REPRODUCTIVE STUDIES AND SAFETY ASSESSMENT OF AN ETHANOLIC SEED EXTRACT OF *PICRALIMA NITIDA* ((STAPF) TH. &H. DURAND)

A THESIS SUBMITTED IN FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF PHILOSOPHY

In the

Department of Pharmacology,
Faculty of Pharmacy and Pharmaceutical Sciences

By

LYDIA FRANCISCA OTOO

KWAME NKRUMAH UNIVERSITY OF SCIENCE & TECHNOLOGY,

KUMASI

MARCH, 2015

DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST. This work has not been submitted for any other degree.

…………………………..

Lydia Francisca Otoo

(PG 7866412)

……………………………

Rev. Prof. Charles Ansah

Supervisor

…………………………….

Dr. George Asumeng Koffuor

Supervisor

DEDICATION

To Austin junior

The task is not so much to see that which no one has yet seen, but to think about that which no one has yet thought about; that which everyone sees.

ABSTRACT

Almost all parts of *Picralima nitida* are widely used in Ghana and other West African countries for the treatment of various ailments, including diabetes, malaria, stomach problems, pneumonia, jaundice, measles, cough, typhoid fever and gonorrhea. Captured in the population patronizing the plant product are men and women of child bearing age. Womenare susceptible to disorders during pregnancy and sometimes require treatment to protect themselves and theirdeveloping fetus. The Thalidomide catastrophe sparked the focus into reproductive studies to ensure that xenobiotics are not toxic to both parents and unborn child.The effect of the ethanolic seed extract of *Picralima nitida* on reproduction

in both males and females and its safety for use was,therefore, investigated in mice and chicks.

The effect ofthe extract on reproductive indices in female mice was studied after pretreatment with 30 – 300 mg/kg orally for fourteen days prior to mating. Possible alterations in estrous cycle or hormonal imbalance after ingestion of the extract was also studied.The chick uterotrophic assay to assess the estrogenic effect of the extract was conducted after seven-day treatment of chicks with 30-300 mg/kg of extract. The number of mounts, sniffing and licking of female, genital and non-genital grooming in an orientation behaviour test to study the aphrodisiac potential of the *Picralima nitida* extract in male mice when treated with the extract was also conducted. Androgenic effect of the extract was also assessed by studying comb growth in White Leghorn chicksafter a six-day treatment with 50-500 mg/kg. The safety for use of the extract was also assessed. Results indicated that mating index and gestation period were not affected. At a dose of 300 mg/kg female fertility decrease from 100 % to 25%.Litter size wasmoderately reduced with increasing doses.

Daily administration of the extract to female rats prolongedsignificantly($p<0.001$) the duration of estrous at a dose of 300 mg/kg.The extract showed estrogenic effect as there was significant ($p<0.01$) increase in uterus weight to body ratio.The extract caused a significant boost in attempted mounts($p<0.001$), sniffing and licking of female($p<0.05$), genital and non-genital grooming($p<0.05$)at a dose of 300 mg/kg. There was however no significant ($p>0.05$) changes in the above parameters upon continuous administration of the extract for 14 days. Although sperm numbers diminished significantly ($p<0.001$) especially at a dose of 300 mg/kg, sperm motility, viability and morphology remained unchanged.The extract caused a significant ($p<0.01$) dose dependent increase in comb

growth, similar to, Testosterone propionate. However, the extract antagonized the effects of testosterone when administered together.Safety studies revealed insignificant ($p>0.05$) changes in haematological and biochemical parameters, making the extract safe up to the doses used.Per the findings the ethanol seed extract of *Picralima nitida*has antifertility effects in females and enhances sexual ability in males.It could be detrimental to females who have plans of conception whilst using the extract to treat a particular ailment but inure to the benefit of others who desire contraception. In males, the extract could be used as an aphrodisiac.

ACKNOWLEDGEMENTS

All praise and thanks to God Almighty for His unlimited mercies and guidance.

It is a matter of pride and utmost satisfaction for me to pay the most sincere gratitude to Rev. Professor Charles Ansah and Dr. George Asumeng Koffuor from the depth of my heart for their consistent guidance, zealous supervision, valuable advice and discussion throughout the period of this research work. Their inspiration, encouragement and sympathetic attitude enabled me to overcome the obstacles during my research work.

I am also grateful to Dr. Kwesi Boadi Mensah for his inputs and invaluable suggestions.

My special thanks to Mr Thomas Ansah and Prince Okyere of the Department of Pharmacology for their technical assistance.

It is also my pleasure to acknowledge the good wishes of my colleagues and friends especially Charles Benneh, who helped me during this project.

Finally, to my husband and family, I want to say God richly bless you all for your fervent prayers, love and support during this entire study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE INTRODUCTION

1.1 OVERVIEW

Natural products have long been recognized as an important source of therapeutically effective medicines. It is known that natural-product structures have great chemical diversity, biochemical specificity, and other molecular properties that make them favourable lead structures (Koehn & Carter, 2005). They play an invaluable role in the management of diseases worldwide especially in developing countries. They offer the advantages of easy accessibility, ready availability and affordability as compared to the conventional drugs. Most advocates of herbal preparations use them under the potentially false assumption that “natural” is synonymous with “safe”. Herbal preparations may have potent pharmacological effects, hence the risk of adverse effects should be considered (Ernst, 2007). Recent studies have shown that certain herbal preparations can be toxic at specific doses. This goes to emphasize the famous Paracelsus theory that "all substances are poison, and nothing is without poison; only the dose permits something not to be poisonous”.

In the late 1950s and early1960s, thalidomide, a drug that was produced as a sedative and as an anti-emetic for pregnant women was found to be teratogenic.Teratogens are compounds that cause insult to a developing conceptus, and in doing so, induce the development of congenital defects (Keeler, 1984). Thalidomide caused the birth of infants with phocomelia, deformed eyes, hearts, alimentary and urinary tracts, blindness and deafness(Hood, 2006).The results of this catastrophe aroused the development of more structured drug regulations addressing the possible adverse impact of any new chemical on the developing embryo and fetus.*Picralima nitida* has gained popularity in Ghana and other Western African countries for the treatment of various ailments. Notable among such ailments are malaria, stomach problems, pneumonia, jaundice, measles, cough, typhoid fever and gonorrhoea. Captured in the population patronizing the plant product are men and women of child bearing age.Women, especially are frequent herbal medicine users in many countries(Hare, 1993; Himmel *etal.*,1993; MacLennan *et al.*, 1996).They are susceptible to illnesses during pregnancy and sometimes require treatment to protect themselves and the foetus.In other circumstances, a woman may be taking a medication before she is aware of her pregnancy, or become pregnant while undergoing a treatment. Safe use of herbal medicines during pregnancy is not only aimed at the well-being of the expectant mothers, but relevant to the health of the foetus as well. However, only a few reports of the scientific evaluation of the safety of herbal medicines in pregnancy have been identified (Jurgens, 2003).

Despite the widespread use of *Picralima nitida*traditionally,there is very limited data on its effects on reproduction and foetal developmental and toxicity. Reproductive studies are geared at structural and functional alterations that affect reproductive competence whilst developmental studies assess the effect of the test agent on the first and second filial(F_1 or F_2)generations (Tyl & Marr, 2006).

This research,seeks to assess the effect of the ethanolic seed extract of*Picralima nitida* on reproduction in both male and female and its safety for use.

1.2 PICRALIMA NITIDA

1.2.1 **Description and Distribution**

Picralima nitida belongs to the Apocynaceae family. The species was primarily described and characterized by T. & H. Durand in 1909 (Meyer *et al.*, 2006). It is common in most forest regions of Africa; Ghana, Nigeria, Cameroon, Zaire, Tanzania, Democratic Republic of Congo and Uganda (Adjanohoun *et al.*, 1996; Keay *et al.*, 1964). *Picralima nitida* can flower and fruit throughout the whole year.Seeds can be dried and stored for about 2 years without losing the pharmacological activity.

It is a deciduous tree that can grow to about 30 m in height with a dense crown (Irvine, 1961). It has a bitter, hard pale yellow bark with a circumference of about 50 cm. The leaves at maturity are simple and entire; opposite; stipules absent; petiole 1-2 cm long; oblong to elliptical blade, cuneate base; acuminate apex and pinnately veined with tough 14-20 pairs of thin lateral veins. The leaves measure 6-20 cm long and 3-10 cm broad.

The white fragrant flowers are about 3cm long, bisexual, regular and open during the day. The solitary or couple fruits are ovoid, glabrous, smooth,yellow to orange and measure about 15 cm long and 10 cm in diameter at maturity. It has a hard outer layer,bearing many seeds. Seeds are flattened, ovate, smooth, brown to orange, 2.5-4.5 cm long and embedded in soft white pulp.

Figure 1.1 A picture of the dried seeds of *Picralima nitida*(Phytoextractum, 2013)

1.2.2 **Traditional Uses**

Almost all parts of *Picralima nitida* are very useful traditionally. Dosage form is either as a decoction or in powdered form. The seeds have a reputation as a treatment for malaria, febrifuge, leucorrhoea, abscesses, pneumonia, chest and stomach pains. The seeds are crushed and eaten with lemon juice to treat hernia, diarrhoea or vomiting. In Ghana, the seeds are chewed as a tonic and stimulant and its decoction used as an enema and analgesic.

The bark or root decoction is reported to cure jaundice, malaria,hernia,as a purgative and to cure sterility in men (Arens *et al.*,1982a;Ayensu, 1978).The leaf is taken orally or used topically to treat measles, otitis and guinea worm. Fruit decoction can be used to treat cough and typhoid fever.Seeds,fruit pulp and roots can be used for arrow poisoning. Wood obtained from the bark can be used for paddles,combs, walking sticks,pestles, mortars and spoons. In Ghana, immature fruits are pounded and scattered into water as fish poisoning.

1.2.3 **Previous Work on the Plant**

The aqueous extract of the *P. nitida* seed when analysed gave positive chemical reactions for glycosides, saponins, tannins, flavonoids, alkaloids, proteins and carbohydrates (Aguwa*et al*., 2001; Vladmir& Ludmila, 2001). From laboratory analysis, Meyer *et al*., (2006) reported that the active principle of *Picralima nitida* is formed by more than ten indole alkaloids(Henry & Sharp, 1927; Henry, 1949).Clinquart first isolated akuammine the major alkaloid of the plant (Clinquart, 1927). Other alkaloids that have been isolated from the seeds, bark and fruits includeakuammicine, akuammidine, akuammigine, pseudoakuammigine and picraline (Peralta *et al.*, 1990; Arens *et al.*, 1982b; Ezekwesili, 1983). At maturity, the seeds contain large amounts of alkaloids with akuammine, being the principal alkaloid.Their names derive from the local name "Akuamma" (Okunji *et al.*, 2005).

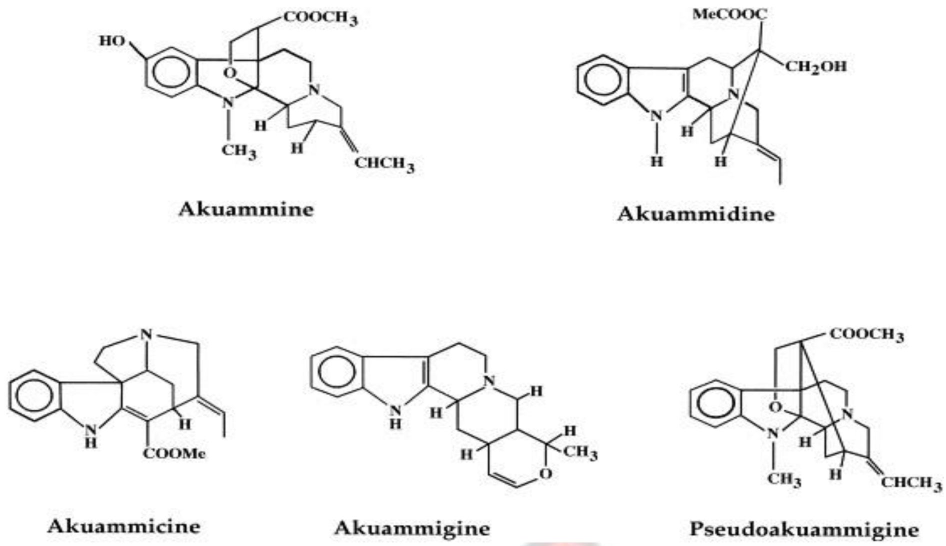


Figure 1.2Major alkaloids isolated from *Picralima nitida*

Pseudo-akuammigine, akuammine, akuammidine, and akuammicine isolated from the seeds have been reported to have anti-inflammatory effects in rodents (Duwiejua *et al.*, 2002; Obiri, 1994; Ezeamuzie *et al.*, 1994).The analgesic effect of some isolated alkaloids has been reported to act through the opioid receptors (Duwiejua *et al.*, 2002; AnsaAsamoah& Amposo, 1986; Menzies *et al.*, 1998).The seed oil has been reported to reduce blood sugar in rats when administered orally (Nwakile& Okore, 2011). The antidiabetic effect was demonstrated in both normoglycaemic and hyperglycaemic rabbits (Aguwa *et al.*, 2001; Okonta& Aguwa, 2007).The aqueous and ethanolic leaf extract have been reported to record high mortality rates against the larvae of *Anopheles gambiae*(Ubulom *et al.*, 2012). Also, constituents of *Picralima nitida* displayed pronounced inhibitory activities against asexual erythrocytic forms of *Plasmodiumfalciparum* in vitro (Francois *et al.*, 1996).Both aqueous and ethanolic leaf extracts exhibited antifungal effects on *A. flavus* and *C. albicans* (Ubulom *et al.*, 2011).The antibacterial studies performed on the stem bark and fruit rind confirmed the traditional use of the plant for various diseases. Extract was active against strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis* and *Escherichia coli*. (Nkere&

Iroegbu,2005; Obasi *et al.*, 2012). The antidiarrheal activity has also been reported (Kouitcheu *et al.*, 2006).

1.3 REPRODUCTIVE SYSTEM

Reproduction involves a wide range of physiological processes and associated behaviours involved in the production of the next generation and the survival of a particularspecies of animal (Senger, 2003). Processes involved in reproduction mainly include:

- Gametogenesis and the pre- and peri-pubertal changes leading to its onset
- Release of gametes (sperm transport and maturation, penile erection and ejaculation, copulation between a male and female of same species and ovulation of oocytes)
- Formation of zygote (sperm storage, capacitation, and other processes leading to fertilization of a single sperm with an ovum).
- Embryonic and foetal development during pregnancy(gestation) where there is the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation, neurulation and organogenesis.
- Parturition and lactation

1.3.1 **Male Reproductive Anatomy and Physiology**

The anatomical structures associated with reproduction in the male usually include paired testes located outside the abdominal cavity in most species; an excurrent duct system (efferent ducts, paired epididymis, vas deferens and urethra); accessory sex glands (ampullae, seminal vesicles, prostate and bulbourethral glands); a scrotum and its associated thermoregulatory functions to protect the testes from mechanical and thermal insult and a penis with a mechanism for protrusion, erection, emission of glandular secretions and ejaculation of sperm. The primary functions of the testis are spermatogenesis and steroidogenesis(Senger, 2003).

The main role of the epididymis are transport and sustenance of sperm, reabsorption and secretion of fluid; spermatozoal acquisition of motility and fertile potential; recognition and elimination of defective spermatozoa; sperm storage prior to ejaculation and secretory contributions to the seminal fluid (Sutovsky *et al.*, 2001). The epididymal transit time varies with different species, but is generally approximately 7–14 days in length, depending on several factors including ejaculation frequency.

There are a number of accessory sex glands that contribute to the composition of the seminal fluid in mammals. These glands include the ampullae, seminal vesicles, prostate and bulbourethral glands (Senger, 2003).Laboratory rodents have an additional gland referred to as the preputial gland, which appears to have a role in the production of pheromone (Creasy & Foster, 2002). These accessory sex glands in the male are generally considered to be androgen dependent, with conversion of testosterone to dihydrotestosterone occurring in the prostate and seminal vesicles of many species(Creasy& Foster, 2002; Senger, 2003). The weights of the accessory sex glands can be used as an indirect measure of testosterone concentrations or exposure to antiandrogens(Thomas& Thomas, 2001; Senger, 2003).

The external genitalia of the male consist of the penis, the prepuce, which protects the penis from environmental and mechanical injury, and the scrotum for testes positioned outside of the body. Penile structure is extremely species variable, with some species even having a special penile bone, but the shaft of the penis generally consists of erectile tissue (corpus cavernosum and corpus spongiosum) which surrounds the pelvic urethra. The glans penis is homologous to the female clitoris, and stimulation of the glans is the primary factor involved in the initiation of ejaculation (Senger, 2003). The scrotum protects the testes from mechanical injury and, in conjunction with the tunica dartos, cremaster muscle and pampiniform plexus, plays a major thermoregulatory role with respect to temperature sensitive, testicular spermatogenesis. Xenobiotics, which cause hyperthermia (ergopeptine alkaloids), have the potential to adversely affect spermatogenesis.

Spermatozoa are highly specialized haploid cells equipped with a self-powered flagellum to facilitate motility, as well as an acrosome to mediate penetration of the zona pellucida. Spermatogenesis occurs within the seminiferous tubules and consists of all the changes germ cells undergo in the seminiferous epithelium in order to produce adequate numbers of viable spermatozoa each day and to continuously replace spermatogonial stem cells(Thomas& Thomas, 2001; Senger, 2003). The duration of spermatogenesis varies with species but generally ranges between 4 and 8 weeks in domestic and laboratory animals and is approximately 75 days in humans.Spermatogenesis can be subdivided into three phases or stages referred to as proliferation, meiosis and differentiation. During each of these phases, sperm precursors or male germ cells undergo specific, stepwise changes as they develop into spermatozoa which will eventually be released into the excurrent duct system.

- Proliferation:This phase is also termed as themitosis or spermatocytogenesis and occurs within the basal compartment of the seminiferous tubule. Proliferation represents all of the mitotic divisions involving spermatogonia (Senger, 2003). A large number of B-spermatogonia result from the mitoses of several generations of spermatogonia (Senger, 2003; Genuth, 2004). Stem cell renewal is accomplished during proliferation by the reversion of some spermatogonia to more primitive germ cells (Senger, 2003). Germ cell mitosis during spermatogenesis ends with the transformation of B-spermatogonia into primary spermatocytes, and this process is particularly susceptible to toxicants, such as chemotherapeutic agents and radiation, which target rapidly dividing cells.

- Meiosis: This process takes place within the adluminal compartment of the seminiferous tubules and involves the participation of primary and secondary spermatocytes in a total of two meiotic divisions. The chromosomal reduplication, synapsis and crossover, as well as cellular division and separation, which occur during this phase of spermatogenesis, are extremely complex and guarantee genetic diversity (Senger, 2003; Genuth, 2004). The meiosis phase of spermatogenesis is considered by some to be most susceptible to toxic insult (Thomas& Thomas, 2001)and ends with the production of haploid round spermatids (Senger, 2003).

- Differentiation: Spermatozoa have been characterized as sophisticated,selfpropelled packages of DNA and enzymes(Senger, 2003). Differentiation or spermiogenesis is concerned with all the changes occurring within the adluminalcompartment, which transform round spermatids intospermatozoa possessing an acrosome for penetration ofthe zona pellucida and a tail or flagellum to facilitatemotility (Genuth, 2004).Differentiation can be subdividedinto the Golgi, cap, acrosomal and maturationphases, which correspond respectively to acrosomalvesicle formation; spreading of the acrosomal vesicle overthe nucleus; elongation of the nucleus and cytoplasm andfinal assembly involving the formation of the post nuclearcap organization of the tail components (Senger, 2003).Following the nuclear and cytoplasmic reorganizationwhich characterizes the changes to germ cells duringspermiogenesis, differentiated spermatozoa are releasedfrom Sertoli cells into the lumen of the seminiferous tubulesby a process referred to as spermiation. The complexsignaling pathways and genomic imprinting involved inregulating the differentiation of round spermatids intospermatozoa can serve as potential targets for Endocrine Disrupting Chemicals (EDCs).

1.3.1.1 Endocrine Regulation of Spermatogenesis

In males, the gonadotropin releasing hormone (GnRH) surge center is diminished, unlike in females where the hypothalamus has fully developed tonic and surge centers for GnRH, especially prior to ovulation. Thus the anterior pituitary gland of the male does not experience surges in GnRH stimulation (Senger, 2003). This gender-specific alteration in the hypothalamus facilitates the normal endocrine milieu which maintains continuous spermatogenesis and stimulates normal sexual behavior. The tonic pulsatile release of GnRH induces the anterior pituitary to produce pulses of leutinizing hormone(LH) and follicle stimulating hormone(FSH) several times during the day and facilitates adequate LH-dependent testosterone production and, depending on the species, normal FSHdependent Sertoli function, both of which are essential for spermatogenesis to occur continuously in the seminiferous tubules(Senger, 2003; Genuth, 2004). Testosterone stimulates Sertoli cells to produce several androgen-regulated proteins, including androgen-binding protein, which is required for spermatogenesis(Creasy& Foster, 2002; Senger, 2003).

Positive and negative feedback mechanisms help maintain an endocrine environment which is conducive to normal male reproductive function. The Sertoli cell can produce activin and inhibin which respectively increase and decrease the secretion of FSH by gonadotropes and, in some species, GnRH release from the hypothalamus(Creasy&Foster, 2002). Testosterone, dihydrotestosterone (DHT) and estradiol all provide negative feedback to the hypothalamus with respect to GnRH release, and testosterone can also directly inhibit LH secretion by gonadotropes(Creasy& Foster, 2002; Senger, 2003). Xenoestrogens and xenoandrogens have the potential to disturb the hypothalamic–pituitary– gonadal axis (O‟Donnell *et al.*, 2001). It is currently thought that antiandrogens and a variety of other xenobiotics can interfere with these feedback loops and possibly other endocrine pathways, resulting in Leydig or interstitial cell hyperplasia(O'Connor *et al.*, 2000; Thomas& Thomas, 2001).

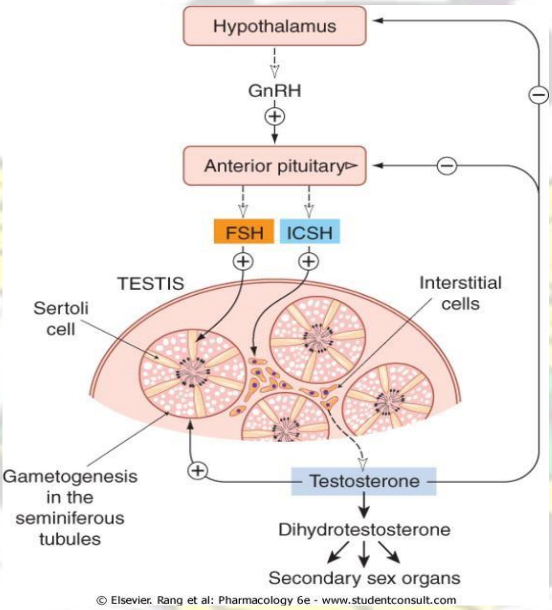


Figure 1.3Hormonal Control of the Male Reproductive System(Rang *et al.*, 2003)

1.3.2 **Female Reproductive Anatomy and Physiology**

The female reproductive tract generally consists of paired ovaries and the tubular genitalia, which comprises the paired oviducts and uterine horns contiguous with a uterine body and cervix, vagina, vestibule and vulva(Senger, 2003; Evans *et al.,* 2007). These organs function to produce the oocyte, facilitate its fertilization, provide a conducive environment for embryonic and fetal development and transport the foetus to the external environment.The primary functions of the ovary are oogenesis andsteroidogenesis.The ovaries of most domestic mammals consist ofa peripheral parenchymatous zone, containingvarious stages of follicular and luteal gland developmentand a central vascular zone, comprised of collagenousconnective tissue rich in blood vessels(Senger, 2003). Follicles are the structural and functional unit of the ovary and they are classified as primordial, primary, secondary and tertiary folliclesbased on their stage of development(Evans *et al.,* 2007). Aprimary oocyte surrounded by a single, flattened celllayer is a primordial follicle. A basal lamina separates thesingle layer of what will become granulosa cells from theadjacent stromal tissue which eventually develops into the theca cells. The granulosacells homologous to the Sertoli cells in the testis,and the theca interna cells are the female equivalent of theLeydig cells (Senger, 2003). Following the appropriateendocrine stimulation, primordial follicles are recruitedto undergo possible further differentiation into estrogenproducingantral follicles and ultimately ovulation, whichresults in the release of a secondary oocyte and formation of a corpus luteum (CL) whichproduces progesterone(Ginther, 1992; Senger, 2003; Evans *et al.*, 2007).

1.3.2.1 Endocrine Regulation of Female Reproductive System

The follicular and luteal phases of the estrous cycle describe the predominant ovarian structures and the corresponding gonadal steroid concentrations which result from the follicular secretion of estrogens or the luteal secretion of progesterone, respectively (Ginther, 1992; Senger, 2003; Evans *et al.*, 2007). Both the follicular and luteal phases can generally be further subdivided into two stages each, proestrous and estrous for the follicular phase and metestrous and diestrous for the luteal phase (Senger, 2003). Proestrus represents the period of transition from the diestrus dominance of progesterone to the dominance of estrogens during estrus, while metestrus represents the opposite shift in the endocrine milieu(Evans *et al.,* 2007; Senger, 2003)

The general sequence of endocrine and morphological changes occurring during the estrous cycle involves a variety of positive and negative feedback loops affecting the hypothalamic–pituitary–gonadal axis and leads to the development of antral follicles, the primary source of estrogens, and, eventually, the formation of corpora lutea, which produce progesterone. When females are exhibiting reproductive cyclicity, there are cyclic alterations in the pattern of hypothalamic GnRH secretion from the tonic and surge centers, which interact with the anterior pituitary to influence the relative amounts of FSH and LH secreted by anterior pituitary gonadotropes. Over the course of the ovulatory season, many primordial follicles leave the reserve pool in a cyclic fashion and enter the active pool of follicles undergoing growth and differentiation and eventually atresia or ovulation (Evans *etal.,* 2007; Senger, 2003). The oocyte in the developing follicle grows in size, the zona pellucida is formed and the granulosa cells surrounding the oocyte undergo mitosis and further differentiation (Senger, 2003). A primary follicle is transformed into a secondary follicle when there are several layers of granulosa cells. Preantral follicles become tertiary follicles, when fluid from the granulosa cells of secondary follicles coalesces to form an antrum (Evans *et al.,* 2007). Cyclic increases in FSH concentrations facilitate recruitment of antral follicles. Granulosa cells can produce activin which is thought to provide positive feedback to the anterior pituitary, further increasing gonadotropic FSH secretion (Senger, 2003; Wilker& Ellington, 2006). Recruited antral follicles, which are gonadotropin sensitive, undergo several waves of follicular development beginning in metestrus and ending in proestrus(Ginther, 1992; Senger, 2003; Evans *et al.,*2007). The final wave of one or more dominant follicles, destined for ovulation, rather than atresia, produces the large amounts of estrogens typical of estrous and required for sexual receptivity and the preovulatory estrous surges in GnRH and LH secretion (Senger, 2003).

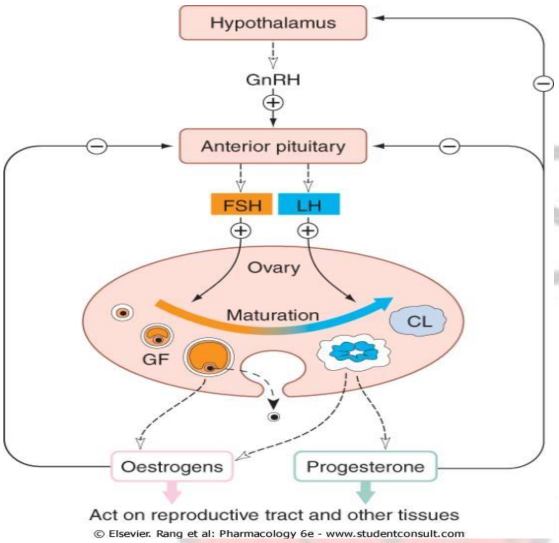


Figure 1.4Hormonal Control of the Female Reproductive System(Rang *et al.*, 2003)

The granulosa cells in the one or more dominant estrous follicles cease to divide shortly prior to ovulation and undergo further differentiation, with upregulation of LH receptors which will be responsive to the estrogen-induced preovulatory LH surge(Evans *et al.*, 2007; Senger, 2003). As LH increases, granulosa cells continue to convert pregnenolone to progesterone, but estradiol production decreases, resulting in a slight preovulatory decline in estradiol (Evans *et al.*, 2007). The preovulatory LH surge is associated with increased follicular pressure, degeneration of theca cells and weakening of the follicular wall, completion of the first meiotic division within the oocyte and, finally, ovulation of a secondary oocyte arrested in metaphase II (Senger, 2003; Evans *et al*., 2007).

1.3.3 **Reproductive and Developmental Toxicity**

1.3.3.1 Reproductive Toxicity

Reproductive toxicity represents adverse effects of xenobiotic exposure on the physiological processes, associated behaviours and anatomical structures that are involved in animal reproduction, especially in the parent generation. Often times, the effect of the agent may be primarily on the maternal system which then affects the developing organism and causes adverse effects. Major sectors affected are fertility, parturition and lactation.

Fertility:Fertility is one„s ability to conceive if the person is female or to induce conception if the individual is male. Fertility is not constant but changes with respect to time, particularly in females. Various factors affecting fertility include age, diet and natural products, weight, stress and even drugs.

Parturition: Xenobiotics have the ability to increase or decrease the onset and duration of parturition. Gestation period may be increased mainly by hormonal imbalances.In animals, the time interval between pup deliveries can be studied.

Lactation: If the fetus escapes the toxic effect, if any, of the xenobiotic, milk from the mother can serve as a source of exposure. Xenobiotics can also affect the quality of milk produced by the dam.

1.3.3.2 Developmental Toxicity

Developmental toxicity refers to any adverse effect on the developing organism associated with either pre-conception parental exposures to toxicants or post-conception xenobiotic exposures to the embryo, fetus or pre-pubertal offspring(Hodgson *et al.*, 2000;Eaton& Klaassen, 2001). Developmental toxicity can also be termed as the ability of a chemical or physical agent to cause any of the manifestations of adverse developmental outcome (death, malformation, growth retardation, functional deficit), individually or in combination. Such substances are generally referred to as teratogens.Full appreciation of the potential deleterious impact of exogenous substances on human embryonic and fetal development came from the discovery in 1960 in Europe of a large number of newborns with limb malformations, later linked to the use of the sedative/hypnotic drug, thalidomide by pregnant women (Hood, 2006).

The manifestations of developmental toxicity vary depending on the timing of exposure, dose, and the underlying processes that are occurring, as well as on the time of observation. These manifestations are likely to be interrelated. Although death, malformation, and permanent functional impairment all clearly represent equally unacceptable outcomes, the significance of effects on growth or on the incidence of structural variations is sometimes questioned, particularly if these only occur at maternally toxic doses or can be shown to be reversible. However, many agents capable of producing death and malformation can also affect growth, depending on the dose and time of exposure. Growth retardation is often associated with functional impairment. While structural variations do not appear to adversely impinge on the health or longevity of affected fetuses, an increase in their incidence may indicate that the embryotoxic threshold is being approached(Kimmel & Wilson, 1973). The significance of these more subtle developmental effects also depends on the context in which they occur. For example, deficient or irregular patterns of ossification that are generalized throughout the fetal skeleton may simply reflect transient growth retardation, but such effects take on added significance when they occur without corresponding reductions in fetal weight or are more prominent in structures in which malformations also occur.

With respect to teratogenesis, there are six basic tenets of teratology, first defined by Wilson in 1959, which need to be kept in mind whenever gestational exposure to a teratogenic xenobiotic is suspected or when a chemical is being evaluated for its teratogenic potential. The general principles proposed have become the backbone of developmental studies:

- Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with environmental factors.
- Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
- Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis.
- The final manifestations of abnormal development are death, malformation, growth retardation and functional disorder.
- The access of adverse environmental influences to developing tissues depends on the nature of the influences.
- Manifestations of deviant development increase in degree as dosage increases from no effect to the totally lethal level(Wilson, 1977).

1.3.4 **Animal Models of Reproductive and Developmental Studies**

1.3.4.1 Effects on Fertility and Early Embryonic Development

Fertility studies provide information about the test substance on the performance and the integrity of the adult male and female reproductive systems (OECD, 2009). It involves the treatment with the test substance before mating (males and/or females), during cohabitation and mating, and until implantation which occurs on gestation day (GD) 6. For females this study design detects effects on the libido, estrous cycle, ovulation, mating behavior, development of pre implantation stages of the embryo, and implantation. In males this study design targets detection of functional effects on libido, mating, and sperm quality (motility, count, and morphology). Males are treated with the test substance 2, 4, or 10 weeks prior to cohabitation and mating, and until termination. Females are treated 2 weeks prior to cohabitation, through cohabitation and mating, and until implantation. Reproductive endpoints evaluated include mating,fertility, pregnancy rate, implantation sites, conceptus viability, corpora lutea, pre and postimplantation losses, and litter size. Internal organs are also cross examined macroscopically for any pathological changes and further histo-pathological analysis. In male or female fertility studies, the test substance is administered only to males or females, respectively (Hood, 2006).

1.3.4.2 Effects on Embryo and fetal Development

These studies, sometimes referred to as Developmental toxicity or teratology studies are used to detect adverse effects of treatment on the pregnant female and development of the embryo and fetus from implantation to closure of the hard palate (GD 15). However, more recently the treatment period has been extended to GD 20, with necropsy and fetal evaluation on GD 21. The endpoints analyzed include maternal toxicity (body weight, clinical and necropsy observations) embryo-fetal death (early and late resorptions), growth retardation, and structural alterations. Functional deficits cannot be determined using this paradigm (Hood, 2006).

1.3.4.3 Effect on Pre and Postnatal Development

This model detects adverse effects on the pregnant and lactating female and on development of the conceptus and the offspring following treatment of the female from implantationthrough to weaning. This study is designed to provide information concerning the effects of a test substance on male and female reproductive capacity, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity. The study design is the one-generation study for the Parental (P1) and first Filial(F1) generations. However, the test substance is administered postweaning to selectedF1 offspring (usually one male and one female per litter) during their growth into adulthood, during cohabitation and mating, and during gestation, parturition, and birth, continuing until the second Filial(F2) generation is weaned (Hood, 2006).

1.3.4.4 Developmental Neurotoxicity Study

The developmental neurotoxicity study providesinformation for use in evaluating the potential for neurotoxic effects in offspring after exposure toa test substancein uteroand/or via maternal milk or by direct dosing of the pup during the lactation period. Females are administered the test substance once daily beginning on GD 6 and continuingthrough post natal day (PND) 21. If there is no lactational transfer of the test substance or active metabolites, thendirect dosing of the pups beginning as early as PND 4 may be required. Females will be evaluatedfor adverse clinical signs observed during parturition, and for the duration of gestation, for littersize, live litter size, and pup viability at birth. F1generation males and females are tested as pupsand as adults for spontaneous locomotor activity, learning and memory, reflex development, andtime to sexual maturation. The functional observational battery (FOB) for pups and adults is performed at specified intervals. Neurohistopathological examination and morphometry are performedon selected brain regions(Hood, 2006).

1.4 JUSTIFICATION OF PRESENT STUDY

Picralima nitida seeds, sold as Picap capsules have been traditionally accepted in Ghana and other African countries for the treatment of a number of ailments. Notable among such ailments are malaria, stomach problems, pneumonia, jaundice, measles, cough, typhoid fever and gonorrhea. Whereas a number of studies have been made to ascertain its efficacy with regards to such claims, there are limited data on its safety profile specifically in the areas of reproduction. The safety of *Picralima nitida*in pregnancy and foetal development is largely unknown. In view of the fact that amongst the population using the plant product are males and females of child bearing age, it is expected that the exposure of these groups should not be underestimated.

Traditional medicine has, in the last few decades, gained popularity worldwide, especially in developing countries. Despite many achievements in human health care in the twentieth century, many of the world"s population in developing countries lack regular access to affordable essential drugs. Traditional medicines, however, have the advantages of being easily accessible and available; even in remote regions. Furthermore, the scarcity of allopathic practitioners in such deprived communities makes it difficult for the rural population to access modern health care.

The cost effectiveness of plant product cannot be under estimated, thus increasing patronage among rural folks. Traditional medicines are also widely acceptable among people living in developing areas, partly due to the inaccessibility of modern medicines and primarily based on the fact that traditional medicines blend easilywith the sociocultural life of these people.Thus, it is popular in many developing countries because it is firmly embedded with wider belief systems(Sofowora, 1982;WHO, 2002). Furthermore traditional medicine remains popular because the traditional medicine practitioners have wisely formed an important economic contract to the mutual benefit of their practice and the population they serve (Leonard, 2001).

However, in spite of all these pros, traditional medicines pose some problems which need to be addressed to maximize their potential as a source of health care (WHO, 2002). The major setback is the lack of scientific proof of its efficacy and safety profile. In fact,there is no thorough scientific investigation on most of the claims made by the traditional medicine practitioners (Sofowora, 1982). A large number of people assume that since herbal medicines are not manufactured in laboratories, they are safe but the use of herbs may expose the patient to certain untoward effects (Magee & Loiacono, 2004). The safety of herbal medicines is of great importance because the majority of these products areselfprescribed and used to treat minor and often chronic ailments (Hussin, 2001).For example aristolochic acid and other components within herbs can cause adverse renal effects and renal toxicity (Wojcikowski *et al.*, 2004). Some herbal remedies used to treat liver disease may be hepatotoxic themselves (Chituri & Farrell, 2000) whilst others cause drug-drug interactions. The most published and potentially worst herb-drug reaction is that of St John"s wort (*Hypericum perforatum*) and drugs metabolized by cytochrome P450, CYP 3A4 isoenzymes. Often without the knowledge of their physicians, patients use St John"s wort to treat symptoms of depression. This may have varied effects. For example women taking oral contraceptives and St John"s wort have been shown to experience breakthrough menstrual bleeding. It is also reported that unexpected pregnancy may result when St

John"s wort is regularly used with oral contraceptives(Schwarz *et al.*, 2003).

There is growing concern within the scientific community and amongst government regulatory agencies about the effects of reproductive toxicants on human fertility.It took the revelation in the early sixties that thalidomide, a drug promoted as a sedative and antiemetic, was a potent human teratogen to arouseinterest in testing for potential developmental toxicity. Phytochemical screening has revealed many bioactive as well as toxic agents of plant extracts that can affect the regulation of oestrus cycle, conception and reproduction(Benie*et al*., 2003; Yakubu*et al.*, 2005)

Subjecting a plant product or drug to reproductive and developmental studies usually provides salient information, which are not tackled in general toxicity studies.In this research work, the reproductive and developmental safety or otherwise of *Picralima nitida* seeds areinvestigated.

1.5 AIMS AND OBJECTIVES

1.5.1 **Aims**

The purpose of this study is to evaluate the effects of the ethanolicseed extract of*Picralima nitida* on reproductive and developmental indices and to ascertain its safety for use.

1.5.2 **Objectives**

The aim would be specifically achieved by:

- Assessing the effects of the extracton mating, fertility, gestation period, live birth and weaning after pretreatment of female mice.
- Studying the outcome of estrous cycle upon administration of the extract.
- Examining the aphrodisiac potential of the extract in male mice.
- Examining the effect of the extract on fertility, caudal epidydimal sperm number, body and reproductive organ weights and testicular histology in male mice.

- Assessing estrogenic or androgenic effectsof the extract using chick uterotrophic and comb growth tests.
- Evaluating effect of extract on haematological and biochemical parameters

CHAPTER TWO

MATERIALS AND METHODS

2.1 PLANT COLLECTION AND EXTRACTION

The fresh pods of *Picralima nitida*(collected from the KNUST Botanical Gardens in January 2013) authenticated at the Department of Pharmacognosy, KNUST, Kumasi, Ghana, by Dr Kofi Annan, were cut open and the seeds air dried. The dried seeds were milled into powder.A 3 kg quantity of the powder was extracted with 70 % ethanol by cold maceration technique for two consecutive seventy-two (72) hour periods. The extract was concentrated using a rotary evaporator(Rotavapor R-215, BUCHI Labortechnik AG, Flawil, Switzerland) at 60 ℃ to yield a syrupy mass which was subsequently dried at 40 °C, in a hot air oven. The solid mass obtained (279.9 g: percentage yield 12.44%), labelled as *Picralima nitida*seed extract (PNE), was reconstituted in normal saline for dosing in this study.

2.1.1 Animals

ICR mice (20-30 g) and Sprague-Dawley rats (120-145 g) were obtained from Noguchi Memorial Institute for Medical Research, Accra, Ghana. White leghorn day old chickswere purchased from Akati Farms, Kumasi Ghana. All animals were housed at the Animal House of the Department of Pharmacology, KNUST, Ghana. They were housed in stainless steel cages (34 × 47 × 18 cm^3) with soft wood shavings as bedding. The rodents and birds were fed *ad libitum* with commercial pellet diet and chick starter mash (Agricare Ltd, Tanoso, Kumasi) and water. All animals used were handled in accordance with the

National Institute of Health Guidelines for Care and Use of laboratory animals and were approved by the Departmental Ethics Committee.

2.2 PRIMARY SCREENING

2.2.1 Irwin Test

The procedure described by Irwin (1968)was employed in the primary screening of PNE to help in the selection of doses for the subsequent experiments, as well as assess the safety profile of the extract. ICR mice were randomly distributed to eight groups (n=6) and left to acclimatize for 48 hours. Animals were fasted overnight but had free access to water. Groups I-VII were treated with PNE in oral doses of 10, 30, 100, 300, 100, 1000, and 2000 mg/kg while animals in Group VIII (the control group) received 1 ml/kg distilled water. They were observed for changes in behavioural and clinical signs of acute toxicity, including mortality, at 0 to 15, 30, 60, 120, 180 minutes and at 24 hours. They were further observed for signs of delayed toxicity for up to 14 days.

2.3 REPRODUCTIVE STUDIES IN FEMALE MICE

2.3.1 Fertility Studies

The effect of PNE on mating, pregnancy, gestation, litter size, live birth, and weaning were evaluated in this study. Five (5) groups of female mice (n=5) weighing 20-30 g were administered withdistilled water (Group I, Control) or 30, 100, 300 and 1000 mg/kg PNE (Group II-V) for two weeks. The females were then introduced into individual cages housing males of proven fertility in the ratio of 1:2, and allowed to co-habit for up to 21 days. Every morning, the females were examined for the presence of vaginal plugs or otherwise the presence of sperms deposited in the vagina of the female. Evidence of vaginal plugs or the presence of sperm-positive lavage indicates successful mating. Males were removed from cages and females were allowed to complete their full term of pregnancy. The number of mice that mated and the number that became pregnant were noted. The mating index (MI), fertility index (FI), gestation period (GP), litter size, live birth index (LBI), and the weaning index (WI) were estimated and the results analyzed to assess the effect PNE on female fertility.

2.3.2 Effectof*Picralima nitida*on Estrous Cycle

To assess the effect of PNE on the estrous cycle, the methods described by Sachin Jain *et al.*, 2012; Long *et al.*, 1922; Mandl, 1951was used. Female Sprague-Dawley rats, weighing between 120-145 g were pre-screened daily for fourteen (14) days in which process vaginal smears was examined microscopically every day, at specific times (8-10 a.m), to obtain animals with regular estrous cycles. In doing so, a drop or two of physiological saline were drawn into a new clean dropping pipette and gently depressed to expel the contents into the vagina of each rat. The saline was gently drawn back into the dropping pipette and its content delivered onto a clean glass slide. Each vaginal lavage sample was then examined using a a Leica DM 750 microscope (Leica Microsystems CM5 GmbH, Wetzlar - Germany) at an objective magnification of ×40for leucocytes, cornified and epithelial cells.

The types of cells present were used for the determination of the estrous cycle phases.The length of each estrous cycle and the number of days spent at each stage of cycle were determined.Animals with regular estrous cycles were selected, and put into four groups (n=5).Group I, served as control, and was treated with distilled water only. Groups II, III and IV received 30, 100 and 300 mg/kg PNE respectively, for twenty one (21) days. Vaginal smears were evaluated similarly during treatments and the length of the cycle was determined. The estrous index was estimated as the ratio of estrous days to the study period. PNE treatments were suspended and cycle was again studied.

Cornified (keratinized) cells - large and irregularly shaped, mostly non-nucleated when mature. They are mainly seen at the estrous stage.

Epithelial cells – much more rounded in shape but not as large as the cornified cells. Those seen at the pro-oestrous stage are mostly nucleated whilst those seen at metestrous phase are non-nucleated.

Leucocytes – very small, rounded cells. They can be observed at the diestrous stage.

2.4 REPRODUCTIVE STUDIES IN MALE MICE

2.4.1 Acute treatment of PNE on the orientation behaviourin males

Orientation behaviours studiedwere mounting, sniffing and licking of the female and genital and non-genital grooming in the male (Singh *et al.,* 2013). Male mice weighing between 20 – 30 g, treated with saline and PNE (30 -300) mg/kg, *po* and placed individually in a plexiglass cage. After about 15 minutes of acclimatization, a non-estrous female was introduced into the cages and the number of attempted mounts, sniffing, licking, and genital grooming were recorded for 15 minutes.

2.4.2 Sub-acute treatment of PNE on the orientation behaviour in males

To assess the sub-acute effects on PNE on orientation behaviour, the males were treated with PNE (30-300 mg/kg) and saline (control) for 14 days. The males were then placed in individual Plexiglas cage and allowed to acclimatize for 15 minutes. The non-estrous female was introduced into the area and the number of attempted mounts, sniffing, licking and genital grooming were recorded for a period of 15 minutes on day 14.

2.4.3 Determination of hesitation time and attraction towards female

In this study, a modified method described by Singh *et al.,* (2013) was used. The female was placed in a cage which had a wooden barrier of 10 cm separating it and the male. A motivated and determined male should be able to cross over to the female. Male mice put into four Groups; I-IV. Group I was administered normal saline while Groups II, III, IV were administered 30, 100, or 300 mg/kg PNE.After one hour after treatments, the hesitation time,which is the time (in seconds) taken by the male before making any attempt to cross the wooden barrier/partition (i.e. latency to cross) to reach a female was monitored (within ten-minute) and recorded.

2.4.4 Epididymal Sperm Analysis

For this experiment, themethod described by Cheesebrough, (2006) was employed. Four groups of male mice (n=5) were used. Group I served as the control group, receiving distilled water only. Groups II-IV received 30, 100 and 300 mg/kg of PNE respectively for 14 days. On the last day of administration, the animals were weighed, euthanized by cervical dislocation and the wet weights of both epididymis and testes recorded. The left cauda epididymis was used for the sperm count analysis. The organ was minced and homogenized in 10 µL of diluent. The diluent is prepared by adding to distilled water 50g sodium bicarbonate ($NaHCO_3$) and10 ml 35% (v/v) formalin.Using a Pasteur pipette, 5 µL of the homogenate was diluted with 95 µL of diluent to give a 1 in 20 dilution. An aliquot of the final solution was then dropped onto an improved Neubauer Haemocytometer. To facilitate counting, the loaded haemocytometer was placed in a humid area for approximately 5 minutes so the sperm heads can settle to a common focal plane. The number of sperm heads was counted with the help of a microscope at a magnification of

200 to 400. The number of spermatozoa per cauda epididymis was calculated using the formula below:

Sperm number = $C_m \times F)/V$

Where Cm = mean count, F= dilution factor, V = Volume of counting chamber

Sperm Morphology:

Sperm Morphology refers to structural evaluation of the sperm. A thin smear of the liquefied well mixed semen form the above test was placed on a slide. While still wet, the smear was fixed with 95% v/v ethanol for 5-10 minutes, and allowed to air-dry. The smear was then washed with the sodium bicarbonate – formalin solution to remove any mucus which may be present. The preparation was then examined for normal and abnormal spermatozoa. Hundred spermatozoa were counted and the percentage estimated. Normal spermatozoa: Each consists of an oval-shaped head, a short middle piece, and a long thin tail. In normal semen, at least 50% of spermatozoa should show normal morphology. Most specimens contain no more than 20% abnormal forms.

Abnormal spermatozoa: Head may be greatly increased or decreased in size. It may also present with an abnormal shape and tapering head (pyriform). Acrosomal cap may be absent or abnormally large. Nucleus contains vacuoles or chromatin is unevenly distributed. Sperm may have two heads and additional residual body.Middle piece may be absent or markedly increased in size. Could appear divided or angled where it meets tail.Tail may be absent or markedly reduced in length, bent or coiled. Some may also present double tails(Cheesebrough, 2006).

Sperm Motility

One drop of the well mixed liquefied semen on a slide and covered with a slip. Using the

40× objective, several fields were examined to assess motility: whether excellent (rapid and progressive) or weak (slow and non-progressive). A total of 100 spermatozoa were counted and the percentage of motile and non-motile sperms estimated.

Normal motility: Over 50% of spermatozoa are motile within 60 minutes of ejaculation. The spermatozoa remain motile for several hours (Cheesebrough, 2006).

Sperm Viability

One drop of liquefied semen was mixed a drop of 0.5% eosin solution (0.1g of eosin in 20 ml of fresh physiological saline) on a slide. The preparation was then examined with the microscope after about 2 minutes. Viable spermatozoa remain unstained, non-viable spermatozoa stain red.

Normal viability: 75% or more of spermatozoa should be viable. A large proportion of non-motile but viable spermatozoa may indicate a structural defect in the flagellum (Cheesebrough, 2006).

2.4.5 Biochemical and Haematological Analysis

Blood samples were collected by cardiac puncture from the experimental animals in 2.4.4. The samples were analyzed for various haematological and biochemical parameters using the Sysmex KX 21N™ Automated Haematoanalyzer and Vital Scientific Flexor Junior Chemistry Analyzer respectively. Haematological outlook included red blood cell count, white blood cell count, platelets and haemoglobin content whereas biochemical parameters measured weretriglycerides, cholesterol and alanine aminotransferase.

2.4.6 Histological Studies

After the animals were sacrificed by cervical dislocation, testes and epididymis were removed for histological studies. They were initially fixed in Bouin‟s solution for 24 hours and later transferred into 10% formalin solution. These were later embedded in paraffin and stained with hematoxylin and eosin (H&E). Digital images were acquired and studied.

2.5 HORMONAL ASSAY

2.5.1 Chick Uterotrophic Assay

This test is based on the principle that elevated levels of natural oestrogens and phytoestrogens (plant-derived xenoestrogens) in female animals during the early stages of development, dose dependently, increases the uterine/body weight ratio (Dorfman, 1969; Lerner et al., 1958; Tullner & Hertz, 1956).Day old Rhode Island Red layer chicks were purchased, housed and on day 7, the chicks were randomly assigned to seven groups (n=6). Groups 1-3 were treated subcutaneously with0.1, 0.3, or 0.9 µg of 17- β-estradiol twice daily. Groups 4-6 were treated orally with 30, 100, and 300 mg/kg of PNE respectively.Group 7, the control group, was treated subcutaneously with 0.2 % v/v corn oil (vehicle) control. Dosing was done 12 hourly for 6 continuous days. During treatments, chicks were weighed every other day before feeding. On the sixth day of treatment, the chicks were weighed, euthanized with ether, dissected and the oviduct was isolated; whiles carefully removing any attached connective tissue. The oviduct was immediately weighed. The weights were then normalized with the final body weight of the chick and expressed as a percentage using the formulas below.

$$\text{Percentage oviduct} - \text{chick weight} = [\text{oviduct weight(g)} \div \text{chick weight (g)}] \times 100$$

The percentage change in weight was then plotted against the log of concentration of the various treatments to obtain log concentration response curves.

2.5.2Chick Comb Test

The method described by Dorfman (1969) was used with slight modifications to study the androgenic effect of PNE. Day old white leghorn chicks (male chicks), after 14 days of acclimatization to the experimental laboratory conditions, were randomly assigned to ten groups (n=10). The length and height of the combs of each of the chicks in the various groups were measured and recorded. Doses were administered as follows for seven (7) days. Group I, the control group, was treated orally with distilled water (vehicle),Groups II-IV were treated intramuscularly with 0.5, 1.0, 1.5 mg/kgTestosterone propionate respectively.Groups V-VII were treated orally with 3, 10, 30mg/kgCyproterone acetate respectively whilesGroups VII-X received, orally50, 100, 500 mg/kg PNE respectively.

The length and height of the combs of each chick was measured and recorded 24 h after the last drug administration.

2.5.3 Effect of Cyproterone on PNE induced comb growth

To estimate the effects of cyproterone on PNE, twenty white leghorn chicks were randomly divided into four groups (n=5) and treated as follows; Chicks in Group 1 were administered PNE (30 mg/kg; p.o). Group 2 was administered testosterone (0.6 mg/kg; i.m)and PNE(30 mg/kg;p.o). Group 3 was administeredCyproterone (10 mg/kg; p.o) and PNE (30 mg/kg; p.o), and Group 4 received PNE (30 mg/kg; p.o) andcyproterone (30 mg/kg; p.o). Doses of testosterone and PNEused in this studywere ED_{50} estimated from the

Chick Comb Test. Twenty four hours after the last administration, change in comb growth (length and height) were measured.

2.5.4 Effect of PNE on Testosterone induced comb growth

To evaluate the effects of PNE on testosterone, twenty single comb white leghorn chicks were grouped into four (n=5) and treated as follows; Chicks in Group 1 were administered testosterone (0.6 mg/kg; i.m). Group 2 was administered testosterone (0.6 mg/kg; i.m) and PNE(50 mg/kg; p.o). Group 3 was administered testosterone (0.6 mg/kg; i.m) and PNE (100 mg/kg; p.o) while Group 4 received testosterone (0.6 mg/kg; i.m) and PNE (500 mg/kg). Twenty four hours after the last administration, change in comb growth (length and height) were measured.

2.6 STATISTICS

All results are presented as mean ± SEM. Data was analyzed using one-way analysis of variance (ANOVA). When ANOVA was significant, multiple comparisons between treatments were done using Newman Keuls or Dunnett"s*post hoc* test when comparing control with treatment groups. GraphPad Prism for Windows Version 5 (GraphPad Software, San Diego, USA) was used for all statistical analyses.

CHAPTER THREE

RESULTS

3.1 PRIMARY SCREENING

3.1.1 Irwin Test

There were no signs of physical, pharmacological, neurotoxicity, autonomic or CNS related toxic symptoms in mice treated with 10, 30, 100 and 300 mg/kg relative to control mice. However, reduced activity and mild sedation was observed at doses 1000 and 2000 mg/kg. No mortality was recorded at any dose. Thus the No-Observable-Adverse-effect-Level (NOAEL) was less than 1000 mg/kg PNE and the LD_{50} was less than 2000 mg/kg.

Table 3.1Primary Observation Test of *Picralima nitida* in mice

DOSE (mg/kg)	MORTALITY	EFFECT
	D/T	
Control	0/6	No observable effect
PNE 10	0/6	No observable effect
PNE 30	0/6	No observable effect
PNE 100	0/6	No observable effect
PNE 300	0/6	No observable effect
PNE 1000	0/6	Sedation (+) and hypo activity(+)
PNE 2000	0/6	Sedation (++) and hypo activity (++)

D/T: number of deaths /number of mice treated. Grade of signs observed: (+) present or slightly increased, (++) moderately increased.

3.2 Reproductive Studies in female mice

3.2.1 Fertility Studies

Treatment with PNE doses of 100, 300 and 1000 mg/kg did not affect mating and gestation period but caused a dose dependent decrease ($p > 0.05$) in fertility index. Treatments also affected litter size and survival, Weaning index was considerably reduced(Table 3.2).

Table 3.2:Reproductive and developmental indices for female mice pre-treated with ethanolic seed extract of *Picralima nitida* fourteen days prior to mating.

Dose (mg/kg)	№ mated	№ pregnant	MI	FI	GP	LS	LBI	WI
Control	5	5	100	100	19.6±0.24	7.8±0.17	100	100
30	5	3	100	60	19.6±0.33	4.8±0.89 ns	100	100
100	5	4	100	80	20.33±0.33	3.75±0.74 **	93.3	100
300	5	3	100	60	19.5±0.50	2.25±1.00 ***	100	100
1000	5	3	100	60	19.5±0.30	3.0±0.95**	100	100

Values for GP and LS are means + SEM, n=5. **$p \leq 0.01$, ***$p \leq 0.001$ (one-way ANOVA followed by Dunnetts, post-hoc test.

Mating Index (MI) = (no of cohabited females/no of females mating) × 100; Fertility Index (FI) = (No of pregnant females/no of females mated) × 100; Live Birth index (LBI) = (No of offspring delivered alive/No of offspring delivered) × 100; Weaning Index (WI) = (No of offspring at day 21/ No of Offspring delivered) ×100;LS= Litter size,

3.2.2Effect of *Picralima nitida* onestrous cycle

PNE, at all doses, prolonged the estrous cycle in Sprague-Dawley rats seen as the significant increments ($p \leq 0.01 – 0.001$) in the estrous index (Table 3). The cycle became irregular upon the administration of PNE. Estrous cycle could last for as long as 20 days instead of the expected 4 to 5 days. The cycles returned to normal upon withdrawal of PNE treatment.

Table 3.3: Effect of PNE on estrous cycle in rats

Treatment Group	**Estrous Index**
Control	0.298±0.01
PNE 30 mg/kg	0.595±0.06**
PNE 100 mg/kg	0.774±0.06***
PNE 300 mg/kg	0.809±0.07***

Estrous index= estrous days/ total study period. Data are presented as mean ± SEM. Statistical analysis is by one way ANOVA followed by Dunnetts *post hoc*. Significant difference from control is depicted by; *** p < 0.001 and **p<0.01

3.3 Reproductive Studies in Male Mice

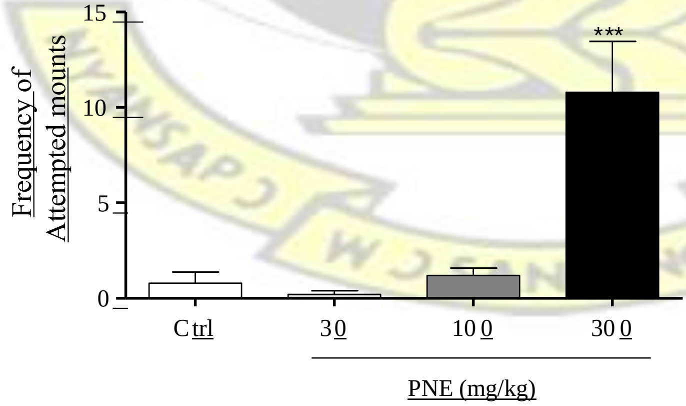
3.3.1 Acute study of the orientation behaviour in males

Treatment of male mice with PNE increased the number of attempted mounts in a dose dependent manner but was significant ($p \leq 0.001$) at a dose of 300 mg/kg (Figure 3.1a). PNE at doses 30, 100 mg/kg produced moderate increases in sniffing and licking of the female anogenital organ by the males. However a more significant ($p \leq 0.05$) increase was observed in animals treated with 300 mg/kg (Figure 3.1b). There was a marginal increasein genital and non-genital grooming in animals treated with 30 mg/kg PNE but at doses of 100 and 300 mg/kg the frequency of genital and non-genital grooming increased significantly ($p \leq 0.05$) (Figure 3.1c)

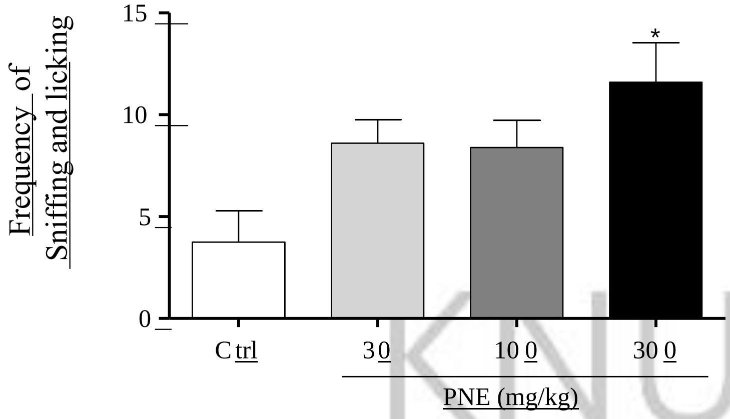
3.3.2 Sub-acute study of the orientation behaviour in males

After 14 days of PNE administration, the frequency of attempted mounts was not significantly different from the control group (particularly at doses 30 and 100 mg/kg). Generally sniffing and licking of the female was moderately increased but showed no significance. However, genital and non-genital grooming which had hitherto increased in the acute study was not affected at all doses PNE (Figure 3.2c).

(a)



(b)



(c)

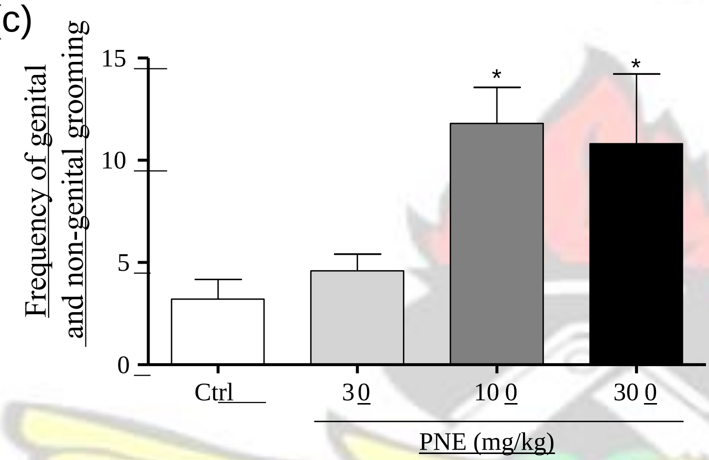
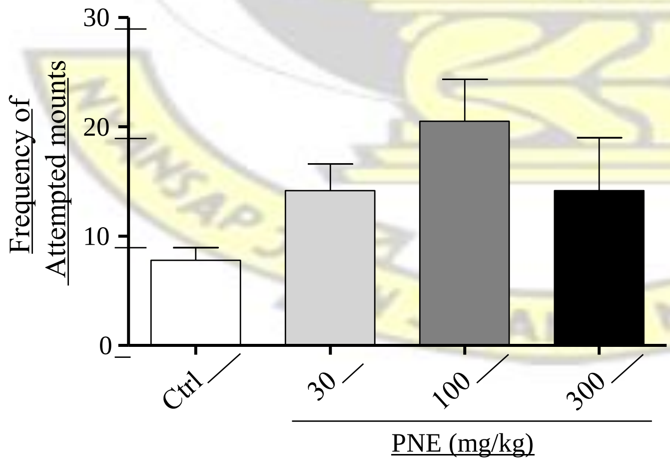
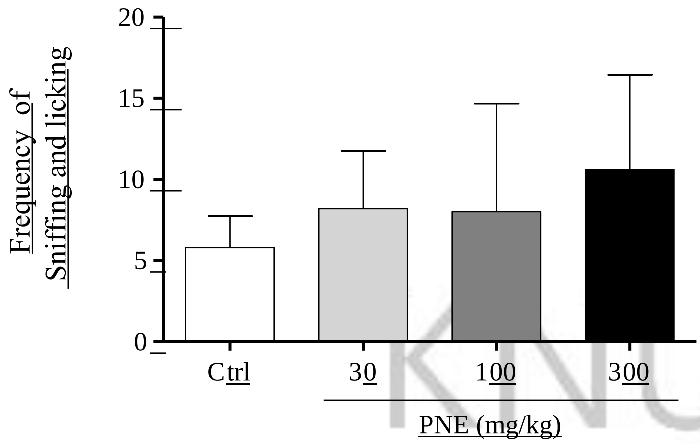


Figure 3.1 Outcome of one hour PNE treatment on (a) attempted mount; (b) sniffing and licking; (c) genital and non-genital grooming. Statistical significance * p< 0.05, *** p< 0.001was by one way ANOVA followed byNewman-Keuls*posthoc* test

(a)



(b)



(c)

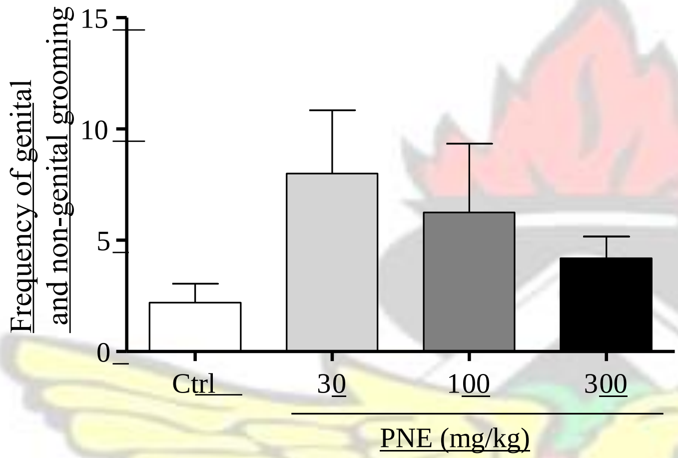


Figure 3.2Effect of 14 day PNE treatment on (a) attempted mount; (b) sniffing and licking; (c) genital and non-genital grooming. Ns implies p>0.05 (one-way ANOVA using Newman-Keuls *post hoc* test)

3.3.3 Determination of hesitation time and attraction towards female

PNE at all administered doses significantly reduced the latency (hesitation time) to cross the wooden barrier, compared with the control group (Table 3.4). This indicates an increased attraction to the female. Males in the control group showed little interest in crossing the wooden barrier.

3.3.4 Sperm Analysis

Sperm count also decreased significantly at doses of 100 ($p< 0.05$) and 300 ($p< 0.001$) mg/kg PNE (Table 3.5).There was however no significant ($p>0.05$) changes in the morphology, motility and viability of the analysed sperms indicating that the quality of the sperm was not affected.The combined mean wet weights of both epididymis and testes of the PNE treated groups were marginally reduced ($P > 0.05$) compared to the control group.

Table 3.4 Effect of *Picralima nitida*Extract on hesitation time of male mice towards females

	Control	PNE30 mg/kg	PNE100 mg/kg	PNE300 mg/kg
Hesitation Time (s)	406±118.80	98.25±43.77*	55.40±18.87**	53.80±14.76**

Data are presented as group means ± SEM. Statistical analysis is by one way ANOVA followed by Dunnetts post hoc. Significant difference from control is depicted by; ** $p < 0.01$ and *$p<0.05$

Table 3.5Effect of *Picralima nitida* treatment on Epididymal Sperm Assay

Group	Mean weight of organs (g)	Sperm count ($\times 10^6$ sperms/µL)	Morphology (%)	Motility (%)	Viability (%)
Control	0.25±0.0024	6.8±0.261	82.0±1.23	68.0±3.74	80.0±4.18
30 mg/kg	0.21±0.0075	5.52±0.445	84.0±2.45	75.0±3.16	79.0±2.45
100 mg/kg	0.22±0.0085	4.65±0.499*	67.5±2.50	60.0±0.00	75.0±2.89
300 mg/kg	0.23±0.0130	3.48±0.504***	78.0±2.00	74.0±2.45	77.0±2.00

Data are presented as mean ± SEM. Statistical analysis is by one way ANOVA followed by Dunnetts post hoc. Significant difference from control is depicted by; *** $p < 0.001$ and *$p<0.05$

3.3.5Effect of sub-acute PNE treatment on Biochemical and HaematologicalParameters

No significant changes were observed in biochemicaland haematological parameters when compared to control mice. All haematological parameters were in normal ranges in the control and testedanimals. Both RBC and WBC were increased but insignificant compared to control.At a lower dose of 30 mg/kg, cholesterol and triglyceride levels were slightly increased but levels decreased with higher doses. Aspartate transaminase (AST) and Alanine transaminase (ALT) levels, although slightly increased were within the acceptable range (Table 3.6).

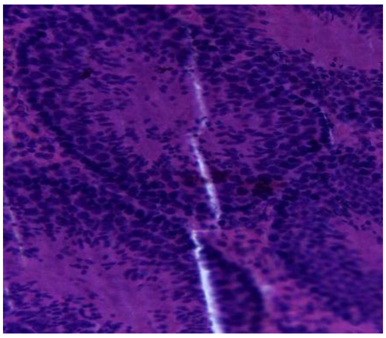
Table 3.6 Effect of *Picralima nitida* on some haematological and biochemical parameters in mice

Parameters	Control	PNE 30 mg/kg	PNE 100 mg/kg	PNE 300 mg/kg
WBC (×10^3/uL)	4.36±0.62	5.68±0.93	6.9±0.70	6.58±0.54
RBC (×10^6/uL)	8.40±0.17	9.25±0.36	9.67±0.29	9.32±0.16
Hematocrit (%)	42.68±0.87	48.48±2.48	47.67±0.29	47.85±0.95
Hemoglobin (g/dL)	12.82±0.34	13.95±0.73	13.97±0.27	13.78±0.32
MCV (fl)	50.88±1.17	52.35±0.78	49.33±1.19	51.35±0.17
MCH (pg)	15.28±0.50	15.05±0.31	14.43±0.24	14.8±0.09
MCHC (g/dL)	30.02±0.50	28.78±0.40	29.3±0.40	28.78±0.18
Cholesterol (mmol/l)	2.87±0.13	4.03±0.62	2.54±0.16	2.77±0.15
Triglycerides (mmol/l)	1.53±0.05	2.05±0.51	0.93±0.12	1.20±0.16
AST (u/L)	40.24±17.73	67.97±35.41	33.40±14.88	80.37±34.03
ALT (u/L)	4.42±2.33	5.65±1.69	4.9±2.77	5.93±1.15

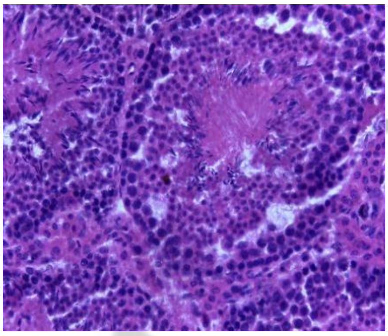
Data expressed as mean± SEM. Statistical analysis is by one way ANOVA using Dunnetts *post hoc*. WBC - White Blood Cells, RBC – Red Blood Cells, MCV – Mean Corpuscular Volume, MCH – Mean Corpuscular Hemoglobin, MCHC – Mean Corpuscular Hemoglobin Concentration, AST – Aspartate Transaminase, ALT – Alanine Transaminase

3.3.6 Effect of sub-acute PNE treatment on the histology of the testes

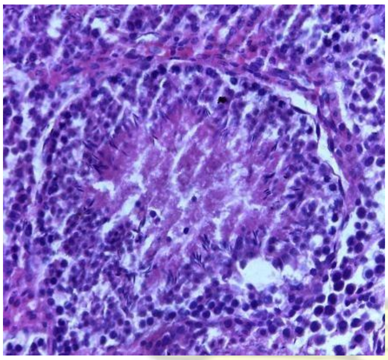
Histopathological examination of the testes in control animals showed semnifeous tubules of the testes possessed epithelia containing the sertoli cells and the germ cells at various stages. Photomicrographs of the transverse sections of the testes of male mice treated with (30-300 mg/kg; po) PNE shows structural disorganization and disruption of the histoarchitecture of the semniferous tubules. (Figure 3.3).



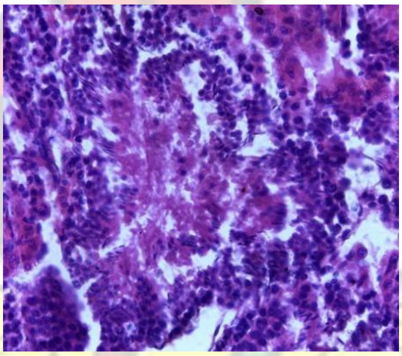
Control



PNE 30 mg/kg



PNE 100 mg/kg



PNE 300 mg/kg

Figure 3.3Photomicrographs of transverse sections of the testes of male mice treated with either distilled water or *Picralima nitida* extract (30-300 mg/kg; *po*)

3.4 HORMONAL ASSAY

3.4.1 Chick Uterotrophic Assay

A 6-day continuous administration of 17- β oestradiol and PNE increased dosedependently ($p \leq 0.001$) the percentage oviduct-chick weight ratio. However, no significant differences in body weights were observed (Figure 3.5). The efficacy exhibited by both treatments were not significantly different but 17- β oestradiol was more potent at increasing oviduct weight compared to PNE as indicated in the estimated ED_{50}‟s (Estradiol: 0.25 μg, PNE:1.5 mg/kg) (Figure 3.7).

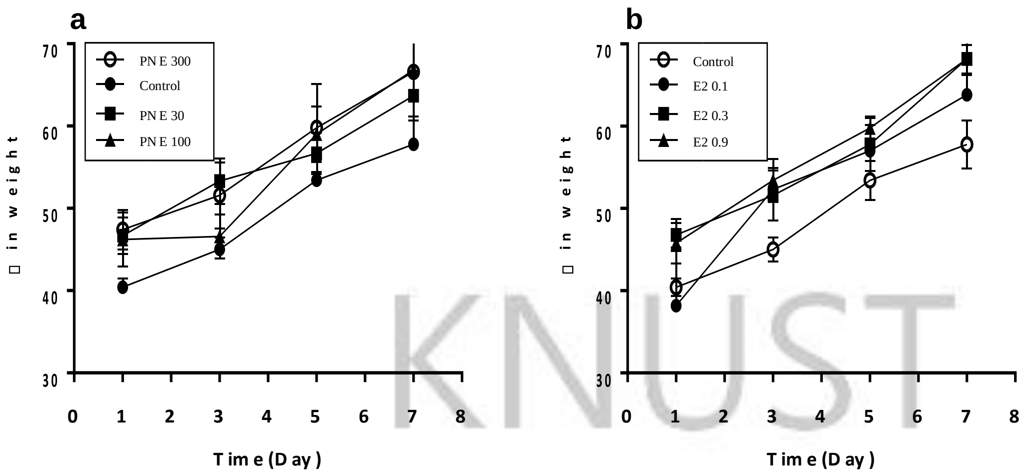


Figure 3.4 Time Course curve of the effects of PNE (30-300 mg/kg) and 17- ß oestradiol (0.1-0.9 µg) on weight change. Values are presented as mean ± SEM. Statistics was by one way ANOVA followed by Newman-Keuls *post hoc* test

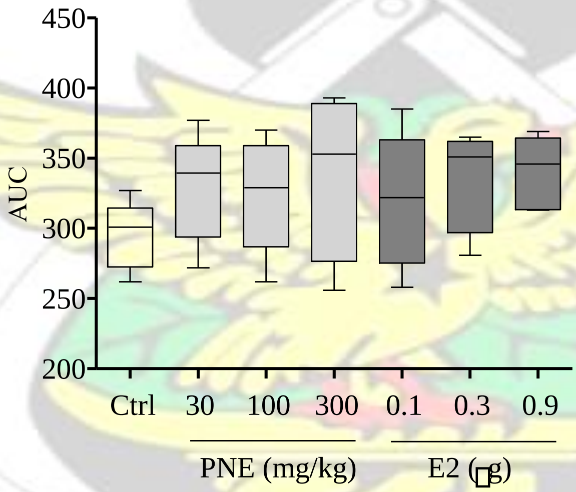


Figure 3.5 Effects of PNE and 17- ß oestradiol on body weight changes in chick uterotrophic assay. The values are represented as mean ± SEM. The lower and upper margins of the boxes represent the 25th and 75th percentiles, with the extended arms representing the 10th and 90th percentiles respectively. The median line is shown as the horizontal line within the box.

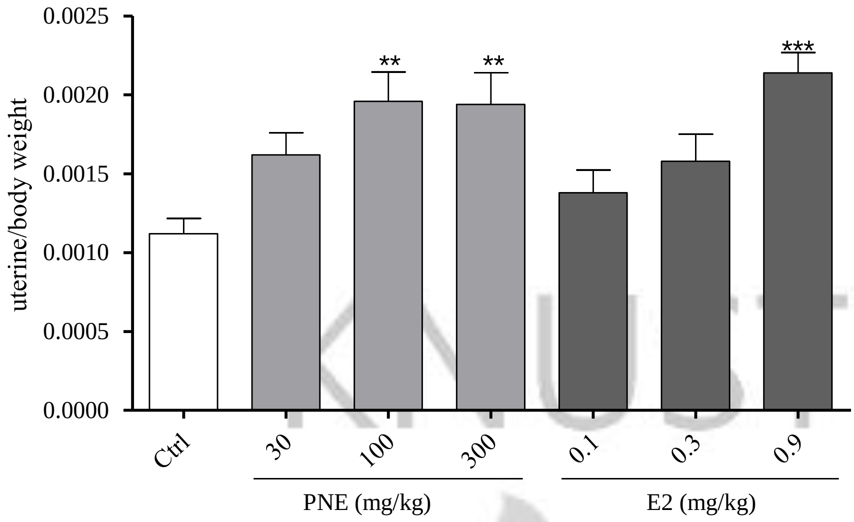


Figure 3.6Effect of PNE (30-300 mg/kg) and17- ß oestradiol (0.1-0.9µg) on chick uterine to body weight ratio. Data expressed as mean± SEM. Significantly different from control: ** p < 0.01; *** p< 0.001. Statistical analysis is by one way ANOVA followed by Newman-Keuls *post hoc* test.

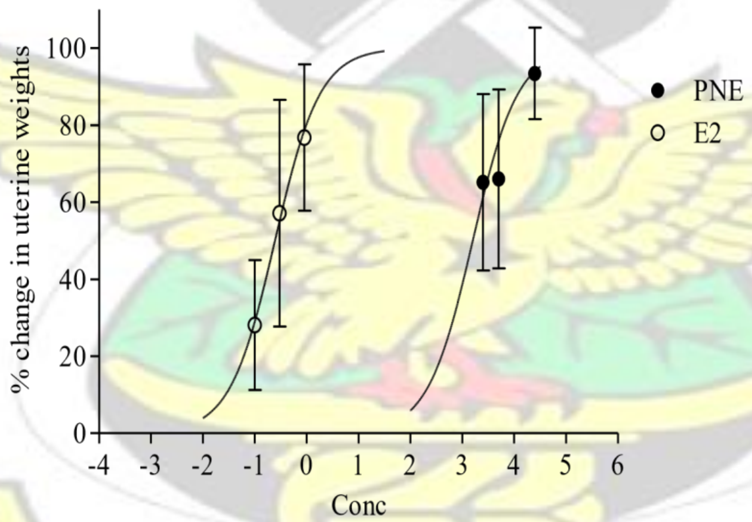


Figure 3.7Dose-response curves of PNE (30-300 mg/kg) and 17- ß oestradiol (0.1-0.9 9 µg) with respect to % change in uterine to body weight in the chick uterotrophic assay. Each point represents the mean ± SEM.Potency ratio of17- ß oestradiol to PNE is approximately 1: 6376

3.4.2 Chick Comb Test

Seven-day treatment with PNE (30-300 mg/kg) and testosterone (0.5 -1.5 mg/kg) increased significantly (p≤0.01; p≤0.001) while Cyproterone acetate significantly decreased

($p \leq 0.001$) comb growth (size and length) in a dose dependent manner; compared to the vehicle treated chicks. Qualitative assessment revealed that chicks treated with testosterone (1.0, and 1.5 mg/kg) and all doses of PNE had relatively brighter red combs, and well developed feathers. Using the comb length as the response, the ED_{50} of testosterone and PNE was estimated to be 0.6, and 27.37 mg/kg respectively. Cyproterone acetate inhibited comb growth at all dose levels with the highest inhibition was observed at 30 mg/kg. The effects of PNE are more comparable to testosterone with the latter being 45.62 times more potent than the extract (figure 3.8).

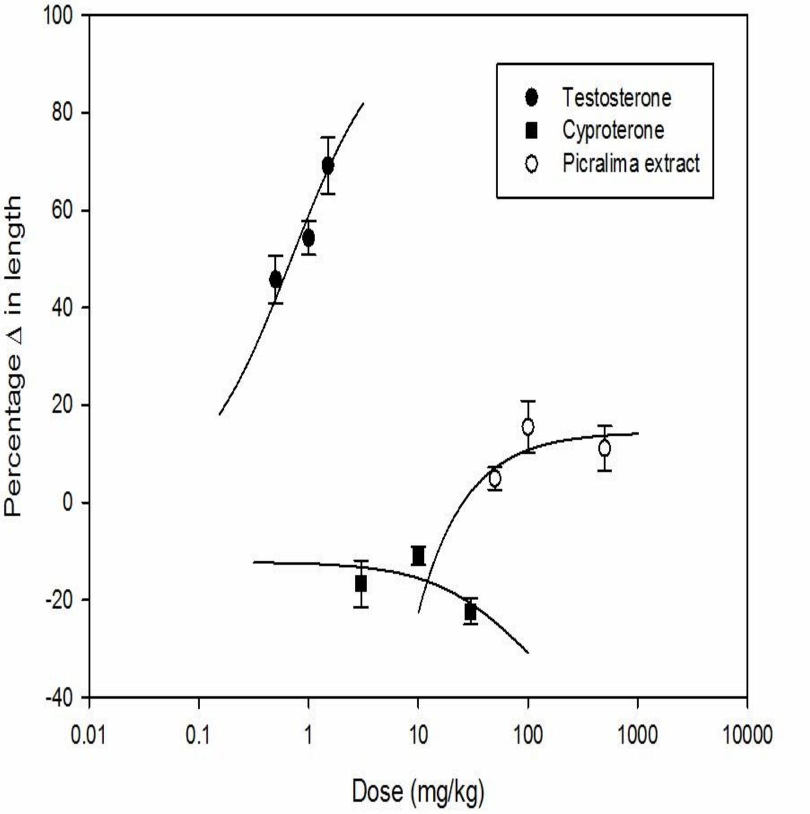


Figure 3.8 Dose response curves of Testosterone, PNE and Cyproterone acetate. ED_{50} of Testosterone was 0.6 mg/kg, PNE 27.37 mg/kg. Potency ratio of Testosterone to PNE is approximately 1:45.62

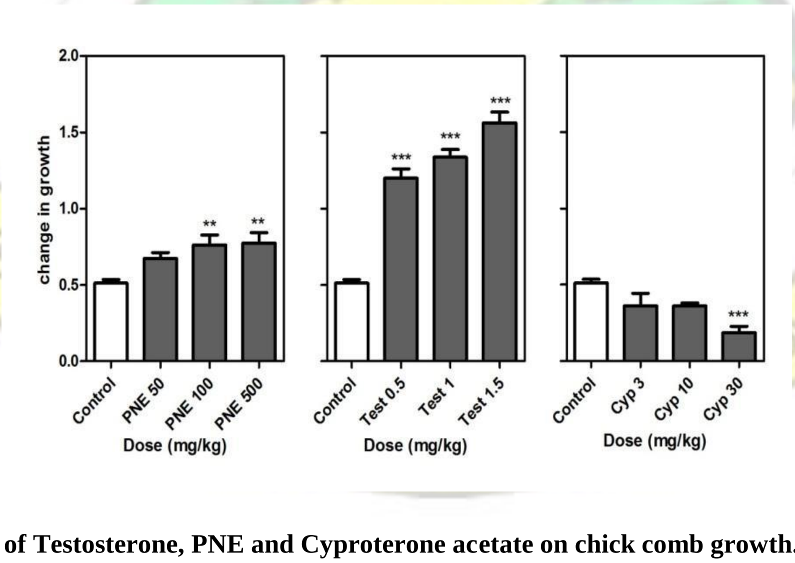


Figure 3.9 Effect of Testosterone, PNE and Cyproterone acetate on chick comb growth. Data expressed as mean± SEM. Statistical analysis is by one way ANOVA followed by Newman keuls post hoc *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared to control.

Figure 3.10Representative images of chick combs after seven days of treatment with PNE, Testosterone, and Cyproterone acetate.

3.4.3Effect of Cyproterone on PNE induced comb growth

This study estimated the effects of cyproterone on *Picralima nitida* induced comb growth. It was observed that cyproterone was able to cause a significant ($p< 0.001$) inhibition of comb growth in both testosterone and PNE groups. Combs were pale red as compared to chicks receiving the corresponding drugs only. Appearance of wattle was also inhibited until the last day of treatment.Figure 3.11 below gives a vivid explanation.

3.4.4 Effect of PNE on Testosterone induced comb growth

Co-administration of 0.6 mg/kg testosterone with (30-300) mg/kg of PNE yielded some interesting results. PNE, when administered alone increased comb growth at all dose levels. However, when given together with testosterone, comb growth was significantly ($p < 0.001$) decreased (figure 3.12). This indicates that the extract antagonised the more potent testosterone propionate. Animals receiving both drugs developed light pink combs and wattles were generally absent.

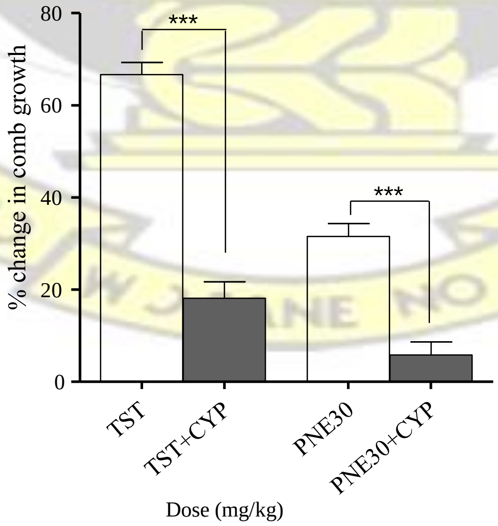


Figure 3.11Effects of cyproterone acetate on testosterone propionate and PNE induced comb growth. Results presented as mean ± SEM. Statistical analysis is by one way ANOVA followed by Newman-Kuels *post hoc* test. * means(p < 0.001) when compared to the corresponding drug only.**

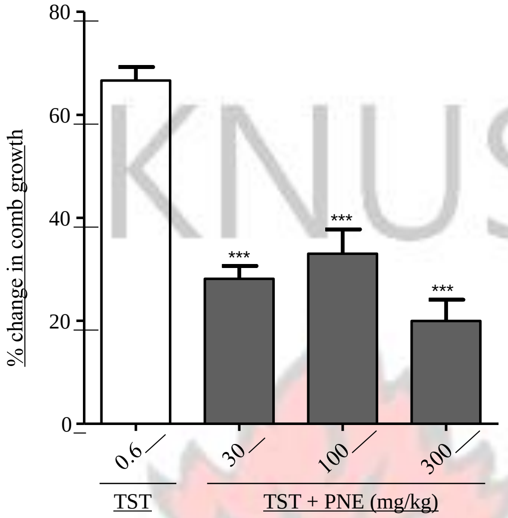


Figure 3.12Effects of PNE on testosterone propionate induced comb growth. Results presented as mean ± SEM. Statistical analysis is by one way ANOVA followed by Newman-Kuels *post hoc* test. * means (p < 0.001) when compared to testosterone only.**

CHAPTER FOUR

DISCUSSION

Plants have become a rich source of biologically active agents that in one way or the other, affect reproductive indices in both males and females. In the last few decades, most plants have been systemically screened for a variety of fertility regulating activities like antiovulatory, anti-implantation, estrogenic/ anti-estrogenic in females and anti-spermatogenic, androgenic/ anti-androgenic in males(Pincus, 1965). Although results from animal toxicological assays cannot be directly extrapolated to humans, it provides the foundation for the prediction of human doses and sets targets for other preclinical and clinical studies. *Picralima nitida* is used extensively across countries in Africa for the treatment of an appreciable number of ailments (Ayensu, 1978). Notable among such ailments are malaria, stomach problems, pneumonia, jaundice, measles, cough, typhoid fever and

gonorrhea.Whereas a number of studies have been conducted to ascertain its efficacy with regards to such claims, there is limited data on its safety profile specifically in the areas of reproduction. In view of the fact that amongst the population using the plant product are males and females of child bearing age, it is expected that the exposure of these groups should not be underestimated.

The Primary Observation test which was proposed by Irwin in 1968 provides an ordered procedure for analyzing both behavioral and physiological function qualitatively. It gives an insight into the potential toxicity or otherwise of the investigational drug and can lead to novel therapeutic agents discovery (Irwin, 1968;Porsolt *et al.*, 2002).It was used to assess the safety of doses to be used on experimental animals in this study, by establishing the NOAEL. The NOAEL lower than 1000 mg/kg indicates that there was no observable physical, pharmacological, neurotoxicity, autonomic or CNS related symptoms. Some of the physical symptoms include lacrimation, pupil size and swelling. Pharmacologically, toxicity could be revealed with symptoms such as sedation, tremor and convulsion (Moser, 2011). An LD_{50} greater than 2000 mg/kg indicates that the extract is relatively safe.

Female mice become anestrus in the absence of a male for a period of time. The estrous cyclicity can be revived by housing the females near males or in cages with soiled bedding from the male cage. The cyclicity is induced by a pheromone in the urine of the male mouse (Whitten, 1956). The females exhibit a 4 to 5-day estrous cycle and become receptive to mating during the estrous stage.

Mating is very important in reproduction and hence both female and male should have favourable mating behaviours. Negative mating behaviour in females occurs as a result of physical impairment, hormonal imbalance, estrous cycle disruption or diminished libido which does not allow for pregnancy. However, treatmentof female mice with PNE for 14 days before mating had no effect on the mating behaviours, suggesting that alterations with respect to other indices may not be due to an adverse effect on mating.

The dose-dependent reduction in pregnancy, and fertility index recorded with PNE treatement, although mating index was not affected affected, could be due to alterations in the estrous cycle, impaired implantation, or destruction of the formed embryo (Hood, 2006). The decrease in the occurrence of pregnancy may also be due to a dysfunctional period of the endocrine functions that might lead to decreased secretion of progesterone which is needed for endometrial alteration at the time of implantation and is vital for successful impregnation (Choudhary& Steinberger, 1975).

A look at the phytochemical components of *Picralima nitida* shows the presence of phytochemicals such as alkaloids, saponins, tannins and flavanoids (Aguwa *et al*., 2001;Vladmir & Ludmila, 2001). According to literature, flavonoids and saponins are known to exhibit antifertility activity (Vaidya *et al.*, 2006; Badami *et al.*, 2003). The antifertility effects of*Balaites roxburghil*may be attributed to its estrogenic activity (Vaidya *et al.,* 2006).Some alkaloids and flavonoids have been shown to reduce plasma concentrations of leutinizing hormone (LH), estradiol and follicle stimulating hormone (FSH) (Browning *et al*., 1998;Lauritzen *et al*., 1997; Bianco*et al*., 2006).The inhibitory effect of steroidal saponins on the estrous cycle has also been reported (Tamura *et al.*, 1997).

The litter size also decreased dose dependently comfirming a negative effect on fertility. Factors affecting litter size include ovulation rate, fertilization rate, implantation rate and postimplantation survival rate (Hood, 2006). Pup survival endpoints can be affected by toxicity of the test substance directly on the offspring such as structural or functional developmental defects, low birth weights or impaired suckling ability. The effect could also be indirect, through effects on the dam resulting in lactational inability or maternal neglect (Hood, 2006). All litter obtained from both treated and control groups, were alive (LBI and WI were 100 %), normal (physical appearance and birth weight) and grew up normally without having any physical abnormalities, giving credence that PNE is not likely to be teratogenic.

Variations in gestational period can be caused by hormonal imbalances and fetal growth retardation. Decreased gestational length is usually associated with decreased pup weight and viability, whiles prolonged gestational period is associated with higher birth weights (Hood, 2006).

The chick uterotrophic assay is based on the principle that elevated levels of natural oestrogens and phyto-estrogens in female animals during the early stages of development, dose dependently, increase the uterine / body weight ratio(Dorfman, 1969; Lerner et al., 1958; Tullner & Hertz, 1956). The uterus responds to estrogens in two ways. The initial response is an increase in weight due to water imbibition. This response is followed by a weight gain due to tissue growth. PNE caused a significant increase in the uterine/ body weight ratio when compared with the control group.In the estrous cycle study, PNE prolonged the estrous cycle, seen as significant dose-dependent increments in the estrous index. Estrous cycle could last for as long as 20 days even though the duration of the estrous cycle in rats is normally 4–5 days (Mandl, 1951). The presence of specific cells depicts different stages in the estrous cycle. The cornified cells are mainly seen at the estrous stage whiles the diestrous stage comprises leucocytes. The epithelial cells present at proestrous are mostly nucleated whilst those seen at the metestrous phase are nonnucleated.

Estrous cycle in mice is controlled by the neuro-endocrine system. Neuroendocrine actions are involved in the control of ovulation, and the cyclical preparation of the reproductive tract for fertilization and implantation (Goodman & Gilman, 2006). Thus alterations in these hormonal levels can affect the reproductive output of the female. Increased hormonal levels could however exert a negative feedback effect on the hypothalamus-pituitary axis. This results in decreased release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Inhibition of follicular development and the absence of LH surge prevent ovulation (Goodman & Gilman, 2006), and hence pregnancy.

A large number of antifertility plant extracts are known to exhibit estrogenic activity. Such phytoestrogens may disrupt the functional equilibrium between endogenous estrogen and progesterone which can result in failed pregnancy. In species such as mice and rats that exhibit facultative delay of implantation, nidatory estrogen secreted within about 24 hours of initiation of implantation is necessary for endometrial receptivity to blastocyst signal. Estrogen, within a narrow range, determines the duration of window of uterine receptivity(Ma *et al.,* 2003). While this window remains opened for an extended period at lower estrogen levels, it closes rapidly at high levels of estrogen (Ma *et al.*, 2003) resulting in decreased fertility.

Furthermore, the cascade of events that lead to the rupture of an ovarian follicle is characteristic of tissue responses to inflammation. Inflammatory-like changes first occur in the theca interna and granulosalayers of the follicle in response to gonadotropic stimulation of the luteinisation process (Espey, 1994). Redness, swelling and sporadic pain, which are glaring expressions of inflammation are displayed by the pre-ovulatory follicle.As such any potent non-steroidal anti-inflammatory drug(NSAID) will inhibit ovulation if administered during the first 80% of the ovulatory process(Espey *et al.*, 1981). Recent in vitro studies shows that *Picralima nitida* seed extract significantly supresses PGE_2 and COX 2 production by targeting multiple pathways involving NF-kB and MAPK signalling (Olajide *et al.*, 2014). It is believed that prostaglandins, mainly E_2 stimulate ovum release (Espey *et al.*, 1992). Thus PNE could be reducing fertility index by inhibiting ovulation.The significant differences in reproductive indices between the control and pre-treated groups suggests that PNE has a negative effect on the reproductive system of nulliparous female mice, affirming its folkloric use as an antifertility agent.

The reproductive study of PNE on males involved its acute and chronic effect on sexual behaviour and mating, sperm analysis, and histolopathological studies. *Picralima nitida* showed prospects as a potential aphrodisiac, giving credence to its local use (Ayensu, 1978). The word „aphrodisiac" is a term used to describe any agent, whether food or drug that arouses sexual desire. Based on their mode of action, they can be categorized into three groups; substances that increase libido, potency or pleasure (Sandroni, 2001).Acute treatment of male mice with PNE increased the number of attempted mounts in a dose dependent manner which is usually preceded by sniffing and licking of the female anogenital organ by the males, as well as genital and non-genital grooming. Hesitation time to attraction towards the female also decreased; which may be attributed to the fact that PNE increased the exploratory desires of the animals.

Gonadal steroids play a key role in sexual behaviour, as is evident from the suppression of sexual behavior caused by castration, and its restoration by subsequent testosterone treatment (McGinnis and Dreifuss, 1989). Work done by Gaworski et al., showed that the significant decrease in libido score of rats treated with nicotine was associated with decrease in the serum testosterone level. Other studies have shown that testosterone is associated with increased sexuality, physical and mental energy, stamina and vitality (Mooradian *et al.*, 1987); increased sperm count, fertility and sex drive (Yamamoto, *et al.*, 1998) . The effects of testosterone on sexual behavior are mediated in the brain directly *via* the androgen receptor (AR) and indirectly (after local aromatization to estradiol) *via* estrogen receptors, primarily estrogen receptor alpha (ERα) (Wersinger et al., 1997).

The aphrodisiac effect may also be attributed to the presence of certain indole alkaloids present in the extract, specifically akuammicine and akuammine.Akuammine is structurally related to yohimbine, an α_2 adrenergic blocking agent. Yohimbine hydrochloride has been found to increase sexual motivation in rats (Clark *et al*., 1984). Akuammicine has also been proven to antagonize α_2-adrenoceptors in mouse vas deferens (Demichel & Roquebert, 1984). Blockade of α_2-adrenergic pathway has been shown to be a mechanism by which some aphrodisiacs correct problems of erectile dysfunction probably by causing vasodilation (Andersson, 2001).Penile erection is a hemodynamic event in the penis. It is the end result of relaxation of the cavernous tissue that involves both the central nervous system and the local factors (Sexual Function Health Council, 2004).

Sub-acute treatment with PNE did not significantly affect sniffing and licking of females but also caused a dose-dependent decrease in genital and non genital grooming. These findings suggests that the continuous use of the extract leads to reduction in sexual behaviour. This could be a negative feedback effect by testosterone.

In males, testosterone regulates the hypothalamic-pituitary-gonadal axis at both the hypothalamic and pituitary levels. A negative feedback effect results in the substantial decrease in the release of GnRH by the hypothalamus, and a subsequent release of FSH and LH by the anterior pituitary. The resultant effects are; the decrease in testosterone production and secretion by the gonads which eventually affects libido or sex drive, and the decrease in spermatogenesis resulting in a low sperm count.

Sperm release is an androgen dependant function of the Sertoli cell and this will also be disturbed by decreased testosterone levels. The disturbance results in spermatid retention (delayed spermiation). Decrease in sperm count often results due to the interference in the spermatogenesis process and the elimination of sperm cells at different stages of development (Abdel Aziz, *et al.*, 1997). Since testosterone plays a pivotal role in maturation, spermatogenesis and the maintenance of accessory sex organs (Sharpe *et al.*, 1992), it was therefore not surprising that prolonged treatment with PNE decreased sperm count significantly.

Spermatogenesis involves the formation of highly differentiated haploid spermatozoa from diploid spermatogonia. This regulated process comprises a series of mitotic divisions of spermatogonia to yield spermatocyte. The spermatocyte is the cell that undergoes the long process of meiosis beginning with duplication of its DNA and finally culminating in reduction divisions to produce the haploid spermatid (Creasy, 1997).

Photomicrographs of the testes show that PNE treatment may affect most of the stages of spermatogenesis due to the effects on the semniferous tubules.It is possible that PNE; causes damage to the gonadal cell (spermatogonia and leydig cells) which will not only affect spermatogenesis but also testosterone production leading to reduced sperm production and decreased libido, or has toxic effect on the spermatocyte. In the testes, increased or decreased testosterone secretion will dictate the morphologic pattern of changes. Since stages VII and VIII of the spermatogenic cycle are the most visibly androgen dependent, these may be the only tubules to exhibit morphologic changes in response to endocrine disruption.

This is not suprising because sex steroids, notably estrogen and steroidal antiandrogens have been found to produce testicular atrophy when administered to laboratory animals for long periods (Russell *et al.*, 1981). *Picralima nitida*,having shown to possess estrogenic effects and its ability to antagonize testosterone contributes to the overall reduction in testicular weight and inhibition of spermatogenesis.

Sub acute treatment of male mice with PNE resulted in an insignificant reduction of body and sexual organ weights when compared with the control. The weight of the testis is mostly related to the number of spermatids and spermatozoa present in the tisssue (Gupta *et al.*, 2005). The weight, size, and secretory function of testes, epididymis and seminal vesicles are closely regulated by androgens (Agrawal *et al.*, 1986) as such any slight change in testosterone levels can alter the weights of reproductive organs. Androgen deprivation,apart from suppressing spermatogenesis, leading to low sperm concentration, also alters the epididymal milieu which renders it hostile for maturation and survival of the spermatozoa (Rao, 1988; Setty, 1979).

In males, testosterone regulates the hypothalamic-pituitary-gonadal axis at both the hypothalamic and pituitary levels, and its negative feedback effect is mediated to a substantial degree by estrogen formed *via* aromatization. Thus, exogenous estrogen administration decreases LH and testosterone levels in men (Goodman & Gilman, 2006). *Picralima nitida* in this present study has been proven to possess estrogenic effects. It is therefore not surprising the outcome obtained in the sperm analysis. This result may raise concerns about the safety of PNE as an anti-malarial or analgesic in men because of its effects on male reproduction on prolonged use. Caution should be exercised in men susceptible to recurrent malaria attacks who may use the extract more frequently than the general population.

In the chick comb test, PNE caused a significant ($p<0.01$) increase in comb growth at doses 100 mg/kg and 500 mg/kg when compared with the control group . Comb growth in chicks is highly androgen dependent.Testosterone is the primary androgen for testicular spermatogenesis. Generally, sexual behaviour is enhanced by elevated testosterone levels.The most characteristic symptom of low testosterone is diminished libido, which, taken inconjunction with signs of testicular atrophy on physical examination, is highly specific for hypogonadism when observed in patients with erectile dysfunction (Hellstrom *et al.*, 2010). Sexual symptoms due to low testosterone include erectile dysfunction, difficulty achieving orgasm, reduced intensity of orgasm, diminished ejaculatory volume, and reduced sexual sensation in the genital region, particularly the penis. Traditionally, *Picralima nitida* is believed to possess aphrodisiac properties(Ayensu, 1978; Oliver, 1960).

Comparing the dose response curves of testosterone and the extract, the extract could be a partial agonist. This was confirmed when the extract antagonised the action of testosterone. This is highly expected because for a crude extract,containing myriads of components, whereas some components may contribute to the overall androgenic effect, others may have inhibitory effects.Administration of androgens to males results in inhibition of GnRH and LH at the level of the hypothalamus and pituitary, through normal negative feedback mechanisms. This results in decreased testosterone secretion from the Leydig cells, which produces changes in spermatogenesis that are characteristic of low testosterone. However, the secondary sex organs respond to the exogenously administered androgen with enlargement and increased secretion (O'Connor *et al.*, 2000).

Opioid receptors in the hypothalamus/pituitary are affected by both endogenous and exogenous opioids. These opioids have the propensity to inhibit GnRH,leading to a decrease in LH secretion and thereby causing a reduction in estradiol and testosterone levels. Previous studies have demonstrated that morphine induces a long lasting decrease in testosterone, which persists during the therapy even if treatment lasts for months or years (Aloisi, *et al*., 2009). Pseudoakuammigine, an isolate of *Picralima nitida* has been reported to exhibit analgesic properties which are mediated via interaction with opioid receptors (Duwiejua *et al*., 2002). The prescence of pseudoakuammigine could possibly contribute to the relatively lower comb growth observed with the extract treament as compared to testosterone.

Administration of herbal products has the propensity to cause significant changes in the structure, function, metabolic transformations and concentraion of biomolecules and enzymes. Such changes may lead to different biochemical transformation yielding various pathological and clinical changes (Murray *et al.*, 2000).Assessment of the hematological parameters helps to determine the extent of damaging effect xenobiotics have on blood. The non significant increase in WBC count may probably be due to normal immune responses to foreign bodies. Furthermore, normal ranges of red blood cell count, hemoglobin and hematocrit suggest that PNE is unlikely to cause anaemia at the dose range used in this study. It could be that the extract has the potential to stimulate erythropoetin release in the kidney, which is the humoral regulator of red blood cell formation. This confirms previous studies by Unakalamba *et al.*,(2013) on the hematological effects of *Picralima nitida* saponin extracts.

There were no significant changes in levels of cholesterol, triglycerides and alanine aminotransferase. Alanine aminotransferase is found in plasma and in various body tissues, but common in liver. Elevated levels of ALT usually suggests the presence of liver damage, viral hepatitis and bile duct problems. Aspartate aminotransferase is found in the liver, heart, kidneys and red blood cells.Alanine aminotransferase and aspartate aminotransferase are liver associated enzymes that are indirect measures of liver homeostasis. Hepatocellular injury leading to permeability of intracellular enzymes into the bloodstream is accompanied by elevated ALT and AST. AST is also present in red cells, cardiac and skeletal muscles, therefore not specific to the liver. Aspartate aminotransferase was slightly increased than those in the control group. Thus the insignificant increment observed may be due to these other tissues containing AST. ALT, which is specific to liver cells, remained within the accepted range, thus PNE is not hepatotoxic at the dose levels used.

CHAPTER FIVE

CONCLUSIONS

The research showed that oral administration of the ethanolic seed extract of *Picralima nitida*leads to an increase in sexual abilities in males. However, prolonged intake of the extract does not continue to significantly enhance the sex drive but may rather decrease sexual abilities and significantly decrease sperm quality.

Our studies also showed that PNE possesses estrogenic andantifertility effects in females.Thus it could be detrimental to an individual who desires conception but inure to the benefit of another who desires contraception.

The no-observerbable-adverse-effect-level (NOAEL)is ≤ 300 mg/kg.

RECOMMENDED FURTHER STUDIES

- Evaluate the main alkaloidal components responsible for the anti-fertility and aphrodisiac properties.

- Estimate the effect of PNE treatment on serum hormonal levels (LH, FSH, prolactin)

REFERENCES

Abdel Aziz, A.H., Shouman, S.A., Attia, A.S.&Saad, S.F. (1997) A study on the reproductive toxicity of erythrosine in male mice. *Pharmacol Res.*35(5):457–62.

Adjanohoun, J., Aboubakar, N., Dramane, K., Ebot, M., & Ekpere, J. (1996). Contribution to ethnobotanical & Floristic studies in Cameroun. *Traditional Medicine & Pharmacopoeia*, 61-65.

Agrawal, S., Chauhan, S., & Mathur, R. (1986). Antifertility effects of embelin in male rats. *Andrologia*, 18:125-131.

Aguwa, C., Ukwe, C., Inya-Agha, S., & Okonta, J. (2001). Antidiabetic effect of Picralima nitida aqueous seed extract in experimental rabbit model. *1(2)*(135-139).

Aloisi, A., Aurilio, C., Bachiocco, V., Biasi, G., Fiorenzani, P., Pace, M. & Ceccarelli, I. (2009). Endocrine consequences of opioid therapy. *Psychoneuroendocrinology*, 34:
5162-168.

Andersson, K. (2001). Pharmacology of Penile Erection. *Pharmacol Rev*, 53:417-450.

Ansa-Asamoah, R., & Amposo, A. (1986a). Analgesic effect of crude extracts of Picralima nitida seeds. *Afr. J. Pharmacol*, 1:35-38.

Arens, H., Borde, H., Illorich, B., & Stockight, J. (1982). Detection of pericine., A new CNS active indole alkaloid from Picralima nitida cell suspension culture by opiate receptor binding studies. *Planta Medica*, 46:210-214.

Arens, H., Ulbrich, B., & Stokigt, J. (1982b). Determination of pericine, a new CNS-active indole alkaloid. *Planta Med.*, 46(4): 210-214.

Ayensu, E. (1978). *Medical Plants in West Africa*. Algonac, Michigan: Reference Publications Inc.

Badami, S., Aneesh, R., Sankar, S., Sathishkumar, M.N., Suresh, B. & Rajan, S. (2003) Antifertility activity of Derris brevipes variety coriacea. *J Ethnopharmacol.* 84:99–104.

Benie, T., Duval, J., & Thieulant, M. (2003). Effects of some traditional plant extracts on rat oestrous cycle compared with clonid. *Phytother Res,* 17: 748-755.

Bianco, F., Basini, G., & Grasselli, F. (2006). The plant alkaloid Sanguinarine affects swine granulose cell activity. *21*(335-340).

Browning, R., Schrick, F. N., Thompson, F., & Wakefield, T. (1998). Reproductive hormonal responses to ergotamine and ergonovine in cows during the luteal phase of the estrous cycle. *76*(1448-1454).

Cheesebrough, M. (2006). *District Laboratory Practice in Tropical Countries.* Cambridge: Cambridge University Press, The Edinburgh Building, Cambridge CB2 8RU, UK.

Chituri, S., & Farrell, G. (2000). Herbal Hepatotoxicity: An expanding but poorly defined problem. *Journal of Gastroenterology and Hepatology*, 15: 1093-1099.

Choudhary, A., & Steinberger, E. (1975). Effect of 5a-reduced androgen on sex accessory organs,irritation and maintenance of spermatogenesis in the rat. *Biol. Reprod*, 12:609-617.

Circosta, C., Sanogo, R., & Occhiuto, F. (2001). Effects of Calotropis procera on estrous cycle and on estrogenic functionality in rats. *Farmaco*, 56:373-8.

Clark, J., E.R., S., & Davidson, J. (1984). Enhancement of sexual motivation in male rats by Yohimbine. *Science*, 847-9.

Clinquart, E. (1927). Alkaloids of Picralima Kleineana. *J. of Pharmacol. Belg*, 9:107.

Creasy, D. M. (1997). Evaluation of Testicular Toxicity in Safety Evaluation Studies: The Appropriate Use of Spermatogenic Staging. *Toxicol Pathol*, 25: 119.

Creasy, D., & Foster, P. (2002). *Male reproductive system. In Handbook of Toxicologic Pathology, vol. 2, Haschek WM, Rousseaux CG, Wallig MA (eds).* San Diego, CA: Academic Press.

Demichel, P., & Roquebert, J. (1984). Effects of raubasine stereoisomers on pre- and postsynaptic α-adrenoceptors in the rat vas deferens. *Br. J. Pharmacol*, 83, 505-510.

Dorfman, R. I. (1969). *Antiestrogens. In: Dorfman RI (ed) Methods in hormone research, Vol IIA.* New York, pp 121-149: Academic Press.

Duwiejua, M., Woode, E., & Obiri, D. (2002). Pseudo-akuammigine, an alkaloid from Picralima nitida seeds, has anti-inflammatory and analgesic actions in rats. *J Ethnopharmacol*, 81(1):73-9.

Eaton, D., & Klaassen, C. (2001). *Principles of toxicology. In Casarett and Doull's Toxicology: The Basic Science of Poisons, 6th edn.* New York: McGraw-Hill,.

Environmental Protection Agency (1998). Health Effects Test Guidelines OPPTS 870.3700 Prenatal Developmental Toxicity Study. *Prevention, Pesticides and Toxic Substances*.

Ernst, E. (2007). „„„First, do no harm"" with complementary and alternative medicine. *Trends Pharmacol Sci*, 28: 48-50.

Espey, L. L. (1994). Current Status of the Hypothesis that Mammalian Ovulation is Comparable to an Inflammatory Reaction. *Biology of Reproduction*, 50, 233-238.

Espey, L. L., Coons, P. J., Marsh, J. M., & LeMaire, W. J. (1981). Effect of indomethacin on preovulatory changes in the ultrastructure of rabbit graafian follicles. *Endrocrinolgy*, 108, 1040-1048.

Espey, L. L., Tanaka, N., Stacy, S., & Okamura, H. (1992). Inhibition of ovulation in the gonadotropin-primed immature rat by exogenous PGE2. *Prostaglandins*, 43: 67-74.

Evans, T., Constantinescu, G., & Ganjam, V. (2007). *Clinical reproductive anatomy and physiology of the mare. In Current Therapy in Large Animal Theriogenology, Younquist RS, Threlfall WR (eds), 2nd edn.* St. Louis: Saunders Elsevier.

Ezeamuzie, I., Ojinnaka, M., Uzogara, E., & Oji, S. (1994). Anti inflammatory, antipyretic and antimalarial activity of a West African medicinal plant-Picralima nitida. *African Journal of Medicine and Medical Science*, 23 (1): 85-89.

Ezekwesili, J. O. (1983). *Biostudies of Picralima nitida (Staph) Th. & Hel. Dur. M.Sc. Dissertation.* Nigeria: Universitty of Nigeria.

Francois, G., Assi, L., Holenz, J., & Bringmann, G. (1996). Constituents of Picralima nitida display pronounced inhibitory activities against asexual erythroctic forms of Plasmodium falciparum in vitro. *Journal of Ethnopharmacology*, 54: 113-117. Gaworski, C.L., Carmines, E.L., Faqi, A.S. & Rajendran, N. (2004). In utero and lactation exposure of rats to 1R4F reference cigarette mainstream smoke: effect on prenatal and postnatal development. *Toxicol Sci*.79(1):157–69

Genuth, S. (2004). *The reproductive glands. In Physiology, 5th edn Berne RM, Levy MN, Koeppen BM, Stanton BA (eds).* St. Louis, MO: Mosby Inc.

Ginther, O. (1992). *Reproductive Biology of the Mare: Basic and Applied Aspects, 2nd edn.* Cross Plains, WI.: Equiservices.

Goodman, & Gilman. (2006). *The Pharmacological Basis of Therapeutics.* New York: McGraw Hill Companies.

Gupta, R., Chaudhary, R., Yadav, R., Verma, S., & Dobhal, M. (2005). Effects of saponins of Albizzia lebbeck (L.) benth bark on the rerproductive system of male albino rats. *J. Ethnopharmacol*, 96:31-36.

Gupta, R., Kachhawa, J., & Rakhi, S. (2006). Antispermatogenic effects of Nyctanthus arbortristis in male albino rats. *Pharmacology online,* 2: 261-273.

Hare, M. (1993). The emergence of an urban United States Chinese medicine. *Med Anthropol Q*, 7: 30-49.

Hellstrom, W., Laduch, D., & Donatuccie, C. (2010). Importance of hypogonadism and testosterone replacement therapy in current urologic practice: a review. *Int Urol Nephrol*, In Press.

Henry, T. A. (1949). *The Plant Alkaloids* . London.

Henry, T. A., & Sharp, T. M. (1927). The alkaloids of Picralima klaineana. *J. Chem. Soc*, 25, 1950-1959.

Himmel, W., Schulte, M., & Kochen, M. (1993). Complementary medicine: are patients‟ expectations being met by their general practitioners? *Br J Gen Pract*, 43: 232-235.

Hodgson, E., Mailman, R., Chambers, J., & Dow, R. (. (2000). *Dictionary of Toxicology, 2nd edn.* New York: Grove‟s Dictionaries Inc.

Hood, R. (2006). *Developmental and Reproductive Toxicology.* CRC Press.

Hussin, A. H. (2001). Adverse effects of herbs and drug-herbal interactions. *Malaysian Journal of Pharmacy*, 1 (2): 39-44.

Irvine, F. (1961). *Woody plant of Ghana.* London: Oxf. University Press.

Irwin S. (1968) Comprehensive observational assessment: la. A systemic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacology* 13: 222-257.

Jain, S., Choudhary, G. P., & Jain, D. K. (2012). Pharmacological evaluation of antifertility activity of ethanolic extract of Jatropha gossypifolia leaf in albino mice. *Asian Pacific Journal of Tropical Biomedidcine*, 1671-1674.

Jurgens, T. M. (2003). Potential toxicities of herbal therapies in the developing fetus. *Birth Defects Res B Dev Reprod Toxicol,* 68:496–498.

Keay, R., Onochie, C., & Stemfield, D. (1964). *Nigeria trees.* 2; 378-396: Ibadan Federal Department of Forest Reserve. Ann. Rep.

Keeler, R.F. (1984) Teratogens in plants. *J Anim Sci* 58: 1029-39

Kimmel, C. A., & Buelke-Sam, J. (2001). *Reproductive and Developmental Toxicology. Patty's Toxicology*. . John Wiley & Sons, Inc.

Kimmel, C., & Wilson, J. (1973). Skeletal deviations in rats: Malformations or variations? *Teratology*, 8:309-316.

Koehn, F. G. (2005). The evolving role of natural products in drug discovery. *Nature*, 4,206-220.

Koehn, F., & Carter, G. (2005). The evolving role of natural products in drug discovery. *Nature*, 4,206-220.

Koneri, R., Balaraman, R., & Saraswati, C. D. (2006). Antiovulatory and abortifacient potential of the ethanolic extract of roots of Momordica cymbalaria Fenzl in rats. *Indian J Pharmacol*, 38:111-4.

Kouitcheu, M., Penlap, B., Kouam, J., Ngadjui, B., Fomum, Z., & Etoa, F. (2006). Evaluation of antidiarrhoeal activity of the fruit rind of Picralima nitida. *African Journal of Traditional, Complementary and Alternative Medicines*, 3 (4): 66-73.

Krum, A. E., Morris, M. D., & Benett, L. L. (1964). *Endocrinology.*

Lauritzen, C., Reuter, H., Repges, R., Bohnert, K., & Schmidt, U. (1997). Treatment of premenstrual tension syndrome with Vitex agnus castus controlled double-blind study versus pyridoxine. *4*(183-189).

Leonard, K. L. (2001). *African Traditional Healers: Are they good at Economics as they are at Medidcine?* (Unpublished report).

Lerner, L., Holthaus, F., & Thompson, C. (1958). A non-steroidal estrogen antagonist 1(p-2-diethylaminoethoxyphenyl)- 1-phenyl-2-p-methoxyphenyl ethanol. *Endocrinology*, 63: 295-318.

Long, J., & Evans, H. (1922). The estrous cycle in the rat and its associated phenomena. *Memories of University of California*, 6: 1-148.

Ma, W.G., Song, H., Das, S.K., Paria, B.C.&Dey, S.K. (2003). Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation.*Proc Natl Acad Sci* , 100(5): 293-8

MacLennan, A., Wilson, D., & Taylor, A. (1996). Prevalence and cost of alternative medicine in Australia. *Lancet*, 347: 569-573.

Magee, K., & Loiacono, C. (2004). A review on common herbs and potential interactions. *International Journal of Dental Hygiene*, 2: 111-121.

Mandl, A. M. (1951). The phases of the oestrous cycle in the adult white rat. *Journal of Experimental Biology*, 28: 576-584.

McGinnis, M.Y & Dreifuss, R.M.(1989). Evidence for a role of testosterone-androgen receptor interactions in mediating masculine sexual behavior in male rats. *Endocrinology*. 124:618–626.

Menzies, J. R., Paterson, S. J., Duwieja, M., & Corbett, A. D. (1998). Opioid activity of alkaloids extracted from Picralima nitida . *European Journal of Pharmacology*, 350, 101-108.

Meyer L., Pesta N. and Ramsay A. 2006. Méthodes d‟extraction et de purification des alcaloïdes de Picralima nitida Intérêts de la CPC. UE Documentation 06/07, PPT 2.

Mooradian, A.D., Morley, J.E. & Korenman, S.G. (1987). Biological actions of androgens. *Endocr Rev*. 8(1):1–28.

Moser, V.C. (2011). Functional Assays for Neurotoxicity Testing. *Toxicol Pathol*, 39: 36-45

Murray, R., Granner, K., Mayer, P., & Rodwell, V. (2000). *Harper's Biochemistry.* London: Mc Graw Hil.

Nkere, C., & Iroegbu, C. (2005). Antibacterial screening of the root, seed and stembark extracts of Picralima nitida. *African Journal of Biotechnology*, 4 (6): 522-526.

Nwakile, C., & Okore, V. (2011). Picralima nitida Seed Oil I: Hypoglycaemic Activity. *Journal of Advanced Pharmacy Education & Research*, 2: 147-153.

O‟Donnell, L., Robertson, K., Jones, M., & Simpson, E. (2001). Estrogen and spermatogenesis. *Endocrine Rev*, 22(3): 229-318.

Obasi, N., Okorie, U., Enemchukwu, B., Ogundapo, S., & Otuchristian, G. (2012). Nutritional Evaluation, Phytochemical Screening and Antimicrobial Effects of Aqueous Extract of Picralima nitida Peel. *Asian Journal of Biological Sciences*, 5: 105-112.

Obiri, D. D. (1994). *Studies on anti-inflammatory activity of extracts from seeds of picralima nitida.* Accra, Ghana: KNUST.

O'Connor, J., Davis, L. G., Frame, S. R., & Cook, J. C. (2000). Evaluation of a Tier I screening battery for detecting endocrine-active compounds (EACs) using the positive controls testosterone, coumestrol, progesterone, and RU486. *Toxicol Sci*, 54:338-354.

Okonta, J. M., & Aguwa, C. (2007). Evaluation of Hypoglycemic activity of Glycosides and Alkaloids Extracts of Picralima nitida Stapf (Apocynaceae) Seed. *International Journal of Pharmacology*, 3 (6): 505-509.

Okunji, C. O., Iwu, M. M., Ito, Y., Smith, P.L. (2005). Preparative Separation of Indole Alkaloids from the Rind of Picralima nitida (Stapf) T. Durand& H. Durand by ph-Zone- Refining Countercurrent Chromatography. *Journal of Liquid Chromatograpy & RelatedTechnologies,* 28(5) 775-783

Olajide, O. A., Velagapudi, R., Okorji, U. P., Sarker, S. D., & Fiebich, B. L. (2014). Picralima nitida seeds supress PGE2 productionby interfering with multiple signalling pathways in IL-1B-stimulated SK-N-SH neuronal cells. *Journal of Ethnopharmacology*, 152(2): 377-383.

Peralta, R., Terenzi, H. F., & Jorge, J. A. (1990). β-D-Glucosidise activities of Humicola grisea: biochemical and kinetic characterization of a mullet-functional enzyme. *Biochimica et Biophsica Acta*, 1033:243-249.

Pincus, G. (1965). *Control of fertility.* New York: Academic Press.

Porsolt, R.D., Lemaire, M., Dürmüller, N. & Roux, S. (2002). New perspectives in CNS safety pharmacology. *Fundamental & clinical pharmacology***16**(3)**:** 197-207.

Rang, H. P., Dale, M. M., Ritter, J. M., & Moore, P. K. (2003). *Pharmacology.* London: Elsevier Science.

Rao, M. (1988). Effect of alcoholic extract of Solanum xanthocarpum seeds in adult male rats. *Indian J. Exp. Biol.*, 26: 95-98.

Russell, L. D., Malone, J. R., & Karpas, S. L. (1981). Morphological pattern elicited by agents affecting spermatogenesis by disruption of its hormonal stimulation. *Tissue and Cell,* 13, 369-380.

S, I. (1968). Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. . *Psychopharmacologia 13 (3)*, 222-257.

Sandroni, P. (2001). Aphrodisiacs past and present: a historical review. *Clin Auton Res*, 11:303-307.

Schwarz, U. I., Buschel, B., & Kirch, W. (2003). Unwanted pregnancy on self- medication with St John"s wort despite hormonal contraception. *British journal of Clinical Pharmacology*, 53: 112-113.

Senger, P. (2003). *Pathways to Pregnancy and Parturition, 2nd edn.* Moscow: Current Conceptions, Inc.

Setty, B. (1979). Regulation of Epididymal function and sperm maturation- endocrine approach to fertility control in male. *Endocrinology,* 74: 100-117.

Sexual Function Health Council. (2004). *American Foundation for urologic disease.* Baltimore: Center for Reconstructive Urology.

Sharpe, R.M., Maddocks, S., Millar, M., Kerr, J.B., Saunders, P.T.&McKinnell, C. (1992) Testosterone and spermatogenesis. Identification of stage-specific, androgen-regulated proteins secreted by adult rat seminiferous tubules. *J Androl.* 1992;13(2):172–84.

Singh, R., Ali, A., Jeybalan, G., Semwal, A., & Jaikishan. (2013). An overview of the current methodologies used for evaluation of aphrodisiac agents. *Journal of Acute Disease*, 85-91.

Sofowora, A. (1982). *Medicinal Plants and Traditional Medicine in Africa.* New York: John Wiley and Sons,Chichester West Sussex.

Sutovsky, P., Moreno, R., Ramahlho-Santos, J., Dominko, T., & Thompson, W. (2001). A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci*, 1665-75. Tamura, K., Honda, H., Mimaki, Y., Sashida, Y. & Kogo, H. (1997). Inhibitory effect of a new steroidal saponin, OSW-1, on ovarian functions in rats. *Br J Pharmacol.* 121:1796–802

Thomas, M., & Thomas, J. (2001). *Toxic responses of the reproductive system In Casarett and Doull's Toxicology: The Basic Science of Poisons, 6th edn, Klaassen CD (ed.).* New York: McGraw-Hill.

Tullner, W., & Hertz, R. (1956). The effect of 17-alpha-hydroxy-11- desoxycorticosterone on estrogen-stimulated chick oviduct growth. *Endocrinology*, 58: 282-283.

Tyl, R., & Marr, M. (2006). *Developmental Toxicity Testing — Methodology, in Hood R. D. (ed) Developmental and Reproductive toxicology-a practical approach.* New York: NW Taylor & Francis Group (CRC Press) 2nd edition.

Ubulom, P., Akpabio, E., Udobi, C., & Mbon, R. (2011). Antifungal Activity of aqueous and ethanolic extracts of Picralima nitida seeds on Aspergillus flavus, Candida albicans and Microsporum canis. *Research in Pharmaceutical Biotechnology,*, 3(5), 57-60.

Ubulom, P., Imandeh, N., Udobi, C., & Ilya, I. (2012). Larvicidal and Antifungal Properties of Picralima nitida (Apocynaceae) Leaf Extracts. *European Journal of Medicinal Plants*, 2(2): 132-139.

Unakalamba, B., Ozougwu, J., & V.C., E. (2013). Preliminary evaluation of the haematological effects of Picralima nitida saponin extracts on Rattus novergicus. *International Journal Of Biology and Biological Sciences*, 28-32.

Vaidya, P., Padmashali, S., Vagdevi, H.M. & Sathyanarayana, N.D. (2006). Antifertility effect of the plant Balanites roxburghii (Balanitaceae) in female rats. Ind J *Pharm Sci*.3:347–51

Vladmir, K., & Ludmila, M. (2001). Glycosides in medicine:The role of glycosidic residue in biological activity. *Curr. Med. Chem*, 8:1303-1328.

Wersinger, S.R, Sannen, K., Villalba, C., Lubahn, D.B., Rissman, E.F., De Vries, G.J. (1997). Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor alpha gene. *Horm. Behav*.32:176–183.

Whitten, W. K. (1956). Modification of the oestrus cycle of the mouse by external stimuli associated with the. *J. Endocrinol*, 13:399-404.

World Health Organization (2002). *Traditional Medicine Strategy 2002-2005*. Geneva: WHO.

World Health Organization (2009). *Handbook: good laboratory practice (GLP).* Switzerland: WHO.

Wilker, C., & Ellington, J. (2006). *Reproductive toxicology in the female companion animal. In Small Animal Toxicology, 2nd edn, Peterson ME, Talcott PA(eds).* St. Louis, MO: Elsevier Saunders.

Wilson, J. (1977). *Current status of teratology: general principles and mechanisms derived from animal studies. In Handbook of Teratology, vol. 1,*. New York: Plenum Press.

Wojcikowski, K., Johnson, D., & Gobe, G. (2004). Medicinal herbal extracts- renal friends or foe? Part One: The toxicity of medicinal herbs. *Nephrology*, 15: 1093-1099.

Yamamoto, Y., Isoyama, E., Sofikitis, N. & Miyagawa, I. (1998). Effects of smoking on testicular function and fertilizing potential in rats. *Urol Res*. 26(1):45–8).

Yakubu, M., Akanji, M., & Oladiji, A. (2005). Aphrodisiac potentials of the aqueous extract of Fadogia agrestis (Schweinf. Ex Hiern) stem in male albino rats. *Asian J Androl*, 7: 399-404.