because it is a component of the chlorophyll molecule, present in green leaves of various medicinal plants; hence it is present in nature in abundance (Rontani and Volkman, 2003).

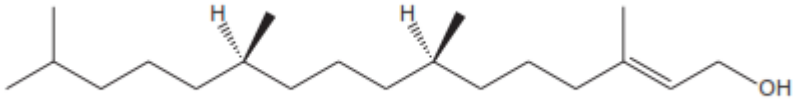


Figure 2.1. Chemical structure of phytol (Santos *et al.,* 2013).

2.2.2 Biological activities of Phytol

In medicinal fields, phytol has demonstrated antioxidant, antinociceptive and anticonvulsant activities (Costa *et al.,* 2012b; Santos *et al.,* 2013) as well as anti-inflammatory and antiallergic effects (Ryu *et al.,* 2011). Studies have revealed that phytol is an excellent immunostimulant, superior to a number of commercial adjuvants in terms of long-term memory induction and activation of both innate and acquired immunity (Lim *et al.,* 2006). Additionally, phytol and its derivatives have no cumulative inflammatory or toxic effects even in immuno-compromised mice (Chowdhury and Ghosh, 2012).

Costa *et al.,* (2014), reported that acute administration of phytol exerts an anxiolytic-like effect on mice. Furthermore, they support the idea that phytol interacts with the $GABA_A$ receptor, probably at the receptor subtypes that mediate benzodiazepines effects, to produce sedative and anxiolytic activities.

Phytol is a known antioxidant (Costa *et al.,* 2012b) and capable of increasing GSH level (Santos *et al.,* 2013). The antioxidant activity of phytol and its ability to reduce free radicals may be attributed to its structural feature. The hydroxyl group (OH) present in phytol

molecule might probably react with free radical, donates hydrogen atom with unpaired electron (H), converting free radicals into less reactive species (Guimaraes *et al.*, 2010)

2.3 Male Reproductive System

In human reproduction, two kinds of sex cells or gametes are involved. Sperm, the male gamete, and a secondary oocyte (along with first polar body and corona radiata), the female gamete must meet in the female reproductive system to create a new individual. For reproduction to occur, both the female and male reproductive systems are essential. It is a common misnomer to refer to a woman's gametic cell as an egg or ovum, but this is impossible. A secondary oocyte must be fertilized by the male gamete before it becomes an "ovum" or "egg".

While both the female and male reproductive systems are involved with producing, nourishing and transporting either the oocyte or sperm, they are different in shape and structure. The male has reproductive organs, or genitals, that are both inside and outside the pelvis.

The male reproductive system consists of the testes and a series of ducts and glands. Sperm are produced in the testes and are transported through the reproductive ducts. These ducts include the epididymis, ductus deferens, ejaculatory duct and urethra. The reproductive glands produce secretions that become part of semen, the fluid that is ejaculated from the urethra. These glands include the seminal vesicles, prostate gland, and bulbourethral glands.

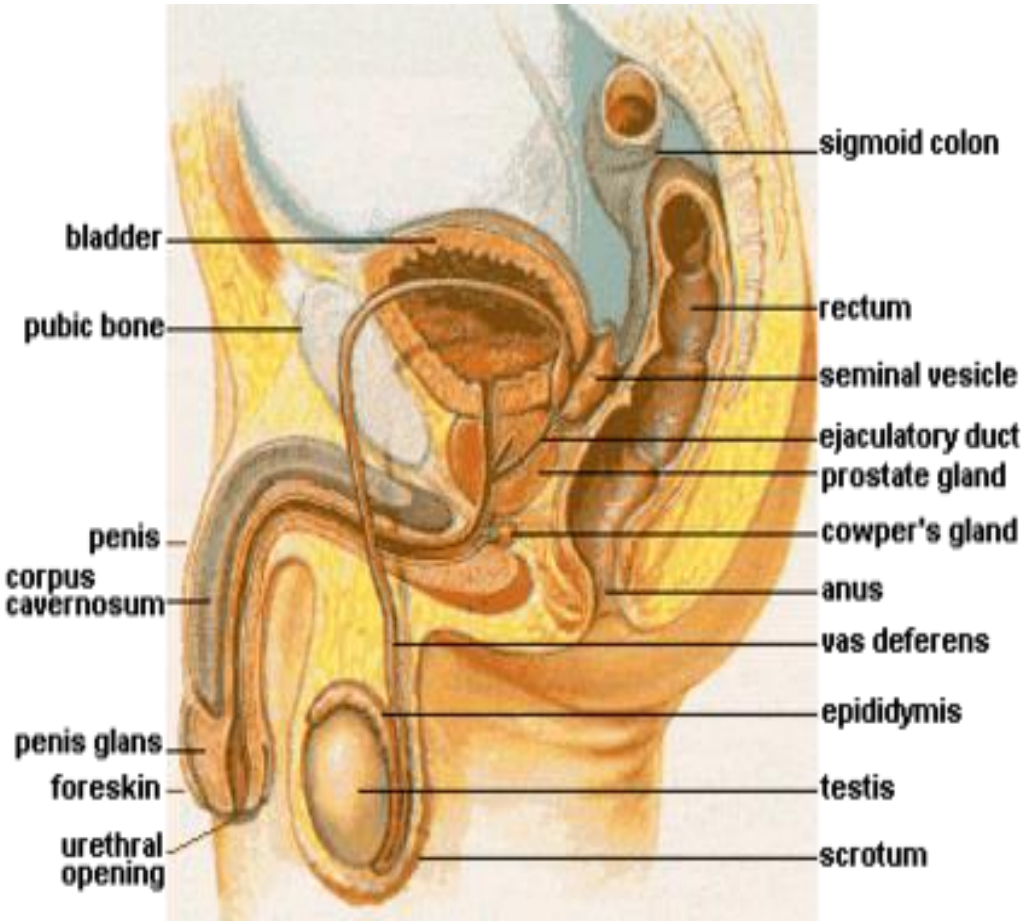


Figure 2.2 The human male reproductive system (Guyton and Hall, 2011)

2.3.1 Organs of Male Reproductive System

The testes (singular, testis) are located in the scrotum (a sac of skin between the upper thighs). In the male fetus, the testes develop near the kidneys, then descend into the scrotum just before birth. Each testis is about 1 1/2 inches long by 1 inch wide. Testosterone is produced in the testes which stimulates the production of sperm as well as give secondary sex characteristics beginning at puberty (Guyton and Hall, 2011).

The two testicles are each held in a fleshy sac called the scrotum. The major function of the scrotal sac is to keep the testes cooler than thirty-seven degrees Celsius (ninety-eight point six degrees Fahrenheit). The external appearance of the scrotum varies at different times in the same individual depending upon temperature and the subsequent contraction or relaxation of two muscles. These two muscles contract involuntarily when it is cold to move the testes closer to the heat of the body in the pelvic region. This causes the scrotum to appear tightly wrinkled. On the contrary, they relax in warm temperatures causing the testes to lower and the scrotum to become flaccid. The temperature of the testes is maintained at about thirty-five degrees Celsius (ninety-five degrees Fahrenheit), which is below normal body temperature. Temperature has to be lower than normal in order for *spermatogenesis* (sperm production) to take place (Guyton and Hall, 2011).

The two muscles that regulate the temperature of the testes are the dartos and cremaster muscles: The dartos muscle is a layer of smooth muscle fibers in the subcutaneous tissue of the scrotum (surrounding the scrotum). This muscle is responsible for wrinkling up the scrotum, in conditions of cold weather, in order to maintain the correct temperature for spermatogenesis. The cremaster muscle is a thin strand of skeletal muscle associated with the testes and spermatic cord. This muscle is a continuation of the internal oblique muscle of the abdominal wall, from which it is derived.

Each testis contains over 100 yards of tightly packed seminiferous tubules. Around 90% of the weight of each testis consists of seminiferous tubules. The seminiferous tubules are the functional units of the testis, where spermatogenesis takes place. Once the sperm are produced, they moved from the seminiferous tubules into the rete testis for further maturation.

In between the seminiferous tubules within the testes, are instititial cells, or, *Cells of Leydig*. They are responsible for secreting the male sex hormones (i.e., testosterone).

A Sertoli cell (a kind of sustentacular cell) is a 'nurse' cell of the testes which is part of a seminiferous tubule. It is activated by follicle-stimulating hormone, and has FSH-receptor on its membranes. Its main function is to nurture the developing sperm cells through the stages of spermatogenesis. Because of this, it has also been called the "mother cell." It provides both secretory and structural support. Other functions During the Maturation phase of spermiogenesis, the Sertoli cells consume the unneeded portions of the spermatazoa. The sperm are transported out of the testis and into the epididymis through a series of efferent ductules. The testes receive blood through the testicular arteries (gonadal artery). Venous blood is drained by the testicular veins. The right testicular vein drains directly into the inferior vena cava. The left testicular vein drains into the left renal vein (Guyton and Hall, 2011).

The seminiferous tubules join together to become the epididymis. The epididymis is a tube that is about 2 inches that is coiled on the posterior surface of each testis. Within the epididymis the sperm complete their maturation and their flagella become functional. This is also a site to store sperm, nourishing them until the next ejaculation. Smooth muscle in the wall of the epididymis propels the sperm into the ductus deferens. Vasa efferentia from the rete testis open into the epididymis which is a highly coiled tubule. The epididymis has three parts- 1) head or caput epididymis- it is the proximal part of the epididymis. It carries the sperms from the testis. 2) body or corpus epididymis- it the highly convoluted middle part of the epididymis 3) tail or cauda epididymis- it is the last part that takes part in carrying the sperms to the vas deferens. The cauda epididymis continues to form less convoluted vas deferens.

The ductus (vas) deferens, also called sperm duct, or, spermatic deferens, extends from the epididymis in the scrotum on its own side into the abdominal cavity through the inguinal canal. The inguinal canal is an opening in the abdominal wall for the spermatic cord (a connective tissue sheath that contains the ductus deferens, testicular blood vessels, and nerves.

The smooth muscle layer of the ductus deferens contracts in waves of peristalsis during ejaculation.

The pair of seminal vesicles is posterior to the urinary bladder. They secrete fructose to provide an energy source for sperm and alkalinity to enhance sperm mobility. The duct of each seminal vesicle joins the ductus deferens on that side to form the ejaculatory duct. There are two ejaculatory ducts. Each receives sperm from the ductus deferens and the secretions of the seminal vesicle on its own side. Both ejaculatory ducts empty into the single urethra. The prostate gland is a muscular gland that surrounds the first inch of the urethra as it emerges from the bladder. The smooth muscle of the prostate gland contracts during ejaculation to contribute to the expulsion of semen from the urethra. The bulbourethral glands also called Cowper's glands are located below the prostate gland and empty into the urethra. The alkalinity of seminal fluid helps neutralize the acidic vaginal pH and permits sperm mobility in what might otherwise be an unfavorable environment (Guyton and Hall, 2011).

The penis is an external genital organ. The distal end of the penis is called the glans penis and is covered with a fold of skin called the prepuce or foreskin. Within the penis are masses of erectile tissue. Each consists of a framework of smooth muscle and connective tissue that contains blood sinuses, which are large, irregular vascular channels. The urethra, which is the last part of the urinary tract, traverses the corpus spongiosum and its opening, known as the meatus, lies on the tip of the glans penis. It is both a passage for urine and for the ejaculation of semen.

2.3.2 Composition of human semen

The components of semen come from two sources: sperm, and "seminal plasma". Seminal plasma, in turn, is produced by contributions from the seminal vesicle, prostate, and bulbourethral glands. Seminal plasma of humans contains a complex range of organic and inorganic constituents. The seminal plasma provides a nutritive and protective medium for the spermatozoa during their journey through the female reproductive tract. The normal environment of the vagina is a hostile one for sperm cells, as it is very acidic (from the native microflora producing lactic acid), viscous, and patrolled by immune cells. The components in

the seminal plasma attempt to compensate for this hostile environment. Basic amines such as putrescine, spermine, spermidine and cadaverine are responsible for the smell and flavor of semen. These alkaline bases counteract the acidic environment of the vaginal canal, and protect DNA inside the sperm from acidic denaturation (Guyton and Hall, 2011).

Table 2.1. The components and contributions of semen are as follows:

GLAND	APPROXIMATE %	DESCRIPTION
Testes	2-5%	Approximately 200- to 500-million spermatozoa (also called sperm or spermatozoans), produced in the testes, are released per ejaculation
seminal vesicle	65-75%	Amino acids, citrate, enzymes, flavins, fructose (the main energy source of sperm cells, which rely entirely on sugars from the seminal plasma for energy), phosphorylcholine, prostaglandins (involved in suppressing an immune response by the female against the foreign semen), proteins, vitamin C
Prostate	25-30%	Acid phosphatase, citric acid, fibrinolysin, prostate specific antigen, proteolytic enzymes, zinc (serves to help to stabilize the DNA-containing chromatin in the sperm cells. A zinc deficiency may result in lowered fertility because of increased sperm fragility. Zinc deficiency can also adversely affect spermatogenesis.)
bulbourethral glands	< 1%	Galactose, mucus (serve to increase the mobility of sperm cells in the vagina and cervix by creating a less viscous channel for the sperm cells to swim through,

and preventing their diffusion out of the semen. Contributes to the cohesive jelly-like texture of semen.), pre-ejaculate, sialic acid

World Health Organization 1992 described normal human semen as having a volume of 2 ml or greater, pH of 7.2 to 8.0, sperm concentration of $20x10^6$ spermatozoa/ml or more, sperm count of $40x10^6$ spermatozoa per ejaculate or more and motility of 50% or more with forward progression (categories a and b) of 25% or more with rapid progression (category a) within 60 minutes of ejaculation.

The erection of the penis is its enlarged and firm state. It depends on a complex interaction of psychological, neural, vascular and endocrine factors. The term is also applied to the process that leads to this state.

A penile erection occurs when two tubular structures that run the length of the penis, the corpora cavernosa, become engorged with venous blood. This is a result of parasympathetic nerve induced vasodilation. This may result from any of various physiological stimuli. The corpus spongiosum is a single tubular structure located just below the corpora cavernosa, which contains the urethra, through which urine and semen pass during urination and ejaculation, respectively. This may also become slightly engorged with blood, but less so than the corpora cavernosa.

Penile erection usually results from sexual stimulation and/or arousal, but can also occur by such causes as a full urinary bladder or spontaneously during the course of a day or at night, often during erotic or wet dreams. An erection results in swelling and enlargement of the penis. Erection enables sexual intercourse and other sexual activities (sexual functions), though it is not essential for all sexual activities.

2.4. PHYSIOLOGY OF HYPOTHALAMO-PITUITARY-GONADAL AXIS

2.4.1 The hypothalamus is an almond shape size sit below the thalamus and above the brainstem in the brain. It extends from the preoptic area and fornix anteriorly to the mammillary bodies posteriorly. It is a collecting centre for information concerning the internal well-being of the body, and much of this information is used to control secretions of many globally important pituitary hormones. It functions to secrete releasing and inhibiting hormone that stimulate or inhibit production of hormone in the anterior pituitary.

The gonadotropin-releasing hormone (GnRH) is one of the releasing hormone from the hypothalamus, Regions of the hypothalamus that are involved in the regulation, production, and secretion of GnRH receive extensive direct connections from the cerebral hemispheres, especially from the temporolimbic structures that are commonly involved in epilepsy, and most notably from the amygdala. GnRH travels along the hypothalamic-hypophyseal portal system and binds to secretory cells of the anterior pituitary (adenohypophysis). GnRH binds to its receptor on gonadotrope cells, stimulating the biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH travel through the peripheral circulation, which in turn stimulate hormone production in peripheral glands (e.g. oestrogen, testosterone). Kisseptin is a principal regulator of the secretion of gonadotropins. It acts upstream on GnRH and, following paracrine stimulatory and inhibitory inputs from neurokinin B and dynorphin (KNDy neuropeptides), signals directly to GnRH neurons to control pulsatile GnRH release. Kisspeptin is a recently discovered neuromodulator that influence GnRH secretion. (Skorupskaite, *et al.,* 2014)

2.4.2 The human pituitary is situated inferiorly to the hypothalamus within the pituitary fossa, above the sphenoid sinus. A stalk connects the pituitary and hypothalamus. Hypothalamic regulatory hormones are secreted into the portal vessel for transport to the pituitary (Dawson 1958; Daniel and Prichard 1975; Bonneville *et al.,* 1989). The anterior pituitary is concerned with HPG-axis. The release of each of the pituitary hormones is under the control by at least one hypothalamic hormone, and hypothalamic hormones are controlled in turn by neurotransmitters. Furthermore, the release of both hypothalamic and pituitary hormones is regulated by concentrations of hormones secreted by the peripheral target glands,

i.e.by a feedback mechanism. The hypothalamic hormones are released in a pulsatile manner. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are secreted by the anterior pituitary. The gonad produces oestrogen and testosterone.

2.4.2.1 Luteinizing hormone (LH) also known as lutropin or interstitial cell-stimulating hormone is a glycoprotein of molecular mass 29.4 kDa. The hormone is released by the anterior pituitary gland (adenohypophysis) in response to gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone (LH-RH).The generic term "gonadotropins" is used for LH and follicle-stimulating hormone (FSH) because those two hormones control the functioning of the gonads.

The LH molecule is a heterodimeric (noncovalently-linked) glycoprotein. Each Monomeric unit is a glycoprotein molecule; one alpha and one beta subunit form the full functional protein. The alpha subunits of LH, follicle-stimulating hormone (FSH), and human chorionic gonadotropin (HCG) are identical. The beta subunits are specific to the respective hormone (Pierce and Parsons, 1981). LH has a beta subunit of 121 amino acids (LHβ) that determines its specific biological action and is responsible for its specific interaction with the LH receptor.

The production and secretion of both LH and FSH is stimulated by gonadotropin-releasing hormone from the hypothalamus. GnRH release is pulsatile; resulting in episodic release of both LH and FSH from the pituitary gland (Levine, *et al.*, 1985).The production and secretion of these hormones is regulated by a complex interaction of endocrine feedback systems. There are two separate feedbacks Centres in the hypothalamus: a tonic negative feedback centre in the basal medial hypothalamus, and a cyclical positive feedback centre in the anterior regions of the hypothalamus. Low concentrations of estradiol during the follicular phase affect the negative feedback centre, whereas high concentrations of estradiol before the midcycle LH peak trigger the positive feedback centre. LH production is again suppressed during the luteal phase by the negative feedback from progesterone combined with estradiol (Burtis *et al.*, 2006)

Luteinizing Hormone is essential for reproduction in both males and females. In pre-pubescent children, the peripheral levels of gonadotropins and gonadal steroids are very low. During puberty, pituitary sensitivity to GnRH increases, resulting in elevated gonadotropin

secretion, gonadal steroid secretion stimulation, and development of secondary sex-specific characteristics. LH encourages the development of sperm cells and regulates the production of testosterone by the testes.

2.4.2.1 Follicle-stimulating hormone

Follicle Stimulating Hormone binds to specific receptors on the plasma membrane of the follicular cells in the ovaries and the Sertoli cells in the testes. Adenylate cyclase acts as the intracellular messenger. FSH promotes follicular growth and prepares the follicles for the ovulation inducing action of LH, and enhances the LH-induced release of oestrogens. In the male, FSH binds to the Sertoli cells, where it induces the synthesis of an androgen-binding protein involved in the transport of testosterone to the seminiferous tubules and epididymis. This is important for achieving the high local levels of testosterone required for spermatogenesis. FSH stimulates seminiferous tubules and testicular growth and is important in initiating spermatogenesis (Skorupskaite, *et al.*, 2014).

2.4.3 Testosterone

Testosterone is synthesized in the Leydig cells in the testicles, under the influence of LH. Testosterone is the major hormone produced in the foetus, but testes only produce aldosterone after birth. The ability to produce testosterone is restored at puberty and continues throughout life. The androgens (principally testosterone and 5-alpha-DHT) are involved in sexual differentiation, spermatogenesis, development of secondary sexual characteristics, anabolic metabolism, and in masculine behaviour patterns. Testosterone is important for sexual fantasizing and desire, but is not involved in achieving an erection.

Serum testosterone exists in three forms:

a) Free testosterone (FT, 2% to 3% of total)
b) Albumin-bound testosterone (55%)
c) Sex hormone-binding globulin (SHBG)-bound (43% to 45%)

(Isojarvi *et al.*,1990).

The SHBG-bound fraction of testosterone in the body is not biologically active, but the albumin-bound and free fractions are active. Decrease in free but not total testosterone is associated with diminished libido, and since testosterone is necessary for the initiation and regulation of spermatogenesis, fertility would be affected (Isojarvi *et al.,* 1990). Testosterone raises potency and libido, whereas estradiol reduces it in men. Although estradiol constitutes only 1% of male gonadal steroids, it exerts almost 50% of the negative feedback on male LH secretion (Bauer *et al.,* 2004).

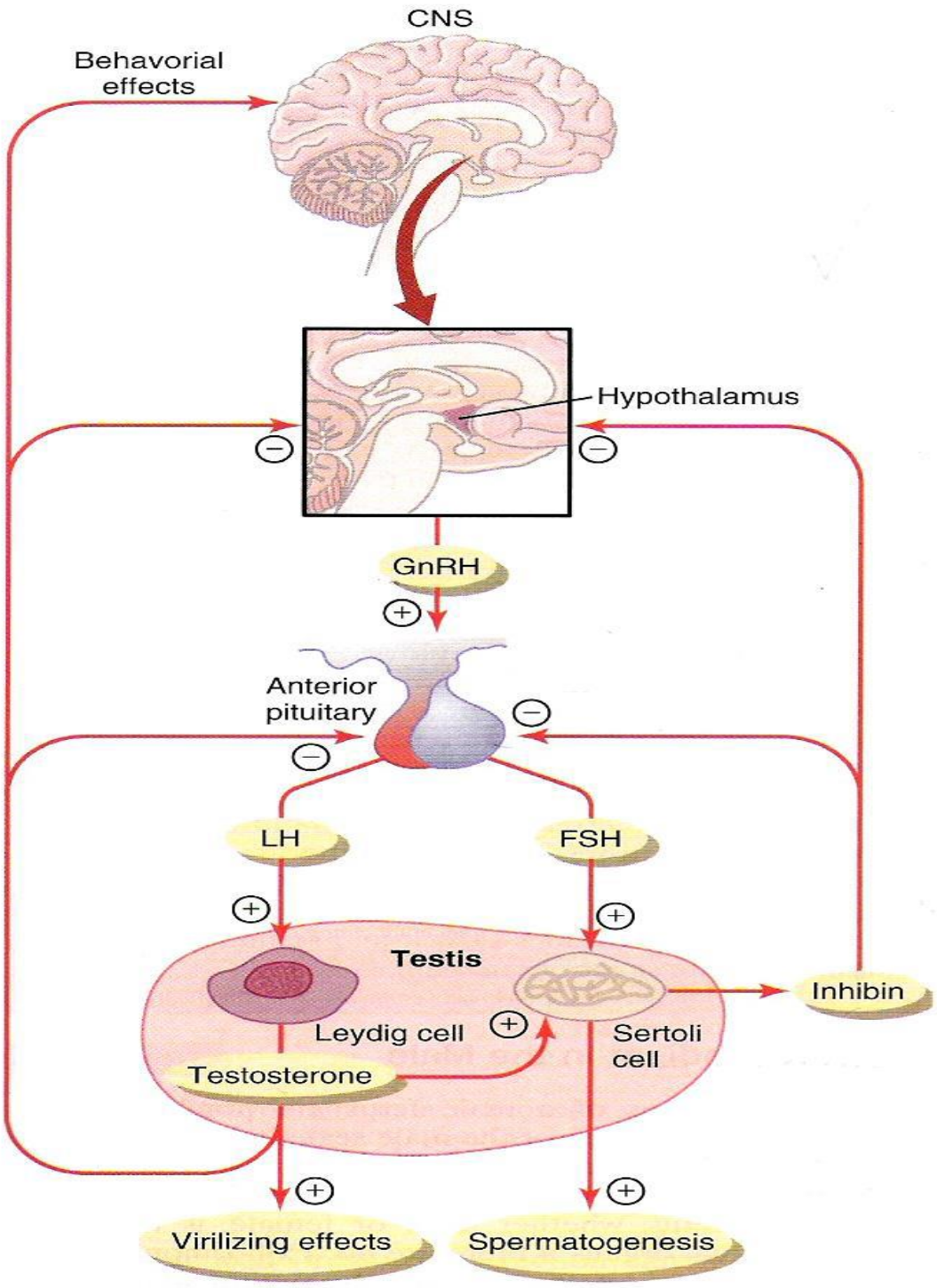


Figure 2.3. Feedback control in hypothalamo-pituitary-gonadal axis (Vadakkadath *et al*., 2005)

2.5. Spermatogenesis

The process by which spermatozoa are produced from spermatogonial stem cells by way of mitosis and meiosis is called spermatogenesis. The initial cells in this pathway are called spermatogonia, which yield primary spermatocytes by mitosis. The meiotic division of primary spermatozytes (Meiosis I) yielded two secondary spermatocytes. Each secondary spermatocyte divides into two spermatids by Meiosis II, which later develop into mature spermatozoa, also known as sperm cells. Thus, the primary spermatocyte gives rise to two cells, the secondary spermatocytes, and the two secondary spermatocytes by their subdivision produce four spermatozoa. Spermatozoa are the mature male gametes in many sexually reproducing organisms. Thus, spermatogenesis is the male version of gametogenesis, of which the female equivalent is oogenesis. In human and other mammal spermatogenesis takes place in the seminiferous tubules of the male testes step by step. For spermatogenesis to occur, certan conditions must be fulfilled for the process to occur correctly, and is essential for sexual reproduction. DNA methylation and histone modification have been implicated in the regulation of this process. It starts at puberty and usually continues uninterrupted until death; although a slight decrease can be discerned in the quantity of produced sperm with increase in age (Chang *et al*., 1998; Sembulingam and Sembulingam, 2012).

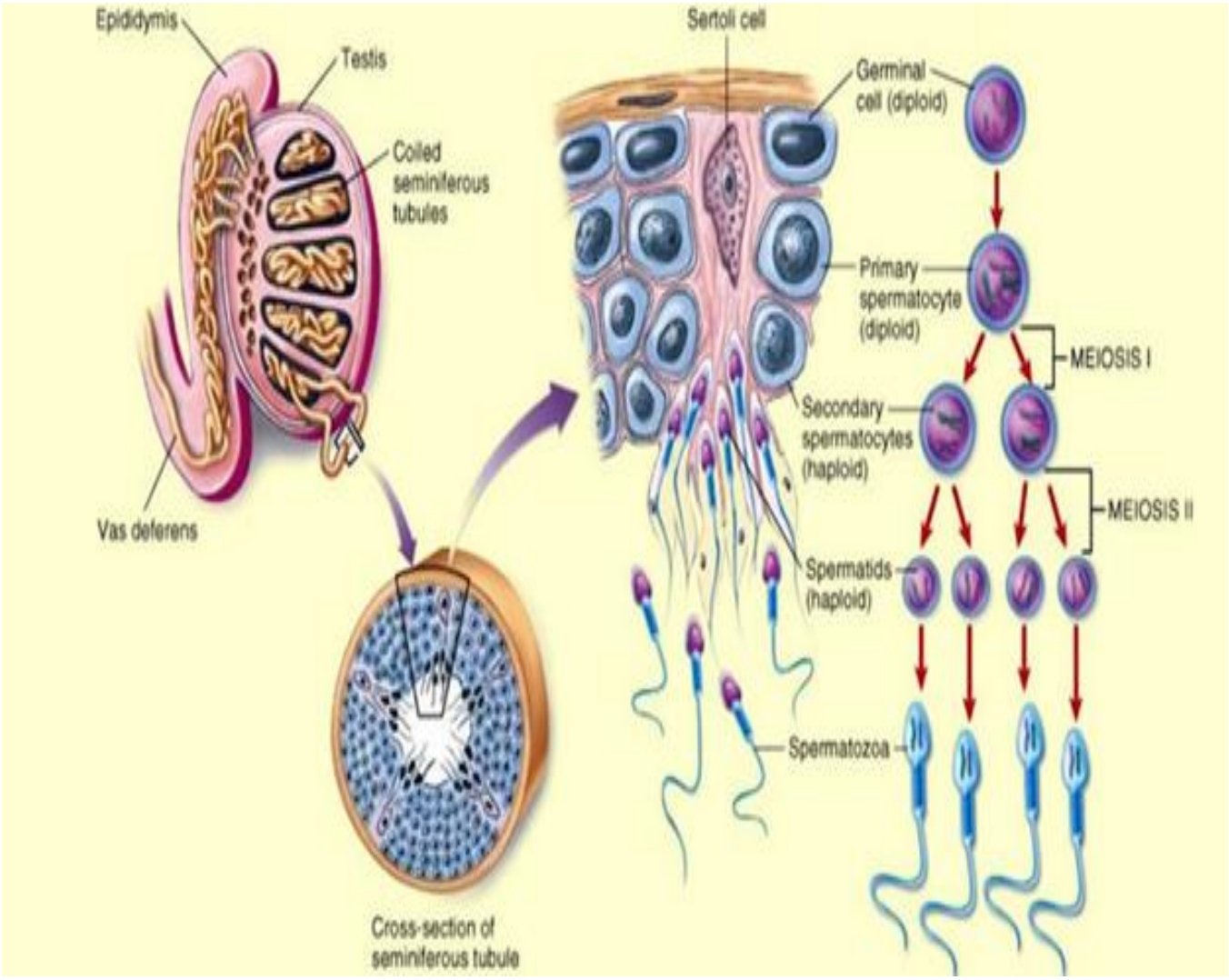


Fig.2.4. The process of spermatogenesis (Guyton and Hall, 2011)

2.5.1 Purpose, Location and Duration of Spermatogenesis

Spermatogenesis produces mature male gametes, commonly called spermatozoa, which have the ability to fertilize the counterpart female gamete, the oocyte, during conception to produce zygote, a single-celled individual. This is the cornerstone of sexual reproduction and involves the two gametes both contributing half the normal set of chromosomes (haploid) to result in a chromosomally normal (diploid) zygote.

The initial stage of Spermatogenic process takes place within the testes and later progress to the epididymis where the developing gametes mature and are stored until ejaculation. Within seminiferous tubules of the testes are the starting points for the process, where spermatogonial stem cells adjacent to the inner tubule wall divide in a centripetal direction—beginning at the walls and proceeding into the innermost part, or lumen —to produce immature sperm. Maturation occurs in the epididymis. Testes/Scrotum location is specifically important because for the production of viable spermatozoa during spermatogenesis, temperature of 2°-8 °C lower than normal body temperature of 37 °C (98.6 °F) is required.

For humans, the entire process of spermatogenesis is variously estimated as taking 74 days (according to tritium-labeled biopsies) and approximately 120 days (according to DNA clock measurements). Including the transport on ductal system, it takes 3 months. Testes produce 200 to 300 million spermatozoa daily. However, only about half or 100 million of these become viable sperm (Chang *et al*., 1998; Sembulingam and Sembulingam, 2012; Skorupskaite, *et al.*, 2014).

2.5.2 Stages in Spermatogenesis

The entire process of spermatogenesis have several distinct stages, each corresponding to a particular type of cell in human.

The processes of spermatogenesis are broken down into four distinct stages as follows.

Stage 1: The Diploid spermatogonia are situated in the seminiferous tubules which includes twice the total number of chromosomes. This replicates mitotically in interphase before the method of meiosis 1 to create 46 pairs of sister chromatids .

Stage 2: In this stage, the chromatids allow the exchange of genetic information through the synapsis process. It is done before dividing into haploid spermatocytes through meiosis.

Stage 3: In this division, the new two daughter cells will further divide into 4 spermatids, having unique chromosomes that are approximately half in number to the original spermatogonium.

Stage 4: In this, the cells will now move from the lumen of the testes to the epididymis. They get mature and developed into four sperm cells with the growth of microtubules on the centrioles to develop an axoneme. Some of the centrioles are there that elongate to develop the sperm tail.

Sperm cells are male reproductive cells. They have a head, which is the body of the cell, and a tail, which is the flagellum that propels the cell. Because they need to be efficient, they contain lots of mitochondria for energy and a nucleus to deliver DNA. Once a sperm reaches the egg, the female reproductive cell, it makes a totipotent zygote, which will divide and eventually make up all of the cells in the body (Chang *et al*., 1998; Guyton and Hall, 2011).

2.5.3 Functions of Sperm cell

A sperm cell consists of two parts, the head and the tail. The overall structure of the sperm makes it perfectly designed to carry out its function. The primary function of sperm is to pass on the necessary genetical information required to produce a new organism. In humans the data that makes an organism is contained in a tiny molecule called DNA. DNA is basically a recipe book used to make human. All physical characteristics are determined by the information contained in DNA. The DNA, or biological information the sperm needs to pass on, is contained in its head. The tail of the sperm helps give it the momentum it needs to reach the egg cell, so it is able to create the embryo in the first place.

2.5.4 Factors Affecting Spermatogenesis

This process of spermatogenesis seems to be very sensitive and can easily be affected by the little change in the hormone level. For Example- testosterone is developed through the hypothalamus, leydig cells and pituitary gland. As this process is very sensitive to changes in

temperature, deficiency in diet, alcoholism, exposure to drugs, hormones and presence of disease can affect the rate of sperm formation adversely.

Testosterone is required in large local concentrations to maintain the process, which is achieved via the binding of testosterone by androgen binding protein present in the seminiferous tubules. Testosterone is produced by interstitial cells, also known as Leydig cells, which reside adjacent to the seminiferous tubules. Seminiferous epithelium is sensitive to elevated temperature in humans and some other species, and will be adversely affected by temperatures as high as normal body temperature. Consequently, the testes are located outside the body in a sack of skin called the scrotum. The optimal temperature is maintained at 2 °C (man)–8 °C (mouse) below body temperature. This is achieved by regulation of blood flow and positioning towards and away from the heat of the body by the cremasteric muscle and the dartos smooth muscle in the scrotum.

Dietary deficiencies (such as vitamins B, E and A), anabolic steroids, metals (cadmium and lead), x-ray exposure, dioxin, alcohol, and infectious diseases will also adversely affect the rate of spermatogenesis. In addition, the male germ line is susceptible to DNA damage caused by oxidative stress, and this damage likely has a significant impact on fertilization and pregnancy. Exposure to pesticides also affects spermatogenesis (Chang *et al*., 1998; Guyton and Hall, 2011).

2.5.5 Hormonal control of spermatogenesis

Hormonal control of spermatogenesis varies among species. In humans the mechanism is not completely understood; however it is known that initiation of spermatogenesis occurs at puberty due to the interaction of the hypothalamus, pituitary gland and Leydig cells. If the pituitary gland is removed, spermatogenesis can still be initiated by follicle stimulating hormone (FSH) and testosterone. In contrast to FSH, LH appears to have little role in spermatogenesis outside of inducing gonadal testosterone production.

FSH stimulates both the production of androgen binding protein (ABP) by Sertoli cells, and the formation of the blood-testis barrier. ABP is essential to concentrating testosterone in levels high enough to initiate and maintain spermatogenesis. Intra-testicular testosterone levels are 20–100 or 50–200 times higher than the concentration found in blood, although

there is variation over a 5- to 10-fold range amongst healthy men. FSH may initiate the sequestering of testosterone in the testes, but once developed only testosterone is required to maintain spermatogenesis. However, increasing the levels of FSH will increase the production of spermatozoa by preventing the apoptosis of type A spermatogonia. The hormone inhibin acts to decrease the levels of FSH. Studies from rodent models suggest that gonadotropins (both LH and FSH) support the process of spermatogenesis by suppressing the proapoptotic signals and therefore promote spermatogenic cell survival.

The Sertoli cells themselves mediate parts of spermatogenesis through hormone production. They are capable of producing the hormones estradiol and inhibin. The Leydig cells are also capable of producing estradiol in addition to their main product testosterone. Estrogen has been found to be essential for spermatogenesis in animals. However, a man with estrogen insensitivity syndrome (a defective ERα) was found produce sperm with a normal sperm count, albeit abnormally low sperm viability; whether he was sterile or not is unclear. Levels of estrogen that are too high can be detrimental to spermatogenesis due to suppression of gonadotropin secretion and by extension intra-testicular testosterone production. Prolactin also appears to be important for spermatogenesis (Chang *et al*., 1998; Guyton and Hall, 2011).

2.5.6 Testosterone

Androgens are essential for male fertility and the maintenance of spermatogenesis. Testosterone is the androgen in the testis that is responsible for supporting spermatogenesis. In the absence of testosterone or functional androgen receptors (AR), males are infertile because spermatogenesis rarely progresses beyond meiosis. This is the male sex hormone. Testosterone is a steroid hormone that is produced in the leydig cells within the testes. A relatively high concentration of testosterone is maintained within the testicular tissue and testosterone is circulated around the body by diffusion of the hormone from the spermatic cord into the testicular veins and arteries. The primary action of testosterone is anabolic growth, spermatogenesis promotion and promotion of secretion from the accessory sex glands.

Male sex hormones are regulated by negative feedback systems that operate at various levels within the male sex hormone system. The starting point for the production of testosterone (and therefore the production of spermatozoa) is the hypothalamus. The hypothalamus contains neuroendocrine cells that are capable of secreting a substance called Gonadotropin-releasing hormone or GnRH. GnRH stimulates basophilic cells in the adenohypophysis, via the "portal system" to secrete two intermediate hormones within the male sex hormone cycle; Luteinizing hormone (LH) and Follicle-Stimulating Hormone (FSH).

The secretion of GnRH is pulsatile and can vary greatly throughout the day and/or year, and therefore the secretion of LH and FSH is also pulsatile (although the plasma concentration of FSH does not fluctuate as much as LH due to the effect of Inhibin). The activity of GnRH neuroendocrine cells is determined by spontaneous rhythms and by sensory impulses. Cycles such as seasonal sexual activity are controlled by this pulsatile system. In male animals there are generally 4 to 12 GnRH pulses per day (Chang *et al.,* 2004; Sembulingam and Sembulingam, 2012).

2.5.6.1 Testosterone Regulation

Intratesticular testosterone is thought to play a very important role in spermatogenesis; however, it is very rarely measured. It is now established that lowering of intratesticular testosterone concentration results in the apoptotic death of some germ cells (e.g., pachytene spermatocytes) in association with nuclear DNA fragmentation in the dying cells (Chang *et al.*, 2004) Previous studies of spermatogenesis in the rat have shown that the experimental reduction of intratesticular testosterone to low enough levels results in germ cell loss (De Gendt, 2004) and that the re-administration of testosterone restores spermatogenesis (Awoniyi 1990). Androgen receptor expression in the seminiferous epithelium is restricted to the somatic Sertoli cells (Sar *et al.,* 1990). Therefore, in response to changes in intratesticular testosterone concentration, the Sertoli cell presumably communicates a signal to the attached and developing germ cells, which lack the androgen receptor, resulting either in the loss or restoration of germ cells (Show *et al.*, 2004). When LH binds to the Leydig cells, it stimulates the cellular messenger cAMP to activate protein kinase A. Protein kinase A undergoes a series of phosphorylation that in turn activate a series of enzymes that synthesis testosterone from the cholesterol base molecule. A portion of the testosterone produced in the Leydig cells

diffuses into the Sertoli cells that are positioned adjacent to the Leydig cells in the testes but separated by a basal lamina. This secreted testosterone is converted to the female sex hormone estradiol in the Sertoli cell and as with the testosterone; a proportion diffuses into the blood, becoming part of the negative feedback system for LH.

Testosterone inhibits the secretion of GnRH from the hypothalamus and therefore secretion of LH from the pituitary gland. If the testes are removed via castration, blood concentrations of LH and FSH will increase as there is only limited negative feedback.

2.5.6.2 General effects of testosterone

Testosterone plays a crucial role in the development of male sex organs during fetal growth where increased production of testosterone causes penis growth and development of accessory sex glands during puberty. Testosterone also affects a number of other characteristics of the male, often called the "secondary sex characteristics". Testosterone is able to bind to receptors in the cytosol of cells in the same manner as other steroid hormones and these hormone-receptor complexes are then able to bind to DNA in the nucleus resulting in alterations in the level of transcription of specific genes.

Testosterone has a number of anabolic effects stimulating the development and growth of the skeleton and skeletal muscles. Muscle masses show a general increase and in certain body regions such as the neck of stallions or bulls there is obvious hypertrophy. Testosterone also alters behaviour in terms of increasing the degree of sex drive and as a result of the action in several areas of the brain, behaviour can become more aggressive. The larynx of males also enlarges during puberty and the vocal cords lengthen resulting in a deeper and stronger voice.

Testosterone also causes an increase in the level of pheromones to be secreted by glands in the skin which attract and evoke sexual behaviour in females. Glands use in scent marking and territorial marking are also activated by testosterone. In certain species, tusks, antlers and horns are also stimulated to develop.

2.5.7 Endocrinology of spermatogenesis and the hypothalamus-pituitary-testis axis

This circuit or axis represents the core unit for the maintenance of the endocrine balance and fertility. Testicular functions, i.e. production of testosterone and of spermatozoa, are entirely

subject to regulation by endocrine factors derived from the brain. Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus and stimulates the synthesis and release of the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. LH acts on testicular Leydig cells and governs the production and secretion of testosterone by these cells. Within the testis, testosterone acts on peritubular cells that surround the seminiferous tubules and on the somatic Sertoli cells within the seminiferous epithelium. Beyond that, testosterone exerts a variety of physiological effects in the periphery and, in fact, androgen receptors have been detected in about 40 organs of the cynomolgus monkey (Dankbar *et al.*, 1995).

2.6. The Metal Lead (Pb)

Lead belongs to group 14 of the periodic table, which also includes C, Si, Ge and Sn. Lead has the most metallic characteristics of this group.The element has an atomic number of 82, anatomic mass of 207, two oxidation states (+2 and+4) and four naturally occurring isotopes (204Pb, 206Pb, 207Pb and 208Pb), of which 208Pb is the mostabundant at 52% of the total mass. Lead is the most abundant of the transition metal elements (Greenwood and Earnshaw 1984). Lead is a chalcophile metallic element forming several important minerals including galena PbS, anglesite $PbSO_4$, cerussite $PbCO_3$ and minimum Pb_3O_4. It is also widely dispersed at trace levels in a range of other minerals, including K–feldspar, plagioclase, mica, zircon and magnetite. Lead is one of the seven metals known in antiquity, because of its relative ease of extraction as a metal. The Roman civilisation, in particular, used large quantities of lead for plumbing purposes. The Pb_2^+ ion (119 pm) is intermediate in size between K^+ (138 pm) and Ca_2^+ (100 pm) and so it replaces these ions in K-feldspar, mica and, to a lesser extent, plagioclase and apatite. As a consequence, it is enriched in felsic igneous rocks relative to mafic rocks, and Pb is mobile in late-stage magmatic processes (MacDonald *et al.,* 1973). The enrichment in felsic igneous rocks is confirmed by the values quoted by Mielke (1979): ultramafic 1 mg kg-1; basaltic 6 mg kg-1; granitic 15–19 mg kg-1; syenite 12 mg kg-1; and a crustal abundance of 13 mg kg-1.

In sedimentary rocks, the distribution of Pb is controlled by the presence of primary detrital minerals, such as feldspar, mica and sulphides, clay minerals (Heinrichs 1974, Heinrichs *et al.,* 1980) and organic matter. Pure limestone (ca. 5 mg kg-1) and quartzitic sandstone (ca.10 mg kg-1) are typically depleted relative to shale and greywacke (ca. 23 mg kg-1). The sedimentary rocks with the highest concentrations are black shale, reflecting the affinity of Pb for organic matter. Loess has an average Pb content of 13 mg kg-1 (McLennan and Murray 1999). Around 35% of Pb in stream sediment occurs in the sand fraction, but the majority is found in the silt and clay fractions, associated with kaolinite and mica, and secondary iron oxide precipitates (Song *et al.,* 1999). The average concentration of Pb in river particulates is 150 mg kg-1, a value that is definitely affected by anthropogenic factors (McLennan and Murray 1999).

Lead is used as a pathfinder for Sedex and VHMS deposits, and also for gold under some conditions, though not in lateritic terrains. The natural lead content in soil is, of course, related to the composition of the parent rock. Although the species of lead vary considerably with soil type, it is mainly associated with clay minerals, Mn oxides, Fe and Al hydroxides and organic matter. In some soil types, lead may be highly concentrated in carbonate particles or in phosphate concentrations (Kabata-Pendias 2001). A baseline lead value for surface soil on the global scale has been estimated to be 25 mg kg-1; levels above this suggest an anthropogenic influence (Kabata-Pendias 2001). In areas of sulphide mineralisation, lead is mobilised by acidity derived from the weathering of galena and other sulphide minerals. The oxidation of lead sulphides results in high concentrations of lead in stream water, particularly around sites of base-metal mining. Lead is generally present in the aqueous environment as Pb_2^+ (aq) below pH 6, but it also forms complexes with organic anions, chloride and hydroxide, and insoluble or poorly soluble compounds with sulphide, sulphate, hydroxy carbonate and phosphate anions. Lead mobility is restricted by sorption on clay, organic matter, secondary iron and manganese oxides, and the formation of secondary minerals with low solubilities such as anglesite $PbSO_4$, cerussite $PbCO_3$ and pyromorphite $Pb_{10}(PO_4)_6C_{l2}$. Biofilms also strongly adsorb lead and can significantly influence the distribution of lead in solution (Nelson *et al.,*1995). Although lead is more soluble in non-calcareous soil below pH 5.2, it is adsorbed on iron oxides in preference to Cu and Zn (O'Day *et al.,* 1998) and, therefore, does not migrate readily to groundwater (Martínez and Motto, 2000). Lead concentrations in unpolluted rainwater and snow are about 1 µg l-1, which is also typical for most unpolluted surface and ground waters (Hem, 1992). In lower alkalinity and pH water, however, the dissolved lead concentration can be significantly higher (Hem, 1992).

Lead from vehicle exhausts, in the form of tetraethyl lead, was, until recently, a significant source of contamination. In urban environments, road dusts can contain very high levels of lead (Archer and Barret, 1976), although the introduction of unleaded petrol has reduced this potentially toxic hazard in developed industrialised countries. In addition, metalliferous mining (especially sulphide ores), metallic detritus, lead -bearing glass and pottery glazes, batteries, old lead-based paints, the corrosion of lead pipes in areas of soft water and sewage sludge are all potential sources of lead. Anthropogenic sources of pollution may cause local enhancement of lead levels in surface water by an order of magnitude compared to background values (Patterson, 1965).

Lead has no known biological role in plants or animals and is highly toxic to mammals and aquatic life. It can cause mental impairment in young children, causing neuropathy and hypertension in adults and may be lethal at high levels, e.g., over 25 µg kg-1 of body weight (WHO 1996). Lead is a particularly dangerous chemical, as it can accumulate in individual organisms, but also in entire food chains. Thus, lead pollution is a worldwide issue.

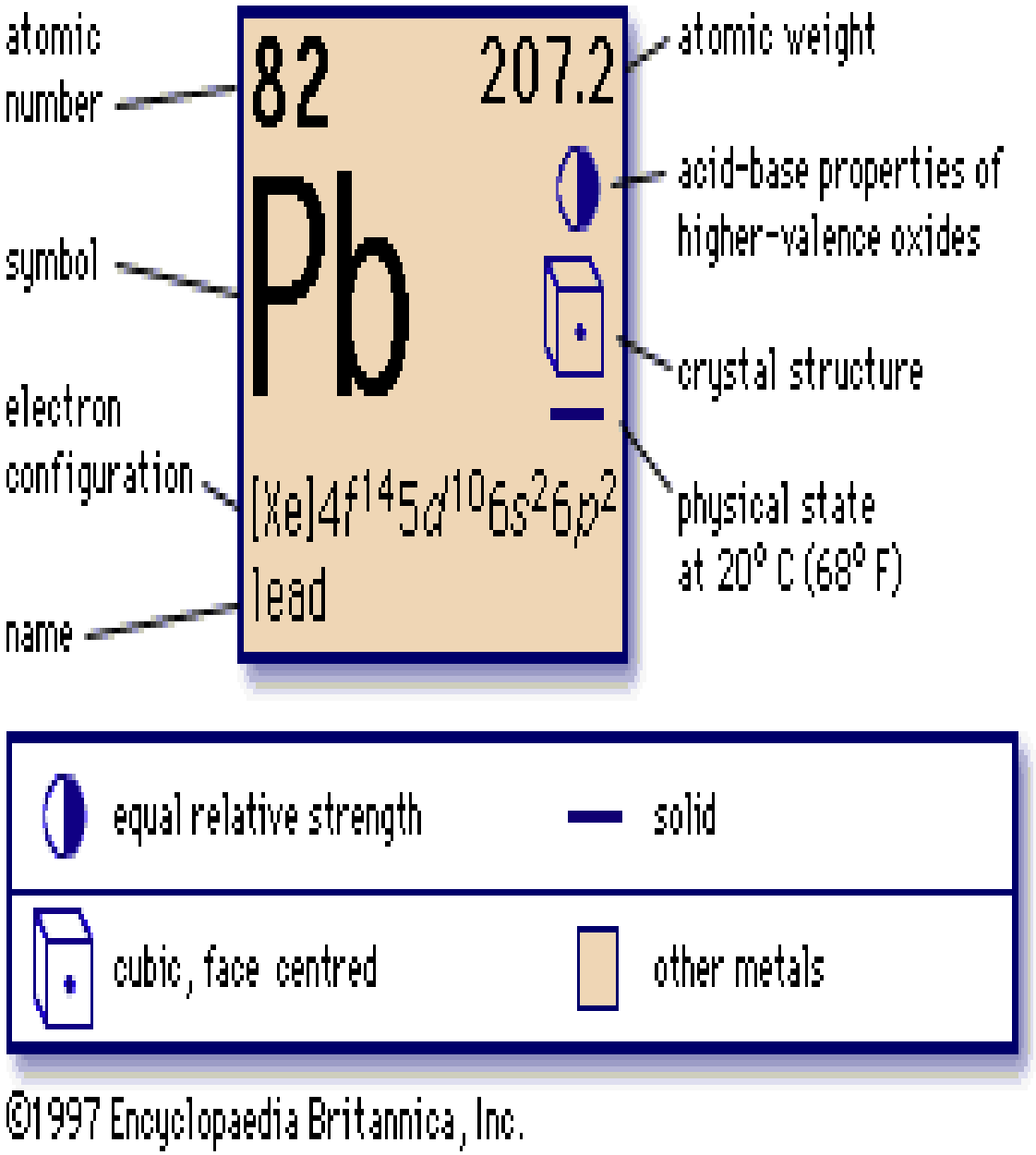


Figure 2.6. Chemistry of Lead (htpp://en.wikipedia.org/wiki/lead)

2.6.2 Sources of lead

Lead can be found in many products and locations. Some you might never have thought of, including some imported candies, toys, and traditional medicines. The most common cause of lead poisoning is dust and chips from old paint. However, some non-paint sources, though less common, can cause severe cases of lead poisoning.

- Paint

Lead was used in paint to add colour, improve the ability of the paint to hide the surface it covers, and to make it last longer. In 1978 the federal government banned lead paint for use in homes. Homes built before 1978 probably contain lead-based paint. Painted toys and furniture made before 1978 may also contain lead-based paint. Lead-based paint becomes a concern when it chips, turns into dust, or gets into the soil.

- Dust

Lead dust is the most common way that people are exposed to lead. Inside the home, most lead dust comes from chipping and flaking paint or when paint is scraped, sanded, or disturbed during home remodelling. Chipping and peeling paint is found mostly on surfaces that rub or bump up against another surface. These surfaces include doors and windows. Young children usually get exposed to lead when they put something with lead dust on it into their mouths. Lead dust may not be visible to the naked eye (Goldfarb *et al*,. 2005)

- Soil

Starting in 1973, the federal government started a gradual phase-down of lead content in gasoline, and by 1996, banned the sale completely. However, lead from car exhausts mixed with soil near roads and is still there today. Homes near busy streets may have higher levels of lead in the soil. Today, lead still comes from metal smelting, battery manufacturing, and other factories that use lead. This lead gets into the air and then mixes with the soil near homes, especially if the home is near one of these sources. Flaking lead-based paint on the outside of buildings can also mix with the soil close to buildings. Lead-based paint mixing with soil is a problem during home remodelling if workers are not careful. Once the soil has lead in it, wind can stir up lead dust, and blow it into homes and yards (Goldfarb *et al*,. 2005).

- Drinking Water

Lead seldom occurs naturally in water supplies like rivers and lakes. Lead enters drinking water primarily as a result of the corrosion, or wearing away, of materials containing lead in the water distribution system and household or building plumbing. These materials include lead-based solder used to join copper pipe, brass and chrome plated brass faucets, and in some cases, pipes made of lead that connect houses and buildings to water mains. In 1986, Congress banned the use of lead solder containing greater than 0.2% lead, and restricted the lead content of faucets, pipes and other plumbing materials to 8.0%. Older construction may still have plumbing that has the potential to contribute lead to drinking water (Ramos and Rosenberg, 2012).

- Air

Lead can be present in outdoor and indoor air. Lead in outdoor air comes mainly from industrial sources (e.g., smelters, waste incinerators, utilities, and lead-acid battery manufacturers). Wind-blown soil and road dust also may contain naturally occurring lead as well as lead from industrial sources, deteriorated paint, and the combustion of leaded gasoline and aviation fuel. Sources of lead in indoor air include outdoor air, suspended dust, and some hobbies (e.g., making stained glass objects using lead solder, shooting using lead bullets at indoor firing ranges) (Ramos and Rosenberg, 2012).

- Folk medicines, ayurvedics and cosmetics

Some folk medicines contain lead. They often are imported from the Middle East, Southeast Asia, India, the Dominican Republic, or Mexico. Two examples are Greta and Azarcon. Azarcon is a bright orange powder also known as Maria Luisa, Rueda, Alarcon, and Coral. Greta is a yellow powder. They are used to treat an upset stomach. Pay-loo-ah also contains lead. It is a red powder used to treat a rash or a fever. Other folk medicines that contain lead include Bala (or BalaGoli), Golf, Ghasard, and Kandu. Some cosmetics such as Kohl (Alkohl) and Surma also contain lead.

Ayurveda is a traditional form of medicine practiced in India and other eastern Asian countries. Ayurvedic medications may contain herbs, minerals, metals, or animal products. These medicines may come in both standardized and non-standardized formulations.

Ayurvedic medications are typically imported into the United States by both practitioners and followers of Ayurvedic medicine (Fisher *et al*., 2006).

- Children's jewellery and toys

Lead has been found in inexpensive children's jewellery sold in vending machines and large volume discount stores across the country. It also has been found in inexpensive metal amulets worn for good luck or protection. Some costume jewellery designed for adults has also been found to contain lead. It is important to make sure that children don't handle or mouth any jewellery (Fisher *et al*., 2006).

- The workplace and hobbies

People exposed to lead at work may bring lead home on their clothes, shoes, hair, or skin. Some jobs that expose people to lead include: home improvement; painting and refinishing; car or radiator repair; plumbing; construction; welding and cutting; electronics; municipal waste incineration; lead compound manufacturing; manufacturing of rubber products, batteries, and plastics; lead smelting and refining; working in brass or bronze foundries; demolition; and working with scrap metal.

Some hobbies also use lead. These hobbies include making pottery, stained glass, or refinishing furniture. Hunters who make their own bullets or anglers who make their own fishing sinkers can be exposed to lead fumes if they don't follow good practices. Fishing tackle (especially sinkers and jig heads) often contains lead. It important to keep all lead objects away from children. Wash hands with soap and water after holding or using lead sinkers and jig heads or reloading lead bullets or shot. Never bite down on lead sinkers (Papp, 2013).

- Lead-glazed ceramics, china, leaded crystal, pewter

Lead may get into foods or liquids that have been stored in ceramics, pottery, china, or crystal with lead in it. Lead-glazed dishes usually come from other countries.

- Imported candies or foods, especially from Mexico, containing chili or tamarind

Lead can be found in candy, wrappers, pottery containers, and in certain ethnic foods, such as chapulines (dried grasshoppers).

- Imported food in cans that are sealed with lead solder

In 1995 the United States banned the use of lead solder on cans. But lead solder can still be found on cans made in other countries. These cans usually have wide seams, and the silver-gray solder along the seams contains the lead. Cans containing lead may be brought to the United States and sold. Over time the lead gets into the food. This happens faster after the can has been opened. Foods that are acidic cause lead to get into the food faster (Fisher *et al*., 2006).

- Firearms with lead bullets

People can also be exposed to lead by eating venison and small game harvested with lead shot and lead bullets. Recent research indicates that small lead fragments are often present in venison from deer harvested with lead bullets. Some bullets shatter into small pieces that can be too small to detect by sight, feel, or when chewing the meat. People can also be exposed to lead when it is released into the air when a gun is fired particularly in indoor shooting ranges. Lead particles are also formed as the lead bullet spirals through the barrel. These particles of lead can get into your body when you breathe or swallow, and lead dust can get on your food, cigarettes, or other items that you eat, drink, or put in your mouth (Papp, 2013).

- Mini-blinds

Some non-glossy, vinyl mini-blinds from foreign countries contain lead.

- Some other common sources of lead

Batteries, radiators for cars and trucks, and some colours of ink also contain lead.

2.6.3 Health Effects of Lead Exposure

- Introduction

Lead poisoning is the presence of too much lead in the body. Lead poisoning is one of the most common and preventable paediatric health problems in the United States today. Lead poisoning, particularly its negative impact on children during the early growth years, is a public health problem of continuing importance. As an understanding of the ramifications of lead poisoning has continued to evolve, public health advocates have pushed for legislation that has decreased the amount of lead in gasoline, residential paint, metal solder and plumbing components. As a result, few children suffer from the most serious effects of lead poisoning such as seizures, comas or death. However, a great deal of old leaded paint still exists in older housing, and thousands of children continue to be exposed to lower doses of lead that can result in subtle but serious health problems.

- Lead in the Body

Lead is stored in the bone for decades, causing long-term internal exposure. Lead enters the body primarily through inhalation and ingestion of lead containing dust. Once in the body, lead travels in the blood to soft tissues such as the liver, kidneys, lungs, brain, spleen, muscles, and heart. The body does not change lead into any other form. Once it is taken in and distributed to the organs, the lead that is not stored in the bones is eliminated slowly from the body by the kidneys and gastrointestinal tract; negligible amounts of lead are lost through perspiration. About 99% of the amount of lead taken into the body of an adult will leave in the waste within a couple of weeks, but only about 32% of the lead taken into the body of a child will leave in the waste (Barry, 1975). The half-life of lead varies from about a month in blood, 1-1.5 months in soft tissue, and about 25-30 years in bone (ATSDR 2007). Lead in bone is considered a biomarker of cumulative exposure because lead accumulates in bone over the lifetime and most of the lead body burden resides in bone. Some of the lead can stay in the bones for decades; however, some lead can leave the bones and reenter the blood and organs under certain circumstances, for example, during pregnancy and periods of breast-feeding, after a bone is broken, and during advancing age.

- Effects of Lead

There is no biologic function or need for lead. There is no such thing as a "normal" lead level, only that level which we are willing to tolerate. Lead is a toxic substance that poses a variety of dangers for humans. Lead has been shown to affect virtually every organ and/or system in the body in both humans and animals. Young children and the developing fetus are particularly at risk. Lead damages the central and peripheral nervous system, the kidneys and the body's ability to regulate vitamin D. Lead negatively affects the formation of red blood cells. Very high levels of lead can cause seizures, coma and death. At lower levels of exposure, a child can suffer from developmental delay, lower IQ, hyperactivity, learning disabilities, behavioural problems, impaired hearing and stunted growth. Many of these effects are irreversible. Increasing the problem of lead poisoning is the fact that signs of lead poisoning are not always obvious. At low lead levels, a child may show no symptoms at all. Many children who are lead-poisoned look and act healthy. Sometimes the vague symptoms may be mistaken for other illnesses. Young children, especially those under the age of 6, are at greatest risk for lead poisoning. Children explore their environment by putting their toys, hands and other objects in their mouths. Children may chew on painted windowsills, railings, furniture and toys. In addition, they spend a lot of time on the floor where sources of lead are likely to be found. Through normal play, they may eat lead that has come from deteriorating paint, paint chips, or dust. Young children also absorb lead more easily than adults. Up to 50% of the lead a child ingests can be absorbed, compared to only 10% in adults. Children also are not as efficient in eliminating the lead they have absorbed. When a child has an iron deficiency, lead is more easily absorbed from the gastrointestinal tract. Children have more trouble than adults in sequestering lead in the bones, so a larger fraction of any lead that might be present in the body is available to a child's targeted organs. A child's developing brain and central nervous system are more susceptible to the toxic effects of lead since the blood-brain barrier is not complete until approximately 36 months of age and during that time there is extensive neuron development. Many of these effects are irreversible. Blood lead levels as low as 2 μg/dL may not cause distinctive symptoms but are associated with decreased intelligence and slower neurobehavioral development in the form of cognitive and language deficits. Many other effects can begin to occur at these low blood levels. Recent studies suggest that lead absorption is harmful at any concentration and that no safe level of lead

exposure exists (Lanphear *et al.,* 2005b). Absorption of lead increases with pregnancy. Pregnant women absorb up to 85% of the lead to which they are exposed. Research indicates that lead in pregnant women can cross the placenta, affecting children even before they are born. Maternal cord blood lead levels of 10 μg/dL to 15 μg/dL appear to be associated with an increase in premature births, reduced birth weight, decreased stature and inability to maintain steady posture. Studies show that babies exposed to lead before birth may have learning and behaviour problems that can last a lifetime. High levels of lead exposure during pregnancy may cause harm to the fetus, including birth defects, brain damage, hearing loss or even death.

- Signs and Symptoms

Lead metal foreign bodies in eye or orbit in humans have been considered to cause little reaction and rarely any toxic effect. Clinical experiences with various intraocular foreign bodies presented in detail with histological studies indicated that lead metallic foreign bodies caused minimal inflammatory reaction, mainly mechanical injury. In another report it was concluded that lead fragments in patients were well tolerated in the eye and in the orbit and that they should not be removed unless they were in the anterior chamber. A case is described in which a small lead shot was allowed to remain in the vitreous, the vision returned to normal as blood in the vitreous absorbed or settled in the course of a year. In one case, which appears to have been quite exceptional, a patient with a lead shot behind one globe had impaired vision in that eye. This was assumed to be due to a toxic effect of lead. A significant improvement of vision was reported when systemic and topical treatment with 2,3-dimercaptopropansulfonate sodium was started 5 yr after the injury.

2.7 Lead and male reproduction

- Spermatogenesis

The most frequent causes of male infertility are associated with spermatogenesis. Because it is relatively easy to conduct, non-invasive and inexpensive to perform, semen analysis (sperm count, semen volume, sperm morphology andassessments of functional parameters) is one of the first laboratory tests commonly performed for infertile couples. Studies on occupationally lead-exposed men have shown multiple sperm parameters being affected as seminal plasma or blood lead concentrations rise, usually at levels of >40 μg/dl, but sometimes even at levels of

<10 μg/dl. For instance, reductions in sperm count and sperm concentration or density (Naha *et al.,* 2005), decreased volume of ejaculation (Naha *et al.,*2005), as well as correlations with asthenospermia, hypospermia, and teratospermia (53 μg/dl) (Lancranjan *et al.,*1975) have been reported in male workers. Furthermore, higher percentages of immature and abnormal spermatozoa such as wide, round, and short sperm in lead exposed workers have been reported at both high (40 μg/dl) and low (<15 μg/dl) blood lead levels (Telisman *et al.,*2007). Many studies on reproductive system of male animals have documented lead as a toxicant for testicular tissue and functions (Hsu *et al.,* 1998) such as significant reductions in the number of spermatozoa within the epididymis in mice administered lead acetate (0.25% and 0.50%) in drinking water (Wadi *et al.,* 1999) and halted spermatogenesis in rats (Batra *et al.,* 2001). Many studies suggest spermatogenesis problems caused by lead, although, some researchers have failed to demonstrate correlations between lead and semen volume, pathologic sperm and sperm concentration among workers exposed to high lead levels (Bonde *et al.,*. 2002), or abnormalities in sperm count and/or the sperm morphology in rabbits (Willems *et al.,*. 2002).

Macroscopic changes in accessory sex organs such as diminished weight of testes, seminal vesicles, epididymis, and ventral prostate have been demonstrated in various studies using experimental animals (Ronis *et al.,*1996). Microscopic changes, histological as well as macroscopic ones, have been induced by increasing lead levels in lead exposed male rats (Adhikari *et al.,*2001) including changes in the testicular tissues morphology (Batra *et al.,*2001) and decreased germ cells layer population (Adhikari *et al.,*2001). In addition, two studies conducted on lead exposedmice demonstrated seminiferous tubule degeneration (Graca *et al.,*2004), and seminal abnormal cytology (Eyden *et al.,*1978). Similarly, electron microscopic analysis has revealed that lead-exposed monkeys, when exposed during infancy, can induce testicular alterations, which persist in later life even when blood lead concentrations had decreased considerably (Foster *et al.,* 1998). Due to ethical limitations, many studies on reproductive organs have been performed using high lead levels with experimental animals, which have revealed lead's effect at the cellular level.

Sperm functional parameters Successful fertilization of an ovum by spermatozoa depends not only on sperm count and morphology but is also relevant to functional parameters. Lead has been shown to incur detectable negative effects on blood, semen and/or spermatozoa quality

in workers, such as inducing prolonged liquefaction time and decreasing sperm motility. It has been negatively associated with sperm motility and viability (blood lead levels ≤ 10 µg/dl) (Hernandez-Ochoa *et al.,* 2005), and a reduction in the functional maturity of sperm among men with mean blood lead levels of 45µg/dl (Wildt *et al.,*1983).

On the other hand, concomitantly, significant improvements in the number of motile sperm has been reported after mean blood lead decreased from 42 µg/dl to 20 µg/dl among the lead factories workers (Viskum *et al.,*1999). Reduced semen quality such as prolonged latency of semen melting have also been reported amongst lead exposed workers, without directly measuring blood /or semen lead concentrations (Xuezhi *et al.,* 1992). As a general rule however, numerous studies have demonstrated that sperm functional disorders induced by lead, are related to the sperm's interactions with oocytes and implantation, such as premature acrosome loss (Benoff *et al.,* 2003), and strong negative correlation between seminal plasma lead levels and artificial insemination rates in humans (Benoff *et al.,* 2003). Two studies of mice and one of rats, have shown that there may be a dose-dependent decrease in the number of sperm attaching to ova (Chowdhuri *et al.,* 2001), reducing the ability of spermatozoa to penetrate the corona radiata and the zonapellucida of the oocyte (Johansson *et al.,* 1989), and an increased frequency of post-implantation loss of embryos (Al-Hakkak *et al.,* 1988) (at 0-2 µg/ml lead acetate, 40 µg/dl blood lead levels and 25-50 mg/kg in chow, respectively). According to these results, lead significantly induces the sperm function disorders in exposure cases before and after ejaculation.

- Hormonal disruption

Reproductive hormones play an important and complicated role in the regulation of spermatogenesis and sperm development. The results of experimental studies in rats have shown that the effects of lead involve multiple sites on male reproductive hormones although the most important part of these disorders probably occurs in the hypothalamic-pituitary-testosterone (HPT) axis (Ronis *et al.,* 1996). For example, depending on lead exposure levels and duration, signals within and between the rat's hypothalamus and pituitary gland appear to be disrupted by lead (Sokol *et al.,* 2002). In a study of lead-exposed rats hyper responsiveness to stimulation with both gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) was demonstrated (Sokol *et al.,* 1987). Another study on male rats administered lead

acetate in water showed a dose-related increase in GnRH mRNA and no effects on the serum concentrations of hypothalamic GnRH or LH, suggesting there may be a compensatory mechanism in the HPT axis (Sokol *et al.,* 2002). In addition to animal experiments, McGregor (1990) reported a positive correlation between serum LH levels and duration of occupational lead exposure (McGregor *et al.,* 1990), a finding which was confirmed one year later in another study of workers with mean blood lead levels of 35µg/dl (Ng *et al.,* 1991). Testosterone, the main male sex hormone, is formed and secreted by Leyding cells in testes in response to stimulation by of LH. Semen lead concentrations at a mean of 2 µg/dl have been reported to be inversely related to serum testosterone among occupationally-exposed men (Alexander *et al.,* 1998). Suppression of testicular testosterone levels and increasing steroid binding globulin levels related to increased duration of exposure to lead has been also demonstrated among mice exposed to lead for 30 days (Rodamilans *et al.,* 1988). The suppression of testosterone levels in the epididymal cells and increased androgen binding protein levels of rats have been also noted (Ronis *et al.,* 1996). However, there are some reports describing increased serum testosterone concentrations on lead exposed men from low (median 5 µg/dl) (Telisman *et al.,* 2007) to relatively high (more than 40µg/dl) blood lead levels (Gustafson *et al.,* 1989). These findings suggest that it might involve other hormonal and/or hormonal feedback pathway(s) than disruption of testosterone secretion in the reproductive hormonal axis by lead, such as a lack of reflex in response to plasma testosterone, direct inhibitory androgen biosynthesis in Leydig cells (Wiebe *et al.,* 1983), or defects in LH regulation at the pituitary level (Sokol *et al.,* 1985). Molecular mechanisms underlying histopathological nexamination have revealed disturbance degeneration in Leydig cells among rats (Saxena *et al.,* 1986), thereby suggesting Leydig cells as a target for lead intoxication.

On the other hand, due to imbalances in the HPT hormonal axis induced by lead exposure, pituitary cells release inappropriate levels of LH and change the steroid negative feedback loop (Ronis *et al.,* 1996), usually at the hypothalamus level (Grattan *et al.,* 1996). Increased concentrations of other reproductive hormones, such as follicle stimulation hormone (FSH), secreted from the pituitary gland, have been observed following lead exposure in men (Ng *et al.,* 1991) and in lead treated rats (Petrusz *et al.,* 1979). However, unchanged concentrations between workers exposed to high and low-levels lead and unmodified levels in mice treated

lead with acetate in drinking water have also been shown. These differences in FSH secretion levels might relate to differing lead levels and/or the duration of exposure among subjects (Assennato *et al.*, 1987; Pinon-Lataillade *et al.*, 1995).

On the other hand, inappropriate inhibin B overproduction in excessively lead exposed subjects may be induced by a Cell of Sertoli dysfunction, which suggests spermatogenesis impairment (Mahmoud *et al.*, 2005). Research on male monkeys has shown that alterations in Sertoli cell function may occur due to decreases in inhibin/FSH (Foster *et al.*, 1993), rather than by a direct effect on the cells. Such findings are consistent with a failure to find significant microscopic alterations in rat's Sertoli cells, except for increased lysosomal size, verified by ultrastructural examination on the rat's cells (Nathan *et al.*, 1992). Thus, the Sertoli cells may be not a direct target of lead toxicity and lead's effects on FSH disruption is the more likely cause of reproductive dysfunction rather than by a direct effect on the cells.

- Mechanisms of lead reproductive toxicity

At a conceptual level, the mechanisms of lead toxicity on male reproductive system have not yet been fully elucidated. There are a number of probable pathways to explain how lead exposure may reduce male fertility. For instance, multiple calcium and potassium channel isoforms in human testes and spermatozoa, may be involved in early events of acrosome reactions (Benoff *et al.*, 2000). In addition, some enzymes activities, such as alkaline phosphatase and sodium potassium ATPase, have been shown to be reduced in the reproductive organs of lead-exposed rats (Batra *et al.*, 2001, Saxena *et al.*, 1989). Another issue in lead's reproductive toxicity might relate to the excessive generation of Reactive Oxygen Species (ROS), an issue which has been paid more attention recently. ROS inhibits the production of sulfhydryl antioxidants, inhibits enzyme reactions, damages nucleic acids and inhibits DNA repair, as well as initiating lipid peroxidation in cellular membranes. Lead induces oxidative stress and promotes the generation of hydrogen peroxide (Vaziri *et al.*, 2007). The negative wide-ranging of effects due to an increase of ROS levels in tissues have been postulated as a major contributor of disorders related to lead exposure (Patrick *et al.*, 2006). An epidemiological study of the male reproductive system has demonstrated positive correlations between seminal plasma lead and spermatozoa ROS levels (Kiziler *et al.*, 2007).

On the other hand, in people with protracted exposure to lead, increased activity of superoxide dismutase has been observed, which suggests an adaptive mechanism against the increased amount of ROS production induced by lead (Kasperczyk *et al.*, 2004). This may result in oxidative cell in the damage in reproductive tissues closely associated with ROS production. For example, a study on rat sperm exposed to ROS in vitro has demonstrated premature acrosome reactions and reduced penetration rate in the zona-intact (Hsu *et al.,* 1999). However, from low to high doses, there are known to be different responses of lead-induced oxidative stress in various target sites, including sperm (Hsu *et al.*, 2002).

Studies on lead-exposed rats have demonstrated that lead influences sperm function, decreases serum testosterone levels and produces early onset of capacitation by activating pathways of ROS generation (Hsu *et al.,* 1998). Additional evidence where rats were chronically exposed to lead has reported an elevation in the concentration of lipid peroxide in reproductive organs (Marchlewicz *et al.,* 2007). Results of studies suggest therefore, that lead-induced ROS is an important molecular mechanism for male reproductive disorders, either in the hormonal stages or during spermatogenesis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection, Identification and Preparation of *Tithonia diversifolia*

The leaves of *Tithonia diversifolia* (sunflower) was collected from the campus of the University of Ibadan and identified at the Forestry Research Institute of Nigeria (FRIN), Ibadan with the specimen No. FHI. 110652, where a voucher specimen was also deposited. Fresh leaves of *Tithonia diversifolia* were collected and air-dried at room temperature (25 ± 5°C) under shade after which they were pulverized into powder using the laboratory mill and the powdered was stored in a container at room temperature.

3.1.1 Extraction and Partitioning of *Tithonia diversifolia* Leaf

Two (2) Kg powder of *Tithonia diversifolia* leaf was soaked with 20 L of methanol (90%) in a stoppered container for 48 hours. The suspension was then decanted and filtered several times with each addition of fresh solvent using Whatman filter paper No. 1. The filtrate collected was evaporated to dryness in rotary evaporator at 40–50°C under reduced pressure to obtain the methanol extract of *Tithonia diversifolia* leaf.

The percentage yield of extract was calculated using the formula below.

$$\% \text{ Yield} = \frac{\text{Extract yield}}{\text{Starting material}} \times 100$$

$$= \frac{255.6}{2000} \times 100$$

$$= 12.78\%$$

The fractionation of methanol extract of *T. diversifolia* leaf was done using solvent-solvent partitioning. The solvent used were, dichloromethyl, ethylacetate and water.

3.1.2 Gas Chromatography Mass Spectroscopy (GC-MS) Analysis of *Tithonia diversifolia* Leaf Extract

Gas Chromatography analyses were performed on *Tithonia diversifolia* leaf extract. Double focusing gas chromatography system fitted with two capillary columns coated with cp – sil- 5 and cp – sil – 9 (Fused silica, 25 um x 0.25 mm, 0.15 µm film thickness) and flame ionization detector (FID). The volume of *Tithonia diversifolia* leaf extract injected was 0.2 ml, and the split ratio was 1: 30. Oven temperature was programmed from 50 – 230 ° C at 3°C / min using hydrogen as the carrier gas. Injection and detector temperatures were manufactured at 200 ° C and 250 ° C, respectively. Qualitative data were obtained by electronic integration of FID area percent without the use of correction factors.

Mass spectroscopy

An agilent gas chromatography, interfaced with a VG analytical 70 – 250 S double focusing mass spectrometer was used. Helium was used as the carrier gas at 1.2 ml/min. The MS operating conditions were ionization voltage 70 ev, ion source 230 ° C. The Mass Spectrometry data were acquired and processed by on – line desktop with a computer equipped with disk memory. The percentage compositions of the *Tithonia diversifolia* leaf extract were computed in each case from Gas Chromatography peak areas. The identification of the components was based on comparison of retention indices and mass spectra with those of authentic samples and with data from literature (Adams, 2007).

Identification of Components

Interpretation of mass spectrum Gas Chromatography/Mass Spectroscopy was carried out by comparing the database peaks of National Institute Standard and Technology (NIST) library with those reported in literature (Nicolas *et al.,* 2005; Tsivou *et al.,* 2006). The spectrum of the unknown compound was compared with the spectrum of known compounds stored in the NIST library.

3.1.3 Phytochemical Screening of Methanol Extract *Tithonia diversifolia* Leaf

Phytochemical screening of methanol extract was carried out using standard procedures as described by Evans, (1999); and Edeoga *et al.,* (2005).

Test for Tannins

Dried sample (0.5g) of the extract was boiled in 20 ml of distilled water in a test tube, filtered and few drops of 0.1% ferric chloride were added. The formation of a brownish-green precipitate indicated the presence of tannins.

Test for Phlobatannins

Dried sample (0.5g) of the extract was added each into 20 ml of distilled water in a different test tube and then filtered, 2 ml of the extract was added to 2 ml of 1% hydrochloric acid and the mixture was boiled. Deposition of a red precipitate showed the presence of phlobatannins.

Test for Saponins

Two gram of plant extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for stable persistence froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously

after which it was observe for the formation of emulsion that indicated the presence of saponins.

Test for Flavonoids

Two gram of the powdered sample of *Tithonia diversifolia* leaf extract was heated with 10 ml of ethyl acetate over a steam bath for 3 mins. The mixture was filtered, and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. Yellow colorations that disappeared on standing indicated the presence of flavonoids.

Tests for Anthraquinones

Dried sample (2.0 g) of *Tithonia diversifolia* leaf extract was added to 20 ml of distilled water in a test tube and then filtered, 3 ml of the extract filtrate was boiled with 3 ml of sulphuric acid and filtered while hot. Benzene (3ml) was added to the filtrate and shaken. The benzene layer will be separated and 3ml of 10% NH_3 (ammonium) added. A pink, red or violet colouration in the ammonical (lower) phase showed the presence of anthraquinone derivatives.

Tests for Terpenoids

Dried powdered sample of *Tithonia diversifolia* leaf extract (0.5 g) and its partitioned will be added into 20ml of distilled water in a test tube and then filtered, 2 ml of the extract was dissolved in 2 ml of chloroform and evaporated to dryness. Concentrated sulphuric acid (3ml) was added to form a thin layer. A reddish brown colouration at the interface indicated positive result for terpenoids.

Tests for Sterols

Dried sample (2.0 g) of *Tithonia diversifolia* leaf extract and its partitioned was added into 20 ml of distilled water in a test tube and then filtered. Then 2 ml acetic anhydride was added to

0.5 g methanol extract of each sample with 2 ml of H_2SO_4. The colour change from violet to green showed the presence of sterols.

Test for Alkaloids

Dried sample (0.5 g) of the extract and its partitioned was added into 20 ml of distilled water in a test tube and then filtered. Then 3 ml of the extract was stirred with 3 ml of 1% HCl on a steam bath. Mayer's and Wagner's reagents were added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Test for Glycosides

The dried powdered (2.0 g) sample was boiled in 20 ml of distilled water in a test tube and then filtered, 5 ml of extract was dissolved in 2 ml of glacial acetic acid containing 1-2 drops of 2% ferric chloride ($FeCl_3$) solution. The mixture was transferred into test tube containing 2 ml of concentrated tetraoxosulphate-6-acid (H_2SO_4). A brown ring at the interface indicated the presence of a deoxy sugar, characteristic of cardenolides. A violet ring was appeared below the brown ring.

Test for Phenols

Dried sample (0.5 g) of the extract and its partitioned was added into 20 ml of distilled water in a test tube and then filtered. One ml of filtrate was treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicated the presence of phenols.

3.1.4. *In vitro* Antioxidant Activity of the Extract of *Tithonia diversifolia* leaf and its Fractions of using DPPH Free Radical Scavenging Activity

The antioxidant activity of the three fractions of the extract of *Tithonia diversifolia* leaf were determined using the 2, 2-dipheny-2-picrlhydrazyl hydrate (DPPH) according to the method of Blois (1958) and described by Brace *et al.*, (2001). One ml of 0.3mM DPPH in methanol was added to the extract and standard (trolox) in a test tube. The mixture was vortexed and then incubated in a dark chamber for 30 minutes after which the absorbance was measured at 517 nm against a DPPH control containing only 1ml of methanol in place of the extract. The antioxidant activity was then calculated using the formula:

[(Ao-Ae)/Ao] x100

Ao = Absorbance without extract

Ae = Absorbance with extract

The quantity of sample necessary to react with one half of the DPPH is expressed in terms of the relative amount of trolox reacted. Antioxidant activity of a sample is expressed in terms of micromole equivalents of trolox (TE) per 100 grams of sample, or simply trolox units per 100 gm or TE/100g.

3.2 Acute oral toxicity test in Rats

The acute toxicity of *Tithonia diversifolia* leaf extract was estimated using the newer method of fixed dose procedure (Guideline 423) of the Organisation for Economic Cooperation and Development (OECD) for testing chemicals (OECD, 2001; Botham, 2004). Female rats (150 – 180 g) were used and kept in the experimental cages for 5 days for acclimatisation to laboratory conditions. The animals were fasted for 3–4 hours prior to administration of drug.

Individual weight of the animals were determined weekly. Varying doses of *Tithonia diversifolia* leaf extract (500, 1000, 2000, and 5000 mg/kg) were given orally in accordance to the single dose toxicity test guideline for pharmaceuticals. Rats were maintained for observation for 72 hours (acute) for any toxic symptom and 14 days (sub-acute) for mortality rate (Behrens and Karber, 1953). The number of deaths in each group within 24 hours was recorded and various signs of toxicity exhibited by the rats were noted. The oral LD_{50} of *Tithonia diversifolia* leaf extract was estimated to be greater than 5000 mg/kg.

3.3 Animals

A total of fifty (50) adult male Wistar rats weighing between 120-150 g were used for the study. The rats were purchased from the Central Animal House of the Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan. The rats were randomly divided into ten (10) groups, with five (5) rats per group. They were fed with vita feed rat chow diet purchased at Orogun, Ibadan, Oyo state and water was given *ad-libitum* during the 14 days of acclimatization before the start of experiment. The experiments were conducted in conformity with guiding principles in the care and use of laboratory animals approved by the council of America Physiological Society.

Chemicals used: Analytical grade Phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) and solvent (methanol, ethylacetate and dichloromethyl) were purchased from Sigma Aldrich, Germany.

3.4 Experimental Design

3.4.1 Animal Grouping and Experimental Protocol

Study 1: Effects of methanol extract of Tithonia diversifolia leaf on lead acetate induced reproductive toxicity in male Wistar rats

The animals were treated as shown below:

Group 1: Control animals were given 0.9 % normal saline orally for 56 days.

Group 2: Animals were administered with 15 mg/Kg body weight of Lead acetate intraperitonially/week.

Group 3: Animals were administered with 50 mg/Kg body weight of methanol extract of *T. diversifolia* leaf orally for 56 days.

Group 4: Animals were administered with 15 mg/Kg body weight of Lead acetate+ 50 mg/Kg body weight of methanol extract of *T. diversifolia* leaf orally for 56 days.

Group 5: Animals were administered with 100 mg/Kg body weight of methanol extract of *T. diversifolia* leaf orally for 56 days.

Group 6: Animals were administered with 15 mg/Kg body weight of Lead acetate+100 mg/Kg body weight of methanol extract of *T. diversifolia* leaf orally for 56 days.

Study 2: Effects of phytol on lead acetate induced reproductive toxicity in male Wistar rats

Group 1: Control animals were given olive oil orally for 56 days.

Group 2: Animals were administered with 15 mg/Kg body weight of Lead acetate intraperitonially/week.

Group 3: Animals were administered with 50 mg/Kg body weight of phytol orally for 56 days.

Group 4: Animals were administered with 15 mg/Kg body weight of Lead acetate+50 mg/Kg body weight of phytol orally for 56 days.

3.4.2 Collection and preparation of samples

Blood samples

Twenty four hours after the last administration of the extract, chemical and normal saline, all rats were weighed. Animals were anesthetized by thiopental (50 mg/kg I.P) (Pereda *et al.*, 2006) and blood sample was collected from the heart through cardiac puncture, allowed to clot, then centrifuged at 3000 rpm for 10 minutes for separation of serum. Serum was used for biochemical and hormonal studies. Blood samples kept in EDTA bottles were used for analysis.

Sperm Samples

Sperm samples were collected from caudal epididymis and used for sperm analysis.

Organ Specimens

Organs of interest like testis, epididymis, seminal vesicle, heart, kidney and liver were collected, cleared of adherent tissues and weighed immediately with an electronic weighing balance, model DT 1000 England with a capacity of 0.1 to 1000 gram. Testes, liver and hypothalamus were perfused with PBS (Phosphate buffer saline), collected in clean dry plastic bags and kept at -40ºC for the determination of lipid peroxidation (TBARS) and antioxidant

status. Specimen from testes, epididymis, liver and hypothalamus were fixed in bouin's fluid and 10 % neutral buffer formalin and were used for the histopathological study.

3.5 Determination of Haematological Indices

3.5.1 Determination of Packed Cell Volume (PCV)

The micro capillary tube method was employed (England *et al.,* 1972). The blood was drawn into the capillary tube by capillary attraction until the tube was about three–quarters filled with blood. The flame of the Bunsen burner was used to seal off the end free of blood. These sealed tubes were centrifuged for 5 minutes at 3,000 r.p.m. The haematocrit was then read off directly using the micro–haematocrits reader

3.5.2 Determination of Haemoglobin (Hb) Concentration

The sahli's method was used to estimate the haemoglobin concentration and it involves converting a known volume of blood to acid haematin and diluting until the colour of the solution matches that of the standard provided. The equivalent of 100 % on the sahli scale (X) is engraved on the stand carrying the tube (Gibson and Harrison, 1945). The dilution tube was filled to the mark 10 with freshly prepared 0.1 NHCl, 20 mm^3 of blood was sucked into the pipette, and the outside of the pipette was wiped. This was introduced into the dilution tube and was blown 2 or 3 times to mix thoroughly. Then, it was allowed to stand for 5 minutes and acid haematin is formed. Distilled water was added drop by drop, stirring with the glass rod between each addition. The test and standard tubes were compared against bright diffuse daylight and also while holding them against a sheet of white paper. The distilled water was added until the colour in the diluting tube was just slightly darker than that of the standard.

The reading was taken. More distilled water was added until the colour is just lighter than that of the standard. The second reading was taken. The average of the two readings was estimated (Y).

3.5.3 Estimation of Red Blood Cell (RBC) Count

Visual method using improved Neubauer counting chamber was used (Rowan, 1983) in the estimation of red blood cell count. The method is based on counting the number of cells in a known small volume of accurately diluted blood. The blood is diluted in the Haymen's solution in a red cell pipette and the red blood corpuscles in a small volume of the dilute blood are counted in a counting chamber.

Blood was drawn into the red cell pipette and allowed to fill to 0.5 marks. The pipette was drawn and kept horizontal to prevent blood shifting. The outside of the pipette was wiped with filter paper. Haymen's solution was drawn into the pipette to the 101 mark, being careful not to overshoot. The pipette was placed in a horizontal position and the rubber tubing was removed. The pipette was rotated between the finger and thumb for about 1 minute to thoroughly mix blood with the diluting fluid in the bulb of the pipette. The counting chamber and cover slip were cleaned. The tip of the pipette was brought in contact with the exposed part of counting chamber and the pipette was raised vertically until diluted blood flowed under the cover slip mounted on the haemocytometer (counting chamber). The cover slip was slipped into the final position on the chamber. The slide was placed on the horizontal stage of the microscope for counting. The number of cells in each of 80 small squares that is, 5 sets of small squares were counted. All cells overlapping the top and left hand sides of a square were counted while those overlapping the bottom and right hand side are not counted.

3.5.4 Estimation of Total White Blood Cell count

The principle for the white blood cell count is the same as the principle of the red blood cell count (Visual method) but only with few differences (Becton-Dickson, 1998). The WBC pipette is marked 1 below and 11 above its bulb; it also contains a white bead. The blood was drawn to the mark of the stem corresponding to 0.5. The diluting fluid was 2 % acetic acid with a dye, methyl violet. The acid destroyed the red cell membrane so that red cells were not seen making the white cells more prominent. The dye stained the white cell nuclei. The small squares of the Thoma ruling were not used for counting the WBC, as these were too scarce. Instead, all the WBC lying on the entire square area were counted including those on the triple rulings forming the boundaries of the large squares using the microscope (x40) to identify the white cells.

3.5.5 Estimation of Differential White Blood Cell Count

Leishman's stain method was used (Bessman, 1986). Two clean dry thin slides were used. The blood was collected by one end of a slide and was quickly placed on the bench, held in position with the thumb and index finger of the left hand. The narrow end of the second slide was placed in the drop and held there until the blood has spread across it. It was then drawn slowly over the whole length of the first slide, being held at an angle 45 º. There was no pressure whatsoever between the two surfaces. After the blood has spread, it was air dried to prevent shrinkage of the cells. It was the fixed with ethanol and stained with Leishman's stain. It was allowed for 10–15 minutes before washing off and allowed to dry. On microscopic examination, the slide was mounted using oil immersion and was viewed under microscope

(X100). A total of 100 white blood cells were counted and they were differentiated into various white blood cell types.

3.5.6 Estimation of Platelet Count

The EDTA blood was allowed to stand at room temperature until a few millimetre of plasma is visible. A dilution of 1 to 10 (1:10) of the plasma was made by adding 0.1 ml of plasma to 0.9 ml of 1 % aqueous ammonium oxalate. It was gently rotated for about 1 minute and then filled into the counting chamber; this preparation was allowed to stand for 30 minutes just to allow all the platelets to settle before counting. The result was adjusted by multiplying with (1- PCV) which allows for the fact that the count was carried out in plasma (Brecher and Cronkite, 1950; Bull *et al.,* 1965).

3.6 Oxidant and Antioxidant enzymes assays

3.6.1 Estimation of Malondialdehyde Levels in Tissue Homogenate

Principle

Malondialdehyde level was estimated by the method described by Kartha and KrishnaMurthy (1978).This was measured as an indicator of lipid peroxidation and by extension reactive oxygen species. Testes and epididymal homogenate prepared were placed in micro-centrifuge tube and incubated with thiobarbituric acid (TBA). Following the incubation, samples were centrifuged (2000 rpm, 10 min) and the absorbance of the pink clear supernatant was measured at 532 nm in duplicate samples. Malondialdehyde bis (dimethyl acetate) was used as the external standard. Thiobabutric acid reactive material was expressed in terms of nanomoles of MDA /gm wet tissue. Lipid peroxidation was determined by measuring the

thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation. This was carried out by the method of Varshney and Kale (1990).

3.6.2 Determination of serum and tissue Superoxide Dismutase Activities

Superoxide Dismutase **(**SOD) was estimated by the technique explained by Fridovich (Beauchamp and Fridovich, 1971). The activity was expressed as unit/mg protein. The level of SOD activity was determined by the method of Misra and Fridovich, (1972).

Principle for the determination of tissue Superoxide Dismutase Activities

The ability of superoxide dismutase to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for this dismutase. Superoxide ($O_2^{\bullet -}$) radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per $O_2^{\bullet -}$ introduced increased with increasing pH (Valerino and McCormack, 1971) and also increased with increasing concentration of epinephrine. These results led to the proposal that autoxidation of epinephrine proceeds by at least two distinct pathways, only one of which is a free radical chain reaction involving superoxide ($O_2^{\bullet -}$) radical and hence inhabitable by superoxide dismutase.

Protocol for the determination of serum and tissue Superoxide Dismutase Activities

1 ml of sample was diluted in 9 ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2 ml of the diluted sample was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of

freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds.

Calculation

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

Where A_0 = absorbance after 30 seconds

A_3 = absorbance after 150 seconds

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

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

3.6.3 Determination of Serum and Tissue Catalase Activities

Catalase (CAT) activity was determined according to the method of Sinha, 1971.

Principle for the determination of serum and tissue catalase activities

This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchromic acid as an unstable intermediate. The chromic acetate then produced is measured colorimetrically at 570 – 610 nm. Since dichromate has no absorbency in this region, the presence of the compound in the assay mixture does not interfere at all with the colorimetric determination of chromic acetate.

The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at a particular time by the addition of dichromate/acetic acid mixture and the remaining H_2 is determined by measuring chromic acetate colorimetrically after heating the reaction mixture.

Colorimetric Determination of H_2O_2

Different amount of H_2O_2, ranging from 10 to 100 µmoles was taken in small test tubes and 2 ml of dichromate/acetic acid was added to each. Addition of the reagent instantaneously produced an unstable blue precipitate of perchromic acid. Subsequent heating for 10 minutes in a boiling water bath changed the colour of the solution to stable green due to formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3 ml and the optical density measured with a spectrophotometer at 570 nm. The concentrations of the standard were plotted against the absorbance.

Table 3.1. Protocol for the estimation of hydrogen peroxide/Catalase Standard Curve

Test tube	1	2	3	4	5	6	7
H_2O_2 (ml)	0.05	0.10	0.15	0.20	0.30	0.40	0.50
Dichromate/acetic acid (ml)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Distilled water (ml)	0.95	0.90	0.85	0.80	0.70	0.60	0.50
H_2O_2 concentration (µmoles)	10	20	30	40	60	80	100
Absorbance (570nm)	0.049	0.095	0.145	0.195	0.291	0.385	0.484

Determination of catalase activity of samples

1 ml of supernatant fraction of the testicular, liver or hypothalamic homogenate was mixed with 19 ml distilled water to give a 1:20 dilution. The assay mixture contained 4 ml of H_2O_2 solution (800 µmoles) and 5 ml of phosphate buffer, pH 7.0 in a 10 ml flat bottom flask. 1 ml of properly diluted sample was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. 1 ml portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide contents of the withdrawn sample were determined by the method described above.

Calculation

Catalase activity was calculated by plotting the standard curve and the concentration of the remaining H_2O_2 was extrapolated from the curve.

H_2O_2 consumed = 800 µmoles – H_2O_2 remaining

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

3.7 Sperm Analysis

3.7.1 Sperm Count, Motility, and Kinetics

Sperm count, motility, and kinetics assessment were done immediately following anaesthetized the animals. The right epididymis was immediately excised with care to minimise blood contamination and placed into a pre-warmed (37 ^{0}C) Petri dish containing two mL of a phosphate buffer saline solution (pH 7.4). The caudal portion was punctured twice with the tip of a scalpel blade to release sperm, commencing a 3-minute "swim-out" period. After the swim-out, the dish was gently swirled, and a 9 µL sample from a relatively dense portion of the sperm cloud was placed onto counting chamber. The sperm samples were analysed with the aid of computer-aided sperm analyser (CASA, JH-6004 Sperm Quality Analyser). The spermatozoa were assessed for total sperm detected, sperm count, total motile

sperm, progressive, non-progressive and immotility, average path velocity, curvilinear velocity, straight line velocity, amplitude of lateral head, beat cross frequency, percent of line moving, linearity, straightness, wobble and mean move angle degree (Tannenbaum *et al.,* 2003; WHO, 2010).

3.7.2 Sperm Viability and Morphology Analysis

A drop of epididymal content of each rat was mixed with an equal drop of eosin nigrosine stain prepared in accordance with Barth and Oko (1994). Thin films were made by spreading the stained content onto clean slides and quickly dried. This was used for sperm viability and morphology. Viable sperm remains colourless. One hundred sperm cells per rat were scored for determining the viability percent.

3.8 Estimation of Serum Sex Hormones Level

3.8.1 Estimation of Serum Gonadotropin Releasing Hormone

Principle

This ELISA kit used Competitive-ELISA as the method. The micro ELISA plate provided has been pre-coated with GnRH. During the reaction, GnRH in the sample or standard competed with a fixed amount of GnRH on the solid phase supporter for sites on the Biotinylated Detection Ab specific to GnRH. Excess conjugate and unbound sample or standard were washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then the Substrate Reagent was added to each well. The enzyme-substrate reaction was terminated by adding Stop Solution and the color change can be measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentration of GnRH in samples were calculated by comparing the OD of the samples with the standard curve.

3.8.2 Estimation of Serum Testosterone Level

Blood samples were spin at 2500 rpm for 10 minutes in a table top centrifuge. The serum samples obtained were analysed to determine the concentration of testosterone level. The analysis was carried via the tube-based enzyme immunoassay (EIA) method. The protocol used for the hormone was according to the method described for the kit (Immunometrics Limited UK) and meet the WHO standards in research programme for reproductive study. Testosterone–HRP conjugate for a constant amount of rabbit anti–testosterone. In the incubation, goat anti–rabbit IgG–coated wells are incubated with 10 µl of testosterone standards, controls, animal samples, 100 µl testosterone–HRP conjugate reagent and 50 µl rabbit anti–testosterone reagent at 37 ºC for 90 minutes. During the incubation, a fixed amount of HRP–labelled testosterone competes with the endogenous testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific testosterone antibody. Thus, the amount of testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of testosterone in the specimen increases. Unbound testosterone peroxidase conjugate is then removed and the wells washed. Next, a solution of TMB reagent is then added and incubated at room temperature for 20 minutes, resulting in the development of blue colour. The colour development is stopped with the addition of 1N HCl, and the absorbance is measured spectrophotometrically at 450 nm. The intensity of the colour formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled testosterone in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The testosterone concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve.

3.8.3 Estimation of serum luteinizing hormone

The luteinizing hormone analysis is based on the principle of a solid phase enzyme–linked immunosorbent assay. The assay system utilizes anti–LH antibody for solid phase (microtiter wells) immobilization and a mouse monoclonal anti–LH antibody in the antibody enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in LH molecules being sandwiched between the solid phase and enzyme–linked antibodies. After 60 minute incubation at room temperature, the wells are washed with water to remove unbound labelled antibodies. A solution of TMB is added and incubated for20 minutes, resulting in the development of a blue colour. The colour development is stopped with the addition of 2N HCl, and the colour is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of LH is directly proportional to the colour intensity of the test sample (Shome and Parlow, 1974).

3.8.4 Estimation of follicle stimulating hormone

The follicle stimulating hormone analysis is based on the principle of a solid phase enzyme–linked immunosorbent assay. The assay system utilises a polyclonal anti–FSH antibody for solid phase (microtiter wells) immobilization and a mouse monoclonal anti–FSH antibody in the antibody enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between the solid phase and enzyme–linked antibodies. After 60 minute incubation at room temperature, the wells were washed with water to remove unbound labelled antibodies. A solution of TMB was added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is stopped with the addition of 2N HCl, and the colour

changed to yellow and measured spectrophotometrically at 450 nm. The concentration of FSH is directly proportional to the colour intensity of the test sample (Abraham, 1981).

3.9 Estimation of Serum Nitrotyrosine

This ELISA kit uses Competitive-ELISA as the method. The micro ELISA plate provided has been pre-coated with 3-NT. During the reaction, 3-NT in the sample or standard competes with a fixed amount of 3-NT on the solid phase supporter for sites on the Biotinylated Detection Ab specific to 3-NT. Excess conjugate and unbound sample or standard were washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then the Substrate Reagent is added to each well. The enzyme-substrate reaction is terminated by adding Stop Solution and the colour change can be measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of 3-NT in samples can be calculated by comparing the OD of the samples with the standard curve.

3.10. Estimation of Serum Tumour Necrotic Factor-alpha in Rats

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided has been pre-coated with an antibody specific to Rat tumour necrotic factor-alpha (TNF-α). Standards or samples were added to appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibodies specific for Rat TNF-α and Avidin Horseradish Peroxidase (HRP) conjugate were added to each micro plate well successively and incubated. After incubation, free components were washed away. Then the Substrate Reagent was added to each well, only those wells that contain Rat TNF-α, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in colour. The

enzyme substrate reaction was terminated by adding Stop Solution and appears yellow in colour. The optical density (OD) was measured with spectrophotometry at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat TNF- α. The concentration of Rat TNF- α in samples were calculated by comparing the OD of the samples with the standard curve.

3.10.2 Estimation of Serum Interleukin-1 Beta

This ELISA kit used Sandwich-ELISA as the method. The micro ELISA plate provided has been pre-coated with an antibody specific to rat interleukin-1 beta (IL-1ß). Standards or samples were added to appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibodies specific for rat IL-1ß and Avidin Horseradish Peroxidase (HRP) conjugate were added to each micro plate well successively and incubated. After incubation, free components were washed away. Then the Substrate Reagent was added to each well, only those wells that contain rat IL-1ß, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in colour. The enzyme substrate reaction was terminated by adding Stop Solution and appears yellow in colour. The optical density (OD) was measured with spectrophotometry at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat IL-1ß. The concentration of Rat IL-1ß in samples was calculated by comparing the OD of the samples with the standard curve.

3.11 Histological analysis

Animals were decapitated and dissected immediately after 56 days of administration of treatments, testis, epidydimis, liver and hypothalamus were removed from treated and control

groups. Reproductive organs were fixed in Bouin's solution while other organs were fixed in 10% formalin. The following processes were then carried out sequentially.

Grossing; The tissues were observed and cut into small pieces of not more than 4mm thick into pre-labelled cassettes. These were further immersed in 10% formalin saline for 24 hours to fix.

Tissue processing; this is done automatically using automatic tissue processor (leica tp1020). The tissue were allowed to pass through various reagents including; station one containing 105 formalin saline, station 3 to station 7; alcohol (70%, 80%, 90%, 95%,absolute 1 & absolute 11) for the purpose of dehydration. The tissues continued to pass through station 8 and station 9 containing two changes of xylene for the purpose of clearing and finally transferred into three wax baths for infiltration/impregnation. The machine has been programmed to run for 12 hours, tissues stayed in each station for 1hour.

Embedding; each processed tissue was given a solid support medium (paraffin wax) and this is done using a semi-automatic tissue embedding center. The molten paraffin wax was dispensed into a metal mold and the tissue was buried and oriented in it, a pre labeled cassettes was placed on this and are transferred to a cold plate to solidify.

The tissue block formed was separated from the mold.

Microtomy: the blocks were trimmed to expose the tissue surface using a rotary microtome at 6 micrometer. The surfaces were allowed to on ice before sectioning.

The tissues were sectioned at 4 micrometer (ribbon section)

Floating; the sections were floated on water bath set at 55^0C and these were picked using clean slides. The slides were labelled.

Drying; the slides are dried on a hotplate (raymonlamb) set at 60^0C for 1hour.

Staining; the staining technique used is haematoxylin and eosin technique

3.12 Statistical analysis

The mean values of the measured variables were determined and values were expressed as Mean±Standard Error of Mean (SEM). Analysis of Variance (ANOVA) was used for the analysis. $P<0.05$ was considered to be statistically significant. Graphpad prism5 statistical package was used.

CHAPTER FOUR

4.0 RESULTS

4.1. Phytochemical Screening of Methanol Extract of *Tithonia diversifolia* Leaf

The result of phytochemical screening of the methanol extract of *Tithonia diversifolia* leaf. The results showed the presence of phenol, flavonoids, terpenoids, saponins, glycosides, phylobatannins, resins and tannins while steroids and reducing sugar were absent (Table 4.1).

Table 4.1: Phytochemical screening of methanol extract of *Tithonia diversifolia* leaf

S/N	**Chemicals**	**Present/Absent**
1	Saponins	+
2	Flavonoids	+
3	Tannins	+
4	Phylobatannins	+
5	Steroids	-
6	Cardiac glycosides	-
7	Alkaloids	+
8	Reducing sugar(Carbohydrates)	-
9	Phenol	+
10	Terpenoids	+
11	Glycosides	+
12	Resins	+

+ Present

- Absent

4.2. Characterisation of methanol extracts *Tithonia diversifolia* by Gas Chromatography-Mass Sectroscopy (GCMS) Study

The Gas Chromatography-Mass Sectroscopy GC-MS) of the methanol extract showed that it contained twenty (20) compounds (Figure 4.1 and Table 4.2). The result showed the presence of **3,7,11,15-Tetramethyl-2-hexadecen-1-ol** (phytol) as antioxidant with the highest percentage of 14.67%.

Table 4.2. GC-MS analysis of methanol extract of *Tithonia diversifolia* leaf used in this study

NO	RT	Name of the compound	Molecular Formula	MW	Peak Area %	Compound Nature	Activity
1	5.59	4-Methyl-2-pentyne	C6H10	82	0.71		Antitumor activity
2	9.09	1,2-cyclopentanedione	C5H6O2	126	1.42	Aliphatic	No activity
3	9.32	Phosphoric acid, trimethyl ester	C3H9O4P	140	2.43	Diprotic Acid	Acidic properties
4.	12.76	Phenol	C6H6O	94	6.40	Phenolic Compound	Antioxidant, Antibacterial
5.	14.52	Phenol, 2-methoxy-	C10H12O3	164	1.45	Phenolic Compound	Pheromones precursor
6.	15.26	2-Furanmethanol	C5H6O2	98	1.03	Furan alcohol	Antioxidant
7.	21.12	Benzofuran, 2,3-dihydro-	C8H8O	120	1.01	Benzofuran	Antimicrobial
8.	22.58	2-Methoxy-4-vinylphenol	C9H10O3	150	2.47	Phenolic Compound	Antioxidant, antiinflamatory
9.	29.36	Phenol, 2, 4bis (1,1-dimethylethyl)	C14H22O	206	0.75	Phenolic Compound	Antioxidant
10	30.56	Cis-5,8,11-Eicosatrienoicacid	$C20H34O_2$	320	1.77	Trienoic acid	Anti-inlammatory
11.	30.88	2-Cyclopenten-1-one, 3,4-dimethyl-	C7H10O	110	3.25	Carbonyl compound	No activity
12.	37.49	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-,(1.alpha.,2.beta.,5.alpha.)	C10H18	138	17.05	Sidyltransferases	Anticancer activity
13.	**37.97**	**3,7,11,15-Tetramethyl-2-hexadecen-1-ol**	**C20H40O**	**296**	**14.67**	**Terpene alcohol**	**Antioxidant, antiinflammator**
14.	38.25	Phthalic acid, 2-(2-fluorophenyl) ethyl tridecyl ester	C25H38O4	402	2.12	Phthalic acids	Endocrine disruptors
15.	38.40	Methyl palmitate	C17H34O2	270	5.47	Fatty acid methylesters	Acaricidal activity, anti-inflammatory
16.	39.62	9,12-Octadecadienoic acid, (Z,Z)- methyl ester	C18H32O2	280	2.29	Linoleic acid	Antiinflammatory, hypocholesterolemia,
17.	39.67	9,12,15-Octadecatrienoic acid methyl ester, (Z,Z,Z)-	C19H32O2	292	3.85	Fatty acid methyl ester	Antimalarial, antioxidant
18.	41.49	9-Propanoyl-1,2,3,4,5,6,7,8-octahydrophenathrene	C17H22O	242	5.79	Acid Halides	Respiratory Toxicity

19	42.13	Phthalic acid, dodecyl 2-ethylhexy 1 ester	C16H22O4	278	2.62	Phthalic acids	Endocrine disruptors
20	43.04	Borane, diethyl [1-ethyl-2-(trimethylstannyl)-1-propenyl]-	C12H27BSn	300	23.45	Boranes	antineoplastic,anti viral, hypolipidemic

4.3. Antioxidant Activities of Different Fractions of Methanol Extract of *Tithonia diversifolia* Leaf using 2,2-dipheny-2-picrlhyrazyl Hydrate (DPPH) Radical Scavenging Assay

Antioxidant activities of dichloro methyl (DCM), aqueous and ethyl acetate fractions of *T. diversifolia* leaf were examined. The DCM fraction has the highest DPPH median inhibitory concentration (IC_{50}) when compared with the standard. The DCM fraction therefore has the highest antioxidant activities (Figure 4.2).

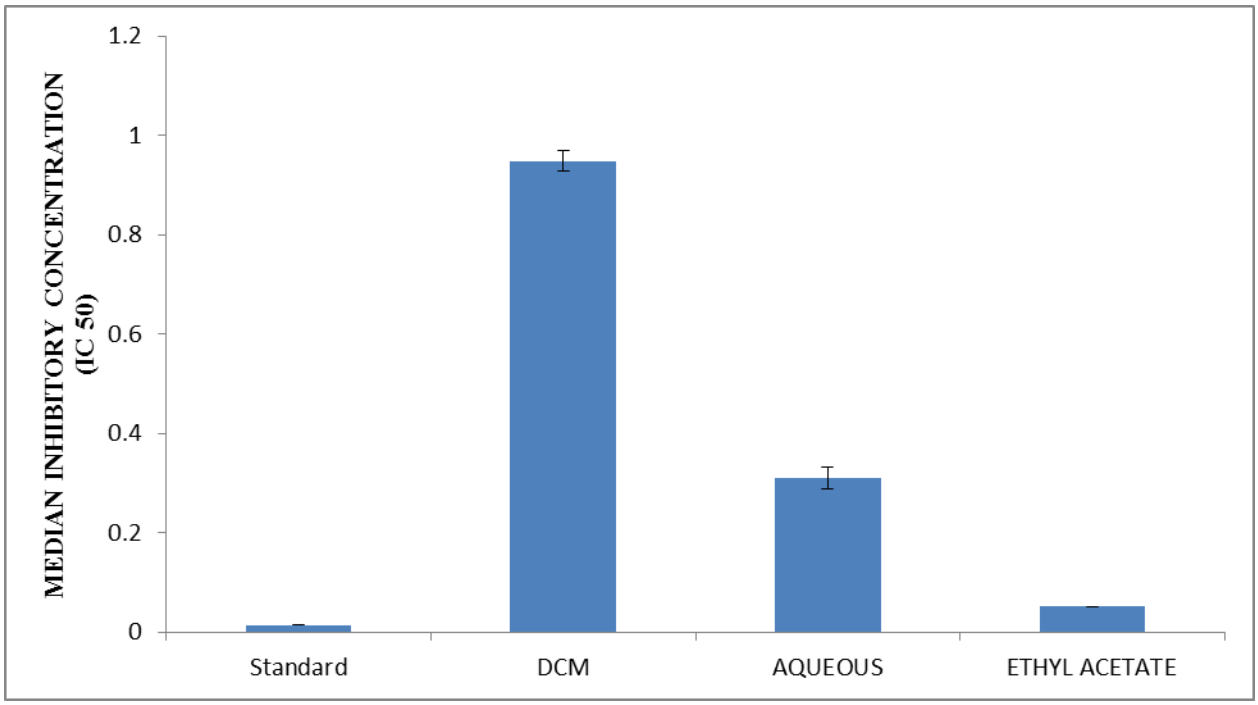


Figure 4.1. Antioxidant activities of different fraction of *T. diversifolia* plant using DPPH radical scavenging assay

4.4. The Acute Oral Toxicity Results of *T. diversifolia* Leaf

At up to 5,000 mg/kg body weight, the rats did not produce any mortality. This shows that acute oral toxicity of methanol extract of *T. diversifolia* leaf is more than 5,000 mg/kg and has a wider marging of safety (Table 4.3).

Table 4.3. Acute Oral Toxicity of methanol extract of *T. diversifolia* leaf

Treatment Group	Survival (%)	Death (%)
500 mg/kg	100	0
1000 mg/kg	100	0
2000 mg/kg	100	0
5000 mg/kg	100	0

4.5. Effects of Oral Administration of Methanol Extract of *Tithonia diversifolia* Leaf on Weekly Percentage Body Weight Changes of Lead Acetate Treated Wistar Rats

There were no significant changes in body weight of all the treated groups (PbA, 50 mg/kg TD, 50 mg/kg TD +PbA, 100 mg/kg TD, 100 mg/kg TD + PbA) compared to the control (Table 4.4).

Table 4.4: Effects of oral administration of methanol extract of *Tithonia diversifolia* leaf (TD) on percentage body weight changes of lead acetate (PbA) treated Wistar rats.

Groups	Control	PbA	50mg/kg TD	50mg/kg TD + PbA	100mg/kg TD	100mg/kg TD + PbA
Week One (%)	11.00±1.00	12.50±0.50	13.50±1.50	12.50±0.50	10.50±1.50	11.60±2.04
Week Two (%)	9.00±1.00	9.50±0.50	7.50±0.50	9.00±1.00	7.50 ±0.50	7.80± 2.52`
Week Three (%)	11.00±1.00	9.00±0.05	8.50±0.50	11.00±1.00	7.50±0.50	8.50 ± 1.48
Week Four (%)	7.50±0.50	7.00±0.50	8.00±0.50	7.50±1.00	8.50±1.00	9.40 ± 2.58
Week Five (%)	3.38±0.80	5.20±1.24	4.83±1.68	3.00±1.21	5.20±1.18	4.63 ± 1.28
Week Six (%)	6.20±2.78	5.00±1.95	4.17±1.62	6.00±1.48	5.00±1.64	5.20 ± 0.98
Week Seven (%)	5.50±1.57	4.67±1.52	5.20±1.28	4.50±1.30	5.40±1.58	4.50±1.50
Week Eight (%)	3.40±1.44	4.27±1.22	3.20±1.20	4.20±1.20	4.50±1.50	3.17±1.54

Values are presented as Mean ± SEM, n=5

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.6. Effects of Oral Administration of Methanol Extract of *Tithonia diversifolia* Leaf on Visceral Organs weight of Lead Acetate Treated Wistar Rats

The study showed significant ($p<0.05$) increase in the weights of the liver, kidney heart and spleen of PbA treated groups compared to the control. There was a significant ($p<0.05$) decrease in the weight of 50 mg/kg TD and 100 mg/kg TD treated groups when compared with PbA group but no significant difference when compared with the control in all organs weighed. Groups treated with 50 mg/kg TD +PbA and 100 mg/kg TD + PbA showed significant ($p<0.05$) decrease in the weight of the liver, kidney and spleen when compared with the PbA treated group (Table 4.5).

Table 4.5: Effects of oral administration of methanol extract of *Tithonia diversifolia* leaf on visceral organ weight of lead acetate treated Wistar rats

	LIVER (g)	KIDNEY(g)	HEART(g)	SPLEEN(g)
CONTROL	2.912 ± 0.130	0.605 ± 0.038	0.337 ± 0.019	0.267 ± 0.015
PbA	4.074 ± 0.340[a]	0.845 ± 0.044[a]	0.387 ± 0.034[a]	0.434 ± 0.033[a]
50 mg/kg TD	3.122 ±0.136[b]	0.586 ± 0.025[b]	0.314 ± 0.020[b]	0.248 ± 0.020[b]
50 mg/kg TD +PbA	3.678 ± 0.187[ab]	0.812 ± 0.023[a]	0.351 ± 0.025	0.423 ± 0.024[a]
100 mg/kg TD	3.120 ± 0.135[b]	0.618 ± 0.031[b]	0.355 ± 0.023	0.270 ± 0.025[b]
100 mg/kg TD + PbA	3.478 ± 0.264[b]	0.768 ±0.043[ab]	0.356 ± 0.023	0.475 ± 0.027[a]

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.7. Effects of oral administration of Methanol extract of *T. diversifolia* leaf (TD) for 56 days on liver malondialdehyde (MDA) and antioxidants level in control and lead acetate (PbA) treated groups.

The study showed a significant ($p<0.05$) increase in MDA level in the group treated with 15 mg/kg of lead acetate (PbA) when compared with control (Table 4.6) whereas a significant ($p<0.05$) decrease was also observed in the level of liver MDA in the group pre-treated with 15 mg/kg of lead acetate and TD (50 mg/kg and 100 mg/kg) when compared with the lead acetate group. However, a significant ($p<0.05$) decreased in antioxidant activity of superoxide dismutase (SOD) and catalase were observed in the group treated with lead acetate (PbA) compared to control whereas in the group pre-treated with 15 mg/kg of lead acetate and TD (50 mg/kg and 100 mg/kg) showed a significant ($p<0.05$) increase in the antioxidant activity of SOD and catalase when compared. Groups treated with TD (50 mg/kg and 100 mg/kg) treated groups showed no significant ($p<0.05$) difference in the antioxidant activity of SOD and catalase when compared with control (Table 4.6).

Table 4.6: Effects of oral administration of methanol extract of *T. diversifolia* leaf (TD) on liver malondialdehyde (MDA) and antioxidants level in control and lead acetate (PbA) treated groups.

	MDA (µm/mg tissue $x10^3$)	SOD (µm/mg tissue)	CATALASE(µm/mg tissue)
CONTROL	2.5 ± 0.94	20.21 ± 1.34	2.21 ± 0.18
PbA	7.2 ± 0.42[a]	14.42 ± 1.29 [a]	0.58 ± 0.007 [a]
50 mg/kg TD	2.5 ± 0.58[b]	21.54 ± 1.22 [b]	2.23 ± 0.17 [b]
50 mg/kg TD +PbA	3.4 ± 0.12 [b]	21.12 ± 1.43 [b]	2.25 ± 0.16 [b]
100 mg/kg TD	2.6 ± 0.15 [b]	22.72 ± 1.46 [b]	2.73 ± 0.19 [b]
100 mg/kg TD +PbA	3.9 ± 0.21 [b]	22.92 ± 1.23 [b]	1.35 ± 0.19 [b]

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.8. Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on liver enzymes in control and lead acetate (Pb) treated groups.

In this study, the level of liver enzymes ALT, AST, ALP and bilirubin in the serum obtained were significantly ($p<0.05$) increased in lead acetate (PbA) treated group when compared with the control (Table 4.7). The groups pre-treated with 15 mg/kg of lead acetate and TD (50 mg/kg and 100 mg/kg) showed a significant ($p<0.05$) decrease in the level of ALT, AST, ALP and bilirubin when compared with PbA group. *T. diversifolia* (50mg/kg and 100mg/kg) treated groups showed no significant ($p<0.05$) difference when compared with the control (Table 4.7).

Table 4.7. Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on liver enzymes in control and lead acetate (PbA) treated groups.

	ALT (U/L)	AST (U/L)	ALP (IU/L)	BILIRUBIN(U/L)
CONTROL	21.48 ± 1.06	34.25 ± 1.49	34.27 ± 1.63	1.69 ± 0.17
PbA	43.56 ± 1.49 [a]	59.55 ± 2.52 [a]	67.38 ± 1.74 [a]	3.96 ± 0.10 [a]
50 mg/kg TD	20.64 ±1.27 [b]	33.35 ± 0.72 [b]	35.89 ± 2.10 [b]	1.67 ± 0.04 [b]
50 mg/kg TD +PbA	27.74 ± 1.99 [b]	38.75 ± 2.52 [b]	38.37 ± 1.75 [b]	1.92 ± 0.14 [b]
100 mg/kg TD	21.74 ± 1.38 [b]	33.35 ± 1.40 [b]	33.52 ± 2.43 [b]	1.68 ± 0.14 [b]
100 mg/kg TD + PbA	23.00 ± 0.86 [b]	43.15 ± 2.06 [b]	40.89 ± 1.93 [b]	2.30 ± 0.12 [b]

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.9: Effects of Oral Administration of Methanol Extract of *T. diversifolia* Leaf on Histology of the Lead Acetate Treated Wistar Rat's Liver

The histology of the liver showing normal architecture in control, 50 mg/Kg TD and 100 mg/Kg TD groups. Their central venules and portal tracts appear normal (white thick arrows), the hepatocytes morphology were normal (blue thick arrows) and the sinusoids appearred normal (back slender arrow). In 50 mg/Kg TD+PbA and 100 mg/Kg TD+PbA groups, there were mild infilteration of inflammatory cells and moderate congestion of central venules. The liver histology of the lead acetate group shows severe haemorrhage within the parenchyma, vessels are congested (black thick arrows), there are sinusoids with lysed cells (black slender arrow) (Plate 4.1).

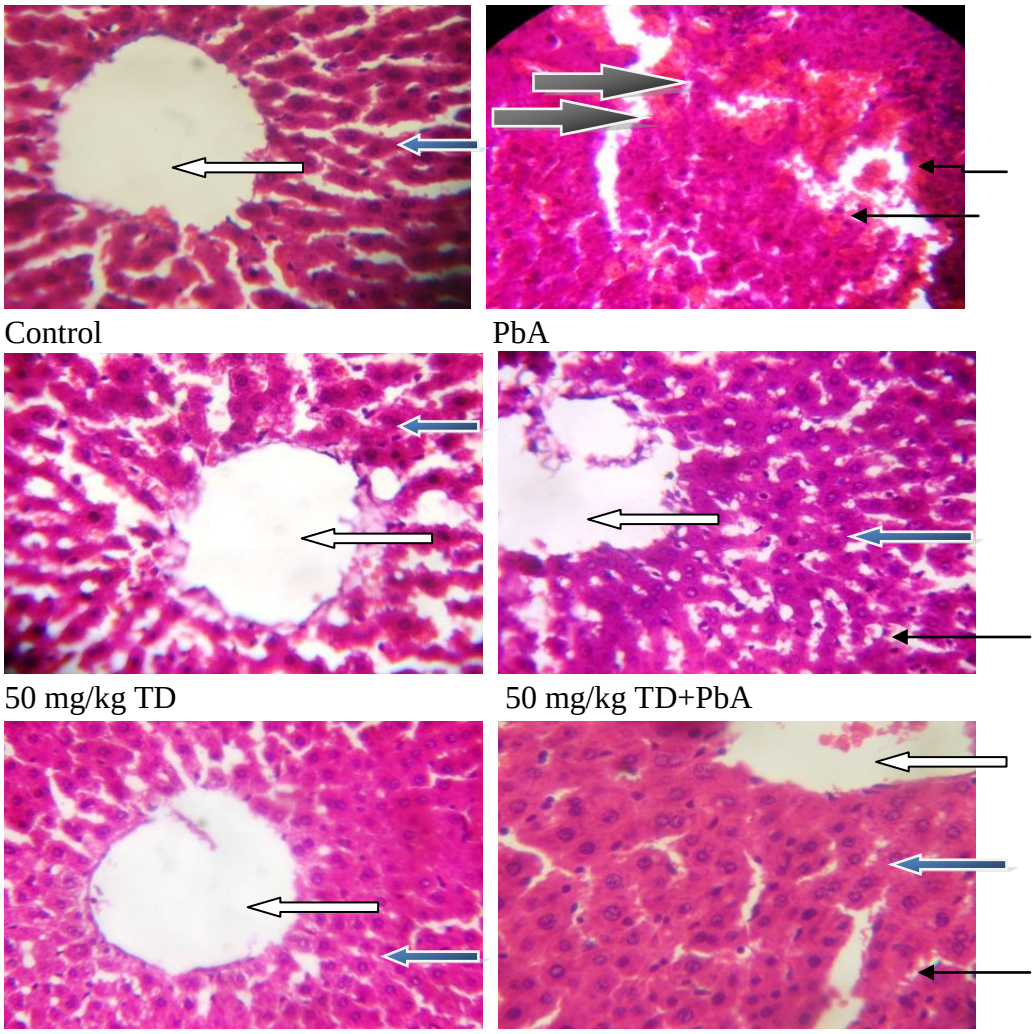


Plate 4.1. Photomicrograph of liver section stained by H&E, showing the effect of oral administration of methanol extract of *T. diversifolia* leaf on histology of the lead acetate treated Wistar rat's liver (× 400)

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

White thick arrow = normal central venules

Black slender arrow = sinusoids with lysed cells

Blue thick arrow = normal hepatocytes morphology
Black thick arrow = congested vessels/severe haemorrhage

4.10. Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on kidney biomarkers in control and lead acetate (PbA) treated groups.

This present study showed a significant ($p<0.05$) increase of serum urea and creatinine concentration in lead acetate treated group when compared with control and other treated groups (figure 4.3 and 4.4). Moreover, urea and serum creatinine concentration significantly ($p<0.05$) reduced in groups pre-treated with 15 mg/kg of lead acetate and concurrent administration of TD (50 mg/kg and 100 mg/kg) when compared with the PbA group. In the groups treated with TD (50 mg/kg and 100 mg/kg), there were no significant difference when compared with the control (figure 4.3 and 4.4).

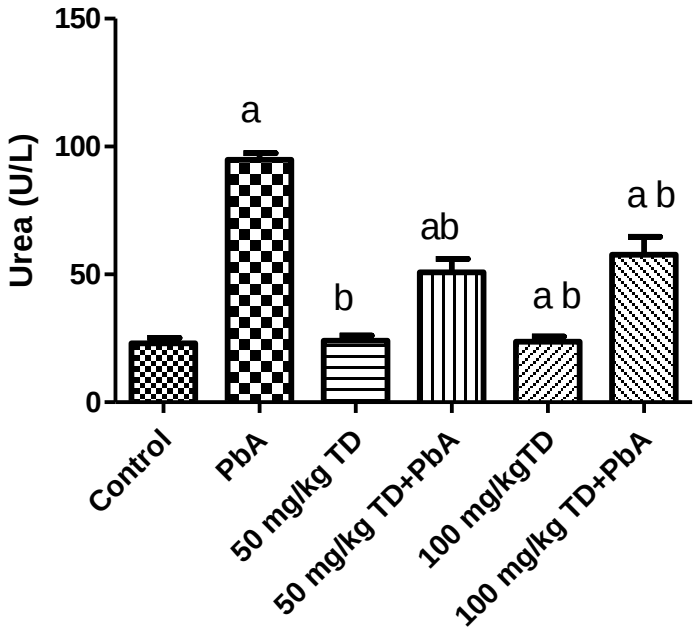


Figure 4.2: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on serum urea in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

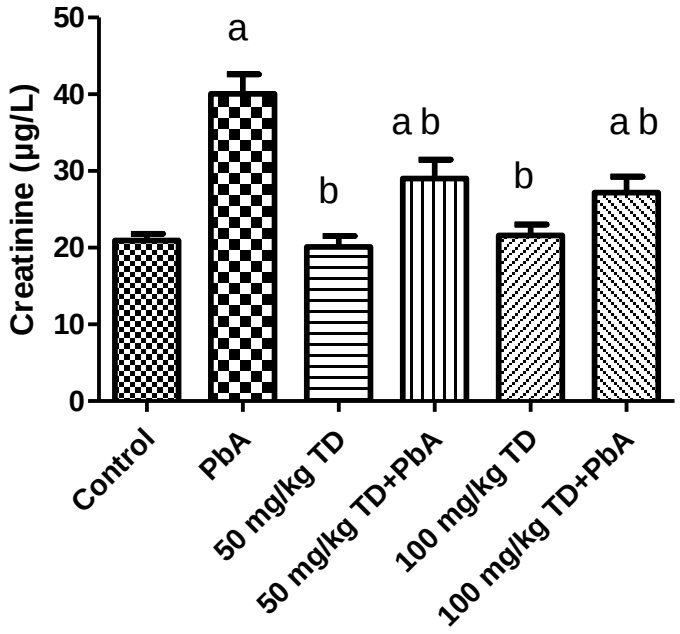


Figure 4.3: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on serum creatinine in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.11. Effects of Oral Administration of Methanol Extract of *Tithonia diversifolia* Leaf (TD) on Haematological Indices of Lead Acetate (PbA) Treated Wistar Rats

This study showed significant ($p<0.05$) decrease in PCV, haemoglobin. RBC and WBC count in lead acetate treated group when compared with the control while a significant ($p<0.05$) increase in all other treated group was observed in PCV when compared with PbA treated group. In TD (50 and 100 mg/kg) treated group, there was a significant increase in haemoglobin, RBC and WBC when compared with the control and PbA treated groups while in 50 mg/kg+PbA and 100 mg/kg+PbA there was a significant increase in haemoglobin, RBC and WBC count when compared with PbA treated group and control (Table 4.8).

Table 4.8: Effects of oral administration of methanol extract of *Tithonia diversifolia* leaf on Haematological Indices of lead acetate treated Wistar rats

	CONTROL	PbA	50 mg/kg TD	50 mg/kg TD +PbA	100 mg/kg TD	100 mg/kg TD +PbA
PCV (%)	42.88 ± 1.76	34.20± 2.37[a]	41.60± 2.64[b]	40.00± 4.04[b]	42.50± 2.22[b]	38.40± 2.90[b]
Haemoglobin (g/dl)	14.88± 0.60	10.72 ±1.81[a]	16.76 ±0.91[ab]	12.44± 1.38[ab]	16.28 ±0.78[ab]	12.46 ±1.90[ab]
RBC(10^6/µl)	7.48 ±0.29	4.45± 0.96[a]	8.90 ±0.53[ab]	5.11± 0.69[ab]	8.24± 0.44[ab]	6.00± 0.89[b]
WBC ($\times10^3$/µl)	4.69±0.31	3.89 ± 0.21[a]	6.22±0.49[ab]	6.02±0.74[a,b]	6.72± 0.41[ab]	6.57±0.51[ab]
Platelet ($\times10^3$/µl)	89.29±3.50	96.00±6.51	123.00±6.94[ab]	110.60±5.29[ab]	107.20±5.29	115.80±5.28[ab]
LYM (%)	62.88±2.57	59.20±2.29	66.2±2.01	61.40±3.78	63.60±28.00	66.80±3.73[b]
NEUTROPHIL (%)	32.75±2.90	37.40±2.14	30.2±2.56	34.60±4.07	30.40±2.31	30.20±4.09
MONOCYTES (%)	2.50±0.19	1.60±0.25[a]	1.40±0.25[a]	2.20±0.37	2.20±0.37	1.20±0.20[a]
EOSINOPHIL (%)	1.88±0.58	1.80±0.37	2.20±0.74	1.80±0.37	2.60±0.68	3.0±0.84

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf PbA = Lead acetate

4.12. Effects of Oral Administration of Methanol Extract of *Tithonia diversifolia* Leaf on Reproductive Organs weight of Lead Acetate Treated Wistar Rats

There were no significant ($p<0.05$) difference in testis, epididymis, seminal vesicle and prostate weights of all the treated groups (PbA, 50 mg/kg TD, 50 mg/kg TD +PbA, 100 mg/kg TD, 100 mg/kg TD + PbA) compared to the control (Table 4.9).

Table 4.9. Effects of oral administration of methanol extract of *Tithonia diversifolia* leaf on reproductive organ weight of lead acetate treated Wistar rats

	Testes (g)	**Epididymis (g)**	**Seminal Vesicle (g)**	**Prostate (g)**
Control	1.178 ± 0.079	0.614 ± 0.032	0.398 ± 0.043	0.204 ± 0.029
PbA	1.298 ± 0.069	0.598 ± 0.034	0.336 ± 0.045	0.186 ± 0.034
50 mg/kg TD	1.090 ±0.021	0.532 ± 0.019	0.46 ± 0.011	0.192 ± 0.017
50 mg/kg TD + PbA	1.112 ± 0.041	0.482 ± 0.019	0.312 ± 0.034	0.126 ± 0.024
100 mg/kg TD	1.076 ± 0.088	0.664 ± 0.043	0.386 ± 0.030	0.224 ± 0.014
100 mg/kg TD + PbA	1.146 ± 0.081	0.544 ± 0.035	0.356 ± 0.032	0.180 ± 0.033

Values are presented as Mean ± SEM, n=5

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.13 Effects of Oral Administration of Methanol Extract of *Tithonia diversifolia* Leaf on Sperm Parameters of Lead Acetate Treated Wistar Rats

4.13.1 Effect of Methanol Extract of *Tithonia diversifolia* Leaf on Sperm Concentration of Lead Acetate Treated Wistar Rats

The sperm concentration was significantly decreased ($p<0.05$) in lead acetate group compared to the control group. The animals treated with *T. diversifolia* (50 mg/kg and 100 mg/kg) showed no significant difference ($p>0.05$) in sperm concentration when compared with control, whereas a significant increase ($p<0.05$) was observed in animals treated with *T. diversifolia* + PbA (50 mg/kg and 100 mg/kg) compared to lead acetate group (Figure 4.5).

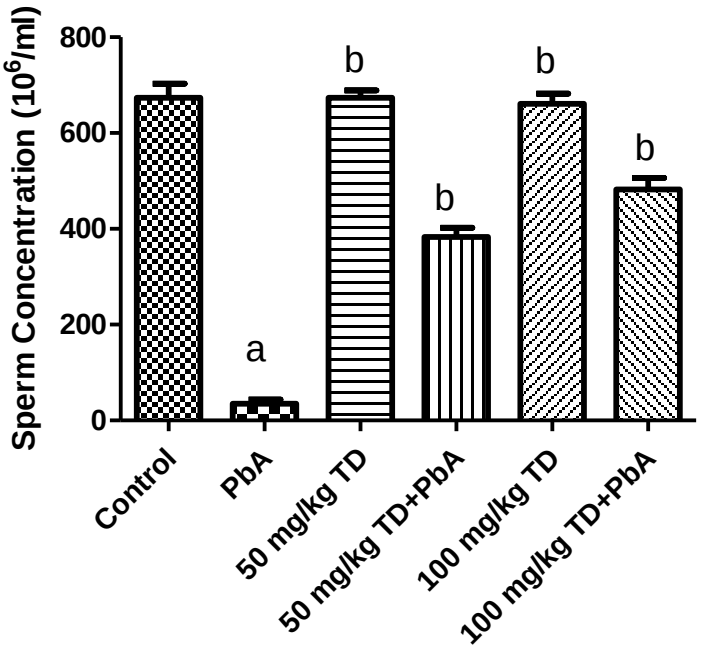


Figure 4.4. Effects of methanol extract of *T. diversifolia* leaf on sperm concentration of lead acetate treated Wistar rats

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.13.2. Effect of Methanol Extract of *Tithonia diversifolia* Leaf on Percentage of Motile Sperm of Lead Acetate Treated Wistar Rats

The percentage of motile sperm was significantly decreased ($p<0.05$) in the group treated with lead acetate when compared to control group. There was no significant difference ($p>0.05$) in animals treated with *T. diversifolia* (50mg/kg and 100mg/kg) compared to control, but a significant increase ($p<0.05$) was observed in animals treated with *T. diversifolia* + PbA (50mg/kg and 100mg/kg) compared to lead acetate group (Figure 4.7).

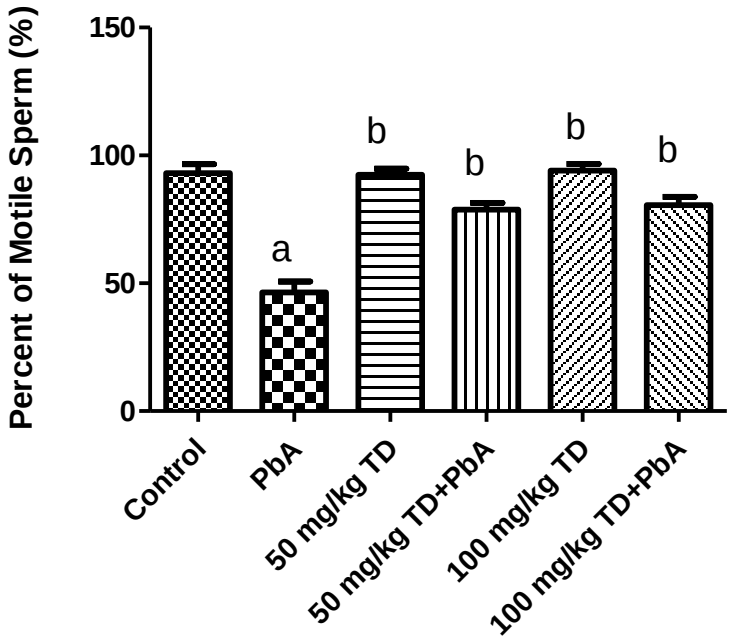


Figure 4.5. Effects of methanol extract of *T. diversifolia* leaf percentage of motile sperm in control and lead acetate treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.13.3. Effects of methanol extract of *Tithonia diversifolia* leaf (TD) on progressive, non-progressive and immotility in control and lead acetate (PbA) treated groups.

The result in figure 4.8 showed a significant ($p<0.05$) decrease in progressive and significant ($p<0.05$) increase in non-progressive and immotility in lead acetate treated group when compared with the control. TD groups (50 mg/kg and 100 mg/kg) showed no significant ($p<0.05$) difference in progressive, non-progressive and immotility when compared with the control. However, groups treated with PbA + TD (50 mg/kg and 100 mg/kg) showed significant ($p<0.05$) increase in progressive and significant ($p<0.05$) decrease in non-progressive and immotility when compared with PbA alone treated group

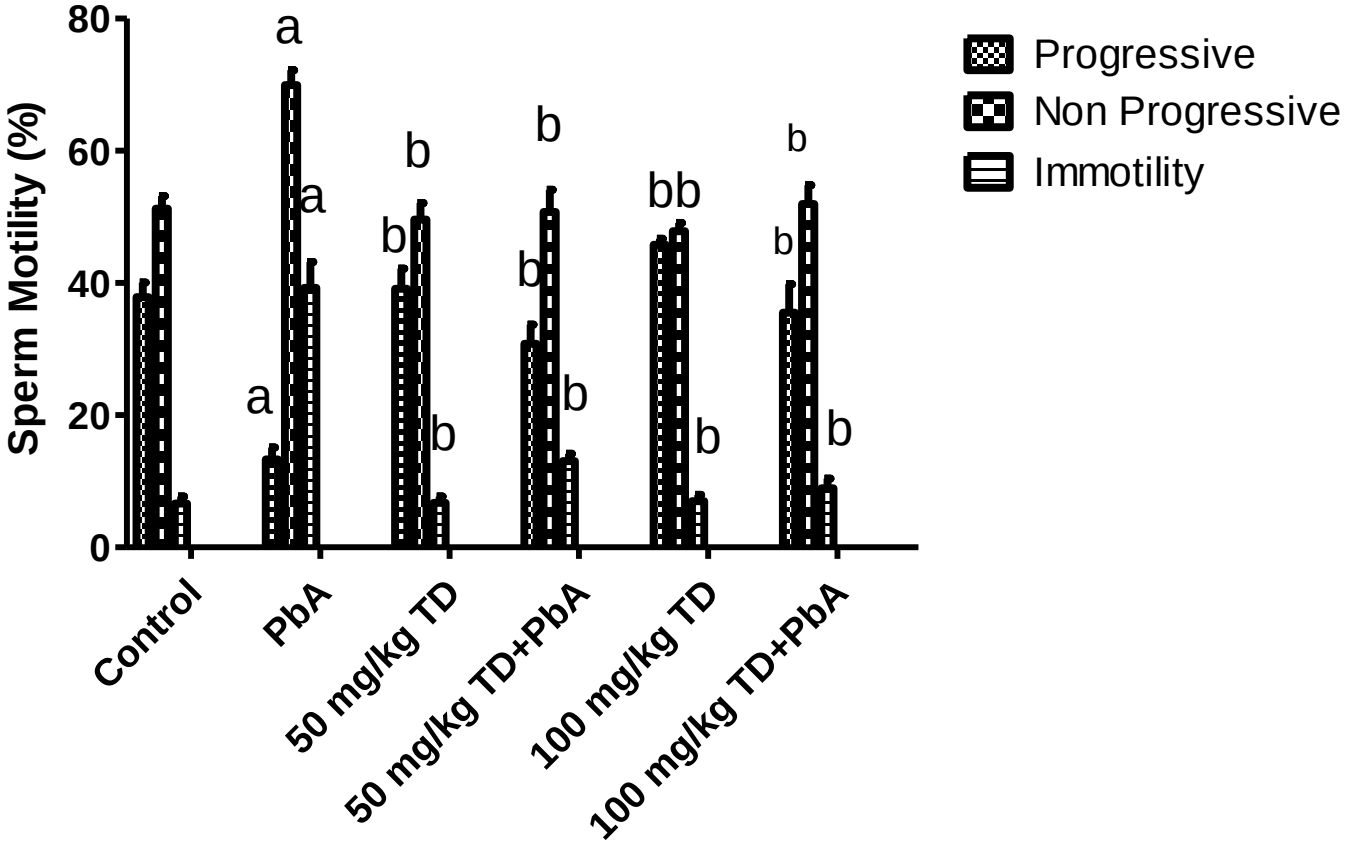


Figure 4.6. Effects of methanol extract of *T. diversifolia* leaf (TD) on progressive, non-progressive and immotility in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.13.4. Effects of methanol extract of *T. diversifolia* leaf (TD) on sperm kinetics in control and lead acetate (PbA) treated groups.

In this study, average path velocity, curnilinear velocity, straight-line velocity, amplitude lateral head , beat cross frequency, line moving and mean move decrease significantly ($p<0.05$) in the lead acetate (PbA) treated group compared to control and other experimental groups. Moreover, groups treated with *T. diversifolia* (50 mg/kg and 100 mg/kg) showed no significant ($p<0.05$) difference compared to control whereas a significant ($p<0.05$) increased was observed in animals treated with *T. diversifolia* + PbA (50 mg/kg and 100 mg/kg) compared to PbA group (Table 4.10).

Table 4.10. Effects of methanol extract of *T. diversifolia* leaf (TD) on sperm kinetics in control and lead acetate (PbA) treated groups.

Groups	Control	PbA	50 mg/kg TD	50 mg/kg TD+PbA	100 mg/kg T. D	100 mg/kg TD+PbA
Average Path Velocity (µm/s)	14.65±1.90	6.51±0.53 [a]	13.37±1.43[b]	9.68±1.23[b]	15.30±.88[b]	14.26±1.16[b]
Curnilinear velocity (µm/s)	21.36±1.03	7.34±0.61[a]	20.46±1.00[b]	14.39 ±1.28[b]	20.42 ±1.4[b]	17.50±.83[b]
Straight-line velocity (µm/s)	7.14±0.59	4.53±0.46[a]	7.25±.045[b]	6.52±0.50[b]	6.44±1.13[b]	7.29±.73[b]
Amplitude Lateral Head (µm)	0.73±0.05	0.32±0.02[a]	0.70±0.04[b]	0.54±0.03[b]	0.69±0.06[b]	0.70±0.05[b]
Beat Cross Frequency (Hz)	3.48±0.44	0.67±0.13[a]	3.54±0.33[b]	2.29±0.31[b]	3.86±0.39[b]	3.16±0.55[b]
Line Moving (%)	31.01±3.81	16.01±0.42[a]	37.50±2.10[b]	29.41±3.29[b]	40.43±3.14[b]	35.8±5.22[b]
Mean Move angle °)	12.34±0.81	2.31±0.16[a]	12.09±0.87[b]	9.65±0.44[b]	13.23±1.31[b]	10.44±0.90[b]

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.14: Effects of methanol extract of *T. diversifolia* leaf on hormonal level in control and lead acetate (PbA) treated groups.

4.14.1. Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on follicle stimulating hormone (FSH) in control and lead acetate (PbA) treated groups.

Significant ($p<0.05$) decrease in the concentrations of serum follicle stimulating hormone (FSH) was observed in the group treated with lead acetate (PbA) as compared with the control. Significant ($p<0.05$) increase was observed in animals treated with *T. diversifolia* + PbA (50 mg/kg and 100 mg/kg) when compared with lead acetate (PbA) whereas *T. diversifolia* (50 mg/kg and 100 mg/kg) treated groups has no significant ($p<0.05$) difference compared to the control (Figure 4.9).

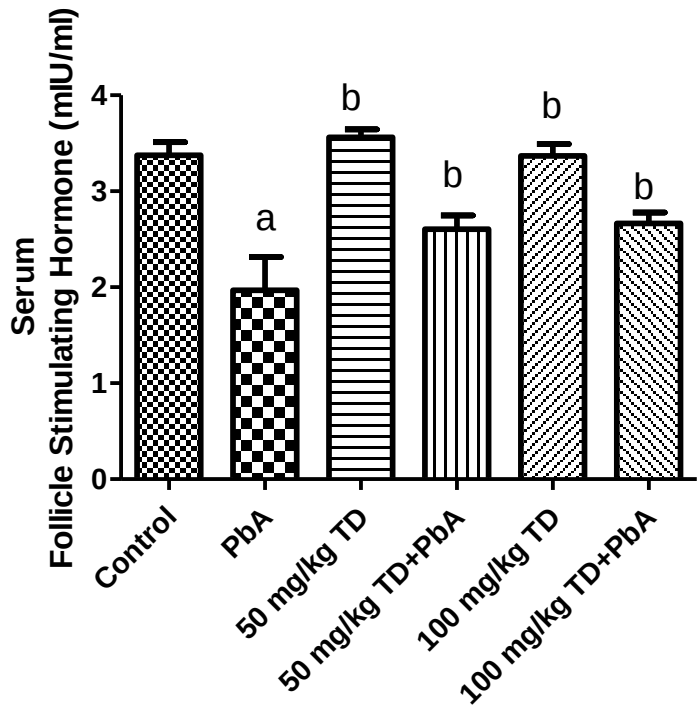


Figure 4.7: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on follicle stimulating hormone (FSH) in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.14.2. Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on luteinising hormone (LH) in control and lead acetate (PbA) treated groups.

Significant ($p<0.05$) decrease in the concentrations serum luteinizing hormone was observed in the group treated with lead acetate (PbA) as compared with control. Significant ($p<0.05$) increase was observed in animals treated with *T. diversifolia* + PbA (50 mg/kg and 100 mg/kg) when compared with lead acetate (PbA) whereas *T. diversifolia* (50 mg/kg and 100 mg/kg) treated groups has no significant ($p<0.05$) difference compared to the control (Figure 4.10).

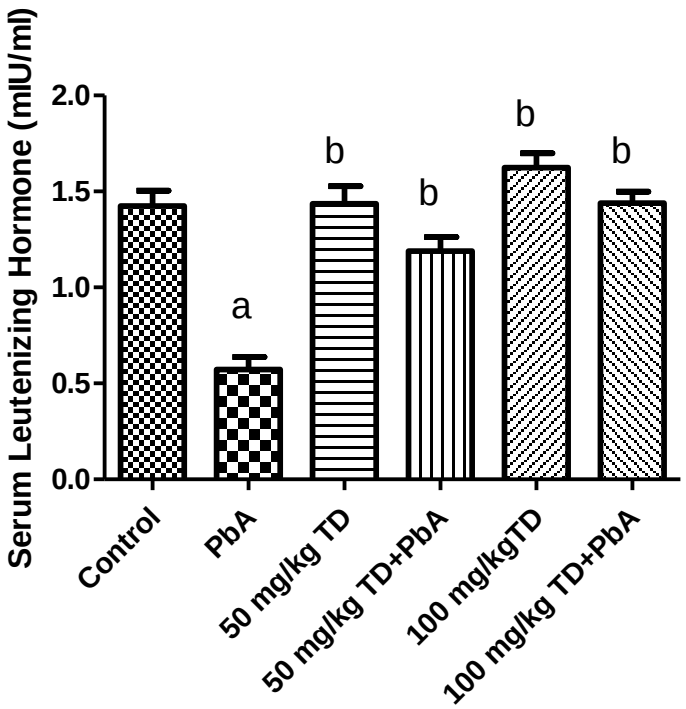


Figure 4.8: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on luteinising hormone (LH) in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.14.3. Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on testosterone in control and lead acetate (PbA) treated groups.

Significant ($p<0.05$) decrease in the concentrations of serum testosterone was observed in the group treated with lead acetate (PbA) as compared with the control. Significant ($p<0.05$) increase was observed in animals treated with *T. diversifolia* + PbA (50 mg/kg and 100 mg/kg) when compared with lead acetate (PbA) whereas *T. diversifolia* (50 mg/kg and 100 mg/kg) treated groups has no significant ($p<0.05$) difference compared to the control (Figure 4.11).

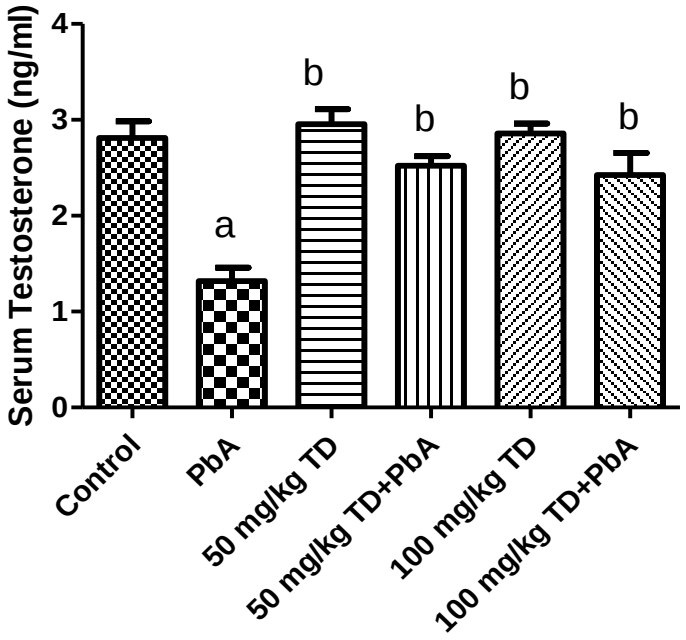


Figure 4.9: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on testosterone in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.15. Effects of oral administration of Methanol extract of *T. diversifolia* leaf (TD) for 56 days on testicular malondialdehyde (MDA) levels in control and lead acetate (PbA) treated groups.

Lead acetate (PbA) treated group showed a significant ($p<0.05$) increase in testicular level of MDA when compared with control group and all other treated groups (Figure 4.12). Significant ($p<0.05$) decrease was observed in testicular level of MDA in the group pre-treated with 15 mg/kg of lead acetate and TD (50 mg/kg and 100 mg/kg) when compared with the lead acetate group (Figure 4.12)

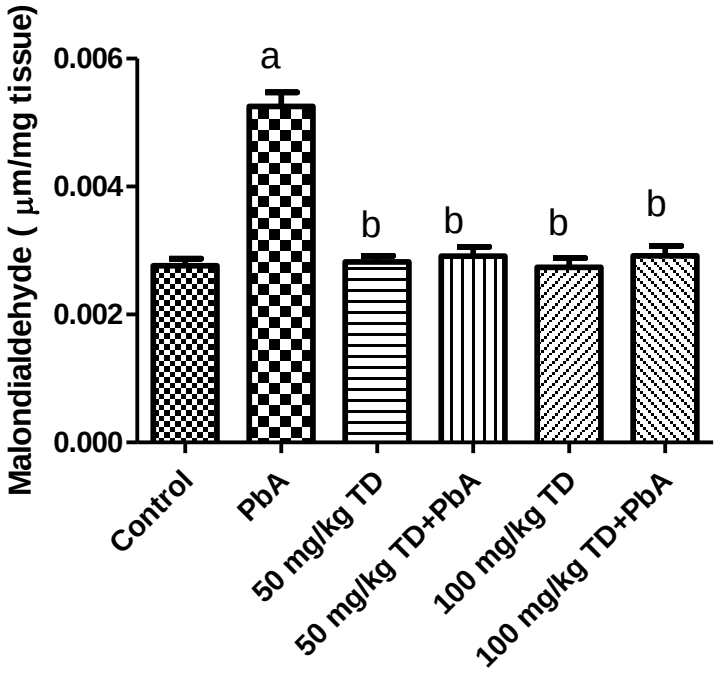


Figure 4.10: Effects of oral administration of Methanol extract of *T. diversifolia* leaf (TD) for 56 days on testicular malondialdehyde (MDA) levels in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.16. Effects of oral administration of methanol extract of *T. diversifolia* for 56 days on antioxidants level in the testis of the control and lead acetate (PbA) treated groups.

This study showed a significant ($p<0.05$) decrease in the level of antioxidant activity of superoxide dismutase (SOD) (figure 4.13) and catalase (CAT) (figure 4.14) in the testes of lead acetate (PbA) treated group compared with control but a significant ($p<0.05$) increase was observed in PbA + *T. diversifolia* (50 mg/kg and 100 mg/kg) and *T. diversifolia* (50 mg/kg and 100 mg/kg) when compared with PbA treated group.

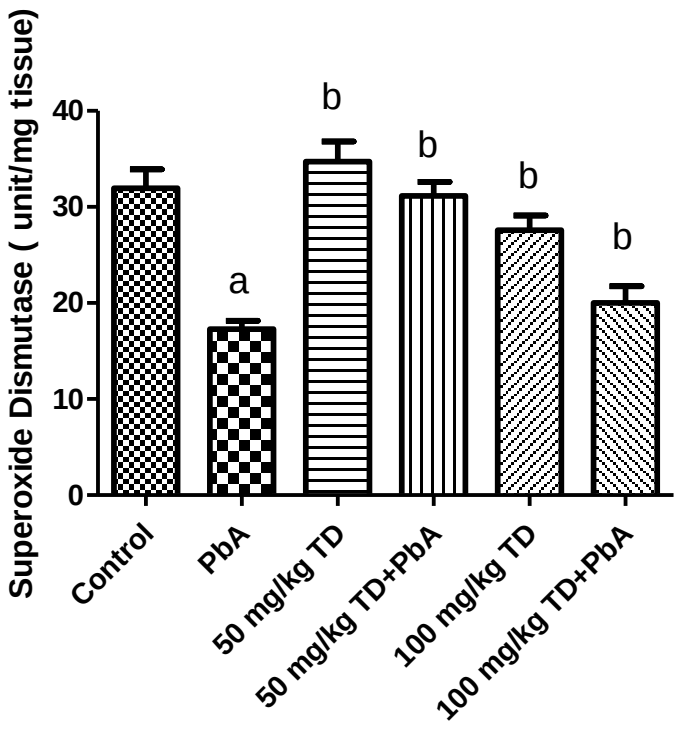


Figure 4.11: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on testicular SOD level in control and lead acetate treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

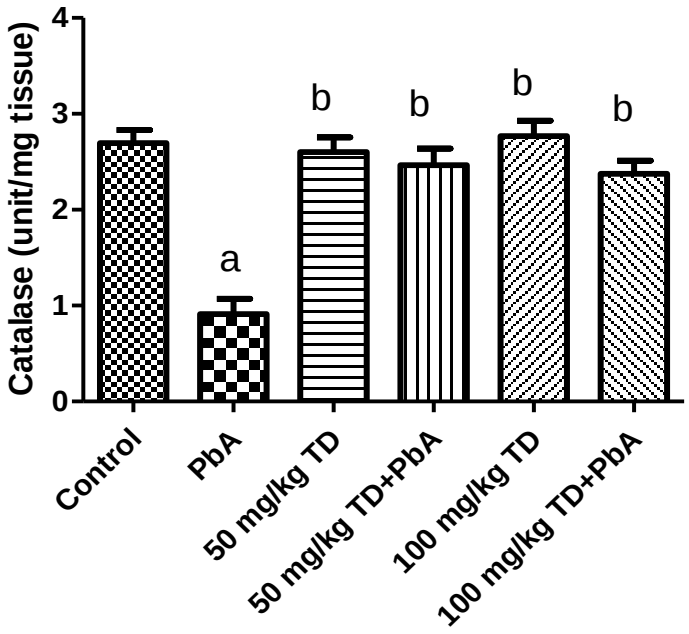


Figure 4.12: Effects of oral administration of methanol extract of *T. diversifolia* leaf for 56 days on testicular catalase level in control and lead acetate (PbA) treated groups.

Values are presented as Mean ± SEM, n=5

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.17. Effect of Oral Administration of Methanol Extract of *T. diversifolia* Leaf on Histology of the Lead Acetate Treated Wistar Rat's Testes.

Lead acetate (PbA) treated group showing abnormal seminiferous tubules with maturation arrest (White thick arrow); the lumen appear widened without spermatozoa, while control and other treated groups (50 mg/kg TD; 50 mg/kg TD + PbA; 100 mg/kg TD and 100 mg/kg TD + PbA) showing normal seminiferous tubules (Black thick arrow) with normal spermatogonia cell, the lumen appear normal with presence of spermatozoa. The interstitial spaces and Leydig cells appear normal (Slender arrow) (Plate 4.2).

HISTOLOGY OF THE TESTES

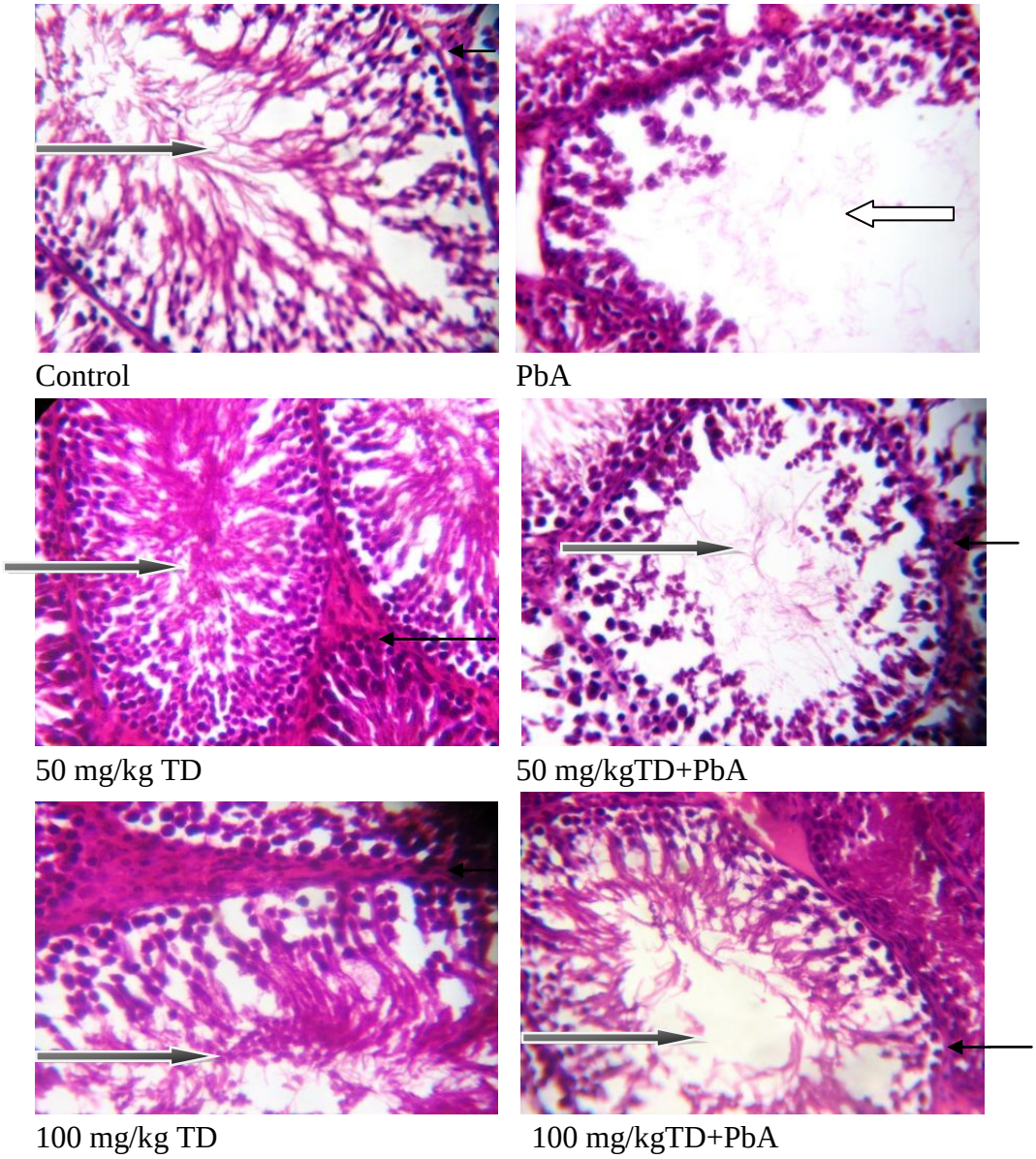


Plate 4.2. Photomicrograph of testicular section stained by haematoxylin and eosin, showing the effect of oral administration of methanol extract of *T. diversifolia* leaf on histology of the lead acetate treated Wistar rat's testis (× 400)

TD = Methanol extract of *Tithonia diversifolia* leaf

PbA = Lead acetate

4.18. Effects of oral administration of phytol for 56 days on reproductive organ weight in control and lead acetate (PbA) treated groups.

The result of this study showed no significant ($p<0.05$) difference in testis, epididymis, seminal vesicle and prostate weights of all the treated groups (PbA, 50 mg/kg Phytol, 50 mg/kg Phytol + PbA) compared to the control (Table 4.11).

Table 4.11: Effects of oral administration of phytol for 56 days on reproductive organ weight in control and lead acetate (PbA) treated groups Weight.

	Testes (g)	Epididymis (g)	Prostate (g)	S. Vessicle (g)
Control	1.10 ± 0.05	0.42 ± 0.02	0.16 ± 0.04	0.78 ± 0.11
PbA	1.13 ± 0.03	0.40 ± 0.03	0.11 ± 0.02	0.55 ± 0.12
Phytol (50 mg/kg)	1.04 ± 0.10	0.40 ± 0.05	0.12 ± 0.01	0.48 ± 0.11
Phytol (50 mg/kg) + PbA	1.05 ± 0.05	0.42 ± 0.04	0.13 ± 0.01	0.58 ± 0.08

Data represents Mean ± SEM. n=5.

PbA = Lead acetate

4.19. Effects of oral administration of phytol for 56 days on weight on visceral organ weight in control and lead acetate (PbA) treated groups.

In this study, administration of lead acetate (PbA) alone showed a significant ($p<0.05$) increase in weight of the liver compared to control while Phytol (50 mg/kg) and Phytol (50 mg/kg) + PbA treated rats significantly ($p<0.05$) decrease compared to the Pb group (Table 4.12). However, only Phytol (50 mg/kg) + PbA treated group showed a significant ($p<0.05$) decrease in weight of hypothalamus compared with control whereas Phytol (50 mg/kg) and Phytol (50 mg/kg) + PbA groups showed no significant ($p<0.05$) difference in weight of hypothalamus when compared with the control (Table 4.12).

Table 4.12. Effects of oral administration of phytol for 56 days on the weight of the liver and hypothalamus in control and lead acetate (PbA) treated groups.

	Liver (g)	Hypothalamus (g)
Control	6.22 ± 0.43	0.08 ± 0.04
PbA	7.72 ± 0.40[a]	0.09 ± 0.02
Phytol (50 mg/kg)	6.00 ± 0.61[b]	0.09 ± 0.02
Phytol (50 mg/kg) + PbA	5.90 ± 0.24[b]	0.06 ± 0.01 [a]

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

4.20. Effects of oral administration of phytol for 56 days on sperm motility and viability in control and lead acetate (PbA) treated groups.

The result of this study showed a significant ($p<0.05$) decrease in sperm motility and viability of a group treated with lead acetate (PbA) when compared to control. Whereas, animals treated with Phytol (50 mg/kg) showed no significant ($p<0.05$) difference compared to control but a significant ($p<0.05$) increase sperm motility and viability were observed in animals treated with Phytol (50 mg/kg) + PbA compared to PbA group (figure 4.15 and 4.16).

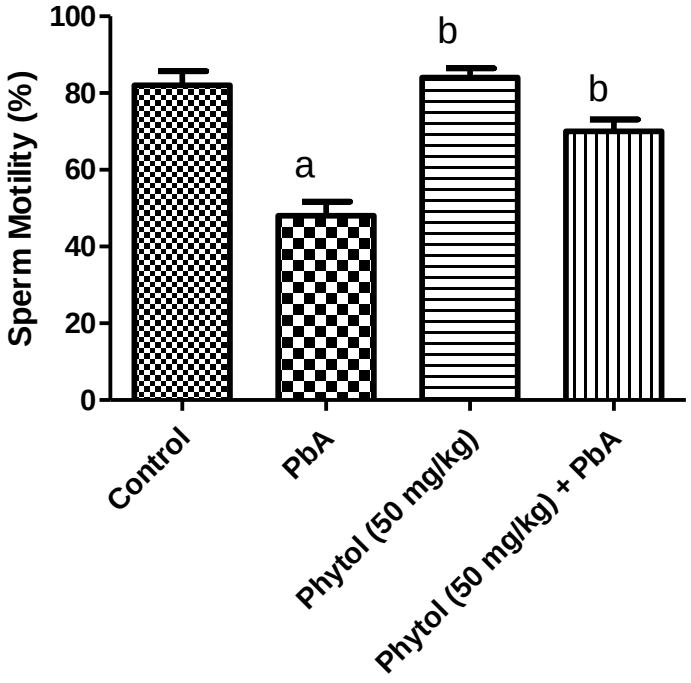


Figure 4.13: Effects of oral administration of phytol for 56 days on sperm motility in control and lead acetate (PbA) treated groups.

Data represents Mean ± SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

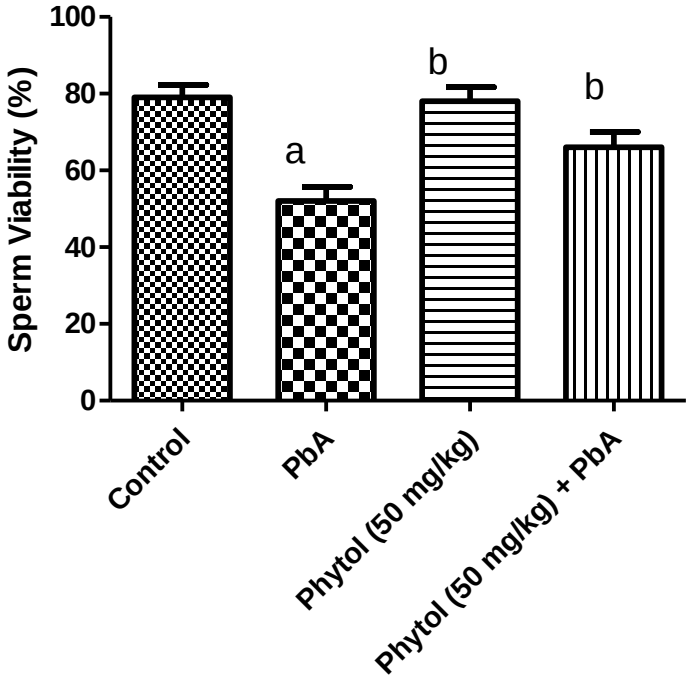


Figure 4.14: Effects of oral administration of phytol (PHY) for 56 days on sperm viability in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PHY = Phytol

PbA = Lead acetate

4.21. Effects of oral administration of phytol for 56 days on hypothalamo-pituitary-gonadal axis in control and lead acetate (PbA) treated groups.

Significant ($p<0.05$) decrease in the concentrations of gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), serum luteinizing hormone (LH) and testosterone were observed in the group treated with lead acetate (PbA) as compared to control (figure 4.17, 4.18, 4.19 and 4.20). Also, a significant ($p<0.05$) increase in GnRH, FSH, LH and testosterone were observed in animals treated with Phytol (50 mg/kg) + PbA compared to PbA group (figure 4.17, 4.18, 4.19 and 4.20) whereas Phytol (50 mg/kg) treated rats showed no significant ($p<0.05$) difference in the concentrations of GnRH, LH and testosterone compared to control (figure 4.17, 4.19 and 4.20) but a significant ($p<0.05$) increase was observed in the concentration follicle stimulating hormone (FSH) compared to control (figure 4.18).

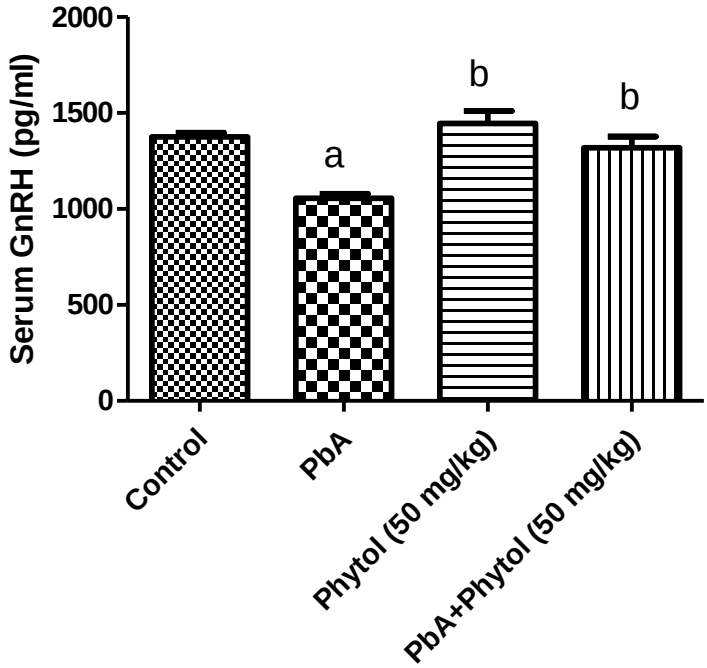


Figure 4.15. Effects of oral administration of phytol for 56 days on gonadotropin releasing hormone in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

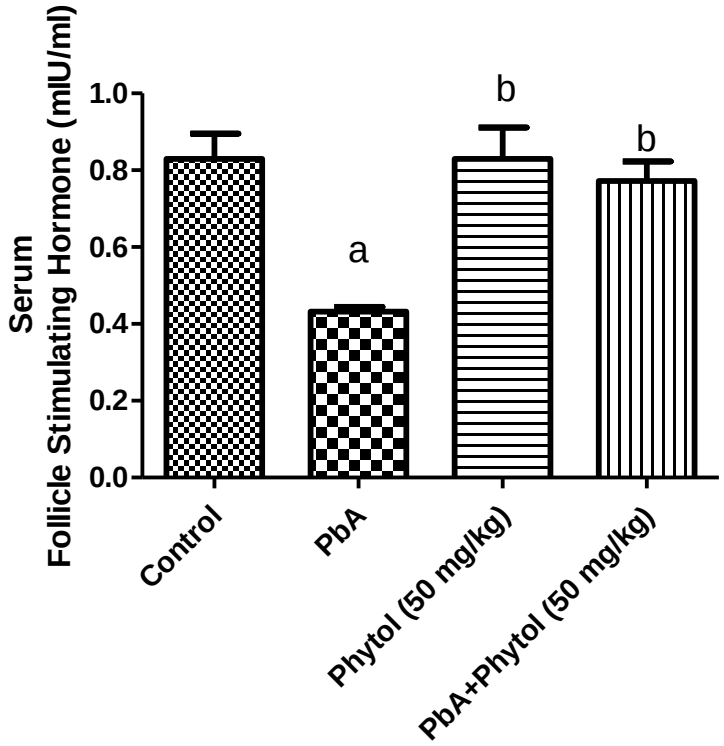


Figure 4.16. Effects of oral administration of phytol for 56 days on follicle stimulating hormone (FSH) in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

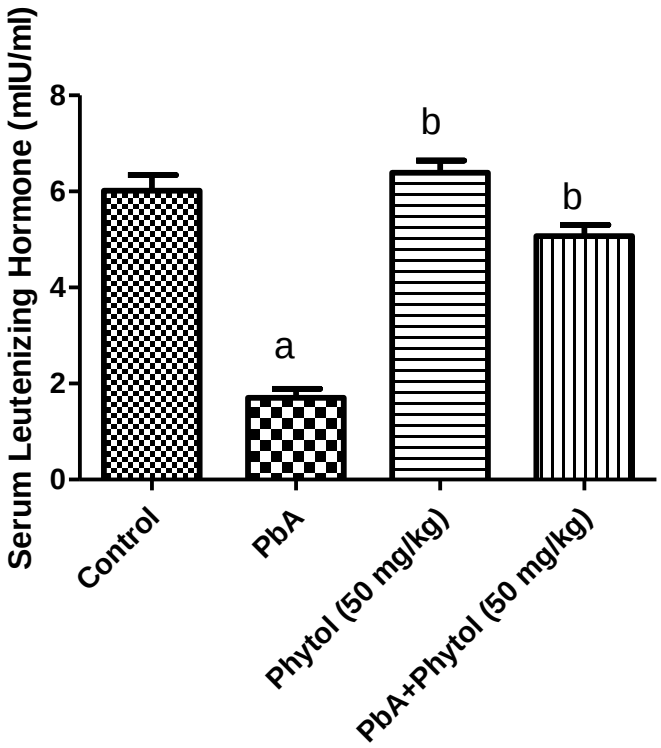


Figure 4.17. Effects of oral administration of phytol for 56 days on leutenizing hormone (LH) in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

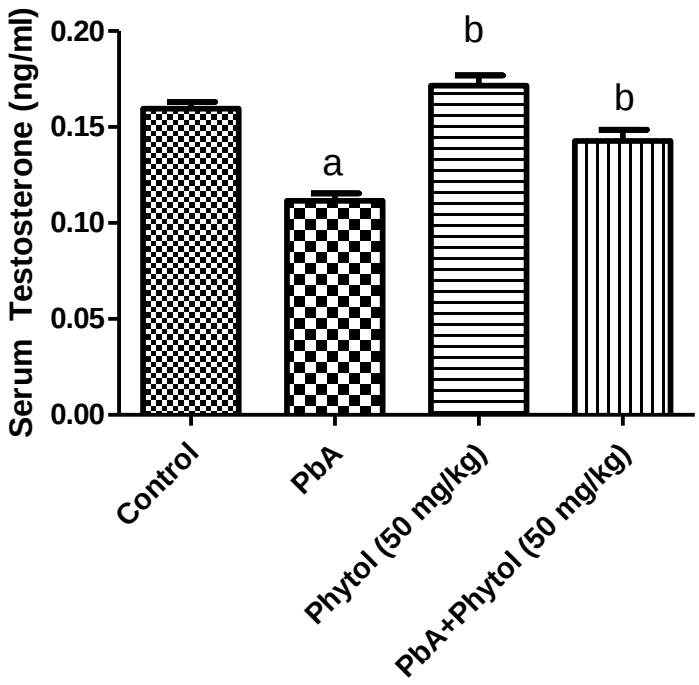


Figure 4.18. Effects of oral administration of phytol for 56 days on testosterone in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

4.22. Effects of oral administration of phytol for 56 days on inflammatory markers (nitrotyrosine, Interleukin 1 beta and Tumor necrotic factor alpha) in control and lead acetate (PbA) treated groups.

Serum nitrotyrosine level significantly ($p<0.05$) increased in lead acetate treated group when compared with the control. Nitrotyrosine level in the group administered with Phytol (50 mg/kg) + PbA significantly ($p<0.05$) decreased when compared with PbA group. However, Phytol (50 mg/kg) treated group showed no significant difference when compared with the control (figure 4.21).

Interleukin 1 beta (IL1-β) equally increased significantly ($p<0.05$) in lead acetate (PbA) treated group when compared to control Group pre-treated with lead acetate and concurrent administration with phytol (50 mg/kg) showed a significant ($p<0.05$) decrease in IL1-β level when compared with Pb group. Whereas, group treated with Phytol (50 mg/kg) showed no significant ($p<0.05$) difference compared with control (figure 4.22).

Tumor necrotic factor alpha (TNF-α) level significantly ($p<0.05$) increased in lead acetate treated group when compared with the control. TNF-α level in the group administered with Phytol (50 mg/kg) + Pb significantly ($p<0.05$) decreased when compared with Pb group. However, Phytol (50 mg/kg) treated group showed no significant difference in the level of TNF-α when compared with the control (figure 4.23).

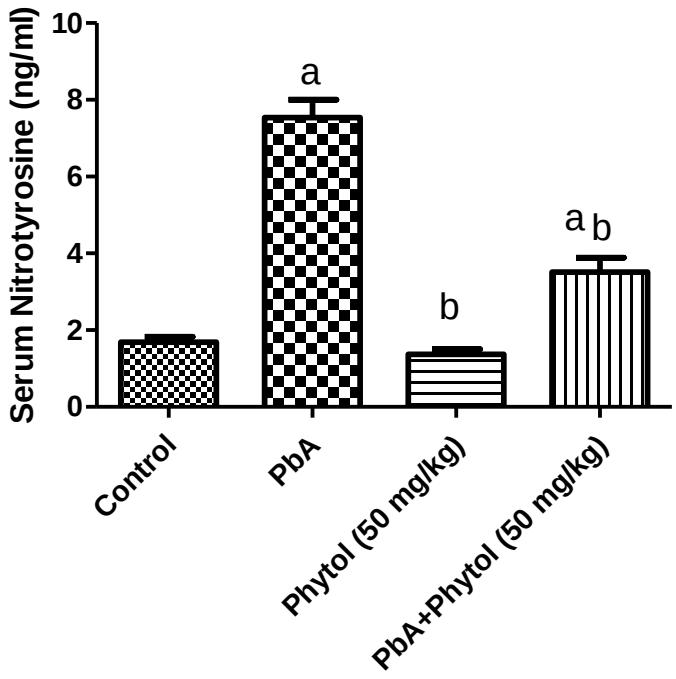


Figure 4.19. Effects of oral administration of phytol for 56 days on nitrotyrosine level in control and lead acetate (PbA) treated groups

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

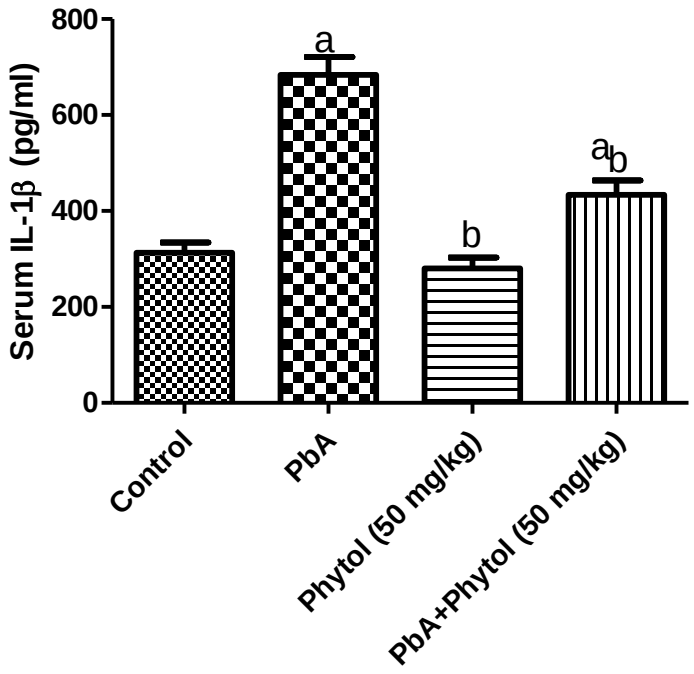


Figure 4.20. Effects of oral administration of phytol for 56 days on interleukin 1 beta (IL 1-β) level in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

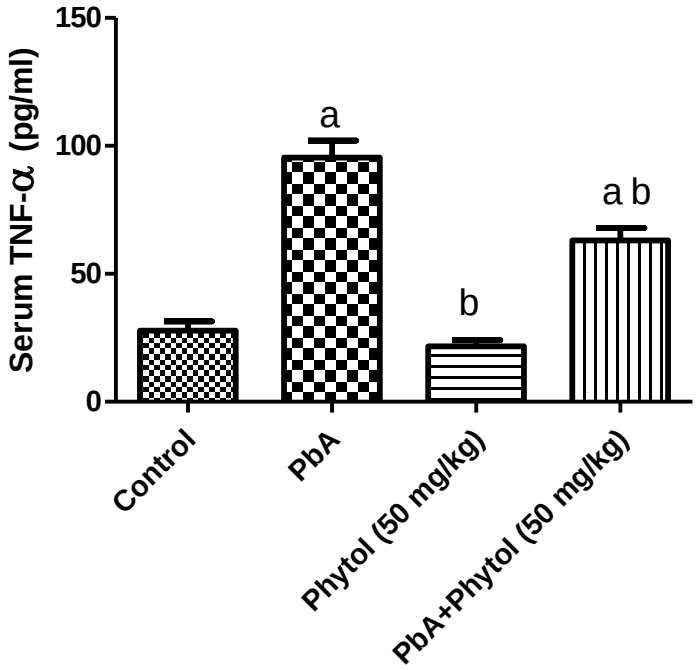


Figure 4.21. Effects of oral administration of phytol for 56 days on tumor necrotic factor alpha (TNF-α) level in control and lead acetate (PbA) treated groups

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

4.23. Effects of oral administration of phytol for 56 days on malondialdehyde (MDA) level in hypothalamic homogenate in control and lead acetate (PbA) treated groups

Lead (PbA) treated group showed a significant ($p<0.05$) increase in level of malondialdehyde (MDA) of the hypothalamus when compared with the control. The level of MDA in the group treated with 15 mg/kg of Pb and 50 mg/kg of Phytol significantly decreased when compared with Pb group. Phytol (50 mg/kg) treated groups showed no significant difference when compared with control (figure 4.24).

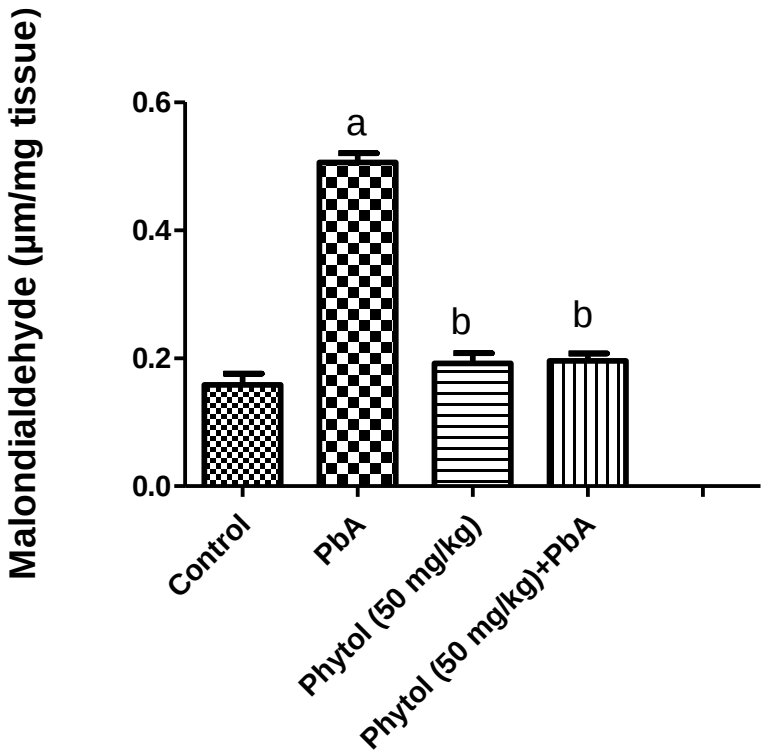


Figure 4.22. Effects of oral administration of phytol (PHY) for 56 days on malondialdehyde (MDA) level in hypothalamic homogenate in control and lead acetate (PbA) treated groups

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

4.24. Effects of oral administration of phytol for 56 days on antioxidant level in hypothalamic homogenate in control and lead acetate (PbA) treated groups.

This study showed a significant ($p<0.05$) decrease in the level of superoxide dismutase (SOD), catalase (CAT) and reduce glutathione (GSH) in the hypothalamus of lead acetate (PbA) treated group compared with control. However, there was a significant ($p<0.05$) increase in SOD, CAT and GSH groups treated with Phytol (50 mg/kg) and Phytol + PbA when compared with Pb group but no significant different when compared with control (figure 4.25, 4.26 and 4.27).

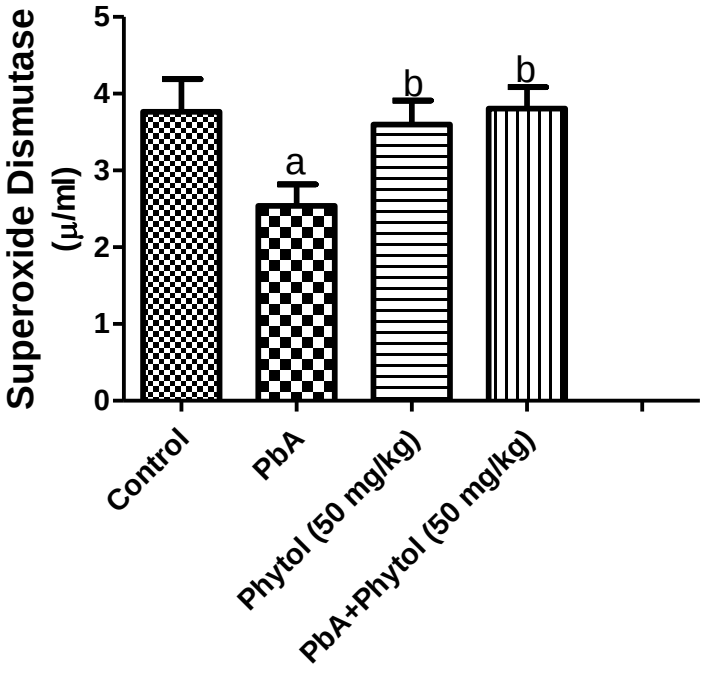


Figure 4.23. Effects of oral administration of phytol for 56 days on SOD level in hypothalamic homogenate in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

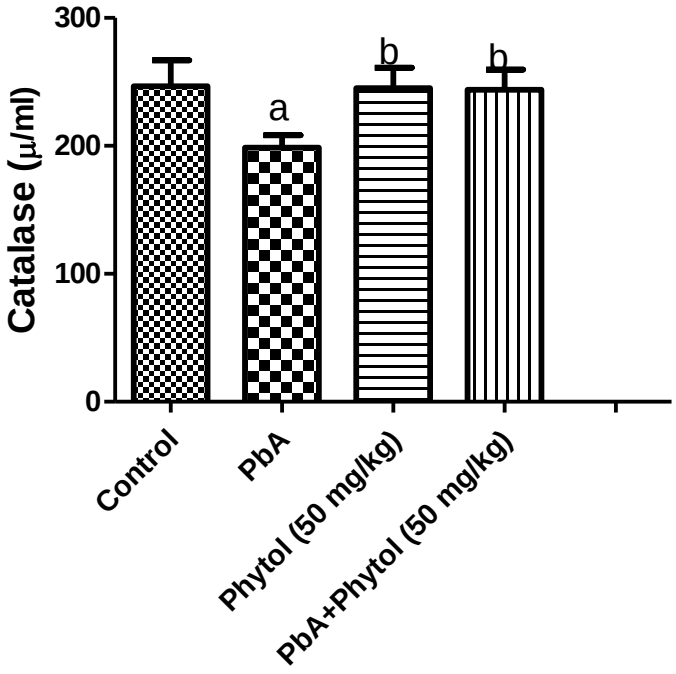


Figure 4.24. Effects of oral administration of phytol for 56 days on catalase level in hypothalamic homogenate in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

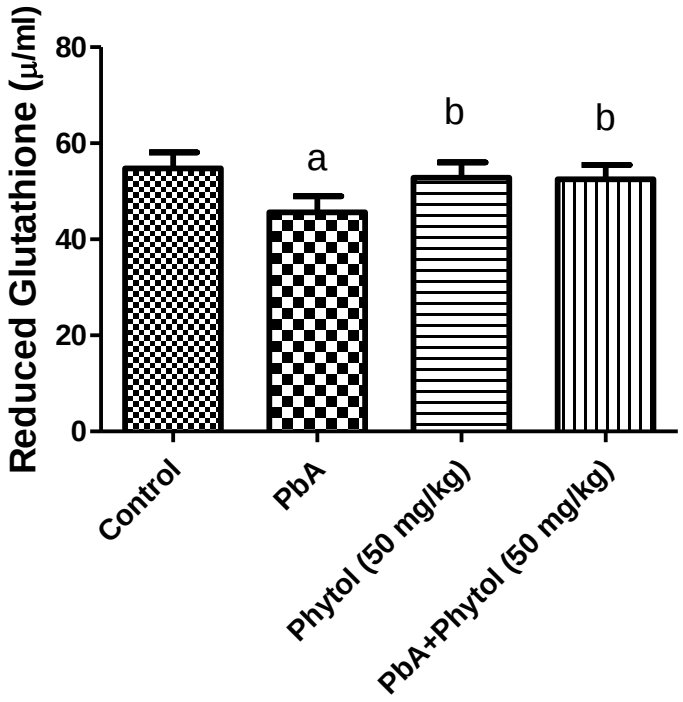


Figure 4.25. Effects of oral administration of phytol for 56 days on reduced gluthatione level in hypothalamic homogenate in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

4.25. Effects of oral administration of phytol on testicular homogenate for 56 days on malondialdehyde (MDA) level in control and lead acetate (PbA) treated groups.

Lead acetate (PbA) treated group showed a significant ($p<0.05$) increase in level of malondialdehyde (MDA) of the testes when compared with the control. The level of MDA in the group treated with 15 mg/kg of PbA and 50 mg/kg of Phytol significantly decreased when compared with PbA group. Phytol (50 mg/kg) treated groups showed no significant difference when compared with control (figure 4.28).

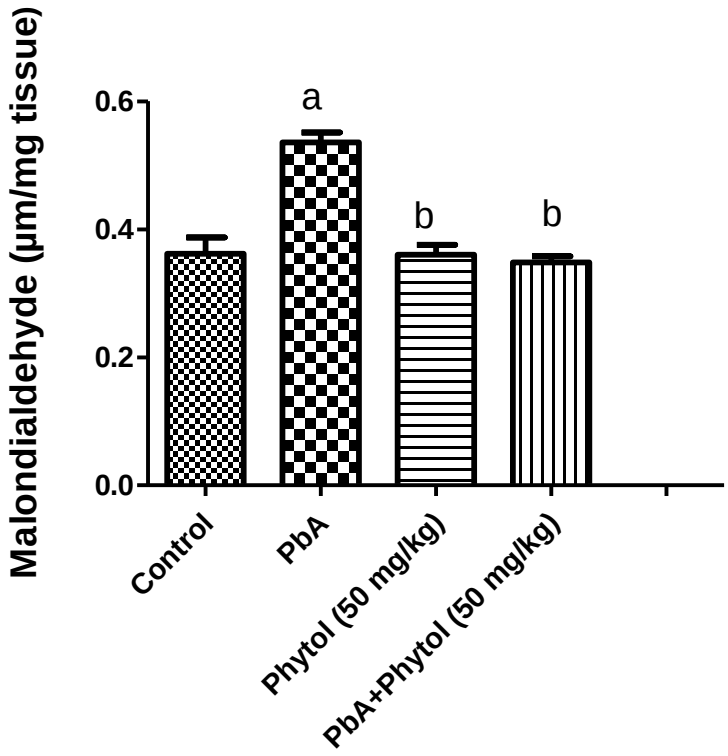


Figure 4.26. Effects of oral administration of phytol for 56 days on malondialdehyde (MDA) level in the testes in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

4.26. Effects of oral administration of phytol for 56 days on testicular antioxidant status in control and lead acetate (PbA) treated groups.

This study showed a significant ($p<0.05$) decrease in the level of superoxide dismutase (SOD), catalase (CAT) and reduce glutathione (GSH) in the testes of PbA treated group compared with control (figure 4.29, 4.30 and 4.31). Meanwhile, there was a significant ($p<0.05$) increase in the level of SOD, CAT and GSH of Phytol (50 mg/kg) and Phytol (50 mg/kg) + Pb treated groups when compared with PbA group (figure 4.29, 4.30 and 4.31). However, a significant ($p<0.05$) increase was observed in the level of reduce glutathione (GSH) in the testes of PbA + Phytol (50 mg/kg) treated group compare with control (figure 4.31).

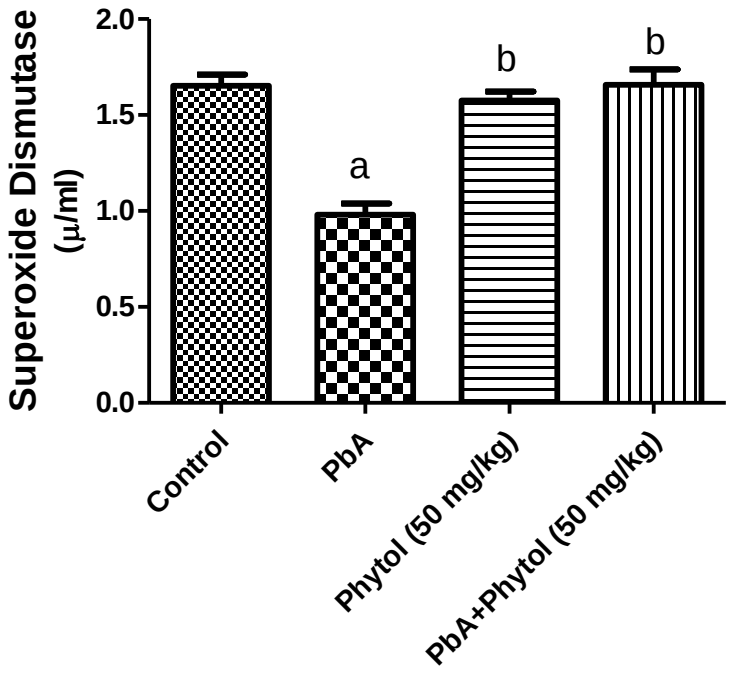


Figure 4.27. Effects of oral administration of phytol for 56 days on superoxide dismutase (SOD) in the testes in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

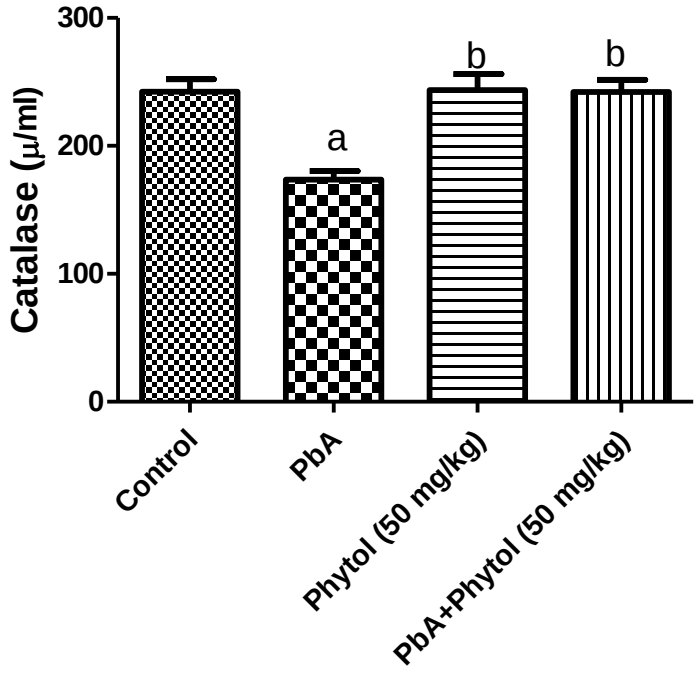


Figure 4.28. Effects of oral administration of phytol for 56 days on catalase (CAT) in the testes in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

PbA = Lead acetate

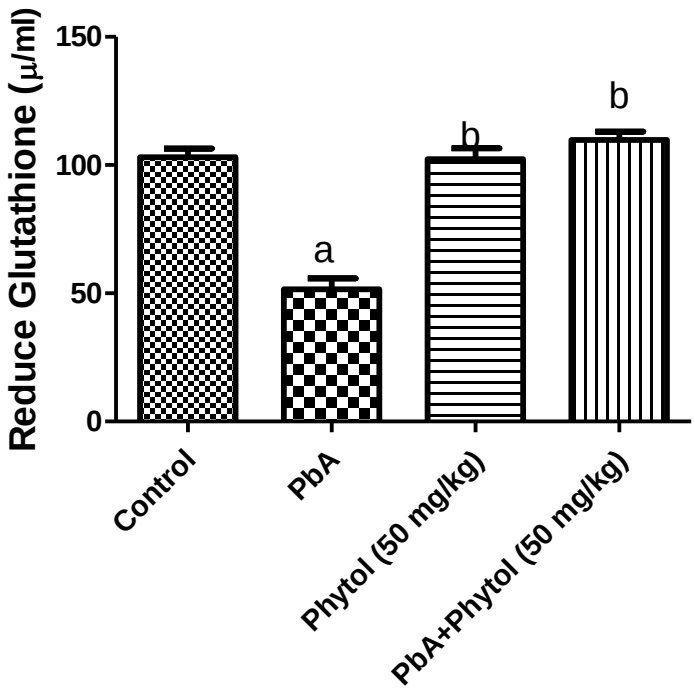


Figure 4.29. Effects of oral administration of phytol for 56 days on reduced glutathione (GSH) in the testes in control and lead acetate (PbA) treated groups.

Data represents Mean±SEM. n=5.

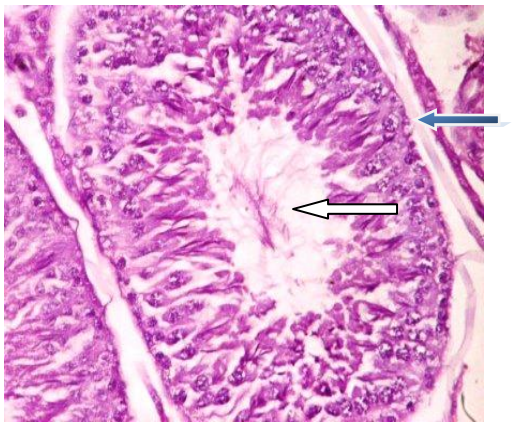
[a] $p<0.05$ was considered significant relative to control group

[b] $p<0.05$ was considered significant relative to lead acetate group

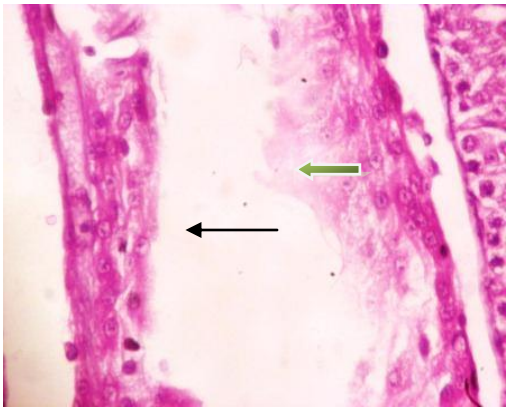
PbA = Lead acetate

4.27. Effects of oral administration of phytol for 56 days on histology of the Lead Acetate Wistar rat's Testes

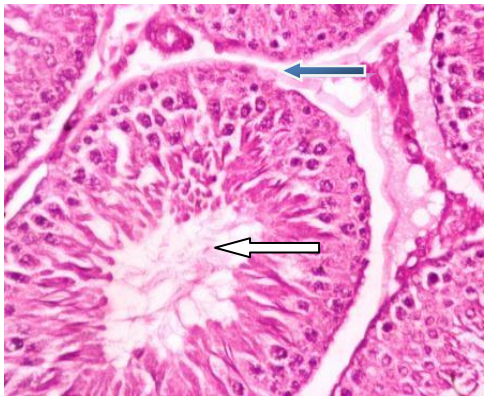
In the histology of the testes, control, Phytol and Phytol+PbA groups showed normal testicular architecture. Normal seminiferous tubules with presence of spermatozoa (white arrow).The interstitial spaces appear normal and leydig cells appear normal (blue arrow).In Lead acetate (PbA) group, there are maturation arrest, the luminal spaces of seminiferous tubules appear widened without spermatozoa (green arrow).There were congestion and oedema in the interstitial tissue(slender arrow) (plate 4.3).



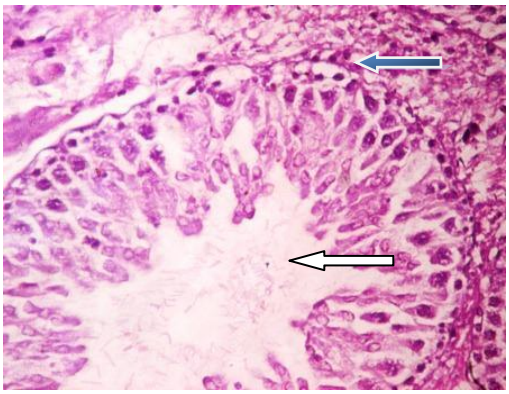
CONTROL



PbA



PHYTOL



PHYTOL+PbA

Plate 4.3: Photomicrograph of a testicular section stained by H&E, showing effects of oral administration of phytol for 56 days on histology of the lead acetate (PbA) treated Wistar rat's testes (X400)

PbA = Lead acetate

White arrow = normal seminiferous tubules

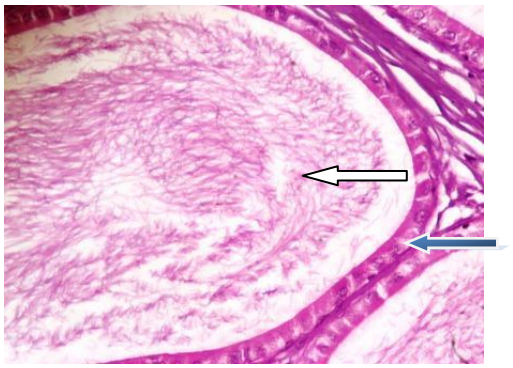
Blue arrow = normal leygid cell

Green arrow = seminiferous tubules without spermatozoa

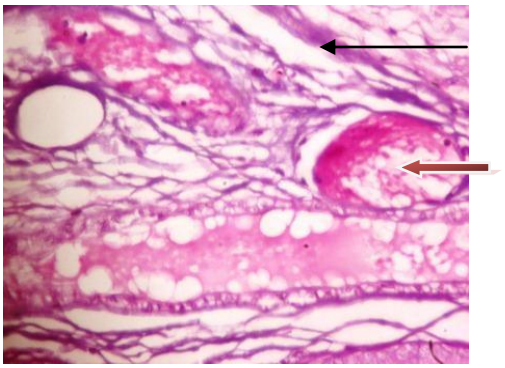
Slender arrow = congestion and oedema in interstitial tissue

4.28. Effects of oral administration of phytol for 56 days on histology of the lead acetate wistar rats epididymis

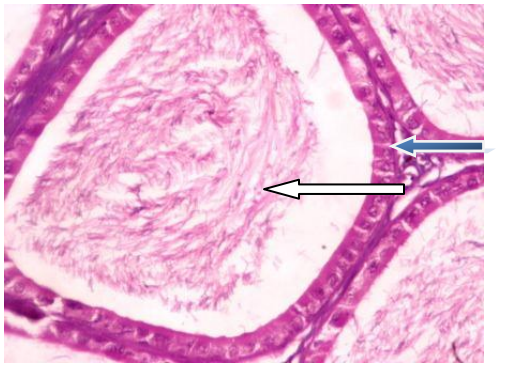
In the cytoachitecture of the epididymis, the Control, Phyol and Phytol+PbA showed epididymal ducts with normal smooth muscle layer and epithelial layers (blue arrow), the epidydimal ducts shows stored content of spermatozoa within the lumen(white arrow). The interstitial spaces appear normal.In PbA group, the interstitial spaces show increased connective tissues (slender arrow) and vascular congestion (red arrow) (plate 4.4).



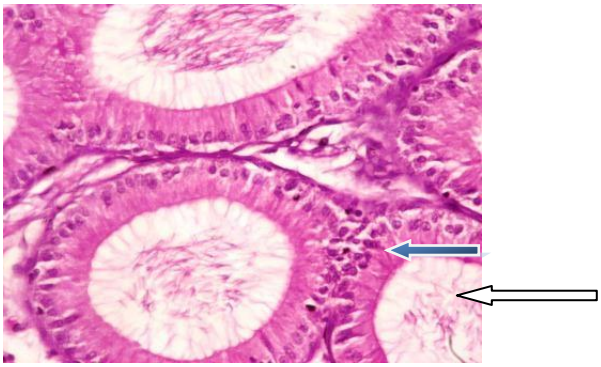
CONTROL



PbA



PHYTOL



PHYTOL+PbA

Plate 4.4 Photomicrographs of epididymal sections stained by H&E, showing the effects of oral administration of phytol for 56 days on histology of the lead acetate (PbA) treated Wistar rat's epididymis (X400)

PbA = Lead acetate

Blue arrow = Normal epididymal duct

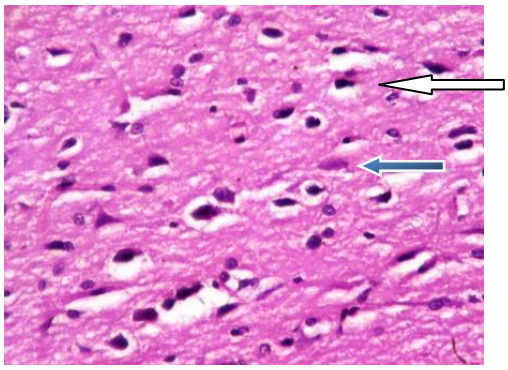
White arrow = Stored spermatozoa

Slender arrow = Abnormal interstitial spaces

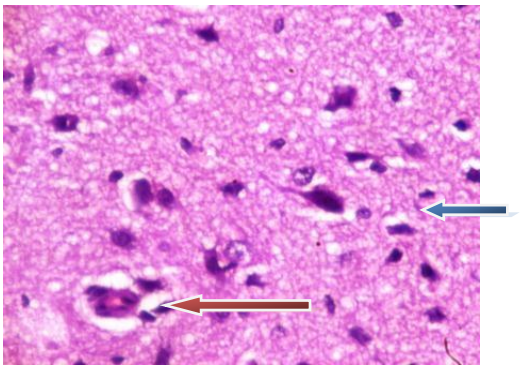
Red arrow = Vascular congestion

4.29. Effects of oral administration of phytol for 56 days on histology of the Lead Acetate Wistar rat's hypothalamus

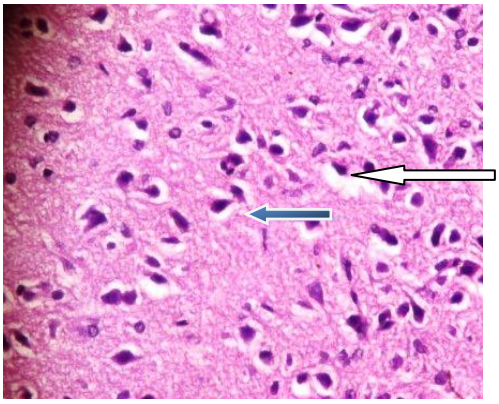
Control, Phytol and Phytol+PbA groups showed normal neuronal cells (blue arrow) on a normal stroma. There are other normal supraoptic and paraventricular nuclei seen (white arrow). The hypothalamus is normal without any pathologhical lesion. In the PbA group, there are normal neuronal cells (blue arrow) and few with hyperchromic nuclei (red arrow) on a normal stroma (plate 4.5).



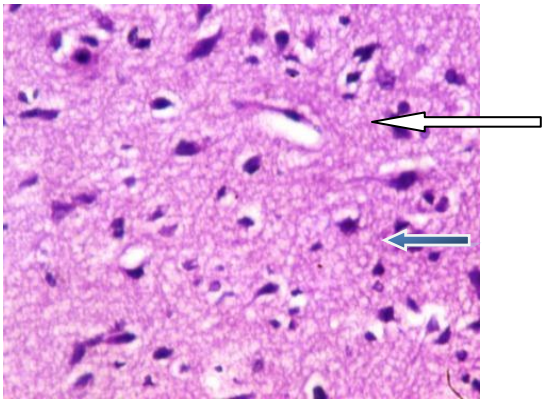
CONTROL



PbA



PHYTOL



PHYTOL+PbA

Plate 4.5. Photomicrograph of section of hypothalamus stained by H & E, showing effects of oral administration of phytol for 56 days on histology of the lead acetate (PbA) treated Wistar rat's hypothalamus (X400).

PbA = Lead acetate

Blue arrow = normal neuronal cells

White arrow = normal supraopic and paraventricular nuclei

Red arrow = hyperchromic nuclei

CHAPTER FIVE

5.0. DISCUSSION

5.1. The phytochemical screening, acute oral toxicity, GCMS analysis and antioxidant activities of different fractions of methanol extract of *Tithonia diversifolia* using DPPH.

The phytochemical screening of methanol extract showed the presence of phenol, flavonoids, terpenoids, saponins, glycosides, phlobatannins, resins and tannins. This is in line with the submission of Menut *et al.,* (2002); Ezeonwumelu *et al.,* (2012) and John-Dewole and Oni, (2013). These compounds have biological and pharmacological activities which have been responsible for their chemical synthesis of drugs in modern medicine and their medicinal uses traditionally (Sofowora, 2001; Okogun, 1996). The presence of these compounds gives *Thitonia diversifolia* the ability to scavenge free radicals by acting as an antioxidant (Lopes-Lutz; *et al.,* 2008; Araújo, *et al.,* 2010)

Acute oral toxicity test of *T. diversifolia* leaf in Wistar rats showed a high LD_{50} of more than 5000 mg/kg body weight which suggests that methanol extract of *T. diversifolia* leaf may be generally safe for consumption even at 5000 mg/kg body weight. This is in line with the study of Ezeonwumelu *et al.,* (2012) who reported LD_{50} of *T. diversifolia* extract to be more than 10,000 mg/kg body weight. The high LD_{50} suggests that the methanol extract of the leaves of *T. diversifolia* may be generally regarded as safe. This may be the reason why it is popular use locally.

The result of antioxidant activities of different fractions of *T. diversifolia* using DPPH radical scavenging assay demonstrated dichloromethyl (DCM) fraction to have the highest antioxidant activities when compared with the standard, aqueous fraction and ethylacetate fraction of *T. diversifolia.* The DCM fraction was therefore used for study one because the pathophysiology of lead and other metals is via oxidative stress (Sharma *et al.,* 2012).

The Gas chromatography Mass Spectrometry result of methanol extract of *T. diversifolia* leaf revealed the presence of about 20 compounds. Out of these compounds, 3,7,11,15-tetramethyl

hexadec-2-en-1-ol (Phytol) is the antioxidant that has the highest peak percentage (14.8%). Phytol is a diterpene, a member of the group of branched-chain unsaturated alcohols (Gloerich *et al.,* 2007 and McGinty, *et al.,* 2010). It is the product of chlorophyll metabolism in plants; hence, phytol is abundantly available in nature. It is found in vitamin K, vitamin E, and other tocopherols (Vetter *et al.,*. 2012). Phytol was therefore used for study two.

5.2 Effects of methanol extract of *T. diversifolia* leaf on body and visceral organs on control and lead acetate-induced toxicity in male Wistar rat

No significant changes in body weight were observed in the entire groups treated with lead, *T. diversifolia* or the combination. This suggests that neither lead nor *T. diversifolia* plant has any significant effect on the weight of the animals. However, the weight of the liver, kidney, heart and spleen significantly increased in lead treated group. This is in line with previous studies which showed the toxicity of lead to visceral organs and its ability to store in soft tissue (Holstege *et al.,* 2013). In groups that were treated with 50 mg/kg + Pb and 100 mg/kg + Pb showed no significant changes in the weight of the liver, kidney, heart and spleen compared with the control. This shows that *T. diversifolia* may probably ameliorate the inflammation caused by lead. This corroborates the report of Owoyele *et al.,* (2004) that T. *diversifolia* has antiinflammatory potential.

5.3 Effects of methanol extract of *T. diversifolia* leaf on liver and kidney functions and haematological parameters on control and lead acetate-induced toxicity in male Wistar rat

Liver enzymes such as alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP) and Bilirubin can serve as markers of hepatocellular injury (Arika et al., 2016). All these enzymes significantly increased in lead-treated group. However, this was significantly reduced with the administration of *T. diversifolia*. This shows the liver protective and anti-inflammatory effects of *T. diversifolia*. This is in line with the report of Lin *et al.,*, (1993) which showed that hepatic lesion caused by CCL_4 were improved by treatment with *T. diversifolia* extract.

Photomicrograph of liver section in lead acetate-treated group showed severe haemorrhage within the parenchyma, vessles are congested, there are sinusoids with lysed cells. While in

control, 50 mg/Kg TD and 100 mg/Kg TD treated groups showed normal architecture. The central venules and portal tracts appear normal, the hepatocytes showed normal morphology and the sinusoids appear normal. In 50 mg/Kg TD+Pb and 100 mg/Kg TD+Pb there are mild infilteration of inflammatory cells and moderate congestion of central venules. This demonstrated the ameliorative and protective effects of methanol extract of *Tithonia diversifolia* leaf.

Urea and creatinine significantly increased in lead-treated group compared with the control, while the administration of *T. diversifolia* extract reduces the level of urea and creatinine in the blood. Increase in blood creatinine and urea is an indication of kidney damage, ameliorating this with *T. diversifolia* treatment indicates that is kidney protective. This is in contrast with the submission of Passoni *et al.,*. (2013) which stated that administration of *T. diversifolia* extract causes kidney damage. This difference may be as a result of different fractions of *T. diversifolia* used during the study.

Increase in some heamatological parameters (RBC, heamoglobin, WBC and plateletes) were observed in *T. diversifolia* treated groups. This is in line with the report of Owoyele *et al.*, (2015). Increase in RBC might indicate its ability to stimulate the production of erythropoietin from the kidney which inturn induce the production of red blood cells. Significant increase in WBC count is an indication that *T. diversifolia* might boost immune system.

5.4 Effects of methanol extract of *T. diversifolia* leaf on reproductive functions of lead acetate-induced toxicity in male Wistar rat

No significant changes were observed in the relative reproductive organ weight i.e, there were no significant changes in the relative weight of the testes, epididymis, seminal vesicle and prostate gland.

There was a significant reduction in all sperm parameters and kinetics in lead treated group. The negative effects of lead on sperm parameters have been reported by several authors (McGivern *et al.*, 1991; Singh, *et.al,* 1993; Nadia *et al.,* 2013). The mechanism by which lead reduces sperm parameters are via the interruption of hypothalamic control of pituitary gland

which in turn reduces the secretion of testosterone and affects spermatogenesis (Sokol, 1987; Singh *et al.,* 1993; Nadia *et al.,* 2013) and generation of oxidative stress i.e increase in ROS and RNS, and reduction in the antioxidant enzymes (Mohsen *et al.,* 2011; Anjum and Reddy, 2015). The negative effects of lead were however ameliorated with the administration of methanol extract of *T. diversifolia* leaf. This result is in line with the submission of Ajayi *et al.,* 2009 which showed that inclusion of *T. diversifolia* leaf meal mixture diet supported optimum caudal epididymal sperm characteristics and testicular morphometrics in rabbits. Ajayi *et al.,*. (2012) equally reported a significant increase in percentage sperm profiles of the rats treated with 50 mg/kg of aqueous extract of *T. diversifolia* leaf. This result is in contrast with the report of Olukunle *et al.,* (2015) which showed a decrease in sperm parameters at a dose of 1600 mg/kg. This difference may be due to the fraction of the extract, the dosage used and the period of administration. The ability of *T. diversifolia* to reverse lead toxicity may also be due to its ability to scavenge free radicals as a result of its antioxidant properties. Extract of *T. diversifolia* may therefore promote the process of spermatogenesis.

The decrease in FSH, LH and Testosterone in lead treated group is in line with previous studies (Gabuchyan, 1987, Chattopadhyay *et al.,*, 2005, Al-Attar 2011 and Sharma, *et al.,* 2012). These decreases in hormones were ameliorated with the administration of antioxidant fraction of methanol extract of *T. diversifolia* leaf. This suggests that *T. diversifolia* leaf might stimulate the hypothalamus for the discharge of GnRH which in turn increases the production of FSH and LH and then Testosterone. Together with gonadotropins, testosterone is necessary for the development, growth and normal functioning of the testes, male accessory reproductive glands and spermatogenesis (Prins *et al.,* 1991; Weinbauer *et al.,* 2010). The secretion of testosterone by the Leydig cells is dependent upon the secretion of LH by the pituitary gland (Uzun *et al.,* 2009). Therefore, methanol extract of *T. diversifolia* leaf stimulates steroidogenic enzymes activities in Leydig cells and in turn improve spermatogenesis.

Normal cytoachitecture of the testis is essential for the maintanance of optimal reproductive function. Testicular achitecture of lead acetate-treated group showed abnormal seminiferous tubules with maturation arrest and the lumen appear widened without spermatozoa. This is in line with the earlier report that lead induced testicular damage and alter testicular tissues (Muthu and Krishnamoothy, 2012; Hamadouche *et al.,* 2013). Administration of *Tithonia*

diversifolia ameliorated the negative effects of lead acetate by showing normal seminiferous tubules with normal spermatogonia cell, the lumen appear normal with presence of spermatozoa. The interstitial spaces and leydig cells equally appeared normal.

5.5 Effects of methanol extract of *T. diversifolia* leaf on testicular lipid peroxidation and antioxidant status on control and lead acetate-induced toxicity in male Wistar rat

Reactive oxygen species (ROS) are autonomously generated in cells during metabolism and are capable of causing oxidative damage when produced in immoderate quantities (Aseervatham *et al.,* 2014). ROS have been implicated in the pathogenesis of a wide variety of human diseases (Jeong *et al.,* 2007). Lead-treated group demonstrated a significant increase in testis and liver malondialdehyde (MDA), and significant reduction in superoxide dismutase (SOD) and Catalase (CAT) in both testis and liver. However, these were ameliorated with the administration of methanol extract of *T. diversifolia* leaf. This shows the antioxidant properties of methanol extract of *T. diversifolia* leaf. The increase in MDA concentration in the testis and liver suggests lipid peroxidation since MDA is a marker of lipid peroxidation which is an index of the extent of oxidative damage to cellular structure (Sharma and Agarwal, 1996). Catalase is an enzyme found mainly in mitochondria and peroxisomes that catalysis the decomposition of hydrogen peroxide to water and oxygen (Rodrigues *et al.,* 2013). The SOD enzyme plays a key role in detoxifying superoxide anions in hydrogen peroxide and oxygen. It can scavenge the superoxide anion by catalysing it to H_2O_2 and O_2 which prevents the oxidative stress-induced cellular damage (Kiasalari *et al.,* 2013). A significant increase in antioxidant enzymes with the administration of *T. diversifolia* and a significant decrease in MDA when compared with lead acetate-treated alone suggest that *T. diversifolia* leaf has potent antioxidant enzymes that can scavenge free radicals and prevent oxidative stress. This is in line with previous studies that suggest that; *Tithonia diversifolia* is capable of scavenging reactive oxygen species (ROS) and reduce oxidative stress (Di Giacomo *et al.,* 2015; Mayara *et al.,* 2016).

5.6 Effects of phytol on reproductive functions of lead acetate-induced toxicity in male Wistar rat.

Tithonia diversifolia has been reported to contain terpenoids (John-Dewole and Oni, 2013). Phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) is a diterpene, a member of the group of branched-chain unsaturated alcohols (Gloerich *et.al*, 2007 and McGinty, *et.al*, 2010). It is present in vitamin K, vitamin E, and other tocopherols (Vetter *et al.,* 2012).

Phytol has no significant effect on reproductive organ weight and visceral organ weight. This may indicate that phytol is non toxic and has high stability (Muhammad *et al.,* 2015). Phytol significantly ameliorated the negative effects of lead acetate on hypothalamo- pituitary-gonadal-axis. There was a significant increase in GnRH, FSH, LH and testosterone in a group treated with both phytol and lead acetate when compared to lead acetate-treated group alone. GnRH is a regulatory hormone secreted from the hypothalamus which stimulates the biosynthesis and secretion of the gonadotropins. LH stimulates the leydig cells for the production of testosterone. While FSH stimulates sertoli cells for the production of androgen binding globulin (ABG) and inhibin. ABG binds with testosterone while inhibin helps to support spermatogenesis and inhibits production of GnRH, FSH and LH. Testosterone increases potency and libido and is necessary for initiation and process of spermatogenesis (Toone *et al.,* 1983; Isojarvi *et al.,* 1990).

5.7 Effects of phytol on hypothalamic and testicular lipid peroxidation and antioxidant status on control and lead acetate-induced toxicity in male Wistar rat.

The significant increase in MDA in hypothalamic and testicular tissue homogeneate in lead acetate-induced toxicity was significantly ameliorated with the administration of phytol thereby reducing oxidative stress. Phytol has been shown to have a significant reduction in TBARS production and have a scavenging activity on hydroxyl radicals and nitrite production thereby inhibiting the cell damage caused by this radical (Santos *et al.,,* 2013). Hydroxyl radical (OH^-) is an extremely reactive species capable of causing damage to DNA, proteins and lipids (Huang *et al.,* 2005; Shukla *et al.,* 2009; Serafini *et al.,* 2011). This showed that phytol's antioxidant potential can protect lipid biomolecule (Serafini *et al.,* 2011). Phytol equally ameliorated the negative effects of lead acetate on antioxidant enzymes (SOD, CAT

and reduced GSH). Phytol is a known antioxidant (Costa *et al.,* 2012b) and capable of increasing GSH level (Santos *et al.,* 2013). The antioxidant activity of phytol and its ability to reduce free radicals may be attributed to its structural feature. The hydroxyl group (OH) present in phytol molecule might probably react with free radical, donates hydrogen atom with unpaired electron (H), converting free radicals into less reactive species (Lima and Cardoso, 2007; Guimaraes *et al.,* 2010)

5.8 Effects of phytol on inflammatory biomarkers (TNF-α, IL-1β and nitrotyrosine) on control and lead acetate-induced toxicity in male Wistar rat.

There was a significant increase in TNF-α, IL-1β and nitrotyrosine in lead acetate-treated group. This was however reversed with the administration of phytol. This is in line with the report of Santos *et al.,*. (2013) and Elmazar *et al.,* (2013), that phytol has the ability to decrease tumour necrosis factor alpha (TNF-α) and IL-1β level. Significant reduction in nitrotyrosine with the administration of phytol indicates that phytol reduces nitrite stress caused by lead acetate. TNF-α is a multifunctional cytokine with effects on pro-inflammatory response (Fiers, 1991), immunoregulatory response (Beutler, 1995) and apoptosis (Baker and Reddy, 1998). IL-1β is known to cause inflammation and induce the expression of pro-inflammatory peptides including TNF-α (Dinarello, 1996). Increase in TNF-α and IL-1β observed in this study may suggest an injury in the testes as previously discussed by Lysiak, (2004). TNF-α and IL-1β are apoptotic enhancers that are released during inflammation. Accumulation of these can lead to neuronal cell death in the hypothalamus and in turn decrease the release of GnRH. Administration of Phytol decreased TNF– α and IL-1β.

All parameters in groups treated with Phytol appear to be within normal physiological range. This is in line with earlier report that Phytol and its derivatives have no cumulative inflammatory or toxic effects even in immuno-compromised mice (Chowdhury and Ghosh, 2012).

5.9 Conclusion

Administration of *Tithonia diversifolia* ameliorated the reproductive toxicity induced by lead. Phytol, a diterpene alcohol is an active component of *Tithonia diversifolia* which possesses potent antioxidant and anti-inflammatory activities. It is suggested that *Tithonia diversifolia* and phytol ameliorated lead-induced reproductive toxicity through mitigation of oxidative stress. Phytol is therefore, a compound that has potential in treatment of Lead-induced reproductive toxicity.

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5.10 Contribution to knowledge:

1. *Tithonia diversifolia* possesses potent anti-inflammatory and antioxidant properties.
2. Both *Tithonia diversifolia* and Phytol were found to be non-toxic in rats.
3. *Tithonia diversifolia* and Phytol can protect against reproductive toxicity induced by lead acetate.
4. *Tithonia diversifolia* and Phytol enhance reproductive functions by improving hypothalamo-pituitary-gonadal hormones which in turn improve sperm quality.
5. Phytol can ameliorate inflammation induced by lead.

6.0 REFERENCES

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7.0 Appendices

Appendice I

Assay procedure for Gonadotropin Releasing Hormone (GnRH)

1. Add **Standard working solution** of different concentrations to the first two columns: Each concentration of thesolution is added into two wells side by side (50 µL for each well). Immediately add 50µL of **BiotinylatedDetection Ab working solution** to each well. Cover the plate with sealer provided in the kit. Incubate for 45 min at37℃.

Note: solutions should be added to the bottom of micro ELISA plate well, avoid touching the inside wall and foaming aspossible.

2. Aspirate or decant the solution from each well, add 350 µL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3times.

Note: a microplate washer can be used in this step and other wash steps.

3. Add 100 µL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.

4. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.

5. Add 90 µL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C.Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but notmore than 30 min.

6. Add 50 µL of **Stop Solution** to each well. Note: the order to add stop solution should be the same as the substrate solution.

7. Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm.

Appendice II

Assay Procedure for serum testosterone estimation in rats

1. The desired number of coated wells were secured in the holder.

2. 10 µl of standard, specimens, and controls were dispensed into appropriate wells.

3. 50 µl of rabbit anti–testosterone reagent dispensed to each well.

4. Thoroughly mixed for 30 seconds. It is very important to mix them completely.

5. 100 µl of testosterone–HRP conjugate reagent dispensed into each well.

6. Incubated at 37 ºC for 90 minutes.

7. Rinsed and flicked the microwells for about 5 times with washing buffer (IX).

8. 100 µl of TMB substrate dispensed to each well. Gently mixed for 10 seconds.

9. Incubated at room temperature (18–22 ºC) for 20 minutes.

10. The reaction was stopped by adding 100 µl of stop solution to each well.

11. Gently mixed for 30 seconds. It is important to make sure that all the blue colourchanges to yellow colour completely.

12. The absorbance was read at 450 nm with a microtiter well reader within 15 minutes.

Appendice III

Assay procedures for serum luteinizing hormone estimation in rats

1. The desired number of coated wells was secured in the holder.

2. 50 µl of standard, specimens, and controls were dispensed into appropriate wells.

3. 100 µl Enzyme conjugate reagent was dispensed into each well.

4. Thoroughly mixed for 30 seconds. It is very important to have a complete mixing in this setup.

5. Incubated at room temperature (18–22 ºC) for 60 minutes.

6. The incubation mixture was reconstituted by flicking plate content into a waste container.

7. Rinsed and flicked the microtiter wells 5 times with washing buffer (1X).

8. The wells were sharply striked onto absorbent paper or paper towels to remove all residual water droplets.

9. 100 µl of TMB solution was dispensed into each well. Gently mixed for 5 seconds.

10. Incubated at room temperature in the dark for 20 minutes.

11. The reaction was stopped by adding 100 µl of stop solution to each well.

12. Gently mixed for 30 seconds. It is important to make sure that all the blue colourchanges to yellow colour completely.

13. Optical density was read at 450 nm with a microtiter well reader within 15 minutes.

Appendice IV

Assay procedures for serum follicle stimulating hormone estimation in rats

1. The desired number of coated wells were secured in the holder.

2. 50 µl of standard, specimens, and controls are dispensed into appropriate wells.

3. 100 µl Enzyme conjugate reagent was dispensed into each well.

4. Thoroughly mixed for 30 seconds. It is very important to have a complete mixing in this setup.

5. Incubated at room temperature (18–22 ºC) for 60 minutes.

6. The incubation mixture was removed by flicking plate content into a waste container.

7. Rinsed and flicked the microtiter wells 5 times with washing buffer (1X).

8. The wells were striked sharply onto absorbent paper or paper towels to remove all residual water droplets.

9. 100 µl of TMB solution was dispensed into each well. Gently mixed for 5 seconds.

10. Incubated at room temperature in the dark for 20 minutes.

11. The reaction was stopped by adding 100 µl of stop solution to each well.

12. Gently mixed for 30 seconds. It is important to make sure that all the blue colourchanges to yellow colour completely.

13. The optical density was read at 450 nm with a microtiter reader.

Appendice V

Assay procedure for serum nitrotyrosine

1. Add **Standard working solution** of different concentrations to the first two columns: Each concentration of thesolution is added into two wells side by side (50 µL for each well). Immediately add 50µL of **BiotinylatedDetection Ab working solution** to each well. Cover the plate with sealer provided in the kit. Incubate for 45 min at37℃.

Note: solutions should be added to the bottom of micro ELISA plate well, avoid touching the inside wall and foaming aspossible.

2. Aspirate or decant the solution from each well, add 350 µL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times.

Note: a microplate washer can be used in this step and other wash steps.

3. Add 100 µL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.

4. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.

5. Add 90 µL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C.Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but notmore than 30 min.

6. Add 50 µL of **Stop Solution** to each well. Note: the order to add stop solution should be the same as the substrate solution.

7. Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm.

Appendice VI

Assay procedure for Serum Tumor necrotic factor-alpha

1. Add **Standard working solution** of different concentrations to the first two columns: Each concentration of thesolution is added into two wells side by side (100 µL for each well). Add samples to other wells (100 µL for eachwell). Cover the plate with sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to thebottom of micro ELISA plate well, avoid touching the inside wall and foaming as possible.

2. Remove the liquid of each well, do not wash. Immediately add 100 µL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.

3. Aspirate or decant the solution from each well, add 350 µL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against

clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.

4. Add 100 µL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.

5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.

6. Add 90 µL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C.Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but notmore than 30 min.

7. Add 50 µL of **Stop Solution** to each well. Note: the order to add stop solution should be the same as the substrate solution.

8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

Appendice VII

Assay procedure for serum interleukin-1 beta

1. Add **Standard working solution** of different concentrations to the first two columns: Each concentration of thesolution is added into two wells side by side (100 µL for each well). Add samples to other wells (100 µL for eachwell). Cover the plate with sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to thebottom of micro ELISA plate well, avoid touching the inside wall and foaming as possible.

2. Remove the liquid of each well, do not wash. Immediately add 100 µL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.

3. Aspirate or decant the solution from each well, add 350 µL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.

4. Add 100 µL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.

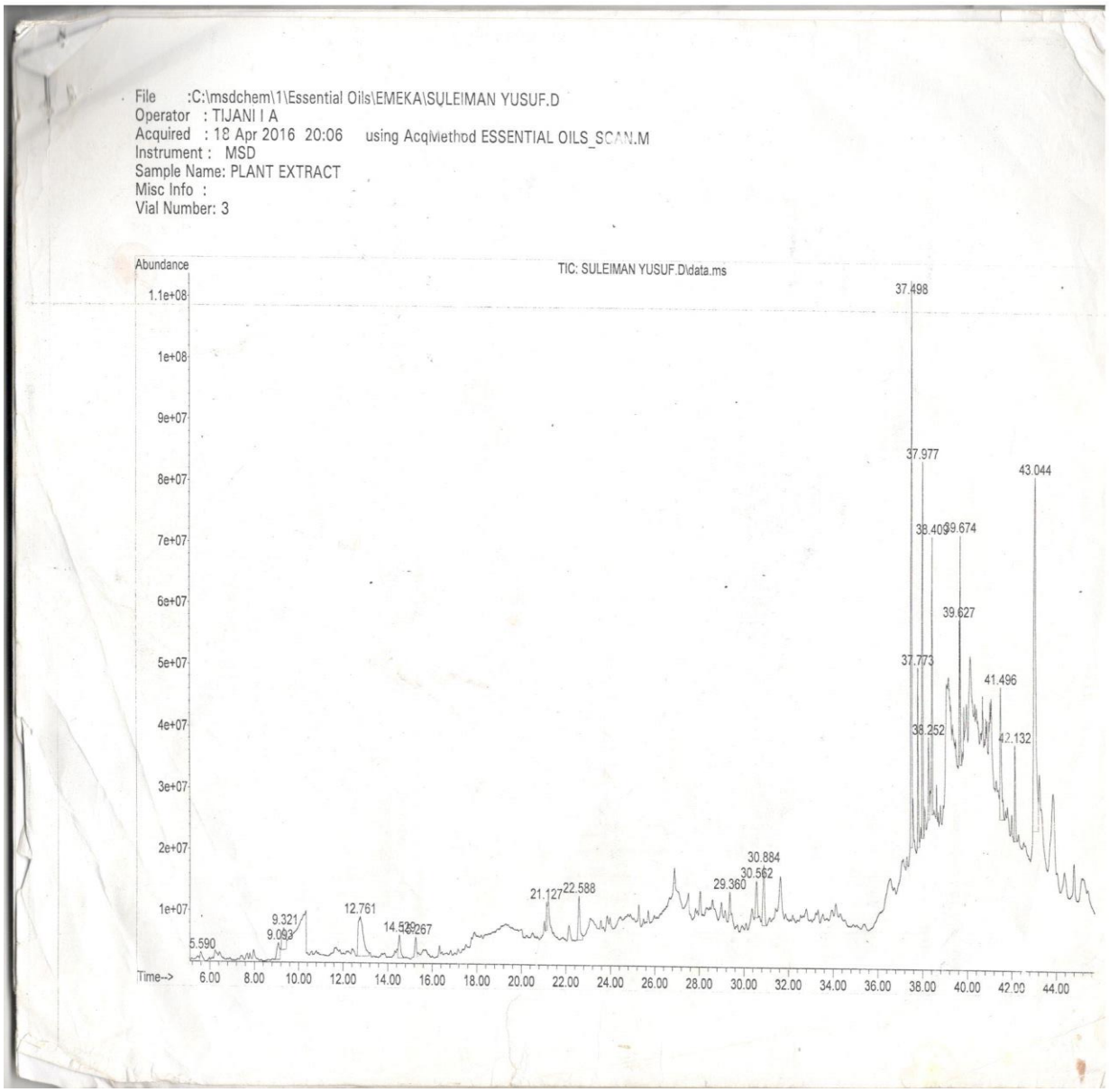
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.

6. Add 90 µL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C.Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but notmore than 30 min.

7. Add 50 µL of **Stop Solution** to each well. Note: the order to add stop solution should be the same as the substrate solution.

8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

Appendice VIII



GC-MS analysis of methanol extract of *Tithonia diversifolia* leaf