Khat-induced Reproductive Dysfunction in Male Rabbits.

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A thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Science in Comparative Mammalian Physiology of the University of Nairobi.

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DECLARATION

This thesis is my original work and has not been submitted for a degree in any University.

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DEDICATION

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SUMMARY

The psychostimulating herbal drug khat (*Catha edulis* Forsk) is habitually used by inhabitants around the horn of Africa, including East Africa and Arabian Peninsula, but is also imported in other countries. Khat has been shown to cause reproductive dysfunction in humans and experimental animals. However, the mechanism by which it influences reproductive parameters is not known. This study investigated the mechanism of action of khat on reproductive function in male rabbits. Twenty five male New Zealand White rabbits were divided into 5 groups of 5 animals each and administered with 0, 1.5, 4.5, 13.5 and 40.5 g/kg body weight of khat extract, respectively, via intra-gastric tube. Blood samples were collected at 15 minutes interval for up to 3 hr after khat administration and plasma assayed for levels of luteinising hormone (LH), testosterone and cortisol using radioimmunoassay technique. For histopathological examination, animals except for 4.5 g/kg body weight of khat extract were used. Two animals from each group were hemicastrated 7 days after khat extract administration to study acute effects while the rest of the animals were sacrificed after 58 days of khat administration and pituitary, testes and epididymis harvested to study chronic effects. The hormonal, haematological as well as data on clinical observation for difference in means among groups and over experimental time period was analysed by two-way ANOVA followed by Tukey’s multiple comparison post hoc test using SPSS statistical package.

Khat extract significantly decreased the pulse frequency, pulse amplitude, area under LH curve and mean plasma bioactive LH concentration (P<0.05) in all khat-treated rabbits as compared to saline-treated controls. Pulsatilite pattern of LH was observed in controls over 3-hour experimental period. The mean plasma testosterone and area under response
curve decreased significantly (P<0.05) at all dosages in khat-treated rabbits as compared to controls. Khat extract also produced a dose-dependent decrease in food consumption, water intake and body weight gain. There was a significant increase in core body temperature in khat-treated rabbits (P<0.05) as compared to saline-treated controls. The haematological parameters (i.e. Packed cell volume, red blood cell count and haemoglobin concentration) showed a significant decrease (P<0.05) in khat-treated rabbits as compared to controls, whereas the white blood cell count showed a significant increase in khat-treated rabbits (P<0.05) as compared to controls.

Histopathological examination of the testes revealed vacuolation as well as degeneration of the zygotene spermatocytes. There were no observable lesions in cauda and caput epididymis of khat-treated rabbits. The pituitary of khat-treated rabbits revealed no histopathological changes in khat-treated rabbits when compared to saline-treated controls. It is concluded that khat extract may have caused hypothalamo-hypophyseal and testicular hypofunction, which resulted into the observed reproductive endocrine changes.
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CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Khat, *Catha edulis* Forsk (family *Ceslestraceae*), is a flowering evergreen shrub or small tree that grows wild or is cultivated in certain regions of East Africa and Southern Arabia (Patel, 2000). Emigrants from these countries try to maintain this habit of khat chewing (Rousseau *et al*., 1998; Ahmed and Salib, 1998), and large quantities of fresh khat are imported into other areas of the world (Mayberry *et al*., 1984; Kalix and Braenden, 1985; Kalix, 1994).

Khat is used as a recreational drug (i.e during leisure time) by inhabitants of areas where it is grown, and fresh leaves and shoots of this plant, which contain the naturally occurring alkaloid, cathinone, provides euphoric and psychostimulant effects to the user (Luqman and Danowski, 1976; Baasher, 1980; Brenneisen *et al*., 1990; Kalix, 1992).

Since only fresh leaves have the desired effects, the khat use is abundant in areas where it is grown. However, because of the possibilities of modern transportation, khat is now becoming reasonably accessible in many parts of Europe (Al Motarreb *et al*., 2002). A much larger section of the population in many parts of the world now use khat and it is estimated that, at present, between five and ten million people chew khat each day. Many of them have become compulsive khat users and develop psychic dependence on the drug. Furthermore, with the development of international air travel, khat use has spread to countries far away from the areas of cultivation, in particular through the movement of emigrants. (Kalix, 1984b).
The medical problems that arise from khat consumption are partly due to its effect on mental health, and partly as a result of the sympathomimetic effects of the drug. The effects of khat were described centuries ago in the Arabic medical literature, and knowledge of the khat habit came to Europe mainly through the travel accounts of Carsten Niebuhr at the end of the eighteenth century (Krikorian, 1983). Toxic effects of khat in humans have been studied over the last twenty years and include multiple symptoms such as euphoria, anorexia, insomnia, gastritis, hypertension and impotence (Hill and Gibson, 1987). Cocaine, amphetamine and other psychostimulants have been shown to influence negatively on reproductive function via alteration of mesocorticolimbic dopamine neurotransmission as a result of stress (Marinelli and Piazza, 2002). Dopamine inhibits gonadotropin release by binding to receptors on basophils thereby activating a membrane inhibiting G protein which is negatively coupled to adenyl cyclase and phospholipase system (Yen, 1991). Findings from several prior investigators have shown that stress and sympathomimetic agents suppress gonadal function leading to reduced testosterone production (Gray et al., 1978). Cocaine, amphetamine and other psychostimulants have been shown to have a negative effect on reproductive function via alteration of mesocorticolimbic dopamine transmission due to stress (Marinelli and Piazza, 2002). Findings from several prior investigators have also shown that plasma levels of glucocorticoids and ACTH are increased by acute administration of amphetamine in both rodents and humans (Halbreich et al., 1981; Jacobs et al., 1989; Smith et al., 2004; Swerdlow et al., 1993).
Although khat has been reported to cause impotence in male addicts (Halbach, 1979), not much is known about its mechanism of action on the neuroendocrine system. Thus, the aim of this study was to investigate the effect of crude khat extract on reproductive hormones (LH and testosterone) as well as cortisol and examine the morphological features of the pituitary gland, testes and epididymides for any pathology.

1.2 LITERATURE REVIEW

1.2.1 KHAT: BACKGROUND, GEOGRAPHICAL DISTRIBUTION AND FREQUENCY OF USE

1.2.1.1 Background

Historically, the original source of khat seems obscure but it is agreed that its use was prevalent in Ethiopia and from there, around the fifteenth century, the habit of khat chewing spread to the south west of the Arabian Peninsula (Peters, 1952). Arab sources suggest that khat was introduced in Yemen in the sixteenth century by Ethiopian conquest. Earliest reference to this plant appears to be dated around 973 to 1053 A.D by Al- Biurni, who meticulously compiled information on all contemporary drugs, and named the drug qat, imported from Turkistan. The earliest scientific report on khat presented to a western country was in the eighteenth century, when Peter Forskal, identified the plant in Yemen and called it *Catha edulis* (Dhaifalah and Šantavý, 2004).

Khat has various local names, for instance, it is known as miraa in Kenya. Other names include: qat, tschat, African salad, African tea, Abyssynian tea, kuses-salahin and tohai. Some chewers also refer to it as ‘gomba’. The leaves come from a small evergreen tree,
which grows at high altitudes extending from East to Southern Africa, as well as Afghanistan, Yemen and Madagascar (Krikorian, 1984; Kennedy, 1987). In Kenya, there are different types of miraa namely: Giza, kangeta and kata. Giza has short stems and is said to be more potent. Kangeta has long stems, while kata is dry khat. The cost of the khat varies with the quality and the season, with the most expensive being around Kshs.350/= ($4.73) ‘a kilo’ and the cheapest around Kshs. 250/= ($3.38) a bundle.

Historically, khat has been used for medicinal purposes (Kennedy et al., 1983) as well as an aphrodisiac (Margetts, 1967; Krikorian, 1984), though it was also used for recreational purposes (Kennedy, 1987). It is also valued for its stimulant effects (Baasher, 1980). Though chewing is the commonest mode of administration, it has also been taken as tea and even smoked (Hodgkinson, 1962; Kennedy, 1987). Khat must be chewed while fresh and, usually, it is wrapped in fresh banana leaves immediately after picking to preserve its potency (Elmi, 1983a).

### 1.2.1.2 Geographical Distribution

Khat plant is widely distributed in Africa. It grows in habitats varying from evergreen submontane forest to deciduous woodlands and is an indigenous plant in Ethiopia, Kenya, Somalia, Djibouti, Uganda, Tanzania, Zambia, South Africa and Yemen. In Ethiopia and some parts of East Africa, it is the basis of life style and plays a dominant role in celebrations, marriages, and political meetings. In the United States it is now a controlled substance (Dalu, 2000; Al Motarreb et al., 2002). Cases of khat-induced psychoses have been reported in the United States and Great Britain (Giannini and Castellani, 1982; Gough and Cookson, 1984).
1.2.1.3 Frequency and distribution of use

The use of khat often starts at a young age (about ten years old) and can develop into a compulsive daily habit lasting a lifetime. It is estimated that there are five to ten million regular khat users worldwide (Kalix, 1984) and the number of users is increasing (Kennedy, 1987). The stimulating and euphorogenic effects of khat can provide a strong inducement for the user to obtain daily supply and to have long khat chewing periods causing psychic dependence in the user (Kennedy et al., 1980). The prevalence varies widely among the various khat using countries (Mancioli and Parrinello, 1967; Omolo and Dhadphale, 1987a). In Somalia, it is estimated that about 18% in the South and 55% in the North are consumers (Elmi, 1983a, b). The ‘khat party’, which is described in detail by Baasher (1980; 1983; Kalix, 1984a; Weir, 1985; Giannini et al., 1986; Kennedy, 1987), is a predominantly male pastime, though women are occasionally involved (Elmi, 1983a; Luqman and Danowski, 1976). Kennedy et al., (1983) estimates that approximately 50 to 60% of women chew khat more than once a week compared to 80 to 85% of men. In countries such as Yemen and Somalia many houses have a room specifically for chewing khat (Baasher, 1980).

While Muslims are the most avid chewers (Krikorian, 1984), many Christians and Yemenite Jews in Israel use khat (Kalix, 1987). In Yemen, khat is regarded as beneficial although it is considered desirable to prevent the younger generation from developing the habit (McKee, 1987). Newspaper reports suggest that khat has only recently been introduced to the United Kingdom (Hogg and Rogers, 1985; Busby, 1987). Its use is confined to those ethnic communities accustomed to it particularly in East London, the
Somali community in Liverpool (Gough and Cookson, 1984, 1987) and the Yemeni community in South Wales (Mayberry et al., 1984). There are, presently, no legal restrictions to its sale.

In 1973, the World Health Organization Expert Committee on Drug Dependence included khat type preparations of *Catha edulis*, in the group of ‘dependence-producing drugs’. In their 22nd report (WHO, 1985), cathine was assessed as having a central stimulating action similar to amphetamine but 7 to 10 times less potent. Similarly, cathomone was regarded as a central nervous system stimulant about half as potent as amphetamine. It was therefore felt that both compounds met the criteria for control under the Convention on Psychotropic Substances (Pantelis et al., 1989). Like other drugs of abuse, its effects are mediated through alteration of brain neurotransmitters in the areas of the limbic system which have been associated with the pleasure reward mechanism of the brain (Nahas, 1981). Khat is deeply rooted in the socio-cultural traditions of several countries, where it was practised by a limited segment of the population in a well-defined and stable social setting. In recent years, however, khat is being used by many people far from areas where it is grown. This phenomenon can be explained, not only by the advent of modern transportation, but also by social and cultural changes that have taken place in these countries in the 20th century (Dhaifalah and Šantavỳ, 2004).

Eddy et al. (1965) assessed khat as causing a moderate but often persistent psychic dependence with no physical dependence or tolerance. This is in contrast with the marked tolerance associated with amphetamine abuse (Kennedy et al., 1980; Kennedy, 1987).
This lack of any appreciable tolerance to khat may be due to the physical limits on the amount that can be chewed (Halbach, 1972). Tolerance may, however, develop to the sympathomimetic effects of khat (Nencini et al., 1984a). Mydriasis, hyperthermia and dryness of the mouth occur as a result of sympathomimetic effect of khat (Dhaifalah and Šantavý, 2004). Kennedy (1987) proposed that heavy khat chewers experience true withdrawal symptoms, albeit relatively weak, of profound lassitude, anergia, difficulty in initiating their normal activities and a slight trembling, several days after ceasing to chew. In addition, he reported extremely unpleasant dreams often of a paranoid nature of being attacked, strangled or followed. These he interpreted as rebound phenomena rather than a specific abstinence syndrome. It is considered that there are no physical symptoms on withdrawal from khat (Luqman and Danowski, 1976; Giannini et al., 1986). The effect of khat withdrawal on health is generally beneficial with improvement in sleep and appetite, fewer problems due to constipation and decrease in alcohol consumption (Luqman and Danowski, 1976).

1.2.2 SOCIO-ECONOMIC EFFECTS OF KHAT

In primitive societies, religion, culture and tradition were the primary regulatory factors in controlling the use of mind-altering drugs. The cultivation and use of khat has profound socio-economic consequences for the countries concerned and has made a considerable impact on the life of individuals. Khat is consumed widely in the countries around the horn of Africa. More than one thousand dollars change hands daily in the 3,000 square metre market area of Awedaye in Harar-Ethiopia, which attracts more than 2,500 traders from the surrounding regions. In Kenya, khat sales fetch at least $ 820, 512 annually, according to the report by Nyambene Miraa Traders Association published in a
local daily newspaper. It is the main source of livelihood to many peasant farmers in Meru District, Kenya. Khat has also been used traditionally as a social drug and this still is the case in Yemen (Weir, 1985).

Khat chewing is more prevalent in males (Al Motarreb et al., 2002), often resulting into family strains through negligence of family duties and dissipation of family income. Many of them spend money on khat while neglecting their vital needs, which indicates psychic dependence on the drug (Ahmed and Salib, 1998). At khat gatherings, guests are distributed in accordance with their familial, social, or political importance and prestige. Each side of the room accommodates six to ten persons. One or two communal tobacco pipes or “hubble bubbles” stand in the centre, and a flexible tube is passed from guest to guest. During this time drinks such as cola, weak black tea, or just cold water are available. Here chewers begin to chew the leaves one by one as they swallow the juice while spitting the residues. Usually a participant takes between 100 to 200 g of khat a day. The young leaves or twigs are favoured because of their potency and tenderness (Luqman and Danowski, 1976).

Khat has been implicated as a causal factor for family instability (Elmi, 1983b), divorce (Baasher and Sadoun, 1983), encouragement of prostitution and criminal behaviour (Elmi, 1983b). Baasher (1980) estimated that in cities of Somalia and Yemen, a consumer spends about 25% of his daily earnings on khat. The cultivation of khat results in decreased production of other more essential crops like cereals thereby promoting malnutrition and disease (Murad, 1983). It also leads to low productivity due to
absenteeism and the after-effects of its use (Halbach, 1972, 1979; Elmi, 1983b; Giannini et al., 1986; Kalix, 1987). At the same time it is a major source of revenue. The concomitant use of alcohol to counteract the stimulant and insomniac effects of khat (Kennedy, 1987; Omolo and Dhadphale, 1987b) raises the risk of complications from both these drugs (Omolo and Dhadphale, 1987b).

The khat session also serves an important social function in Yemeni society (Weir, 1985; Kennedy, 1987). Despite the reduction in working hours spent on chewing khat (Eddy et al., 1965; Halbach, 1979), these sessions can provide an arena for communication where serious exchange of ideas and information take place (Kennedy, 1987). In modern times, secular rulers have attempted to curtail the use of these drugs by laws and edict. During this century, these regulations have been channelled through the offices of the United Nations and its specialized agencies, which have singled out “dependence producing drugs” (Nahas, 1981). The medical and socio-economic impact of khat use on society generates regular, at times heated debate on whether or not khat should be considered a drug of abuse and be banned, or it should be looked upon as an innocuous stimulant like caffeine conducive to social interaction. The medical problems that arise from khat consumption are partly due to the sympathomimetic effects of the drug and partly as a result of its effect on mental health (Balint and Balint, 1994). Because of this, the status of khat and cathinone (a labile substance present mainly in young fresh leaves) varies among countries. Most countries of eastern Africa and Arabian Peninsula allow free possession and use of khat while in the United States, khat is classified as a Schedule IV substance and cathinone as a Schedule I drug by the Drug Enforcement Agency. The

1.2.3 ACTIVE INGREDIENT OF KHAT

The first attempts to isolate the active principles of khat were made about a century ago (Flückiger and Gerock, 1887). It was Wolfes who in 1930 identified the presence of an alkaloid, S, S-(+)-norpseudoephedrine (NPE) also known as cathine in khat leaves. Until the beginning of 1960s, this substance was generally considered to be the main active principle responsible for the effects of khat (Alles et al., 1961), although it had been stated in 1941 by Von Brüke that the amount of NPE present in the khat is insufficient to account for the symptoms produced (Kalix, 1984a).

The variance in these findings led to the reinvestigation of the plant. Fresh and freeze-dried material obtained from Yemen, Kenya and Madagascar were analysed by United Nations Narcotics Laboratory culminating in the isolation of a keto analogue of cathine from fresh leaves of khat in 1975 (United Nations Narcotic Laboratory, 1975). The name S-(-)-α-aminopropiophenone (cathinone) was suggested as the main psychoactive alkaloid of khat (Szendrei, 1980; Schorno et al., 1982; Kalix, 1988, 1990, 1992). Later it became evident that cathinone is a labile compound that is mainly present in young fresh leaves and is reduced by three days after leaves are removed from khat tree. This is in complete agreement with the fact that khat is usually consumed as fresh and not as dried material (Balint and Balint, 1994).

Most of the pharmacological effects of khat chewing are attributed to cathinone
S-(-)-α-aminopropiophenone content (Schorno and Steinegger, 1979), which resembles amphetamine in chemical structure and biological activity. The only difference being that oxygen double bond substitutes the two hydrogen on the first carbon of the amphetamine side chain.

The chemical structure of Cathinone (CTN); Amphetamine (APA); (+)-norpseudoephedrine (NPE).
Since the effects of khat had been described earlier as being similar to those of amphetamine, cathinone was examined first for amphetamine-like effects (WHO Advisory Group, 1980). In a wide variety of in vitro and in vivo experiments (-)-cathinone has been shown as an indirectly acting sympathomimetic alkaloid having catecholamine-releasing properties at both central dopaminergic (Pehek et al., 1990) and serotonergic (Kalix, 1984a) synapses as well as at peripheral noradrenaline storage sites (Kalix, 1983b). It operates through the same mechanism as amphetamine which explains the observed central nervous system stimulant effects in the khat chewer (Kalix, 1992). The behavioural effects observed were of particular interest because the effect desired by khat consumers involves central nervous system (CNS) stimulation. Thus, for example, drug-conditioned animals do not distinguish between cathinone and amphetamine (Zelger et al., 1980). Clinical experiments have also shown that cathinone in human beings produces amphetamine-like objective and subjective effects. The mechanism of action of cathinone-induced male sexual dysfunction is far from clear (Islam et al., 1990). Since the effect of khat has been described as being reminiscent of those of amphetamine, (-)-cathinone has been examined for amphetamine-like effects, with initial screening being carried out by an Advisory Group of the WHO. The experimental studies have demonstrated that cathinone is a potent amphetamine-like drug and is the constituent of khat, which is mainly responsible for the CNS-effects; it can also be assumed to be the dependence-producing constituent of khat. When the somatic effects of the new alkaloid were tested, it was found that (-)-cathinone, when administered to anaesthetized cats or rats, causes substantial increase in blood pressure, and that it has a positive ionotrophic and chronotropic effect on isolated guinea pig heart muscles (Gugelmann et al., 1985). Like
amphetamine, cathinone has also been found to stimulate release of $^3$H-serotonin from rat corpus striatum. It is, however, a third-fold less potent than amphetamine in causing this release (Kalix, 1984a). Furthermore, constrictions of isolated rabbit ear artery have been potentiated by (-)-cathinone, whose potency in this respect is similar to that of (+) amphetamine (Kalix, 1984a).

It is worth mentioning that in addition to cathinone, and less psychoactive phenylpropanolamine diastereomer cathine [S, S- (+)-norpseudoephedrine], khat leaves also contain norephedrine [R, S- (-)-norephedrine] as well as the so-called cathedulins or khatamines (Baxter et al., 1979). These are pure polyester alkaloids and have until now been isolated only from Kenyan and Ethiopian khat samples. Their molecular weights are in the range of 600 to 1200 and they can be subdivided according to their complexity and molecular weight into three groups. Apart from cathedulins, new partially pharmacologically active constituents have been obtained and their properties published since 1984. They include: (+)-merucathinone, (+) – merucathine and (-)-pseudomerucathinone which occur only in the khat found in the Meru area of Kenya (Geisshüssler and Brenneisen, 1987; Kalix, 1988). Nearly 20 other compounds have been isolated from khat such as tannins (7 to14%), vitamin C (15 mg/100 mg) and trace amounts of thiamine, niacin, riboflavin and carotene (Patel, 2000). According to the preliminary investigations, these compounds seem to facilitate the sympathomimetic action of the other components of khat leaves (Balint and Balint, 1994).
1.2.4 THE PHARMACOLOGY AND PHARMACOKINETICS OF KHAT

The stimulant effect of the plant khat was originally attributed to S, S- (+)- pseudonorephedrine (cathine), a phenylalkylamine-type substance isolated from the plant. In 1975, the alkaloid cathinone [S- (-) - α-aminopropiophenone] was isolated by United Nations Narcotic Laboratory. Cathinone is fairly unstable, being metabolized relatively quickly to cathine and norephedrine [R, S-S (-) norephedrine], which are more stable and less potent molecules. Cathine and cathinone are phenylisopropylamine derivatives, which resemble the amphetamines in chemical structure and biological activity (Schorno and Steinegger, 1979).

The stimulating effects of khat is perceived by individuals as an increase in alertness and energy, relief from fatigue, feeling of elation, improved ability to communicate, enhanced imaginative ability to associate ideas, and finally as an increase in self-confidence. Objectively, khat use induces a state of mild euphoria and excitement often accompanied by loquacity; the associated behavioural syndrome can be described as hypomania (Laurent, 1962; Margetts, 1967). High doses may induce hyperactivity and frank manic behaviour; in exceptional cases, khat consumption may result in toxic psychosis. Khat is an effective anorectic and its use results in constipation. Mydriasis, which is prominent during khat consumption, reflects the sympathomimetic effects of the drug. A state of drowsy hallucinations may result from khat use as well. There may also be arrhythmias and increase in blood pressure depending on the amount and quality of the material absorbed (Halbach, 1972; Nencini et al., 1984a), while the cardiovascular response to effort is exaggerated after khat consumption (Galkin and Mironychev, 1964; LeBras and
Frétilière, 1965). Withdrawal symptoms that may follow prolonged khat use include lethargy, mild depression, nightmares and slight tremors. These different effects of khat are, of course, difficult to quantify since the leaves are a non-standardized material, the potency of which depends on freshness and origin, and there are certainly inter-individual differences in the efficiency of the mastication process (Kalix, 1991).

According to the findings of World Health Organization (1985), khat is rapidly absorbed after mastication, metabolized in the liver with only a small fraction appearing in urine (Kalix and Braenden, 1985). The more rapid and intense action of cathinone compared to cathine, is explained by its higher lipid solubility, facilitating quicker access into the central nervous system (Zelger et al., 1980). In four human subjects who chewed khat leaves for 1 hour, then spat out the residues and subsequently had their urine samples tested for the presence of cathinone and its metabolites, cathinone was detected for up to approximately 26 hours, while cathine and norephedrine were detected for at least 80 hours (Toennes and Kauert, 2002). Earlier studies on excretion of khat alkaloids in humans showed that both cathine and norephedrine are slowly absorbed and metabolised (Maitai and Mugera, 1975). In another controlled study by Toennes et al., (2003) involving four healthy non-drug using volunteers (two males and two females, age range 26 to 57 years) who chewed four portions of a total of 0.6 g authentic khat leaves per kilogram body weight for 1 hr, plasma concentration-time data for khat alkaloids was described using a two-compartment model with two-segment absorption. In this model, it was found that the mucosa of the oral cavity is the first absorption segment where the major proportion of the alkaloids is absorbed.
In animal studies, cathinone has positive ionotropic and chronotropic effects on the heart, a presser effect on the arteries, increased blood pressure and constriction of the vas deferens (Kalix and Braenden, 1985; Knoll, 1979) as well as producing excitation and hyperactivity (WHO, 1980; Kalix and Braenden, 1985). It increases metabolic rate and oxygen consumption, causes hyperthermia and has an analgesic effect via activation of monoaminergic pathways mediating conception (Yanagita, 1979; WHO Advisory Group, 1980; Ahmed et al., 1983; Nencini et al., 1984b, c; Kalix and Braenden, 1985). Cross-tolerance has been demonstrated between d-amphetamine and d-cathinone (Schuster and Johanson, 1979) and both have similar stimulant effects (Rosecrans et al., 1979) with a self-administration pattern resembling that of cocaine (Yanagita, 1979). At cellular level, cathinone has a similar effect to amphetamine at the central dopaminergic synapses (Kalix, 1983a, b; Kalix and Braenden, 1985), as well as similar effects on other central and peripheral neurotransmitters (Knoll, 1979; Nencini et al., 1984c; Kalix and Braenden, 1985). Subsequent to the initial studies of the WHO Advisory Group (1980), it was shown that the locomotor stimulation induced by cathinone in mice is characterised by a dose-effect relationship resulting in a curve of inverted U-shape (Kalix, 1991); a feature that is reminiscent of hypermotility of the amphetamine type, and that the locomotor effect of cathinone could be antagonized by the same substances as that of amphetamine (Valterio and Kalix, 1982). It was also found that cathinone is similar to amphetamine with regard to induction of stereotypical behaviour in rodents (Zelger et al., 1980).
The similarity between the animal effects of cathinone and those of amphetamine raised the question as to whether or not cathinone acts through the same mechanism as amphetamine, that is, as an indirect sympathomimetic that induces the release of catecholamine neurotransmitters from presynaptic storage sites. In various experiments carried out using rabbit brain tissue prelabelled with tritiated dopamine, it was found that addition of low concentrations of amphetamine to the solution superfusing the tissue slices causes a rapid increase of the efflux of radioactivity, and that cathinone could substitute for amphetamine for this reaction (Kalix, 1988). The amphetamine-like releasing effect of cathinone was also demonstrated on tissue from nucleus accumbens (Kalix, 1982), a brain region thought to be critically involved in the expression of amphetamine hypermotility (Teitelbaum et al., 1979). It was also shown that substances known to block the releasing effect of amphetamine prevented the induction of release by cathinone (Kalix, 1981, 1982). These findings led to the conclusion that cathinone acts like amphetamine at dopaminergic synapses of the CNS, a view that is supported by neurochemical (Wagner et al., 1982) and electrophysiological (Mereu et al., 1983) data. Taken together, these findings indicate that cathinone is the constituent of khat that is mainly responsible for the CNS effects of the plant, and that it is a potent amphetamine-like compound (Kalix, 1991).
1.2.5 EFFECT OF KHAH ON BODY ORGAN SYSTEMS

1.2.5.1 Effect on neuroendocrine system

The disturbances of neuroendocrine function in khat chewers have been reported by several authors (Nencini et al., 1983; Parmar and Tariq, 1984). Cathinone has been shown to inhibit the release of several anterior pituitary hormones (Wagner et al., 1982). On the other hand, amphetamine produces a stress-like syndrome resulting in massive release of catecholamines (Kalix and Glennon, 1986; Knych and Eisenberg, 1979). Dopamine inhibits gonadotropin release by binding to its receptors on basophils thereby activating a membrane inhibiting G protein which is negatively coupled to adenyl cyclase and phospholipase system (Yen, 1991). Sympathomimetic agents are also known to suppress gonadal function and therefore decrease in testosterone production (Gray et al., 1978). Cathinone particularly acts by releasing catecholamines from presynaptic storage sites (Kalix, 1992). The above findings suggest that both cathinone and amphetamine have an effect in the brain and at gonadal level.

In a study on male rats (Islam et al., 1990), it was found that cathinone causes a significant decrease in the plasma testosterone. This may be explained as a result of cytotoxic effect on Leydig cells causing androgenic deficiency in rats (Tariq et al., 1987). Male sexual behaviour was assessed in another experiment (Kalix et al., 1995) by recording erectile responses (grooming of genitals, yawning/stretching and homosexual mounting) in cathinone and caffeine-treated male rats. The copulatory pattern of male rats (mounting, intromissions, ejaculation and refractory period) was recorded after introduction of female rats in the rat cages. Oral treatment of cathinone (5 mg/kg/day),
caffeine (50 mg/kg/day) and their combinations for 15 days increased arousal (motivation) in male rats as evidenced by increased mounting performance and anogenital investigatory behaviour. Erectile and ejaculatory responses showed no stimulant effect, hence it is conceivable that cathinone modified masculine pattern behaviour and caffeine also changed the effect of cathinone when administered concomitantly.

Chronic use of khat produces undesirable side effects including sleeplessness, nightmares, nervousness and increased libido (Halbach, 1979); impotence, spermatorrhoea, loss of appetite and constipation (Dalu, 2000; Pantelis et al., 1989). Like other amphetamine class of psychostimulants, cathinone, an amphetamine analogue, is a potent indirect-acting sympathomimetic that causes release of the excitatory neurotransmitters (dopamine, serotonin and norepinephrine) from storage vesicles in the central nervous system and blocks neuronal uptake (Kalix, 1992). In vitro studies in mice revealed that high levels of 6-hydroxydopamine inhibit testosterone production by Leydig cells (Wango et al., 1995). Several experimental studies carried out both in man and laboratory animals have shown varying findings of khat effect on the reproductive system.

1.2.5.1.1 Effect of Khat on Female Reproductive System

Khat chewing has been associated with a wide range of pathological manifestations in women and female laboratory animals. Islam et al., (1994) reported that female rats given extract from khat leaves from day 6 to 15 of their 21 – day pregnancies eat less and gain
less weight. The treatment also increases the number of stillbirths and leads to smaller litter size in rats. Reports by Jansson et al., (1988a, b) showed that female guinea pigs treated with khat extract in their last trimester also gave birth to smaller pups, and this was correlated with decreased blood flow to the uterus. Eriksson et al., (1991) reported similar effects that babies born to women who chew khat habitually are smaller and their mothers produce less milk. These findings were similar to those of Ghani et al., (1987), who reported khat chewing as being associated with decreased birth weight. It was also shown that norseudoephedrine is secreted into breast milk and transferred to the suckling infant (Kristiansson et al., 1987). A study on the genotoxic potential of khat on female mice using a single dose regime (Tariq et al., 1990) showed a significant increase in post-implantation embryo loss.

1.2.5.1.2 Effect of Khat on Male Reproductive System

There are several contradictory reports regarding the association of khat chewing and sexual disorders in males. Khat has been reported to be used as a medicament for premature ejaculation (Luqman and Danowaski, 1976) and for increasing sexual desire (Elmi, 1983a). In a study on effect of khat extract in Olive male baboons, it was found that khat also causes penile erection, increase in plasma testosterone and decrease in cortisol and prolactin levels (Mwenda et al., 2006). Recent findings have shown that catheine and norephedrine directly affect mammalian sperm function, accelerating capacitation and inhibiting spontaneous acrosome loss. These could be a possible source of infertility associated with khat chewing. These responses correlated with initial stimulation and subsequent inhibition of cyclic AMP production (Adeoya-Osiguwa and Fraser, 2005). Other reports have shown it causes impairment of sexuality (Halbach,
1972; W.H.O, 1980); inability to sustain erection (Elmi, 1983b) and loss of libido (Krikorian, 1984) as well as testicular cellular infiltration, lymphatic engorgement and atrophy of Sertoli and Leydig cells (Islam et al., 1990).

Cathinone may cause anatomical pathologies of the male genital organs (testes, seminal vesicles, epididymis and ventral prostate), which may be responsible for the observed reproductive impairment. In a study on male wistar rats, administration by injection of three doses (5, 10 and 30mg/kg body weight) of pure cathinone intraperitoneally was said to cause degenerative changes in testicular morphology and reduction in plasma levels of testosterone (Islam et al., 1990). In a controlled study involving humans, semen parameters in two groups of Yemeni male, khat ‘addicts’ and ‘non-addicts’, were compared. Sperm concentration, morphology and motility were reported to be significantly poorer in the ‘addicts’. Aflagellate heads- either with single or double heads associated with voluminous cytoplasmic droplets, headless flagella and dead spermatozoa with disappearance of the plasma membrane, dislocation of acrosome, karyolysis and structurally abnormal mitochondria and dense fibres plus disappearance of microtubules of both proximal centriole and the axoneme were observed (El-Shoura et al., 1995).

Khat extract and cathinone have been shown to depress cell proliferation and inhibition of RNA, DNA and protein synthesis in dividing cells, which may be responsible for reduced spermatogenesis (Al-Meshal et al., 1989; De Hondt et al., 1984; Hammouda, 1971). Hammouda, (1978) also found out that khat reduces sperm output, sperm count and fertilizing ability in roosters. However, chronic use of khat has also been shown to cause spermatorrhoea in humans (Halbach, 1979; Pantelis et al., 1989).
Cathinone also increases the frequency of abnormal sperm in mice treated with different doses 30 and 36 hours before sacrificing (Qureshi et al., 1988). It was also observed that an aqueous solution of khat administered orally at doses of 5, 20 or 40 mg/kg body weight, respectively, to three different groups of male mice for six weeks produced a dose-dependent reduction in fertility with total sterility at a dose of 40 mg/kg body weight. The mutagenicity of a methanolic extract of khat on germ cells in male albino mice which were allowed to mate with two different groups of female mice was evaluated by Tariq et al, (1990) using a Dominant Lethal Test. It was found that khat extract produced a dose-dependent reduction in the rate of fertility as well as post-implantation loss during the first week following treatment. Recent findings have shown that khat induces apoptosis in human leukaemia cell lines (HL-60, NB4, Jurkat) within 8 h of exposure and the effect was dependent on de novo protein synthesis demonstrated by co-treatment with cycloheximide (Dimba et al., 2004).

1.2.5.2 Effect on gastrointestinal and urinary system

In a study on long-term khat consumption in humans, it was found to cause genetic damage and oral malignancy, especially when accompanied by alcohol and tobacco consumption (Kassie et al., 2001). Earlier studies in humans have shown that it causes keratosis of buccal mucosa as a pre-cancerous lesion and that 2 to 12% of individuals with such lesions develop oral cancer (Sankaranarayanan et al., 1995). In another study, gastric emptying of a radiolabelled semisolid meal was measured on 12 healthy volunteers, who chewed khat leaves or lettuce for 2 hours before the study. Gastric emptying was significantly prolonged after chewing khat compared with lettuce. It was concluded that the sympathomimetic action of cathinone in khat could have caused the observed delay in gastric emptying.
(Heyman et al., 1995). Gastritis is common among khat users (Halbach, 1972). Constipation, often accompanied by haemorrhoids has been attributed to high tannin content present in khat leaves, which has astringent effect on the bowel (Halbach, 1972). This tannin has also been shown to be hepatotoxic hence causing liver cirrhosis. Khat use may be a factor in the brownish colouration of teeth. Cathinone has been reported to produce a dose - dependant decrease in food consumption suppressing the body weight gain (Islam et al., 1990).

1.2.5.3 Effects on cardiovascular and respiratory systems

In controlled studies, where six drug naive volunteers received doses of khat corresponding to 0.8 mg/kg body weight as well as alkaloid free khat as a placebo, maximal plasma concentrations of cathinone (127 ± 53 ng/ml) were attained after 127 ± 30 min. The area under the plasma concentration-time curve from 0 to 9 hr was 415 ± 207 ng/ml.hr and terminal elimination half-life was 260 ± 102 min (Widler et al., 1994). Since the effects of khat had been described earlier as being reminiscent of those of amphetamine, (-) cathinone was examined for amphetamine-like effects, with the initial screening being carried out by an Advisory Group of the Word Health Organisation (WHO Advisory Group, 1980). When the somatic effects of the new alkaloid were tested, it was found that (-) cathinone, when administered to anaesthetized cats or rats, caused a substantial increase in blood pressure, a positive ionotropic and chronotropic effects on isolated guinea pig myocardium. Furthermore, constrictions of isolated rabbit ear artery were potentiated by (-) cathinone, whose potency, in this respect, was similar to that of (+) - amphetamine. Several studies on khat effects in humans have shown elevated blood
pressure and increase in heart rate (Drake, 1988; Brenneisen et al., 1990; Hassan et al., 2000). Increase in blood pressure, heart rate and cardiac contractile force in anaesthetized dogs (Kohli and Goldberg, 1982) and positive ionotropic and chronotropic actions in isolated atria (Gugelmann et al., 1985) have been reported after the administration of the active ingredient, (-)-cathinone. Incidences of acute myocardial infarction among regular khat chewers in Yemen have recently been reported (Al-Motarreb et al., 2005). Recent findings have shown that khat stimulates the respiratory centre and bronchodilation, which explains the feeling of comfort for asthmatic users (Dhaifalah and Šantavý, 2004). Taken together, these observations suggest that (-)-cathinone is responsible for the sympathomimetic effects observed after khat consumption (Kalix, 1980).

Since the consumption of khat causes a number of sympathomimetic symptoms especially at the cardiovascular level, an investigation was carried out to determine whether or not these cardiac changes were due to release of noradrenaline from sympathetic nerve endings. Experiments on slices of rabbit heart prelabelled with \( ^3 \)H-noradrenaline showed that superfusion with (-)-cathinone caused a substantial increase in the release of radioactivity (Kalix 1984b).

### 1.2.5.4 Psychoactive effects of khat abuse

The pleasurable effects of khat are considered beneficial (Kalix, 1987) and are similar to those of amphetamine and include euphoria, increased alertness and excitement (Giannin et al., 1986). Khat users believe they think more clearly and quickly and are fairly alert, though concentration and judgement are objectively impaired (Pantelis et al., 1989).
Methcathinone is a derivative of cathinone, and like amphetamine, multiple administrations of this compound decreases vesicular uptake of dopamine and dihydrotetrabenazine binding (Brown et al., 1990); an effect associated with dopaminergic and not serotonergic neurons. In mid western United States, methcathinone, the synthetic form of cathinone, has been produced illegally since 1989. It is relatively easy to produce and contains the same chemicals found in over-the-counter (OTC) asthma and cold medicines, paint solvents and thinners and drain openers such as drano. The addiction is similar to that of cocaine (Larson, 2002).

1.3 JUSTIFICATION OF THE STUDY

Cathinone, a psychoactive component of khat plant has been shown to produce reproductive dysfunction in both man and experimental animals. Earlier investigators reported that khat causes impairment of sexuality (Halbach, 1972; W.H.O. 1980); inability to sustain erection (Elmi, 1983b) and loss of libido (Krikorian, 1984) as well as testicular cellular infiltration, lymphatic engorgement and atrophy of Sertoli and Leydig cells (Islam et al., 1990). Recent findings have shown that cathine and norephedrine directly affect mammalian sperm function, accelerating capacitation and inhibiting spontaneous acrosome loss (Adeoya-Osiguwa and Fraser, 2005).

Although the W.H.O played an important role earlier on in encouraging and funding scientific studies designed to understand the active constituents of khat and health problems associated with its use (WHO, 1980), little attention has been paid in studying its pharmacological influence on reproductive function in rabbits. The aim of this study,
therefore, was to investigate the effect of khat on reproductive neuroendocrine system and the accompanying histopathology of the pituitary, testes and epididymides in khat-treated male rabbits.

1.4 RESEARCH OBJECTIVES

1.4.1 Overall objective

To investigate the mechanism of action of crude khat extract on reproductive function.

1.4.2 Specific objectives

a) To assess effect of fresh crude khat extract on plasma luteinising hormone, testosterone and cortisol levels in male rabbits after treatment with khat extract.

b) To determine effects of fresh crude khat extract on histology of pituitary gland, testes and epididymides of male rabbits.

c) To observe the clinical manifestations in khat-treated rabbits as compared to saline-treated controls.

1.5 HYPOTHESIS

Crude khat extract does not cause neuroendocrine disturbances in the pituitary-gonadal axis and histological changes in the pituitary and testes.
CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 STUDY AREA AND EXPERIMENTAL ANIMALS

This study was conducted at Chiromo Campus, University of Nairobi, Department of Veterinary Anatomy and Physiology, Reproductive Biology Unit. This research was conducted in accordance with the internationally accepted principles for laboratory animal use and care (NIH publication # 85 – 23, revised in 1985). Twenty-five (25) disease and parasite-free sexually mature New Zealand White male rabbits from Njoro farm aged between 9 and 12 months and weighing 1.5 to 3 kg were used. Standard rabbit pellets (Unga Feeds, Nairobi), green vegetables, carrots and fresh tap water were supplied ad libitum. The animals were kept under natural lighting conditions (approximately 12:12 hours light/ dark) at an average room temperature of 23 ± 1°C with a humidity of approximately 60%. Each animal was housed singly in cages (40.5 cm x 40.5 cm x 61 cm) put on a raised surface from the floor, in the animal house. Their beddings (wood shavings and dry grass) were changed every after two days.

2.2 CLINICAL ASSESSMENT

2.2.1 Body weight

This was measured using a weighing balance each day prior to and after administration of crude khat extract and recorded over the period of the experiment. Body weight gain or loss (g) was determined by subtracting the body weight of rabbits at the start of the experiment from any additional or reduced body weight for different khat doses over the experimental period. This gave body weight gain or loss at different khat doses and over
the experimental period. Food (g) intake in both test and control animals were monitored daily. Food intake per day was determined by subtracting the weight of food remaining from weight of food given to the animal the previous day. The weight was determined using a 3 lever beam balance. Feeding troughs were anchored on thre wire mesh of the cage at a raised surface to avoid spillage.

2.2.2 Body temperature

Rectal temperature was measured using a clinical thermometer daily for 2 weeks before and every 3hr after khat extract administration for 5 weeks.

2.3 EXPERIMENTAL DESIGN

A total of 25 male New Zealand White rabbits used in this study were habituated to handling and insertion of intragstric tube before the start of the experiment. These were divided into five groups (I to V) each comprising five (5) animals. Group I animals served as controls; each given 5 ml normal saline (0.9% sodium chloride) via intra-gastric tube twice a week for 5 weeks. Groups II, III, IV and V received 1.5 g/kg, 4.5 g/kg, 13.5 g/kg and 40.5 g/kg body weight of khat extract, respectively, via intra-gastric tube. Each rabbit in respective groups received khat extract twice a week for 5 weeks. These khat doses were prepared as described below.

2.4 CRUDE KHAT EXTRACT PREPARATION

Fresh khat was bought each time before conducting the experiment. Four different doses (1.5 g/kg, 4.5 g/kg, 13.5 g/kg, and 40.5 g/kg body weight) of khat extract were prepared from fresh leaves of khat blended in normal saline. Each dose was given in 5 ml volume.
For control, 5 ml of normal saline was used. The khat doses (1.5 g/kg, 4.5 g/kg, 13.5 g/kg and 40.5 g/kg body weight of khat extract) were prepared as follows: For example to prepare a dose of 1.5 g/kg body weight, 15 g of fresh khat leaves were blended in 10 ml normal saline to give a concentration of 1.5 g/ml. For a rabbit weighing 2 kg, it required a total of 3 g of khat extract. So 2 ml of the stock solution containing 1.5 g/ml was mixed with 3 ml of normal saline to make a total volume of 5 ml, which was given via an intra-gastric tube. 30 g of fresh khat leaves were blended in 10 ml of normal saline to give a concentration of 3 g/ml. For a rabbit weighing 2 kg, 3 ml of this solution was taken and mixed with 2 ml of normal saline to give a dose of 4.5 g/kg body weight and administered. To prepare 13.5 g/kg, 60 g of khat leaves were blended in 10 ml of normal saline to give a concentration of 6 g/ml. For a rabbit weighing 2 kg, 4.5 ml was taken and 0.5 ml normal saline added. For 40.5 g/kg, 140 g of fresh khat leaves were blended in 8 ml of normal saline to give a concentration of 17.5 g/ml. For a 2 kg rabbit, to 4.6 ml of this concentration, 0.4 ml of normal saline was added to give a total of 5 ml to be given via an intra-gastric tube.

2.5 BLOOD SAMPLING
Rabbits were adequately restrained physically by use of a restraint box. They were habituated by placing them in the restraint box for about 10 min, daily for two weeks before blood collection. The marginal ear vein of the rabbits was cannulated using a 22 G blood vessel cannula two days before blood collection to habituate them. The marginal ear vein was made visible by shaving the area of the ear using a surgical blade and swabbed with 70% alcohol. The cannula was inserted in the vein and heparinised saline
introduced into the cannula to prevent blood clotting inside the lumen. The adhesive tape was used to anchor the cannula to the ear to prevent from dropping. Thereafter, 1 ml blood samples for hormonal assays were collected into heparinized LP3 tubes every 15 min for 3 hr, 10 min after khat extract administration. A total volume of 12 ml of blood was collected by the end of the 3 h sampling period. This volume was within the physiological limit of 6 – 10% of blood volume or 7 – 13 ml of blood. The samples were then centrifuged and plasma stored at -20°C until assayed for LH, testosterone and cortisol.

For observation of haematological parameters (red blood cell count, white blood cell count, haemoglobin concentration and packed cell volume), 0.5 ml blood samples were collected once in heparinized capillary tubes at the end of 3-hr sampling period.

2.6 TISSUE SAMPLING FOR LIGHT AND ELECTRON MICROSCOPY
This involved harvesting of pituitary, testes and epididymides for light and electron microscopy. Tissue samples were harvested from animals in all groups except III in which the pilot hormonal assay showed no appreciable difference from that of group II. Therefore, the inclusion of group II animals was considered sufficient to represent group III animals as well. Two animals from each of these groups were hemi-castrated 7 days after khat extract administration, to study acute effects while the remaining animals in all groups were sacrificed 58 days later to study chronic effects. Hemi-castration was done after anaesthetising the animals first by pre-medication using 5 mg/kg body weight xylazine (Chanizine, Chanelle Pharmaceuticals Manufacturing Ltd., Galway, Ireland)
intramuscularly to reduce stress followed by 10 mg/kg body weight ketamine (Ketaset, Fort Dodge Laboratories Inc., Fort Dodge, Iowa) intravenously after 5 min. To harvest tissues for morphological studies, animals were euthenised with diethyl ether (R.P. Normapur™ AR, Fontenay S/Bois, EEC) and fixed through intra-cardiac perfusion beginning with normal saline followed 2.5% 0.1 M phosphate buffered glutaraldehyde (pH 7.4). In brief, the procedure was performed as follows: a mild incision was made from the neck to the groin, and thoracic and visceral organs exposed. The intercostal muscles were severed to allow introduction of a 22G needle (connected to the flushing apparatus) into the heart through the apex of the left ventricle and clamping it in place. Physiological saline (0.9% sodium chloride) was then introduced slowly into the left ventricle of the heart while taking care not to introduce the air bubbles. An outlet was made at the caudal vena cava to flush out all the blood and saline as the heart pumped. Flushing was continued until the fluid coming out was as near clear as the saline being introduced.

After flushing, 2.5% glutaraldehyde formulated in 0.1M phosphate buffer was introduced through the same needle into the heart to perfuse all the systems of the body. At this time the incision at the caudal vena cava was clamped to allow the fixative/perfusate to be retained in the body tissues to achieve maximum fixation. After perfusion was complete, which was characterised by hardening of tissues, the testes, epidydimis and pituitary were harvested. A little of the fixative was injected right beneath the tunica albuginea before immersing the tissues into sample bottles containing 2.5% glutaraldehyde. To harvest the pituitary gland, the neck was severed at the level of occipital bone and all muscles of the
head removed using a surgical blade. The maxillae and the hard palate were carefully removed using a bone cutter to access the sphenoid bone, which was then removed using forceps. The pituitary was then harvested and immersed in sample bottles containing 2.5% glutaraldehyde. These samples were kept in the fixative for about 24 hours for proper fixation then removed and stored in phosphate buffered saline until processing.

2.7 SAMPLE PROCESSING

2.7.1 Haematological parameters

The haematological parameters measured were packed cell volume (PCV), haemoglobin concentration, red blood cell counts and white blood cell counts. The PCV was measured using the standard haematocrit method. Blood samples (0.5 ml) were put in LP3 tubes containing heparin and gently mixed to avoid clotting. Non-heparinised capillary tubes were filled three-quarter with heparinised blood sample. The tubes were then exposed to non-luminous flame to expel the air inside and seal the tips. Thereafter, they were placed in wells of microhaematocrit centrifuge with sealed tips facing centripetally and spun at 1500 x g for 5 minutes. The length of the column of packed cells and that of packed cells plus plasma was measured and the ratio calculated as a percentage.

Haemoglobin concentration was measured using the acid haematin method. In this method, 0.1 N HCL was pipetted up to 20% mark of the haemoglobin graduated tube and 20 µl of heparinised blood sample added and mixed vigorously using a mixing rod. Acid haematin forms after about 3 min when haemoglobin reacts with HCL. By use of a Pasteur pipette, distilled water was added drop by drop to the haemoglobin tube in the
haemoglobinometer and stirred each time using a mixing rod until the colour of the solution matched that of the haemoglobinometer. This gave the amount of haemoglobin in g/l.

Red blood cell and white blood cell counts were done with a haemocytometer. For RBC, Hayem’s solution (containing 0.9% sodium chloride) was used while for WBC, the dilution fluid (2% Glycerol acetate and Gentian Violet or Crystal Violet) was used. For the WBC, the cells in the four peripheral and central big squares of the haemocytometer were counted and multiplied by a factor of 40. For RBC the 4 peripheral smaller squares and 1 central smaller square in the central big square of the haemocytometer were used and the number multiplied by a factor of 10,000.

2.7.2 Hormonal analysis

The WHO Matched Reagent Program provided the reagents for radioimmunoassay (RIA) of testosterone and cortisol, and luteinizing hormone (LH) assay (bioassay).

2.7.2.1 Radioimmunoassay for testosterone

Plasma testosterone was analysed by radioimmunoassay (RIA). The method is based on competition for a limited number of binding sites on the testosterone specific antibody by the hormone in the sample and labelled testosterone (1,2,6,7-3H Testosterone, TRK 402 B 99, Amersham Life Science, England) and the technique utilizes dextran-charcoal for separation of free from antibody-bound hormone (WHO, 1996).
The working tracer solution was prepared by taking 150 µl of stock solution into a tube and solvent evaporated by heating on hot plate at 40 ºC. This was then re-dissolved with 15 ml assay buffer (2.35 g NaH₂PO₄, 11.6 g Na₂HPO₄, 8.8 g NaCl, 1 g gelatine and 0.1 g NaN₃ in 1 litre of double distilled water, pH 7.4) and allowed to stand for 30 minutes.

The antiserum (Batch no. K200710, WHO) was prepared by reconstituting the contents of one bottle with 10 ml of assay buffer saline and allowed to stand for 10 minutes and vortex mixed before use.

Testosterone standards: Before running the assay, testosterone standards (Batch no. K079810, concentration: 220 nmol/l, WHO Reagent Programme) were prepared as follows:

The residue from 100 µl of ethanolic solution was reconstituted with 10 ml phosphate assay buffer, heated to 40ºC for 30 minutes, mixed vigorously and allowed to cool to room temperature before use.

1. A rack containing 5 test tubes (10 ml) labelled 1-5 was set up and, using the same pipette and tip, 2 ml of buffer was added to each tube.

2. Using the same pipette and tip, 2 ml of testosterone standard (from 1 above) was added to tube 1 and carefully mixed.

From tube 1, serial dilutions were done by carefully drawing 2 ml from tube 1 into tube 2 and vortexed, then 2 ml from tube 2 into tube 3, and finally transferring 2 ml from tube 4 into tube 5. Each withdrawal was preceded with careful mixing.

Sample preparation: Plasma samples were thawed and vortexed; 200 µl was taken from each sample and 200 µl of assay buffer added to each tube. Then 2 ml of diethyl ether
was added to each tube, vortexed and thereafter shaken vigorously for 3 minutes using a Hover motor shaker.

The aqueous phase was frozen using dry ice and the supernatant decanted into 7.5 ml glass tubes. The solvent was evaporated in a vacuum and 2 ml of the phosphate assay buffer added to each tube. The tubes were vortex mixed, left to stand for 20 minutes, and then vortex mixed again. From each of the re-dissolved sample extracts, 500 µl aliquots were taken in triplicates and each put into labelled LP_3 assay tubes. In each tube, 100 µl of tritiated testosterone working solution and 100 µl of testosterone antiserum were added, then vortexed and incubated overnight at 4 °C. Included in each assay were standard tubes (S), tubes for the determination of non-specific binding (NSB) to which no antiserum was added, zero antigen tubes (B0) which contained 500 µl of phosphate buffer, total count tubes (Tc approximately 10,000 cpm), high and low quality control (Qc) samples were also included in each assay.

After incubation, the samples were transferred to ice baths and 200 µl of ice-cold dextran coated charcoal (0.625 g charcoal, 0.0625 g dextran in 100 ml phosphate assay buffer) was added to each tube except the Tc tubes. The tubes were vortexed then incubated for 15 minutes at 4 °C. Centrifugation was done 1500 x g for 10 minutes at 4 °C using Beckman Model TJ-6 Centrifuge connected to a TJ-Refrigeration Unit. The supernatant from each tube was decanted into labelled scintillation vials then 4 ml of toluene scintillation fluid added and counted in a Packard PRIAS Counter. Results from samples were calculated from the standard curve. The intra assay coefficient of variation (CV) for the assay was 13.7% (n = 5) at 1.36 nmol/l. The inter assay coefficient of variation (CV) for the assay
was 7.4% (n = 5) at 1.36 nmol/l. The sensitivity (detection limit) of the assay, defined at 94% confidence limit was 0.35 nmol/l.

2.7.2.2 Radioimmunoassay for cortisol

The procedure was set by preparing cortisol standards, working tracer solution (1, 2, 6, 7-
$^3$H Cortisol, TRK 407 Batch no. 37- Amersham, England), antiserum (Batch no. K577410- WHO Reagent Programme) and samples. This was as follows:

Cortisol tracer:

This was prepared by taking 150 µl into a tube to evaporate the ethanolic solution at 40
°C and re-dissolve it with 15 ml assay buffer and allowed to stand for 30 minutes.

Antiserum:

This was prepared by reconstituting the contents of one tube with 10 ml of assay buffer in
a vial and stood for 10 minutes. This solution was carefully mixed before use.

Standards:

1) The residue from 100 µl of ethanolic solution was reconstituted with 10ml of
assay buffer and heated to 40 ºC for 30 minutes, mixed vigorously and allowed to
cool to 4ºC before use.

2) A rack containing 5 test tubes (10 ml) was labelled.

3) To all the 5 tubes, 1 ml of assay buffer was added using the same pipette and tip.

4) To tube 1, 1 ml of reconstituted solution in 1 above was added using same pipette
and tip and vortexed.
5) Another 1 ml was taken from tube 1 to tube 2 using same pipette and tip and carefully vortexed. The procedure was completed by transferring 1 ml from tube 4 to tube 5 and vortexed.

Sample treatment:
Unlike testosterone extraction where ether was used, in cortisol assay a simple non-extraction procedure was used that relied on the principle of isolation of cortisol hormone from binding proteins known as cortisol binding globulin (CBG). 50 µl of plasma was pipetted in assay tube and 450 µl of double distilled water added and vortexed. This was then incubated at 60°C for 30 minutes in the LAB-LINE/ DUBNOFF INCU- SHAKER to denature the CBG. The samples were then centrifuged at 1500 x g for 10 minutes.

Into each tube, 100 µl of tritiated cortisol working solution and 100 µl of cortisol antiserum were added, then vortexed and incubated at room temperature for 30 minutes. The samples were then incubated at 37°C for 1 hour. Included in each assay were standard tubes (S), tubes for the determination of non-specific binding (NSB) to which no antiserum was added, zero antigen tubes (B0) which contained 500 µl of phosphate buffer, total count tubes (Tc approximately 10,000 cpm), high and low quality control (Qc) samples were also included into each assay.

After incubation, the samples were cooled to 4 °C for 15 minutes and rapidly 200 µl of ice-cold dextran-coated charcoal suspension (0.625 g charcoal and 0.0625 g dextran dissolved in double distilled water) added to all the tubes except for Tc tubes. This was followed by
incubation at 0 °C in ice-cold water for 10 minutes. Centrifugation was done 1500 x g for 10 minutes at 4 °C using Beckman Model TJ-6 Centrifuge connected to a TJ-Refrigeration Unit. The supernatant from each tube was decanted into labelled scintillation vials then 4ml of toluene scintillation fluid added and counted in a Packard PRIAS Counter. Results from samples were calculated from the standard curve. The intra assay coefficient of variation (CV) was 5.1% (n = 5) at 23.3 nmol/l. The inter assay CV was 15.9% (n = 6) at 23.3 nmol/l. The detection limit of the assay, defined at 96% confidence limit was 4.32 nmol/l.

2.7.2.3 Luteinizing hormone Assay (Bioassay)

Plasma LH was determined by bioassay as described below using materials provided by WHO Matched Reagent Programme (WHO, 1996).

2.7.2.3.1 Interstitial Cell Preparation:

Testes were removed from adult Swiss mice of 5 to 7 weeks old after cervical dislocation, decapsulated and fat trimmed off, then rinsed in ice-cold 10ml Basal Medium Eagle (BME) media (0.919 g BME, 0.161 g NaHCO₃, 3 ml calf serum and 2% Bovine Serum Albumin dissolved in 100 ml double distilled water) in plastic Petri dish. The media was aerated with medical gas (95% O₂: 5% CO₂) in ice-cold water before use. Testes were minced with a pair of scissors and cells dispersed mechanically using a Fin pipette by sucking in and out several times. These minced tissues were transferred to a 50 ml conical flask containing 100 ml medium, and then mixed gently using a magnetic stirrer for 15 minutes at 4 °C. The suspension was then filtered through fine cotton gauze to
obtain interstitial cells then transferred into 100ml conical flask and incubated for 15 min in 5% carbon-dioxide water bath at 4 ºC. The cell suspension was then divided among 6 LP3 tubes and spun at 1500 x g at 4 ºC for 10 min and the supernatant discarded. The base of the LP3 tubes was tapped to free cell pellets from adhering to the base of the tubes, then each tube added 2 ml BME media, sucked in and out to wash the cells and recentrifuged at 1500 x g at 4 ºC for 10 min. The procedure was repeated 3 times and then cells in all tubes pooled into a conical flask. The cells were isolated by sieving the suspension through cotton gauze, diluted with the incubation media and counted using a haemocytometer to obtain a working concentration of approximately $2 \times 10^5$ cells/ml to be used in the bioassay.

2.7.2.3.2 Bioassay of LH

The samples were first prepared by pipetting 50 µl of plasma sample and adding 450 µl double distilled water into assay tubes, vortexed and incubated at 60 ºC for 30 minutes in water bath incubator shaker to isolate LH from peptide binding proteins. Samples were then centrifuged at 1500 x g for 10 minutes and aliquots pipetted into LP3 tubes.

LP3 tubes containing 200 µl of interstitial cell suspension and 100 µl of standard hormone (Batch no. K201010, Concentration; 50 mU- WHO) solution or plasma sample in 100µl media were then incubated at 37º C under 95%:5% O₂: CO₂. The standard tubes were incubated in triplicate and plasma samples assayed in duplicate after incubation for 3 hours with shaking at 150 cycles /minute using LAB-LINE/DUBNOFF INCU-SHAKER.
After incubation, 1 ml of assay buffer was added to all tubes to dilute the cells then centrifugation done at 1500 x g for 15 minutes. From each assay tube, 500 µl aliquots were pipetted in labelled LP₃ assay tubes similar to that of testosterone radioimmunoassay described previously. Bioactive LH levels were estimated as testosterone in the sample vials and compared with that of standard preparations. The intra assay CV for the bioassay was 9.1% (n = 7) at 4.15 IU/L. The inter assay CV for bioassay was 8.7% (n = 7) at 4.15 IU/L. The sensitivity of the bioassay at 94% confidence limit was 0.624 IU/L.

2.7.3 Light and electron microscopy sample processing

Tissue processing for light and transmission electron microscopy (TEM) was done with an aim of embedding the tissues in a solid medium firm enough to allow tissue sectioning. Tissues of the pituitary, epididymides and testes were trimmed into sufficiently small size (1 mm³) to permit proper fixation and processing. They were then appropriately labelled using tags and post-fixed in 4% Osmium tetroxide for 2 hours. These were again rinsed in phosphate buffer before dehydration in ascending concentrations of ethanol (20, 40, 50, 60, 70, 80, 90, and 100 [absolute]), three times every 15 minutes. Tissue blocks were then cleared using propylene oxide twice every 15 minutes. This was followed by infiltration with epoxy-resin mixture (13 ml Epon-812, 7 ml MNA, 8 ml DDSA and 16 drops of DMP-30):

1) Firstly as 2 parts of propylene oxide to 1 part of epoxy-resin mixture for 30 minutes.
2) Secondly as 1 part of propylene oxide to 2 parts epoxy-resin mixture for 30 minutes

3) Finally, infiltrate in pure epoxy-resin mixture without accelerator (DMP-30) overnight.

4) Then embed in resin mixture as previously prepared, comprising of 100 ml resin mixture and 1.8 ml DMP (accelerator) in plastic capsules or aluminium foil boats then incubated in the oven at 60°C. Tissue sectioning was then done using a Sorvall® ultra microtome. 1 µ thick sections were cut using glass knives, floated on moistened and clean glass slide and fixed by heat on a hot plate at 70 ºC. These sections were then stained with 3% toluidine blue in 1% Borax, cleaned and examined under light microscope. These sections served for observation of histological changes as well as for general screening of the tissue to locate the required area for electron microscopy.

2.8 **STATISTICAL ANALYSIS**

All values were expressed as mean ± standard error of mean (SEM). Hormonal, haematological and data on clinical observations for difference in means among groups and over the experimental period was analysed by two-way ANOVA at 95% confidence interval followed by Tukey's multiple comparison post hoc test. Statistical significance was set at P<0.05. For LH peak determination, LH values that exceeded the previous value by 3 standard deviations followed by either a decline or no significant increment was taken as LH peaks while amplitude was calculated by subtracting concentration at onset of pulse of LH from peak plasma LH concentration. LH frequency was calculated
by counting the number of peaks. The area under the curve that gave total amount of LH released was calculated using trapezoid rule.
CHAPTER 3

3.0 RESULTS

3.1 CLINICAL OBSERVATIONS

None of the rabbits showed adverse clinical signs throughout the experimental period. The clinical signs examined included: demeanour (state of haircoat), state of alertness, general body condition, posture, any discharges through natural orifices and general responsiveness to handling. The mean body weight gain between the different groups of rabbits given different doses of khat extract was significantly different (P< 0.05) (Fig. 1a). The mean body weight gain over 5 week experimental period was also significantly decreased (P<0.05). Post hoc test with Tukey's multiple comparison showed that the change in mean body weight of the saline-treated controls (1.886 ± 0.168 kg) was significantly higher (P<0.05) than the khat extract-treated rabbits at the end of the experiment and the decrease was significant with doses 13.5 g/kg and 40.5 g/kg body weight of khat extract (Fig. 1b).

For all doses, khat extract suppressed food consumption in the rabbits, P<0.05 (Fig. 2a). Dose 40.5 g/kg body weight showed the highest decline in food consumption (51.47 ± 9.716 g), while the dose 1.5 g/kg body weight showed the least drop in food consumption (109.82 ± 1.484 g) as shown in Fig. 2b. The rectal temperatures remained within normal range (38 to 39.5°C) in saline-treated controls while increasing significantly (P<0.5) to 40.3 ± 0.13°C and 41.1 ± 0.19°C at doses of 13.5 g/kg body weight and 40.5 g/kg body weight, respectively (Fig. 3a). At doses 1.5 g/kg and 4.5 g/kg body weight, there was no
significant increase in mean body temperature (38.8 ± 0.02°C and 38.9 ± 0.04°C). The increase in rectal temperature was dose-related (Fig. 3b).

The haematological parameters (red blood cell count, white blood cell count, haemoglobin and packed cell volume) showed a significant difference (P<0.05) between saline-treated controls and khat-treated rabbits. Khat extract reduced RBC count at all dosages of khat extract (Fig. 4a). The mean RBC count between khat doses was significantly different (P<0.05) and mean RBC count over 5 week experimental period reduced significantly (P<0.05). The greatest suppressive effects were seen with the highest two doses (13.5 g/kg and 40.5 g/kg body weight), whereas the least suppression was observed at the dose level of 1.5 g/kg body weight (Fig. 4b). It was noted that the effects of khat extract on RBC count was dose-related.

Haemoglobin concentration showed a similar trend (Fig. 5a). The mean haemoglobin concentration between khat doses were significantly different manifesting a significant reduction over the experimental period (P<0.05). The highest two doses (13.5 g/kg and 40.5 g/kg body weight) showed the greatest decrease in haemoglobin (10.86 ± 0.558 g% and 9.998 ± 0.768 g%, respectively). The effects of khat extract on haemoglobin were dose-dependent (Fig. 5b). There was a significant increase in WBC count in khat-treated rabbits compared to saline-treated controls (Fig. 6a). The mean WBC count between khat doses was significantly different (P<0.05) and the counts over the experimental period also increased significantly (P<0.05). There was a dose-dependent increase in WBC count with the highest dose (40.5 g/kg body weight of khat extract) showing highest increase while the controls showed the least increment (Fig. 6b). Mean PCV, however,
decreased with increase in khat dose, showing a significant difference between khat doses (P<0.05). It was also shown that mean PCV over the period of the experiment significantly decreased (P<0.05) (Fig. 7a). The suppressive effect was dose-related (Fig. 7b) reducing from 35.64 ± 0.087% in controls to 32.81 ± 0.80% at 40.5 g/kg body weight. Another clinical observation in khat-treated rabbits was mydriasis, which occurred about 30 to 45 min and lasted for up to 2 hr 30 min after khat administration. The degree of dilation was highest at a dose of 40.5 g/kg body weight of khat extract.

3.2 HORMONE ANALYSIS

3.2.1 Effect of khat extract on bioactive luteinizing hormone

The changes in plasma bioactive LH in saline-treated controls and khat-treated rabbits in each group are presented in Figures 8a and b. Pulsatile release pattern of LH was observed in samples collected at 15 minutes interval for 3 hours in all experimental animals (Fig 8a). However, these patterns were lowered in the highest two doses (13.5 g/kg and 40.5 g/kg body weight of khat extract). The pulse frequency in the controls (2.6 ± 0.24) did not show any significant difference (P>0.05) during sampling periods. These pulse frequencies declined progressively with increasing doses of khat extract; 2.2 ± 0.2, 2.0 ± 0.32, 1.8 ± 0.2 and 1.4 ± 0.25 at doses 1.5, 4.5, 13.5 and 40.5 g/kg body weight of khat extract respectively (Table 1). Changes in pulse frequency in khat-treated rabbits were associated with changes in pulse amplitude, which also decreased in a dose-dependent manner. The mean pulse frequencies were significantly different between khat doses (P<0.05). The pulse amplitude did not differ significantly between doses 4.5 and 13.5 g/kg body weight of khat extract, with mean values as 30.6 ± 6.5 IU/L and 31.1 ±
4.3 IU/L respectively. There was a significant decline in mean area under LH curve in khat-treated rabbits as compared to controls. The mean values were, however, not significantly different among khat dosages in khat-treated rabbits (213.9 ± 16.9 IU, 167.4 ± 40.5 IU, 187.9 ± 13.1 IU, 168.8 ± 14.4 IU), (P>0.05). At all dosages, khat extract suppressed plasma LH levels when compared to saline-treated controls. The mean plasma LH concentrations over the 3 h sampling period were 20.37 ± 0.39 IU/L, 19.43 ± 0.32 IU/L, 18.68 ± 0.43 IU/L, 16.57 ± 0.49 IU/L and 15.45 ± 0.64 IU/L for doses 0, 1.5, 4.5, 13.5 and 40.5 g/kg body weight of khat extract, respectively. These mean LH levels were significantly different between khat doses and over 3 hr sampling period (P<0.05). Tukey’s multiple comparison test showed that effects of 13.5 and 40.5 g/kg body weight of khat were significantly different from those with 4.5 g/kg body weight of khat dose, which was significantly different from controls (P<0.05). The largest suppressive effects were seen at the highest two doses of 13.5 g/kg and 40.5 g/kg body weight of khat, whereas the least suppression was observed at a dose level of 1.5 g/kg body weight (Fig. 8b). It was noted that the effects of khat extract on plasma LH was dose-related.

3.2.2 Effect of khat extract on plasma testosterone

The changes in mean plasma testosterone concentrations in controls and khat-treated rabbits are shown in Fig. 9a. The mean plasma testosterone levels were significantly different between khat dosages and over 3 h sampling period (P<0.05). The pulsatile release pattern of testosterone was altered with the highest dose of 40.5 g/kg body weight of khat showing the most suppression of 1.27 ± 0.44 nmol/l at 1 hr 35 min after khat administration. There was suppression of plasma testosterone at all levels of khat extract,
with the greatest suppressive effects at the highest dose of 40.5 g/kg body weight of khat extract and least at a dose level of 1.5 g/kg body weight of khat extract (Fig. 9b). The mean levels of plasma testosterone were 2.06 ± 0.06 nmol/l, 1.89 ± 0.06 nmol/l, 1.79 ± 0.05 nmol/l, 1.68 ± 0.06 nmol/l and 1.58 ± 0.06 nmol/l for doses of 0, 1.5, 4.5, 13.5 and 40.5 g/kg body weight of khat extract respectively (Table 2). It was observed that the effect of 40.5 g/kg body weight of khat extract on plasma testosterone was significantly different from that of 13.5 g/kg body weight of khat extract, which in turn was significantly different from 1.5 g/kg body weight and 4.5 g/kg body weight of khat extract (P<0.05). Khat effects on plasma testosterone in rabbits were observed to be significantly different from those of saline-treated controls. (Fig. 9b). These effects were seen to be dose-related. The area under response curve indicating total amount of hormone secreted showed a steady decline in plasma testosterone levels in khat-treated rabbits compared to controls. The mean values (21.8 ± 1.2 nmol, 20.8 ± 1.7 nmol, 19.6 ± 2.7 nmol, 18.4 ± 0.9 nmol and 17.3 ± 1 nmol) were significantly different between doses of khat extract (P<0.05).

3.2.3 Effect of khat extract on plasma cortisol

Plasma levels of cortisol in rabbits over the experimental period are depicted in Fig 10a. At all dosages, khat extract elevated plasma cortisol levels when compared to the saline-treated controls. The mean plasma cortisol levels were significantly different between doses and over the 3 hr sampling period (P<0.05). Mean cortisol levels at doses 13.5 g/kg and 40.5 g/kg body weight of khat extract were significantly higher (P<0.05) than those of doses 1.5 g/kg and 4.5 g/kg body weight of khat extract. The mean levels of the saline
controls (26.72 ± 0.46) were significantly lower from those of khat-treated rabbits (P<0.05) as shown in Fig 10b.
Fig. 1a. Effect of khat extract on body weight gain in saline-treated and khat-treated rabbits over the experimental period of 5 weeks. Khat extract suppressed body weight gain, with the highest doses of 13.5g/kg and 40.5g/kg body weight of khat extract showing significant suppression (P<0.05) as compared to saline-treated controls. n = 25.
**Fig. 1b.** Body weight gain in saline-treated controls and khat-treated rabbits as a function of khat dose over the experimental period of 5 weeks. At doses 4.5g/kg, 13.5g/kg and 40.5 g/kg body weight, khat extract caused a significant decrease in body weight gain (P<0.05) with the highest suppression at dose 40.5 g/kg body weight of khat extract as compared to saline-treated controls; n = 25 rabbits.
Fig. 2a. Effect of khat extract on weekly food consumption in saline-treated and khat-treated rabbits. Food intake was significantly reduced (P<0.05) at all dosages of khat extract as compared to controls. The highest dose of 40.5g/kg body weight of khat extract showed the highest suppression of food intake. n = 25 rabbits.
Fig. 2b. Food consumption in both controls and khat-treated rabbits as a function of khat dose. Khat extract significantly suppressed (P<0.05) food intake in khat-treated rabbits, with the highest suppression at dose 40.5g/kg body weight of khat extract as compared to saline-treated controls. n = 25 rabbits.
**Fig. 3a.** Effect of khat extract on core body temperature 2 weeks before and 5 weeks after khat treatment. Body temperature measurements in saline-treated controls and khat-treated rabbits were taken after 3 h of khat administration. Body temperature increased at all dosages with significant increment (P<0.05) at 13.5g/kg and 40.5g/kg body weight of khat extract as compared to controls. n = 25.
**Fig. 3b.** Mean body temperature of rabbits 2 weeks before and 5 weeks after khat treatment as a function of khat dose. Khat extract caused a transient increase in body temperature of rabbits at all dosages and which subsided at about 5 h after khat administration. Significant increase was seen in rabbits treated with 13.5g/kg and 40.5g/kg body weight of khat extract (P<0.05) compared to controls; n = 25.
**Fig. 4a.** Mean Red Blood Cell (RBC) count in saline-treated and khat-treated rabbits as a function of khat dose. RBC count decreased significantly (P<0.05) at all dosages in a dose-dependent manner. The decrease in the highest dose, however, was within the physiological limit. n = 25 rabbits.
**Fig. 4b.** Mean Red blood cell count in saline-treated and khat-treated rabbits as a function of khat dose. Khat extract suppressed RBC count in a dose-dependent manner, with 40.5g/kg body weight of khat extract causing the highest suppression (P<0.05) compared to controls. n = 25.
Fig. 5a. Mean haemoglobin (Hb) levels in saline-treated and khat-treated rabbits as a function of khat dose. Hb levels showed a significant decrease in khat-treated rabbits in a dose-related manner (P<0.05; n = 25 rabbits).
**Fig. 5b.** Effect of khat extract on Haemoglobin (Hb) content in rabbits as a function of khat dose. Khat extract suppressed Hb content in a dose-dependent manner, with the greatest suppression at dose 40.5g/kg body weight of khat extract (P<0.05) compared to saline-treated controls. n = 25.
Fig. 6a. Mean white blood cell (WBC) count in saline-treated controls and khat-treated rabbits over a 5-week experimental period as a function of khat dose. Khat- treated rabbits showed increased WBC counts which, however, were within the physiological limit. Significant increase in counts ($P<0.05$) was seen in rabbits treated with 13.5g/kg and 40.5g/kg body weight of khat extract compared to controls. $n = 25$. 
Fig. 6b. Mean white blood cell (WBC) count in saline-treated controls and khat-treated rabbits as a function of khat dose. Khat extract increased WBC counts at all dosages of khat extract and significant increase (P<0.05) was seen in rabbits treated with doses 13.5g/kg and 40.5g/kg body weight of khat extract compared to saline-treated controls. n = 25.
**Fig. 7a.** Mean packed cell volume (PCV) in saline-treated controls and khat-treated rabbits as a function of khat dose. PCV levels declined at all dosages of khat extract. Significantly decline (P<0.05) was seen in rabbits treated with doses 13.5 g/kg and 40.5 g/kg body weight of khat extract compared to controls. n = 25 rabbits.
**Fig. 7b.** Packed cell volume (PCV) levels in saline-treated controls and khat-treated rabbits as a function of khat dose. Khat caused a general decrease in PCV in a dose-related manner. Significant reduction (P<0.05) was seen in rabbits treated with 40.5g/kg body weight of khat extract compared to saline-treated controls. n = 25.
Fig. 8a. Pattern of plasma bioactive luteinising hormone (LH) concentration in saline-treated controls and khat-treated rabbits as a function of khat dose. Plasma LH levels were significantly suppressed (P<0.05) at all dosages of khat extract compared to controls. Significant LH suppression was seen in rabbits treated with 13.5g/kg and 40.5g/kg body weight of khat extract. n = 25.
Fig. 8b. Plasma luteinising hormone (LH) levels in saline-treated controls and khat-treated rabbits as a function of khat dose. Plasma LH levels were significantly depressed at all dosages of khat as compared to controls (P<0.05; n = 25 rabbits)
**Fig. 9a.** Pattern of mean plasma testosterone levels in saline-treated controls and khat-treated rabbits over a 3-hour period as a function of khat dose. Testosterone levels were suppressed at all dosages of khat extract, with the highest of 13.5g/kg and 40.5g/kg body weight showing a significant suppression (P<0.05) as compared to controls. Dose 40.5g/kg body weight of khat extract showed a maximum decline at the 1 hr 45 min. n = 25.
Fig. 9b. Mean plasma levels of testosterone in saline-treated controls and khat-treated rabbits as a function of khat dose. Testosterone levels were significantly depressed (P<0.05) at all dosages of khat as compared to controls. n = 25.
**Fig. 10a.** Pattern of plasma cortisol levels in saline-treated controls and khat-treated rabbits as a function of khat dose. Plasma cortisol increased at all dosages of khat as compared to controls. The significant increase (P<0.05) was seen in rabbits treated with 13.5g/kg and 40.5g/kg body weight of khat extract and reached maximum levels after 2 hr 30 min of khat administration. n = 25.
Fig. 10b. Mean plasma cortisol in saline-treated and khat-treated rabbits over the 3 hour period as a function of khat dose. Cortisol levels showed a significant increase (P<0.05) at all dosages of khat as compared to saline-treated controls. n = 25.
PLASMA LUTEINIZING HORMONE (LH) PARAMETERS OVER 3 HOUR PERIOD AFTER KHAT EXTRACT ADMINISTRATION

<table>
<thead>
<tr>
<th></th>
<th>0g/kg bwt</th>
<th>1.5g/kg bwt</th>
<th>4.5g/kg bwt</th>
<th>13.5g/kg bwt</th>
<th>40.5g/kg bwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse amplitude (IU/L)</td>
<td>41.9 ± 7a</td>
<td>37.3 ± 6.3b</td>
<td>30.6 ± 6.5c</td>
<td>31.1 ± 4.3c</td>
<td>21.3 ± 4.4c</td>
</tr>
<tr>
<td>Pulse frequency</td>
<td>2.6 ± 0.2a</td>
<td>2.2 ± 0.2b</td>
<td>2.0 ± 0.3c</td>
<td>1.8 ± 0.2d</td>
<td>1.4 ± 0.3c</td>
</tr>
<tr>
<td>Area under curve (IU)</td>
<td>224 ± 18.8a</td>
<td>214 ± 16.9b</td>
<td>167.4 ± 40.5c</td>
<td>187.9 ± 13.1c</td>
<td>168.8 ± 14.4c</td>
</tr>
<tr>
<td>Mean LH (IU/L)</td>
<td>20.4 ± 0.4a</td>
<td>19.4 ± 0.3c</td>
<td>18.7 ± 0.4c</td>
<td>16.6 ± 0.5d</td>
<td>15.5 ± 0.6e</td>
</tr>
</tbody>
</table>

Table 1. Plasma LH levels measured over a 3 hr experimental period at 15 min interval, 10 min after khat extract administration in controls and test rabbits. For each parameter, similar superscripts denote no significant difference. Abbreviation ‘bwt’ stands for body weight.

PLASMA TESTOSTERONE PARAMETERS OVER A 3 HOUR PERIOD AFTER KHAT EXTRACT ADMINISTRATION

<table>
<thead>
<tr>
<th></th>
<th>0g/kg bwt</th>
<th>1.5g/kg bwt</th>
<th>4.5g/kg bwt</th>
<th>13.5g/kg bwt</th>
<th>40.5g/kg bwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area under curve (nmol)</td>
<td>21.0 ± 1.2a</td>
<td>20.8 ± 1.7b</td>
<td>19.6 ± 2.7c</td>
<td>18.4 ± 0.9d</td>
<td>17.3 ± 1.0e</td>
</tr>
<tr>
<td>Mean testosterone (nmol/l)</td>
<td>2.06 ± 0.1a</td>
<td>1.89 ± 0.1b</td>
<td>1.79 ± 0.1c</td>
<td>1.68 ± 0.1d</td>
<td>1.58 ± 0.1e</td>
</tr>
</tbody>
</table>

Table 2. Plasma testosterone levels measured over a 3 hr period at 15 min interval, 10 min after khat extract administration in both control and experimental rabbits. For each parameter, similar superscripts denote no significant difference. The abbreviation ‘bwt’ stands for body weight.
3.3 EFFECTS OF KHAT EXTRACT ON TESTICULAR, EPIDIDYMAL AND ADENOHYPOPHYSEAL MORPHOLOGY

3.3.1 Testes

In controls, the testicular parenchyma comprised of seminiferous tubules and sparsely distributed interstitial tissue (Fig. 1). The seminiferous tubules bounded by the boundary tissue or limiting membrane, provided structural underpinning on which the Sertoli cells and cells of the basal compartment of the seminiferous epithelium rested. The epithelium comprised of Sertoli cells with their irregularly outlined nucleus near the base and their cytoplasmic processes projecting into the lumen (Fig. 1). Various forms of spermatogenic cells occurred in the lateral recesses formed by Sertoli cell branches. Type A spermatogonia were present and frequently found lying with their long axis parallel to and contacting the basement membrane. They contained oval to round nuclei. Two generations of primary spermatocytes were present: the zygotene and pachytene spermatocytes. The two cell populations contained condensed chromatin material in their nuclei but zygotene spermatocytes were identified by their thick condensed strands of chromatin material surrounded by pale cytoplasm while pachytene spermatocytes appeared comparatively larger with indistinct nuclear membrane. Elongate spermatids were also observed in the tubular wall. The interstitial tissue comprised of interstitial (Leydig) cells, generally pleiomorphic in shape but mostly polyhedral or polyangular in outline, among which were few scattered blood and lymphatic vessels, fibroblasts and macrophages (Fig. 1). The Leydig cells contained a large, ovoid nucleus with peripherally marginated heterochromatin. Electron dense bodies, possibly lipid droplets, were observed in the cytoplasm.
In animals treated with 1.5 g/kg body weight of khat extract, both the acute (Fig. 14) and chronic (Fig. 15) phases did not show any appreciable structural differences with the controls. Seminiferous epithelium retained the two primary spermatocyte generations namely zygotene and pachytene spermatocytes. Spermatogonia type A were present lying with their long axis parallel to and in contact with the basement membrane. Spermatids in their cap phase were observed in the tubular wall while the lumen contained spermatozoa (Fig. 15). Leydig cells appeared not to have been affected by the treatment since they displayed similar structural characteristics as in the normal. This feature was observed in all the treatments suggesting that Leydig cells were unresponsive to khat extract exposure.

At a dose of 13.5 g/kg body weight, the acute phase showed degeneration of chromatin material accompanied by vacuolation in spermatogonia and zygotene spermatocytes (Fig. 16). Pachytene spermatocytes and round spermatids were normal and the tubular lumen contained spermatozoa. In the chronic phase, there was further degeneration of chromatin material and vacuolation in zygotene spermatocytes (Fig. 17). Spermatogonia appeared to be undergoing mitotic division. However, pachytene spermatocytes and the acrosomal phase spermatids appeared normal and the tubular lumen was devoid of spermatozoa.

In the acute phase of the highest dose, 40.5 g/kg body weight, there was continued degeneration of zygotene spermatocytes (Fig. 18). Pachytene spermatocytes and spermatids appeared normal and the tubular lumen was devoid of spermatozoa. The
chronic phase showed an enhanced degeneration of zygotene spermatocytes accompanied by vacuolation (Fig. 19). Pachytene spermatocytes and elongate spermatids appeared normal.
**Fig. 11.** Testicular tissue of control rabbit showing normal testicular morphology, consisting of seminiferous tubules (S) and interstitium (I). The seminiferous tubule is bounded by the boundary tissue (B). (Toluidine blue x 400).

**Fig. 12.** Seminiferous tubule of control rabbit showing Sertoli cells (S), type A spermatogonia (A), zygotene spermatocytes (Z), pachytene spermatocytes (P) and elongate spermatids (E). (Toluidine blue x 1,000)

**Fig. 13.** Interstitial tissue of control rabbits. Leydig cells (L) with marginations of heterochromatin in the nucleus (N) and lipid droplets (D). Lymphatic vessels (L) surrounded by endothelial cells (E) and peritubular myoid cells (M), blood capillaries (C) and fibroblasts (F) are present. (Toluidine blue x 1,000).

**Fig. 14.** Seminiferous tubules in acute phase of 1.5 g/kg body weight of khat extract. Note the similarity of type A spermatogonia, zygotene and pachytene spermatocytes with controls (Fig. 14). Leydig cells (L) appear not to have been affected by the treatment. (Toluidine blue x 1,000).

**Fig. 15.** Chronic phase of 1.5 g/kg body weight of khat extract. Type A spermatogonia and pachytene spermatocytes appear similar to those in Fig. 14. (Toluidine blue x 1,000).
**Fig. 16.** Acute phase of 13.5 g/kg body weight of khat extract showing degeneration and vacuolation in spermatogonia and zygotene spermatocytes (arrows). Pachytene spermatocytes (P) and cap phase spermatids (CS) appear normal. The lumen contains spermatozoa (S). (Toluidne blue x 1,000).

**Fig. 17.** Seminiferous tubule of rabbit testis in chronic phase of 13.5 g/kg body weight of khat extract. Similar vacuolations (arrow head) in zygotene spermatocytes as those observed in acute phase are present. Pachytene spermatocytes (P) and acrosomal phase spermatids (AS) appear normal. Type A Spermatogonia (A) appears to be undergoing mitotic division. (Toluidine blue x 1,000).

**Fig. 18.** Seminiferous tubule of rabbit treated with 40.5 g/kg body weight of khat extract in its acute phase. Vacuolations (arrow head) in zygotene spermatocytes is evident. Pachytene spermatocytes (P) and cap phase spermatids (arrow) appear normal. Type A spermatogonia (A) are present. (Toluidine blue x 200, x 1,000).

**Fig. 19.** Seminiferous tubule of rabbit in chronic phase of 40.5 g/kg body weight of khat extract. Notice enhanced degeneration accompanied by vacuolations (arrow head) in zygotene spermatocytes. Type A spermatogonia (A), Sertoli cell (SC), Pachytene spermatocytes (P) and elongate spermatids (E) appear normal. (Toluidine blue x 1,000).
3.3.2 Epididymis

In this study, varied doses of khat extract appeared not to have any significant effect on the epididymal structure. In the caput segment the epithelium consisted of principal cells, basal cells and lymphocytes (Fig. 20). The epididymis had a pseudostratified columnar epithelium with stereocilia. The nucleus was ovoid with regular outline and contained little heterochromatin while the cytoplasm contained numerous micropinocytotic vesicles, secretory granules and multivesicular bodies. Basal cells and lymphocytes were scattered basally between principal cells. The epithelial cell height in the corpus appeared slightly reduced and with short apical microvilli in comparison to the caput epididymis (Fig. 21). The cytoplasm contained numerous secretory granules, prominent Golgi apparatus and multivesicular bodies. The amount of micropinocytotic vesicles was reduced. In cauda epididymis, the epithelial height was considerably reduced in comparison to caput and corpus. The principal cells contained ovoid nuclei and prominent Golgi apparatus in the apical cytoplasm (Fig. 22). The amount of multivesicular bodies was reduced.

3.3.3 The pituitary gland

Khat extract appeared not to have any effect on the adenohypophysis in all groups of animals treated. The adenohypophysis of rabbits (both treated and controls) showed clusters of glandular cells (basophils, acidophils and chromophobes) mainly around blood capillaries but others were scattered in the adenohypophysis (Fig. 23). The cytoplasm of
basophils appeared light stained with less secretory granules while that of acidophils appeared deeply stained with abundant secretory granules. Chromophobes appeared to have light-staining cytoplasm and they were relatively smaller than basophils.
**Fig. 20.** Caput epididymal epithelium of khat-treated rabbit with tall principal cells (P). Heterogeneous dense bodies (arrow), apical and basal micropinocytotic vesicles (V) and well developed Golgi apparatus (G) characterize these cells. Basal cells (B) and intracubal epithelial lymphocytes (L) are scattered between principal cells (Toluidine blue x 1,000).

**Fig. 21.** Corpus epididymis of khat-treated rabbit showing slightly reduced epithelial height and comparatively shorter apical microvilli in comparison with the caput epididymis. Secretory granules (arrow), micropinocytotic vesicles (V) and Golgi apparatus (G) are present. (Toluidine blue x 1,000).

**Fig. 22.** Cauda epididymal epithelium of khat-treated rabbit showing considerably reduced epithelial cell height in comparison to the caput and corpus. The amount of multivesicular bodies is reduced. Golgi apparatus (G) are present. (Toluidine blue x 400).

**Fig. 23.** Khat-treated rabbit adenohypophysis showing clusters of glandular cells mainly around blood capillaries (C). Basophils (B) with light staining cytoplasm, acidophils (A) with deeply stained cytoplasm and abundant secretory granules and chromophobes (C) with light-staining cytoplasm and smaller than basophils, are present. (Toluidine blue x 1,000).
CHAPTER 4

4.0 DISCUSSION

4.1 CLINICAL OBSERVATIONS

The results of clinical observations in the present study indicate a significant effect of khat extract particularly on vital parameters monitored *vis a vis* body weight gain and daily food intake both in khat-treated rabbits and saline-treated controls. The results show reduced body weight gain and food consumption in khat-treated rabbits in a dose-dependent manner. This is in agreement with earlier reports on khat (Halbach, 1972; Luqman and Danowski, 1976; Baasher and Sadoun, 1983; Kalix, 1991; Patel, 2000) and (-)-cathinone, (W.H.O, 1980; Zelger *et al*., 1980; Islam *et al*., 1990), showing appetite suppressant activity. In this study, it was noted that increase in body weight in rabbits became progressively smaller with higher doses of khat extract. This observation is in line with other findings of Islam *et al*, (1990) in the study on effect of cathinone enantiomers in male Wistar rats, which reported (-)-cathinone, active ingredient of khat, as having reduced body weight gain in a dose-dependent manner.

Several researchers have reported khat as a psychoactive drug, whose effects are principally centred in the nervous system and particularly in the brain. Khat, like other psychoactive drugs, has been shown to have effects in several rat brain regions such as nucleus accumbens, striatum and caudate nucleus (Kalix, 1984a; 1988). The impairment of reproductive function associated with psychoactive drugs is mediated by their action on the hypothalamus (Nahas, 1981). As it is located outside of the blood-brain barrier, it is exposed to drugs and other compounds in the systemic circulation. Rabbits, unlike
most mammals, have a direct arterial supply to the pars distalis (Pietsch, 1930; Popa and Fielding, 1933). On the other hand, the hypothalamus has been shown to be involved in central appetite regulation and, in particular, the arcuate nucleus (Neary et al., 2004). It does this through Agouti-related protein (AgRP), found exclusively in the arcuate nucleus and which increases food intake through antagonism of the melanocortin MC3 and MC4 receptors and thus blockade of the inhibition of the anorexigenic agonist α-melanocyte stimulating hormone (α-MSH). Virtually all AgRP neurones co-secrete neuropeptide Y (NPY), (Goldstone et al., 2002), which have Y2R - a presynaptic inhibitory autoreceptor. The arcuate nucleus also contain galanin-like peptide (GALP)-containing neurones, which plays a critical role in the regulation of mammalian energy balance and reproduction (Gottsch et al., 2004), and several discrete populations of neurones in this nucleus are targets for the regulatory hormone leptin (McMinn et al., 1998, 2000). In rodents and primates, arcuate nucleus contains mRNA of neuropeptides involved in feeding and/or reproduction including neuropeptide Y (NPY), α-melanocyte stimulating hormone (α-MSH), agouti-related protein (AgRP), and galanin (Cone et al., 2001). GALP infusions have been shown to stimulate luteinizing hormone (LH) release in a dose-related manner in male rats, mice and macaques (Cunningham et al., 2004; Krasnow et al., 2003, 2004; Matsumoto et al., 2001) and this LH release is likely mediated via GnRH-1-dependent pathways (Cunningham et al., 2004; Matsumoto et al., 2001). It could have been that khat extract in the gut stimulated release of PYY that resulted in reduction of feeding in the rabbits or interfered with mRNA of neuropeptides in arcuate nucleus involved in regulation of feeding and reproduction. It is also possible
that khat interfered with synthesis or release of GALP in the arcuate nucleus or preoptic area resulting in the observed effects but these, however, remain speculative.

Cathinone has been shown to act on the catecholaminergic synapses to increase levels of dopamine, serotonin and noradrenaline in the brain (Kalix, 1981; 1982, 1984; 1988; 1991; Calcagnetti and Schechter, 1993). Serotonin (5-HT) is a short-acting widespread neurotransmitter which acts on a number of receptor subtypes found at high density in the limbic system and raphe nuclei as well as in the hypothalamus (Blundell, 1984). Agonists at the 5-HT$_{2c}$ receptor show the most consistent inhibition of food intake and the 5-HT$_{2c}$-knockout mouse is hyperphagic and obese (Tecott et al., 1995). In another study on investigation of involvement of 5-HT$_{2C}$ receptors in the regulation of food intake in the Siberian hamsters treated with fenfluramine, it was observed that fenfluramine exerted a potent hypophagic effect (Schuhler et al., 2005). Because fenfluramine is known to increase 5-HT release and inhibit its reuptake, these observations demonstrated the involvement of the serotonergic system in the regulation of food intake in the hamster. In general, agonists at the 5-HT receptors and drugs that inhibit the uptake of serotonin reduce feeding. Additionally, 5-HT stimulates noradrenaline release and modifies behaviour and mood (Neary et al., 2004). However, at high doses, the massive release of 5 –HT not only gives rise to acute psychotic symptoms but also cause chemical damage to the cells that release it (Kalant, 2001). From these observations, it is possible that the observed effects of khat on food intake in rabbits may have been due to their effect on the arcuate nucleus or as a result of increased levels of 5-HT in the brain. In this study since food and water intake showed positive correlation, both appeared to affect body weight gain significantly.
The results on body temperature showed a significant increase in a dose-dependent manner throughout the experimental period. Khat has been shown to cause central nervous system stimulation, presumably as a consequence of the sympathomimetic effects of cathinone (Balint and Balint, 1994). Central thermoregulatory disturbances from sympathomimetics may arise from complex interactions between dopamine and 5-HT in the brain stem and hypothalamus (Ricuarte et al., 1980; Yamawaki et al., 1983; Callaway and Clark, 1994). There are several reports linking khat to increase in body temperature (Luqman and Danowski, 1976; Kalix, 1991, Patel, 2000). Previous studies on rats have shown that a nonselective dopamine receptor agonist, such as apomorphine, when given in the presence of a D2 dopamine receptor antagonist, such as haloperidol, can elicit an effect on 5-HT receptors resulting in hyperthermia (Yamawaki et al., 1983). Hyperthermia also commonly follows increased heat production from physical activity. It has been shown by earlier investigators that khat at high doses causes, among other effects, hyperactivity (Kalix, 1991; Pantelis et al., 1989; Patel, 2000) and this may have led to the observed changes in core body temperature in khat-treated animals. Both lack of central dopaminergic activity and serotonergic hyperstimulation have been associated with altered hypothalamic thermoregulatory control as well as abnormal central sympathetic and motor activity that may influence peripheral body heat production and dissipation (Lee et al., 1985; Nimmo et al., 1993; Parada et al., 1995; Schwartz et al., 1995). Dopamine injection into the preoptic nuclei of anterior hypothalamus decreases core body temperature in animal models, suggesting that decreased dopaminergic activity at this level may precipitate hyperthermia (Yamawaki et al., 1983). An earlier study
investigating the role of cathinone in the brown tissue thermogenesis obtained evidence suggesting that beta-adrenergic receptors might be involved in the responses obtained (Tariq et al., 1989).

Another proposed mechanism of sympathomimetic-induced hyperthermia suggests that these agents may initiate neuronal damage and increase hypothalamic concentrations of interleukin-1 beta commonly known as the cytokine of fever (Albers and Sonsall, 1995). Peripherally, sympathomimetics such as 3, 4-methylenedioxymphetamine have been shown to raise metabolism up to 118% while elevating core temperature (Gordon et al., 1991). Seizures, hyperkinetic muscle action and motor excitability may also contribute to a rise in core temperature. Khat contains sympathomimetic compounds that cause vasoconstriction of peripheral vessels in the rabbit (Kalix, 1991) and cardiac effects (Balint and Balint, 1994) thereby impairing heat dissipation. Although the exact mechanism remains unknown, clinical data and animal studies indicate that ambient temperature, motor activity, metabolic regulation, autonomic vascular changes and central disturbances in thermoregulation all contribute to profound elevations in core body temperature.

From the foregoing, it is likely that the hyperthermia associated with khat administration to rabbits in the present study may be due to 1) the sympathomimetic effect of catecholamines on the hypothalamus and peripheral circulation or both via the beta adrenergic receptors or 2) effect on central thermoregulatory control.
Mydriasis associated with khat could be as a result of stimulation of the lateral hypothalamic areas, which have similar effects as those of peripheral sympathetic fibres (Balint et al., 1991) or indirect sympathomimetic effect on the pupil via noradrenaline release at the nerve terminals in the iris (Kalix, 1983b). In the present study, khat extract increased WBC counts while decreasing RBC counts and haemoglobin concentration in a dose-dependent manner. Earlier findings have shown that cathinone and amphetamine induce stress that leads to lymphocytosis, neutropenia and eosinophilia in the user (Marinelli and Piazza, 2002). It is possible that elevated WBC counts had an effect on hormonal parameters measured since stress has been shown to activate glucocorticoid secretion (Hoebel et al., 1989; Salamone et al., 1994; Westerink et al., 1994; Wilson et al., 1995; Taber and Fibiger, 1997; Bassareo and Di Chiara, 1999). Stress has also been shown to inhibit the secretion of GnRH from the hypothalamus resulting in a reduced release of LH and FSH from the pituitary and subsequent secretion of the sex steroids from the gonads (Marinelli and Piazza, 2002).

4.2 HORMONAL ANALYSIS

4.2.1 Effect of khat extract on bioactive luteinizing hormone

The findings of the present study have shown that crude khat extract caused changes in bioactive LH and the response was dose-related. The results show that khat extract had depressive effects on bioactive LH compared to saline-treated controls by its action on the hypothalamo-pituitary-gonadal axis. In this regard, some reports have implicated cathinone to inhibit the release of several anterior pituitary hormones (Wagner et al., 1982).
The finding of highly variable plasma levels of LH in ovariectomized rhesus monkeys led to the first demonstration that gonadotropins tend to be released in pulsatile fashion (Dierschke et al., 1970). The concentrations of circulating gonadotropins are known to depend on the frequency and amplitude of GnRH pulses discharged into the pituitary portal vessels, which in turn depend on the endocrine status. This is in agreement with the present study which has shown pulsatile pattern of LH release. This pattern was, however, significantly affected by two high doses: 13.5 g/kg body weight and 40.5 g/kg body weight of khat extract. Similarly, pulse amplitude showed a dose-dependent decline, indicating that khat extract possibly had an effect on the hypothalamic areas such as medial basal hypothalamus or acted directly on the pituitary gland. Multiple pulses of GnRH in the portal blood flow have been shown to be accompanied by increased frequency of pulsatile LH release, the magnitude of which is governed initially by the responsiveness of the anterior pituitary after being primed with estrogen (Gallo, 1981). Another possible mechanism by which khat may have caused a decrease in bioactive LH is via its sympathomimetic effect which initiates neuronal damage thereby resulting in large amounts of hypothalamic concentrations of interleukin-1 beta (Albers and Sonsall, 1995). This speculation was evidenced with consistent increase in core temperatures in khat-treated rabbits. Kaira et al., (1990) reported that interleukin-1 (IL-1) inhibits the release of hypothalamic GnRH in the rat. It is now known that the secretion of anterior pituitary hormones such as growth hormone, prolactin, thyroid stimulating hormone, luteinizing hormone and follicular stimulating hormone are usually influenced by cytokines such as IL-1α and β, IL-6, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (INF-γ) (Scarborough, 1990). It has been reported that the pituitary
cells express cytokine receptors for IL-1, IL-2 and IL-6 (Marquette et al., 1990). Based on this observation, it is also possible that high doses of khat caused release of cytokines, which may have played a role in the hormonal changes observed in this study. However, this hypothesis remains speculative as work should be carried out to establish the cytokine production, if any, and its potential effects on GnRH secretion in the hypothalamus following khat administration. Shin et al., (1974) reported the detection of dopamine receptors on pituicyte membranes. Dopamine inhibits gonadotropin release by a mechanism involving binding of dopamine to its receptors on basophils. Dopamine receptor complex activates a membrane inhibiting G protein, which is negatively coupled to adenyl cyclase and phospholipase system thereby reducing gonadotropin release and mRNA transcription (Yen, 1991).

4.2.2 Effect of khat extract on plasma testosterone

In this study, it was noted that khat extract had depressive effects on plasma testosterone levels in rabbits when compared to the saline-treated controls. The high doses (13.5 g/kg body weight and 40.5 g/kg body weight of khat extract) significantly suppressed the pulsatile release pattern of testosterone. The pulse frequency and pulse amplitude were affected in a similar manner suggesting that the effect could either be indirectly from the hypothalamus following reduced bioactive LH release from the pituitary gland or directly on the cellular components or specific receptors in the testicular tissue. Reports from investigators (Wagner et al., 1982) have shown cathinone to be an inhibitor of the release of several anterior pituitary hormones. It is, however, not clear if LH is affected in the same way. Following the well-known negative feedback mechanism of
steroid secretion, a fall in plasma LH is likely to affect steroidogenesis. On the other hand, cathinone and amphetamine have been shown to induce stress, which interacts with the rewarding properties of these drugs by altering mesocorticolimbic dopamine transmission (Marinelli and Piazza, 2002). This stress affects the hypothalamo-pituitary-gonadal axis, thus inhibiting the secretion of GnRH from the hypothalamus resulting in a reduced release of LH and FSH from the pituitary and subsequent secretion of the sex steroids from the gonads. Cathinone has also been shown to have indirect sympathomimetic effects (Kalix, 1991) which are known to suppress gonadal function leading to a decrease in testosterone levels (Gray et al., 1978).

Other studies done to investigate effect of khat on reproductive function, have reported impairment of sexuality (W.H.O, 1964; Maitai, 1983), loss of libido (Krikorian, 1984), inhibition of spermatogenesis and reduced semen output in roosters treated with khat extract (Hammouda, 1978) and decrease in plasma testosterone as well as atrophy of Sertoli and Leydig cells in male Wistar rats (Islam et al., 1990). Several contradictory reports have cited khat as an aphrodisiac (Margetts, 1967; Krikorian, 1984), a medicament for premature ejaculation (Luqman and Danowski, 1976; Pantelis et al., 1989) and increasing sexual desire (Elmi, 1983a) and boosting sperm power (Adeoya-Osuguwa and Fraser, 2005). These varying findings about khat effects on reproductive function have left a lot of speculations among many investigators. The differences in observations, however, may be as a result of different varieties of khat used, animal models and route of administration as well as different methodologies in carrying out these experiments. Perhaps more importantly, it should be mentioned that the observed
effects in the khat user are partly as a result of the varying proportions of catecholamines released after khat use. The effects of dopamine and serotonin on sexual behaviour are antagonistic, such that dopamine is facilitative whereas serotonin is inhibitory (Hull et al., 1999). Low levels of dopamine in the medial preoptic area (MPOA) may facilitate parasympathetically mediated erections by stimulating D1-like receptors (Hull et al., 1992; Markowski et al., 1994), whereas higher doses will facilitate sympathetically mediated ejaculation via D2-like receptors (Bazzett et al., 1991). On the other hand, serotonin levels increase markedly in the anterior lateral hypothalamus then diffuse to the preoptic area after ejaculation (Lorrain et al., 1997). This shows that the neurotransmitter released in large amounts or the concentration of that neurotransmitter will determine, in part, the clinical manifestations of the drug.

In this study, giza variety of khat (known to be the most potent) was used and intragastric administration was preferred to parenteral administration, since many of its psychoactive effects result from its metabolites. The study did not, however, focus on evaluating neurotransmitter concentrations as a result of khat treatment. The findings of this study suggest that khat may have interfered with the reproductive parameters by exerting its effect on the MPOA integrative system in the hypothalamus. The MPOA is critical for male sexual behaviour in all vertebrates studied (Meisel and Sachs, 1994). Kalix, (1991) reported the similarity between animal effects of cathinone and amphetamine to be sympathomimetic in nature. The increase in neurotransmitter concentrations (e.g. dopamine) probably result from dopamine uptake blockade, reverse transport of dopamine through the dopamine transporters (Schmitz et al., 2001) and possible action on endogenous opioid systems (Schad et al., 2002). In in vitro studies on
mouse Leydig cells, increasing doses (15 - 1000 µg/ml) of 6-hydroxydopamine (a β-adrenergic blocker) stimulated, then suppressed testosterone production (Wango et al., 1995). Recent findings on effect of khat extract on isolated mouse interstitial cells have shown that high concentrations (30mg/ml and 60mg/ml) of khat extract adversely affected cell viability while low concentrations (0.06mg/ml, 0.6mg/ml and 6mg/ml) caused minimal effect. These higher doses affected the viability of interstitial cells by about 50%. (Nyongesa et al., 2006). Previous studies investigating the role of cathinone in brown adipose tissue thermogenesis yielded evidence suggesting that β adrenergic receptors may be involved in the responses obtained (Tariq et al., 1989).

Earlier studies attempting to identify a mechanism of action of phenylpropanolamines in somatic cells focused mainly on either responses in the central nervous system or effects on ventricular contraction. Amphetamine derivatives and cathinone were shown to potentiate the actions of noradrenaline (NA) on rat right ventricular contraction; it was suggested that this occurred as a result of these derivatives or cathinone preventing the uptake of NA from the nerve terminal by the action that involved competitive blockade of the NA transporters (Cleary et al., 2002; Cleary and Docherty, 2003). Rothman et al., (2003) and Tariq et al., (1989) obtained evidence suggesting that β-adrenergic receptors were involved in cathinone and amphetamine-induced thermogenesis in brown adipose tissue. These findings provide some support for the possibility that cellular responses induced by cathinone and its metabolites might involve adrenergic receptors. In the present study, it is possible that one of the mechanisms of action of khat is through its effects on β-adrenergic receptors on the Leydig cells and interference with the operations of cyclic adenosine monophosphate (cAMP) thereby impairing the process of
steroidogenesis. However, cAMP was not measured in this study. The results on food intake in the present study recorded suppressive effect in a dose-dependent manner, with the highest doses (13.5 g/kg and 40.5 g/kg body weight of khat extract) suppressing food intake significantly. Earlier reports have shown that partial or total starvation affect male reproductive system and produce atrophy of testes and epididymis (Howland et al., 1974; Fanjul et al., 1981). Khat may also have interfered with reproductive parameters including steroidogenesis due to its impairment of food intake as is evident in the present findings. On the other hand, cytotoxic effect of cathinone has been reported to be responsible for androgenic deficiency in mice (Tariq et al., 1987). A significant mitodepressive activity of khat and cathinone has also been reported (Al-Meshal et al., 1987). Khat extract and cathinone have been shown to depress cell proliferation and inhibit RNA, DNA and protein synthesis in dividing cells, which may be responsible for reduced spermatogenesis (Al-Meshal et al., 1989; De Hondt et al., 1984 and Hammouda, 1971). Recent reports assessing the effects of khat on male reproductive tract showed that feeding rabbits for 3 months with food containing different amounts of dried, ground khat leaves stimulated spermatogenesis yet cauda epididymides and Leydig cells remained normal, when compared to equivalent tissue sections from controls (Al Mamary et al., 2002). These findings are at variance with those of the present study due to use of dried leaves rather than fresh leaves as used by khat users. It is therefore difficult to conclude that food preparation given together with khat had an effect on its potency or as a result of different mechanisms of action of khat in both in vivo and in vitro environments. More recently, studies in male baboons have shown that khat up-regulates plasma testosterone production and suppresses cortisol and prolactin levels (Mwenda et al., 2006).
The present study did not show any significant decrease in the size of Leydig cells in khat-treated rabbits compared to the controls. These results are at variance with those of Castro et al., (2002) who reported a strong correlation of both plasma and tissue levels of testosterone with the percentage volume of Leydig cell nuclei as well as the number of Leydig cells per gram of testis. Other findings of Ewing et al., (1979) cited species differences in testosterone secretion to be due to some qualitative differences in Leydig cell structure and/or function. The same investigators speculated that there might be a strong correlation between testosterone secretion and amount of membranous structures present in either the mitochondria and/or smooth endoplasmic reticulum (SER) of Leydig cells in different species. This view was confirmed by Zirkin et al., (1980) who reported a positive linear correlation between testosterone secretion and the volume density of SER in five mammalian species, including the rabbit. This study is considering ultrastructural studies of Leydig cells as a subsequent objective to augment earlier findings. Another factor involved in testosterone synthesis by Leydig cells is the direct relationship between the binding activity of androgen receptors and the number and size of Leydig cells, as demonstrated by Tripepi et al., (2000) in the domestic pig at different biological stages of development. These findings are in agreement with those of the present study, which seems to indicate a direct effect of khat on Leydig cell steroidogenesis in the rabbits. This finding is significant in that it points to the possible mechanism of action of khat in the body.
4.2.3 Effect of khat extract on plasma cortisol

Increasing doses of khat extract given to rabbits in this study showed an increase in plasma cortisol levels in a dose-related manner compared to controls. Since the saline-treated rabbits were handled in the same manner as the khat-treated rabbits, it is logical to attribute the elevation of cortisol levels to khat treatment. Additionally, blood sampling was done at constant times of the day (in the morning hours); each time blood was serially collected for cortisol assay. This was to avoid influence of time of day on cortisol levels as glucocorticoid hormones are characterized by a circadian cycle (Akana et al., 1986). Thus, the observed pattern in cortisol levels in this study is unlikely to be due to variations in time of handling.

Findings from numerous clinical studies have confirmed popular beliefs that stress contributes to the development, maintenance and outcome of substance of abuse disorders in humans (Brewer et al., 1998; Brown et al., 1990; Cole et al., 1990; Karlsgodt et al., 2003; King et al., 2003; Sussman and Dent, 2000). It has also been shown that psychostimulants increase corticosterone secretion (Mello and Mendelson, 1997). The locomotor response induced by the injection of psychostimulants in the nucleus accumbens is dopamine-dependent (Vezina and Stewart, 1984; Delfs et al., 1990), and it has been shown that this response is decreased by suppressing corticosterone and re-established by restoring basal levels of the hormone (Marinelli et al., 1994). Suppression of glucocorticoids by adenolectomy reduces extracellular concentrations of dopamine in the nucleus accumbens both in basal and in response to psychostimulants (Piazza et al., 1996; Barrot et al., 2000). This follows considerable evidence suggesting the possibility
of drugs of abuse acting via mechanisms involving mesocorticolimbic dopamine pathways. There is growing evidence that glucocorticoids are important mediators of the relationship between stress and drug-seeking behaviour in rodents (Marinelli and Piazza, 2002; Piazza and Le Moal, 1998). High levels of glucocorticoids have also been shown to increase rates of response to psychostimulant drugs (Goeders and Guerin, 1996b; Piazza et al., 1991) though mechanism modulating the rise in plasma cortisol remains speculative. In this regard, reports showing that adrenolectomy attenuate psychostimulant self-administration (Deroche et al., 1997; Goeders and Guerin, 1996a) and reduction in the amount of alcohol intake in alcohol-preferring rats (Fahlke et al., 1994) may be relevant; these effects can be reversed by exogenous corticosterone replacement. In this study, however, the adrenal glands were neither weighed nor examined histologically. The mechanism underlying the relationship between stress and drug-seeking behaviour prevalent in khat chewers is not well understood; it is hypothesised that stress interacts with the rewarding properties of drugs of abuse by altering mesocorticolimbic dopamine neurotransmission (Marinelli and Piazza, 2002). This finding is in line with that of the present study, showing significant increase in plasma cortisol with higher levels of khat extract, indicating enhanced interaction with the pleasurable effects of the drug thereby altering dopamine neurotransmission in the mesocorticolimbic system. In several other investigations designed to examine the relationship between cortisol levels, mesolimbic dopamine release and subjective drug response to amphetamine in healthy volunteers, it was found that increase in cortisol levels was associated with greater amphetamine-induced dopamine release in several regions of striatum, including left ventral striatum (LVS), left dorsal putamen (LDP) and right dorsal putamen (RDP) (Oswald et al., 2005).
Glucocorticoids are not only activated by stress but their secretion also precedes many goal-seeking behaviours such as food-seeking (Hoebel et al., 1989; Salamone et al., 1994; Westerink et al., 1994; Wilson et al., 1995; Taber and Fibiger, 1997; Bassareo and Di Chiara, 1999) and drug-seeking behaviour (Cadoni et al., 2003; Marinelli and Piazza, 2002).

Further investigations on amphetamine have shown that plasma levels of glucocorticoids and adrenocorticotrophic hormone (ACTH) are increased by its acute administration in rodents and humans (Halbreich et al., 1981; Jacobs et al., 1989; Smith et al., 2004; Swerdlow et al., 1993). The results in this study have demonstrated that khat indeed has an effect on plasma cortisol levels. Even so, the mechanism modulating the rise in plasma cortisol values remain speculative. However, since cathinone has been described as a natural amphetamine (Kalix, 1984a, 1988, 1991, 1992; Kalix and Glennon, 1986), having a pharmacological profile resembling that of amphetamine while causing similar euphoric and stimulatory effects (Brenneisen et al., 1990; Kalix, 1992), it is possible that it also exerted effect on plasma levels of glucocorticoid and ACTH after administration, hence the increased plasma cortisol concentrations observed in khat-treated animals compared to controls.
4.3 EFFECTS OF KHAT EXTRACT ON TESTICULAR, EPIDIDYMAL AND ADENOHYPOPHYSEAL MORPHOLOGY

In the present study, testicular, epididymal and adenohypophyseal effects of khat extract at different dose levels were studied in rabbits. In normal rabbits, the volume of interstitial tissue was small comparable to what is known in the chinchilla, guinea pig, rat and mouse (Fawcett et al., 1973). The morphological results of this study suggest that, cellular components of the interstitial tissue including Leydig cells, macrophages, fibroblasts and endothelial cells associated with blood and lymphatic vessels are unaffected by khat extract exposure, but the drug has a profound influence on the process of spermatogenesis. This finding is at variance with what is known in the rat (Islam et al., 1990) where cathinone causes atrophy of Leydig and Sertoli cells as well as degeneration of spermatocytes and spermatids but similar to those reported in baboons where, Leydig cells were unaffected by khat extract (Mwenda et al., 2006). However, in the study on baboons, like in the present study, the ultrastructure of Leydig cells was not examined perhaps causing variation in results. Earlier studies have shown that khat extract and cathinone depress cell proliferation and inhibit RNA, DNA and protein synthesis in actively dividing cells (Al-Meshal et al., 1989; De Hondt et al., 1984; Hammouda, 1978). The vacuolation in zygotene spermatocytes of khat-treated rabbits observed in this study confirms earlier findings of impaired spermatogenesis reported in roosters (Hammouda, 1978), mice (Qureshi et al., 1988; Tariq et al., 1990), rat (Islam et al., 1990), humans (El-Shoura et al., 1995). It is apparent that khat primarily affected the premature forms (spermatogonia and zygotene spermatocytes) while pachytene spermatocytes, spermatids and spermatozoa were unaffected. Premature germ cells (spermatogonia and zygotene
spermatocytes) undergo active mitotic cell division, hence, exhibit the greatest dynamic transformation of chromatin. In these stages there is abundance of histones that are highly susceptible to toxic agents but in the final stages of sperm formation (maturation stages), chromatin is highly condensed due to the displacement of lysine rich histones by small arginine and cysteine-rich protamines (Carlson, 1999) which are unlikely to be affected by these insulting agents. In the present case, therefore, chromatin condensation and subsequent vacuolation in zygotene spermatocytes all point towards a serious influence of khat on cell division and development. Vacuoles in the seminiferous epithelium are frequently encountered where there is disruption in the process of spermatogenesis. Similar findings were reported in rats treated with heptachlor (Wango et al., 1997) and goats treated with ethane dimethanesulphonate (Onyango et al., 2001a). The present study is currently focussing on the ultrastructure of Leydig cells to observe any possible cytological changes caused by khat extract and correlate these findings with results on testosterone measurements obtained in this study.

The histology of the epididymis in khat-treated rabbits did not show any observable lesions compared to saline-treated controls. In the caput segment, numerous micropinocytotic vesicles, secretory granules and multivesicular bodies were observed in the cytoplasm of normal epithelial cells similar to those observed in epididymis of khat-treated rabbits. This finding compares favourably with other reports in rabbits (Jones et al., 1979), equines (Arrighi et al., 1993) and goats (Onyango et al., 2001b) where similar dense bodies and micropinocytotic vesicles were reported in normal principal cells. Under normal conditions, principal cells of the caput perform both secretory as well as
endocytic functions but with a net secretory preference (Moore and Bedford, 1979; Hermo et al., 1998). The cytoplasm of principal cells in the corpus contained numerous secretory granules, prominent Golgi apparatus and multivesicular bodies. The cauda epididymis showed similar morphological characteristics although the cytoplasm of the principal cells contained less secretory granules and multivesicular bodies. Epithelial cells of cauda epididymis of khat-treated rabbits showed similar findings. The results of the present study are in agreement with those in baboons (Mwenda et al., 2006), where the epididymides were unaffected by khat exposure.

The histology of the adenohypophysis of saline-treated controls and khat-treated rabbits showed similar features in the gonadotropes. They appeared as large rounded less staining cells close to the blood capillaries and contained irregular nucleus with numerous secretory granules. These were distinguished from acidophils which appeared deeply staining and with numerous secretory granules in the cytoplasm. The results on hormonal assays, however, showed that khat extract had a suppressive effect on LH levels. This is in agreement with earlier findings that cathinone inhibit the release of several anterior pituitary hormones (Wagner et al., 1982). The mechanism by which it does this, however, remains poorly understood. A possible mechanism may involve dopamine. Dopamine receptors have been reported on basophil cells (Shin et al., 1974). Dopamine inhibits gonadotropin release by a mechanism involving binding of dopamine to its receptors on basophils. Dopamine receptor complex activates a membrane inhibiting G protein, which is negatively coupled to adenyl cyclase and phospholipase system thereby reducing gonadotropin release and mRNA transcription (Yen, 1991). Khat may have
interfered with dopamine receptor expression on basophils or mRNA transcription thereby leading to the observed suppression of LH secretion.
CHAPTER 5.

5.0 GENERAL DISCUSSION

The present study focussed on the mechanism of action of khat extract on reproductive function accompanied by clinical manifestations in rabbits. The results provide an indirect in-sight into human ‘khat addicts’, who have been chewing khat for a long period of time. The findings on hormonal analysis indicate that crude khat extract caused changes in bioactive LH, plasma testosterone and cortisol levels and the response to khat was dose-dependent. The LH and testosterone levels in khat-treated rabbits decreased compared to saline-treated controls. This is in agreement with earlier findings which indicated that cathinone, an active ingredient of khat, inhibits release of several anterior pituitary hormones (Wagner et al., 1982). Whether khat extract affected LH levels in a similar manner remains to be elucidated. Ultrastructural studies in these khat-treated rabbits are currently underway to elucidate possible effects of khat on cellular components involved in the synthesis and release of LH. Previous studies in humans (Kalix and Braenden, 1985; Balint et al., 1991) and rats (Islam et al., 1990) also showed that khat causes a decrease in plasma testosterone. This observed decrease in plasma testosterone may have been as a result of direct effect of khat extract on Leydig cells or indirectly as a consequence of decreased LH levels thereby affecting steroidogenesis. It may also have been as a result of increased plasma cortisol levels. The latter possibility follows previous report by Hall, (1994) that high cortisol levels cause a reduction in testosterone levels. Cathinone has also been implicated in having indirect sympathomimetic effects (Kalix, 1991) which are known to suppress gonadal function. This is in contrast with a recent study in Olive baboons (Mwenda et al., 2006) which
showed that khat causes an increase in plasma testosterone levels. The study, however, showed a transient or short-term effect of khat as opposed to long-term effects in human ‘khat addicts’. Khat extract seem to have a similar effect as betel quid (a combination of nut or fruit from *Areca catechu*, leaf from the *Piper betel* and paste of bark from *Acacia catechu*) and they all seem to be derivatives of amphetamine. In a study on effects of aqueous extracts of betel quid and its constituents on testosterone production by dispersed mouse interstitial cells, it was shown that lower doses of the extract arecoline, alone or in combination with 10 ng/ml ovine luteinising hormone significantly stimulated testosterone production over the basal level. However, when co-cultured with cAMP it became inhibitory (Yang *et al.*, 2004). This is in agreement with earlier findings showing effects of 6-hydroxydopamine (a β-adrenergic blocker) on mouse Leydig cells. Increasing doses (15 - 1000 µg/ml) of 6-hydroxydopamine stimulated, then suppressed testosterone production (Wango *et al.*, 1995). At cellular level, cathinone has a similar effect to amphetamine at the central dopaminergic synapses (Kalix, 1983a, b; Kalix and Braenden, 1985), as well as similar effects on other central and peripheral neurotransmitters (Knoll, 1979; Nencini *et al.*, 1984 c; Kalix and Braenden, 1985).

In the present study, khat had marked degenerative changes on testis and these changes occurred in zygotene spermatocytes, which are fast dividing cells. This influence of khat extract on premature forms of germ cells where dynamic transformation of chromatin is greatest, points towards susceptibility of lysine rich histones to insulting compounds resulting in disruption of the process of spermatogenesis. Chromatin in mature forms such as spermatids and spermatozoa is highly condensed due to displacement of histones
by protamines (Carlson, 1999) and therefore resulted in these forms being unaffected by khat exposure. These findings are similar to those reported in roosters (Hammouda, 1978), mice (Qureshi et al., 1988; Tariq et al., 1990), rats (Islam et al., 1990) and humans (El-Shoura et al., 1995). As in this study, the findings of cathinone in rats and khat in humans showed an accompanied decrease in plasma testosterone levels suggesting that khat affected both steroidogenesis and spermatogenesis. Whether or not both processes influence one another to give the observed outcome is not clear. These findings are at variance with those of Adeoya-Osiguwa and Fraser (2005) who reported that khat accelerates sperm capacitation and inhibit spontaneous acrosome loss in rabbits. The study, however, used dried khat leaves to study effects of cathine and not cathinone found in fresh khat leaves. In the present study, there was no structural alteration in the epididymis and pituitary gland at histology. These findings are similar to those reported in Olive baboons (Mwenda et al., 2006).

The clinical manifestations of decreased body weight gain, food and water intake as well as elevated body temperature all point towards indirect sympathomimetic effect of khat (Kalix, 1991). This could also have contributed to the observed mydriasis in khat-treated rabbits.

5.1 CONCLUSION

This study has provided evidence suggesting that long-term use of khat extract has a negative influence on the reproductive function and these effects can be detrimental if the compound reaches toxic levels in the body. The study has demonstrated that khat extract lowers LH and testosterone levels while elevating cortisol levels accompanied by overt
structural alterations in the seminiferous epithelium of testis of rabbits. It has shown that these effects are dose-dependent. Studies are currently underway to determine the ultrastructural changes in Leydig cells and gonadotropes that may be attributed to khat use.
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APPENDIX 1

1.1 UNITS

µl   Microlitre
ml   Millitre
g    Gram
SEM  Standard error of mean
ºC   Degree Celsius
nmol/l Nanomol/litre
IU   International units
%    Per cent
g%   Grams per cent
pH   The negative log of H⁺
hr   Hour
min  Minutes
L    Litre
µ    Micron
cpm  counts per minute
mU   Millunit
APPENDIX 2

2.0 PREPARATION OF REAGENTS

2.1 STEROID ASSAY BUFFER

It contains the following chemicals and their mass, dissolved in 1 litre (1L) of double distilled water.

2.35 g Sodium dihydrogen phosphate (anhydrous) [NaH$_2$PO$_4$ (MW 120)]

11.60 g Disodium hydrogen phosphate (anhydrous) [Na$_2$HPO$_4$ (MW 142)]

8.80 g Sodium chloride (Nacl)

0.10 g Gelatin

1.00 g Sodium azide.

Gelatin was weighed and dissolved in 100ml warm double distilled water. The rest of the reagents were weighed and dissolved in 75ml double distilled water and stirred vigorously to dissolve the particles. The two solutions were then mixed together and volume made up to 1 litre then stirred to dissolve all the particles. The pH was adjusted to 7.4 by addition of either sodium hydroxide or hydrochloric acid. The buffer was then stored at 4 °C until required for use.

2.2 0.1 M PHOSPHATE BUFFER SALINE, pH 7.2

A stock solution of 0.1 M phosphate buffer was prepared by dissolving 17.6 g Na$_2$HPO$_4$.H$_2$O, MW 178.05 in 500 ml double distilled water to make first stock solution.

A second stock solution was prepared by dissolving 13.8 g NaH$_2$PO$_4$.H$_2$O, MW 138.01 in 500 ml double distilled water. The final stock solution of 0.1 M was made by mixing 360
ml of the first stock solution with 140 ml of second solution and diluting it to 1 litre with double distilled water to give 0.1 M phosphate buffer saline at pH 7.2.

2.3 TESTOSTERONE TRACER

This is (WHO RIA matched reagent programme) in amounts of 9.25 MBq (250 µCi) in sealed ampoules. The contents of the ampoule were emptied into a 25 ml volumetric flask, the ampoule rinsed with a solution of toluene: ethanol (9: 1 by volume) and the ‘washing’ added to the contents of the flask. The volume of the stock solution was made to 25 ml with the toluene: ethanol (9: 1) solution. This stock solution (370 kBq or 10 µCi/ml) was then transferred to a dark bottle with airtight seal and stored at -20 ºC. When required, 150 µl of the stock solution was pipetted into a clean vial, solvent evaporated and residue re-dissolved in 15 ml steroid assay buffer. The solution was stood for 30 min before use.

2.4 TESTOSTERONE ANTISERUM

This is provided (WHO RIA matched reagent programme) in lyophilized form. The contents of antiserum are reconstituted with 10 ml of steroid assay buffer, vortex mixed and left standing for 5 – 10 min before use. The resultant solution is enough for one assay.
2.5 TESTOSTERONE STANDARD

This is provided (WHO RIA matched reagent programme) in ethanolic solution at a concentration of 220 nmol/L. To prepare this standard, a vial containing 100 µl of ethanolic solution was reconstituted with 10 ml steroid assay buffer and heated to 40 ºC for 30 min then mixed vigorously and allowed to cool to 4 ºC before use. This solution contained testosterone at a concentration of 2.2 nmol/L and is known as solution B. A rack of 5 test tubes properly labelled was set and in each, 2 ml of steroid assay buffer added. Into tube 1, 2 ml of solution B was pipetted and vortex mixed. Using the same pipette tip, a further 2 ml from tube 1 was pipetted into tube 2 and vortex mixed. The procedure was repeated until tube 5 where 2 ml withdrawn from tube 4 was pipetted and vortex mixed. Together with the stock solution, this gave six serially diluted standard points for the assay.

2.6 CORTISOL TRACER

This is provided (WHO RIA matched reagent programme) in amounts of 9.25 MBq (250 µCi) in sealed ampoules. The contents were transferred to a 25 ml volumetric flask and ampoule rinsed with a solution of toluene: ethanol (9: 1) and ‘washings’ added to the contents of the flask. The volume of the stock solution was made up to 25 ml (containing 370 kBq or 10 µCi per ml) with the toluene: ethanol (9: 1) solution. This solution was then transferred to a dark bottle with an airtight seal and stored at -20 ºC until required for use.
2.7 CORTISOL STANDARD

This is provided (WHO RIA matched reagent programme) in ethanolic solution at a concentration of 6.0 µmol/L. To prepare the standard, 3 x 100 µl aliquots were transferred into glass bottles with screw-on caps. Into each glass bottle, 10 ml of steroid assay buffer was added and heated to 40 ºC for 30 minutes. The solution was then vortex mixed and cooled to 4 ºC before use. This is known as solution B containing 60 nmol/l. A rack of 5 test tubes properly labelled was set and in each, 1 ml of steroid assay buffer added. Into tube 1, 1 ml of solution B was pipetted and vortex mixed. Using the same pipette tip, a further 1 ml from tube 1 was pipetted into tube 2 and vortex mixed. The procedure was completed by transferring 1ml from tube 4 into tube 5 and vortex mixed. Together with the stock solution, this gave six serially diluted standard points for the assay.

2.8 CORTISOL ANTISERUM

This is provided (WHO RIA matched reagent programme) in lyophilized form and stored at 4 ºC until required. The contents of one vial were reconstituted with 10 ml of steroid assay buffer and stood for 10 minutes. This was then vortex mixed before use.

2.9 BASAL MEDIUM EAGLE INCUBATION MEDIUM

The incubation medium contains the following reagents dissolved in 100 ml of double distilled water.

0.919 g Basal Medium Eagle

0.161 g NaHCO₃
2% Bovine Serum Albumin
3 ml Calf serum.
The reagents were carefully mixed to dissolve the particles and stored in ice-cold.

3.0 LUTEINISING HORMONE STANDARD
This is provided (WHO RIA matched reagent programme) in ethanolic solution and one vial contains 50 mU. This was reconstituted with 2 ml incubation medium, vortex mixed and stood for 10 min. To prepare the top standard, 480 µl of this stock solution was pipetted into a test tube and volume made up to 1000 µl using incubation medium giving a required concentration of 12 mU/ml. A rack of six (6) well-labelled tubes was set up and into each, 500 µl of incubation medium was pipetted. Into tube 1, 500 µl of top standard was pipetted and vortex mixed Using the same pipette tip, a further 500 µl from tube 1 was pipetted into tube 2 and vortex mixed. The procedure was completed by transferring 500 µl from tube 5 into tube 6 and vortex mixed. Together with the top standard solution, this gave seven serially diluted standard points for the bioassay.

3.1 CHARCOAL-DEXTRAN SUSPENSION
This was prepared by weighing the following reagents
0.625 g activated charcoal
0.0625 g Dextran
100ml Steroid assay buffer.
Charcoal and dextran were provided by WHO RIA matched reagent programme.
Dextran was dissolved in 100ml steroid assay duffer in a stopper container. Activated charcoal was then added and shaken vigorously to disperse the particles. The suspension was then stored at 4 ºC and when required, it was vigorously stirred for 1 min before use.

### 3.2 SCINTILLATION FLUID

30 g 2, 5-diphenyloxazole (PPO) was transferred in to a 5 litre bottle and 4 litre toluene added and mixed to dissolve the particles.

### 3.3 2% OSMIUM TETROXIDE (OsO4)

Into a flask containing 50 ml double distilled water, 1 g of Osmium tetroxide was added, shaken vigorously and flask wrapped with aluminium foil and stored at 4 ºC until required for use.