REPRODUCTIVE AND DEVELOPMENTAL TOXICITY OF THE AQUEOUS ROOT EXTRACT OF THE ANTIMALARIAL HERBAL *CRYPTOLEPIS SANGUINOLENTA* (LINDL.) SCHLTR (PERIPLOCACEAE) IN EXPERIMENTAL ANIMALS.

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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.

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Nun danket alle Got

"Now thank we all our God, with heart and hands and voices;

Who wondrous things hath done, In whom his world rejoices;

Who, from our mother's arms, hath blessed us on our way

With countless gifts of love, and still is ours to-day"

(MHB 10) *Martin Rinkart* (1586 – 1649)

If I can see this far is because I stood on the shoulders of Giants". *Isaac Newton*

My giants are all members of the Department of Pharmacology particularly Dr Ansah, Prof Woode, Mr. Ansah.

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DEDICATION

To my nephew, Nana Yaw Boakye - Mensah and my niece, Efua Gyemfa Bamfo

ABSTRACT

Cryptolepis sanguinolenta, the West African antimalarial plant, is a known cytotoxic to mammalian cells *in vitro*, a DNA intercalator and topoisomerase II inhibitor. Malaria is endemic in Africa. The high cost of conventional antimalarial agents would suggest that pregnant women particularly in rural West Africa could be exposed to cryptolepis. Recently, cryptolepis containing products have appeared in Ghanaian pharmacies and herbal shops as male sexual enhancers. Paternal exposure to xenobiotics has an equal chance of having an adverse effect on the health of the conceptus as that of the mother. Using animal models, the effects of cryptolepis on reproduction, foetal development and *in vivo* mutagenesis is extensively studied in this project.

Cryptolepis extract (62.5- 1000 mg/kg) reduced female fertility from 100 % in the control group to 0 % at the highest dose of 1000 mg/kg when given during mating and gestation. However, in females unlike males, pretreatment before mating did not have adverse outcomes on reproduction. Further studies showed that inhibition of ovulation is one the mechanisms of cryptolepis reproductive toxicity.

In developmental studies, cryptolepis did not induce malformations (monstrosity) or structural abnormalities. However, early treatment of pregnant mice with cryptolepis resulted in terminations of pregnancy which was as high as 66 % when cryptolepis (100 mg/kg) was introduced on the day of mating till the end of gestation. Intrauterine growth inhibition was significant ($p < 0.01$) for cryptolepis treatment at 100 - 500 mg/kg. The developmental toxicity observed was largely influenced by the time of treatment with cryptolepis and the developmental stage of the conceptus.

In functional toxicity studies, prenatal treatment with cryptolepis delayed the development of sensori-motor systems, caused hyperactivity, anxiety, reduced same sex pair interactions and affected object recognition in first generation animals. It however had minimal effect on spatial and reference memory.

In the mounting behavioural test to study the aphrodisiac potential of cryptolepis in mice, acute dosing had insignificant effect on all mounting parameters except penile licking which was very significant ($p < 0.001$) at all doses tested. All aphrodisiac properties were reversed below control

values with subacute treatments. Histological studies showed that it affected the histoarchitecture of the testes. Cryptolepis also reduced serum testosterone in male mice significantly at all doses of treatment thus consistent with the lack of aphrodisiac property observed with subacute treatments.

Cryptolepis exhibited potent anti-androgenic effects by the chick comb method. It was approximately 31.05 times less potent than the cyproterone acetate used as the standard drug. Furthermore it inhibited exogenous testosterone induced comb growth in the white leghorn chick, *Gallus domesticus*. Cryptolepis (100 – 1000 mg/kg *p.o*) also induced significant renal damage. In the dominant male lethal assay for genotoxicity and mutagenicity, cryptolepis (62.5-1000 mg/kg) did not induce significant increase in post implantation losses.

Overall, the study shows that the aqueous extract of *Cryptolepis sanguinolenta* (Periplocaceae) affects reproduction and foetal development in experimental animals. Though results from this study cannot be directly extrapolated to man, caution should be exercised in the use of aqueous extract of *Cryptolepis sanguinolenta* in pregnancy for the treatment of malaria.

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ABBREVIATION

AVPV	Anteroventral Periventricular Nucleus
BBB	Blood Brain Barrier
BMI	Body Mass Index
CNS	Central Nervous System
COX	Cyclooxygyenase
CRYPTOLEPIS	Aqueous extract of *Cryptolepis sanguinolenta* roots
DNA	Deoxyribonucleic Acid
DDT	Dichlorodiphenyl trichloroethane
EDC	Endocrine Disrupting Chemicals
F_1	First Generation mice
FDA	Food and Drugs Administration
FSH	Follicle Stimulating Hormone
HHG/Th	Hypothalamus Hypophysis Gonad/Thyroid axis
GD	Gestation Day
LH	Lutenizing Hormone
IL	Interleukin
MPN	Medial Preoptic Nucleus
NF-kB	Nuclear Factor Kappa Beta
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
eNOS	Endothelial Nitric Oxide Synthase
PC	Post coitus
PG	Prostaglandins
PND	Post Natal Day
SDN-POA	Sexually dimorphic Nucleus of the Preoptic Area

TNF	Tissue Necrosis Factor
USEPA	United State Environmental Protection Agency
UNICEF	United Nations International Children Emergency Fund
VMH	Ventromedial Hypothalamus
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 OVERVIEW

Herbal medicines form an integral part of health systems in the world particularly in Africa and Asia where it is estimated that up to 80% of sick people use herbal medicines (Sofowora, 1982; WHO, 2008). Depending on the laws of a country, herbal medicines are considered as medicines, ethnomedicines, contemporary and alternative medicines, traditional medicines or health foods. Whilst most people accept that they are indeed efficacious and can be helpful in some cases where conventional drugs have failed or are too expensive and inaccessible, controversy exists on their safety as medicines. Most proponents and users of herbal medicines believe that they are from nature and hence are free from toxic effects. Others believe that as alternative medicines, only controlled and restricted usage can actually distinguish them from poisons. Indeed Paracelsus said "all substances are poison, and nothing is without poison; only the dose permits something not to be poisonous". Against this background, Talalay and Talalay (2001) recommended that all natural products used in therapeutics must be subjected to safety tests by the same methods for new scientific drugs.

In Ghana it is estimated that 3.5 million people contract malaria every year and approximately 20,000 children die from malaria each year (UNICEF, 2007). The cost and accessibility of malaria treatment in Ghana always creates the need for people to seek alternative medicines. In fact, The National Malaria Control Programme in 2007 reported that 70% of Ghanaians used herbal medicines in treating malaria (Siaw, 2011).

The aqueous extract of *Cryptolepis sanguinolenta*, previously known as *Cryptolepis triangularis* (Asclepiadaceae), is used traditionally as an antimalarial, antidysentery and febrifuge remedy in Central and West Africa (Sofowora, 1982; Boye and Ampofo, 1983; Michel *et al.*, 2008). In addition, it possesses a wide range of pharmacological actions. Several cryptolepis based products have appeared in Ghanaian pharmacies and herbal shops for the treatment of malaria and other ailments.

Although *Cryptolepis sanguinolenta* has been used in traditional medicine for many generations there is limited data on its toxicity profile. In recent experiments, Ansah and Gooderham, (2002) reported *in vitro* cytotoxicity. *In vivo* general toxicological assay showed that cryptolepis is apparently safe (Addy, 2003; Ansah *et al.*, 2008a; Ansah *et al.*, 2009a). There are however, a number of concerns about its apparent safety.

Cryptolepis is cytotoxic (Ansah and Gooderham, 2002) and cytotoxics have the tendency to cross the placenta exerting toxic effects on the developing foetus (Cardonick and Iacobucci, 2004). The safety of *Cryptolepis sanguinolenta* in pregnancy and foetal development is largely unknown. In view of the fact that pregnant women and children are more prone to malaria and subsequent recurrent attacks compared to the general population, it is expected that the exposure of these groups to cryptolepis especially amongst the rural population could be underestimated.

Cytotoxics exert inhibitory effects on processes involving rapid cell proliferation such as spermatogenesis, haematopoiesis and foetal development (Allan *et al.*, 1988; Cai *et al.*, 1997; Cardonick and Iacobucci, 2004). Cryptolepis has been shown to be cytotoxic to some

mammalian cells (Ansah and Gooderham, 2002). Effects of drugs on male fertility is now of primary health concern in Ghana. Furthermore, cryptolepis containing products have been used and marketed as male sexual enhancers in Ghana, a claim not proven scientifically. Paternal exposure to chemicals can result in adverse outcomes on the survival and health of the offspring (Green *et al.*, 1985; Russell and Shelby, 1985; Shelby, 1994).

Subjecting a drug to reproductive and developmental studies provide key and salient information, which are not normally seen in general toxicity assay. This include transgenerational effects and slow onset toxicities.

In this project, cryptolepis, a popular antimalarial plant, is subjected to reproductive and developmental toxicity assessment.

1.2 CRYPTOLEPIS SANGUINOLENTA

Botanical source – aqueous *Cryptolepis sanguinolenta* is obtained from the roots of *Cryptolepis sanguinolenta* of family Periplocaceae or Asclepiadaceae. It is locally known as nibima (Twi), Kadze (Ewe), gangamau (Hausa) or Ghana quinine, probably because of its bitter taste and its substitution for quinine as an antimalarial.

Fig 1.1 The dried roots of *Cryptolepis sanguinolenta*

The plant is a thin-stemmed and scrambling shrub. It can be found dispersed in open areas (Luo *et al.*, 1998; Paulo and Houghton, 2003). The leaves are petiolate, glabrous, elliptic or oblong-elliptic, and up to 7 cm long and 3 cm wide. The blades have an acute apex and symmetrical base. The inflorescence cymes, lateral on branch shoots, are few flowered, with a yellow corolla tube up to 5 mm long. Its fruits are paired in linear follicles and are horn-like. The seeds are oblong in shape, small (averaging 7.4 mm in length and 1.8 mm in the middle), and pinkish, embedded in long silky hairs (Irvine, 1961; Addy, 2003). Cryptolepis has a sweet fragrance when dry. The root has a light brown colour and bitter taste. The root varies from 0.4 - 6.6 cm long and 0.31-1.4 cm wide. It is hard and brittle.

1.2.1 Traditional uses of *Cryptolepis sanguinolenta*

In Ghana, decoctions of the dried roots of the plant is used to treat various forms of fevers, including malaria, urinary and upper respiratory tract infections, rheumatism and venereal diseases (Boakye –Yiadom, 1979a; Michel *et al.*, 2008)**.** Amongst the Fulani's in Guinea-Bissau, the aqueous extract of the plant is used to treat jaundice and hepatitis (Silva *et al.*, 1996). In Zaire and the Casamance district of Senegal, infusions of the roots are used in the treatment of stomach and intestinal disorders (Kerharo and Adam, 1974). In Congo the aqueous extract of the roots is used in amoebiasis (Tona *et al.*, 1998). In northern Nigeria the hot aqueous decoction is used as a source of yellow dye for goat hide.

1.2.2 Biological Effects Of *Cryptolepis sanguinolenta* and its constituents.

Cryptolepine is the major alkaloid in the aqueous extract (Dwuma-Badu *et al.*, 1978). Most of the major pharmacological activities exhibited by cryptolepis are due to the alkaloid, cryptolepine. Cryptolepine is unique as synthesis by Fichter and Boehringer in 1906 first preceeded the isolation by Clinquart, (1929); Gellert and Raymond-Hamet, (1951) and later by Dwuma-Badu *et al.*, (1978). Cryptolepine exhibits antimalarial (Noamesi *et al.*, 1991b; Kirby *et al.*, 1995; Greiller *et al.*, 1996) and antiplasmodial activity (Paulo *et al.*, 2000); hypotensive activity (Raymond-Hamet., 1938; Bamgbose and Noamesi, 1978); preferential presynaptic alpha adrenoceptor blocking actions (Noamesi and Bamgbose, 1980; Noamesi and Bamgbose, 1982) antimuscarinic property (Rauwald *et al.*, 1992); antiplatelet, antithrombotic, and fibrinolytic activity (Oyekan *et al.*, 1986; Oyekan and Okafor, 1989; Oyekan and Ablordeppey, 1993a; Oyekan and Ablordeppey, 1993b). Recently, it has been shown that cryptolepine and its

derivatives are able to inhibit hemozoin polymerization (Onyeibor *et al.*, 2005). Cryptolepine also has antibacterial (Boakye-Yiadom and Heman-Ackah, 1979 b; Cimanga *et al.*, 1996), antimycobacterial (Gibbons *et al.*, 2003) and antifungal activities (Cimanga *et al.*, 1998; Sawer *et al.*, 2005).

Cryptolepis reduces plasma glucose (Luo *et al.*, 1998) and cryptolepine enhanced insulin-mediated glucose disposal in a mouse model of diabetes and in an *in vitro* system using the 3T3-L1 glucose transport assay (Luo *et al.*, 1998). It has potent antiinflammatory activity without inducing gastric ulceration (Bamgbose and Noamesi, 1981; Olajide *et al.*, 2009). Both cryptolepis and its main alkaloid, cryptolepine, are cytotoxic (Ansah and Gooderham, 2002) with low genotoxicity (Ansah *et al.*, 2005). The molecular mechanisms include DNA intercalation and inhibition of topoisomerase II (Bonjean *et al.*, 1998; Lisgarten *et al.*, 2001).

1.3 REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Concerns about the safety of chemicals in the environment, food, cosmetics and medicines have been given much prominence in the current decade and researchers are scrutinizing substances to see if they hold the answers to many of mankind's debilitating conditions such as cancer, diabetes and hypertension. Strict controls and extensive research regulate the introduction of any new substance into public use as food, food additive, medicine, pesticide or insecticide and the manufacturer has the sole responsibility of proving the safety of the product.

WHO and other regulatory bodies such as FDA, however, have much lenient standards for chemical substances, food, food additives or medicines which have been in use before 1980. This stands was partly due to the large number of animal life that would be destroyed if toxicity test

were to be done on all the substances. However, it has led to the existence of several substances with unknown toxicities.

The development of animal friendly models is reversing the trend and has created the need for thorough assessment of the safety of substances used as medicine, food, cosmetics and pesticides. Prior to 1970, safety assessment of chemicals was limited to general toxicity assays. However, the story of thalidomide sensitized the WHO and chemical regulatory bodies such as the FDA about the need to include reproductive studies in safety assessment.

In evaluating the safety of a substance, toxicity may exist beyond the limits of acute and chronic administration of the substance and only a detailed and vigorous investigations will be able to detect effects such as;

- Delayed or slow onset fatalities which may occur after administration of a substance.
- Substances safe in the parent generation but toxic to the offspring e.g. diethyl stilboesterol and thalidomide.
- Toxic effects transferred from generation to generation i.e. transgenerational

Reproductive studies allow the detection of the above effects.

1.3.1 Reproduction and foetal development

1.3.1.1 Embryonic and foetal development in humans

Right after fertilization, the sperm and ovum form a zygote which undergoes rapid division to form a morula. Morula descends into the uterus three to four days after fertilization where it floats freely. It continues to proliferate and differentiate into a blastocyst capable of implantation.

A blastocyst is a single-layer hollow ball of about 50 cells encircling a fluid-filled cavity, with a dense mass of cells grouped together at one side. This dense mass, known as the inner cell mass, becomes the embryo/foetus. The remaining tissue serves a supportive role during intrauterine life. The thin outermost layer, the trophoblast, accomplishes implantation, after which it develops into the foetal portion of the placenta (Klaassen *et al.*, 1996).

1.3.1.2 Spontaneous abortions (Miscarriages) in humans.

Spontaneous abortion is a clinical condition describing the loss of a foetus weighing less than 500 g before 20 weeks gestation counted from the first day of the last menstrual period. A habitual aborter describes a patient who serially miscarries, at least three or more, pregnancies. Abortion refers to any process by which a pregnancy ends with the death and removal or expulsion of the foetus, regardless of whether it is spontaneous or intentionally induced.

Epidemiological data on spontaneous abortion from different parts of the world vary. However, Hertig (1967) estimated it to be approximately 50% of all conceptions. Wilcox *et al.*, (1985) using sensitive techniques then for detecting pregnancy as early as 9 days postconception, observed that 35% of postimplantation pregnancies ended in an embryonic or foetal loss. More recent data estimate that up to half of all fertilized eggs die and are lost (aborted) spontaneously, usually before the woman knows she is pregnant (Simpson and Jauniaux, 2007). The rate of early miscarriage (<8 weeks gestation) may be even greater as some women may not recognize that they are pregnant, and the miscarriage is simply thought of as a late menstrual period. Most women with a five to ten-day delay in the onset of the menstrual bleeding are very frequently diagnosed through the beta subunit of human chorionic gonadotropin as early spontaneous

abortions (Laurino *et al.*, 2005). Among those women who knew they were pregnant, the miscarriage rate is about 15-20%. Most miscarriages occur during the first 7 weeks of pregnancy (Katz, 2007; Simpson and Jauniaux, 2007).

Generally 10 –15% clinically apparent miscarriages occur during the first trimester which is mainly due to genetic defects and progesterone deficiencies, drug and alcohol abuse, exposure to environmental toxins, infections, obesity, physical problems with the mother's reproductive organs, problem with the body's immune response and smoking (Laurino *et al.*, 2005).

In some cultures, a foetus is recognized as human very early in pregnancy, and a miscarriage requires naming and burial rituals (Chalmers and Meyer, 1992a; Chalmers and Meyer, 1992b). In other cultures where the foetus is not considered human until the second trimester or later, early pregnancy loss is not mourned in the same way as later pregnancy loss. Moreover, beliefs about the causes of miscarriage vary between cultures, and include angering ancestral spirits, witchcraft, eating forbidden foods or breaking other social or religious taboos, family conflicts, and misconduct by the woman or by her partner (Cecil, 1996). Following a miscarriage, it is customary in some cultures to perform rituals or use charms or medicines to prevent another miscarriage.

1.3.1.3 Neuro-endocrine activity controls reproduction and development

The hypothalamus–hypophysis–gonad and foetal development

The hypothalamus–hypophysis–gonad/thyroid (HHG/Th) axis controls sexual maturation and behaviour by integrating impulses from other pathways in the body. Maturation of neuro-endocrine systems is complex and can be permanently affected by exogenous substances. Development of the reproductive system in humans starts with a gonad which has the capability to become ovary or testis and an undifferentiated hypothalamus–hypophysis (HH) axis. Due to genetic signals, the gonads develop specifically as either male or female and begin to produce hormones causing programmed development of the axis in the male or in the female. Between infancy and puberty, the axis undergoes an interval of quiescence (Miller *et al.*, 2004).

The Hypothalamus-Hypophysis-Gonad axis and sexual differentiation of the brain in mammals

The brain is the most important part of the reproductive system, because of its critical roles in endocrine function, sexual and parental behaviours. Circulating steroidal hormones from the gonads induce functional and anatomical changes in the developing nervous system. This leads to sexual dimorphism.

In mammals, the ovaries of developing females are generally quiescent, and development of the female brain and reproductive tract occurs in the absence of estrogens.

In male rodents, testosterone secreted by the gonads in the foetal period is converted by aromatase to oestradiol at the brain level, where it alters brain development. These sex

differences in hormone synthesis and exposure result in the development of distinct male and female brain. The Medial Preoptic Nucleus (MPN) is a key area of the brain with marked sexual dimorphism. A region of the MPN, the Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA), is approximately five times greater in volume in male than in female rats (Gorski *et al.*, 1978) and contains more neurons due to the prevention of programmed cell death by testosterone locally aromatized to estradiol (Davis *et al.,* 1996).

The Anteroventral Periventricular Nucleus (AVPV) is also sexually dimorphic, in that females have larger AVPV than males. AVPV is a part of the hypothalamus regulating ovulatory cycles. Differentiation of the AVPV is due to the actions of aromatized metabolites of testosterone on oestrogen receptors, which modulate apoptosis early in life. In contrast to the SDN-POA, masculinization of the AVPV is due to testosterone induced apoptosis (Simerly, 2005). Hormonal manipulations during critical periods of development affect brain organization and hence sexual behaviour and development.

Non-reproductive actions of gonadal hormones

Gonadal hormones, and, in particular, oestrogens, have many effects on the nervous system that extend beyond their role in reproduction. Non-reproductive actions of oestrogen involve brain regions outside of the hypothalamus, such as the hippocampus, the basal forebrain, the brainstem and the spinal cord. In these regions gonadal hormones generally appear to modulate behaviours not directly linked to reproduction, such as cognition, memory and the stress response (Mc Ewen and Alves, 1999). Oestrogens influence cerebral cortical development. Sex differences in cortical connectivity might underlie sex differences in complex behaviours such as cognitive and

mnemonic functions and explain sex differences in vulnerability to some neurological and psychiatric diseases.

Thyroid hormones and sexual reproduction

Thyroid hormones are also essential for normal brain development during critical periods beginning *in utero* and extending through the first 2 years postpartum in humans. They regulate neuronal proliferation, migration and differentiation in specific brain areas. In addition they regulate development of cholinergic and dopaminergic systems serving the cerebral cortex and hippocampus (Porterfield, 2000). Thyroid hormones regulate somatic growth, metabolism, brain development and other crucial processes in developing and adult animals.

In males, triiodothyronine normally inhibits Sertoli cells proliferation directly while stimulating their differentiation into Leydig cells; thyroid hormone regulates testosterone production mediated by responsiveness to trophic hormones. The thyroid action on the ovary is less understood; perturbation of the thyroid homeostasis leads to inhibition of ovarian function, alteration of follicular maturation, granulosa cell differentiation as well as ovulation (Cooke *et al.*, 2004).

The Hypothalamus Hypophysis and Ageing

After adulthood, the HHG axis activity is modified again and the whole organism is directed toward the ageing process leading to a decrease in neuro-endocrine function. Changes in hormonal levels associated with ageing influences brain function as well. A number of *in vitro*

studies have indicated that oestrogens protect nerve cells from oxidative stress (Manthey and Behl, 2006). Thus, it has been hypothesized that in the female loss of ovarian hormones may increase vulnerability of brain cells to damage and degeneration, and that steroids may confer protection from neurodegenerative diseases (Manthey and Behl, 2006).

Other neuropeptides augment the activity of Gonadal hormones

Hypothalamic and hypophysis neuropeptides such as oxytocin, vasopressin and prolactin are involved in the regulation of reproductive functions and modulation of a number of behavioural abilities such as learning, anxiety and the stress response. In particular, oxytocin, produced primarily in the paraventricular and supraoptic nuclei of the hypothalamus, regulates induction of labour, milk ejection, oestrous cycle, penile erection and ejaculation, and also plays an important role in social behaviour and affective conditions (Bielsky and Young, 2004).

1.3.2 Reproductive toxicity

This evaluates parameters which may affect effective reproduction in the parent generation. The main classes of reproductive toxicity are fertility, parturition and lactation.

1.3.2.1 Fertility

Fertility is one's ability to conceive if the person is female or to induce conception if the individual is male. It is closely linked to fecundity, which is one's ability to produce offspring within a specific time. Issues of fertility are very thorny with both socio-cultural and religious implication particularly in Africa where large families, a product of fertility, is a sign of wealth,

power and social status. Today countless couples are trying everything from medical techniques, to superstition to promote fertility.

Spermatogenesis

The control of spermatogenesis is extremely complex requiring testosterone from the Leydig cells situated within the interstitium and the supportive role of the Sertoli cells within the seminiferous tubules (Sharpe, 1994). During spermatogenesis, spermatogonia divide by mitosis and meiosis producing haploid spermatids which then transform into spermatozoa. The synchronous development of a clone of spermatogonia should give rise to 4096 spermatids. However, the actual numbers fall far short of this, since the average clone contains only about 100 cells (Russel *et al.*, 1990); cell division and cell death are both implicated in controlling the final number of spermatozoa.

Role of sertoli and leydig cells in spermatogenesis

The Sertoli cell is a somatic cell within the seminiferous epithelium of the testis. This cell does not divide and, in general, is not susceptible to toxins although some industrial chemicals are known to pose a threat. The Sertoli cell provides the necessary biomolecules for the growth and development of germ cells and structural support for the germ cells so that they can maintain their cellular associations. In addition, tight junctions between the Sertoli cells serve to exclude potentially harmful substances such as toxins and antibodies thus rendering the seminiferous epithelium 'an immune privileged site'. The integrity of this cell is essential for successful spermatogenesis.

Reports of Sertoli cell apoptosis are few although Allan *et al.*, (1988) reported that 5 Gy irradiation induces death of Sertoli cells by apoptosis in 4-day-old rats. However, when Sertoli cells are cultured, a basement membrane of laminin or matrigel is required for Sertoli cell survival. Sertoli cells cultured on plastic die by apoptosis even in the presence of known regulators of Sertoli cell function (Dirami *et al.*, 1995). One function of Sertoli cells is to engulf and degrade dead germ cells. This phagocytosis is impaired when liposomes containing acidic but not neutral phospholipids are present *in vitro* (Shiratsuchi *et al.*, 1997).

The Leydig cell synthesizes and secretes testosterone thus maintaining the somatic and testicular aspects of male fertility. Like Sertoli cells, somatic Leydig cells do not divide and apoptosis of Leydig cells has not been reported in the normal adult rat testis (Taylor *et al.*, 1998). Toxicants usually exert their effects on Leydig cells by interfering with steroidogenic enzymes and the action of such toxicants is often characterized by their ability to reduce peripheral androgen levels. In addition, toxicants may have indirect effects on Leydig cells by affecting the neuroendocrine axis at the hypothalamus or pituitary to affect luteinizing hormone (LH) levels. Alternatively, Leydig cells may be killed via an indirect action on Sertoli or germ cells since these three cell types depend on paracrine interactions for survival signals (Morris, 1996).

Some toxicants have been described that act directly on Leydig cells, inducing apoptosis and reducing serum testosterone levels. Ethane dimethanesulphonate has a unique cytotoxic action on the Leydig cells of the rat testis and consequently, has been used to investigate the physiological role of the Leydig cell. Early reports showed that interstitial cells of the rat testis may undergo

apoptosis in response to hypophysectomy or ethane dimethanesulphonate (Morris *et al.*, 1986; Tapanainen *et al.*, 1993; Henriksen *et al.*, 1996).

Factors Affecting Fertility

The fertility of an individual does not remain constant but changes with time. This is particularly true in females. The fertility of a matured individual is regulated primarily by the neuro-endocrine system. Inputs from several pathways in the body stimulate the HHG/Th axis to cause the release of hormones to affect organs which determine reproductive state. Physiological, biochemical or anatomical alterations or defects in HHG/Th can impair fertility. The reasons for low fertility include age, stress and drugs.

Age

Generally fertility in humans' start early in adolescence, then peaks up in mid twenties and decline till old age in men or ceases completely in females in the late forties. Delay in childbearing can affect fertility in females because unlike males, females are born with limited number of ova with only about 300,000 being available till puberty as a result of atresia. No new ova are added on throughout the lifetime of the woman as such the end of every menstrual cycle without fertilization signifies a reduction in total ova and the probability of conceiving. In her early twenties the chances of a married woman becoming infertile is 7% as against 29% in early forties. In the same way the chances of an infertile married woman remaining childless is 6% in early twenties as compared to 64% in early forties (Menken *et al.*, 1986). Age is also associated

with increase in aneuploidy, miscarriages and congenital malformations. This is due to the fall in hormone levels and a reduction in natural vigour of the sex cells.

Weight

There is a strong correlation between the body mass index (BMI) of a woman and her chances of conceiving although the actual mechanism involved has not been identified. Irregular or infrequent menstrual cycles, increased risk of birth defects have been associated with obesity. Women with BMI greater than 30 or less than 19 have reduced chance of conceiving and increased risk associated with pregnancy than women of BMI 22 to 24. Weight loss of 5 to 10% dramatically improves ovulation and pregnancy rates (Menken *et al.*, 1986).

Diet and Natural Products

Eating certain foods has been closely linked with temporal loss of fertility. Most of these foods reduce fertility by mimicking endogenous compounds like hormones. An isoflavonoid, genistein from soyabean and structural analogues from other legumes are known to inhibit fertility in humans.

Stress and Anxiety

Fertility tends to be low in women in stressful occupation than women in less stressful jobs. Stress is a main cause of conception failure in *in vitro* fertilization. Although the mechanisms are

yet to be elucidated fully, stress is known to influence the plasma level of glucocorticoids and other neuroendocrine mediators.

Drugs may promote or hinder fertility.

Rampant contraceptive use is attributed with decline in fertility in the population. Steroidal contraceptive regulation of sexual reproduction involves activation and inhibition of numerous transmitters systems in the MPN and the VMH in the rat.

1.3.2.2 Parturition

Reproductive toxicities of xenobiotics on labour and delivery is seen as an increase or decrease in the onset and duration of parturition. In animals it is indicated by the time interval between pups delivery. Gestation period may be increased by hormonal imbalances and substances that relax uterine muscle clinically known as tocolytics.

1.3.2.3 Lactation

Milk may be a source of exposure of offspring to toxins. Xenobiotics may also affect the quality as well as the quantity of milk produced and the frequency of the feeding of the young by the mother. Nursing instincts and litter care can all be affected by exposure to xenobiotics.

1.3.3 Developmental toxicity

It refers to studies into the ability of xenobiotics to cause structural or functional alterations in the first generation (F_1). Developmental toxicology encompasses the study of pharmacokinetics, mechanisms, pathogenesis and outcome after exposure to agents or conditions that lead to abnormal development. Manifestations of developmental toxicity include structural malformations, growth retardation, mortality and functional impairment (Wilson, 1973). It is a relatively new science but its roots are firmly embedded in teratology. Jim Wilson in 1973 proposed the general principles of teratology which became the backbone of developmental toxicity.

- Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with adverse environmental factors
- Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence
- Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis)
- The access of adverse influences to developing tissues depends on the nature of the influence (agents)
- The four manifestations of deviant development are death, malformations, growth retardation and functional deficit**.**
- Manifestations of deviant development increase in frequency and degree as the dose increase from no effect to total lethal effects.

1.3.3.1 Mortality

It measures death from conception to the post weaning stage. Embryolethality occurs in early conception whilst foetal mortality occurs later. Pre and post implantation loss, resorption, stillbirth, spontaneous abortions are positive signals of mortality. Foetal mortality however may not necessary mean toxicity to the foetus but may reflect maternal mortality as well.

1.3.3.2 Malformations

Teratology is the science of malformations. As a descriptive science it precedes written language. Mythological figures such as cyclopes and sirens originated with the birth of severely malformed infants. The word is derived from the Greek word "tetras" meaning monster. The Latin word monstrum from monstrare (to show) or monere (to warn) is derived from the perceived ability of malformed infants to foretell the future. Structural alterations in the skeleton and tissues of the offspring such as cleft palate and abnormal limbs constitute malformations (Klaassen, *et al.*, 1996).

1.3.3.3 Intrauterine growth retardation

Xenobiotics can cause inhibitions in foetal growth *in vivo*. Positive signals include reduce mean foetal length, crown-rump length and ano–genital distance.

1.3.3.4 Functional toxicity

It measures changes in physiological and biochemical functions in the offspring rather than anatomical defects. Development does not stop at birth but continues through childhood, adolescence to adulthood. Hence functional toxicities are slowly detected relative to other developmental endpoints. A functionally toxic agent may produce neuronal, behavioural (particularly sexual), locomotor, fertility, learning and memory deficits in the offspring.

1.3.4 Some Human Developmental Toxicants

Diseases and Nutritional Deficiency

Infections which are detrimental in pregnancy include rubella virus, cytomegalovirus, herpes virus, toxoplasmosis, Venezuelan equine encephalitis virus, rheumatic disease, virilising tumours, hyperthermia, and diabetes. For example diabetes in pregnancies results in nutritional deficiencies and metabolic imbalance which affect foetal development. A well documented example of nutritional deficiency induced developmental toxicity is neural tube defect in infants born to mothers with foliate deficiencies (Klaassen *et al.*, 1996).

Drugs and chemicals

Chemicals used as food, food additives, cosmetics, and pesticides or drugs for recreational purpose can affect reproduction and foetal development. Examples of well-known developmental toxins are; therapeutic drugs - Steriods, angiotensin converting enzyme inhibitors, tetracycline, trimethadone, diphenlhydantoin, methimazole, penicillamine, chlorobiphenyls,

valproic acid warfarin. Recreational drugs such as tobacco, nicotine, cocaine, alcohol and pesticides such as DDT, chlordane, paraquat (Klaassen *et al.*, 1996).

Thalidomide

In 1961, Lenz and Mcbride, working independently saw an overt and unusual form of malformations in infants born between 1956 and 1961(McBride, 1961; Lenz and Knapp, 1962). The affected individuals had amelia (absence of limbs) or phocomelia (reduction of the long bones of the limbs) affecting the arms more than the legs and usually involving both the left and the right sides although to differing degrees. Congenital heart disease; ocular, intestinal and renal anomalies and malformations of the external ears were also involved. They identified thalidomide, a sedative/hypnotic used throughout the world except in the USA, as the cause of the malformation and that subsequently led to withdrawal of the drug. Following that, regulatory agencies in many countries began developing animal testing requirements for evaluating the effects of drugs on pregnancy outcomes that were separate from chronic toxicity studies.

Diethylstilboesterol

It is a non steroidal oestrogen used in the United States to prevent miscarriages by stimulating steroid synthesis in the placenta. An epidemiological case control study found an association between increased clear cell adenocarcinoma in the vagina of young women between 15 to 22 years and prenatal exposure to diethylstilboestrol (Klaassen *et al.*, 1996).

1.3.5 The Endocrine Disrupting Chemicals (EDC)

Neuro-endocrine maturation is an extremely complex process which can be permanently affected by any exogenous substance able to interfere with the hormonal signalling at various levels. The EDCs are a heterogeneous group of chemicals which have the ability of interfering with endocrine homeostasis.

The reproductive health as well as the intrauterine and postnatal development is considered especially vulnerable to endocrine disruption (Mantovani, 2002) due to the major role of endocrine balance in such life stages. EDCs include some groups of pesticides and biocides (e.g. ethylene bisdithiocarbammates, organotins, imidazoles and triazoles), persistent organic pollutants (POPs) (e.g. DDT and metabolites, dioxins, and polychlorinated biphenyl ethers), industrial chemicals (e.g. polybrominated flame retardants, alkyl phenols, phthalates, parabens and bisphenol A) and some metals (e.g. arsenic and cadmium) as well as some natural compounds such as phytoestrogens and mycotoxins.

The nervous system represents one of the main targets of EDC as *in utero* exposure to POPs has been linked to adverse effects on neurological and intellectual function in infants and young children (Jacobson and Jacobson, 1996; Ribas-Fito *et al.*, 2001; Stewart *et al.*, 2005). Several experimental and epidemiological data support a significant interference of different environmental pollutants with endocrine disrupting activity during brain development (Tilson, 1998). The mechanisms by which EDCs affect brain development are still largely unknown.

1.3.5.1 Behavioural endpoints in reproductive and developmental studies

Behavioural analysis is relevant in assessing the interference of xenobiotics with neuro-endocrine maturation. This is because behaviour is a sensitive marker of perturbation of neuro-endocrine functions. There is enough evidence suggesting that the role of steroid hormones on the organization of the brain particularly in the hypothalamus is more important than their regulatory functions on reproductive organs (Palanza *et al.*, 1999). In addition steroidal hormones also affect neuronal function in the adult brain by non-genomic mechanisms, and modulate the expression of behaviours linked to reproductive functions by acting in brain regions outside of the hypothalamus (Mc Ewen and Alves, 1999).

Successful reproduction in laboratory rodents requires competitive aggression between males, territorial behaviour, affiliation and parental care, which are all determined by gonadal and hypothalamic hormones. Such behaviours involved in reproductive functions, classified as sociosexual behaviours, include wider spectrum of behavioural patterns than strictly defined sexual behaviour. In addition, there are non-social behaviours not classically hormone-mediated and expressed by both sexes such as learning capacities, exploration activity, novelty seeking and anxiety levels, which show both qualitative and quantitative differences in the two sexes. These behaviours are typical of each sex, age and with typical patterns determined by genetic, neural, and hormonal experience factors.

The principle of behaviour teratology is that any interference with either the organizational or activational role of gonadal and hypothalamic hormones may result in measurable changes in behavioural patterns associated with reproductive functions that can be analysed using reliable and standardised ethological methods (Calamandrei *et al.*, 2006).

1.4 APHRODISIACS

Aphrodisiac is a word derived from 'Aphrodite', the Greek goddess of love, beauty and sexuality, and aphrodisiacs are the substances that are used to improve an impaired sexual function (Shah, 2002; Ramachandran *et al.*, 2004). These agents have been used since time immemorial as there is enough evidence showing their use by the early Greek and Arab physicians. In ancient Greece, Theophrastus, a philosopher and herbalist, reported that a plant called *satyrion* allows a man to perform 70 consecutive acts of intercourse. Satyrion became extremely popular and was harvested to extinction. In Rome, Roman naturalist Pliny the Elder wrote that ginger was an aphrodisiac when pounded into paste and spread on the stomach, scrotum and anus. In Europe in the 1700s, chemists ground up Spanish fly, a species of the blister beetle, to create a notorious sex stimulant (Preckshot, 1999).

Aphrodisiacs were first sought out as a remedy for various sexual anxieties including fears of inadequate performance as well as a need to increase fertility. Procreation was an important moral and religious issue and aphrodisiacs were sought to ensure both male and female potency. In those times, there were clear distinction between substances that increased fertility and the ones that simply increased sex drive. There was however little consensus on what foods was considered aphrodisiacs or "anaphrodisiacs" (decrease potency).

Unlike ancient writings, modern medical science recognizes a very limited number of aphrodisiacs. According to the Encyclopedia Britannica, most writings on the subject are little more than unscientific compilations of traditional or folkloric material. Of the various foods to which aphrodisiac powers are traditionally attributed, no chemical agent has been identified that has direct pharmacological effect upon the genitourinary tract.

It has been suggested that man's universal attribution of libidinous effects to certain foods originated in the ancient belief in the therapeutic efficacy of signatures: i.e. if an object resembled the genitalia, it was reasoned to have sexual powers and thus the legendary aphrodisiac powers of ginseng root, powdered rhinoceros horn, banana flower, avocado, carrot, and asparagus.

Recently, aphrodisiacs have been classified by Sandroni (2001) into 3 categories according to their mode of action:

- those that increase libido (i.e. sexual desire),
- those that increase potency (i.e. effectiveness of erection), and
- those that increase sexual pleasure.

1.4.1 Substances that increase libido

These aphrodisiacs operate at the level of the central nervous system by changing a particular neurotransmitter or specific sex hormone's concentration. A number of these are useful in both sexes, but most act via increase in testosterone concentration and as a result are male-specific (Sandroni, 2001). Ambrein a chief constituent of *Ambra grisea* is used in Arab countries and is also found in the gut of sperm whales (Taha *et al.*, 1995). Animal studies have shown that this tricyclic triterpene alcohol increases the concentration of several anterior pituitary hormones and serum testosterone, which in turn stimulate dopamine receptor synthesis and sexual behaviour (Taha *et al.*, 1995).

A nonspecific enhancement in sexually oriented behaviour occurs with the ingestion of central nervous system stimulants, such as amphetamine, cocaine, dopaminergic agents, caffeine,

antiserotonin drugs and cannabis. A variety of stimulants are present in beverages and chewable derivatives of the kola nut, guarana, and betel nut, the use of which as leisure and aphrodisiac drugs is widespread in Africa, Asia and Latin America (Morton, 1992).

1.4.2 Substances that increase potency (allow or sustain erection).

This group of aphrodisiacs acts via initiation of vasodilatation, to allow for erection to occur. Such remedies are mainly for males, even though to a minor extent they could be helpful in women (Sandroni, 2001). Sildenafil citrate, an oral drug for men with erectile disorder, produces acceptable erections and improves sexual satisfaction without affecting sexual desire (Jackson, 1998).

1.4.3 Substances that enhance sensory experience during coitus.

These act through irritation of the genital mucosa, therefore enhancing sensation. Not uncommon, they are ingested by an innocent subject through a drink organized by the (potential) partner (Sandroni, 2001). *Cantharidin* ("Spanish fly") is a chemical with vesicant properties obtained from blister beetles, which have been used for millennia as a sexual stimulant by both sexes (Karras *et al.*, 1996). Its mode of action is by inhibition of phosphodiesterase and protein phosphatase activity and stimulation of β-receptors, inducing vascular congestion and inflammation. The ingestion of live beetles (*Palembus dermestoides*) in Southeast Asia and triatomids in Mexico may have a similar rationale (Chu *et al.*, 1977).

1.5 REPRODUCTIVE CELL DEATH

Exposure to toxicants may lead to cell death. The death of a cell was thought for many years to be an uncontrolled, degenerative and catastrophic failure of homeostasis in response to cellular injury and was thus of little scientific interest and death occurred primarily by necrosis. Current scientific studies recognize two forms of cell death; necrosis and apoptosis.

Apoptosis is a term first introduced in the 1970s to define the morphology of dying cells (Kerr *et al.*, 1972) although Glucksmann and colleagues had described the same morphologic alterations in the kidney during the early 1950s (Glucksmann, 1951).

The early pioneering work of Andrew Wyllie and his colleagues described the importance of apoptosis in various tumours and in certain forms of chemical-induced injury. In general, when the toxicant is at high enough concentration to cause gross cellular injury or a major perturbation in the cellular environment the cell dies by necrosis or 'cell murder'. However, it is more common for the affected cells to be deleted by apoptosis or 'cell suicide'. It is now believed that apoptosis is the major form of pathophysiological cell death and that necrosis is much rarer, occurring only in circumstances of gross cell injury (Raffray and Cohen, 1997).

Apoptosis is desirable to the organism as a whole since it provides a mechanism for the disposal of cells damaged by mutagenic chemicals or irradiation without perturbing the homeostatic balance of its environment. In addition to a role during a cellular response to toxicants, apoptosis occurs throughout development and is vitally important in the immune system (Jacobson *et al.*, 1997). Examples include the removal of interdigital cells from a solid limb paddle during digit formation (Hammar and Mottet, 1971) and deletion of potentially lethal self-reactive B- and T-

lymphocytes in the immune system (Macdonald and Lees, 1990). Moreover, apoptosis is of major importance in the pathogenesis of several diseases such as cancer (Dive and Wyllie, 1992; Lee and Bernstein, 1995; Reed, 1995), AIDS (Gougeon and Montagnier, 1993) and neurological disorders such as Alzheimer's and Parkinson's disease (Carson and Ribeiro, 1993; Bredesen, 1995)

1.5.1 Morphology of cells during apoptosis

The execution phase of apoptosis has striking morphological characteristics (Alison and Sarraf, 1995; Raffray and Cohen, 1997; Zamzami *et al.*, 1998) although at present, the latent phases of apoptosis are undetectable. The morphology of the end stages of apoptosis is remarkably similar across a wide variety of tissues; an apoptotic body from the liver may be indistinguishable from an apoptotic body from the crypt of the small intestine (Alison and Sarraf, 1995). Briefly, they include loss of cell contact with neighbouring viable cells, chromatin condensation to form dense compact masses and breakdown of the cytoskeletal scaffolding. The cell surface membrane, which is already contracted, begins to ruffle and bleb. This is followed by breakdown of the cell into membrane-bound fragments or 'apoptotic bodies'. In contrast to necrosis, the plasma membrane and cytoplasmic organelles of the apoptotic cell remain intact. The apoptotic cell is swiftly recognized and engulfed and subsequently degraded by professional or amateur phagocytes (macrophages and neighbouring cells, respectively) (Savill *et al.*, 1993).

In vitro, in the absence of phagocytosis, a secondary non-specific degeneration occur which results in the uptake of vital dyes such as trypan blue and is commonly mistaken for necrosis. This process is often referred to as secondary necrosis. Importantly, the whole apoptotic process

occurs without the development of an inflammatory response and thus facilitates the deletion of cells with minimal disruption to the surrounding cellular environment and tissue architecture. The apoptotic cell is swiftly recognized and engulfed then degraded by professional or amateur phagocytes (macrophages and neighbouring cells, respectively) (Savill *et al.*, 1993). Importantly, the whole apoptotic process occurs without the development of an inflammatory response and thus facilitates the deletion of cells with minimal disruption to the surrounding cellular environment and tissue architecture as reviewed in Gill and Dive, (2000).

1.5.2 Apoptosis in the normal testes

The numbers of apoptotic cells in rodent testes are low but groups of spermatogonia linked by intercellular bridges undergo apoptosis synchronously (Allan *et al.*, 1988; Sharpe, 1994). Indeed, the first clear description was provided by Huckins, (1978) although at the time the phenomenon of apoptosis had not been recognized. Primary and secondary spermatocytes as well as spermatids occasionally undergo apoptosis (Russel and Clermont, 1977; Kerr, 1992; Brinkworth *et al.*, 1995; Blanco-Rodriguez and Martinez-Garcia, 1996). However, A1, Intermediate and type-B spermatogonia rarely degenerate (Huckins, 1978).

Attenuation of LH and follicle stimulating hormone (FSH) by hypophysectomy increases the number of degenerating pachytene spermatocytes (Russel and Clermont, 1977) via apoptosis (Tapanainen *et al.*, 1993; Sharpe, 1994). This can be attenuated by addition of exogenous LH and FSH alone or in combination (Russel and Clermont, 1977; Tapanainen *et al.*, 1993). The observation that testosterone also prevents hypophysectomy induced cell death (Tapanainen *et al.*, 1993) suggests a critical role for LH-stimulated testosterone secretion for the maintenance of

germ cell viability. The exact role of FSH in spermatogenesis is still uncertain (Sharpe, 1994). FSH can prevent programmed cell death of rat pachytene spermatocytes and spermatids *in vitro (*Henriksen *et al., 1996)* whilst at the same time stimulating proliferation of spermatogonia and preleptotene spermatocytes. *In vivo,* specific immunoneutralization of FSH induces apoptosis of spermatogonia and pachytene spermatocytes as early as 24 h after administration of the FSH antiserum (Shetty *et al.,* 1996).

In human testes, apoptosis occurs spontaneously as shown in tissues obtained after orchidectomy for prostate cancer (Brinkworth *et al.*, 1997; Woolveridge *et al.,* 1998) or at autopsy following sudden traumatic death (Sinha-Hikim *et al.,* 1998). Apoptosis occurs in a wide variety of germ cell types but not in Sertoli or Leydig cells and there is no significant relationship between the amount of apoptosis and either the age of men or testis weight (Brinkworth *et al.,* 1997).

Men with azoospermia or severe oligozoospermia have an increased frequency of apoptotic germ cells in their testicular biopsies in comparison to men with normal spermatogenesis, suggesting that programmed cell death may play a role in human male infertility (Lin *et al.,* 1997). In addition, there is an increased frequency in the number of spermatozoa with apoptotic DNA in the ejaculates of infertile men in comparison to fertile controls.

1.6 DNA TOPOISOMERASE II AND GENOTOXICITY

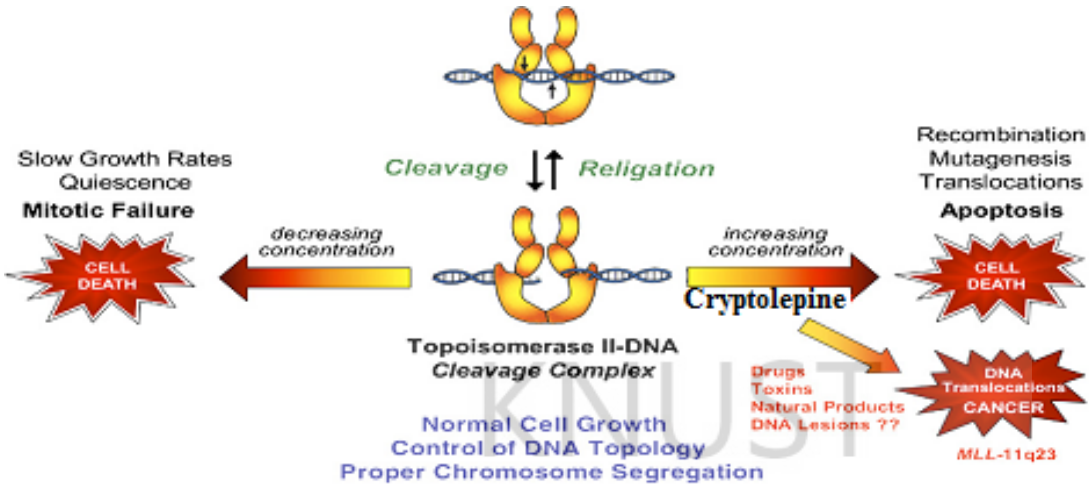


Fig. 1.2 Mechanisms of topoisomerase ll mediated genotoxicity (adopted form McClendon and Osheroff, 2007)

Cryptolepine the main alkaloid of the aqueous extract interferes with the activity of topoisomerase II. Topoisomerase II is an essential, but genotoxic enzyme. The formation of topoisomerase II–DNA cleavage complexes is required for the enzyme to perform its essential cellular functions. If the level of cleavage complexes falls too low (left arrow), cells are unable to undergo chromosome segregation and ultimately die of mitotic failure. If the level of cleavage complexes becomes too high (right arrow) the actions of DNA tracking systems can convert these transient complexes to permanent double-stranded breaks in the genetic material. The resulting strand breaks, as well as the inhibition of essential DNA processes, initiate multiple recombination/repair pathways and generate chromosome translocations and other DNA aberrations. If the DNA strand breaks overwhelm the cell, as in the case of cryptolepine, they trigger apoptotic pathways. This is the basis for the actions of several widely prescribed anticancer drugs as reviewed McClendon and Osheroff (2007).

1.7 AIMS AND OBJECTIVES OF THE STUDY

1.7.1 Aims

The aqueous extract of *Cryptolepis sanguinolenta* is cytotoxic (Ansah and Gooderham, 2002). Cytotoxics affect spermatogenesis and have a plethora of effects on male reproduction (Cai *et al.,* 1997). Furthermore paternal exposure to a chemical can have detrimental effects on the health of the offspring. Products of cryptolepis are used by males to treat malaria and other ailments. Some herbal medicines marketed as male sexual enhancers contain cryptolepis.

Malaria is endemic in West Africa and pregnant women and children are more prone to malaria and recurrent attacks than the general population. The cost of conventional antimalarials, accessibility and side effects compel patients to resort to herbal medicines.

Cryptolepis is a popular West and Central African plant used to treat malaria, dysentery and other febrile conditions (Sofowora, 1982; Boye and Ampofo, 1983; Michel *et al.*, 2008). Cryptolepis is cytotoxic and cytotoxics affect reproduction and foetal development in mammals.

In view of this cytotoxic activity of cryptolepis, it is hypothesized that cryptolepis could affect reproduction and foetal development in mammals.

The aim of this study therefore is to ascertain the effects of cryptolepis on reproduction and foetal development in mammals.

1.7.2 Specific Objectives

Specific Objectives shall include;

1. Examination of the effects of cryptolepis on fertility, gestation period, live birth and sex ratio after pretreatment of female mice.
2. Assessment of the effects of cryptolepis treatment on male fertility.
3. Examination of the aphrodisiac potential of cryptolepis in male mice.
4. Determination of the *in vivo* genotoxicity and mutagenic potential of cryptolepis.
5. Estimation of the effects of cryptolepis (subacute) treatment on reproductive hormonal levels in male mice.
6. Evaluation of the effects of cryptolepis on embryonic and foetal development.
7. Assessment of the effects of time of treatment with cryptolepis on developmental outcome.
8. Determination of the effects of prenatal cryptolepis treatment on functional behaviour in the first (f_1) generation mice.
9. Determination of possible interspecies variation in the effects observed.

CHAPTER TWO

EFFECTS OF AQUEOUS EXTRACT OF CRYPTOLEPIS ON REPRODUCTION IN MICE

CHAPTER TWO

2.1 INTRODUCTION

Cryptolepis sanguinolenta, the West African antimalarial, is a known cytotoxic to mammalian cells *in vitro* (Ansah and Gooderham, 2002). Cryptolepine, the main alkaloid of the aqueous extract responsible for most of its biological activity is cytotoxic, a DNA intercalator and interferes with topoisomerase II (Bonjean *et al*., 1998; Dassonneville and Bonjean, 1999; Lisgarten *et al.*, 2001). The cost of treatment of malaria, especially in rural communities where multiple factors e.g. accessibility to health care, poverty, transportation, ignorance, etc militate against satisfactory health care, creates the need for people to resort to traditional medicine. Because cryptolepis is an antimalarial, there is a high possibility of exposure during pregnancy. However, very little is known on the effects of cryptolepis on reproduction.

Effects of drugs on male sexual health are a health concern in Ghana. Although undocumented, cryptolepis is a major constituent of alcoholic beverages marketed as "bitters" and products containing cryptolepis are marketed as male sex enhancers in pharmacies, herbal and chemical shops; a claim yet to be proven scientifically. Paternal exposure to chemicals can result in adverse outcomes on the survival and health of the offspring (Green *et al.*, 1985; Russell and Shelby, 1985; Shelby, 1994). Studies show that cytotoxics have the potential to affect reproduction and foetal development (Cardonick and Iacobucci, 2004). Using murine models, this chapter evaluates the effects of cryptolepis treatment on reproduction in male and female mice.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

Ethylacetate, methanol, ammonia, chloroform, diethylamine, sodium bicarbonate were obtained from Sigma-Aldrich Inc, St Louis, MO, USA.

Reference cryptolepine and isolated cryptolepine were kind donations from Mr. Edward Ofori of Department of Pharmaceutical Chemistry, KNUST.

2.2.2 Animals

ICR mice (20-30 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana and maintained in the animal house of the Department of Pharmacology, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. They were housed in stainless steel cages (34 x 47x 18 cm) with soft wood shavings as bedding, and fed with normal commercial pellet diet (GAFCO, Tema,Ghana) and given water *ad libitum*. The animals were humanely handled throughout the experiment in accordance with internationally accepted principles for laboratory animal use and care (EEC Directive of 1986: 86/609 EEC). Additionally all animal experiments were approved by the departmental ethics committee.

2.2.3 Source of plant material

Authenticated dried *Cryptolepis sanguinolenta* roots were obtained from the Centre for Scientific Research into Plant Medicine, Mampong-Akwapim, Ghana where it is routinely used as an antimalarial agent at the clinic for patients. A voucher specimen was deposited at the department of Pharmacognosy herbarium (FP/08/069).

2.2.4 Preparation of the aqueous extract of cryptolepis

To simulate the traditional method of preparation, dried cryptolepis roots (1 kg) were milled and extracted by boiling with 10 litres of distilled water for 30 minutes. The solution was filtered and the filtrate after cooling was freeze dried to obtain a yellowish-brown powder referred to subsequently as cryptolepis. The percentage yield was 9.3% w/w. Routinely, cryptolepis was freshly prepared in water and administered by gavage to the experimental animals.

2.2.4.1 Identification of the major constituents of the aqueous extract

Aqueous extract of cryptolepis along with the reference cryptolepine were spotted on precoated analytical thin layer chromatography plates, using ethylacetate: methanol: ammonia (35%) 80: 15: 5 as solvent system.

In a second experiment, powdered roots of cryptolepis were extracted with methanol. The methanolic extract along with reference cryptolepine and an isolated cryptolepine were spotted on precoated analytical thin layer chromatography plate and eluted with the solvent system composed of ethylacetate: chloroform: diethylamine 80: 15: 5.

The thin layer chromatogram of the aqueous extract was compared to that of the methanolic extract.

2.2.5 Primary observation (Irwin) test in the mouse

This experiment was performed as previously described by (Irwin, 1968). Briefly mice of either sex were grouped into four (n=5) and treated with either distilled water or cryptolepis (62.5 -

2000 mg/kg *p.o*) and subsequently observed at times 0, 15, 30, 120, 180 min and 24 h. The animals were assessed for behaviours specifically related to neurotoxicity, such as convulsions and tremor, for behaviours related to central nervous system (CNS) stimulation, such as excitation, Straub tail, jumping, hypersensitivity to external stimuli, stereotypes, and aggressive behaviour, and for behaviours related to CNS depression, such as sedation, rolling gait, loss of balance, loss of traction, motor incoordination, hyposensitivity to external stimuli, decreased muscle tone, akinesia, catalepsy, and hypothermia. Effects on autonomic functions, such as respiration, pupillary diameter, body temperature, salivation and defecation, were also noted, as was the lethality of the test agent.

2.2.6 Reproductive toxicity of *Cryptolepis sanguinolenta* in female mice

The method described by Goldenthal, 1966 was used. Five groups of ICR mice (n=10) were used in the study. Group I was the vehicle control and received distilled water only. Groups II, III, IV, and V received 62.5, 100, 500 and 1000 mg/kg of cryptolepis extract respectively for two weeks representing the pretreatment phase. After the two weeks pretreatment, animals were regrouped by subdividing each group into two to provide ten groups as follows: IA, IB, IIA, IIB, IIIA, IIIB, IVA, IVB, VA and VB. Two male mice were introduced into each of the ten groups. Groups IA and IB were maintained as control groups and continued to receive distilled water only. Treatment with cryptolepis was then discontinued in the A groups i.e. groups IIA, IIIA, IVA and VA. However, the B groups i.e. groups IIB, IIIB, IVB and VB continued to receive cryptolepis till the end of gestation. Formation of vaginal plug was taken as evidence of successful mating. The effect of the extract on reproductive indices; fertility, mating, litter number, litter size, live births, and gestation period were assessed.

Summary of treatment schedule for reproductive toxicity studies in female mice

Pre-treatment with cryptolepis before mating

Group 1A: received only distilled water and served as control.

Group IIA: 62.5 mg/kg cryptolepis extract *p.o*

Group IIIA: 100 mg/kg cryptolepis extract *p.o*

Group IVA: 500 mg/kg cryptolepis extract *p.o*

Group VA: 1000 mg/kg cryptolepis extract *p.o*

Treatment with cryptolepis before mating, during mating and through gestation

Group 1B: received only distilled water and served as control.

Group IIB: 62.5 mg/kg cryptolepis extract *p.o*

Group IIIB: 100 mg/kg cryptolepis extract *p.o*

Group IVB: 500 mg/kg cryptolepis extract *p.o*

Group VB: 1000 mg/kg cryptolepis extract *p.o*

2.2.7 Reproductive toxicity of *Cryptolepis sanguinolenta* in male mice

2.2.7.1 Epididymal sperm assay

For epididymal sperm counts, the method described by Meistrich, (1989) was used. Four groups of male mice (n= 5) were used in the study. Group 1 served as the vehicle control and received distilled water only. The other three groups received 62.5, 500, 1000 mg/kg of cryptolepis daily respectively for 14 days. Animals from each group were then euthanized by cervical dislocation and the wet weight of the left cauda epididymis and testis were taken. For semen analysis, the left cauda epididymis was minced, and homogenized for 4 min in 10 ml of 0.9 % $NaHCO_3$ solution containing 0.1% formalin. The homogenate was allowed to settle at 4^0C, diluted to 50 ml, and lightly stained with 40% eosin solution. After agitation of the stained samples, an aliquot was immediately dropped onto a haemocytometer and sperm heads were counted.

2.2.7.2 Acute effects of *Cryptolepis sanguinolenta* on mounting behaviour in mice

To quantify mounting behaviour, experiments were designed as previously described by Lawler (1984) to measure the libido of the male mice (Taha *et al.*, 1995; Tajuddin. *et al.*, 2005). Mount is operationally defined as the male assuming copulatory position but failing to intromit and an attempted mount is defined as incompetent mounts in which the orientation is wrong, such as mounts of the female's head or side. Four groups of male mice (n=5) were treated with saline (control group) or with cryptolepis (62.5- 1000 mg kg-1, *p.o.*) and placed individually in a plexiglas cage (60 × 75 × 20 cm). After 15 minutes of acclimatization, a non-oestrous female was introduced into the arena and the number of mounts, anal sniffs, penile licks, attempted mounts was recorded during a 15-minute observation period. The female was then separated for

105 minutes and reintroduced and the number of mounts was observed again for 15 minutes as before. The first observation period was designated as the 1st hour and the second observation period as third hour.

2.2.7.3 Subacute effects of *Cryptolepis sanguinolenta* on mounting behaviour in mice

To quantify the subacute effects of cryptolepis on mounting behaviour, four groups of male mice (n= 5) were treated with saline (control group) or with cryptolepis (62.5-1000 mg kg-1, *p.o)* for 14 days. Male mice were placed individually in a plexiglas cage (60 × 75 × 20 cm). After 15 minutes of acclimatization, a non-oestrous female was introduced into the arena and the number of mounts, anal sniffs, penile licks, attempted mounts recorded during a 15-minute observation period.

2.2.8. *In vivo* mutagenecity and genotoxicity studies - The dominant male lethal assay.

The method used was as described by Green *et al.*, (1985). Twenty (20) male ICR mice were grouped into four (n = 5). Group I was the vehicle control group and received distilled water only throughout the duration of the experiment. Groups II, III, IV received 62.5, 500, 1000 mg/kg *p.o.* of cryptolepis dissolved in distilled water respectively throughout the duration of the experiment. Animals were assessed by mating with two females after 1, 2 and 5 weeks. Successful mating was indicated by the formation of vaginal plugs. Females were assessed on gestation day 14 and pregnant females were laparotomized for the determination of early death of foetus recognized as decidual tissue or moles.

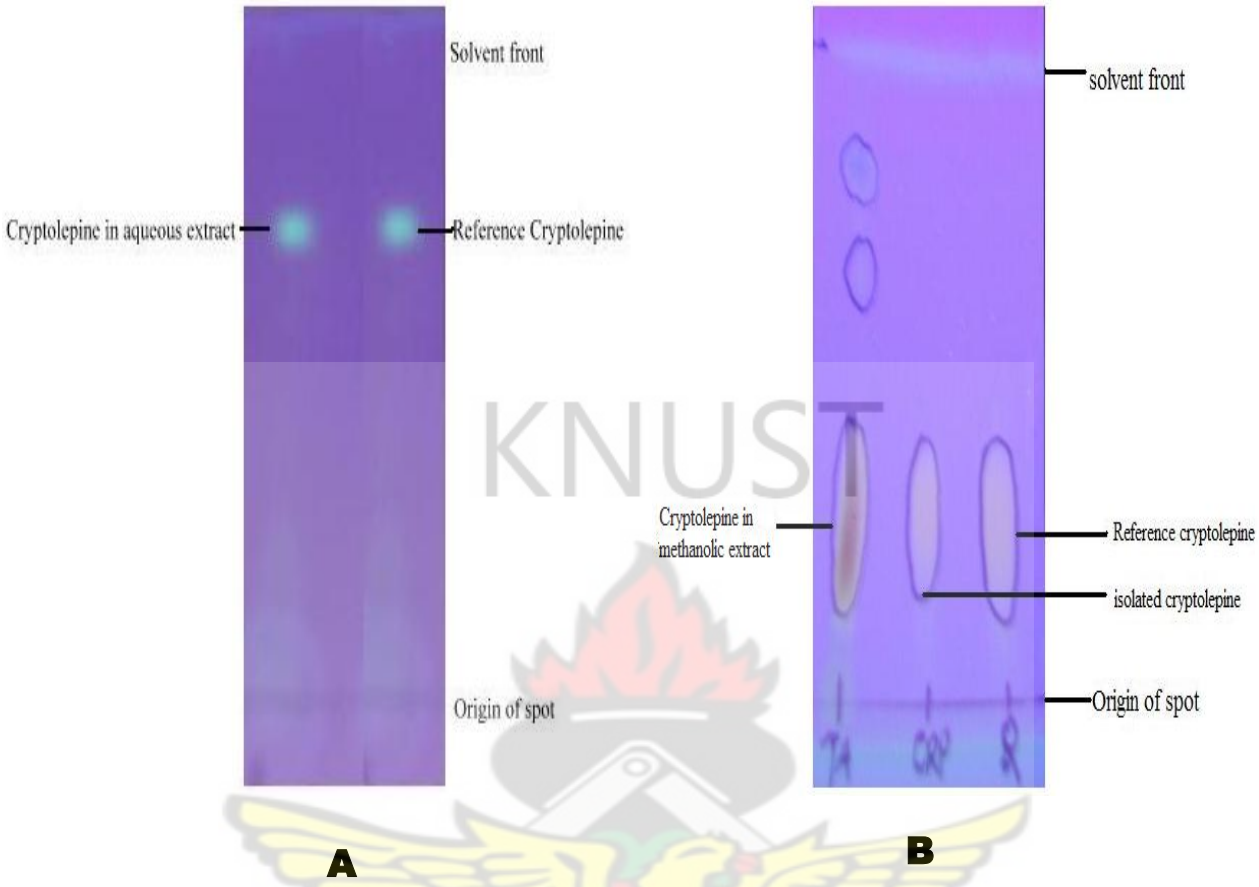
2. 3. RESULTS

2.3.1 Identification of the major constituents of the aqueous extract

Powdered root bark of *Cryptolepis sanguinolenta* was extracted with methanol and spotted on a thin layer chromatography plate. It yielded three major components. The first was identified as cryptolepine (Fig 2.1 B).The other two compounds are likely to be quindoline and CSA-3.

Aqueous freeze dried extract of cryptolepis however yielded one major compound identified as cryptolepine (F 2.1 A). Other components may be minor relative to cryptolepine.

This work confirms earlier work by Dwuma-Badu *et al.*, (1978) that the main alkaloid of the *Cryptolepis sanguinolenta* is cryptolepine.



(Fig 2.1 A) A Thin Layer Chromatogram of aqueous extract of cryptolepis and a reference cryptolepine

B) A Thin Layer Chromatogram of methanolic extract of cryptolepis, an isolated cryptolepine and reference cryptolepine

2. 3.2 Primary observation test

For the Irwin's test, mice received either distilled water or cryptolepis (62.5, 100, 500, 1000, 2000 mg/kg *p.o*) and then observed at times 0, 15, 30, 120, 180 minutes and 24 h. The animals were assessed for behaviours specifically related to neurotoxicity, central nervous system (CNS) stimulation or depression and autonomic functions. There were no signs of neurotoxicity, stimulation of autonomic function or CNS excitation in treated mice relative to controls. Cryptolepis however caused signs of CNS depression in the form of sedation and hypoactivity in treated animals at 500, 1000 and 2000 mg/kg of cryptolepis treatment but not at 62.5 and 100 mg/kg. This effect lasted up to 24 h at 2000 mg/kg (Table 2.1).

Table 2.1 Primary Observation Test (Irwin) of *Cryptolepis sanguinolenta* in mice

Dose mg/kg	Lethality(M/T)	Sign of toxicity observed
Control	0/6	-
62.5 mg/kg	0/6	-
100 mg/kg	0/6	-
500 mg/kg	0/6	Sedation (+) and hypoactivity(+)
1000 mg/kg	0/6	Sedation (+) and hypoactivity (+)
2000 mg/kg	0/6	Sedation (++) and hypoactivity (++)

T/*M*: number of deaths /number of mice treated. Grade of signs observed: (−) no sign of toxicity, (+) present or slightly increased, (++) moderately increased.

2. 3.3. Reproductive toxicity in female mice

Animals pretreated with cryptolepis for two weeks only followed by mating had reproductive indices similar to controls (Table 2.2). Pretreatment with cryptolepis before mating did not affect the mating and fertility index. Neither did it affect the length of gestation, weaning index nor sex ratio.

Cryptolepis treatment for two weeks and through mating and gestation did not affect the mating index but resulted in decreased fertility index at all doses used (Table 2.2). The decrease in fertility was however not dose dependent. At 1000 mg/kg, fertility was completely inhibited although the animals were mated. This affected other parameters such as gestation period, live birth index, weaning index and sex ratio at 1000 mg/kg. There were mortalities in both groups during the pretreatment stage particularly at 1000 mg/kg but not postcoital.

Treatment caused foetal death and reduced litter survival. Stillbirths indicated by the live birth index, was high (Table 2.2). Litter survival until the twenty-first day after birth (weaning index) was considerably reduced (Table 2.2). The sex ratio was affected at cryptolepis treatment of 62.5 and 100 mg/kg of cryptolepis treatment.

The results clearly show that cryptolepis causes reversible inhibition of fertility in female mice.

Table 2.2 Comparison of reproductive indices for female mice pretreated with cryptolepis for 14 days only prior to mating and female mice pretreated with cryptolepis for 14 days prior to mating with continued treatment during mating and gestation.

Treatment	Group/Dose of cryptolepis	No of mated animals	No of pregnant females	Deaths during pretreatment	Mating index	Fertility index	Gestation Period	Live Birth index	Weaning index	Sex ratio
Pre-treatment for 14 days only followed by mating	1A (control)	10	10	0	100	100	20.33±1.414	96.7	100	82
	IIA (62.5 mg/k)	9	9	1	100	100	20.22±0.666	97.3	100	84
	IIIA (100 mg/kg)	10	9	0	100	90	20.01±0.707	98	98	56
	IVA (500mg/kg)	10	10	0	100	100	20.34±1.432	97	100	87
	VA (1000mg/kg)	7	7	3	100	100	20.23±0.365	100	100	62
Pre-treatment for 14 days followed by mating and continued treatment during gestation	1B (control)	10	10	0	100	100	20.11±0.833	100	100	77
	IIB (62.5 mg/kg)	10	4	0	100	40	21.23±1.118	94.1	42	100
	IIIB (100 mg/kg)	10	6	0	100	60	20.78±1.568	86.4	94	66
	IVB (500 mg/kg)	10	3	0	100	30	21.56±0.711	70	85	81
	VB (1000 mg/kg)	5	0	5	100	0	-	-	-	-

Mating Index = (no of cohabited females/No of females mating) x 100

Fertility Index = (No of mated females/No of pregnant females) x 100

Live Birth index= (No of offspring delivered alive/No of offspring delivered) x 100

Weaning Index = (No of offspring at day 21/ No of Offspring delivered) x100

Gestation period is presented as mean ± SD

Sex Ratio = (No of Males / No of Females) x 100

2.3.4. Effects of cryptolepis treatment on epididymal sperm number

Left caudal epididymis and testes of mice exposed to aqueous cryptolepis or distilled water for two weeks were extracted and weighed and further subjected to sperm analysis as previously described (Meistrich, 1989). The combined mean wet weights of left epididymis and left testes of the treated groups were reduced especially at 1000 mg/kg of cryptolepis although it was not statistically significant ($P < 0.05$) compared to the controls (Table 2.3). Sperm numbers also decreased in all treated groups compared to the vehicle-treated group (Table 2.3). This was significant ($p < 0.001$) at cryptolepis treatment of 500 and 1000 mg/kg. Although on treatment day one there were no significant difference between control and treated groups, significant weight differences however occurred between controls and treated groups at the end of the 14 day treatment period (Table 2.3).

Table 2.3 Effects of cryptolepis treatment on epididymal sperm number

Dose	Initial weight	Final weights	Wet weights of organs (g)	Mean number of sperm x 10^4
Control	37.00 ± 1.225	41.00 ± 2.915	0.1478 ± 0.0020	170.5 ± 15.09
62.5mg/kg	33.00 ± 1.225	38.00 ± 1.980	0.1395 ± 0.0006	135.8 ±11.44
500mg/kg	36.00 ± 1.000	34.00 ± 1.871*	0.1455 ± 0.0062	50.74 ±16.05***
1000mg/kg	35.00 ± 0.0 00	33.00 ± 1.100*	0.1350 ± 0.0083	51.00 ± 6.519***

Data are presented as group means ± SEM. Significant difference from control is depicted by; **P*<0.05, ****P*<0.001 by one way ANOVA using Bonferronis test to compare all means.

2. 3. 5 Mounting behaviour test

Male mice were treated with saline (control group) or with cryptolepis (62.5-1000 mg /kg, *p.o.*) and placed individually in a plexiglas cage (60 × 75 × 20 cm). After 15 minutes of acclimatization, a non-oestrous female was introduced into the arena and the number of mounts, anal sniffs, penile licks, attempted mounts recorded during a 15-minute observation period.

Treatment of male mice with cryptolepis did not affect mounting behaviours such as anal sniffing, penile licking, attempted mounts and mounts during the first hour of observation. Generally, anal sniffing decreased by the third hour except at 1000 mg/kg where it increased significantly ($P < 0.05$). Mounts and attempted mounts did not improve with time with the treatment compared to control. Penile licks were significantly (*** $p < 0.001$) higher than control values during the third hour but not during the first hour.

Treatment of male mice with cryptolepis for 14 days caused decreased mounting behaviour relative to controls. Mounting and attempted mounts were significantly different from controls particularly at 62.5 and 1000 mg/kg ($p< 0.001$). Penile licking which hitherto had increased in the third hour showed no significant difference from control after two weeks treatment.

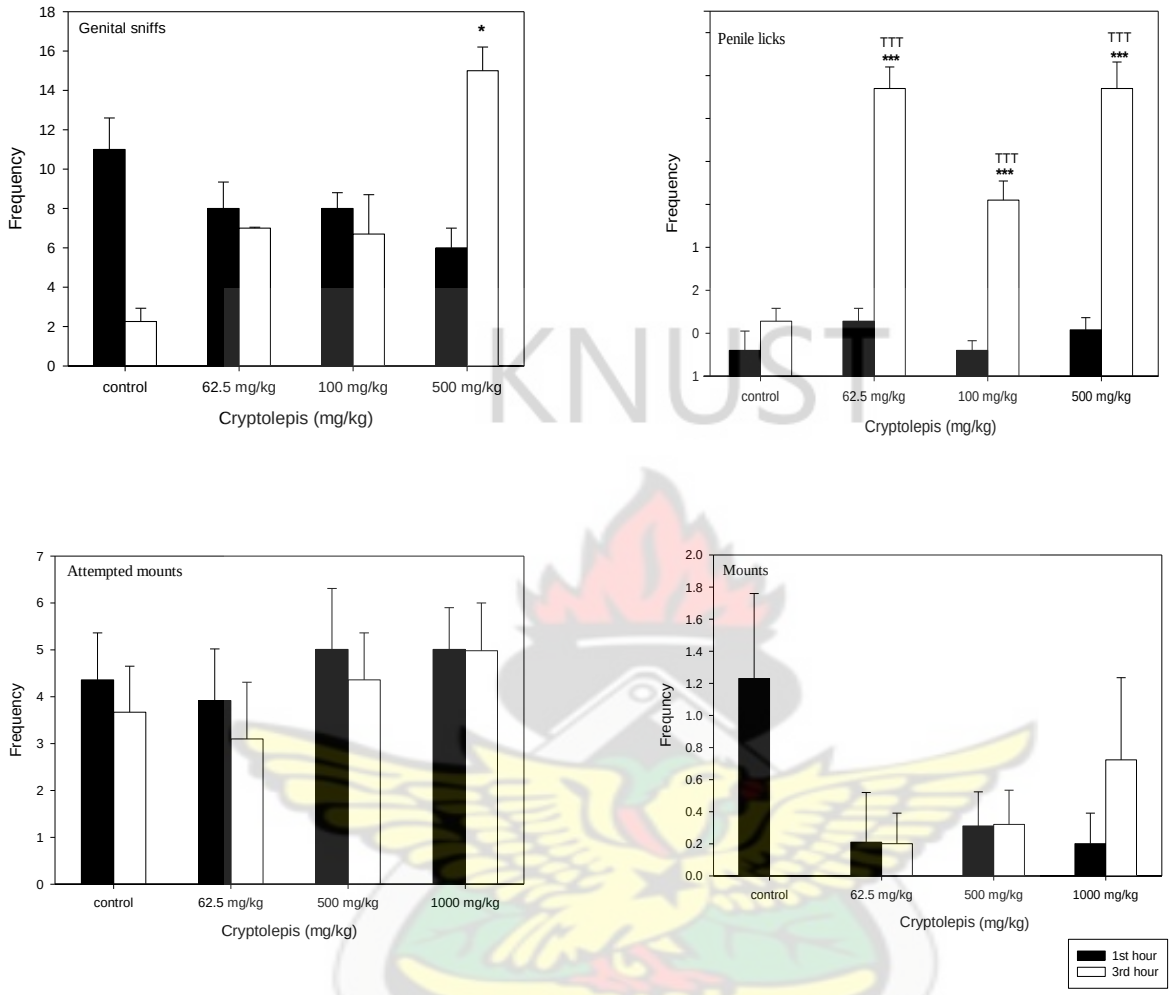


Fig 2.2. Effects of acute cryptolepis treatment on mounting behaviour: anal sniffing, penile licks, attempted mounts, and mounts. Statistical significance is by one way ANOVA Newman-Keuls test * means $p < 0.05$, *** means $p < 0.001$ when compared with control whilst ^{TTT} means $p < 0.001$ when compared with Ist hour**.**

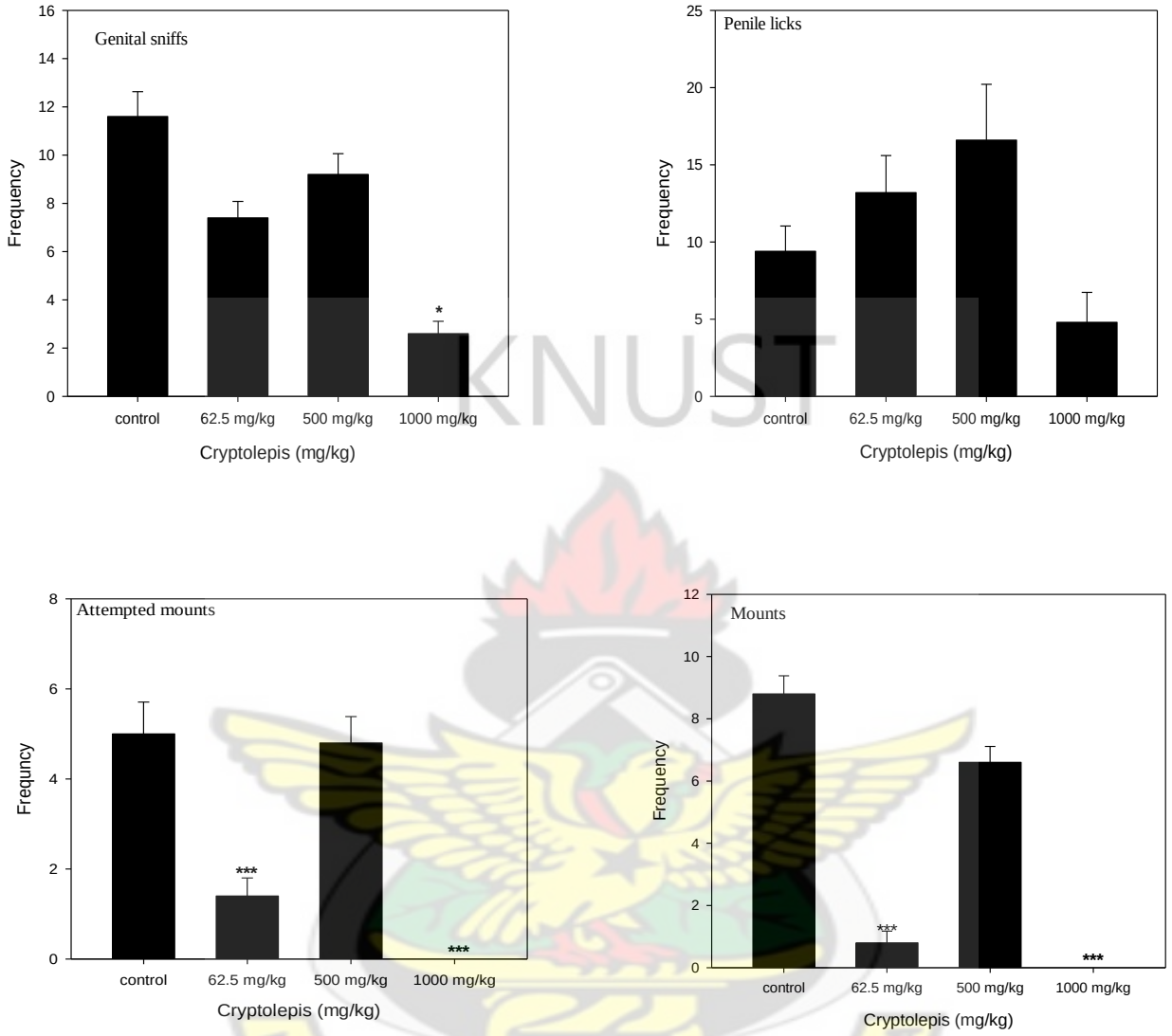


Fig 2.3. Effects of 14 days cryptolepis treatment on mounting behaviour: anal sniffing, attempted mounts, mounts and penile licking. Statistical significance; * $p < 0.05$, *** $p < 0.001$ by one way ANOVA using Newman-Keuls post hoc test.

2.3.6 Male dominant lethality assay

Male mice received 62.5, 500 and 1000 mg/kg cryptolepis for 5 days and each was mated serially on different weeks with two female mice. The pregnant females were kept until 14 days post coitus. For each group, females were analyzed according to the dominant lethal protocol (Greene *et al.*, 1985). Postimplantation loss was estimated as a percent of embryos that died at the early stage of implantation divided by the number of implants. There was no significant difference in post implantation losses between female groups assessed at weeks 1, 2 and 5 (Table 2.4). Treatment however affected male fertility as reflected in decreased female fertility index at all doses of cryptolepis employed (Table 2.4).

This shows that aqueous extract of cryptolepis has a low mutagenicity profile *in vivo*.

Table 2.4 Effects of paternal cryptolepis treatment on fertility and total implants in female mice

Post treatment period (Week) in males	Dose	Female Fertility index	Total implants per female	Total live implants/female	No of deaths per female	%Post implantation loss
ONE	Control	100	11.40±2.171	11.00±2.000	0.40	3.51
	62.5mg/kg	80	10.63±1.923	10.38±1.923	0.25	2.35
	500mg/kg	100	10.20±1.687	10.00±1.826	0.20	1.96
	1000mg/kg	70	11.00±2.380	10.71±2.430	0.29	2.60
TWO	Control	100	9.60±1.955	9.00±1.247	0.60	6.25
	62.5mg/kg	100	11.00±1.491	10.50±1.780	0.50	4.95
	500mg/kg	90	11.22±2.224	10.89±2.088	0.33	4.41
	1000mg/kg	80	8.50±2.828	8.25±2.493	0.25	2.94
FIVE	Control	100	10.30±1.130	9.80±1.317	0.50	4.85
	62.5mg/kg	100	10.20±1.814	9.80±1.678	0.40	4.40
	500mg/kg	90	10.11±2.028	10.00±2.000	0.11	1.10
	1000mg/kg	80	10.25±1.389	9.875±1.458	0.38	3.66

Results for Total implants per female and Total live implants per female are presented as mean ± SD. % Post implantation losses = (No of early Deaths/Total number of implants) x 100.

2.4 DISCUSSION

From the Irwins observation test, the LD_{50} after acute oral administration of cryptolepis is above 2000 mg/kg in mice.The Irwin Primary observation test (Irwin, 1968) was also used to estimate the safety of 62.5, 100, 500, 1000, 2000 mg/kg of cryptolepis in mice. The results of this test were used to predict the maximum dose to be used in subsequent experiments that will not have adverse physiological and behavioural effects. From the test, cryptolepis was safe in mice up to doses of 2000 mg/kg with sedation as the main sign of acute toxicity occurring from 500 mg/kg. Marked sedation and hypoactivity occurred at 2000 mg/kg which lasted for more than 24 hours.

The mouse, though genetically close to the rat, tends to be more susceptible to some compounds than the rat because of metabolic and specie differences. However, similar results have been reported in rats where the LD_{50} was estimated to be above 3000 mg/kg (Ansah *et al.*, 2008a; Ansah *et al.*, 2009a) and mild sedation and reduction in spontaneous locomotor activity as side effects of acute toxicity. Based on the results, doses of cryptolepis up to 1000 mg/kg were selected for all other experiments.

Female mice aggressively resist mating in several phases of the oestrous cycle except during the oestrous phase. Oestrous cycle in mice is controlled by neuroendocrine system and the presence of substances that mimic hormones can alter the cycle and affect mating and conception in the animals. There were clear differences between pretreatment with cryptolepis before mating and treatment with cryptolepis during mating and gestation. There was insignificant difference in reproductive indices between the control and pre-treated groups which suggest that either

cryptolepis has little effect or at worst has temporal and reversible effect on the reproductive system of nulliparous female mice.

Treatment with cryptolepis during mating and gestation did not affect the mating indices, which suggest that the differences in other indices with cryptolepis treatment may not be due to an adverse effect on mating. Cryptolepis causes a reduction in fertility index in female mice. However because there was no effect on mating index the reduction in fertility may be possibly due to mechanisms such as inhibition of ovulation, fertilization, reduction in sperm motility or death of gametes or embryos. A critical look at the pharmacological effects of cryptolepine, the main alkaloid in the aqueous extract, show that cryptolepis has the potential to alter reproduction in mice.

Cryptolepis and cryptolepine, the main alkaloid in the aqueous extract, possess potent anti-inflammatory activity mediated by COX-2 inhibition (Bamgbose and Noamesi, 1981; Olajide *et al.*, 2007a; Olajide *et al.*, 2009; Olajide *et al.*, 2010). In addition cryptolepine directly blocks the activity of prostaglandin E_2 (Bamgbose and Noamesi, 1981). In mammals the COX-2 enzyme is very active in the follicular stages of the ovarian cycle. COX-2 induction precedes ovulation and is mediated by gonadotropin acitivty (Sirois *et al.*, 1992; Sirois *et al.*, 2004). It is believed that prostaglandins, particularly E_2, are involved in ovulation by causing ovum release and the process is very analogous to the inflammatory process (Espey, 1980; Espey and Lipner, 1994).

NSAIDs particularly COX-2 selective inhibitors induce anovulatory condition in mammals where there are clinical signs of ovulation but there is no ovum release. This has also been seen in young women with rheumatoid arthritis on NSAIDs undergoing fertility treatments (Zanagnolo *et al.*, 1996; Skomsvoll *et al.*, 2005). Furthermore, this has also been confirmed in

experiments using COX-2 knockout mice. Mice lacking the COX-2 gene have multiple reproductive, ovulatory and fertilization failure (Lim *et al.*, 1997).

Notwithstanding the above mechanism, it is also possible that the reduction in fertility may be a direct effect of cryptolepis on the developing embryo as cryptolepis and its main alkaloid are cytotoxic causing cell cycle arrest through several mechanisms (Bonjean *et al.*, 1998; Dassonneville and Bonjean, 1999; Lisgarten *et al.*, 2001; Ansah and Gooderham, 2002; Ansah *et al.*, 2005; Zhu *et al.*, 2005; Zhu and Gooderham, 2006; Ansah *et al.*, 2008 c; Ansah and Gooderham, 2009 b). In mice early embryonic death is followed by tissue resorption and this can presented as a reduced fertility index in an *in vivo* experiment.

Prostanoids mediate uterotonic actions of oxytocin during labour. Clinically gestation period can be prolonged by treatment with non selective non steroidal anti-inflammatory drugs such as indomethacin and aspirin but not COX-2 selective agents. This presupposes that the COX-1 isoenzyme, not the COX-2, is involved. Consequently it has been shown that there is a delay in onset of parturition in COX-1 knockout mice; COX-2 knockout mice, although posses multiple reproductive problems, have normal onset of parturition (Langenbach *et al.*, 1995; Gross *et al.*, 1998). In this study, cryptolepis did not have significant effect on the gestation period and the onset of parturition. This reinforces the COX-2 isoenzyme selective activity of cryptolepis and cryptolepine, the main alkaloid of the aqueous extract (Olajide *et al.*, 2007a; Olajide *et al.*, 2009; Olajide *et al.*, 2010).

Pharmacologically, stimulation of α_2 adrenoceptors and muscarinic receptors of the uterus in some animals cause contraction. Cryptolepine, the main alkaloid of the aqueous extract, is an

antagonist on these receptors (Noamesi and Bamgbose, 1980; Noamesi and Bamgbose, 1982; Rauwald *et al.,* 1992) hypothetically, blockade of these receptors should cause relaxation of the uterus, enhance survival and increase the gestation period.

Sex ratio in a population is approximately one is to one. Certain chemicals in the environment can affect probability of an animal being female or male. Presence of endocrine disrupting chemicals can induce feminization or masculinisation thereby distorting this ratio. In all groups, the numbers of females were slightly more than males except in groups of high mortality in the experiment. The effect of cryptolepis on sex ratio appears insignificant and the difference could be due to chance and foetal mortality rather than a possible endocrine effect.

In the mounting behaviour study, cryptolepis did not show prospects as a potentially good aphrodisiac in mice although it is used for such purposes in humans. It did not significantly affect the mounting behavioural parameters with the exception of penile licking. It is possible that the reason for its wide usage as an aphrodisiac may not be an influence on sexual behaviour and libido but a direct effect on the carvenosum of the penile organ as indicated by enhanced penile licks in male mice during treatment. According to the classification of aphrodisiacs, (Sandroni, 2001) activity of cryptolepis may be related to substances that enhance potency; enhancing erection by vasodilation. Blockade of α-adrenergic pathway has been shown to be a mechanism by which some aphrodisiacs resolve problems of erectile dysfunction (Andersson, 2001) probably by causing vasodilation. Theoretically muscarinic receptor antagonists can delay ejaculation thereby prolonging sexual activity. Cryptolepine, the main alkaloid in the aqueous extract, posseses preferential alpha$_2$ (α_2) adrenoceptor blocking activity (Noamesi and

Bamgbose, 1982) and non selective muscarinic activity (Rauwald *et al.*, 1992) which may give scientific credence for the purported usage as an aphrodisiac.

Traditionally, it is the ethanolic extract popularly called 'bitters" that is mostly used as a sex enhancer. Although the effects of alcohol on sexual behaviour are quite controversial, it is widely accepted that at the early stages it enhances sexual activity. This could partly account for the acclaimed aphrodisiac effects of cryptolepis. Thirdly, male sexual dysfunction or weakness may be secondary to other medical diseases. The use of cryptolepis as a male sex enhancer may not be due to a direct aphrodisiac effect but rather its ability to treat sexual problems secondary to other medical conditions. For instance, there is a high correlation between leucocytospermia and reduced fertility in the men. In the absence of clinical symptoms the origin of the leucocytes as well as its role in infertility is unknown. However, research from several fertility clinics show that selective COX-2 inhibitors may enhance sperm numbers and quality in treated males as well as increased pregnancy rates in women with treated partners (Kaleli *et al.*, 2000; Lackner *et al.*, 2006; Piomboni *et al.*, 2007). Cryptolepis is a selective COX-2 inhibitor (Olajide *et al.*, 2010) and hence may be very beneficial to male infertility associated with leucocytospermia.

In mice, subacute treatment however results in the reversal of the apparent aphrodisiac property observed with acute treatment. It appears that subacute treatment rather leads to reduction in libido. Libido is primarily determined by the testeosterone levels in an organism, meaning that cryptolepis treatment may either directly or indirectly affect testosterone levels. On the other hand, cryptolepis is a sedative and causes hypoactivity (Mshana *et al.*, 2000; Ansah *et al.*, 2008 b; Ansah *et al.*, 2008a; Ansah *et al.*, 2009a). Persistent sedation and hypoactivity can affect mounting behaviour parameters. It is also possible that the reduction in mounting behaviour

parameters is in line with the decrease in both testicular and epididymal weight as well as mean epididymal sperm number.

Fertility experiments with treated males consistently showed an apparent decline in fertility with treatment. The sub fertilities were not reversible after treatment particularly at the highest dose of 1000 mg/kg. Antimuscarinic activity (Rauwald *et al.*, 1992), alpha adrenoceptor blockade (Noamesi and Bamgbose, 1980; Noamesi and Bamgbose, 1982), sedation (Mshana *et al.*, 2000; Ansah *et al.*, 2008a; Ansah *et al.*, 2009a) and cytotoxicity (Ansah and Gooderham, 2002) are pharmacological properties exhibited by cryptolepine which can adversely affect male fertility in rodents but with some distinctions between the mechanism and the type of inhibition.

Atropine and other antimuscarinics inhibit male fertility reversibly by unknown mechanism without weight or histopathologic findings on sperm number in the cauda epididymis, and sperm motility in the vas deferens (Ratnasooriya, 1984; Ban *et al.*, 2002). The effects are however reversible on cessation of treatment and fertility returns to control values.

Prazosin, phenoxybenzamine, tamsulosin, terazosin, all alpha adrenoreceptor antagonists, have a potently negative effect on fertility in male rodents by inhibition of sperm emission (Homonnai *et al.*, 1984; Ratnasooriya and Wadsworth, 1990; Ratnasooriya and Wadsworth, 1994). The mechanism, as has been demonstrated for prazosin, (Doggrell, 1981; Bradley and Doggrell, 1985; Solomon *et al.*, 1997) involves an inhibition on both the neurally-evoked contractions on isolated rat vas deferens and sperm transport from the cauda epididymis to the distal vas deferens hence reduced sperm numbers in the ejaculated semen. However this effect has only been demonstrated in rodents.

Cytotoxics have adverse effect on proliferating cells thus affect male fertility by affecting the number of sperms, their morphology as well as the integrity of the organs involved (Cai *et al.*,

1997). Subacute treatment of male animals for 14 days with cryptolepis resulted in a dose dependent reduction in average sperm number in the head of the cauda epidimydis .The combined wet weight of the left testes and epididymis at the highest dose of cryptolepis was less than that of the control. A reduction in caudal epididymal sperm number shows that the mechanism may not be wholly due to alpha adrenoceptors blockade. It is possible that cryptolepis damage to the gonads affects not only spermatogenesis but Leydig cells as well leading to a drop in testosterone levels and a general drop in libido and sexual behaviour as seen in the mounting behavioural studies. It has recently been demonstrated that selective COX-2 inhibitors induce apoptosis in the epididymis which the normal methods of detecting apoptosis in tissues cannot detect (Wong *et al.,* 2002). This also can affect sperm numbers. Cryptolepis showed a decrease in fertility and epididymal sperm numbers in the present study.

In vitro genotoxicity experiments by Ansah *et al.*, (2005) showed that cryptolepis, although a cytotoxic, is at worst a weak mutagen whilst its alkaloid, cryptolepine, is not mutagenic. However because *in vitro* experiments may not include the activity of drug metabolising enzymes, substances which are not mutagenic but are activated to mutagens by metabolism are rarely detected.

Using the male dominant lethal assay, cryptolepis has a low mutagenecity profile. This can be accounted for partly by the mechanism that cryptolepine, the main alkaloid in the aqueous extract, induces apoptosis. It has been shown that cryptolepine is a DNA intercalator and interferes with topoisomerase II activity (Bonjean *et al.*, 1998; Dassonneville and Bonjean, 1999; Lisgarten *et al.*, 2001). Topoisomerase II enzyme is involved in the cleavage of DNA strands. Most anticancer agents and cell poisons that modulate the activity of this enzyme cause cell death either by apoptosis or mitotic failure once cell damage is overwhelming. Recently it has

been shown by Zhu *et al.*, (2005); Zhu and Gooderham, (2006) that cryptolepine exhibits diverse mechanisms for inducing apoptosis and the chosen pathway or mechanism depends partly on the concentration of cryptolepine.

The genesis of mutagenesis is when strand breaks as well as the alteration of essential DNA processes, do not overwhelm the cell to cause death, but rather stimulate the initiation of multiple recombination or repair pathways (Baguley and Ferguson, 1998; Kaufmann, 1998; Fortune and Osheroff, 2000; Walker and Nitiss, 2002; Wilstermann and Osheroff, 2003) leading to chromosomal translocations and other DNA aberrations in surviving populations. Cryptolepis cause apoptosis in certain cell types (Ansah and Gooderham, 2002). It is very probable that because cryptolepis can cause cell cycle arrest and apoptosis by several mechanisms and pathways, prominent amongst them include intercalating of DNA (Lisgarten *et al.*, 2001), interference with topoisomerase II activity (Bonjean *et al.*, 1998; Dassonneville and Bonjean, 1999; Lisgarten *et al.*, 2001), inducing p53 and its target gene p21 (Ansah and Gooderham, 2002), inhibiting proapoptotic factors such as COX-2, iNOS (Olajide *et al.*, 2007a; Olajide *et al.*, 2007b; Olajide *et al.*, 2009; Olajide *et al.*, 2010) cryptolepis treatment may leave mammalian cells with little chance of initiating repairs and hence inducing mutagenesis.

Weight changes and mortalities were seen with subacute cryptolepis treatment in mice. Similar results have earlier on been reported in rats administered cryptolepis although weight changes were not significant (Ansah *et al.*, 2009a). Autopsy examination of the dead rats revealed slight enlargement of liver and kidneys, with congestion and hyperemia in the lungs and muscles (Ansah *et al.*, 2009a). Cryptolepine, the main alkaloid in cryptolepis, is a known non selective muscarinic antagonist at M_1, M_2, M_3 receptors (Rauwald *et al.*, 1992). Pharmacological agents of this class have antisecretory property on salivary, parotid, lacriminal glands as wells as gastric

acid secretions (Eglen and Watson, 1996). It can reduce food intake thereby creating a net negative energy balance and a weight decrease. Exposure to cytotoxics also leads to a high cell turnover rate which can lead to weight losses and death (Hannun, 1997; Komarov *et al.*, 1999; Lakin and Jackson, 1999). These properties could explain why treatment with the extract caused significant weight reductions and death.

In conclusion, *Cryptolepis sanguinolenta* treatment negatively affects reproduction in female as well as male mice.

CHAPTER THREE

EFFECTS OF CRYPTOLEPIS ON FOETAL DEVELOPMENT IN MICE

CHAPTER THREE

3.1 INTRODUCTION

Cryptolepine the main alkaloid of the aqueous extract of *Cryptolepis sanguinolenta* has been shown to cross the placenta and accumulate in some tissues of mice using a labelled carbon (Noamesi *et al.*, 1991a). Despite this, there has been no study on the effects of cryptolepis or cryptolepine on the developing embryo and foetus. In the previous chapter, it was clearly shown that *Cryptolepis sanguinolenta* causes reversible inhibition of female fertility in mice. The mechanism, though not fully elucidated, was attributed to possible inhibition of ovulation and fertilization. There is the likelihood that effects on the embryo and foetus could confound the results of the reproductive studies as early death of embryos cause resorption of embryonic tissues leading to an apparent reduction in fertility in *in vivo* experiments. Consequently thorough developmental studies are warranted.

Developmental toxicology involves studies into the ability of a xenobiotic to cause structural or functional alterations in the first and subsequent generations. Classically, developmental endpoints are structural malformations, growth retardation, mortality and functional impairment (Wilson, 1973). The parameters evaluated are *in utero* demise (resorption, foetal death), foetal body weights, and the size and morphology of external, visceral, and skeletal structures.

Ideally in developmental toxicological studies, treatment starts after implantations because if the test material is teratogenic and the metabolite(s) is not, exposure beginning earlier than implantation can lead to a possible concomitant induction of enzymes which will enhance metabolism resulting in the conceptus being exposed to less of the teratogenic moiety during organogenesis, and the study may be falsely negative. Treatment is also started on day 6 in mice

if interference with implantation is anticipated (Tyl and Marr, 2006). Early treatments are however required when the chemical has slow systemic absorption and is known to have cumulative toxicity, mechanism of toxicity involves depleting essential components, or a chemical which is inert but the metabolite is toxic (Tyl and Marr, 2006). Because of the lack of data on the pharmacokintetic profile of cryptolepis, several dosing regimen and times were used to cater for various possibilities which may be present.

A conceptus susceptibility to a chemical is not static but varies with the stage of development hence the timing of exposure is key to the outcome. Wilson, (1973) argues that susceptibility depends on the genotype of the conceptus and the manner in which it interacts with the chemical. Foetal and embryonic cells are not well developed like adult cells hence their interactions may be different from adult cells. One of the reasons may be their enzyme system, the predominant phase of the cells in the cell cycle.

The placenta has been described as a barrier which limits the access of chemicals to the developing organism. However unlike other barriers, the placenta is not static and its composition and chemical accessibility changes with foetal growth hence some substances which may not cross readily at some time may cross later on in development. Placental blood supply and the nature of the chemical can all affect the developmental outcome.

In this chapter, effects of cryptolepis treatment on embryonic and foetal development and whether the time of treatment affects the outcome are investigated.

3.2 MATERIALS AND METHODS

3.2.1 Chemical

Ammonium sulphide was obtained from Sigma-Aldrich Inc, St Loius, MO, USA.

3.2.2 Animal preparation and treatment

One hundred and sixty female 1CR mice about 8 to 12 weeks old and weighing approximately 15 to 20 g were kept in groups of ten for three weeks to enhance the synchronization of their oestrous cycle in anoestrous. Prior to co-habitation with males, females were housed in the presence of bedding taken from male animal cages. Female mice were introduced into unclean male cages in the late afternoon, and the following morning the females were checked for the presence of copulation plugs in the vagina. Successfully mated females were tagged and the day for mating recorded as gestation day 0.

3.2.3 Effects of cryptolepis on conception

Forty mated female mice were assigned to one of two groups (n=20). Group one received distilled water and group two received 100 mg/kg of cryptolepis *p.o* from the gestation day 0 until the end of gestation. Mice were assessed on gestation day 14 and on gestation day 19 for signs of pregnancy indicated by maternal weight changes. Animals that failed to deliver at the end of gestation were laparotomized. *Uteri* were stained with ammonium sulphide (10%) to visualize any implantation sites that may have undergone early resorption (Tyl and Marr, 2006).

3.2.4 Effects of cryptolepis on implantation

Mated mice were grouped into four (n=10). Group 1 (vehicle treated control) received distilled water only. Groups II, III, IV received 62.5, 500 and 1000 mg/kg of cryptolepis from the fourth day of gestation to end of gestation. Mice were assessed on gestation day 14 and on gestation day 19 for signs of pregnancy indicated by maternal weight changes and evidence of litter at the end of gestation. Litter size, litter weight and life status were assessed (Tyl and Marr, 2006).

3.2.5 Effects of cryptolepis on organogenesis

Forty (40) mated mice were assigned to one of four groups (n=10). Female mice in each group received either aqueous *Cryptolepis sanguinolenta* (62.5, 100, 500 mg/kg *p.o)* or distilled water from gestation day 6 to the end of gestation. On gestation day 18, 5 selected dams from each group were euthanized and laparotomized. *Uteri* were removed and examined for implantation sites. Sites that contain one-horn pregnancies were stained with (10%) ammonium sulphide (Salewski, 1964) to visualize any implantation site that may have undergone very early resorption starting at the left ovarian end and moving, in order to the cervix and up to the right ovary. The status of implantation sites was determined and designated as follows;

Live: Foetus exhibits spontaneous or elicited movement.

Dead: Non live foetus with discernible digits in any or all paws, body weight of 0.3 g for mice

Full: Non live foetus with discernible digits below the weights below 0.3g

Late: Some embryonic or foetal tissue from foetal remains to a full foetus with discernible limb buds but no discernible digits; foetuses may show signs of autolysis or maceration and appear pale or white.

Early: Evidence of implantation sites visualized only after staining fresh uterine preparation with ammonium sulphide (Tyl and Marr, 2006).

3.2.6 External examinations for teratogenecity of cryptolepis

The method employed was as described by (Edwards, 1968). Ten (10) foetuses were selected from each dose level in experiment 3.2.5 and their core body temperature lowered below 25˚C (Lumb and Jones, 1973; Blair, 1979) by placing them on a towel over ice. The foetuses were carefully examined externally beginning from head to tail with the aid of a magnifying lens.

Cranofacial Examinations

The contours of the cranium were noted for profile and full-face view. Eyes and pinnae were checked for bulginess, size, symmetry and position. The alignment of the upper and lower jaw as well as the angle was examined in profile. The nose examined for protruding slightly further than the lower jaw. The upper jaw was checked face-on for notches, furrows, or distortions. The tongue and palate were checked by depressing the tongue while opening a pair of closed forceps that have been inserted between the upper and lower jaws.

The Skin and Posture

The skin covering the head and the rest of the body was checked carefully for continuity, and abnormalities in colour, texture, or tone. Irregular swellings, depressions, bumps, or ecchymoses (subdermal hematomas) are recorded as well. The overall posture of the foetus was observed.

Digits Examinations

The fore- and hind limbs are checked carefully for normal size, proportions, and position. The number and disposition of the digits was noted (four digits plus a dewclaw on the forelimbs, five digits on the hind limbs), and the depth of the interdigital furrows was explored by gently pressing against the paws with the forceps to spread the digits.

The anus, external genitalia

The anal opening was examined for the proper location. The external genitalia were checked for general shape and size.

Tail Examinations

The tail was also examined for normal length and diameter (to detect thread-like tail), as well as abnormal kinking or curling and localized enlargements or constrictions.

3.2.7 Effects of cryptolepis on foetogenesis

Mated mice were grouped into four (n=10). Group 1 (vehicle treated control) received distilled water only. Groups II, III, IV received 62.5, 500 and, 1000 mg/kg of cryptolepis from the 15th day of gestation to end of gestation. Mice were assessed on gestation day 19 for signs of pregnancy indicated by maternal weight changes and evidence of litter at the end of gestation. Litter size, litter weight and life status were assessed (Tyl and Marr, 2006).

3.3 RESULTS

3.3.1 Effects of cryptolepis treatment on conception

When female animals were treated with 100 mg/kg cryptolepis on gestation day 0, conception rate was 60% as against 85% of the vehicle control on examination on gestation day 14. One half of the treated mice that appeared to be pregnant at gestation day 14 were not pregnant on day 19 (Table 3.1). However all control animals pregnant on GD 14 were pregnant on day 19. All pregnant mice i.e. control and treated were allowed to go full gestation.

Laparotomy was performed on all animals pregnant on day 14 but not on day 19 as well as all mice that showed no sign of pregnancy at all during the study. For the control mice that did not show signs of pregnancy during the study, there were no implantation sites indicating an unsuccessful mating upon laparotomy and staining with 10% ammonium sulphide.

Three of the treated showed no implantations after staining with ammonium sulphide indicating unsuccessful mating. Five of the remaining eleven mice had sites of implantations but no foetuses indicating early death of foetuses and tissue resorption. Six of the cryptolepis treated mice had 13 foetuses in total with 2 being alive and 11 dead (Table 3.1).

This study indicates that cryptolepis treatment causes early embryonic death and interferes with fertilization, rapid embryonic cell proliferation or implantation.

Table 3.1. Effects of cryptolepis treatment on conception

DOSE	CONTROL	100mg/kg
% of pregnant mice on Day 14	85	60
% of unsuccessful pregnancies by day 14	15	40
% pregnant on Day 19	85	30
% of unsuccessful pregnancies by day 19	15	70
Gestation Index	100	50
Total number of pups delivered	107	41
No of live births	99	35
No of Still births	8	6
Results of laparotomy for mice with unsuccessful pregnancies		
No of implantation sites per female	0	5.64
No of early death	0	31
Total no of foetus	-	13
No of Late/Full death/dead foetus	-	11
No of Live foetus	-	2

% Pregnant females = (No of pregnant females/No of mated females) x 100;

Gestation Index = (No of pregnant females on GD 14/No of pregnant females on GD 19) x 100

3.3.2 Effects of cryptolepis on implantation

Implantation in rodents occurs on gestation day 5-6 when day of mating is assigned gestation day 0. Administration of cryptolepis (62.5, 100, 500 mg/kg *p.o*) to pregnant mice GD 4 to coincide with implantation caused termination of pregnancies in mice (Table 3.2). There were higher incidences of failed implantations indicated by the reduced pregnancy rates in treated animals in relation to controls (Table 3.2).

Significant weight deficits (Intrauterine growth inhibition).* $p < 0.05$, ** $p < 0.001$, occurred in litters born to cryptolepis treated mothers. Mortality amongst offspring was 12 % at 1000 mg/kg of cryptolepis as against 0.5% of the controls. Treatment did not cause maternal mortality.

This study shows clearly that cryptolepis interferes with implantation or processes of implantation.

Table 3.2 Effects of cryptolepis on implantation

Dose	% of pregnant female on GD 19	% Maternal Mortality	% of Foetal Mortality	Mean weight of litter
Control	100	0	0.5	1.450± 0.124
62.5mg/kg	50	0	3.0	1.361± 0.168
500mg/kg	60	0	5.5	1.251± 0.249*
1000mg/kg	40	0	12.0	1.200± 0.324**

Mean weight of litter is presented as mean±SD (N=20).
% of pregnant female = (No of females mated/No of females pregnant on GD 19) x100;
Statistical significance by one way ANOVA is shown as * $P < 0.05$, **$P < 0.001$ using Neuman-Keuls test Maternal Mortality = (No of dead females/No of pregnant females) x 100;
% Foetal Mortality = (No of pups delivered dead/No of pups delivered) x 100.

3.3.3 Effects of cryptolepis on organogenesis

Organogenesis in mice occur on gestation day 6 and ends on gestation day 15. Treatment of mice with cryptolepis on gestation day 6 (0, 62.5, 100, 500 mg/kg) did not affect conception rate in mice (Table 3.3). Gestation Index was not affected with treatment. Treatment did not induce maternal mortality although it increased foetal mortality. In addition it induced intrauterine growth inhibitions (mean birth weight).

3.3.4 External examinations for teratogenicity of cryptolepis

Ten foetuses were selected from each group for external teratogenicity assay. Cryptolepis did not cause structural malformation to the contours of the cranium, the size, symmetry and position of the eye and pinnae. The upper jaws of foetuses had no notches, furrows or distortions and the noses protruded slightly further than their lower jaw. The skins of all the foetuses were continuous without irregular swellings, depressions, subdermal hematomas. Fore- and hind limbs were of normal size, proportions, and position. Each animal had four digits plus a dewclaw on the forelimbs and five digits on the hind limbs and with normal depth of interdigital furrows.

The anal opening was well located in all mice with a general shape and size of the external genitalia. The tails of treated animals was comparable to controls in length and diameter. There were no thread-like tails, abnormally kinking or curled tails and localized enlargements or constrictions.

Table 3.3 Effects of cryptolepis on organogenesis

Parameter	Control	62.5mg/kg	100mg/kg	500mg/kg
% pregnant on Day 14	90	80	100	90
% of unsuccessful pregnancies by day 14	10	20	0	10
% pregnant on Day 18	90	80	100	90
% of unsuccessful pregnancies by day 18	10	20	0	10
Gestation Index	100	100	100	100
% Maternal Mortality	0	0	0	0
% Foetal Mortality	1.4	3.0	5.1	2.0
Mean Birth Weight	1.47±0.06	1.46±0.157	1.23±0.111***	1.35±0.133*
Results of laparotomy experiment				
Total number of implantations/Female	8.2 ± 0.66	7.60 ± 0.75	8.60 ± 0.81	7.8 ± 1.07
No of early death/female	0.6 ± 0.41	0.40 ± 0.40	0.40 ± 0.24	0.0 ± 0.00
No of foetus/female	7.6 ± 0.51	7.20 ± 0.92	8.20 ± 0.58	7.8 ± 1.07
No of Live foetus/female	7.2 ± 0.37	5.80 ± 0.37	7.00 ± 0.63	7.2 ± 0.86
No of Late/Full death/dead foetus/female	0.4 ± 0.24	1.60 ± 0.67	1.20 ± 0.734	0.6 ± 0.40

Mean weight of litter is presented as mean ± SD (N=20).
% of pregnant female = (No of females mated/No of females pregnant on GD 19) x100;
Maternal Mortality = (No of dead females/No of pregnant females) x 100;
% Foetal Mortality= (No of pups delivered dead/No of pups delivered) x 100.
Statistical significance by one way ANOVA * P < 0.05, ***P<0.001 by Neuman keuls test.
Results for laparotomy are presented as mean ± SEM.

3.3.5 Effects of cryptolepis on foetogenesis

Treatment of pregnant mice with cryptolepis (62.5, 500, 1000 mg/kg) on the gestation day 15 (Table 3.4) did not result in termination of pregnancies as seen with previous experiments. Intrauterine growth was reduced relatively to controls. However reductions were not significant statistically. Foetal mortality was higher compared to controls. There were two maternal deaths with one occurring within 24 hours of cryptolepis treatment and the other after three days of treatment. There was discharge of blood around the genitalia of the animals that died.

Table 3.4 Effects of cryptolepis on foetogenesis

Dose	% of pregnant mice	% Maternal Mortality	% Foetal Mortality	Mean weight of litter
Control	100	0	1.7	1.492 ± 0.133
62.5mg/kg	100	0	1.8	1.442 ± 0.152
500mg/kg	100	20	4.2	1.321 ± 0.211
1000mg/kg	100	20	3.3	1.378 ± 0.253

Mean weight of litter is presented as mean ± SD (N =20);
% of pregnant female = (No of females mated/No of females pregnant on GD 19) x 100;
Maternal Mortality = (No of dead females/No of pregnant females) x 100;
% Foetal Mortality= (No of pups delivered dead/No of pups delivered) x 100

3.4 DISCUSSION

This part of the study evaluated parameters that occur *in utero* such as demise (resorption, foetal death), foetal body weights, and the size and morphology of external structures. Because of the lack of data on the pharmacokinetic profile of cryptolepis and the possibility of having outcome influenced by the timing and stage of development, multiple dosing regimens were used.

The most important period for teratology study in rodents is the period from the beginning of organogenesis (day 6) to the closure of the secondary palate (day 15) (Tyl and Marr, 2006). However all experiments ended on day 19 because if duration of dosing was stopped before the delivery date, there is the possibility that the post dosing recovery period can confound the outcome through compensatory mechanism. Another advantage with this model of dosing is that it is a better model for human exposure than exposure only during a portion of gestation, with abrupt cessation at the end of embryogenesis. The main disadvantage however is the cost burden of an extended experiment and possibility of underestimating foetal malformation because more malformed foetuses may die with prolonged drug dosing (Tyl and Marr, 2006).

The results showed the effects of cryptolepis treatment on conception, implantation and organogenesis and foetogenesis. It was evident that cryptolepis toxicity during pregnancy was influenced by the time of treatment with the extract. Treating mice on gestation day 0 or gestation day 4 resulted in a reduction in number of pregnancy in mice. This however did not occur with treatments on day 6 and day 15. Implantation in mice occurs on gestation day 5 if the day of mating is taken as day 0 whilst organogenesis starts on day 6. This shows the conceptus is much susceptible to cryptolepis treatment before implantation and hence *cryptolepis*-induced developmental toxicity, involves interference with fertilization, inhibition of the rapid cell

proliferation from the one cell stage to the blastocyst, decidualization or implantation. Furthermore all doses of treatment with cryptolepis resulted in embryolethality and intrauterine growth reduction, but no malformations. Chemicals that induce embryolethality and intrauterine growth inhibitions but no malformations tend to have pronounced foetal sensitivity before implantation (Klaassen *et al.*, 1996). This is because the foetus at that stage is a fluid filled cavity (blastocyst) with only the inner mass developing into parts of the organism. The cells at that stage have great restorative potential hence damage which is not overwhelming to cause death is easily repaired (Snow and Tam, 1979). This has been demonstrated for nicotine and DDT (Fabro, 1973; Fabro *et al.*, 1984; Klaassen *et al.*, 1996).

When cryptolepis treatment began on day 0, there were early deaths compared to day 6. When early death occurs there is tissue resorption. It is possible that one of the mechanisms of the reduced fertility observed in the previous chapter is early death caused by cryptolepis treatment and subsequent resorption of the tissue by the mother.

The mechanisms underlying the effects of cryptolepis on embryonic and foetal development may be as a result of the cytotoxic action of cryptolepis on proliferating cells. Cryptolepine, the main alkaloid of the aqueous extract, is cytotoxic and it intercalates DNA (Bonjean *et al.*, 1998; Lisgarten *et al.*, 2001; Ansah and Gooderham, 2002). The use of cytotoxics in early pregnancy is associated with findings such as premature birth, low birth weight, major malformations, spontaneous abortions, and foetal death particularly in the first trimester (Zemlickis *et al.*, 1992; Norgard *et al.*, 2003; Leslie *et al.*, 2005).

Another probable pathway worth considering is COX-2 isoenzyme inhibition in the mother as well as the foetus. COX-2 has also been shown to be important for the implantation process in several mammalian species. This was demonstrated in experiments in which blastocyst were transferred from wild type mice to the *uteri* of COX-2 deficient mice, where there were absolute implantation failure (Lim *et al.*, 1997). Also interference with COX-2 activity in the embryos lead to embryolethality. Embryonic development in COX-2 null mice is associated with early death as a result of severe morphological defects and consequent functional failures in the kidneys (Dinchuk *et al.*, 1995; Morham *et al.*, 1995). Cryptolepis is a COX-2 inhibitor (Olajide *et al.*, 2007a; Olajide *et al.*, 2009; Olajide *et al.*, 2010).

Another mechanism which could account for the effects of cryptolepis in pregnancy in mice is the activity of nitric oxide synthase (NOS). Pregnancy is associated with a reduction in systemic vascular resistance and a blunting of several pressor responses to accommodate the conceptus. Recent studies show that placental and foetal Nitric Oxide synthase (NOS) may play a considerable role in maintaining quiescence of the myometrium in human pregnancy (Batra *et al.*, 2003a; Batra *et al.*, 2003b). Its activity increases at the onset of pregnancy and decreases only at the time of parturition due to a decrease NOS activity. Experiments with NO donor, glyceryl trinitrate, shows it can prolong the duration of pregnancy (Campbell *et al.*, 1994).

Nitric oxide when produced can activate guanylate cyclase in the same cells that produced it (endothelial cells); giving rise to autocrine effects such as vasodilatation and smooth muscle relaxation or it can also diffuse from its site of synthesis and activates guanylate cyclase in neighbouring cells. The resulting increase in cGMP affects protein kinase G, cyclic nucleotide phosphodiesterases, ion channels and possibly other proteins. This inhibits the calcium ion

induced smooth muscle contraction of the myometrium preventing rejection of conceptus. Furthermore NO also hyperpolarises vascular smooth muscle as a consequence of potassium channel activation (Rang *et al.*, 2006).

It has also been shown that an increase in iNOS occurs in both the uterus and the implanting embryo. In pre- and peri-implantation embryos, iNOS are localized in the inner cell mass. Postimplantation embryos showed decreased iNOS localization (Saxena *et al.*, 2000). In the mouse, embryo development begins at the one cell stage, using maternally derived mRNAs. When the embryo progresses to the two-cell stage, it begins to produce embryonically derived mRNAs. NOS inhibitor prevents embryos from developing beyond the two-cell stage (Nathan and Xie, 1994). Paradoxically excess NO will cause apoptosis of the embryo while a loss of NO will stunt development but not cause cell death.

NO is one of the inflammatory mediators whose actions can be affected by cryptolepine treatment. In experiments by Olajide *et al.*, (2007b) cryptolepine-hydrochloride dose-dependently inhibited lipopolysaccharide induced NO production in the murine macrophage cell line RAW 264.7. Effects of cryptolepine on NO may have a genomic component involving a reduced binding of activated NF-kB to DNA (Olajide *et al.*, 2007b) causing a decrease in transcription of the iNOS as well as a non genomic mechanism involving blockade of parasympathetic activation of muscarinic (M_3) receptor which causes activation of eNOS through the activation of calmudolin by calcium. Cryptolepine, a non selective muscarinic receptor antagonist (Rauwald *et al.*, 1992), can block acetylcholine activation of NOS and hence affecting the synthesis of NO.

Treatment with cryptolepis on gestation day fifteen resulted in both maternal and foetal mortality. There were signs of maternal toxicity which could be as result of stress from the later stages of pregnancy potentiating the effects of cryptolepis. There were insignificant differences in the weight of the treated dams relative to the control. There were no terminations of pregnancies.

Cryptolepis affects the implanted foetus. This explains why some animals seemed pregnant on day 14 but not day 19. This could be due to the effects of cryptolepis on growing cells.

Treatment with cryptolepis resulted in growth inhibition at all times of treatment. Multiple genetic and environmental factors contribute to intrauterine growth inhibitions (Bell and Ehrhardt, 2002). Although the foetal genome plays an important role in growth potential *in utero,* the intrauterine environment is the major determinant of foetal growth. Among intrauterine environmental factors, nutrition plays the most critical role in influencing placental and foetal growth (Barker and Clark, 1997). Foetal growth is most vulnerable to maternal dietary deficiencies of nutrients during the peri-implantation period and the period of rapid placental development (Wu *et al.*, 1998). Maternal malnutrition during gestation reduces placental and foetal growth of both domestic animals and humans (Barker and Clark, 1997; Bell and Ehrhardt, 2002). Maternal over nutrition before or during pregnancy may also result in foetal growth restriction and increased risk of neonatal mortality and morbidity in animals and human (Castro and Avina, 2002).

One way that cryptolepis may affect the intrauterine environment is by affecting the activity of nitric oxide (Olajide *et al.*, 2007b) which regulates placental-foetal blood flows and, thus, the transfer of nutrients and oxygen from mother to foetus. Inhibition of NO synthesis by NOS

inhibitors in rats or the absence of nitric oxide synthesis in eNOS-knockout mice results in intra uterine growth inhibition (Hefler *et al.*, 2001). It is also possible that the mechanism for weight loss in adult animals is similar to the mechanism that leads to intrauterine growth inhibition. This may be related to cytotoxicity as seen in patients undergoing cancer chemotherapy because of the inhibition of cells that are rapidly proliferating.

This part of the study has shown clearly that prenatal cryptolepis treatment in mice affects foetal development by inhibiting intrauterine growth and induces both maternal and foetal mortalities without external teratogenesis.

CHAPTER FOUR

EFFECTS OF PRENATAL CRYPTOLEPIS TREATMENT ON POST NATAL GROWTH AND SENSORI-MOTOR DEVELOPMENT.

CHAPTER FOUR

4.1 INTRODUCTION

In the previous chapter it was evident that *Cryptolepis sanguinolenta* treatment caused intrauterine growth inhibition, foetal and maternal mortalities. Not all animals exposed prenatally to cryptolepis died and such animals may survive to adulthood. Though the mice appeared normal, they may exhibit functional deficits. Functional deficits are detected by functional toxicity studies and it is one of the cardinal endpoints of developmental toxicity studies. However unlike other developmental toxicity endpoints such as embryolethality, malformations, and growth retardation, functional toxicity is limited to studies on the developing nervous system. Furthermore it is more sensitive and occurs at much lower doses (Vorhees, 1997).

Developmental neurotoxicity, in functional toxicity, involves studies into the ability of a chemical to cause alterations in behaviour, neurohistology, neurophysiology and neurochemistry of the central nervous system in an offspring as a result of exposure of the mother during pregnancy or during lactation. In adults, neurotoxicants must pass the blood brain barrier to exert their toxic effects. However to reach the foetus, the chemicals must pass through the placenta. Transfer of chemicals across placenta and blood brain barrier is correlated to their lipid solubility (Watanabe *et al.*, 1990). Therefore, many compounds identified as neuroactive in adults have the potential to pass the placenta and to reach foetal circulation upon exposure of the mother. Cryptolepis exhibits central nervous system activity in mice i.e. it is anxiogenic and a sedative (Ansah *et al.*, 2008 b). It has also been shown that cryptolepine, the main alkaloid of the aqueous extract enter foetal tissue when administered on gestation day 16 and accumulates in tissue of the foetus particularly melanin containing tissues of the eye (Noamesi *et al.*, 1991a). However very

little is known about its neuroactivity and whether it constitutes a risk in prenatally exposed animals.

Transplacental passage may occur through active transport, facilitated transport or through passive diffusion. However placental permeability to a chemical is influenced both by placental characteristics (e.g. thickness, area, carrier systems, lipid and protein content) and by chemical characteristics such as the degree of ionization, lipid solubility, protein binding, and molecular weight. Placental blood flow, placental permeability and placental metabolism affect placental transfer of chemicals and they are dynamic parameters which change as pregnancy and gestation progress (Slikker and Miller, 1994).

This chapter considers the functional toxicities by monitoring the effects of prenatal cryptolepis on growth, physical and sensori-motor development.

4.2 MATERIALS AND METHODS

4.2.1 Prenatal treatment of pregnant mice with cryptolepis

Forty female mice were cohabited with 20 male mice and observed for signs of mating by either direct observation of copulation or formation of vaginal plugs. Successfully mated females were tagged and assigned to one of four dose levels (n=7). The day was recorded as gestation day 0. Female mice received either *Cryptolepis* (62.5, 100, 500 mg/kg *p.o)* or distilled water from gestation day 6 to the end of gestation. On gestation day 16, dams were assigned to individual cages containing approximately 3cm of coarse saw dust and treatment continued until delivery. The day of birth (day 0) was determined by examining the cages twice a day; morning and evening. The duration of gestation, the number of pups per dam, weight of pups, crown-rump distance was measured. Pups were culled up to eight per dam.

4.2.2 Effects of prenatal cryptolepis treatment on survival of pups and postnatal growth

Pups (n= 18) from each dose level were monitored daily for the rate of growth, as well as survival rate from postnatal day (PND) 0 to PND 14. All experiments involving weighing were carried out at 9 am.

4.2.3 Effects of cryptolepis treatment on the time of attainment of physical landmarks

Pups (n = 18) were selected from each dose level and time of acquisition of developmental landmarks, such as pinna detachment (pinna unfolding), incisor eruption, eye opening (pups are born blind with eyes shut), hair appearance, testis descent were noted (Buelke-Sam *et al.*, 1985).

Pinna (external ear) detachment or Pinna Unfolding

Beginning on PND 1, pups at each dose level were examined closely for detachment of either the right or left pinna from the side of the head. The criterion was met when the point of either pinna was detached. The observation continued until all pups in the litter met the criterion.

Pilation (hair growth)

Beginning on PND 1, all pups at each dose level were examined daily until bristles appeared on the dorsal surface.

Incisor Eruption

Pups at each dose level were carefully examined for eruption through the gum by either an upper or lower incisor starting from PND 7. Criterion was met when an incisor was visible

Eyelid Separation

Each pup was carefully examined for any break in the membrane connecting the upper and lower eyelids, and criterion was met when there was a break in the membrane of either eye beginning on PND 10.

Testes Descent

Male pups were examined for testis descent beginning on PND 19. Each male rodent was removed from its cage and examined for the presence of one or both testes in the scrotum.

4.2.4 Effects of cryptolepis treatment on sensori-motor development

Rooting reflex

A selected pup was induced to crawl forward, pushing its head in a rooting fashion when the snout was bilaterally between the thumb and forefinger. The number of pups producing this reflex on day 1, 2 and 3 were noted (Caston *et al.*, 2004).

Vibrissae placing response

The animal was suspended by its tail and its vibrissae touched with a pencil, a positive result was when it raised its head and extended its forelimbs in order to grasp the object. The number of animals eliciting this reflex was noted every day from day 1 to day 3 (Caston *et al.*, 2004).

Cliff avoidance

Cliff avoidance was evaluated beginning on PND 1 for all pups. Cliff avoidance is the behaviour of crawling away from an edge of a flat surface edge (cliff). Pups were placed on a table or platform, with their front paws over the edge. The criterion was met when the pup attempted to crawl away from the edge within a 10 s period (Tyl and Marr, 2006).

Righting reflex

When the neonate was placed in the supine position, it immediately turns over to restore its normal prone position. In this test, the pups were placed in a supine position on a plane surface

and then no longer restrained. Time needed to recover to normal prone position (cutoff: 10 s) was noted. Righting reflex was considered to be fully achieved when the pups had turned 180º around their longitudinal axis, their four paws being in contact with the plane surface (Caston *et al.*, 2004; Tyl and Marr, 2006).

Negative geotaxis

Negative geotaxis also called negative geotropism (Crozier and Pincus, 1926) is a postural reaction bringing the animal in the upright position when it is placed downwards. In the experiment, the rat pups were placed on a 20º tilted plane, their snout being downwards. The animals had to turn for 180º in order to bring their snout upwards. The time (cutoff: 180 s) needed by the animal to achieve the 180º rotation was noted.

Pinna reflex

The pinna reflex tests the somatomotor component of the 7th cranial nerve in rats. The presence of the pinna reflex was evaluated daily, beginning on PND 11. The inner surface (near the concha) of the pinna was lightly touched with a filament. The criterion was met with the presence of any movement of the pinna (sudden twitch or flattening of the ear) made in response to the applied stimulus. If the first ear tested did not respond to the stimulus, the opposite ear was tested (Tyl and Marr, 2006).

Auditory Startle Reflex

Beginning on PND 11, all pups were examined daily for the auditory startle reflex. The auditory startle reflex was noted as a sudden flinch or cessation of ongoing movement following a high pitched auditory stimulus. Each nesting box was removed from the study room and placed in a quiet room. Littermates remain outside the testing room in order to mitigate habituation to the auditory stimulus. The pup was placed in a beaker, with approximately 600 ml of bedding material and taken into the testing room. A clicker was held directly above the beaker, but not touching it and the clicker stimulus was delivered. Any observable whole body response (e.g., flinching, jumping, and freezing of activity) meets the criterion for the startle response (Tyl and Marr, 2006).

Air righting reflex

The air righting reflex is the ability of pups to land on all four paws when dropped from an inverted position. All pups in each litter were tested on once each day beginning on PND 11 until all pups in the litter demonstrated the reflex. The pup was held in a supine position above a well-padded surface and then released. Pups were held 17 to 20 cm above the surface. The pup that landed on all four limbs met the criterion (Tyl and Marr, 2006).

Reflex suspension

The ability of the pup to remain suspended by its forelimbs from a 2 mm rod was noted. Attainment of the reflex suspension benchmark was defined as the postnatal day when the pup could remain suspended for a minimum of 20 s (Tyl and Marr, 2006).

4.2.5 Effects of cryptolepis treatment on motor activity

4.2.5.1. Effects of cryptolepis on spontaneous locomotor activity at PND 30

On postnatal day 30, five female and five male mice of the f_1 generation prenatally treated to either distilled water only or cryptolepis (62.5, 100, 500 mg/kg *p.o*) from gestation day 6 till delivery were selected and tested for the effects of prenatal cryptolepis treatment on spontaneous locomotor activity. The spontaneous locomotor activity was measured with an automated mice activity cage (Model 7401, Milan, 19 x 23 x 35 cm). The movement of mice in the cage was detected by 29 stainless steel bars placed 1cm apart on the floor of the cage. Five selected mice of the same group and sex were placed simultaneously into the cage. Ten minutes was allowed for habituation of the animals to the new environment. Activity was measured cumulatively every five minutes for thirty minutes.

4.2.5.2. Effects of cryptolepis on motor coordination at PND 40

The effects of prenatal cryptolepis treatment on motor coordination were assessed with the rotarod apparatus on postnatal day 40. The rotarod apparatus (Model 7600, Ugo Basile, Milan, Italy) consist of a base platform and a rotating rod (3cm diameter) with a non skid surface. The rod, 50 cm high, is divided into five equal sections by six disks allowing for the testing of five mice simultaneously. In this experiment, the rotarod apparatus was set to rotate constantly at 12 rpm. Motor performance was measured as the latency to fall from an accelerating rotarod. Five male mice treated at the same dose level were placed on the rod and the latency to fall off the rod or the endurance time was recorded. Each animal was given four successive trials and the mean

of the last two trials was determined. The experiment was repeated using five female mice. A maximum cut off time of 300 s was used.

4.2.5.3. Effects of cryptolepis treatment on grip strength at PND 80

Measurement of fore limb grip force was made using a grip force analyzer as described (Wilcox *et al.,* 2000). The apparatus measures the magnitude of tensile force an animal exerts against a wire mesh grid with its fore paws, and gently pulled (10 cm/s) in a caudal direction. The peak force exerted by the mouse before it releases its grasp of the wire mesh grid was registered by a force transducer and recorded in grams.

Selected male mice (n=7) prenatally treated with cryptolepis (0, 62.5, 100, 500 mg/kg) were used. Six consecutive hind limb grip force measurements (10 s apart) were obtained for each mouse and the average of the last four measurements recorded.

4.3 RESULTS

4.3.1 Effects of prenatal cryptolepis treatment on pups survival

Pregnant mice from gestation day 6 to the end of gestation received either distilled water or cryptolepis (62.5, 100, 500 mg/kg *p.o.).* Eighteen pups were randomly selected from each dose level to study the effect of prenatal treatment with cryptolepis on growth and pup survival. Mortality was significantly high at 62.5 and 100 mg/kg of treatment compared with controls. It was however not affected at 500 mg/kg of treatment (Fig 4.1). Most of the deaths occurred before postnatal day 6. These results suggest that prenatal cryptolepis treatment decreases postnatal survival.

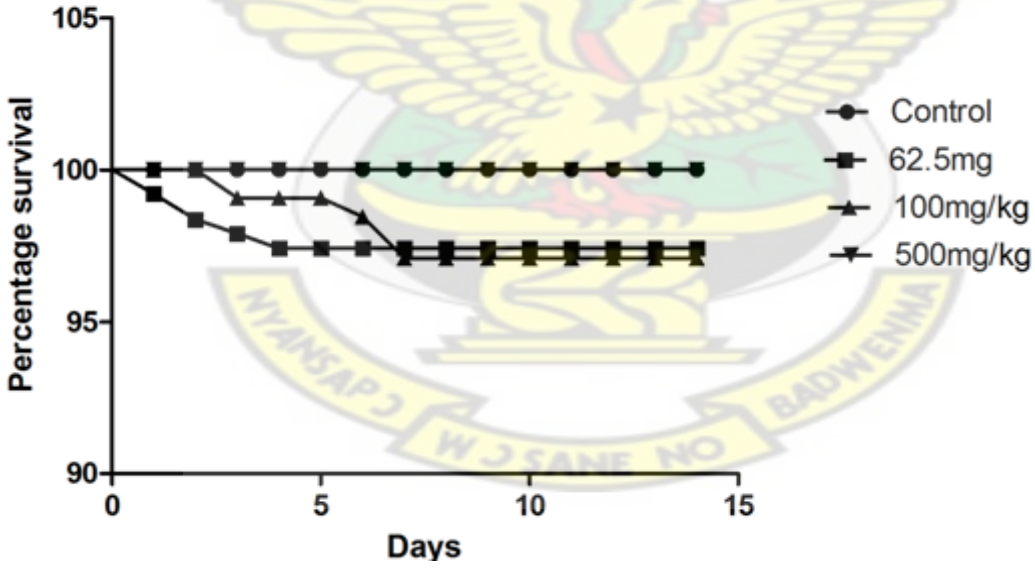


Fig 4.1. Effects of prenatal cryptolepis treatment on postnatal survival of pups. Statistical analysis by log-rank (mantel-Cox) test show that the survival curves are significantly different p value = 0.0108 and a chi square value of 11.18 at Df = 3.

4.3.2 Effects of prenatal cryptolepis treatment on post natal growth

Pregnant mice were either treated with distilled water or cryptolepis (62.5, 100, 500 mg/kg) from gestation day 6 till delivery. Eighteen pups were selected from each dose level and the initial weight recorded. The weight was then monitored daily from PND 1 till PND 15.

In terms of daily weight gain, prenatal treatment with cryptolepis had a biphasic effect on post natal growth. Low dose of 62.5 mg/kg cryptolepis significantly stimulated postnatal growth. Doses of cryptolepis, 100 and 500 mg/kg, inhibited postnatal growth significantly with the effects more pronounced at 100 mg/kg.

At the start of the study pups from cryptolepis treated groups 100 and 500 mg/kg weighed significantly less than control animals. However pups from the 62.5 mg/kg cryptolepis group were of comparable weight to the control. On day 15 of the study, pups from 62. 5 mg/kg of cryptolepis group were significantly bigger than controls ($p < 0.001$) and this effect persisted as at PND 50 (Table 5.4). Pups from the 500 mg/kg group were comparable to controls and pups from the 100 mg/kg weighed less than controls.

To understand the mechanisms further, the rate of growth of each animal was computed. Prenatal cryptolepis treatment stimulated the rate of growth at doses tested (Fig 4.2). This explains why mice comparable in weight to controls at the start of the study (62.5 mg/kg) became heavier than controls whilst mice that weighed less than controls became comparable to controls (Fig 4.3).

These results suggest that prenatal cryptolepis treatment stimulates postnatal growth.

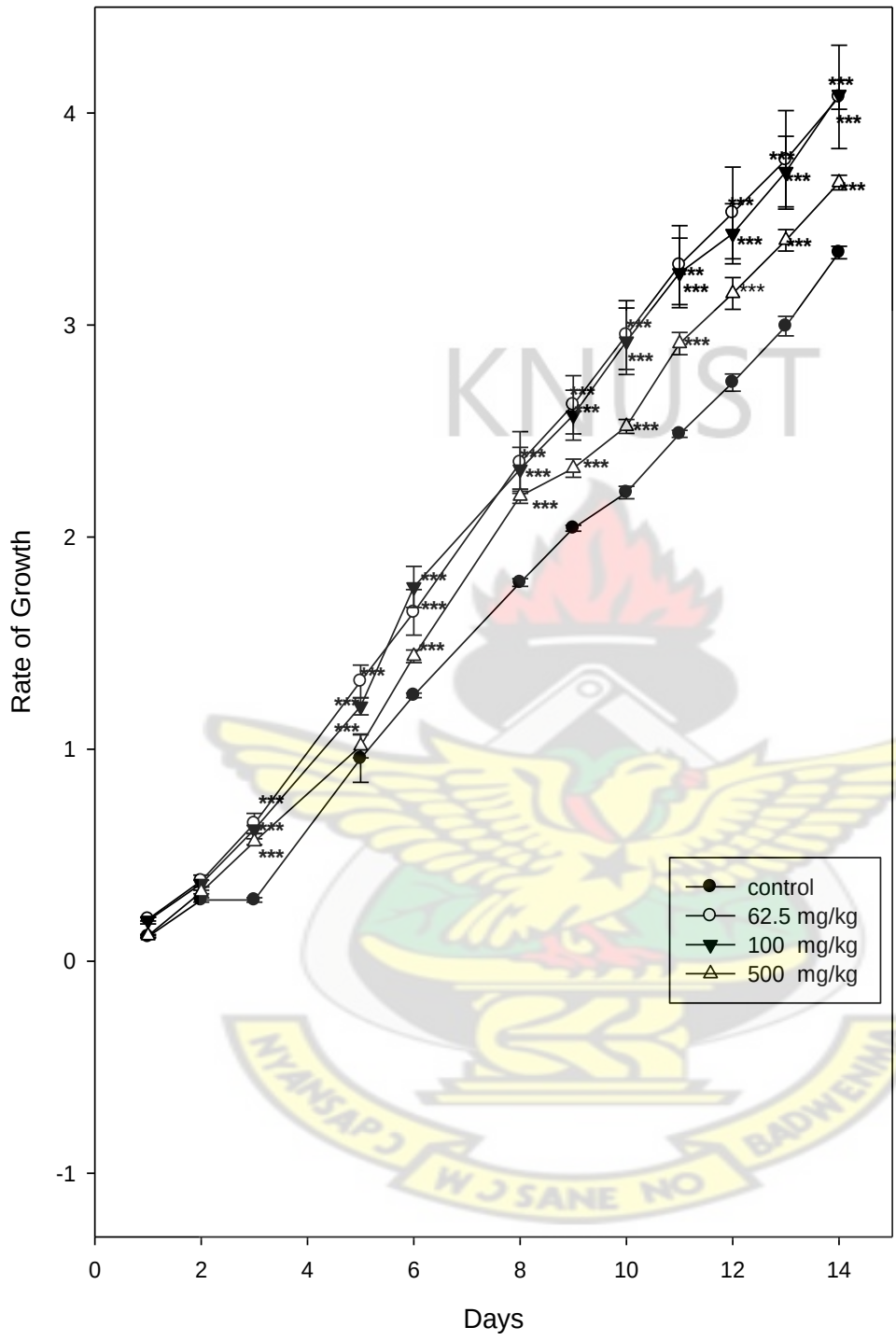


Fig 4.2 Effects of prenatal cryptolepis treatment on postnatal growth. Statistical analysis was by two way ANOVA using Bonferronis post hoc test. *p < 0.05, ** p < 0.01, ***p < 0.001.

4.3.3 Effects of cryptolepis on the time of attainment of physical landmarks

Pups prenatally treated with distilled water and cryptolepis (62.5, 100, 500 mg/kg) were followed daily to see the time of attainment of key physical and developmental landmarks. The study showed that prenatal exposure to cryptolepis could affect the attainment of certain important landmarks. Treatment with cryptolepis caused inhibition in foetal birth weight and crown rump distance. Whilst low dose prenatal treatment with cryptolepis appeared to enhance the appearance of hair, higher doses inhibited it. High doses of cryptolepis appears to promote pinna unfolding, significant ($p < 0.05$) at 500 mg/kg of treatment (Table 4.1). Eruption of incisors and testicular descent were not affected by prenatal cryptolepis treatment. Eye opening was delayed by treatment significantly at 100 and 500 mg/kg.

These results suggest that prenatal cryptolepis treatment delays the time of development of physical landmark such as eye opening, pilation and pinna unfolding.

Table 4.1 Effects of cryptolepis treatment on the time of attainment of physical landmarks

Dose	Control	62.5mg/kg	100mg/kg	500mg/kg
Birth weight	1.472±0.060	1.462±0.157	1.228±0.111***	1.350±0.133*
Crown rump distance(cm)	3.439±0.038	3.267±0.095	2.980±0.051**	2.894±0.085***
Pilation	3.89±0.076	3.778±0.101	4.06±0.056	3.94±0.5556
Pinna unfolding	3.94±0.055	4.000±001	3.78±0.101	3.61±0.1182*
Incisor eruption	10.39±0.118	10.33±0.1143	10.56±0.121	10.61±0.1182
Eye opening	14.11±0.076	14.06 ±0.098	14.89±0.159***	14.61±0.1182**
Testicular decent	23.00±0.365	22.50±0.224	23.67±0.421	23.17±0.3073

The unit of measurement is per litter. Statistical analysis is one way ANOVA using Newman-Keuls post hoc test to compare means to control. * $p < 0.05$,**$p<0.001$, ***$p<0.0001$

4.3.4 Effects of cryptolepis treatment on sensori-motor development

Eighteen pups prenatally treated with cryptolepis (62.5, 100, 500 mg/kg) or distilled water, were monitored for the time of development and the integrity of sensori-motor systems. Pups were tested for the development of rooting behaviour, vibrissae placing response and cliff avoidance on the first 3 days of postnatal life. Prenatal treatment did not appear to affect these behaviours during the first three days of testing (Tables 4.2 - 4.4). Rooting behaviour was however, significantly different from control on the day 2 of the study at 500 mg/kg of cryptolepis treatment.

Selected pups were followed daily from postnatal days 3, 4, 5 and 6 for the development of righting reflex (Table 4.5). Prenatal treatment caused a delay in the development of righting reflex. However, all pups used in the assay met the criteria on day 6. When pups were examined for negative geotaxis on day 6, all animals met the criteria (Table 4.6).

Pup were tested at postnatal day 11, 12, 13 pups were tested for their ability to suspend on 2 mm bar, midair righting, development of the middle ear (auditory startle), and pinna reflex. Prenatal treatment appeared to delay midair righting, pinna reflex and auditory startle but not reflex suspension (Tables 4.6 - 4.10). Delay in mid air righting was significant for all doses of cryptolepis tested.

Prenatal cryptolepis treatment delays the development of, righting reflex midair righting, pinna reflex and auditory startle.

Table 4.2 Effects of cryptolepis treatment on rooting behaviour

Day	CONTROL	62.5mg/g	χ^2	100mg/kg	χ^2	500 mg/kg	χ^2
1	7	8	0.06	5	0.53	6	0.06
2	14	11	2.00	12	0.72	10	3.94*
3	15	18	2.50	16	0.10	17	0.10

Each group represents the number of pups eliciting a fully developed rooting reflex by the 1st, the 2nd and the 3rd postnatal days. Differences between the control and prenatal cryptolepis treated groups were tested by the Chi square test. * means significant at $P < 0.05$

Table 4.3 Effects of prenatal cryptolepis treatment on vibrissae placing response

Day	control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
1	5	9	3.39	8	1.73	6	0.07
2	13	15	0.62	11	0.62	13	0.07
3	15	17	0.90	13	0.90	15	0.10

Each group represents the number of pups eliciting fully developed vibrissae by the 1st, the 2nd and the 3rd postnatal days. Differences between the control group and prenatal cryptolepis treated groups were tested by the Chi square test.

Table 4.4 Effects of prenatal cryptolepis treatment on cliff avoidance or cliff aversion

Day	control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
1	2	2	0.14	5	3.52	4	1.27
2	15	12	2.50	13	0.90	15	0.09
3	17	17	0.26	16	0.26	18	0.26

Each group represents the number of pups avoiding the cliff by the 1st, the 2nd and the 3rd postnatal days. Differences between the control group and each of the cryptolepis treated groups were tested by the Chi square test

Table 4.5 Effects of prenatal cryptolepis treatment on righting reflex

Day	control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
3	1	2	0.26	0	0.26	1	0.26
4	5	6	0.07	1	3.39	2	1.73
5	10	11	0.06	4	6.806**	7	1.41
6	18	18	-	18	-	18	-

Each group represents the number of pups exhibiting a fully developed righting reflex in a time of 10 s on 3rd, 4th, 5th and 6th postnatal days. Differences between the control group and each cryptolepis group were tested by the Chi square test. ** means significant at $p < 0.01$

Table 4.6 Effects of prenatal cryptolepis treatment on negative geotaxis

Dose	Day 6	χ^2
Control	12	0.625
62.5mg/kg	15	1.5625
100mg/kg	14	0.5625
500mg/kg	14	0.5625

Each group represents the number of pups exhibiting negative geotaxis in a time of 180s on 6th postnatal days. Differences between the control group and each cryptolepis group were tested by the Chi square test.

Table 4.7 Effects of prenatal cryptolepis treatment on reflex suspension

Day	Control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
11	6	9	1.56	5	0.06	4	0.56
12	15	17	0.90	15	0.10	16	0.10
13	17	17	0.26	18	0.26	18	0.26

Each group represents the number of pups' with reflex suspension in 11th, 12th, 13th on postnatal days. Differences between the control group and each cryptolepis group were tested with Chi square.

Table 4.8 Effects of prenatal cryptolepis treatment on mid air righting

Day	Control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
11	9	10	0.06	7	0.50	4	4.50*
12	17	14	6.62*	13	12.97***	13	12.97***
13	16	15	0.141	14	0.26	15	0.14

Each group represents the number of pups eliciting mid air righting reflex on 11th, 12th, 13th postnatal days. Differences between the control group and cryptolepis groups were tested by the Chi square test. * means significant at $p < 0.05$ and *** means significant at $p < 0.001$.

Table 4.9 Effects of prenatal cryptolepis treatment on auditory startle

Day	Control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
11	0	0	-	0	-	0	-
12	14	14	0.08	9	6.55*	11	2.01
13	17	18	0.53	14	6.61*	18	0.53

Each group represents the number of pups with auditory startle on 11th, 12th, 13th postnatal days. Differences between the control group and each cryptolepis groups were tested by the Chi square test. * means significant at $p < 0.05$.

Table 4.10 Effects of prenatal cryptolepis treatment on pinna reflex

Day	Control	62.5mg/kg	χ^2	100mg/kg	χ^2	500mg/kg	χ^2
11	10	12	0.51	6	2.76	13	1.41
12	16	18	1.27	9	23.74***	18	1.27
13	18	18	-	18	-	18	-

Each group represents the number of pups eliciting a fully developed pinna reflex by the 11th, 12th and 13th postnatal days. Differences between the control and prenatal cryptolepis treated groups were tested by the chi square test. *** means significant at $p < 0.001$

4.3.5 Effects of prenatal cryptolepis treatment on spontaneous locomotor activity.

Thirty-day old mice treated prenatally with distilled water or cryptolepis (62.5, 100, 500 mg/kg) were selected for the study. Spontaneous locomotor activity was measured for every five up to thirty minutes after allowing a ten minute period of habituation. Animals treated prenatally with cryptolepis had higher spontaneous locomotor actitvity than control animals. Activity was highest in animals in the 100 mg/kg compared to control.

Prenatal treatment of mice with cryptolepis increases spontaneous locomotor activity during adolescence.

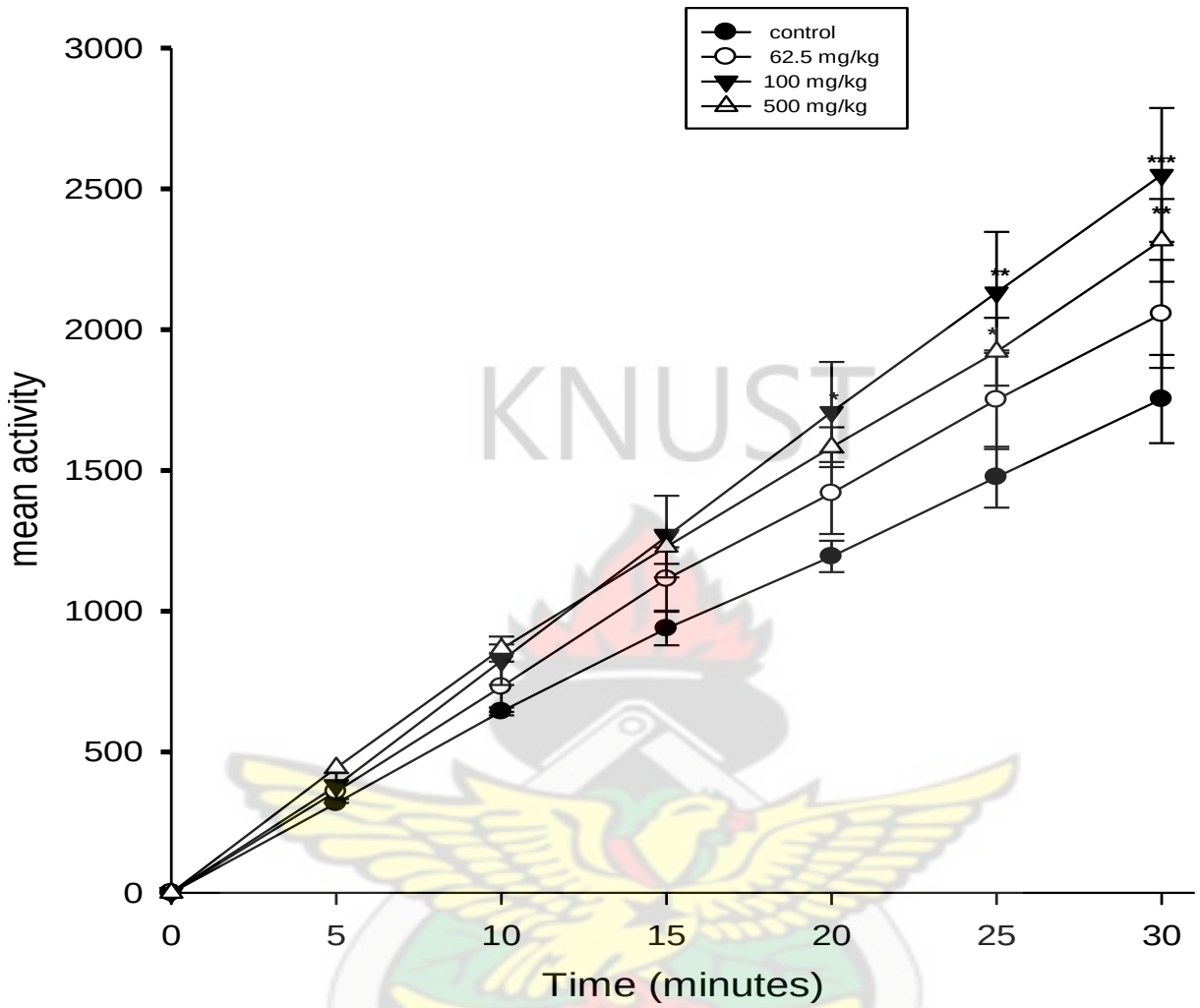


Fig 4.3 Effects of prenatal cryptolepis treatment on spontaneous locomotor activity on PND 30. Statistical analysis is by two way ANOVA using Newman-keuls post hoc to compare means. * $p < 0.05$,**$p<0.01$, ***$p<0.001$

4.3.6 Effects of prenatal cryptolepis treatment on motor coordination

Forty day old mice were assessed for their latency to fall of a rotarod. Five males and five females were selected from each dose level (control or 62.5,100,500 mg/kg *p.o* cryptolepis) and assessed. Within a group, female mice had longer latencies to stay on the rod compared to males. Females prenatally treated with cryptolepis had higher latencies to stay on the rotarod than controls at all doses of treatment though the results were not statistically significant.

However, in males a reduction in latencies of falling from the rotarod was observed. This was significant at all doses of treatment. Male animals prenatally exposed to cryptolepis had altered motor coordination.

Table 4. 11 Effects of prenatal cryptolepis treatment on motor coordination

DOSE	TIME/s	
	FEMALES	MALES
Control	132.2 ± 45.87	43.33 ± 9.208
62.5 mg/kg	152.1 ± 52.57	5.00 ± 1.722 ***
100 mg/kg	225.5 ± 39.10	8.58 ± 2.850 ***
500 mg/kg	118.0 ± 40.44	16.40 ± 3.374 **

Effects of cryptolepis treatment on motor coordination. Statistical analysis is by one way ANOVA using Newman-keuls post hoc test to compare means to control (n=5). ** $p < 0.05$,***$p<0.01$

4.3.7 Effects of cryptolepis on grip strength on postnatal day 80

Mice of approximately 80 days of age were assessed for their strength when holding on to a metal grid. Animals treated prenatally exhibited higher grip strength ($p < 0.05$) relative to controls.

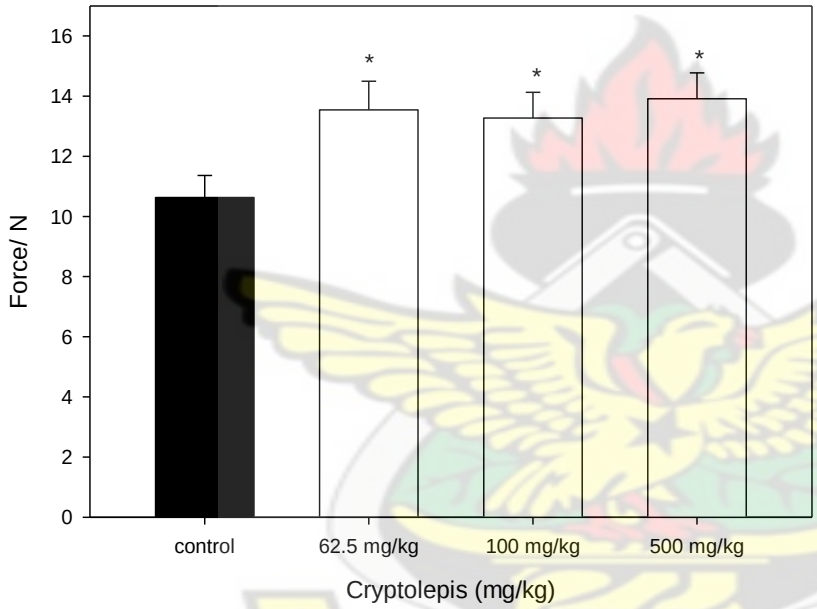


Fig 4.4 Effects of cryptolepis on grip strength on PND 80. Results presented as mean ±SEM. Statistical analysis is by one way ANOVA using Newman-keuls post hoc to compare means. * $p < 0.05$.

4.4. DISCUSSION

In the previous chapter, prenatal treatment with cryptolepis resulted in intrauterine growth inhibition. Prenatally treated pups had reduced weight as well as length depicted by the crown and rump distance. In chapter 3, it was evident that prenatal cryptolepis treatment inhibited growth *in utero*.

Contrary to intrauterine growth, prenatal treatment did not inhibit postnatal growth at all doses of cryptolepis tested but rather appeared to stimulate. This high post natal growth rates appeared to compensate for the intrauterine growth inhibitions. Interestingly, animals in the low dose group became significantly heavier than the controls from postnatal day 6 to postnatal day 14. On postnatal day 50 the difference still existed.

Pups prenatally exposed to cryptolepis had higher postnatal mortalities than controls. It is possible that the high growth rate seen in cryptolepis treated groups were compensatory mechanisms as a result of prolonged intrauterine growth inhibition caused by cryptolepis. Additionally, mortalities in these groups reduced the number of pups per dam hence a reduced intra-litter competition. Similar observation were made in mice exposed to prenatal stress by Morley-Fletcher *et al.*, (2003), where prenatally stressed mice were consistently bigger than controls. This was attributed to metabolic alterations in such animals.

Prenatal treatment with cryptolepis caused a delay in the attainment of certain key physical and developmental landmarks. It is not surprising as animals that are intrauterine inhibited tend to have a delay in appearance of some key physical landmarks (Caston *et al.*, 2004). Furthermore, in developmental studies, a delay in eye opening is a clear indication of an inhibition in the

formation and development of the neural tube (Caston *et al.*, 2004) suggesting alteration in neurogenesis.

Prenatal treatment with cryptolepis also affected motor development. The rooting reflex and the vibrissae placing response, which are grasping behaviours, are related to eating behaviour. Suckling pups have to display rooting and grasping reactions in order to successfully reach for the dam's teats. Treatment with cryptolepis had minimal effect on these behaviours suggesting that poor postnatal nutrition could not account for the differences in growth and appearance of developmental landmarks.

Prenatal cryptolepis treatment delayed the development of righting reflex, auditory startle, midair righting, pinna reflex. Pinna reflex is effected by the 7th cranial nerve. Auditory startle depicts the integrity of the middle ear. In mice, vestibular structures undergo differentiation on gestation day 14 (Anniko, 1983). Prenatal cryptolepis treatment caused a delayed in righting and mid air righting reflexes; two motor assays of vestibular and proprioceptive origin (Roberts, 1967). The 8th cranial nerve or the vestibulochochlea nerve inputs sensory signals of proprioception from the vestibule and the semicircular canals to medulla and the cerebellum of the brain for interpretation. A delay in this process shows alteration in neurogenesis. This study shows that prenatal cryptolepis treatment can affect the development of nervous structures.

In addition to the above, mice prenatally treated with cryptolepis performed poorly in the rotarod experiment of motor coordination than controls. This effect however appears to be sex specific as females performed better than the controls. Studies on grip strength showed that prenatally treated mice exerted much higher grip strength than controls. This suggests that the poor motor

coordination was not due to weakened muscles but probably alterations in cerebellum and associated structures for motor coordination. In addition profound hyperactivity characterized mice treated with cryptolepis at all doses.

Cryptolepis can elicit these effects in several ways. One possible pathway is the interaction with the Wnt signaling pathway which is critical during foetal development. During primary neurulation the ectoderm divides into three components; neural tube, epidermis and neural crest. The neural tube has four distinct areas each developing into portions of the CNS. For example prosencephalon develops into the cerebrum, optic nerves and hypothalamus whilst rhomencephalon develops into the pons, cerebellum, and medulla oblongata. Cryptolepis appears to delay the development of the neural tube indicated by a delay in eye opening (Caston *et al.*, 2004). The dorsal part of the neural tube which develops into the spinal cord controls the development of sensation whilst the ventral controls motor coordination (Jessell, 2000). Wnt proteins are signalling molecules playing very active roles in the development of sensation and sensory systems in the spinal cord of the foetus. Wnt also exerts inhibitory effects on molecules such as the sonic hedgehog (Shh) on the ventral side of the spinal cord controlling motor development and motor coordination (Ulloa and Marti, 2010). In addition to its action in the spinal cord, Wnt canonical signaling induces neural dorsal identities in other regions of developing central nervous system such as the forebrain and the eye (Backman *et al.*, 2005; Solberg *et al.*, 2008; Veien *et al.*, 2008). Wnt activity depends on prostaglandin and substances that interfere with prostaglandins will affect the pathway and alter the development of sensory systems (Goessling *et al.*, 2009). Cryptolepis and cryptolepine antagonises the activity of COX 2 (Olajide *et al.*, 2007a; Olajide *et al.*, 2009; Olajide *et al.*, 2010) and cryptolepine inhibits the

activity of prostaglandin E_2 (Bamgbose and Noamesi, 1981). This suggests that inhibition of prostaglandin synthesis and subsequently Wnt could account for the altered sensori-motor development.

Furthermore, studies with cryptolepine show that cryptolepine accumulates in foetal tissues particularly melanin containing tissues of the eye (Noamesi *et al*., 1991). It is therefore possible that the delay in eye opening in the animals prenatally treated with cryptolepis is also related to the affinity of cryptolepine for the tissues of the eye.

In conclusion prenatal cryptolepis treatment affects foetal growth, sensori-motor development and delays the attainment of physical and developmental landmarks.

CHAPTER FIVE

EFFECTS OF PRENATAL CRYPTOLEPIS TREATMENT ON SOCIAL INTERACTION, MEMORY ACQUISITION AND ANXIETY IN ADOLESCENT AND ADULT FISRT GENERATION MICE.

CHAPTER FIVE

5.1 INTRODUCTION

There is considerable data suggesting that certain classes of chemicals are able to change the permeability characteristics of the blood brain barrier (BBB) in rodents when administered during susceptible periods of the CNS development, and their effects may persist after cessation of exposure (Gupta *et al.*, 1999; Hougaarda *et al.*, 2005; Green *et al.*, 2011). A changed BBB may render the nervous system more vulnerable to other toxicants that would otherwise not be able to cross the blood brain barrier. The enhanced susceptibility of the developing brain seems to depend to a greater extent on disruption of sensitive processes that occur only during development when the CNS undergoes defined periods of maturation (Tilson, 1998). An assumption in neurotoxicology is that damaged neurons have a limited capacity for regeneration. Accordingly, exposure to neurotoxic substances during such critical developmental period can have long lasting consequences and alter CNS function in a manner that does not compromise the growth and viability of the foetus but causes severe neural and behavioural changes. Although the developing nervous system has some capacity to adapt to or compensate for early perturbations, there is clear evidence that such developing nervous system is more vulnerable to many chemical agents than the adult nervous system (Tilson, 1998). In fact, an individual's vulnerability to some biological abnormalities as seen in depression, schizophrenia and anxiety can be related to prenatal perturbation (Huizink *et al.*, 2004; Hougaarda *et al.*, 2005; Green *et al.*, 2011).

It has also been shown in rodents that prenatal neural perturbations of the offspring causes long term effects which can be demonstrated in the adult offspring, such as hyperactivity (Weller *et*

al., 1988), increased emotionality (Poltyrev *et al.*, 1996), cognitive deficits (Lemaire *et al.*, 2000) and neurological and behavioural abnormalities (Stott, 1973; Chapillon *et al.*, 2002). In the previous chapter, it was evident that prenatal treatment with cryptolepis resulted in a delay in the development and appearance of physical landmarks and sensori-motor reflexes in pups; an indication of alteration in neural development. However, very little is known on the extent of this early neural alterations as well as their impact on the adult life.

In the functional toxicity studies, the main route of cryptolepis exposure to the developing foetus was transplacental. One of the biggest problems in reproductive and developmental studies is ascertaining whether an effect is a direct effect of the chemical on the foetus or the effect is indirect as a result of changes in the maternal system. Administration of a drug to a foetus *in vivo* can affect the mother before affecting the foetus hence maternal effects affects pup development. Furthermore, previous studies on functional toxicity (chapter 4) showed that cryptolepis treated animals exhibited functional deficits quite similar to animals born to prenatally stressed animals i.e. hyperactivity (Weller *et al.*, 1988), delay in sensori-motor system (Caston *et al.*, 2004), growth inhibitions and mortalities (Caston *et al.*, 1997). It is therefore possible that some of the observed effects may not be directly due to cryptolepis on the foetus but are mediated by changes in the mother being transmitted to the foetus. One way to confirm this is to study alterations in maternal behaviour.

This chapter evaluates the effects of prenatal cryptolepis treatment on activity, anxiety, social interaction, emotionality, neurological and behavioural abnormalities, and cognitive function in the adolescent and adult mice and estimates the role that maternal exposure may have on functional toxicities due to cryptolepis.

5.2 MATERIALS AND METHODS

5.2.1 Prenatal treatment of mice with cryptolepis

Forty female mice were cohabited with 20 male mice and observed for signs of mating by either directly observing copulation or formation of vaginal plugs. Successfully mated females (n=7) were tagged and assigned to one of four dose levels. The day was recorded as gestation day 0. Female mice received either cryptolepis (62.5, 100, 500 mg/kg *p.o)* or distilled water from gestation day 6 to the end of gestation.

5.2.2 First generation (f_1) same sex pair interaction on PND 45

First generation mice treated prenatally with cryptolepis were kept until post natal day (PND) 45. The mice in each group had been separated into male and female cages on PND 21. On post natal day (PND) 44, mice in the same cage and of the same sex and of comparable body weight (Terranova *et al.*, 1993) were isolated from each other over a 24 hour period. On post natal day 45, each pair underwent a 20 min social encounter. The encounter took place at 7.00 am in a perpex cage supplied with clean sawdust bedding. Behaviour was videotaped under dim light. The behavioural responses were scored with the aid of Jwatcher V 1.0 and then classified in two main groups as described below (non social and social) based principally upon the ethological profile of mouse behaviour described by Grant and Mackintosh, (1963),Van Oortmerssen, (1971) Terranova *et al.*, (1993).

5.2.1.1 Social behaviours

Investigative behaviour

Sniffing— sniffing the ano-genital, body or head region of the partner.

Follow— following the partner around the cage, without any quick or sudden movement.

Squire — following the moving partner while maintaining a constant nose contact with its fur (mostly near the ano-genital area).

Mutual circle — Partners mutually sniffing each other's ano-genital region, while describing tight circles with their reciprocal following movements and maintaining close nose ano-genital contact.

Affiliative behaviours

Social rest— laying flat or standing still (with the eyes closed or opened) while maintaining close physical contact with the partner, which may be, in turn, either inactive or involved in mate-directed activity (i.e., grooming or sniffing the partner).

Allogrooming— grooming partner's body.

Soliciting behaviours. — pushing the snout or the whole anterior part of the body under the partner's body, and then resting.

Crawl over— crawling over the partner's back, crossing it transversally from one side to the other.

5.2.1.2 Non social behaviours

Exploring— moving around the cage, rearing, sniffing the air, the walls, or the sawdust.

Inactive— laying flat or standing still, with the eyes closed or opened in the total absence of movements.

Digging— digging in the sawdust, pushing and kicking it around using the snout and/or both the fore paws and hind paws.

Self-grooming—Wiping, licking, combing or scratching any part of own body.

5.2.3 Behavioural and functional analysis of mouse phenotype on PND 50

The popular behavioural and functional analysis of mouse phenotype model (SHIRPA Protocol) developed by Rogers *et al.*, (1997) and used by pharmaceutical laboratories for the primary screening of drug candidates is based on one originally modelled by Irwin, (1968). This standard method provides a behavioural and functional profile by observational assessment of mice. This test indicates defects in gait or posture, motor control and co-ordination, changes in excitability and aggression, salivation, lacrimation, piloerection, defecation, muscle tone and temperature. All parameters are scored to provide a quantitative assessment which enables comparison of results.

The equipments required for the test are;

- Clear perspex arena (approximate internal dimensions 55 x 33 x18 cm). In the floor of the arena is a perspex sheet marked with 15 squares (11cm). A rigid horizontal wire (3 mm diameter) is secured across the rear right corner for wire manoeuvre. A grid (40 x 20 cm) with 12 mm mesh secured across the width of the box for measuring tail suspension.
- Two clear perspex cylinders (15 x 11 cm) used as viewing jars.
- One grid floor (40 x 20 cm) with 12 mm mesh on which viewing jars stand.
- Four cylindrical perspex supports (3 cm high x 2.5 cm diameter) to raise grids off bench.
- One square (13 cm) stainless steel plate for transfer of animals to the arena.
- A dowel rod with a fine stainless steel wire (15mm length and 0.15mm diameter) attached to end for measurement of pinna and corneal reflexes.
- A plastic dowel rod sharpened to a pencil point to test salivation and biting.

- A pair of dissecting equipment forceps, curved with fine points (125 mm forceps) for the toe pinch.
- A stopwatch.
- Click box is used for testing the preyer and startle responses.
- Scales to measure body weight.
- A ruler.
- A 30 cm clear perspex tube for the contact righting reflex.

Mice treated prenatally with distilled water or cryptolepis (62.5, 100, 500 mg/kg) (n=7) were selected for the study. The test began by observing and scoring undisturbed behaviour in a viewing jar, as well as looking for manifestation of bizarre or stereotyped behaviours, convulsions, compulsive licking, self-destructive biting, retropulsion and indications of spatial disorientation.

Thereafter, the mouse was transferred to an arena for testing of transfer arousal and observation of normal behaviour. This was followed by a sequence of manipulations using tail suspension and the grid across the width of the arena. To complete the assessment, the animal was restrained in a supine position to record autonomic behaviours prior to measurement of the righting reflex. Throughout this procedure vocalization, urination and general fear, irritability or aggression was recorded (Rogers *et al.*, 1997).

5.2.4. Effect of cryptolepis treatment on anxiety

5.2.4.1. Elevated plus Maze on PND 95

The elevated plus maze was a modification of a previous design (Pellow, 1985). It consisted of two open arms (30 cm x 5 cm x 1 cm) and two closed arms (30 cm x 5 cm x1cm) that extended from a central platform (5 cm x 5 cm). Like arms opposed to each other across the central platform. The maze was constructed from opaque plexiglas and was elevated on a woody stand 60 cm above the floor. The test was performed under dim red light in a sound attenuated room and the behaviour was recorded on a videotape with a digital camera placed 100 cm above the maze. First generation mice were treated prenatally with either cryptolepis or distilled water until delivery. The mice were raised until postnatal day 95. Mice were individually tested and only once for 5 min. The maze was cleaned following each trial to remove any residue or odours. Behavioural parameters were scored from videotapes with the aid of a computer program, J watcher V 1.0 for the following:

- number of closed and open arm entries. An arm entry was counted only when all four limbs of the mouse were within a given arm.
- time spent in exploring the open and closed arm of the maze
- number of stretch attend postures- the mouse stretches forward and retracts to original position;
- number of rearing.

5.2.4.2. Light Dark Box on PND 100

The apparatus is based on the initial model described by Crawley and Godwin, (1980) and modified by other workers (Belzung and Le Pape, 1994). It consists of a wooden box (45 x 30 x 30 cm), which is divided into two compartments by a wooden board 7 x 7 cm opening located centrally at the floor level, connecting the compartments. One compartment is painted black and covered with a wooden lid. The uncovered part is painted white and lit by a 60-W light bulb held 30 cm above the box.

In the experiment, mice prenatally treated with distilled water or cryptolepis (62.5, 100, 500 mg/kg) were raised until postnatal day 100. At the beginning of the experiment, mice were placed at the centre of the covered dark compartment and time spent in the area as well as the latency for the animal to move to the illuminated area was recorded for five minutes by means of a video tape.

5.2.5 Effects of cryptolepis treatment on learning and memory acquisition

5.2.5.1. Effects of cryptolepis on recognition memory- Novel Object Identification Task on PND 55

The object recognition test was performed as described by Ennaceur and Delacour (1988) with the exception of the arena which was modified by Blokland *et al.*, (2003). The apparatus consisted of a square arena bottom (40 x 40 cm) and 20 cm high wall with half made of transparent perpex glass (front) and the other half made of opaque perpex glass (back). The light intensity was equal in the different parts of the apparatus. Two objects were placed in symmetrical position about 5 cm away from the wall. Each object was available in triplicate.

Two objects were presented in the first trial and a third one in the recognition trial, to prevent the use of odour cues. In addition, the objects were always thoroughly cleaned between sessions. Four different sets of objects were used each present in triplicates. The different objects were;

- a cone made of wood (diameter 6 cm and total height 3.8 cm),
- a plastic bottle (diameter 2.7 cm, height 8.5 cm) filled with stones,
- a massive metal cube (2.5 cm × 5 cm × 7.5 cm) with two holes (diameter 1.5 cm), and
- a massive ceramic with a tapering top (4.5 cm × 4.5 cm × 8.5 cm).

A mouse could not displace the objects. The order of objects used per subject per session was determined randomly. All combinations and locations of objects were used in a balanced manner to reduce potential biases due to preferences for particular locations or objects. Three days prior to the experiment, the animals were allowed to explore the apparatus (without any objects) twice for 5 min each day. The duration of each trial was 5 min.

Six male animals from groups prenatally exposed to distilled water or cryptolepis (62.5, 100, 500 mg/kg) were selected for the experiment. During the first trial (T_1), the apparatus contained two identical objects (samples). A mouse was placed in the apparatus facing the wall at the middle of the front (transparent) segment to avoid bias. After the first exploration period, the mouse was put back in its cage (habituation). Subsequently, after a one hour delay interval, the mouse was put back in the apparatus for the second trial (T_2), but now with two dissimilar objects; a familiar one (the sample) and a new one (novel object). The time spent in exploring each object during T_1 and T_2 were recorded with a video and scored with jwatcher V1.0. Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting on the object was not considered as exploratory behaviour.

5.2.5.2. Effects of cryptolepis on spatial and reference memory - Morris Water Maze on PND 60

The Morris water maze (Morris, 1984) used was a swimming pool (diameter: 55 cm) that was surrounded by several, clearly visible cues. This is standard procedure used to assess various aspects of learning and memory. Briefly the pool of water is coloured opaque with non-toxic tempera paint, where animals swim to a hidden escape platform. Because the water is opaque, the animals cannot see the platform, and cannot rely on scent to find the escape route. Instead, they rely on external/extra-maze visible cue. As the animals become more familiar with the task, they are able to find the platform more quickly.

In the experiment, the water maze was filled up with tap water with temperature approximately 26°C. Periodically the water temperature was checked so that it was within one degree of 26°C. A video camera with the ability to have all sides of the maze in its field view was mounted above the pool. An escape platform was placed in the northeast quadrant of the maze, 1cm below the surface and not visible to animals during testing. However during training, it was exposed, one cm above the water to teach mice that is the only way to get out of the water. Later, after the animals were trained and ready for testing, the escape platform was then adjusted below the surface of the water.

Water maze testing

On day 5 of training, five male mice from groups treated prenatally with either distilled water or cryptolepis (62.5, 100, 500 mg/kg *p.o.*) were selected. The lighting and water temperature was the same as in the training process and the platform was kept in the northeast quadrant of the maze. Each animal underwent 12 trials; 3 trials for each starting direction. The start directions

were northwest, southwest and southeast. As a precaution no start direction was used twice in a row, and also the same order was not repeated for any of the directions.

Mice were placed in pool facing the wall of the maze, the experimenter seated at a designated place without disturbing the animal. The time to reach the platform was recorded. If the animal does not reach the platform in 60 seconds, it is guided it to the platform, as in training. Mice are then allowed to sit for 10 seconds, dry off and then returned to a holding cage. This was repeated for each animal for all 12 trials, with the experimenter returning to the same designated spot during each trial.

The probe test (without platform test) in morris

The probe trial is performed to verify that the animal understands the platform location, and observe the strategy that the animal follows when it discovers the platform is not there. The mouse was released from the northwest quadrant. The number of times the animal crosses the centre of the pool during the 30 seconds, total distance travelled, distance travelled in quadrants and circles and time spent in quadrants and circles were noted.

5.2.5. Effects of cryptolepis treatment on maternal behaviour on post PND 8

To understand the role of changes in maternal behaviour on functional toxicity in pups as against the direct effects of cryptolepis on functional toxicity, a maternal behaviour study was performed on post natal day 8. The method used was as described in Caston *et al.*, (2002).

Briefly, 20 pregnant mice grouped into four (n=5) were treated with either distilled water or cryptolepis (62.5, 100, 500 mg/kg) from gestation day 6 until delivery. Three days to delivery, animals were isolated into standard cages (30 x15 x 8) cm containing approximately 3 cm of coarse saw dust. The day of birth (day 0) was determined by examining the cages twice a day; morning and evening. Maternal behaviour experiment was carried out on post natal day 8 between 7 am and 12 noon. The experiment began by removing the pups from the nest and putting them on the opposite corner of the cage without changing anything else in the nest. Then for 20 minutes, maternal behaviour was recorded on a video tape. To score maternal behaviour, a behavioural grid modified by Chevalet and Le Pape, (1989) was used. Twelve items were pulled into three different units as below;

- Dam's activity towards the pups (retrieving, sniffing, licking and suckling),
- Dam's activity not directed towards the pups (nest building, digging the nest, moving on the saw dust),
- Dam's activity directed towards itself (eating, self grooming, moving in the cage and resting).

The latency for retrieval of pups and the percentage of pups retrieved were also noted.

5.3 RESULTS

5.3.1 Same sex pair interaction on PND 45

First generations mice treated prenatally with cryptolepis or distilled water were kept until Post Natal day (PND) 45. The mice in each group had been separated into male and female cages on PND 21. On PND 44, mice in the same cage and of the same sex and of comparable body weight (Terranova *et al.*, 1993) were isolated from each other over a 24 hour period. On PND day 45, each pair underwent a 20 min social encounter.

Prenatal exposure of mice to cryptolepis resulted in a general decrease in the frequency and duration of social interaction in treated offspring compared to controls. Prenatal treatment with cryptolepis dose dependently decreased frequency and duration of investigative behaviours such as sniffing, squire, mutual circle and follow (Fig 5.1). It also resulted in significant reduction in the frequency of affiliative behavioural parameters such as allogrooming and social inactivity (Fig 5.2). However the duration of affiliative behaviours were not affected by prenatal cryptolepis exposure (Fig 5.2).

Prenatal treatment of mice led to a dose dependent decrease in frequency and duration of soliciting behaviour (fig 5.3). This was significant (*** $p<0.001$) for frequency of soliciting and for duration (* $P < 0.05$) of soliciting at 500 mg/kg.

Prenatal cryptolepis treatment increased frequency and duration of non social behaviours such as digging, exploring, eating, self grooming dose dependently although the effects were not statistically significant for all assesed parameters except for duration of exploration and duration of digging (** $p< 0.01$) at 500 mg/kg.

Prenatal cryptolepis treatment led to a reduction in social behaviour whilst promoting non social behaviour in mice.

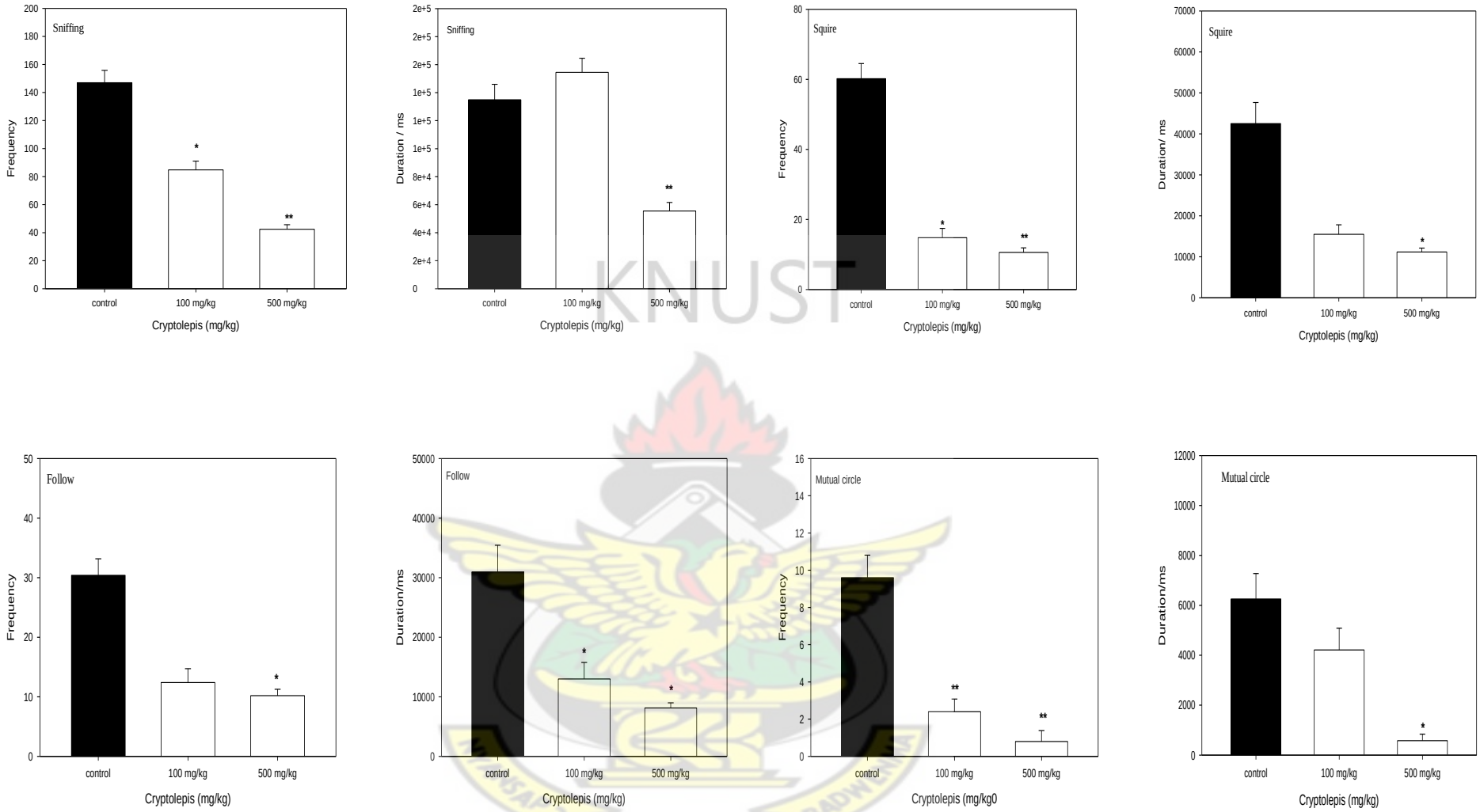


Fig 5.1 Effects of prenatal cryptolepis treatment on investigative behaviour of F_1 generation mice on PND 45. Statistical significance one way ANOVA * $p < 0.05$, ** $p < 0.01$, using Newman-Keuls post hoc test.

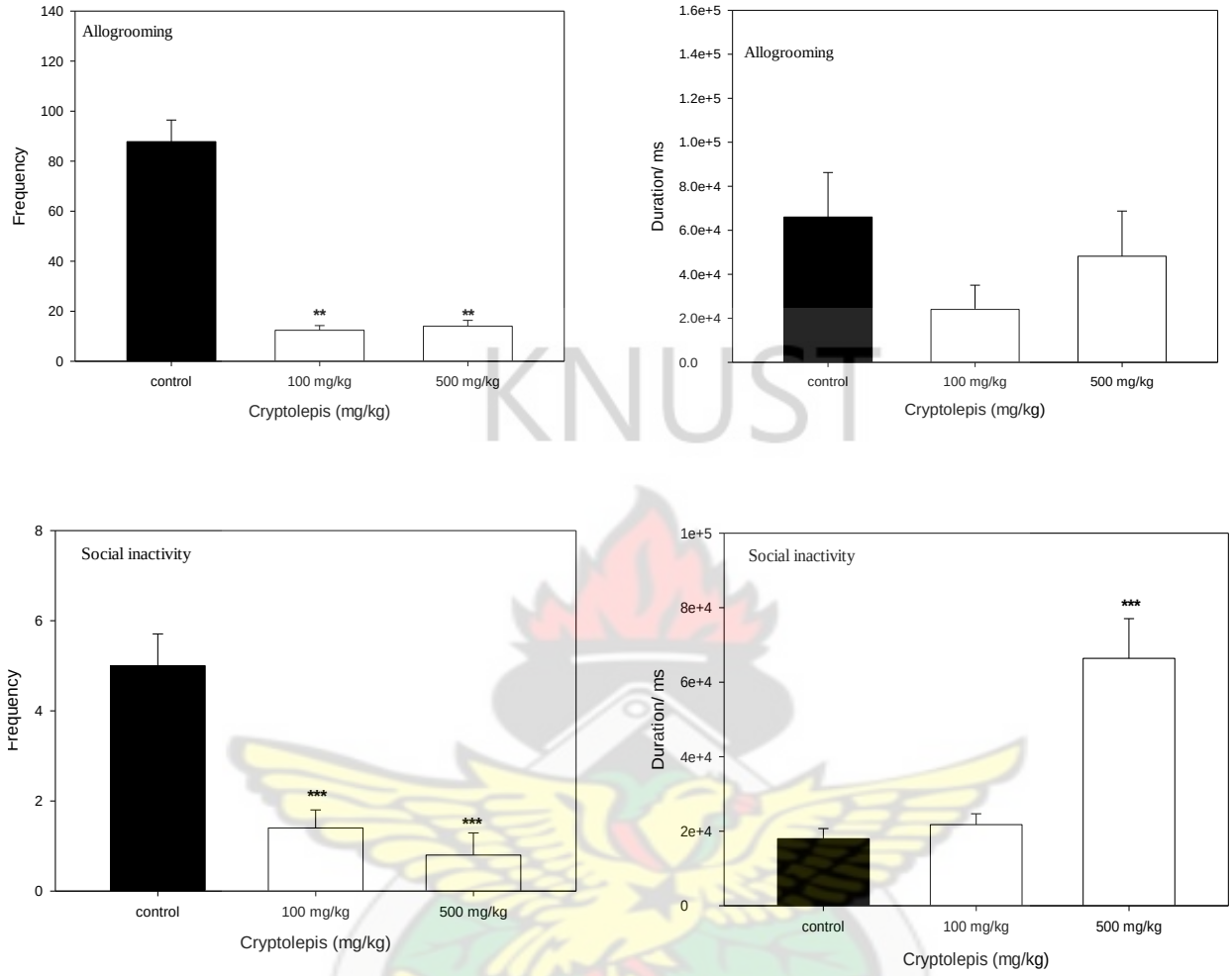


Fig 5.2 Effects of prenatal cryptolepis treatment on affiliative behaviour of F_1 generation mice on PND 45. Statistical significance by one way ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using Newman-Keuls post hoc.

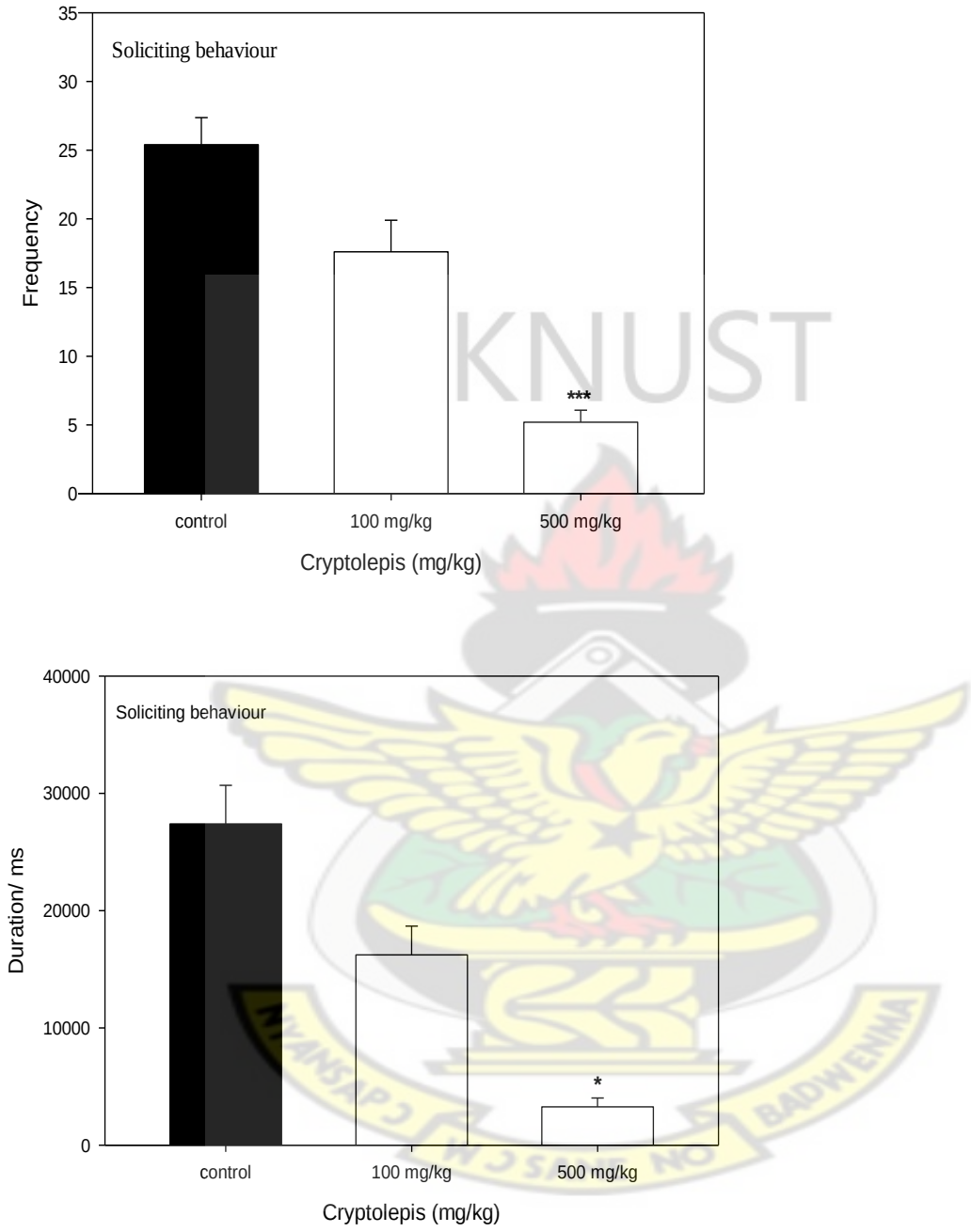


Fig 5.3 Effects of prenatal cryptolepis treatment on soliciting behaviour in F_1 generation mice on PND 45. Statistical significance by one way ANOVA *P< 0.05, ***P<0.001 using Newman-Keuls post hoc

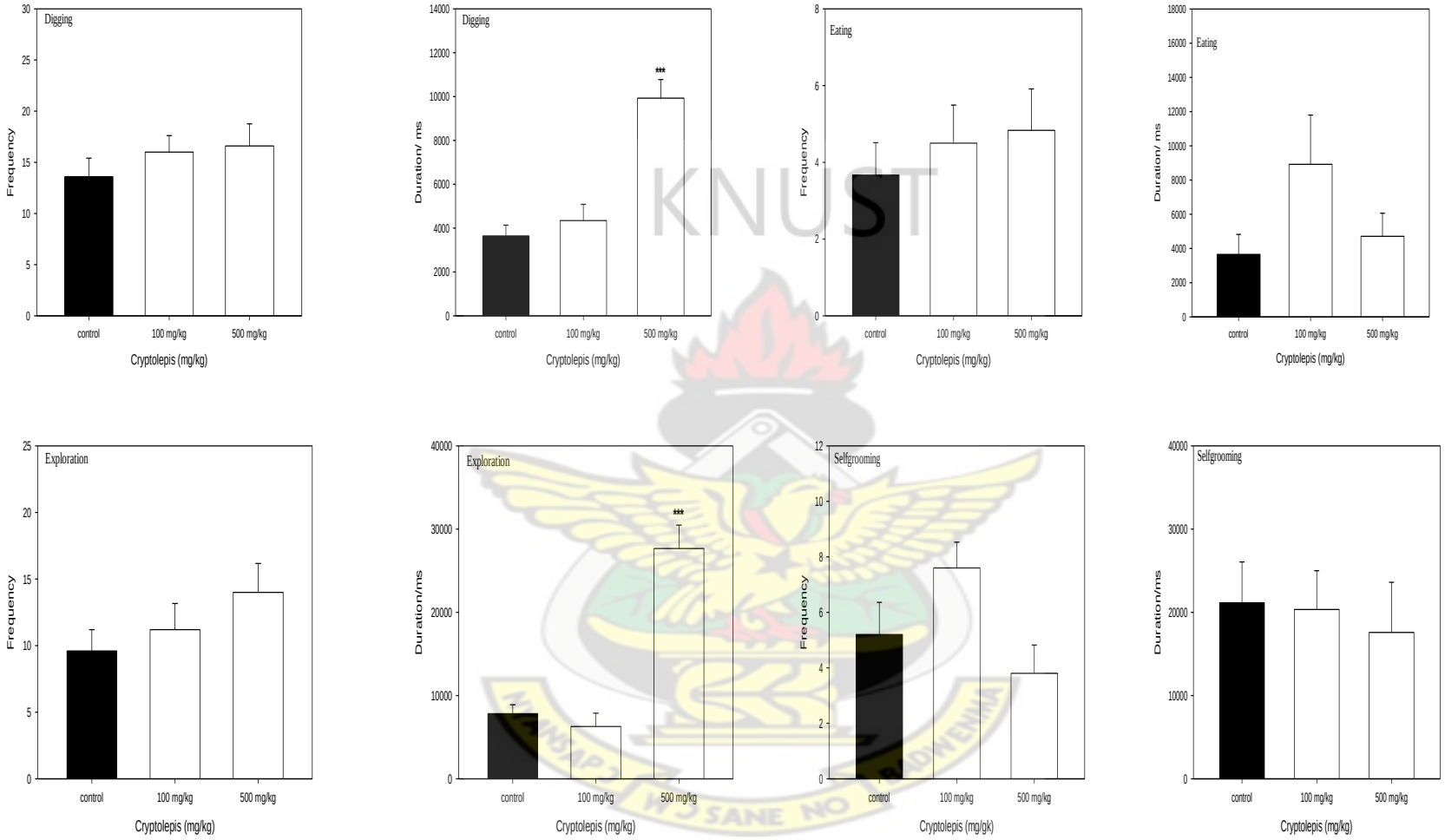


Fig 5.4 Effects of prenatal cryptolepis treatment on non social interaction of F_1 generation mice on PND 45. Statistical significance shown one way ANOVA *** $p < 0.001$ using Newman-Keuls post hoc

5.3.2 SHIRPA protocol

This study is used to assess any new strain of mice for phenotype and behavioural difference from an already existing strain. In this study ICR mice and ICR mice prenatally exposed to cryptolepis were compared.

The test began by observing and scoring undisturbed behaviour in a viewing jar (Table 5.1). There were no differences between animals exposed to cryptolepis (62.5, 100, 500 mg/kg) and control animals in terms of posture, tremors, respiratory rates, bizarre or stereotyped behaviours, convulsions, compulsive licking, self-destructive biting, retropulsion and spatial disorientation.

When animals were transferred to the arena, there was no momentary freeze which indicates the absence of fear. There were no differences in palpebral closure, piloerection, startle response, gait, pelvic elevation, tail elevation. However a distinct difference between control animals and cryptolepis treated animals was observed in the locomotor activity and their response to touch (Table 5.2).

Behaviour recorded above the arena is more of a test for the sensory system. The visual placing tests shows how good the sight of the mouse is by measuring the minimum distance it can locate the wire. Corneal reflex, pinna reflex and toe pinch, test the mice response to an adverse condition. There was no significant difference between mice prenatally treated with cryptolepis and the controls mice in these tests (Table 5.3).

When mice were observed whilst restrained supinely, there were no differences in skin colour as well as autonomic parameters such as lacrimation, salivation, irritability, fear, heart rate. All exhibited a fully developed righting, negative geotaxis and biting when provoked (Table 5.4).

Table 5.1 Behaviour recorded in the Jar

Test	control	62.5mg/kg	100mg/kg	500mg/kg
Body position	3.667±1.033	3.667±1.033	4.000±1.095	3.800±1.095
Spontaneous	2.500±0.837	3.200±0.837	3.600±0.548	3.200±0.837
Respiratory rate	2.000±0.000	2.000±0.000	2.000±0.000	2.000±0.000
Tremor	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000

Results presented as mean ± SEM.

Table 5.2 Behaviour recorded in the arena

Test	control	62.5mg/kg	100mg/kg	500mg/kg
Transfer arousal	4.667±0.5167	4.800±0.4472	4.800±0.4472	4.800±0.447
Locomotor activity	11.60± 5.639	22.00±4.147*	21.33±4.131*	18.83±6.047*
Palpebral closure	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
piloerection	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Startle response	1.000±0.000	1.000±0.000	1.200±0.4472	1.000±0.707
Gait	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Pelvic elevation	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Tail elevation	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Touche escape	1.750±0.463	2.300±0.675*	2.600±0.515*	2.500±0.527*
Positional passivity	0.000±.0000	0.000±.0000	0.000±.0000	0.000±.0000

Results presented as mean ± SEM. Statistical analysis is by one way ANOVA using Neuman-Keuls post hoc test.* means $p < 0.05$.

Table 5.3. Behaviour recorded above the arena

Test	control	62.5mg/kg	100mg/kg	500mg/kg
Trunk curl	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Limb grasping	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Visual placing	2.800±0.422	3.000±0.000	3.300±0.675	2.900±0.567
Body tone	0.000±0.000	0.000±.0000	0.000±.0000	0.000±.0000
Pinna reflex	1.182±0.405	1.364±0.505	1.182±0.405	1.364±0.505
Corneal reflex	1.000±0.000	1.090±0.302	1.000±0.000	1.000±0.000
Toe pinch	3.000±0.667	3.200±0.633	3.100±0.876	2.500±0.972
Wire manoeuvre	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000

Results presented as mean ± SEM.

Table 5.4 Behaviour recorded in supine restraint

Test	control	62.5mg/kg	100mg/kg	500mg/kg
Body length	8.527±0.735	8.690±0.739	8.410±0.580	8.510±0.401
Body weight	22.43±1.042	27.16±1.374***	23.16±1.423	23.08±1.485
Skin colour	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Heart Rate	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Limb Tone	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Abdominal Tone	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Lacrimation	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Salivation	2.000±0.000	2.000±0.000	2.000±0.000	2.000±0.000
Provoked Biting	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Righting Reflex	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Contact Righting Reflex	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Negative Geotaxis	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Fear	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Irritability	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000
Vocalization	1.000±0.000	1.000±0.000	1.000±0.000	1.000±0.000

Results presented as mean ± SEM. Statistical analysis is by one way ANOVA using Newman-Keuls post hoc test.*** means $p < 0.001$.

5.3.3 Effects of prenatal cryptolepis on light-dark exploration

Administration of cryptolepis (62, 100, 500 mg/kg) to pregnant mice caused an increase in the time spent in the dark compartment by the adult offspring whilst decreasing the time spent in the light compartment (Fig 5.5). This effect, though not significant statistically, was dose dependent. Mice treated prenatally with cryptolepis also made more several transitions between compartments relative to controls. Mice prenatally treated with cryptolepis had shorter latencies moving from dark compartment into the light compartment (Fig 5.5).

5.3.4. Effects of prenatal cryptolepis on elevated plus maze

The elevated plus-maze test is a widely used paradigm to investigate anxiety-related behaviour. It is based on the test-induced conflict between aversion of being exposed to an open and elevated platform and motivation to explore the new environment. As a consequence, the less anxious the mice, the more they explore the open arms. In the study, treatment of pregnant mice with cryptolepis resulted in a dose dependent decrease in the time spent in the open arms and a dose dependent increase in the time spent in the closed arms in the adult offspring. The number of open arm entries was also decreased with cryptolepis treatment whilst the number of closed arm entries appeared to decrease except at 100 mg/kg of cryptolepis treatment where it increased (Fig 5.6). Treatment with cryptolepis also decreased the number and duration of rearing and stretch attend withdrawals.

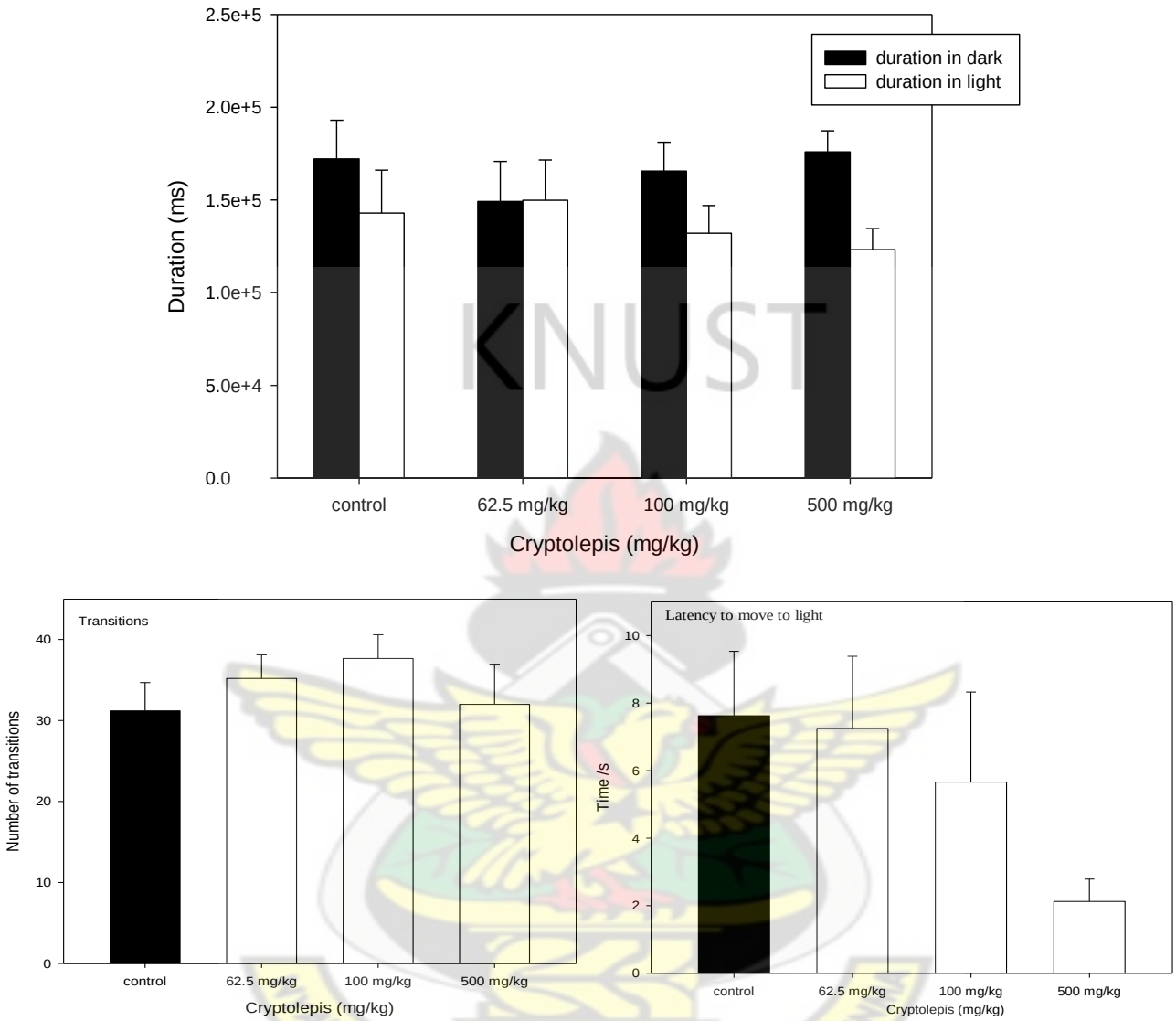


Fig 5.5. Effects of prenatal cryptolepis treatment on light/dark exploration at PND 100.Values are presented as mean ± SEM (n=10)

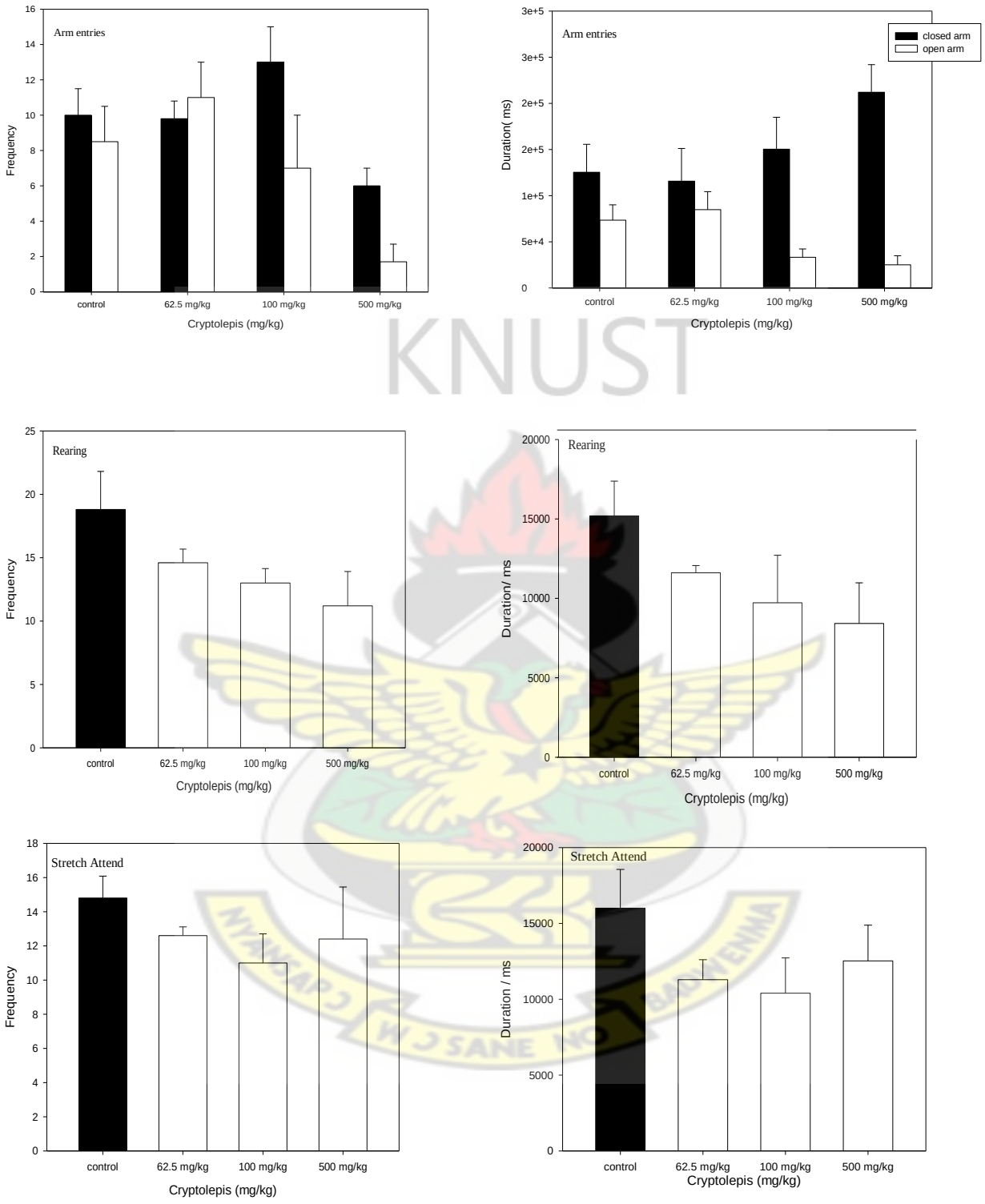


Fig 5.6 Effects of prenatal cryptolepis on parameters of the elevated plus maze. Values are presented as mean ± SEM (n=10)

5.3.5 Effects of prenatal Cryptolepis treatment on recognition memory

The novel object identification task is used to assess recognition memory in animals. Six male mice from each group prenatally treated with cryptolepis (62.5, 100, 500 mg/kg) or distilled water were assessed for recognition memory.

Animals pretreated with cryptolepis prenatally exhibited low discrimination between a familiar object and a novel object. Non treated animals spent more time with the novel object then the familiar object. Fifty percent of animals prenatally treated with cryptolepis at 100 mg/kg could not discriminate between a novel object and a familiar one (Table 5.5).

During the trial phase before the test, mice prenatally treated with cryptolepis spent more time with one familiar object than the other (Table 5.5). This was significant for all doses of cryptolepis in duration and frequency.

This study suggest that prenatal cryptolepis treatment affects object recognition memory.

Table 5.5 Effects of cryptolepis on recognition memory in novel object identification task

THE TRIAL	CONTROL	62.5 mg/kg	100 mg/kg	500 mg/kg
Time spent on left sample (A_1)	97612	111614	113119	145999
Time spent on right sample (A_2)	98163	106761	128950	92929
Total time spent with both samples	195775	218375	242069	238928
E_1 (A_1 + A_2)	32629 ± 588	39950 ± 6911	45670 ± 3001	37761 ± 5781
Difference in time (A_1-A_2)	1887 ± 396	6948 ± 1202**	17950 ± 1331***	25138 ± 1291***
Frequency of left sample exploration (F_1)	12.50 ± 1.020	13.17± 1.52	13.16 ± 1.542	14.71 ± 2.135
Frequency of right sample exploration(F_2)	12.45 ± 1.152	15.17± 1.276	12.50 ± 2.247	11.29 ± 2.032
Total frequency (F_1+ F_2)	149	170	157	182
Differences in frequency (F_1- F_2)	0.500 ± 0.224	4.167± 1.447	4.000 ± 1.751	6.857± 1.657*
THE TEST				
Time spent on sample (A)	131168	144287	92255	74659
Time spent on object (B)	70145	77104	72241	50009
E_2(A + B)	201313	221391	164496	124668
Mean time E_2	33552 ± 2848	36899 ± 4230	27416 ±7076	24943±4982
D_1(b-a)	10171 ± 3693	11197 ± 5228	3336 ± 3947	4930 ± 1821
D_2 (d_1/e_2)	0.3105± 0.114	0.2483 ± 0.1388	- 0.014± 0.237	0.1687± 0.0429
Habituation (minutes)	60	60	60	60
% of animals unable to discriminate	0	16.67	50	20
%Time spent with novel object	65.16	65.17	56.08	59.88

Results presented as mean ± SEM. Statistical significance shown as * p <0.05, **p<0.01, ***p<0.001
a_1 is time in exploring the right identical object, a_2 is the time in exploring the left identical object
e_1 is the total time spent in exploring both identical objects (a_1 and a_2) in the first trial
e_2 is the total time spent in exploring both the familiar (a) and new object (b) in the test
h_1 is the measure of global habituation from trial 1 to trial 2
d_1 and d_2 are the measures of discrimination between the new and familiar object. F_1 frequency of left object, F_2 frequency of right object

5.3.6 Effects of prenatal cryptolepis treatment on spatial and reference memory using the Morris water maze

The Morris water maze was a swimming pool of diameter 55 cm. It was filled with water and made turbid with a non toxic paint. Because the water was opaque, animals were to learn how to locate a hidden platform with the aid of external visible cues. Five male animals from groups treated prenatally with cryptolepis or distilled water were assessed for spatial and reference memory acquisition.

Prenatal treatment with cryptolepis had very little effect on the ability of mice to locate the hidden platform during training (Table 5.6) and the water maze testing (Table 5.7). In the probe trial it did not affect the number of crosses made by animals into the northeast quadrant that contained the platform during training (Table 5.9). Mice treated with cryptolepis spent less time in the northeast quadrant compared to controls but this effect was not statistically significant.

This study shows that cryptolepis treatment had minimal effect on spatial and reference memory.

Table 5.6 Effects of cryptolepis on latency to find the platform during training

Day	CONTROL	62.5 mg/kg	100 mg/kg	500 mg/kg
1	-	-	-	-
2	26.27± 5.068	27.33 ± 5.823	22.74 ± 6.121	24.31 ± 5.026
3	9.00 ± 2.348	16.10 ± 4.285	14.53 ± 3.295	20.03 ± 4.994
4	19.54± 2.789	25.40 ± 5.542	14.85 ± 2.245	20.17 ± 4.445

Latency to find the platform is measured in seconds. Results presented as mean ± SEM

Table 5.7 Effects of cryptolepis on spatial memory in Morris Water maze on Day 5

Quadrant	CONTROL	62.5 mg/kg	100 mg/kg	500 mg/kg
Northwest	19.90± 6.171	27.36 ± 6.292	22.16 ± 6.047	23.92 ± 9.637
Southwest	25.80± 13.39	20.75 ±11.79	16.00 ±12.01	11.30 ± 2.055
Southeast	16.24±5.566	18.60 ±9.379	15.20 ± 9.335	12.04 ± 1.755

Latency to find the platform is measured in seconds. Results presented as mean ± SEM

Table 5.8 Effects of cryptolepis on reference memory in the probe test of Morris water maze

	Control	62.5 mg/kg	100 mg/kg	500mg/kg
Number of crosses into Northeast quadrant	10.00±1.581	7.60 ± 1.965	8.20± 2.396	7.80± 1.594
Total time spent in the Northeast quadrant/s	16.47±1.415	15.48±1.734	15.49±2.159	16.80± 2.029

Results presented as mean ± SEM.

5.3.7 Effects of cryptolepis on maternal behaviour

Pregnant female mice were treated with either distilled water or cryptolepis (62.5 to 500 mg/kg *p.o*) from gestation day 6 till the end of gestation. On post natal day 8, dams were observed for 20 minutes for maternal behaviour. Behaviour was categorised as pup oriented, nest building or maternal activities.

When pups were moved to the other end of the nest, control animals retrieved relatively a higher percentage of the pups back into the nest compared with treated animals. Control animals spent about 44 % of the study time in activities such as suckling, grooming and licking the pups where as cryptolepis treated animals spent less time on such activities (Table 5.9). However, animals treated with cryptolepis spent more time in nest building activities such as carrying saw dust, digging the nest or in activities directed towards the dam such as grooming themselves, eating, climbing the nest (Table 5.9). The study shows that cryptolepis treatment alters maternal behaviour.

Table 5.9 Effects of cryptolepis on maternal behaviour

Parameter	control	62.5 mg/kg	100 mg/kg	500 mg/kg
Latency of pup retrieval	0.3700±0.2015	0.2867±0.1170	3.533±2.157	0.1900±0.0900
Percentage of pups retrieved	79	72	54***	66***
Frequency of pup oriented activities	2.2500±0.6292	3.500±0.500	2.000±0.5774	2.250±0.9465
Duration of pup oriented activity	535.7±176.8	178.1±42.79	184.7±77.49	195.3±39.06
Percentage of time spent on pups	44.6	14.84	15.39	16.27
Frequency of nest building	1.200±0.4899	4.667±0.6667*	5.000±1.528*	3.833±0.7923*
Duration of nest building	90.93±38.05	213.1±111.4	199.5±114	532.2±98.57**
Percentage of time spent on building the next	43.67	63.29	63.82	44.00
Frequency of maternal activities	2.800±0.9165	4.5±0.8660	3.75±0.8539	2.571±0.4286
Duration of maternal activities	771.4±126.5	917.9±22.52	765.9±152.8	482.7±123.4
Percentage of time spent on maternal activities	7.57	20.6	13.86	39.88

Maternal behaviour presented as mean ± SEM. Parametric analysis is by one way ANOVA with Neuman-Keuls post hoc test. Non parametric analysis is by Chi square. * means $p < 0.05$,** $p < 0.01$,*** $p < 0.001$

5.4. DISCUSSION

Alteration in behaviour patterns in laboratory animals can provide relevant information in assessing interference of xenobiotics with neuroendocrine maturation, as behaviour is a marker of perturbation of both nervous and endocrine functions. Although developmental studies showed that cryptolepis treatment does not cause structural malformations and monstrosities, prenatal cryptolepis treatment resulted in a reduction of social interaction amongst adolescent mice of the same sex whilst promoting non social behaviour.

The hypothalamus is the key area involved in modulation and control of sexual, parental and socio-agonistic behaviour in mice hence alteration in behaviour would suggest that there were perturbations in the development of the hypothalamus-hypophysis-gonad axis. File and Hyde, (1979) showed that low scores in social interaction were associated with increased basal plasma corticosterone, adrenocorticosterone and high hypothalamic noradrenaline concentrations and is similar to those found in anxious animals.

In addition, there is also strong correlation between social interaction and anxiety level of an organism and in the social interaction test, the more time a pair of rodents spend in active social interaction the less anxious they are (File and Hyde, 1978). Consequently, anxiolytics promote social interaction where as anxiogenic substances promote non social interaction. Cryptolepis has been shown to be anxiogenic in mice (Ansah *et al.*, 2008 b) and therefore there is a possibility that the reduced social interaction in mice prenatally treated with cryptolepis may be due to anxiety in those animals.

In anxiety experiments, elevated plus maze and light/dark box, cryptolepis treatment promoted anxiogenesis though not statistically significant. Mice prenatally treated with cryptolepis spent more time in the dark during the light/dark exploration but made more transitions between the compartments than controls; a clear indication of anxiety and hyperactivity. This study, unlike the previous anxiety experiment by Ansah *et al.*, (2008b), was not confounded by sedation as animals were not treated on the day of testing. Increase noradrenaline release could be closely related to the provocation of negative emotions such as anxiety and fear and subsequently reduced social interaction. Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety.

In the SHIRPA protocol, it was evident that minimal physical differences existed between animals prenatally treated with cryptolepis and controls confirming the results from the teratology studies. The main differences were that animals prenatally treated with cryptolepis were heavier than controls significant at 62.5 mg/kg. They were hyperactive with increased emotionality; a clear indication of anxiety and probably alterations in the hypothalamus-hypophysis- adrenal axis.

Two main forms of memory were assessed in this study. The Morris water maze measures spatial and reference memory whilst novel object identification task measures recognition memory. In the novel object identification task, treatment with cryptolepis affected recognition memory by reducing the ability of mice to discriminate between a familiar object and a novel one. In the trial test preceding the experiment, animals treated with cryptolepis exhibited an abnormal behaviour. Whilst control animals spent nearly equal time with both samples (left and right), cryptolepis

treated mice spent more time with either the left sample or the right sample. This was significant in duration and frequency. The hippocampus has been shown as the main portion of the brain involved in recognition memory (Bachevalier, 1990) hence it is possible that its function has been compromised as a result of interference with neurogenesis in the hippocampus by cryptolepis.

In the Morris water maze, prenatal treatment with cryptolepis did not significantly affect the latency to locate the platform during training and the water maze testing. In the probe trial on day 6, prenatal treatment with cryptolepis did not affect the total time spent in that the quadrant containing the platform or the number of crosses made into that quadrant. The water maze specifically tests spatial or reference memory (D'Hooge and De Deyn, 2001). It appears that prenatal cryptolepis treatment has little effect on the acquisition of spatial memory. The Morris Water Maze is used to test hippocampal-dependent learning including acquisition of spatial memory, long term memory, and long term spatial memory.

However, there are numerous other components of the task that do not involve spatial memory: the stress involved with the task, the understanding of the rules of the task (that to "escape", the animal must find a hidden platform, and stay on it in order to be "rescued"), and the understanding that there is a means of escaping the task.

Prenatal cryptolepis treatment causes long term effects, such as hyperactivity, increased emotionality, cognitive deficits and neurological and behavioural abnormalities in adult offspring. Cryptolepis has the ability to directly cause these effects by affecting neurogenesis. It can also cause this effect indirectly by inducing changes in maternal behaviour.

It is possible that cryptolepine directly interferes with maturation of neurotransmitter systems in the central nervous system. Cryptolepine antagonises the actions of noradrenaline on alpha receptors with preferential α_2 blocking activity (Noamesi and Bamgbose, 1980; Noamesi and Bamgbose, 1982). Yohimbine, an α_2-adrenoceptor antagonist causes a marked increase in noradrenaline release in several brain regions (Andén *et al.*, 1982). Cryptolepine may cause the release of noradrenaline by similar mechanism, provoking anxiety and subsequently a reduction in social behaviour. Indeed it has been proposed that cryptolepis induced anxiogenesis is possibly mediated by interaction with α_2-adrenoceptor blockade (Ansah *et al.*, 2008b).

Inhibition of neurogenesis and neural development in certain brain centres can also be the cause of the observed reduction in social interaction. NF-kB has been shown to have unique roles in the CNS, in such processes as neuronal plasticity, neurodegeneration and neuronal development. A high molecular-weight complex (110–115 kDa) with NF-kB binding activity was found to be present in nuclei of young rats and mice (up to postnatal day 2); the activity was highest in developing cortex and lowest in cerebellum (Cauley and Verma, 1994) which were present in the brains of young rats but not in adults (Bakalkin *et al.*, 1993). Cryptolepine affects activity of NF-kB by reducing its binding to DNA (Olajide *et al.*, 2007b).

Cryptolepine interferes with the activity of mammalian topoisomerase II enzyme activity (Bonjean *et al.*, 1998). Mammalian DNA topoisomerase IIβ, unlike the topoisomerase IIα isoenzyme, which is specifically expressed in proliferating cells and most likely performs the essential function of intertwined chromosome pairs during mitosis, is apparently dispensable in cell growth. Effects of cryptolepine on the different forms of the enzyme are yet to be elucidated.

Notwithstanding, gene knockout studies have shown that topoisomerase IIβ has a critical role in neural and neuromuscular development (Yang *et al.*, 2000b). Whereas mice lacking the gene have phenotypes indistinguishable from their littermates, they tend to die of breathing impairment at birth owing to neural and neuromuscular defects.

Cryptolepis is used to treat insomnia (Mshana *et al.*, 2000). Studies with cryptolepis shows that it potentiates pentobarbitone induced sleeping time (Ansah *et al.*, 2008a). Sedatives may enhance the inhibitory activity of GABA or impair activity of NMDA**.** In either way it can induce apoptosis in certain brain regions. In addition to their proapoptotic effects in the developing brain, sedatives may also impair cell proliferation and differentiation, synaptogenesis, synapticplasticity, cell migration and axonal arborization (Ikonomidou, 2009).

The results from the maternal behaviour study show that the functional deficits seen in mice prenatally treated with cryptolepis may not only be direct effects of cryptolepis on neurogenesis alone but could also partly involve an indirect effect of cryptolepis affecting the maternal system which subsequently affects the foetus. This partly explains why mice prenatally treated with cryptolepis exhibited behavioural and functional deficits similar to prenatally stressed animals. Treatment with cryptolepis resulted in considerable maternal stress even to the extent of maternal weight reduction and death. Maternal stress can cause an unspecific prenatal stress to the foetus. The mechanisms involve induction of maternal stress in dams leading to hyperproduction of corticotrophin releasing hormone (CRH), adrenocorticotropin (ACTH) and cortisol in blood. Release of maternal cortisol stimulates placental release of CRH which, in turn, stimulates the HPA axis and secretion of more cortisol (positive feedback loop), leading to a further placental

CRH secretion. This leads to alteration in the maturation of the hypothalamus–pituitary–adrenal (HPA) axis in the foetus (Guo *et al.*, 1993; Fameli *et al.*, 1995).

The long term effects of the prenatal stress are reduced expression of juvenile social play (Ward and Stehm, 1991), enhanced behavioural reactivity to emotional challenges (Joffe, 1978; Vallee *et al.*, 1997), persistent impairment of negative feedback regulation of the hypothalamic-pituitary-adrenal axis (Green *et al.*, 2011;Vallee *et al.*, 1997), reduced activity of the opioid, GABA/benzodiazepine, serotonin, and dopamine systems and increased activity of the sympathico-adrenal system (Huizink *et al.*, 2004). The extent to which the direct effects and maternally mediated effects interact to produce neurological deficits in the offspring is presently unknown.

In conclusion, prenatal cryptolepis treatment induces functional toxicity in mice.

CHAPTER SIX

POSSIBLE MECHANISMS FOR CRYPTOLEPIS-INDUCED REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

CHAPTER SIX

6.1 INTRODUCTION

Cryptolepis has been shown to adversely affect reproduction in both male and female mice, interfere with foetal development, and alter behaviour in offspring exposed prenatally during development in the previous chapters. The aqueous extract of *Cryptolepis sanguinolenta* has antiinflammatory activity due to inhibition of cycloxygenase 2 (Olajide *et al.*, 2007a; Olajide *et al.*, 2009; Olajide *et al.*, 2010). Previous studies with the main alkaloid of the aqueous extract show that it inhibits NF-kB and antagonises the actions of prostaglandin E_2 (Bamgbose and Noamesi, 1981; Olajide *et al.*, 2007b). In chapter two in the present study, it was proposed that one of the mechanisms of the reproductive toxicity induced by cryptolepis was inhibition of ovulation. Ovulation is analogous to inflammation. Both processes are mediated by the induction of COX 2 hence substances that reduce inflammation by inhibiting the activity of COX 2 and prostaglandin E_2 inhibit ovulation (Espey, 1980; Espey, 1994; Espey and Lipner, 1994).

The rabbit is a mammal used extensively in reproductive toxicity studies (Foote and Carney 2000). Unlike other mammals, the rabbit possesses certain distinct characteristics which are of importance and can be exploited for reproductive studies. Chief amongst them is that they are *induced ovulators*. An *induce ovulator* means that ovulation occurs 10 to 12 hours after mating. This makes it possible to study effects of substances on ovulation *in vivo* without having to induce it with a chemical which may confound the findings. It also makes it possible to separate events occurring before ovulation from those that occur after ovulation thereby providing a clear evidence of the mechanisms.

One side effect associated with non steroidal antiinflammatory drugs is their ability to cause renal damage as the kidney is one of the few organs where COX-2 has been found to be constitutive (Komhoff *et al.*, 1997; Perazella, 2001). Subacute treatment with cryptolepis leads to a considerable level of mortality in the parent animal as well as foetuses. Embryonic development in COX 2 null mice is associated with early death as a result of severe morphological defects and consequent functional failures in the kidneys (Dinchuk *et al.*, 1995; Morham *et al.*, 1995). It is probable that the observed mortalities in the parent occur partly as a result of renal damage. As part of the determination of mechanisms for the reproductive toxicity of cryptolepis, this work will examine the effects of cryptolepis treatment on renal histology.

Reproductive studies in males show that subacute cryptolepis treatment results in decline in mounting behaviour parameters as well as spermatozoa in the head of cauda epidydimis. Spermatogenesis and libido are regulated by the endocrine system. This study will determine the effects of cryptolepis treatment on serum testosterone, follicle stimulating hormone, luteinizing hormone and prolactin levels as well as testicular histology.

This chapter aims at elucidating some of the probable mechanisms for the observed reproductive and developmental toxicities of cryptolepis.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

Diclofenac injection 3 mg/ml was purchased from Denk pharma, Germany.

Testosterone propionate was a kind donation from Abeth medical laboratories, Kumasi, Ghana.

Cyproterone acetate (Batch No 04094 A) was obtained from Bayer, Germany.

ELISA kit for testosterone, prolactin, follicle stimulating hormone, luteinizing hormone were obtained from Fortress Diagnostics Limited, United Kingdom.

6.2. 2 Effects of cryptolepis on pre and post ovulatory events.

Virgin female New Zealand white rabbits (n = 45) were obtained from (St. Louis Senior High School, Oduom, Kumasi) and kept in the animal house of the Department of Pharmacology for two weeks for acclimatization. Animals were fed on grass and administered water *ad libitum*. The animals were then mated and the time of mating recorded. Animals were then randomly assigned to one of nine groups (n=5) each receiving different treatment as follows;

Group 1 received only distilled water and served as control.

Preovulatory experiment in female rabbits

Group II received 3 mg/kg of diclofenac (*im)* five hours after mating

Group III received 62.5 mg/kg of cryptolepis *p.o* five hours after mating

Group IV received 100 mg/kg of cryptolepis *p.o* five hours after mating

Group V received 500 mg/kg of cryptolepis *p.o* five hours after mating

Post ovulatory experiment in female rabbits

Group VI received 3 mg/kg of diclofenac by *im* injection 20 h and repeated 48 h after mating

Group VII received 62.5 mg/kg of cryptolepis *p.o* 20 h and repeated 48 h after mating

Group VIII received 100 mg/kg of cryptolepis *p.o* 20 h and repeated 48 h after mating

Group IX received 500 mg/kg of cryptolepis *p.o* 20 h and repeated 48 h after mating

Rabbits were kept and fed normal until gestation day 10. On gestation day 10, they were sacrificed. The total number of implants, number of resorption sites and the number of corpus luteum were then counted.

6.2.3. Effects of cryptolepis on reproductive hormone

To measure the effect of cryptolepis on testosterone, FSH, LH and prolactin levels, five groups of male mice (n= 8) were treated with either distilled water or cryptolepis (62.5, 100, 500, 1000 mg/kg *p.o.*) for 14 days. After the treatment period mice were sacrificed and blood was collected into Vacutainer tubes. The blood was centrifuged at 500 *g* for 15 min and serum was collected and stored at -20 ˚ C.

Hormone assays were carried out using competitive immunoenzyme assays as described by the manufacturer. Briefly, the hormone calibrator or specimen was first added to a streptavidin coated well. Biotinylated monoclonal and enzyme labeled antibodies (directed against distinct and different epitopes of the hormorne) were added and the reactants mixed. Reaction between

the various hormone antibodies and native specific hormone forms a sandwich complex that binds with the streptavidin coated to the well. After the completion of the one hour incubation period, the enzyme- hormone antibody bound conjugate was separated from the unbound enzyme-hormone conjugate by aspiration or decantation. The activity of the enzyme present on the surface of the well was quantitated by reaction with a suitable substrate to produce colour and absorbance was measured at 450nm. Several serum references of known hormone levels were used to extrapolate unknown specimen activity.

6.2.4 Effects of cryptolepis on testicular and renal histology

Testicles and kidneys were harvested from male mice in experiment 6.2.3 for histological studies. Transverse as well as longitudinal sections were made for testicles at each dose level. Longitudinal sections were made for kidneys. All sections were fixed in 10% formalin solution. Tissues were processed by dehydration with graded concentrations of alcohol, cleared with xylene and infiltrated with paraffin. These were later embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Digital images were acquired with a camera mounted on a Nikon E400 microscope using a x100 magnification. The numbers as well as morphology of seminiferous tubules were noted.

To quantify the effects of cryptolepis on seminiferous tubules, digital images of the seminiferous tubules of testicular histology were acquired with a camera mounted on a Nikon E400 microscope using a x100 magnification. Sixty seminiferous tubules were randomly selected from control and each dose of cryptolepis (62.5-1000 mg/kg *p.o*) and their diameters measured with the aid of a computer programme Image J 1.44p; java 1.6.0-20.

6.2.5 Chick comb method for androgenic activity

The chick comb method was used with slight modification to evaluate the androgenic activity of aqueous extract of cryptolepis. Day old single comb white leghorn chicks, *Gallus domesticus* were obtained from Asamoah and Yamoah farms at Kegya, Kumasi and kept in the animal house of Department of Pharmacology, KNUST for 14 days for acclimatization. On Day 14 chicks were randomly assigned to one of ten groups (n = 8) and treated as follows;

Group 1; distilled water only

Group II: testosterone 200 μg/kg *im* for five days

Group III: testosterone 0.5 mg/kg *im* for five days

Group IV: testosterone 1 mg/kg *im* for five days

Group V: cyproterone 3 mg/kg *p.o* for five days

Group VI: cyproterone10 mg/kg *p.o* for five days

Group VII: received cyproterone 30 mg/kg *p.o* for five days

Group VIII: received cryptolepis 62.5 mg/kg *p.o* for five days

Group IX: received cryptolepis 100 mg/kg *p.o* for five days

Group X: received cryptolepis 1000 mg/kg p.o for five days

Prior to the beginning of the assay, the length and height of each chick comb was determined. Twenty four hours after the last drug administration, the growth of the comb was estimated as the product of the mean change in length and height. The mean of each group was calculated and plotted as a dose response curve for testosterone, cyproterone and cryptolepis and the potency ratios relative to the standards were calculated.

6.2.6. Effects of Cryptolepis on testosterone-induced chick comb growth.

From experiment 6.2.5 above, the ED_{50} of testosterone, cyproterone and cryptolepis were estimated to be approximately 0.7 mg, 10 mg, 300 mg respectively. To estimate the effects of cryptolepis on testosterone, 30 single comb white leghorn chicks were grouped into five (n=6) and treated as follows;

Group 1: distilled water only

Group II: 0.7 mg/kg testosterone *im*

Group III: 300 mg/kg cryptolepis *p.o.* followed by 0.7 mg/kg testosterone *im* 30 minutes later

Group IV: 600 mg/kg cryptolepis *p.o.* followed by 0.7 mg/kg testosterone *im* 30 minutes later

Group V: 10 mg/kg cyproterone *p.o.* followed by 0.7 mg/kg testosterone *im* 30 minutes later

6.3 RESULTS

6.3.1 Effects of cryptolepis on preovulatory events

Ovulation involves the rapture of a matured Graafian follicle leading to the release of an ovum and the formation of a corpus luteum. If the ovum is fertilized, it develops into a blastocyst which is then implanted after 7 days in rabbits.

Treatment of rabbits five hours after mating with cryptolepis (62.5 to 500 mg/kg) or diclofenac (3 mg/kg) led to an inhibition in ovulation. Total number of corpus luteum was decreased indicating a decrease in the numbers of ova released (Table 6.1). The implantation indices for each dose of treatment were less than the control (Table 6.1). There were also preimplantation losses for both cryptolepis and diclofenac.

Histology of the ovary showed there were dilated unraptured Graafian follicles in the ovary ten days after mating (Plate 1). The effects of cryptolepis were observed to be similar to diclofenac.

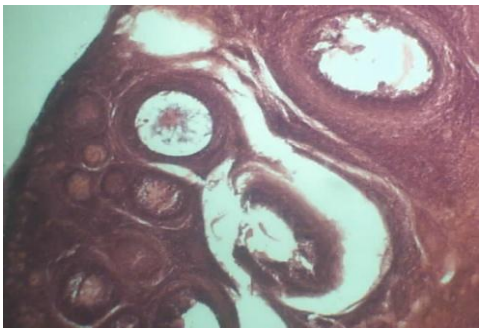
This study shows clearly that cryptolepis treatment (62.5 – 500 mg/kg *p.o*) prior to ovulation leads to ovulatory failure.

Table 6.1 Effects of cryptolepis on preovulatory Events

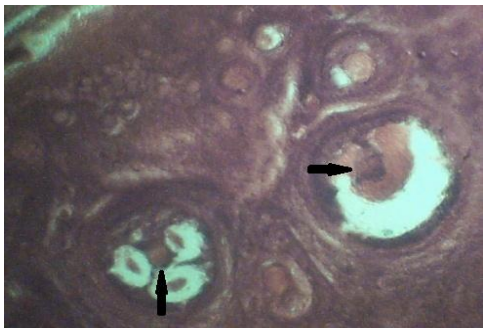
PARAMETER	CONTROL	DICLOFENAC		CRYPTOLEPIS	
		(3.3 mg/kg)	(62.5 mg/kg)	(100 mg/kg)	(500 mg/kg)
Mean maternal weight on Gd 0 (kg)	2.34 ± 0.152	2.26 ± 0.114	2.156 ± 0.256	2.36 ± 0.114	2.18 ± 0.164
Mean maternal weight on Gd 10 (kg)	2.49 ± 0.175	2.32 + 0.085	2.245 ± 0.277	2.420 ± 0.100	2.32 ± 0.183
Maternal weight gain	0.15 ± 0.033	0.59 ± 0.809	0.0895± 0.034	0.325± 0.106	0.22 ± 0.106
No of corpora lutea	8.20 ± 1.304	1.40 ± 0.548***	4.2 ± 1.789***†	2.0 ± 1.581***	2.6 ± 1.949***
No of implants	6.60 ± 1.140	1.00 ± 0.707***	3.20 ± 1.095***	1.30± 0.957***	1.90 ±1.140***
Implantation index (%)	80	71.4	76.2	65	73
Preimplantation loss (%)	19.5	28.5	23.8	35	27

Implantation Index = $\frac{\text{number of implants}}{\text{number of corpora lutea}} \times 100$ Preimplantation loss = $\frac{\text{number of corpora lutea – number of implants}}{\text{number of corpora lutea}} \times 100$

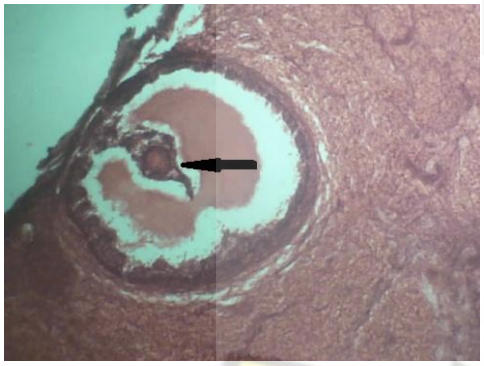
Results presented as mean ± SEM. Statistical analysis is by one way ANOVA using Newman- Keuls post hoc test. *** means $p < 0.001$. † means $p < 0.05$ compared with diclofenac.



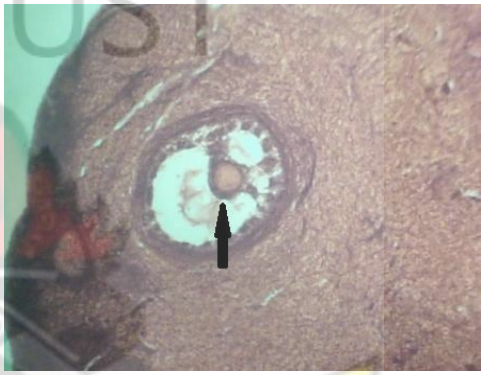
Control



Diclofenac 3 mg/kg



Cryptolepis 62.5 mg/kg



Cryptolepis 100 mg/kg

Cryptolepis 500 mg/kg

Plate 6.1: **Effects of preovulatory cryptolepis treatment on ovarian histology. Arrows show dilated but unraptured Graafian follilcle after cryptolepis or diclofenac treatment five hours before ovulation. Control animal shows corpora lutea.**

6.3.2 Effects of cryptolepis on postovulatory events

Ovulation occurs 10 to 12 hours after mating in rabbits. Treatment of mated rabbits with diclofenac or cryptolepis at the twentieth and forty eighth hours had little effects on the ovulatory process. The number of corpora lutea were not significantly different from controls suggesting that a difference in the number of implants was not due to inhibition in ovulation (Table 6.2).

Histology of the ovary shows that cryptolepis has little effect when administered at the twentieth and forty eighth hour.

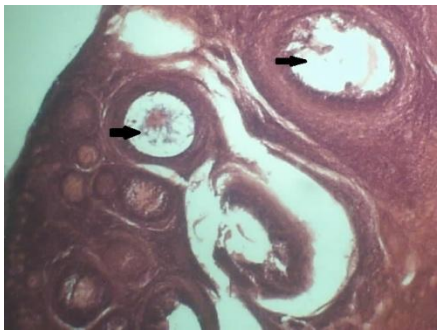
Uteri of cryptolepis treated animals had less number of implantations relative to controls and diclofenac treated animals (Table 6.2). This was confirmed by the low implantation index and the high percentage of preimplantation losses in animals treated with cryptolepis (62.5 – 500 mg/kg).

Table 6.2 Effects of Cryptolepis on Postovulatory Events

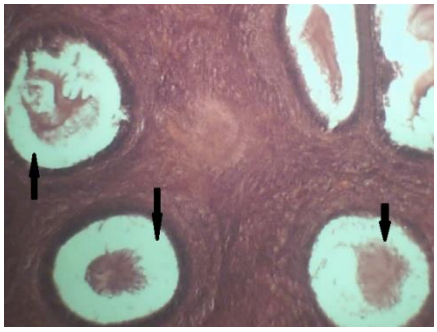
PARAMETER	CONTROL	DICLOFENAC		CRYPTOLEPIS	
		(3.3 mg/kg)	(62.5 mg/kg)	(100 mg/kg)	(500 mg/kg)
Mean maternal weight on Gd 0 (kg)	2.34 ± 0.152	2.340 ± 0.068	2.300 ± 0.105	2.080 ± 0.139	2.44 ± 0.059
Mean maternal weight on Gd 10 (kg)	2.49 ± 0.175	2.450 ± 0.073	2.366 ± 0.778	2.200 ± 0.130	2.49 ± 0.045
Maternal weight gain	0.15 ± 0.033	0.11 ± 0.029	0.0660 ± 0.038	0.120 ± 0.049	0.05 ± 0.038
No of corpora lutea	8.20 ± 1.304	8.60 ± 0.6782	7.8 ± 0.8602	6.800± 0.663	7.60 ± 0.600
No of implants	6.60 ± 1.140	5.80 ± 0.3742	4.00 ± 0.7071	2.00± 0.7071**††	2.40 ± 0.509**†
Implantation index (%)	80	67	51	29	51
Preimplantation loss (%)	19.5	32	48.7	70	68

Implantation Index = $\frac{\text{number of implants}}{\text{number of corpora lutea}} \times 100$ Preimplantation loss = $\frac{\text{number of corpora lutea – number of implants}}{\text{number of corpora lutea}} \times 100$

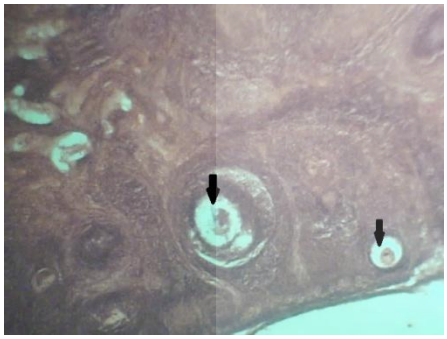
Results presented as mean±SEM. Statistical analysis is by one way ANOVA using Newman-Keuls post hoc test. ** means $p < 0.01$. † means $p < 0.05$ compared with diclofenac.†† means $p < 0.01$ when compared to diclofenac



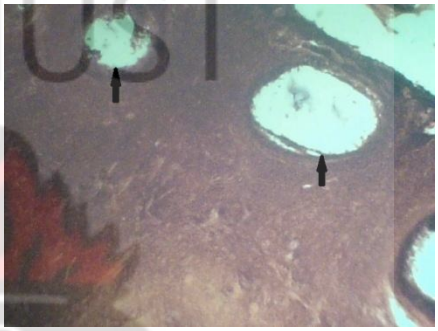
Control



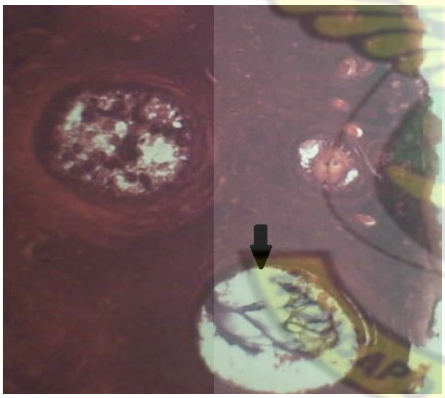
Diclofenac (3 mg/kg)



Cryptoplepis 62.5 mg/kg



Cryptoplepis 100 mg/kg



Cryptoplepis 500 mg/kg

Plate 6.2: Effects of postovulatory cryptolepis treatment on ovarian histology. Arrows indicate corpora lutea. Cryptolepis treatment after ovulation has minimal effects on ovarian histology.

6.3.3 Effects Cryptolepis on reproductive hormones

Male mice were treated with cryptolepis for 14 days. Serum FSH, LH, prolactin and testosterone were the measured. Cryptolepis treatment (62.5 -1000 mg/kg) led to significant ($p < 0.05$) reduction in serum testosterone in treated animals. Cryptolepis treatment did not affect serum prolactin. Its effects on serum FSH and LH were inconsistent. At doses of 62.5 -500 mg/kg, cryptolepis treatment increased serum LH, though not statistically significant and at 1000 mg/kg, it reduced serum LH. Mortalities occurred at all doses except in the control and 62.5 mg/kg of cryptolepis treatment (Table 6.3).

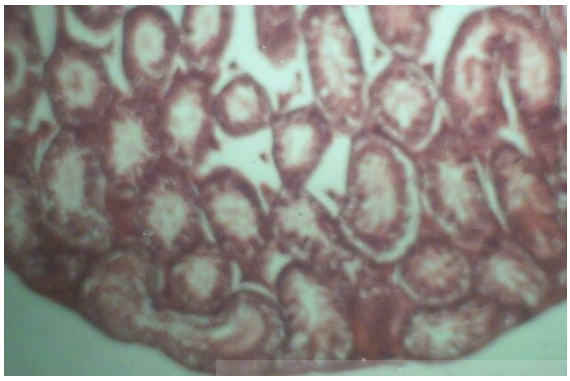
Table 6. 3. Effects Cryptolepis on serum hormone levels

	Control	62.5 mg/kg	100 mg/kg	500 mg/kg	1000 mg/kg
% mortality	0	0	30	15	40
Testosterone(ng/ml)	2.633±0.613	0.948±0.5875*	0.021±0.021*	0.0043±0.004*	0.4200±0.1488*
Prolactin	14.45±0.032	15.23±0.4321	15.67± 0.078	14.89±0.114	15.45±0.034
FSH((mIU/ml)	12.94±3.417	7.11±3.4501	17.61±2.386	11.1±1.012	3.74±5.6130
LH(mIU/ml)	6.67±0.781	5.67±1.001	8.01±3.011	9.89±2.221	3.08 ± 0.5510

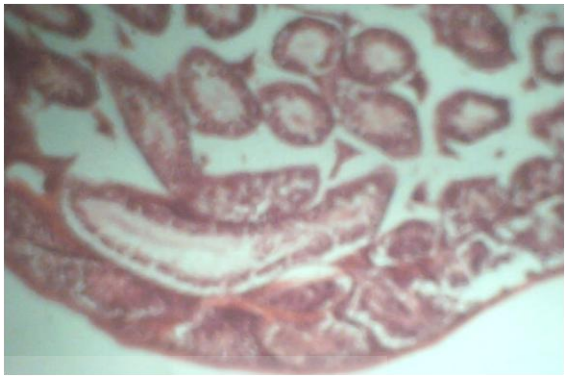
Results presented as mean±SEM. Statistical analysis is by one way ANOVA using Newman-Keuls post hoc test. * means $p < 0.05$

6.3.4 Effects of subacute cryptolepis treatment on the histology of the testes

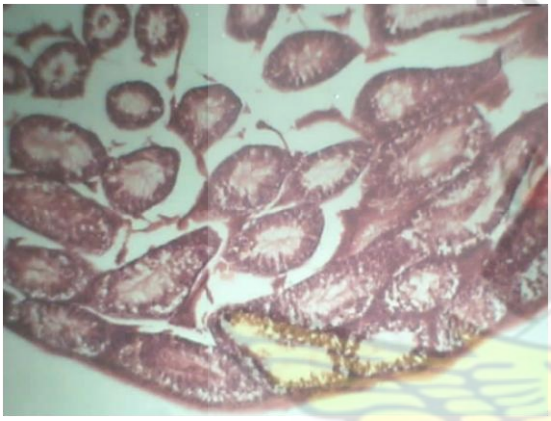
The photomicrographs (plate 6.3 and 6.4) represent the transverse and longitudinal sections respectively of the testes of male mice treated with either distilled water or cryptolpeis (62.5-1000 mg/kg *p.o.*). It was evident that cryptolepis disrupts the histoarchitecture of the testes particularly at 1000 mg/kg. It had insignificant effects on the numbers as well as the diameter of the seminiferous tubules (Fig 6.1).



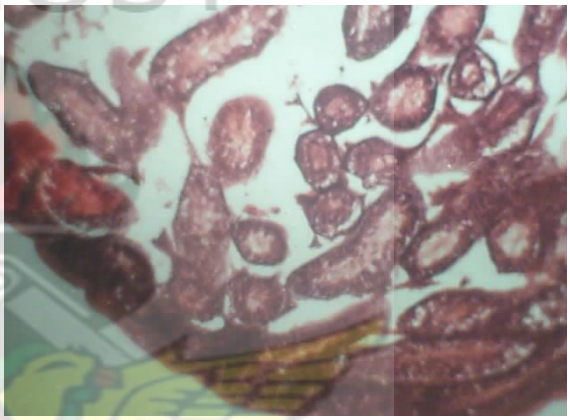
Control



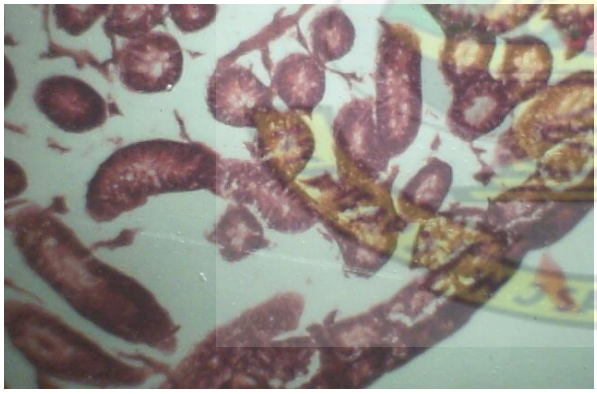
Cryptolepis 62.5 mg/kg



Cryptolepis 100 mg/kg

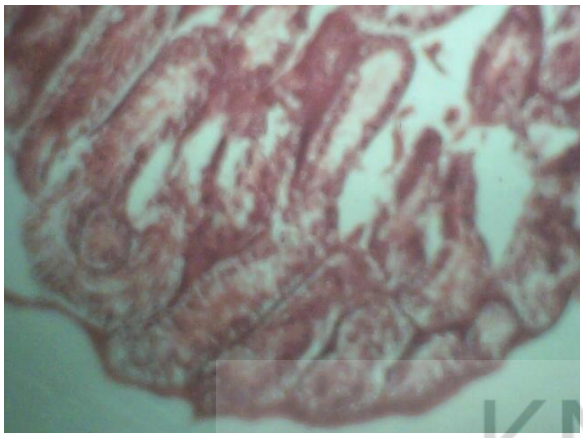


Cryptolepis 500 mg/kg

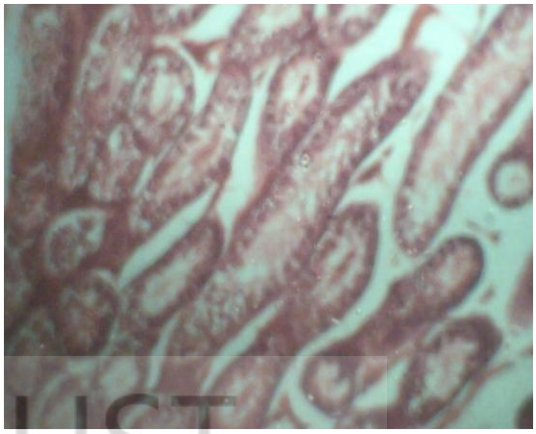


Cryptolepis 1000 mg/kg

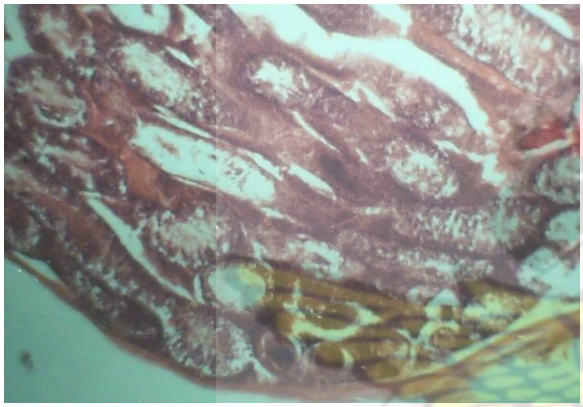
Plate 6.3: **Photomicrogram of tranverse sectioning of testes showing arrangement of seminiferous tubules after subacute treatment with *Cryptolepis sanguinolenta***



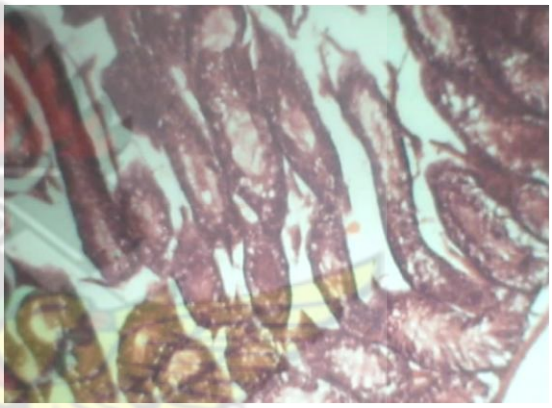
Control



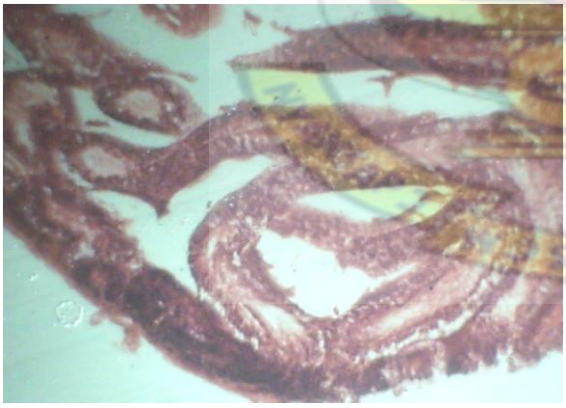
Cryptolepis 62.5 mg/kg



Cryptolepis 100 mg/kg



Cryptolepis 500 mg/kg



Cryptolepis 1000 mg/kg

Plate 4: Photomicrogram of longitudinal sectioning of testes showing arrangement of seminiferous tubules after subacute treatment with *Cryptolepis sanguinolenta*

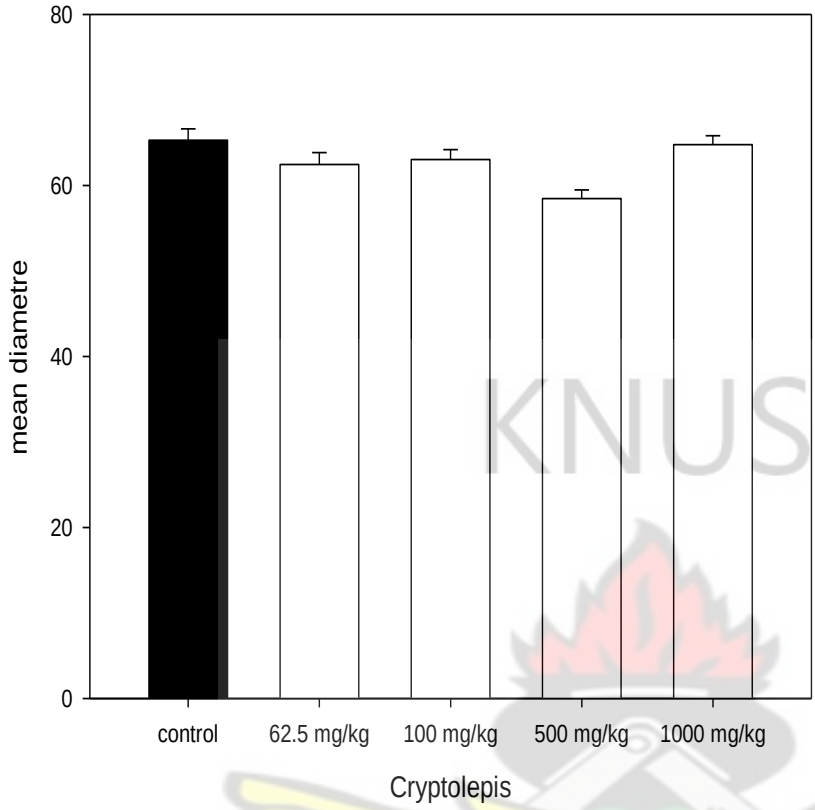
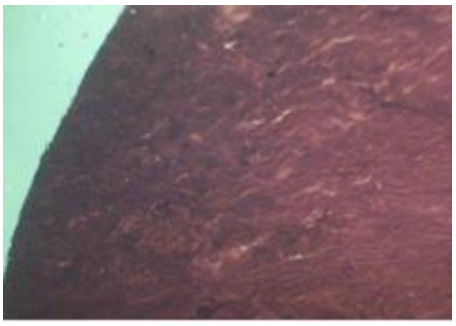


Fig 6.1 Mean diameter of seminiferous tubules (n = 60) after 14 days treatment with cryptolepis (62.5 – 500 mg/kg *p.o*). Results presented as mean ± SEM.

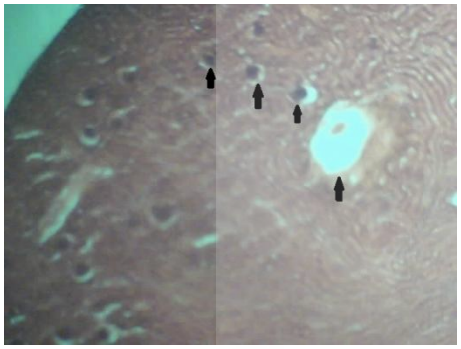
6.3.5 Effects of cryptolepis treatment on renal histology

Treatment of male mice for 14 days with cryptolepis from (62.5 – 1000 mg/kg *p.o*) induces renal damage. The damage induced appears systematic like apoptosis. The process begins with condensation of groups of cells to form a multinucleated cell in tissues of the renal cortex and some medullary regions. These cells pick up stain and appear dark, an indication of a condensation of their nuclear material. There are many groups of cells undergoing this process in the renal cortex but it appears not to disturb the surrounding tissues. With time however, the multinucleated cell disappears thus leaving a massive lesion in the kidney (plate 6.5).

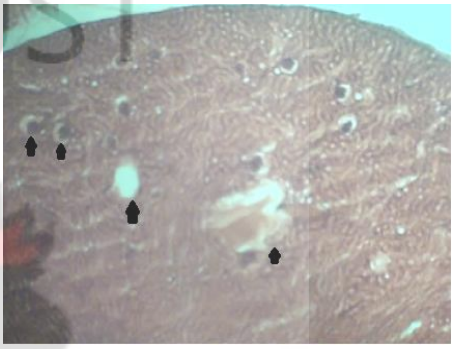
Control



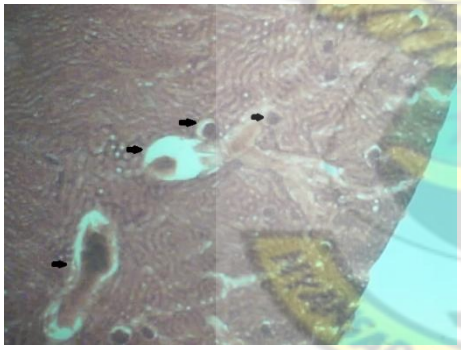
Cryptolepis 62.5 mg/kg



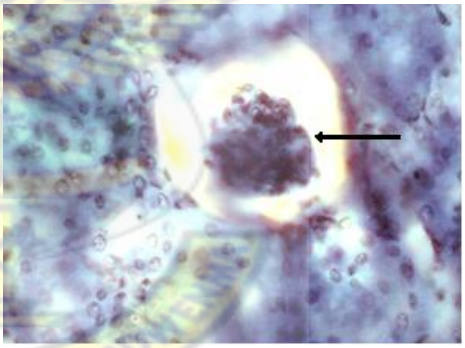
Cryptolepis 100 mg/kg



Cryptolepis (500 mg/kg)



Cryptolepis 1000 mg/kg



Cryptolepis (100 - 1000 mg/kg) x 400

Plate 6.5: Photomicrographs of the kidneys of mice after 14 days treatment with cryptolepis (62.5 – 1000 mg/kg *p.o*). Arrows show systematic renal damage by cryptolepis: condensation of groups of cells to form a multinucleated cell, and subsequent disappearance of the cell leaving a massive lesion.

6.3.6 Androgenic Assay by the chick comb method

Fourteen day old chicks were monitored for rate of growth of their combs after treatment with cryptolepis (62.5–1000 mg/kg *p.o.*), testestorone propionate (20µg/kg – 1mg/kg *im*), cyproterone acetate (3 – 30 mg/kg *p.o*) or distilled water for five days. The comb growth was calculated as the change in the area of the comb.

Treatment with testosterone led to significant comb growth in length and height (Table 6.3). The combs were bright deep red. The combs growth was evident on the third day of treatment. All animals treated with testosterone (0.5 – 1 mg/kg *im*) had fully developed pairs of wattles where as animals treated with 20 µg/kg testosterone had partially developed pairs of wattles. They had higher growth rate compared with controls and cryptolepis treatment but this was not significant statistically. The ED_{50} of testosterone was estimated to be 0.7 mg/kg in the study (Fig 6.2).

Treatment with cryptolepis (62.5 – 1000 mg/kg *p.o*) caused a dose dependent inhibition in comb growth. The combs were pink and there were no wattles at all doses of treatment. The rate of growth was less than testosterone treated groups at all doses tested but was not significant statistically. The effects produced by cryptolepis were more comparable to cyproterone acetate than testosterone (Fig 6.2).

The ED_{50} of cryptolepis was approximately 300 mg/kg as against 10 mg/kg for cyproterone. Cryptolepis was thus 31.05 less potent than cyproterone in the study. Cryptolepis exhibits antiandrogenic effects.

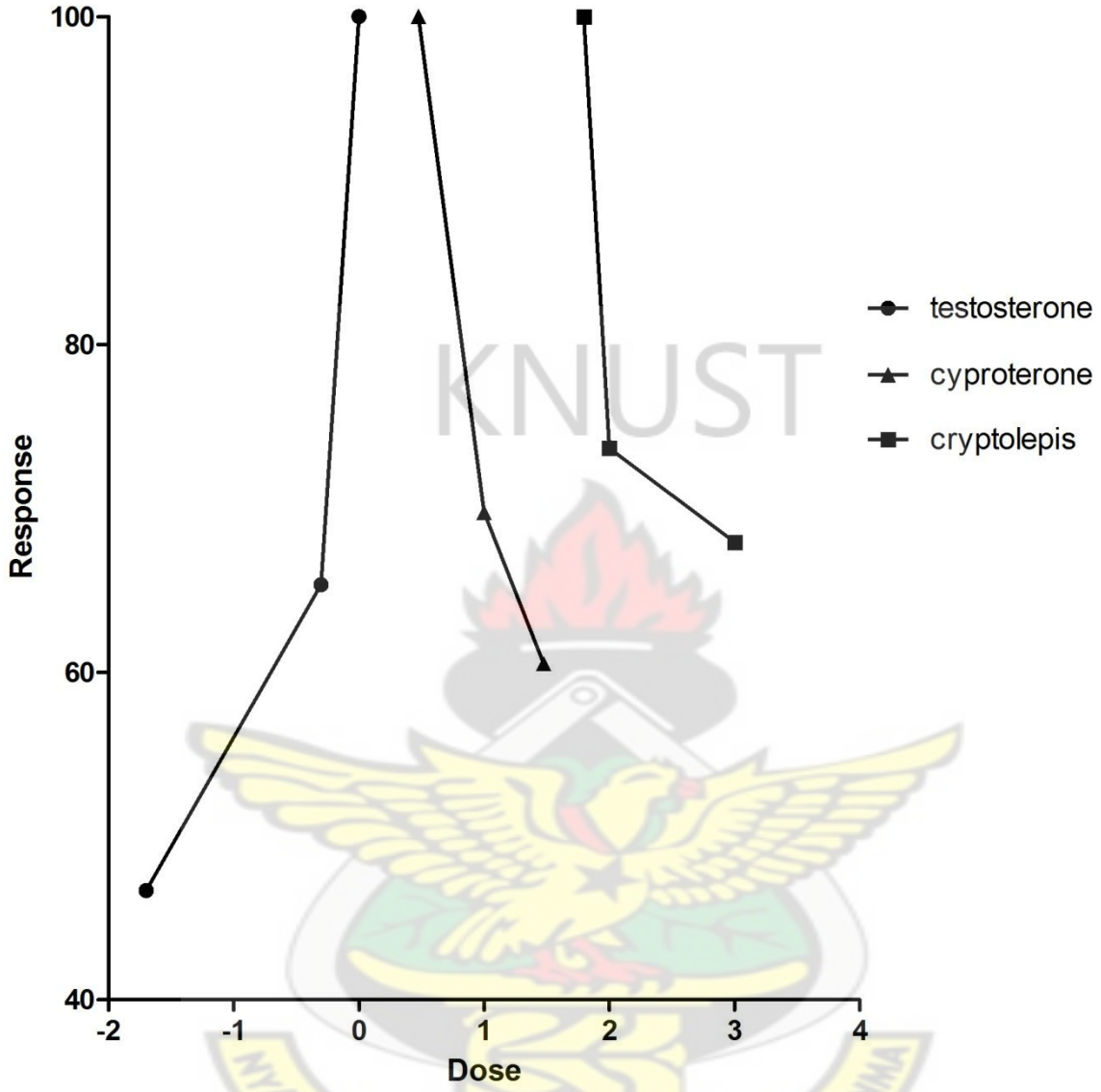


Fig. 6.2 Dose response curves of testosterone, cyproterone and cryptolepis. ED_{50} of testosterone propionate was 0.7 mg/kg, Cyproterone 10 mg/kg, Cryptolepis was 300 mg/kg. Potency ratio of cyproterone to cryptolepis is 1: 31.05

Table 6.3. Comparison of the effects of cryptolepis, cyproterone and testosterone on the chick comb

Dose	% weight change	Initial length	Final length	% change in length	Initial height	Final height	% change in height
Control	16.2± 3.2	1.15 ±0.07	1.58 ± 0.05	38.90 ± 6.730	0.32 ± 0.02	0.55 ± 0.06	144.4 ± 33.79
Testosterone 200 µg/kg	24.6 ±1.97	1.12 ± 0.05	1.85 ± 0.02	66.93 ± 6.130†	0.28 ± 0.03	0.766 ± 0.02	188.9 ± 36.18
Testosterone 0.5 mg/kg	25.0 ±2.97	1.03 ± 0.01	1.83 ± 0.17	78.50 ± 9.448**	0.22 ± 0.05	0.80 ± 0.08	408.3±13.07**
Testosterone 1mg/kg	25.8 ±1.11	1.07± 0.08	2.08 ± 0.13	107.8 ± 12.60***	0.20 ± 0.04	0.88 ± 0.08	420.8±90.47**
Cyproterone 3 mg/kg	20.5 ±2.50	1.23 ± 0.08	1.27± 0.23	34.18 ± 6.283 †††	0.55 ± 0.05	0.55 ± 0.05	72.22±19.56††
Cyproterone 10 mg/kg	24.3 ±2.87	1.20 ± 0.04	1.52 ± 0.05	26.86 ± 5.399 †††	0.57 ± 0.03	0.57 ± 0.03	50.56±11.07††
Cyproterone 30 mg/kg	19.0 ±1.54	1.13 ± 0.07	1.42 ± 0.14	24.61 ± 7.249 †††	0.37 ± 0.03	0.37 ±0.03	35.42±25.50††
Cryptolepis 62.5 mg/kg	17.7 ±5.18	1.20 ± 0.07	1.65 ± 0.04	39.37 ± 6.570 †††	0.67 ± 0.02	0.67 ± 0.02	109.7±20.35††
Cryptolepis 100 mg/kg	18.5 ±2.01	1.22 ± 0.05	1.67± 0.05	37.77 ± 4.599 †††	0.58 ± 0.07	0.58 ± 0.047	77.78 ± 18.09††
Cryptolepis1000 mg/kg	18.2 ±4.25	1.03 ± 0.05	1.43 ± 0.08	38.67± 4.324 †††	0.50 ± 0.06	0.50 ± 0.05	88.89±19.08††

Results is presented as mean ± SEM. Statistical analysis is by one way ANOVA using Newman-Keuls post hoc test.** means $p < 0.01$, *** $p < 0.001$, when compared to control.†† means $p < 0.01$,††† means $p < 0.001$ when compared to testosterone (1 mg/kg)

Control

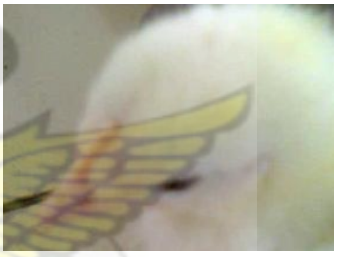
Testosterone 200 μg/kg

Testosterone 0.5 mg/kg

Testosterone 1 mg /kg

Cyproterone 3 mg/kg

Cyproterone 10 mg/kg



Cyproterone 30 mg/kg

Cryptolepis 62.5mg/kg

Cryptolepis 100 mg/kg

Cryptolepis 1000 mg/kg

Fig 6.3 Images of chick combs after a 5-day treatment with testosterone, cyproterone or cryptolepis.

6.3.7 Effects of cryptolepis on testosterone induced growth of chick comb

This study estimated the effects cryptolepis on testosterone-induced comb growth. Treatment of chicks with 300 mg/kg or 600 mg/kg of cryptolepis 30 minutes before the administration of 0.7 mg/kg testosterone resulted in an inhibition of testosterone-induce comb growth. Appearance of wattle was delayed until the fifth day of treatment compared with testosterone only which occurred on the third day. Cryptolepis 300 mg/kg was equipotent with cyproterone 10 mg/kg which was the reference drug. Cryptolepis inhibits testosterone induced comb growth.

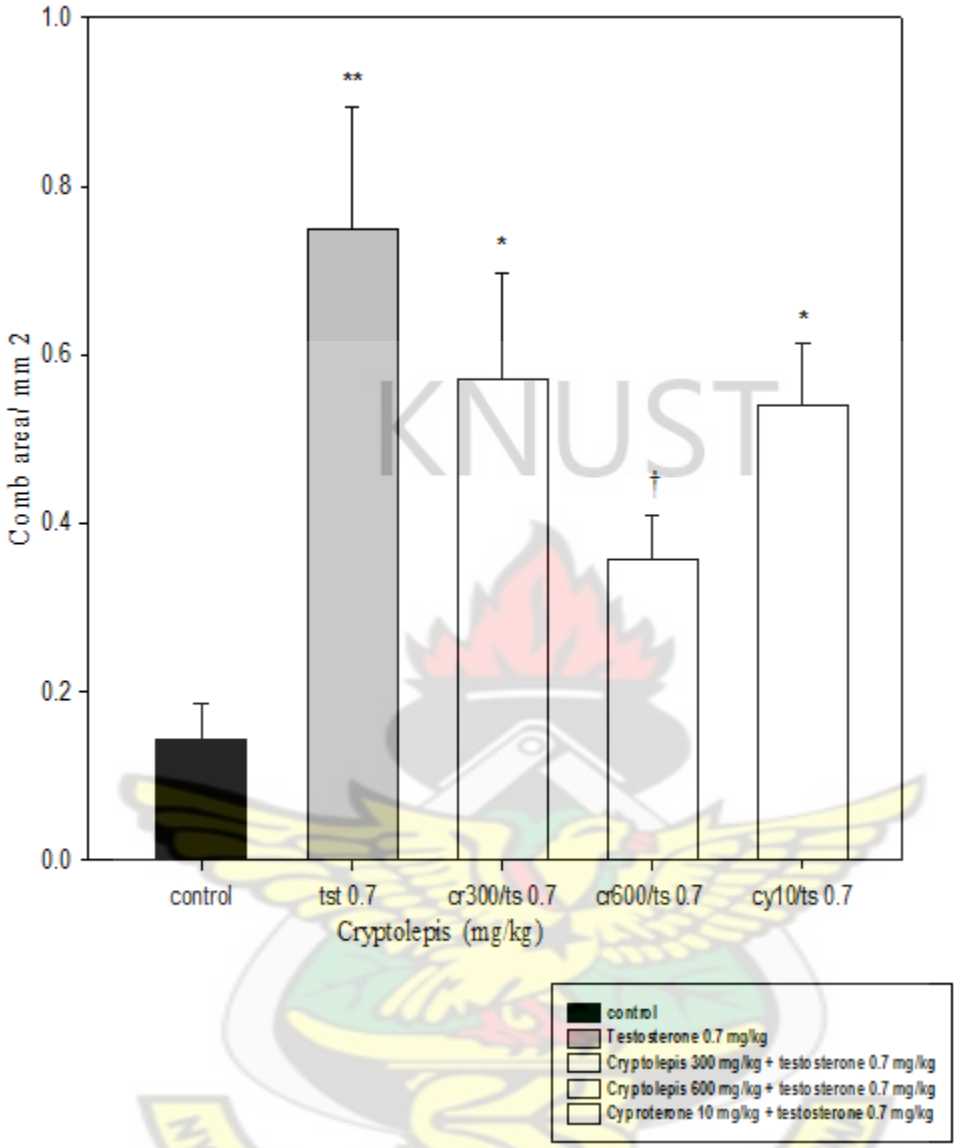


Fig 6.4 Effects of cryptolepis and cyproterone acetate on testosterone propionate. Results presented as mean ± SEM. Statistical analysis is by one way ANOVA. * means ($p < 0.05$) when compared to control. † means ($p < 0.05$) when compared to testosterone propionate

6.4 DISCUSSION

Reproductive toxicity studies require that an effect seen in one kind of animal be demonstrated in another animal of a different species. This increases the likelihood that the observed effect can occur in humans. The rabbit, aside being genetically different from the mouse, possesses some advantages over other animals used in reproductive toxicity studies. The results of the pre ovulatory study show that cryptolepis inhibits ovulation in rabbits. This effect is possessed by substances that can interfere with the activities of prostaglandins. Studies with cryptolepine, the main alkaloid of the aqueous extract, show that it affects the activity of prostaglandin in multiple ways (Bamgbose and Noamesi, 1981; Olajide *et al.*, 2007a; Olajide *et al.*, 2009 Olajide *et al.*, 2010). The activity of cryptolepis was comparable to that of diclofenac which may suggest that the mechanism may be similar as both have potent antiimflammatory activity and interfere with prostaglandin activity. Production of prostaglandin E_2 by cycloxygenase 2 appears to be very essential during inflammation and ovulation and cryptolepis either directly blocks the activity of Prostaglandin E_2 and prevents it from binding to its target site (Bamgbose and Noamesi, 1981) or it reduces the activity of cycloxygenase 2 activity (Olajide *et al.*, 2007a; Olajide *et al.*, 2010) which is essential for prostaglandin E_2 production or both. In addition cryptolepine, the main alkaloid of the aqueous extract, inhibits NF-kB which is essential for inducing the synthesis of cycloxygenase 2 and many other inflammatory mediators (Olajide *et al.*, 2009).

When cryptolepis was administered after ovulation, it had little effect on events in the ovary. However, cryptolepis unlike diclofenac had profound effects on the number of implanted foetuses. There were high number of preimplantation losses with cryptolepis treatment compared to diclofenac treated and controls. This means that cryptolepis could affect fertilization or the

rapid cell division from a zygote to embryo. Cryptolepis is cytotoxic (Ansah and Gooderham, 2002) and cytotoxics almost without exception are toxic to rapidly dividing cells.

Spermatogenesis in the testis is a tightly regulated process. Spermatogonia undergo mitotic proliferation and then meiosis to form primary and secondary spermatocytes (spermacytogenesis). After completion of the second meiotic division, spermatocytes become spermatids and differentiate into spermatozoa (spermiogenesis). In mice, the general guidelines for determining the effects of a chemical on spermatogenesis is that, an effect on progeny outcome after exposure of males to a drug for 1 to 5 days would represent an effect on spermatozoa, most probably those residing in the epididymis (Nomura 1994; Russell 1994). Exposure to a toxicant 10 to 18 days prior to conception would represent an effect on spermatids, while long exposures (35 days or more) prior to conception should represent an effect on spermatogonia.

A two way mechanism for cryptolepis effects on male reproduction is proposed. The first is, cryptolepis induces testicular damage because it is cytotoxic (Ansah and Gooderham, 2002). Photomicrographs of the testes show that cryptolepis treatment may affect virtually all stages of spermatogenesis from spermatogonia through spermatocytes to spermatids because of the effects on seminiferous tubules. Cryptolepis induces damage to the testicles which affects Leydig cells leading to a fall in serum testosterone hence a decrease in spermatogenesis, atrophy and apoptosis of reproductive structures and anti-anabolic effects. This is not surprising because animal data reveal that cytotoxics have a plethora of effects on male germ cells (Cai *et al.*, 1997). Leydig cells are the principal testosterone producing cells. Leydig cells however, unlike other cells, are quite resistant to chemical induced apoptosis (Taylor *et al.*, 1998).

Studies show that androgen withdrawal particularly by orchidectomy leads to atrophy and marked apoptosis in male animal reproductive organs ((Robaire and Fan, 1998). The second mechanism is that cryptolepis interferes with testosterone actions in the body just like cyproterone (Neumann and Von Berswordt-Wallrabe, 1966; Hovatta and Koskimies A., 1978). The drop in serum testosterone seen in mice confirms this mechanism. Furthermore in the chick comb method, cryptolepis inhibited the effects of both endogenous and exogenous testosterone dose dependently. The antagonism to testosterone is manifested by reduction in testicular weight and by the inhibition of spermatogenesis, and apoptosis in the epididymis (Neumann and Von Berswordt-Wallrabe, 1966; Hovatta and Koskimies A., 1978; Robaire and Fan, 1998).

The renal histology shows that cryptolepis treatment induces considerable damage to the kidneys. The bulk of the damage occurred in the renal cortex but major necrotic lesions occurred in the medulla. This appears to be the first demonstration of apoptosis induced by cryptolepis *in vivo*. Apoptosis in renal cells is a little different from that of other organs as apoptosis is usually followed by secondary necrosis.

The reason why the renal cortex is prone to apoptosis is that the cortex constitutes the major portion of the kidney and receives a disproportionately higher percentage (90%) of blood flow compared to the medulla (6–10%) or papilla (1– 2%). Thus, when a blood-borne toxicant is delivered to the kidney, a high percentage of the material is delivered to the cortex and will have a greater opportunity to influence cortical rather than medullary or papillary functions. (Schnellman, 2008)

Renal medullar also presented with necrotic lesions. Unlike the cortical areas that are exposed to blood borne toxicant during each cardiac output, damage to medulla may mean that the tissues are exposed to higher luminal concentrations of toxicants for prolonged periods of time, a

consequence of the more concentrated tubular fluid and the more sluggish flow of blood and filtrate in these regions. Earlier experiments by Ansah *et al.*, (2009a) did not show compromised kidney function or histology with subacute cryptolepis treatment in rats. This may indicate some species differences in the pharmacokinetics of cryptolepis toxicities in animals.

Prostaglandins are essential for renal function. The kidney synthesizes prostaglandins from different location, including afferent and efferent arteriole, glomeruli, and tubular system. It is therefore possible that the effects of cryptolepis may be related to its inhibition of the activity of prostaglandins. COX-2 null mice develop severe nephropathy (Williams *et al.*, 1999; Buttar and Wang, 2000). In humans selective COX-2 inhibition causes sodium and water retention and transient decrease in glomerular filtration rate (Komhoff *et al.*, 1997; Rossat *et al.*, 1999).

Data are limited on whether COX-2 inhibitors are less nephrotoxic than the classical NSAIDs. In COX-2 knockout mice, significantly reduced synthesis of renin is seen in both baseline condition and low-salt intake (Yang *et al.*, 2000a) indicating an association of COX-2 with renin synthesis. COX-2 inhibitors were reported to be associated with acute renal failure, hyperkalemia and increased risk for renal insufficiency (Perazella, 2001).

This chapter has demonstrated that cryptolepis inhibits ovulation in rabbits by inhibition of protaglandins. It also causes preimplantation losses after ovulation.

It affects testicular histology. It affects male reproduction by reducing serum testosterone level and also inhibits testosterone induced comb growth.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

CHAPTER SEVEN

7.1 GENERAL DISCUSSION

Safety of herbal medicines has always been a controversial issue in health partly due to the limited information on them. Users of herbal medicines and researchers alike tend to lay greater emphasis on the therapeutic effects whilst neglecting toxic effects. The argument put forward is that herbs cannot have therapeutic effects without toxic effects. Incidences of herbal toxicities are few in literature probably because most appear to have wide therapeutic windows. Moreover toxicities which are not acute are not easily recognized and reported. This makes it difficult to establish an association of a toxic effect with a particular herb. Unfortunately this has led to the practice of administering herbal medicines without strict dosing regimen as pertains to orthodox medicines.

Results from animal toxicological assays cannot be directly extrapolated to mean outcomes in humans. However, it provides the basis for prediction of possible human doses, toxicity and sets targets for other preclinical and clinical studies. A United State Environmental Protection Agency (USEPA) report in 1991 emphasized the relevance of animal studies in developmental toxicity studies. The USEPA reported that several environmental agents were established as causing developmental toxicity in humans while many others are suspected of causing developmental toxicity in humans on the basis of data from experimental animal studies (USEPA, 1991).

In comparing data for several agents identified as causing human developmental toxicity to the experimental animal data, the USEPA said the agents causing human developmental toxicity in almost all cases were found to produce effects in experimental animal studies and, in at least one

species tested, types of effects similar to those in humans were generally seen. Furthermore, when careful dose response comparisons were done, taking factors such as route, timing, and duration of exposure into account, the minimally effective dose for the most sensitive animal species was generally higher than that for humans, usually within 10-fold of the human effective dose, but sometimes was 100 times or more higher e.g. polychlorinated biphenyls (Tilson *et al.*, 1990; USEPA, 1991). This information provides a strong basis for the use of animal data in conducting human health risk assessments.

The aqueous extract of *Cryptolepis sanguinolenta* has been used for generations for the treatment of malaria and other ailments. After over a decade of meticulous toxicity studies on cryptolepis and its alkaloid cryptolepine, a wide gap has developed as *in vitro* toxicity studies results do not appear to correlate with *in vivo* outcomes. Whilst almost all *in vitro* assays exclusively report on cytotoxicity in animal cells with relevant mechanisms (Bonjean *et al.*, 1998; Dassonneville and Bonjean, 1999; Lisgarten *et al.*, 2001; Ansah and Gooderham, 2002; Ansah *et al.*, 2005; Zhu *et al.*, 2005; Zhu and Gooderham, 2006; Ansah *et al.*, 2008 c; Ansah and Gooderham, 2009 b), *in vivo* studies show its high safety profile (Ansah *et al.*, 2008a; Ansah *et al.*, 2009a).

Cryptolepis is an antimalarial and hence may be used by men, women and children. Animal studies show clearly that cryptolepis adversely affect female reproduction. As to whether cryptolepis should be encouraged in women for treatment of malaria may have to be determined on case by case bases. Cryptolepis appears to have minimal effects on follicular stages of development in the ovary with the exception of the Graafian follicle. Cryptolepis inhibits ovulation by preventing Graafian follicle rupture. For a woman with no intentions of conceiving, cryptolepis treatment may be indicated for malaria and other ailments. For women wanting to conceive or undergoing fertility treatment, it will be advisable to withhold cryptolepis treatments

as treatment may reduce the chances of successful conception. A key point noteworthy about cryptolepis is that unlike other antiinflammatory drugs that can only inhibit ovulation, cryptolepis affects postovulatory processes as well. Consistently it induced embryolethality. The most sensitive period for cryptolepis-induced embryolethality is the time from ovulation to implantation. Implantation in humans occurs between 5 - 7 days after conception. In humans one of the periods of high embryo susceptibility is the period between conception and implantation. Studies using very sensitive early pregnancy tests have found that 25% of embryos are miscarried by the sixth week since the woman's last menstrual period, even if a woman does not realize it (Wilcox *et al.*, 1999; Wang *et al.*, 2003). As to whether women taking cryptolepis consistently abort early pregnancies without being aware is unknown. An early abortion may only be indicated by a five to ten day delay in the onset of the menstrual bleeding (Laurino *et al.*, 2005).

One issue of human reproduction is the obsession of some would-be parents about the sex of the child. The reproductive studies in mice showed that treatment with cryptolepis may not affect the chances of having a male or female offspring. Work by Gupta and Goldman, (1986) showed that agents that block the arachidonic acid cascade at the level of phospholipase A_2 or at the level of cyclooxygenase (indomethacin, aspirin) also block masculine differentiation in male embryos. That is, the masculinisation of male embryos is inhibited and the masculinisation of female embryos produced by exogenous testosterone is also prevented. Cryptolepis interferes with COX -2 (Olajide *et al.*, 2007a; Olajide *et al.*, 2009; Olajide *et al.*, 2010). In addition to that, this study found that it antagonizes the actions of testosterone. Although cryptolepis is unlikely to change the sex of an animal, it has the potential to inhibit musculinization in male animals. This calls for caution in its use in pregnancy.

Treatment with cryptolepis may not lead to teratogenesis of external morphology although it may induce significant intrauterine growth inhibitions. In the present study, cryptolepis was functionally toxic to animals treated prenatally with it. It may affect neurogenesis leading to alterations in behaviour, growth, memory and anxiety in exposed offspring. The mechanism of this could partly be mediated by maternal effects. From functional toxicity studies, neurological structures and functions likely to be altered by prenatal cryptolepis treatment include; 7^{th} and 8^{th} cranial nerves, vestibular apparatus, cerebellum, hypothalamus-hypophysis-gonad axis and the hippocampus.

The study shows that there may be very little justification for using cryptolepis in men for purposes of reproduction as it appears to affect all aspects of male reproduction negatively i.e. reducing sperm count, causing testicular atrophy and reducing serum testosterone level. These effects account for the low sexual behaviour in mounting behavioural studies after subacute treatment with cryptolepis. Furthermore this finding calls for concern on the safety of aqueous cryptolepis as an antimalarial in men. It is proposed that caution be exercised in men susceptible to recurrent malaria attacks who may use cryptolepis more frequently than the general population.

Histopathological studies show that a principal target for cryptolepis toxicity is the kidney. The renal toxicity appears to have an earlier onset than the fourteen days used in the study and occurs at doses as low as 100 mg/kg. There could also be a possible species effect as earlier work by (Ansah *et al.*, 2009a) in rats did not show changes in renal function and integrity. Nonetheless, this is a potential risk as there is also the possibility of people who use cryptolepis for malaria to

add on a non steroidal anti-inflammatory drug to their treatment regimen. This practice may increase renal toxicity induced by cryptolepis and provoke renal failure. This is because NSAIDS inhibit renal prostaglandin synthesis and cryptolepis, and cryptolepine (Dwuma-Badu *et al.*, 1978), the main alkaloid of the aqueous extract have potent anti inflammatory activity likely due to interference with COX-2 activity (Olajide *et al.*, 2010). The kidney is a target because the kidney is one of few organs in the body where COX-2 is a constitutive enzyme and relies heavy on prostaglandins for renal perfusion.

Several mechanisms are believed to be responsible for the reproductive and developmental toxicities of cryptolepis in experimental animals; cycloxygenase 2 inhibition (Olajide *et al.*, 2010), cytotoxicity (Ansah and Gooderham, 2002), inhibition of inducible nitric oxide synthase activity (Olajide *et al.*, 2007b) and inhibition of testosterone. Several studies show that these mechanisms may be linked and are interrelated due the multiple roles of prostaglandins in inflammation, cell proliferation or tumourigenesis and reproduction. Perhaps the main reason why cryptolepis and cryptolepine have shown very potent cytotoxic, anti-inflammatory and reproductive activities is due to its effects on prostaglandin E_2 (first reported by Bamgbose and Noamesi 1981) and NF-kB (Olajide *et al.*, 2007b). Ovulation, inflammation and tumourigenesis are closely linked and are all mediated by prostaglandin E_2 activity.

For example, it is known that inflammation is closely linked to tumour promotion; substances with potent antiinflammatory activities (NSAIDS) exert chemoprotectective effects in tumouriogenenesis particularly in the promotional stages (Thun *et al.*, 1991; Kawamori *et al.*, 1998). The mechanism of this effect is related to the activity of COX-2 and iNOS. PGE_2 is associated with cancers of breast, colon, lung, pancreas and head and neck in humans (Goodwin and Ceuppens, 1983; Qiao *et al.*, 1995; Tsujii and DuBois, 1995). Over expression as well as

inappropriate induction of COX-2, has been observed in many different types of tumours where it promotes Bcl-2 protein expression (antiapoptotic factor), induces iNOS, diminished levels of transforming growth factor beta-2 receptor and E-cadherin and prolongs the survival of malignant and tumour cells (Xie and Herschman, 1995; DuBois *et al.*, 1996; Muller-Decker *et al.*, 1998). Hence Studies show that COX-2 inhibitors suppress proliferation and induced apoptosis in certain cancer cells (Chan *et al.*, 1998; Zimmerman *et al.*, 1999; Grossman *et al.*, 2000) to an extent that even the expression of antiapoptotic factors such as Bcl-2 by the cells is not able to protect them (Hsu *et al.*, 2000).

Ovulation is also very analogous to inflammation. Both processes are mediated by the induction of COX-2. The preovulatory follicle exhibits the redness, swelling and (occasionally) pain that are the gross manifestations of inflammation. Immune cells are present in the mature follicle (Chun *et al.*, 1993), as well as mediators of inflammation such as vasoactive substances, eicosanoids, interleukins, tumour necrosis factor and chemotactic substances (Espey, 1994). COX-2 induction precedes ovulation and is mediated by gonadotropin acitivty (Sirois *et al.*, 1992; Sirois *et al.*, 2004). It is believed that prostaglandins, particularly E_2 cause ovum release (Espey, 1980; Espey and Lipner, 1994). COX-2 selective inhibitors induce anovulatory condition in mammals where there are clinical signs of ovulation but there is no ovum release. This has also been seen in young women with rheumatoid arthritis on NSAIDs undergoing fertility treatments (Zanagnolo *et al.*, 1996; Skomsvoll *et al.*, 2005).

Prostaglandins have varied effects on mammalian reproduction. In males they interact with testosterone, and nitric oxide in modulating reproductive function. For mammals, seminal PGs are produced mainly in the accessory glands of the reproductive tract, and PG have been shown

in *vitro* to affect sperm motility, capacitation, and the acrosome reaction. In general, prostaglandins of the E series stimulate sperm motility, whereas PGF2α inhibits motility (Colon *et al.,* 1998). Studies of human sperm have shown that prostaglandins of the E series promote calcium influx via a receptor-linked mechanism that is capable of inducing the acrosome reaction (Schaefer *et al*. 1998). Neeraja *et al.*, (2003) reported the expression of COX-2 enzyme in testicles of mature and immature rats and the increased expression of the COX-2 gene upon testosterone administration.

It is therefore not surprising that cryptolepis and its main alkaloid cryptolepine in *in vitro* assay increased expression of p53 (proapoptotic) and its target proteins, diminished anti apoptotic factors, decreases the activity of NF-kB (Olajide 2007b) which is responsible for inducing the expressing of genes COX-2 and iNOS and causes multiple reproductive problems *in vivo* as all these effects are linked to inhibition of prostaglandin E_2 and NF-kB (Olajide 2007b).

Now, a possible mechanism for the reproductive and developmental toxicity of cryptolepis is proposed in the schematic diagram in Fig 7.1

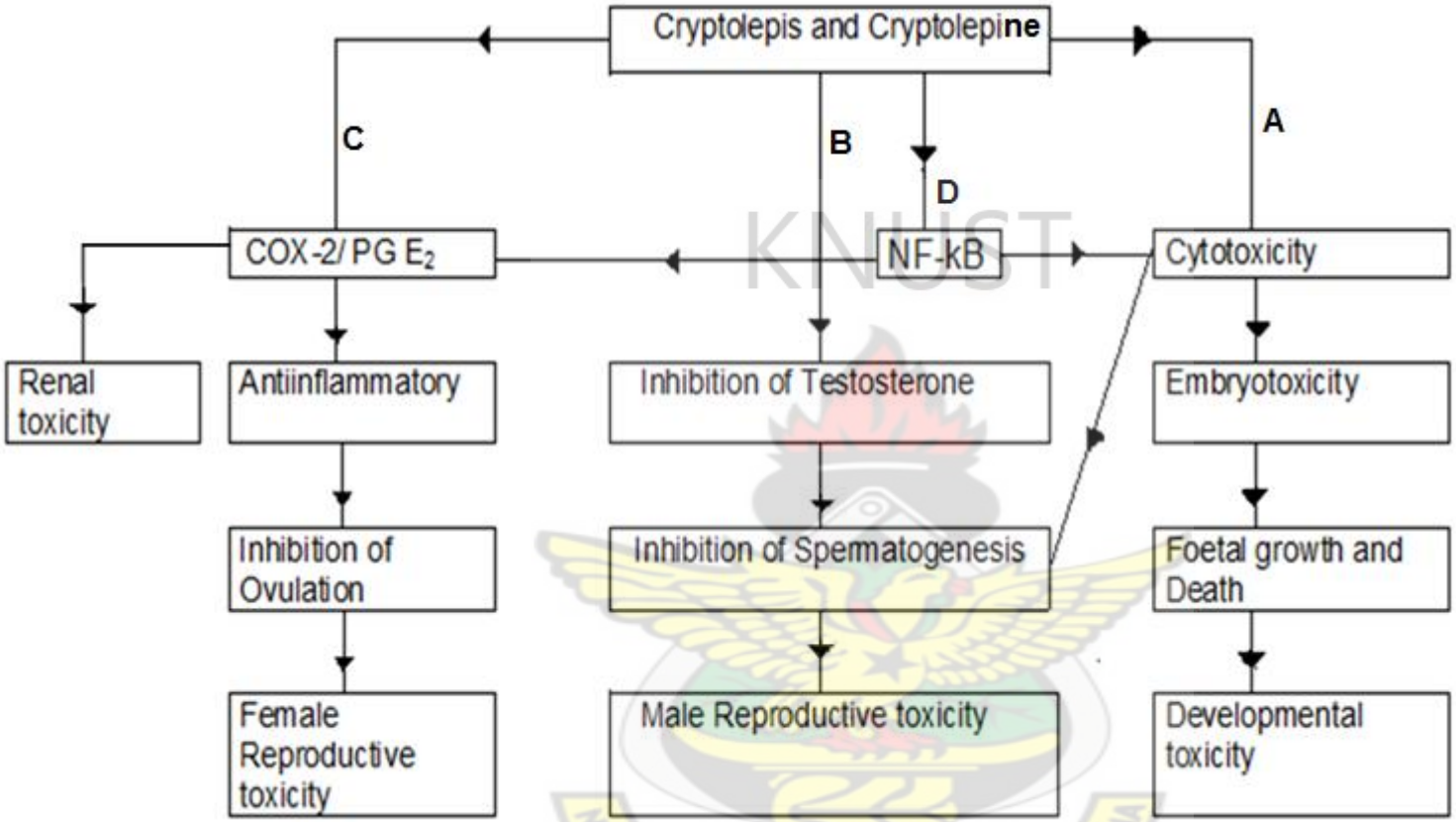


Fig 7.1 Proposed mechanisms of cryptolepis induced Reproductive and Developmental toxicities. Cryptolepis is cytotoxic hence it inhibits foetal growth, spermatogenesis and causes foetal mortalities (A). It also inhibits spermatogenesis by reducing testosterone levels leading to male reproductive toxicity (B). Cryptolepis inhibits ovulation and causes renal toxicity by interfering with COX-2 and PG E_2 activity (C). Cryptolepis also interferes with the activity of NF-kB (D) (an inducer of early inflammatory genes such as COX-2, inducible Nitric oxide synthase and inhibitor of proapoptotic gene, protein 53) leading to cytotoxicity, inhibition of spermatogenesis and ovulation and renal toxicity.

7.2 CONCLUSIONS

- Overall, the studies have shown that pretreatment of female mice with cryptolepis does not affect fertility, gestation period, live birth, sex ratio and gestation period.
- Treatment of female mice with cryptolepis during mating and gestation affects fertility and the number of live births but not the gestation period, sex ratio or mating behaviour.
- Cryptolepis affects embryonic and foetal development in mice by inducing intrauterine growth inhibitions, embryolethality and functional toxicities. However, there was no evidence of teratogenesis in mice.
- Cryptolepis provokes maternal toxicities; maternal mortalities and weight loss and alters maternal behaviour of mice.
- Cryptolepis inhibits ovulation and post ovulatory events in the rabbit possibly by inhibiting the activity of COX-2 and/or inhibiting rapid cell proliferation
- Cryptolepis is a weak mutagen by the male dominant lethal assay.
- Cryptolepis at best has weak aphrodisiac potential in mice
- Cryptolepis affects fertility of male mice by reducing epididymal sperm number and serum testosterone.
- Cryptolepis treatment inhibits comb growth in chicks and exogenous testosterone induced comb growth in chicks by reduction of testosterone levels.
- Cryptolepis causes renal damage in mice by inducing apoptotic-like cell death.

7.3 RECOMMENDATIONS

In view of the reproductive toxicity profile of cryptolepis in experimental animals, this study recommends that caution should be exercised in its use during reproduction and foetal development in females. Chronic use of cryptolepis in males as aphrodisiacs and as antimalarial in men who are prone to recurrent malaria attacks should be discouraged as it may affect spermatogenesis and sperm counts.

Cryptolepis use may lead to renal damage possibly by interaction with prostaglandin synthesis. The use of cryptolepis with NSAIDs in the management of malaria and other ailments may potentiate renal toxicity of cryptolepis as both substances interfere with prostaglandin synthesis.

7.4 FUTURE WORK

Though the results from animal studies cannot be directly extrapolated to man, the results of the present study suggest that cryptolepis and its alkaloids have the potential to be used as contraceptives. The potential of cryptolepis and its alkaloids should be further studied.

The potent cytotoxicity of cryptolepis and its additional anti-androgenic effects demonstrated in the present study suggest that it may have a role in the management of prostate cancer. This potential of cryptolepis should be exploited.

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