EFFECTS OF CRUDE KHAT (*CATHA EDULIS*) extract ON SELECTED CYTOKINE PROFILES AND FOETAL GROWTH AMONG PREGNANT BABOONS (*Papio anubis*)

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A thesis submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science (Immunology) in the School of Pure and Applied Sciences of Kenyatta University

JUNE 2017
DECLARATION

I, Wambua Philomena Nduku, do hereby declare that this thesis is my original work and has not been presented for degree or other awards in any other university

Signature……………………………………….. Date…………………………

Department of Zoological Sciences

Supervisors Approval

We confirm that the work reported in this thesis was carried out by the candidate under our supervision as supervisors

Signature……………………………………….. Date…………………………

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Signature……………………………………….. Date…………………………

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DEDICATION

I dedicate this work to my husband Mr. Ben Kyalo and our sons Peter Mumo and James Muoki.
ACKNOWLEDGEMENTS

For the life within me and the many opportunities at my disposal, God, I am indebted to you. Thank you Father.

I wish to thank my supervisors, Prof. Michael Gicheru of Department of Zoological Sciences, Kenyatta University and Dr. Atunga Nyachieo of the Institute of Primate Research and School of Medicine of University of Nairobi for their guidance and support during my study. Sincere gratitude goes to Mr John Macharia of IPR who helped in data collection and laboratory analysis. I extend my gratitude to the Teachers Service Commission for granting me study leave. Special thanks to the Higher Education Loans Board for awarding me a scholarship that eased my financial burden. Am grateful to my parents Wambua Ndeti and Ruth Mbaika for their tireless efforts of taking me to school. Live long dad and mum. I wish to thank my sister-in-law, Angeline Nduku who played the role of a mother to my sons during my study. God bless you. Finally, I am grateful to my family for the patience which they showed to me during my studies.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACUC</td>
<td>Animal Care and Use Committee</td>
</tr>
<tr>
<td>BCA</td>
<td>B-cell Attracting chemokine</td>
</tr>
<tr>
<td>BLC</td>
<td>B-Lymphocyte chemoattractant</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCL5</td>
<td>Chemokine (c-c motif) Ligand 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Chemokine (c-x-c motif) Ligand 8</td>
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<td>CXCL 10</td>
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<td>CXCL 13</td>
<td>Chemokine (c-x-c motif) Ligand 13</td>
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<tr>
<td>DEA</td>
<td>Drugs Enforcement Act</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas Ligand</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross Domestic Product</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor 1 alpha sub unit</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat shock proteins 70s</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>IP-10</td>
<td>Interferon-gamma Inducible protein 10</td>
</tr>
<tr>
<td>IPR</td>
<td>Institute of Primate Research</td>
</tr>
<tr>
<td>ISERC</td>
<td>Institutional Scientific and Ethical Review Committee</td>
</tr>
<tr>
<td>MHC 11</td>
<td>Major Histocompatibility Complex 11</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometers</td>
</tr>
<tr>
<td>ml</td>
<td>Millitres</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>P38MApk</td>
<td>p38 mitogen activated protein kinases</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>pg</td>
<td>Pictograms</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T-lymphocytes</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF α</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TREM</td>
<td>Triggering receptor expressed on myeloid cells</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitres</td>
</tr>
<tr>
<td>UN</td>
<td>United Nations</td>
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<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Khat (Catha edulis) is a dicotyledonous evergreen shrub of the family Celastraceae whose fresh leaves are habitually chewed for their euphoric properties. The habit of khat chewing is mainly found in Arabia, Somalia, Kenya and has spread to Asia, Europe and other parts of the world due to improvement in transport and communication. Khat consumption was traditionally confined to adult men of the population. However; today khat chewing has become popular among all segments of the population including reproductive aged women. Studies have indicated that khat consumption can be associated with infertility and cardiovascular diseases. However, there have been no such studies on the effects of khat on immunological profiles especially in pregnant females, blood pressure, body weight as well as foetal development during pregnancy. Therefore, the present study sought to determine the effect of crude khat extract on Th 1/Th 2 cytokines, chemokines, blood pressure, weight of pregnant baboons and newborns. The study group comprised of six pregnant olive baboons (Papio anubis) which were divided into two groups; the experimental group (n=3) and, the control group (n=3). During the 3rd and the 4th months of gestation, each study subject in the experimental group was given in addition to normal feed, 50ml of crude khat extract orally once a week and bled once a week for two months. Each study subject in the control group was given in addition to normal feed, 50ml of normal sterile saline orally once a week and bled once a week for the two months. In the 5th and 6th months of gestation, both groups were fed normally and bled once every two weeks for two months till the end of gestation. IFN-γ, IL-10, CXCL 10 and CXCL 13 were then determined using enzyme linked immunosorbent assay (ELISA) technique and compared, blood pressure, temperature and body weight of the pregnant baboons was also determined while the birth weights of newborns were taken. Statistical comparisons between the groups were done using the Students t-test. Administration of khat extract induced a significant decrease in body weight (p=0.0001), in CXCL 13 (p=0.001), and a significant increase in blood pressure (systolic, p=0.049; diastolic, p=0.024), there was no significant difference in body temperature, in IL-10 and IFN-γ (p>0.05). The results suggest that khat consumption decreases levels of IL-10 and body weight; it also elevates blood pressure in baboons. These can be detrimental to pregnancy and the fetus; therefore, it is recommended that pregnant women should be discouraged from consuming khat.
CHAPTER ONE: INTRODUCTION

1.1 General information

Khat (*Catha edulis*) belongs to the sub-order *Rosidae* and family *Celastraceae* (the bitter-sweet family of plants) (Manghi *et al.*, 2009). Khat (*Catha edulis*) is a tall plant, grows to a height of seven meters and spreads over to cover an area of three meters wide. Its natural distribution is limited to East Africa from Ethiopia, Eritrea and Somalia, through to South Africa; and it also spreads to some parts of Rwanda, Democratic Republic of Congo, Malawi and Zimbabwe (Getahun and Krikorian, 1971). Khat plant is also found sporadically in Afghanistan, Israel, Saudi Arabia, Syria and Turkistan (Balint and Balint, 1994). It grows best at high altitudes of 1500-2500 meters above sea level (Corkery *et al.*, 2011). It is best cultivated in high elevations with high rainfall in acidic, well-drained clay soils, but can survive long periods of drought. Khat plant is rarely affected by diseases and can live up to 75-100 years (Lugman and Danowski, 1985; Balint and Balint, 1994).

*Catha edulis* is known by various names in the different regions where it is grown such as Arabian tea, Tchat, Chak, Chaad, Khat, Miraa and Qat. Khat trunk is straight and slender; the bark is thin, smooth and grayish brown in appearance. The plant has a tap-root which grows to a depth of 3 meters or more. The *Catha edulis* plant is polymorphic and the branches have either opposite or alternate leaves. The leaves are 2-5 cm wide and 5-10 cm long (Figure 1.1).
Figure 1.1: Leaves of the khat plant (*Catha edulis*)
The shape of leaves range from oval-lanceolate to elliptical and have serrated edges. Old leaves are leathery in texture, highly polished on their upper surface and deep green in colour. The peduncle is around 3-7 mm long; it has small flowers that are produced on short axillary cymes that are 4-8 cm in length. Each flower is small with five white petals. The *Catha edulis* fruit is an oblong three calved capsule containing one to three seeds (Raman, 1983). The leaf odor is faintly aromatic (Kalix and Braenden, 1985). Its tender twigs and leaves (Figure 1.2) are harvested almost year round (Al Motarreb *et al.*, 2007).
Figure 1.2: Tender shoots and leaves of khat plant harvested by farmers
Khat consumption is a common habit by the communities in the regions where it is grown. However, improvements in transport and communication have enabled spread of khat consumption to almost all parts of the world (Toennes et al., 2003). Khat has huge economic benefits for the farmers, traders and even government in form of taxes from the khat business (Carrier, 2005). Income from khat contributes substantial amounts of Gross Domestic Product (GDP) in countries like Ethiopia, Yemen and Kenya (Al-Hebshi and Skaug, 2005). Khat use has increased steadily over the last two decades (Sparago et al., 1996; Rousseau, 1998) and has become an issue of significant social and medical importance (Al-Motareb et al., 2002; Beckerleg, 2008).

One of the most common forms of drug use and abuse in many East African nations involves chewing parts of the khat plant (Sparago et al., 1996; Rousseau, 1998; World Drug Report, 2001). The amount of khat chewed per user is 100 to 200 g of leaves and stems over 3 to 4 hours (Nencini and Ahmed, 1989; Kalix, 1990; 1994; Al-Motareb et al., 2002). The tender leaves and stems which lose their potency one day after harvest are chewed and the juice swallowed. Large amounts of liquid are consumed while chewing because of the dryness induced by the plants.

Khat leaves have been used in traditional medicine for the treatment of depression, fatigue, hunger, obesity and gastric ulcers (Kennedy et al., 1983). It is a controlled substance in some countries such as United States, Britain, Canada and Germany, while its production, sale and consumption are legal in other nations including Djibouti, Somalia, Ethiopia and Yemen (Elmi, 1983). In Kenya, khat is legal, however, two of its active components, cathinone and cathine were classified effective 1993, cathinone was
placed into Schedule I of the Controlled Substances Act (Federal Register, 1993). Khat is a Schedule I substance when cathinone is present. Cathine was ruled as a Schedule IV substance (Federal Register, 1988). When the plant no longer contains detectable levels of cathinone, as in older leaf samples, but contains cathine, khat is classified as a Schedule IV substance.

Cytokines are a broad category of small proteins approximately 5-20 kDa that are released by cells and affect the behavior of other cells and sometimes the behavior of the releasing cell itself (Alexander, 2001). Cytokines include chemokines, interferons, interleukines, lymphokines, tumor necrosis factor but generally not hormones or growth factors (Horst, 2013). Cytokines are produced by a broad range of immune cells like macrophages, B-lymphocytes, T-lymphocytes and mast cells as well as endothelial cells, fibroblasts and various stromal cells. T-lymphocytes are considered to be the major source of cytokines; they possess a central role not only in pregnancy but in the whole immune system. Cytokines modulate the balance between humoral and cell-based immune responses (Levy et al., 2003). They regulate the maturation, growth and responsiveness of particular cell populations. Some cytokines enhance or inhibit the action of other cytokines in complex ways (Horst, 2013).

Cytokines are important in health and disease specifically in host inflammation, trauma, sepsis, cancer and reproduction (Saito, 2001). Successful pregnancy in vertebrates is secondary to the ability of the maternal immune system to retain the fetus, which is a semi- allograft (Abbas et al., 1994). The rejection of an allograft is mainly mediated by cellular responses (Mason et al., 1986), and the activation of cell mediated immunity is
dependent on the secretion of the type 1 cytokines IL-2, IFN-γ and IL-12 (Mossman et al., 1987, 1989; Romagnani et al., 1991; Swain, 1991; Del Prete et al., 1991). It has been proposed that successful pregnancy in mice is a Th 2 phenomenon (Wegmann et al., 1993; Krishan et al., 1996) and that abnormally elevated concentration of the type 1 cytokines are associated in mice (Wegmann et al., 1993; Krishan et al., 1996) and humans (Hill et al., 1992, 1995; Dudley, 1995) with spontaneous abortions and impaired fetal development. Establishment and maintenance of a proper maternal-fetal interface is essential for successful mammalian pregnancy. Communication between fetal trophoblast cells and maternal immune cells dictates placental development and vasculogenesis during early pregnancy. It also establishes and maintains immune privilege throughout gestation.

Recent studies identify chemokines as critical factors in this process. Chemokines are a family of structurally-related, small, secreted proteins that render their chemoattractant effect through interaction with a subgroup of seven transmembrane domain G-protein-coupled receptors (Balkwill, 2004; Johrer et al., 2008). Traditionally known to recruit immune cells to mediate inflammation, chemokines are now recognized as regulators in central nervous system development and hematopoiesis. A study by Hanna et al. (2003) supports the critical role of inflammatory chemokines in pregnancy. Chemokine 10 is an inflammatory chemokine released by decidual Natural Killer (dNK) cells during pregnancy (Kanellopoulous-Langevin et al., 2003; Hanna et al., 2003). Chemokines regulate multiple events that are closely associated with normal pregnancy including fetal protection and placental development by modulation of homeostasis and functions of
immune and trophoblast cells in a paracrine or autocrine manner (Drake et al., 2007; Hanna et al., 2007). Chemokine 13 belongs to the CXC chemokine family. It is selectively chemotactic for B cells belonging to both the B-1 and B-2 subsets (Legler et al., 1998). It participates in developmental processes such as differentiation and directed migration (Legler et al., 1998).

Effects of khat on reproduction and pregnancy have been studied; however, no studies have been performed to measure cytokine and chemokine profiles of pregnant women who chew khat and the effect of khat on blood pressure and weights of pregnant women.

1.2 Statement of the Problem

Fresh leaves of the khat tree (Catha edulis) are habitually chewed for their euphoric properties in East Africa and parts of the Middle East (Arabian Peninsula) (Al motareb et al., 2002). This deep-rooted socio-cultural tradition has recently spread to East Africa and Middle Eastern communities in Europe and the United States (Griffiths, 1998; Toennes et al., 2003). When khat leaves are chewed, cathine and cathinone are released and absorbed through the mucous membranes of the mouth as well as the lining of the stomach (Brenneisen et al., 1990; Toennes et al., 2003). The action of cathine and cathinone on the re-uptake of epinephrine has been demonstrated in laboratory animals showing that one or both of these chemicals causes the body to recycle these neurotransmitters more slowly resulting in the wakefulness and insomnia associated with amphetamines (Nencini et al., 1984).
Use of khat by women is on the increase (Nakajima et al., 2013). Many reproductive aged women continue to chew khat during pregnancy and lactation (Jansson, 1988; Selassie et al., 1996). Khat chewing by pregnant mothers has been shown to be associated with full-term human newborns with lower birth weight (Abdul-Ghani et al., 1987). Inhibition of lactation (Luqman and Danowski, 1976) has been reported in khat chewing mothers, possibly resulting from increased dopamine production (Laurent, 1962). Even though a lot of research has been done on laboratory animals to investigate the effects of khat on male reproduction, little has been done to investigate the effects of khat on pregnancy. The effects of khat on cytokine and chemokine profiles during pregnancy are not documented, no studies have been carried out to investigate the effects of khat use on blood pressure, body temperature and body weight during pregnancy and even the effects of khat consumption on the growth of the fetus are not well studied.

No study had been done to measure cytokine and chemokine profiles among pregnant baboons and prior to this study, there was no data recorded on the effects of khat on cytokine and chemokine profiles during pregnancy. The current study therefore quantified the profiles of INF-γ, IL-10, CXCL 10 and CXCL 13 in khat-fed pregnant baboons, monitored the blood pressure, body temperature and body weights among pregnant khat fed baboons and weights of their newborns in order to understand the pathophysiology of khat on pregnant female khat-chewers and fetal growth.
1.3 Justification for the study

Pregnancy is a process which requires immunologic responses to allow growth and development of the fetus. Thus, changes in cytokine and chemokine profiles may be observed in the different stages of gestation (Keelan and Mitchell, 2007). Elimination of T-cells during pregnancy in murine experimental models results in increased levels of fetal abortion and significant reduction of placental growth (Chaout et al., 1988; Wegmann et al., 1993). Th1 cytokines such as IFN-γ and TNF-α are necessary during the early stages of pregnancy and during labour (Saito et al., 1999). Th2 cytokines such as Interleukin 10 (IL-10), Granulocyte Macrophage- Colony Stimulating Factor (GM-CSF), Interleukin 3 (IL-3) are important in fetal-placental growth (Athanassakis et al., 1993; Chaouat et al., 1999). Th1/Th2 balance during pregnancy is essential to fetal survival, a balance that leans towards Th1 or Th2 responses according to the stage of pregnancy (Athanassakis et al., 1993; Yui et al., 1994; Saito et al., 2001).

Effects of khat on male reproduction have been studied; however, no such studies have been performed to measure cytokine and chemokine profiles of pregnant women who chew khat as well as the effect of khat consumption on foetal growth. There is no documented data on the effects of khat on blood pressure, body weight and temperature among pregnant women. Analysing cytokines and chemokines profiles in pregnant baboons fed on khat extract in the current study would be relevant for human applications.
The present study was conducted at the Institute of Primate Research (IPR) which is a biomedical and primatology research centre of the National Museums of Kenya. The laboratory is well equipped with basic equipments for biomedical Research. Olive baboons (*Papio anubis*) were used in the study to investigate whether khat consumption has any detectable effects on cytokine and chemokine profiles, blood pressure and body weight during pregnancy and whether khat affects foetal growth. Non-human primates such as baboons are used in the study because they are phylogenetically closely related to humans with similar physiology (Higley *et al.*, 1993; Kaplan *et al.*, 1995). Data from this model would be relevant for humans therefore baboons would form useful models for further studies.

### 1.4 Research questions

i. What are effects of khat on body weight, body temperature and blood pressure of pregnant baboons?

ii. What are the levels of IFN-γ, IL-10, CXCL 10 and CXCL 13 among khat fed baboons during pregnancy?

iii. What is the effect of khat on the birth weight of the newborns among pregnant baboons?

### 1.5 Null hypotheses

i. Khat extract has no effect on body weight, body temperature and blood pressure among pregnant baboons.
ii. Khat extract has no effect on IFN-γ, IL-10, CXCL 10 and CXCL 13 levels during pregnancy.

iii. Khat extract has no effect on the birth weight of the newborns among pregnant baboons.

1.6 Objectives

1.6.1. General objective

To determine the effects of crude khat extract on IFN-γ, IL-10, CXCL 10 and CXCL 13 profiles, blood pressure, body temperature, body weight of pregnant baboons and birth weight of their newborns

1.6.2 Specific objectives

i. To determine the effect of khat extract on pregnant baboons body weight, body temperature and blood pressure

ii. To determine the effect of khat extract on IFN-γ, IL-10, CXCL 10 and CXCL 13 levels among pregnant baboons

iii. To determine the effect of khat extract on the birth weight of newborns among pregnant baboons.

1.7 Significance of the study

Results obtained in this study will provide vital information in understanding the effects of khat on cytokine profiles during pregnancy and whether khat affects blood pressure, body temperature and weight of pregnant baboons and birth weight of newborns. This
information is important for understanding pathophysiology of khat among pregnant women and can be useful in formulating policies with regard to chewing and handling of khat among pregnant women.
2.1 *Catha edulis*

Khat (*Catha edulis*) is an evergreen flowering tree or shrub (Figure 2.1) that grows in the equatorial climates mainly in the Arabian Peninsula and the regions around the horn of Africa (Al-Motarreb *et al.*, 2002). There is controversy as to the exact region where khat use and cultivation originated, with some authors suggesting Ethiopia and others suggesting Yemen (Al-Motarreb *et al.*, 2002; Al-Hebsi and Skaug, 2005).
Figure 2.1: A growing khat tree (*Catha edulis*).
Khat (*Catha edulis*) grows at high altitudes, arid environments and at a temperature range of 5 to 35°C (41 to 95°F) (Corkery *et al.*, 2011). Ethiopia, Yemen and Kenya are the main khat growing countries (Al-Hebshi and Skaug, 2005). In Kenya, khat, commonly called miraa is cultivated on commercial scale around Nyambene Hills found in Meru County, 320 km North East of Nairobi (Carrier, 2005). It is of prime economic importance for the Meru region where it feeds a growing international as well as national market (Carrier, 2005). Khat is harvested throughout the year (Al Motarreb *et al.*, 1987).

The tree requires about 10 years to attain maturity, but the leaves and the shoots are ready for harvest 3-4 years after planting (Nordal, 1980; Al Motarreb *et al*, 2002). The fresh succulent stems and leaves (Figure 2.2) are harvested regularly from the tree and are highly valued for their stimulating properties (Kalix, 1985; Brenneisen, 1990).
Figure 2.2: *Catha edulis* shoots and leaves ready for harvest
The stimulant effect of khat is related to the cathinone content of the leaves (Kalix, 1990). Cathinone has been isolated in variable quantities from Catha edulis fresh leaves (Kalix, 1985; Brenneisen et al., 1990) hence preferred by chewers. This compound is unstable in the presence of oxygen and decomposes (Branneisen and Geisshusler, 1985, 1987) within a few days of picking or if the plant is dried, making fresh leaves the best source of cathinone (WHO, 1980).

For maximum potency, khat must be picked in the morning and chewed that afternoon (Branneissen and Gesshusler, 1985). Fresh khat leaves (100 g) contains approximately 36 mg cathinone, 120 mg norpseudo-ephedrine and 8 mg norephedrine (Branneisen et al., 1990; Widler et al., 1994; Toennes et al., 2003). Farmers and consumers keep the leaves fresh by wrapping them in plastic foil, wet clothes or shawls or banana leaves (Figure 2.3).
Figure 2.3: *Catha edulis* leaves and shoots wrapped in banana leaves to keep fresh.
The harvested young leaves and tender twigs of *Catha edulis* are then arranged in bundles (Figure 2.4) for transportation to the different regions where khat is consumed.
Figure 2.4: *Catha edulis* leaves and shoots in bundles ready for transportation
In addition, they immerse the cut end of the stem in water for one minute or more to prolong freshness. At best, the leaves remain in an acceptable condition for up to five days (Branneisen et al., 1990; Widler et al., 1994). The plant has been targeted by anti-drug organizations such as USA Drugs Enforcement Act (DEA) (DEA, 2006). In 1980, the World Health Organization (WHO) classified khat as a drug of abuse that can produce mild- to- moderate psychological dependence (less than tobacco or alcohol) although WHO does not consider khat to be seriously addictive.

2.2 Habit of Khat chewing

The vast majority of those ingesting *Catha edulis* fresh leaves and young twigs do so by chewing (Sparago et al., 1996; Rousseau, 1998; World Drug Report, 2001). The chewer fills their mouth with leaves and twigs, and then chews slowly and intermittently to release the active components in the juice which is then swallowed with saliva. The plant material is chewed into a ball which is kept for a while in the cheek, causing a characteristic bulge (Nencini *et al*., 1986). During khat chewing sessions, the leaves and the bark of the plant are chewed slowly over several hours and the juice of the masticated leaves is swallowed but not the residues.

Drinking water while chewing helps the alkaloids from the saliva more quickly enter the stomach and thus the blood stream. Chewing khat is both a social and a cultural activity (Toennes *et al*., 2003; Voogelbreinder, 2009) in Yemen. It is said to enhance social interactions, playing a role in ceremonies such as weddings.
Khat is a stimulant and it is used to improve performance, stay alert and increase work capacity (Kalix, 1984). Workers on night shifts use it to stay awake and postpone fatigue. Students have chewed khat in an attempt to improve mental performance before examinations. Yemen khat chewers believe that khat is beneficial for minor ailments such as headaches, colds, body pains, fever, arthritis and also depression (Kennedy et al., 1983). According to several early studies, on average around 100 g - 300 g of khat can be chewed in a 3-4 hour session (Nencini and Ahmed, 1989; Kalix, 1990, 1994).

### 2.3 Pharmacology of khat

Studies of the chemical constituents of the khat plant date back to the late 1800s by European investigators (Zelger et al., 1980). Khat contains three main phenylpropylamine alkaloids; (-)-S-cathinone, (+) - 2S-norpseudoephedrine (cathine) and (-)-1R, 2S-norephedrine (Al-Motarreb et al., 2002). Cathinone is the main psychoactive constituent in fresh *Catha edulis* leaves; it is amphetamine-like stimulant which is said to cause excitement, loss of appetite and euphoria (UN Document, 1975). Cathinone is very unstable and rapidly decomposes into norpseudoephedrine and norephedrine as the leaves and shoots dry up (Brenneissen et al., 1984; Brenneissen and Geisshusler, 1985, 1987). Khat grown in the Meru region of Kenya was found to contain phenylpentenylamine alkaloids like (+)-S-merucathinone, (-)-3S, 4S-pseudomerucathine and (+)-3R, 4S-merucathine (Brenneissen and Geisshusler, 1985). Khat also has another group of alkaloids known as catheludines and these are a group of sesquiterpenes (Sammuelsson, 1992), identified as K1, K2, K6 and K15 from Kenyan khat. The Ethiopian khat contains
E2, E4, E5 and E8 and Y1 from Yemeni khat (Crombie et al., 1979; Al-Motarreb, 2002). Other constituents present in khat leaves include small amounts of essential oils, sterols and triterpenes (Kalix et al., 1985), protein (Braenden et al., 1979, 1985), ascorbic acid (Raman, 1983), tannins (7-14% by weight in dried leaves) and minute amounts of thiamin, niacin, riboflavin, iron and amino acids (Lugman and Danowski, 1976). However, the composition of these constituents depends on where and how khat is cultivated as well as the climatic conditions (Brenneisen et al., 1985).

2.4 Effects of khat on body physiology

2.4.1 Effects of khat on blood pressure

Among humans, significant and progressive rise in systolic and diastolic blood pressure and heart rate was observed during a three hour period of chewing fresh Catha edulis, and levels did not return to baseline one hour after chewing had ceased (Hassan et al., 2000). In anaesthetized dogs, the increase in heart rate, blood pressure and cardiac contractile force induced by both (-)-S-cathinone and amphetamine were inhibited by pretreatment with the neural uptake inhibitor, methylphenidate (Kohli et al., 1982). Similarly, pretreatment with cocaine and desipramine inhibited the efflux of [3H]-noradrenaline from [3H]-noradrenaline-loaded rabbit atria in response to (-)-S-cathinone (Kalix et al., 1983). A study by Nyachieo et al. (2012) demonstrated that high-dose khat administration increased systolic and diastolic blood pressure in male baboons.

However, the effect of khat on blood pressure in pregnant baboons is not yet established. Another study in humans showed an increase in blood pressure and pulse rate in khat
chewers (Hassan et al., 2007). The effect of khat on blood pressure is thought to be mediated by cathinone, which has a similar structure to amphetamine, in inducing the release of catecholamines from presynaptic storage sites, hence mimicking stimulation of the sympathomimetic nervous system (Brenneisen et al., 1990; Toennes et al., 2003; Hassan et al., 2007).

2.4.2 Effects of Khat on body weight and temperature

Baboons’ body weight decreased after high-dose khat (500g/week) administration to male baboons during one month (Nyachieo et al., 2012). This observation was similar to previous studies in baboons receiving a low dose of khat (250g/week) (Mwenda et al., 2005, 2006). There exists a rich body of literature documenting the subjective effects associated with acute khat use (Halbach, 1972; Nencini et al., 1986; Brenneisen et al., 1990), including euphoria, excited mood, increased wakefulness and alertness, and suppression of appetite. Dalu (2000) established that khat chewing in the 3rd trimester significantly reduced the maternal weight gain in humans.

Understanding the appetite and weight effects of Amphetamine-class stimulants, either natural or synthetic, also requires an examination of thermoregulation as well as behavioral neuromodulation. Hyperthermia is a major adverse side effect of acute use of several Amphetamine-class stimulants and may be one mechanism behind the weight loss associated with Amphetamine (Parrott, 2001). It has been known for some time that both Amphetamine and cathinone increase brown adipose tissue and rectal temperature in rats,
though Amphetamine shows a threefold higher temperature response than cathinone (Tariq et al., 1989).

Acute administration of a high dose of cathinone (10 mg/ kg) on metabolic hormones supports the hypothesis that cathinone and Amphetamine may have similar effects on both direct and indirect appetite modulators and metabolism. In one of the studies on appetite hormones, khat chewing, at least by habitual users, decreased subjective feelings of hunger and increased subjective feelings of fullness despite no change in ghrelin or Peptide tyrosine tyrosine (PYY) secretion (Murray et al., 2008). This is in contrast to Amphetamine where leptin has been called into question as a mediator of food restriction sensitization of d-amphetamine's reward effects (Hao et al., 2006).

2.4.3 Effects of Khat on reproduction

Detailed studies on the effects of khat on reproduction are lacking. Most of the studies are based on male reproduction because khat chewing has for long been a habit of men. However, the limited available data reveals that khat chewing has a negative impact on human reproductive health. In pregnant women, consumption of khat affects growth of foetus by inhibiting utero-placental blood flow and as a consequence impairs foetal growth (Kalix, 1987).

Khat is genotoxic and has teratogenic effects on the fetus if regularly consumed by pregnant mothers (Mwenda et al., 2003, 2005). Studies in albino mice show that khat reduces the possibility of pregnancy and increases the possibility of post-implantation abortion (Tariq et al., 1986). In males, khat is thought to cause impotence (Islam et al.,
1990; Hakim, 2002) and several studies have shown degenerative changes in male sexual organs, low testosterone levels (Islam et al., 1990, Nyongesa et al., 2007; Nyongesa et al., 2008), and poor semen and sperm health (El-Shoura et al., 1995). Since low birth weight (Abdul-ghani et al., 1987) is an established risk factor for both perinatal and young infant death, khat chewing during pregnancy may be one of the factors contributing to infant mortality in communities where khat is commonly chewed.

Khat consumption affects the potency of male sexuality (Halbach, 1979); however, the precise mechanisms by which khat affects the male reproductive physiology have not been elucidated. Baboon is a good model of human reproduction. Baboons exhibit menstrual cycle similar to humans (Scott, 2010). Data on effects of khat consumption on pregnancy in baboon would be relevant for understanding khat use and its impact on human reproduction.

2.4.4 Effects of Khat on Cytokines

Peripheral blood mononuclear cells (PBMC) treated with khat in vitro showed an increase in expression of co-stimulatory molecules cluster of differentiation 80 / 86 (CD 80 / 86) and major histocompatibility complex 11 (MHC 11) and pattern recognition receptors, toll-like receptors 2 / 4 (TLR-2 and TLR-4) and triggering receptor expressed on myeloid cells 1 (TREM-1), in the same study, there was also inhibition of secretion of inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), chemokine 5 (CCL 5) and chemokine 8 (CXCL 8) (Murdoch et al., 2011). In PBMC in vitro study, khat induces an increase in the secretion of anti-inflammatory cytokines
such as interleukin-2 (IL-2) and interleukin-10 (IL-10). These khat induced alterations were accompanied by increased expression of transcription factors p 38 mitogen activated protein kinases (p38 MAPk) and Hypoxia-inducible factor 1 alpha subunit (HIF-1α), whereas the expression of Nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) was inhibited (Murdoch et al., 2011). Data on effect of khat on cytokine and chemokine profiles in pregnant baboons would be relevant for pregnant women who chew khat.

2.5 Cytokines and reproduction

The best studied peripheral immune cells in human pregnancy are T-lymphocytes. Within the T-lymphocytes population, helper T-lymphocytes (Th) and cytotoxic T-lymphocytes (Tc) can be distinguished. Th lymphocytes provide help to other immune cells by producing cytokines, whereas Tc lymphocytes can directly kill foreign or infected cells. The numbers of Tc lymphocytes and Th lymphocytes may (Matthiesen et al., 1996; Luppi et al., 2006) or may not (Coulam et al., 1983; Veenstra Van Niewenhoven et al., 2002) differ in pregnant women versus non-pregnant women. T-lymphocytes can also be classified into different functional subsets based on their profile of cytokine production.

Type 1 T cells produce, for example, Interferon-gamma (IFN-γ), Interleukin-2 (IL-2) and Tumor Necrosis Factor-alpha (TNF-α) which promotes cellular immune responses whereas type 2 T-cells produce Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-9 (IL-9), Interleukin-10 (IL-10) and Interleukin-13 (IL-13) that provide optimal help for
humoral immune responses (Mossman et al., 1986). Wegmann et al. (1993) was the first to propose the concept that pregnancy is a Th 2 phenomenon. The shift away from type 1 cytokine production during pregnancy is beneficial for pregnancy since type 1 cytokines (IFN-γ and TNF-α) are harmful for pregnancy; they inhibit embryonic and fetal development (Chaouat et al., 1990; Haimovici et al., 1991) and terminate pregnancy when injected into pregnant mice (Chauat et al., 1990).

Various studies have shown that especially in the third trimester of human pregnancy the ratio of type 1/type 2 cytokine productions of peripheral T-lymphocytes is decreased as compared with non-pregnant women (Ekerfelt et al., 1998; Sabahi et al., 1999; Saito et al., 1999; Veenstra Van Nieuwenhoven et al., 2002). The importance of this relative dominancy of type 2 cytokines over type 1 cytokines may be stressed by the fact that pregnancy loss is associated with less type 2 cytokine production as compared with normal pregnancies (Piccini et al., 1998, 2001). Chemokines are a subgroup of chemo-attracting cytokines that include approximately 50 ligands involved in normal homeostasis (Kunkel, 1999).

Inflammatory chemokines play a critical role in pregnancy (Hanna et al., 2003). Chemokine 10 is an inflammatory chemokine released by decidual Natural Killer (dNK) cells during pregnancy. Successful reproduction relies on limited inflammation during implantation, immune tolerance (anti-inflammation) at mid-pregnancy and inflammation again during parturition (Kanellopoulos-Langevin et al., 2003; Hanna et al., 2003). Arnold et al. (2007) reported that CXCL 13 also participates in T cell homing to germinal
centers of lymphoid tissue (Arnold et al., 2007), induction of migration of immature dendritic cells (Howard, 2005) and maintenance of epithelial cell angiostatic activity.

A study by Nhan-Chang et al. (2011) established the presence of CXCL 13 in amniotic fluid of humans early in gestation and its concentration was stable throughout pregnancy suggesting that chemokine13 may also contribute to the homeostatic balance that it may be important in the maintenance of a normal gestation. Since type 1 cytokines, CXCL 10 and CXCL 13 are essential during the early stages of pregnancy any disruption of these profiles during pregnancy may be detrimental to pregnancy and hence the fetus. Data on effect of khat on cytokine and chemokine profiles among pregnant baboons is lacking.

2.6 Olive Baboon model as models for biomedical research

Baboons (genus Papio) (Figure 2.5) belong to the larger taxonomic grouping; Old world monkeys (super family Cercopithecoidea). The genetic similarity between baboons and humans is evident at the level of Deoxyribonucleic acid (DNA) sequence (Caccone and Powell, 1989). The sequence of specific genes and the arrangement of genetic loci on chromosomes (Graves et al., 1995; Perelygin et al., 1996) reflect the close evolutionary relationship between the two species. In addition, baboons and humans share a broad range of other physiological similarities that distinguish them from other animal models and that make baboons particularly valuable as models for human reproduction (Higley et al., 1993; Kaplan et al., 1995).

Olive baboons have a gestation period of approximately 182 days or six (6) months (Heffernan, 2006). Baboons have an Oestrous/Menstrual cycle of 30-35 days (Scott,
2010). The physiology and biochemistry of female baboons are similar to women as well as their plasma and urine hormone levels during pregnancy (Scott, 2010). A lot of research has been done in baboons to investigate the effects of khat on various aspects of body physiology with results indicating that high-dose khat administration increased systolic and diastolic blood pressure in male adult baboons and also the baboon’s body weight decreased after high-dose khat administration (Nyachieo et al., 2012); Mwenda et al. (2003) demonstrated that oral administration of khat extract on adult olive baboon’s increases testosterone but down regulates prolactin and cortisol levels in blood plasma. There is no information on the effects of khat on profiles of cytokines and chemokines during pregnancy in baboons.

Mwenda et al. (2003) demonstrated that khat is genotoxic and is teratogenic to the fetus if consumed by pregnant mothers. However, data on the effects of khat on immunological profiles during pregnancy is lacking. The present study, therefore, used pregnant olive baboon models to investigate the effects of khat on selected cytokine and chemokine profiles during pregnancy, blood pressure, body temperature, body weight among pregnant baboons and birth weight of their newborns.
Figure 2.5: Olive Baboon (*Papio anubis*) in a cage.
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site

The research work was carried out at the Department of Reproductive Biology in the Institute of Primate Research (IPR), which is a biomedical and primatology research centre of the National Museums of Kenya. The laboratory is well equipped with basic equipments for biomedical Research. The Institute of Primate Research is located in Oloolua forest, Kajiado County, Kenya about 22 kilometers west of Nairobi.

3.2 Collection of *Catha edulis*

Bundles of the fresh plant shoots and young leaves of *Catha edulis* were purchased at a local market from Nyambene, Meru North County, where it is grown in large scale, 320km North East of Nairobi, Kenya. The fresh bundles were packed in plastic bags and transported in an icebox to the laboratory.

3.3 Preparation of the khat extract

Fresh plant shoots and young leaves of *Catha edulis* were used. The extracts were prepared as described by Conner et al., (2000) with some slight modifications. The fresh plant materials were finely chopped using a blender, weight and placed in a flask containing sterile saline. For every 5g of minced leaves, 50ml of saline were added. The extractant were decanted, filtered with whatmann no. 1 filter paper, and then dried using a lyophilizer (Labconco Corporation, USA). The dried extracts were kept in a refrigerator at -20 °C until use.
3.4 Experimental design

The target population comprised of olive baboons (*Papio anubis*) that were maintained in accordance with the Kenyan guidelines for care and use of laboratory animals (Turner, 2009) at the Institute of Primate Research. The study population consisted of six pregnant olive baboons that were maintained in individual cages under natural lightning after they were time-mated. They were fed with commercial monkey cubes, supplemented with fruits and vegetables and water was provided *ad libitum*.

The Institute Scientific Evaluation and Review Committee (ISERC) and the Animal Care and Use Committee (ACUC) of the Institute of Primate Research approved the study protocol (Appendix I). The experimental group consisted of three (3) pregnant olive baboons which were given khat extract and the control group consisted of three (3) pregnant olive baboons which were given normal sterile saline in the 3rd and 4th months of gestation.

During the 3rd and the 4th months of gestation, each study subject in the experimental group was given a dose of 50ml khat extract orally once a week and were bled once every two weeks in a period of two months, each study subject in the control group was given 50ml of normal sterile saline orally once a week and were bled once every two weeks for two months at mid-gestation. From the 5th month of gestation, both groups were fed normally till the end of gestation. For this study, plasma samples collected in the 3rd and 4th months of the gestation period were analyzed for cytokines and chemokines.
3.4.1 Blood collection for plasma preparation

Venous blood samples were collected from each of the study subject once every two weeks in the 3rd and 4th months of the gestation period. Five milliliters of each sample was dispensed into ethylenediaminetetra acetic acid (EDTA) containing tubes (Vacutainer; BD sciences, USA). Plasma was separated, frozen quickly and stored at 20°C until use.

3.4.2 Determination of the pregnant baboons blood pressure

Blood pressure measurements were taken using an electronic sphygmomanometer (VWR International, Leuven, Belgium) once per week in the 3rd and 4th months of gestation. During blood pressure measurements, a baboon was anesthetized and an inflatable cuff placed smoothly around a shaved arm at roughly the same vertical height as the heart while the baboon lied in the recumbent position and the arm supported horizontally (Figure 3.1). The cuff was then inflated until tight, and then slowly the pressure in the cuff was released until stable readings were achieved and recorded as systolic and diastolic pressures.
Figure 3.1: Taking blood pressure of pregnant olive baboon (*Papio anubis*)
3.4.3 Determination of the pregnant baboons body weight

Body weight measurements were taken using a balance once per week in the 3rd and 4th months of gestation. During body weight measurements, a baboon was anesthetized intravenously using 2ml of ketamine, the baboon was placed on the balance, body weight read then recorded.

3.5 Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

Concentrations of IFN-γ and IL-10 were determined by enzyme linked immunosorbent assay (ELISA). Briefly, polystyrene micro-ELISA plates (Dynatech Laboratories, Sussex, UK) were coated overnight with 100 µl of a 2-µg/ml of capture monoclonal antibody to human IFN-γ or IL-10 (MabTech, Sweden) diluted in phosphate buffered saline (PBS). Excess coating buffer was removed, and non-specific binding sites blocked with 1% bovine serum albumin (Sigma, USA) in PBS for one hour at room temperature. The plates were washed four times with 0.05% Tween-20 in PBS and 100 µl of serum or standard was dispensed in duplicates to appropriate wells. Human IFN-γ or IL-10 standards were diluted according to the manufacturer’s instructions and 100 µl diluted serially in duplicates dispensed in appropriate wells.

The plates were incubated at 37°C for two hours. Plates were washed as before. Fifty microlitres (50 µl) of biotinylated secondary monoclonal antibody to human IFN-γ or IL-10, 1/2000 dilution was added, followed by incubation at 37°C for one hour. The plates were washed four times as before, fifty microlitres (50 µl) HRP-conjugated streptavidin, 1/1000 dilution added, and incubated for one hour as before. The plate was washed six
times in PBS-Tween, and 100 µl of tetramethylbenzidine (TMB, SureBlue, KPL) added. The plates were incubated at 37°C in the dark for thirty minutes and absorbance read at 620 nm. Cytokine levels were extrapolated by comparison with the standard.

3.6 Chemokine sandwich ELISA

Concentrations of CXCL 10 and CXCL 13 were determined by quantitative sandwich enzyme immunoassay technique. Pre-coated micro plate (R&D Systems, Inc. USA) for IP-10 or BLC/BCA-1 respectively was equilibrated to room temperature. In each well, 75 µl of assay diluents (RD1-56 and RD1S) were added. Seventy five microliters of standard, control, or sample per well was added then covered with an adhesive tape and incubated for two hours at room temperature. The plates were then washed with 400 µl of wash buffer (Wash Buffer Concentrate, R&D Systems) three times using an auto washer (Dynatech Inc, USA). Two hundred microliters of IP-10 conjugated to horseradish peroxidase (Conjugate, R&D Systems) was added to each well then covered with an adhesive tape and incubated for two hours at room temperature. The plate was washed as before and 200 µl of substrate solution (Color reagent A and B, R&D Systems) was added and the plate incubated at room temperature in the dark for thirty minutes. Fifty microliters of sulfuric acid (Stop Solution, R&D Systems) was added to each well then tapped for uniform mixing. Optical density was read after thirty minutes using a micro plate reader (Dynatech Inc, USA) set at a wavelength of 450 nm. Chemokine levels were extrapolated by comparison with the standard.
3.7 Ethical considerations

The study was approved by the Board of Postgraduate studies Kenyatta University (Appendix I), ethical approval for animal use was sought from Institute of Primate Research (IPR), Institutional Scientific and Ethical Review Committee (ISERC) and Animal Care and Use Committee (ACUC) (Appendix II).

3.8 Statistical analysis

Data processing was performed using SPSS software (19.0 version) (Inc, USA). Data are expressed as mean ± SD for all parameters investigated. Mean values of each set of triplicate were used for statistical analysis. The significance of differences between the groups was determined using Students t - test. All tests were two-tailed and an α-value of 5% (<0.05) was considered significant.
CHAPTER FOUR: RESULTS

4.1 Effect of khat on body weight, blood pressure and temperature among pregnant baboons.

4.1.1 Effect of khat on body weight among pregnant baboons

The experimental group had a body weight mean of 13.07 ± 0.13, whereas the control group had a body weight of mean of 14.98 ± 0.43. When a comparison was done between both groups of baboons, results showed that khat had a significant effect on body weight with baboons fed on khat extract having lower body weight than non khat fed baboons (Figure 4.1, Table 4.2, p < 0.05).

![Body weights (Kgs) for the experimental and control group at mid gestation. Baboons fed on khat had significantly lower body weights compared to non khat fed baboons (p < 0.05).](image-url)
4.1.2 The effect of khat on the birth weight of newborns among pregnant baboons

The birth weights of the infants for the experimental group and control group were taken immediately after delivery and recorded. Average birth weight for the three newborn baboons of the experimental group was 0.88 kg whereas the average birth weight for the three newborn baboons of the control group was 0.83 kg. When the mean weights were compared between the two groups, there was no significant difference (Table 4.1, Table 4.2, p < 0.05) although khat fed group had weighed slightly higher than the non khat fed group.

<table>
<thead>
<tr>
<th>Animal number</th>
<th>Treatment</th>
<th>Infant weights (kg)</th>
<th>Mean wt. ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN 3873</td>
<td>Not fed</td>
<td>0.85</td>
<td>0.83±0.077</td>
<td>0.22</td>
</tr>
<tr>
<td>PAN 3897</td>
<td>Not fed</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAN 3415</td>
<td>Not fed</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAN 4001</td>
<td>Khat fed</td>
<td>0.9</td>
<td>0.88±0.073</td>
<td></td>
</tr>
<tr>
<td>PAN 3355</td>
<td>Khat fed</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAN 3314</td>
<td>Khat fed</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Birth weights of infants for the experimental group and control group
4.1.3 Effect of khat on blood pressure among pregnant baboons

The changes in blood pressure were established in mm Hg from each of the baboons’ systolic and diastolic pressure. Among the experimental group, systolic pressure had a mean of 82±13.67, whereas the diastolic pressure had a mean of 42±4.84. The control group systolic pressure had a mean of 77±11.45, whereas the diastolic pressure had a mean of 35±13.37. When a comparison between the experimental group and the control group was done, the results indicated that khat had significant effect on blood pressure of pregnant baboons with baboons fed on khat extract having significantly higher blood pressure than non khat fed baboons (systole/ diastole) (Figure 4.2, Table 4.2, p < 0.05).

Figure 4.1: Blood pressures (mm Hg) among experimental and control group. The experimental group had a significantly higher blood pressure than the control group (p < 0.05), n=3 for each of the groups.
4.1.4 Effect of khat on temperature among pregnant baboons

The experimental group had a mean body temperature of 36.80 ± 0.15 whereas among the control group had mean body temperature of 36.54 ± 0.08. When a comparison between the experimental group and the control group was done, the results indicated that khat extract had no significant difference in body temperature among the baboons (Figure 4.3, Table 4.2, p > 0.05).

Figure 4.3: Body temperatures (°C) between experimental group and the control group. There was no significant difference in body temperature between the two groups (p>0.05).
4.2 The profiles of IFN-γ, IL-10 and CXCL 10 and CXCL 13 among experimental group and the control group

4.2.1 Profile of IFN-γ among pregnant baboons

Among the experimental group (PAN 4001, PAN 3355, PAN 3314), levels of IFN-γ decreased with a mean of 7.8 ± 0.00 pg/ml to 5.72 ± 1.13 pg/ml during the period of study, whereas, among the control group (PAN 3897 and PAN 3873), levels of IFN-γ decreased with means of 9.75 ± 2.00 pg/ml to 3.9 ± 0.00 pg/ml during the period of study (9-16 weeks of gestation).

Figure 4.4: Profiles of IFN-γ among pregnant baboons at mid-gestation, please note n = 2, for control group because the sample for one baboon was lost.
To compare the profiles of IFN-γ in the two groups of baboons, linear regression analysis was done. The trend of IFN-γ among the experimental group was a decrease ($y = -0.1989x + 6.561$) as the pregnancy progressed, among the control group the trend of IFN-γ was also a decrease ($y = -0.5706x + 7.6814$) as the pregnancy progressed. A t-test showed there was no significant difference in the levels of IFN -γ when the two groups of baboons were compared. (Figure 4.4, Figure 4.5, Table 4.2, p>0.05).

![Graph showing IFN-γ levels in khat fed and non khat fed baboons](image)

Figure 4.1: Mean profile of IFN-γ in khat fed and non khat fed pregnant baboons at mid gestation, n=3 for each of the group.
4.2.2 Profile of IL-10 among pregnant baboons

In the khat fed pregnant baboons (PAN 4001, PAN 3355 and PAN 3314), the profile of IL-10 decreased with a mean of $2.34 \pm 0.00$ pg/ml to $1.78 \pm 0.115$ pg/ml during the period of study. Among the non khat fed pregnant baboons (PAN 3897 and 3873), the profile of IL-10 increased with mean values of $1.52 \pm 0.012$ pg/ml to $2.46 \pm 0.06$ pg/ml during the study period.

Figure 4.6: Profiles of IL-10 among pregnant baboons at mid gestation
To compare the profiles of IL-10 in the two groups of baboons, linear regression analysis was done. The trend of IL-10 among the khat fed baboons was a decrease \((y = -0.0932 + 2.5171)\) as the pregnancy progressed whereas among the non khat fed baboons, an increase in IL-10 levels \((y = 0.1629 + 1.3986)\) was observed. However, the results of t-test indicated that there was no significant difference in IL-10 levels between the two groups of pregnant baboons (Figure 4.7, Figure 4.6, Table 4.2, \(p > 0.05\)).

![Figure 4.7: Mean profiles of IL-10 in khat fed and non khat fed pregnant baboons at mid gestation](image)
4.2.3: Profile of CXCL 10 among pregnant baboons

Among the khat fed pregnant baboons (PAN 4001, PAN 3355 and PAN 3314), Chemokine-10 levels decreased with a mean of $103.87 \pm 6.69\, \text{pg}/\text{ml}$ to $74 \pm 3.25\, \text{pg}/\text{ml}$ over the mid-gestation period, whereas, among the non khat fed baboons, the levels of Chemokine-10 decreased from means of $174.62 \pm 38.5\, \text{pg}/\text{ml}$ to $62.65 \pm 8.25\, \text{pg}/\text{ml}$ during the period of study.

Figure 4.8: Profiles of CXCL 10 in pregnant baboons at mid gestation
To compare the profiles of CXCL 10 in the two groups of baboons, linear regression analysis was done. The trend of CXCL 10 among the khat fed baboons was a decrease \( (y = -5.4689x + 109.64) \) as the pregnancy progressed, whereas, among the non khat fed baboons the trend of CXCL 10 levels was also a decrease \( (y = -17.197 + 174) \). The result of t-test showed that there was no significant difference in the levels of CXCL 10 among the two groups of baboons. (Figure 4.8, Figure 4.9, Table 4.2, \( p > 0.05 \)).

![Figure 4.2: Mean profiles of CXCL 10 in khat fed and non khat fed pregnant baboons at mid gestation, n=3 for each of the groups.](image)

- \( \text{Non Khat fed} \)
- \( \text{Khat fed} \)
4.2.4 Profile of CXCL 13 among pregnant baboons

Among the khat fed pregnant baboons ((PAN 4001, PAN 3355 and PAN 3314) the profile of Chemokine-13 was established at mid gestation. Levels of Chemokine-13 increased with a mean of 9.25 ± 0.693pg/ml to 17.62 ± 0.52pg/ml during the period of study, whereas, among the non khat fed baboons the levels of Chemokine-13 increased with means of 11.21 ± 0.61pg/ml to 18.20 ± 0.51pg/ml during the period of study.

Figure 4.3: Profiles of CXCL 13 in pregnant baboons
To compare the profiles of CXCL 13 in the two sets of baboons, linear regression analysis was conducted. The trend of CXCL 13 levels among the khat fed baboons was an increase \((y = 1.3871x + 7.2943)\) at mid gestation, whereas, among the non khat fed baboons the trend was also an increase \((y = 1.1789x + 9.4586)\). Results of t-test showed that there was a significant difference on CXCL 13 profiles when the groups of baboons were compared. (Figure 4.11, Figure 4.10, Table 4.2, \(p < 0.05\)).

Figure 4.4: Mean profile of CXCL 13 among khat fed and non khat fed pregnant baboons, \(n=3\) for each of the groups.
Table 4.1: Comparison of parameters measured between experimental and control group

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>Khat fed baboons Mean +/-SD</th>
<th>Non-khat fed baboons Mean +/-SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (Kgs)</td>
<td>13.1±0.13</td>
<td>14.97±0.43</td>
<td>0.0001</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>82±13.67</td>
<td>77±11.45</td>
<td>0.049</td>
</tr>
<tr>
<td>Diastolic</td>
<td>42±4.84</td>
<td>37±13.37</td>
<td>0.024</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>5.67±1.15</td>
<td>5.11±1.98</td>
<td>0.510</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>2.14±0.21</td>
<td>2.05±0.38</td>
<td>0.674</td>
</tr>
<tr>
<td>Chemokine 10 (pg/ml)</td>
<td>87.07±12.38</td>
<td>105.07±38.7</td>
<td>0.146</td>
</tr>
<tr>
<td>Chemokine 13 (pg/ml)</td>
<td>12.84±2.89</td>
<td>14.17±2.89</td>
<td>0.0001</td>
</tr>
<tr>
<td>Infant weights after birth (Kgs)</td>
<td>0.88±0.077</td>
<td>0.83±0.073</td>
<td>0.221</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.80±0.15</td>
<td>36.54±0.08</td>
<td>0.123</td>
</tr>
</tbody>
</table>

n=3 for each of the groups.
CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Introduction

Previous investigations of the khat effects have spanned various physiological and metabolic effects. However, most of the studies have been performed in rodents yet the genetic and physiological processes differ greatly with those of humans. As a result most of the experimental observations do not match human studies. The current study investigated the effects of khat extract on selected cytokine and chemokine levels during pregnancy using olive baboon model since khat chewing is on the increase among women (Nakajima et al., 2013). Women use it for its perceived health benefits such relief of headache, weight loss and assisting birth and delivery (Stevenson et al., 1996). Khat consumption has extended to reproductive aged women including pregnant and also lactating mothers. In the current study, levels of selected cytokines and chemokines among pregnant olive baboons (*Papio anubis*) that were fed on khat extract at mid-gestation was evaluated. The effects of khat extract on blood pressure, temperature, body weight and weights of pregnant baboons and newborns were also analyzed.

5.1.2 Effect of khat extract on blood pressure among pregnant baboons

The effect of khat on blood pressure among pregnant baboons at mid-gestation was investigated. These results are in agreement with earlier findings that established khat chewing induces small and transient rises in blood pressure and heart rate in humans (Al Motarreb et al., 2002). Nyachieo et al. (2012) demonstrated high-dose khat
administration increased systolic and diastolic blood pressure in male baboons. The finding was also similar to previous findings in humans showing an increased blood pressure and pulse rate in khat chewers (Hassan et al., 2007).

Although the effect of khat on blood pressure has been investigated, this is the first report on the effect of khat extract on blood pressure among pregnant baboons. The increase in pressure could be due to the fact that khat produces cathinone which has vasoconstriction activity as it was observed in isolated perfused hearts from guinea pigs (Al Motarreb et al., 2003). Cathinone has also be shown to potentiate noradrenaline-evoked contractions of the rat right ventricle and to inhibit the uptake of noradrenaline into ventricular slices by a mechanism involving competitive blockade of the noradrenaline transporter (Cleary et al., 2002; Cleary et al., 2003). The vasoconstriction activity of cathinone explains the increase in blood pressure seen in humans (Brenneisen et al., 1990) and in animals (Kohli et al., 1982), and might be related to the increased incidence of myocardial infarction occurring during khat sessions, that is, during the khat-effective period (Al Motarreb et al., 2002), and associated with heavy khat chewing (Al Motarreb et al., 2005).

The observed increase in pressure due to khat consumption may have serious implications to the survival of pregnancy. One of the complications of pregnancy is increase in blood pressure of the mother (Vest et al., 2012, Deak et al., 2012). Based on the data presented in this study khat may affect pregnancy through increase in blood pressure.
5.1.3 Effect of khat extract on body weight among pregnant baboons

Results of this study indicate that khat caused a decrease in body weight among the experimental group. These results are in agreement with earlier findings that baboon body weight decreased after high-dose khat administration (Nyachiego et al., 2012). Dalu (2000) had earlier established that khat chewing in the 3rd trimester significantly reduced the maternal weight gain in humans. Previous studies on rats fed on Catha edulis extract showed reduced weight gain (Maitai et al., 1977). Retardation of growth rate was considered to be due to decreased absorption of food and not due to decreased food consumption. In pregnant rats, khat reduced food consumption and maternal weight gain, and also lowers the food efficiency index (Al Motareb et al., 2002).

The decreased weight could be due to the fact that amphetamine suppresses appetite by increasing the synaptic availabilities of Norepinephrine (NE) and Dopamine (DA) in hypothalamus, and subsequently activates the Norepinephrine (NE) – and Dopamine (DA)-dependent mechanisms that attenuate the central nervous system control of food intake (Hsieh et al., 2005, 2006). Reduction in weight gain among pregnant women may have adverse effect on foetal development.

5.1.4 The effect of khat extract on the birth weight of newborns among pregnant baboons

The findings of this study are in line with earlier results done on pregnant guinea pigs that resulted to growth retardation in the offsprings (Jansson et al., 1987) although the khat was administered during the last trimester. From literature, experiments by Abdul et al.
(1987) asserted that khat use during pregnancy results to reduced birth weight in offsprings, however, there is contrasting evidence at a population level where the positive economic impact of the khat industry has resulted in increased birth weight in khat production areas in Africa (Seyoum et al., 1986). From literature, it is clear there are contradictory reports on the effect of khat on birth weights of infants, in the current study; the results are in agreement with (Abdul-ghani et al., 1987) where the birth weight of infants decreases among khat chewers.

5.1.5 Profile of Interferon gamma (IFN-γ) and Interleukin 10 (IL-10) among pregnant baboons

The results of the study are in line with the findings of Marzi et al. (1996) that showed reduction of IFN-γ in pregnancy among mice. Marzi et al. (1996) also concluded that Th1 cytokines are harmful during mid-gestation but are necessary at early pregnancy and during labor, possibly, this explains why the levels of IFN-γ were decreasing as the pregnancy progressed. When a comparison of the decrease between the experimental group and the control group was done, the profiles of IFN-γ in the two groups followed the same trend but the decrease in the experimental group was less when the two groups of baboons were compared.

Type I T-cells produce IFN-γ, IL-2 and TNF-α which promote cellular immune responses (Mosmann et al., 1986). IFN-γ are of type I cytokines, these cytokines and the paradigm of Th 1 response is a key element in the early stages of pregnancy, supporting the inflammatory reaction that enables the blastocyst to implant (Raghupaty, 1997; Michie,
1998). IFN-γ has an essential action on angiogenesis and the trophoblastic invasion process. As pregnancy progresses to mid-gestation the levels of IFN-γ start to decrease since type 1 cytokines are harmful for pregnancy because they inhibit embryonic and fetal development (Chaouat et al., 1990; Haimovici et al., 1991) and terminate pregnancy when injected into pregnant mice (Chaouat et al., 1990).

The results of the present study are therefore in agreement with the studies of Wegmann et al. (1993) that during pregnancy there is a shift away from type I cytokines to type 2 cytokines as pregnancy progresses to mid-gestation, probably, this explains why the level of IFN-γ in the baboons were decreasing as the pregnancy progressed. Marzi et al. (1996) studies on human pregnancy showed that pregnancy hormones especially progesterone levels increase in the serum resulting to decrease in established Th 1 clones hence the decrease in the levels of IFN-γ. However, earlier observations by Murdorch et al. (2011) that khat increases the secretion of IFN-γ in peripheral blood mononuclear cells in vitro could possibly account for the slight decrease of IFN-γ among the khat fed pregnant baboons compared to controls and these may have implications on survival of pregnancy.

In contrast, the profile of IL-10 in the control group was increasing slightly as the pregnancy progressed. A lot of information from literature exists citing the importance IL-10 in pregnancy but there exists little data on the mechanisms underlying IL-10 secretion. This is the first study to identify the decreased levels of IL-10 associated with khat consumption during pregnancy. The down regulation of IL-10 among khat fed baboons may have implications in the survival of pregnancy. Since Marzi et al. (1996)
established that IL-10 is beneficial during mid-gestation, its decreasing trend among the khat fed baboons and even the low levels can be detrimental to pregnancy. It is interesting to note IL-10 levels increasing with pregnancy among the non khat fed baboons but khat seems to affect that physiology, this may have adverse effects on pregnancy.

Type 2 T-cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 that provide optimal help for humoral immune responses (Mosmann et al., 1986). The maternal immune response is regulated by a complex array of cytokines to protect the conceptus and promote proper growth and development of the placenta. Wegmann et al. (1993) suggested that during pregnancy there is a T-helper (Th) 2 bias to promote tolerance to the half-foreign fetus and Th1 cytokines are detrimental to the tolerance of the conceptus, similar to allografts in transplant recipients (Suthanthiran et al., 1995; Burns et al., 2005). It has been found that during tolerance induction to an allograft there is a decrease in Th1 cytokines such as interleukin-2 (IL-2) and IFN-γ and an increase in Th2 cytokines including Interleukin-4 (IL-4) and Interleukin-10 (IL-10). Existing data suggest that Th2 bias in pregnancy is an oversimplified model and that during the various stages of pregnancy the pro-inflammatory and anti-inflammatory cytokine milieu is dynamically modulated.

The first stage of pregnancy, which involves a blastocyst implanting into the uterus, is a predominantly pro-inflammatory phase. Localized activation of inflammatory mediators occurs and the mother’s immune system repairs the damage done by the invading blastocyst. The second phase of pregnancy is a predominantly anti-inflammatory phase. Th2 cytokine skewing during the second phase of pregnancy can be systemic or local at
the feto-maternal interface. The last phase of pregnancy is parturition, which causes contraction of the uterus and again, the pro-inflammatory milieu is predominant. Inflammation is tightly controlled during all stages of pregnancy, however excessive and persistent maternal inflammatory responses are associated with adverse pregnancy outcomes (Athanassakis et al., 1989; Armstrong and Chaouat, 1989, Wegmann et al., 1993). IL-10 therefore being a type 2 cytokine maintains pregnancy during the second phase of pregnancy.

In the current study, the levels of IL-10 were slightly increasing at mid-gestation in the non khat fed baboons, this observation would probably be used to extrapolate that the levels of IL-10 continued to increase even as the pregnancy progressed to the third trimester. According to Marzi et al. (1996) IL-10 exerts a beneficial action during mid-gestation, therefore found in the serum in higher levels as pregnancy progresses. The results of this study are in agreement with the various investigations that during pregnancy especially at the third trimester there is a shift away from type 1 cytokine production to type 2 cytokine production (Ekerfelt et al., 1997) as this increase was witnessed at mid-gestation in the current study.

Successful pregnancy is as a result of coordinate production of a number of hormones that favors the establishment of the environment hosting the fetus and allowing its development. More relevant to pregnancy may be the effect of progesterone on Th function and cytokine secretion. A report by Piccini et al. (1996) showed that progesterone hormone progressively increases in pregnancy and this favors’ the development of the type 2 cytokine- producing Th lymphocytes. This information may
partly account for the increase in the levels IL-10 observed among the control group during mid-gestation. Khat administration to the baboons was only done at mid-gestation for a limited period and hence the effect observed could have been transient hence no significant difference with controls.

5.1.6 Profile of CXCL 10 and CXCL 13 among pregnant baboons

The general trend in the decrease of CXCL 10 among khat fed baboons and the non khat fed baboons group may partially be attributed to the fact that CXCL 10 is inflammatory. It is required in early pregnancy then decreases as the pregnancy progresses. It should be noted that khat seems to affect the decrease in CXCL 10 which may have adverse effect on fetal development. Since khat extract was given to the baboons for a short period, the effect observed could have been transient hence no significant difference with the controls.

Chemokine 10 (IP-10) also known as CXCL 10 was originally identified as an IFN-γ – inducible gene (Luster et al., 1987). It is induced in a variety of cells in response to IFN-γ and LPS (Lipopolyssaccharides). Chemokine 10 is an inflammatory chemokine (Released in response to infection), its release is stimulated by pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1β (Mantovani et al., 1996). It has been proposed that this chemokine is also involved in the recruitment and potentiating of T-helper 1 (Th1) responses (Gangur et al., 1998).

The findings of the present study are in agreement with recent study that established that during pregnancy, dNK cells (decidua Natural Killer cells) release CXCL 8/IL-8 and
CXCL 10/IP-10. These chemokines direct CXCR1+ and CXCR3+ trophoblast cells towards endovascular invasion and vascular remodeling, thus, the mutual chemokine-mediated attraction between dNK cells and invading trophoblasts appears necessary for developing a functional maternal-fetal interface early in pregnancy (Kanellopoulous-Langevin et al., 2003; Hanna et al., 2003). Based on this knowledge, it therefore means that CXCL 10 is necessary in early pregnancy and its secretion decreases as the pregnancy progresses.

The profile of CXCL 10 is contrasted by the profile of CXCL 13 that increased at mid-gestation. CXCL 13, a chemokine previously named B-lymphocyte chemoattractant (BLC) in mice (Gunn et al., 1998) or B cell attracting chemokine-1 (BCA-1) in humans (Legler et al., 1998) was first identified in 1998 in liver and lymph nodes. CXCL 13 is a potent chemokine secreted by monocytes, lymphocytes and dendritic cells (Vissers et al., 2001) and is detected in serum (Widney et al., 2005) of normal lymphoid tissue and in acute and chronically inflamed tissue (Carlsen et al., 2004).

The presence of CXCL 13 in serum of pregnant baboons early in gestation and its stable concentration throughout pregnancy suggests that CXCL 13 may also contribute to the homeostatic balance that it may be important in the maintenance of a normal gestation. Decrease in the elevation of CXCL 13 observed in khat fed baboons may have an effect on pregnancy and growth of the fetus. In the current study, khat was administered for a short duration during the pregnancy and for a limited period and since khat chewers do it for long periods, it therefore implies that if khat was administered for a longer period
during the gestation period, it would give a clear picture of the trends of the chemokines and cytokines for the entire gestation period.

5.2 Conclusions

i. This study established that khat use causes changes in body weight and blood pressure in baboons; khat consumption demonstrated a significant decrease in body weight and significant increase in blood pressure among pregnant baboons.

ii. This study established that Cytokines and Chemokines profiles in baboons change during pregnancy and especially at mid-gestation: the levels of interferon-gamma (IFN-γ) and CXCL 10 indicated a gradual decrease at mid-gestation, whereas, the levels of interleukin 10 (IL-10) and CXCL 13 slightly increased. Khat demonstrated a slight decrease in IFN-γ and CXCL 10 when administered to pregnant baboons although the decrease was not significant when compared to controls. IL-10 increased but again there was no significant difference when compared with controls, the levels of CXCL 13 had a significant increase.

iii. Khat use caused an increase in body weight of infants although the increase was not significant when compared to the newborns of non-khat fed baboons.

5.3 Recommendations

5.3.1 Applications of findings

Health education should be given about khat chewing during pregnancy as one of the risk factors to pregnancy and fetal growth since the results of the study have established that
khat changes profiles of cytokines and chemokines and increases blood pressure among pregnant baboons.

5.3.2 Suggestions for future research work

i. The effects of khat on Cytokines and Chemokines profiles should be investigated for the entire gestation period in baboons to give a clear picture of the changes in these cytokines and chemokines profiles in the three phases of pregnancy so that a comparison with the controls can be done.

ii. A considerable amount of work still remains in the study of the appetite effects of khat consumption during pregnancy as such a study will enable researchers to answer the questions on khat and birth weight of newborns.

iii. As this study was carried out, the specific constituents of khat responsible for its effects on cytokines and chemokines during pregnancy were not determined. Therefore, there is need for fractionation of the khat extract and testing of various fractions and khat specific pure constituents in order to determine the actual factors in khat responsible for changes in immunological profiles during pregnancy. In addition, the concentrations at which these constituents induce these changes need to be determined and thereafter compared with the concentration found in the saliva during khat chewing in order to assess their potential effects.
5.4 Study limitation

The investigation was part of an ongoing study at the Institute of Primate Research (IPR), Karen where the baboons are reared. The study was limited by the small number of baboons used in the study, baboons are very expensive to purchase and to time mate, also maintaining them is costly, therefore because of financial constraints the study utilized samples that had already been collected over the mid-gestation period, as a result the entire gestation period was not covered.

Time-mating baboons and synchronizing them is tricky hence the study was limited to six baboons whose plasma was available for the experiments. The number is acceptable for practical and ethical reasons. However, data from the current study is limiting since baboons were given khat extract only at mid-gestation and for a limited period, not all cytokines were studied in the study and since the study was ongoing the current study was limited on sampling points.
REFERENCES


APPENDICES

APPENDIX I: Animal care and use committee (ACUC) approval letter (IPR)

INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: IRC/06/13

Dear Dr. Atunga Nyachieo,

It is my pleasure to inform you that your proposal entitled “The effects of Khat on pregnancy and fetal development” in collaboration with ‘Dr. Daniel Chai’ has been reviewed by the Institutional Scientific and Review Committee (IRC). The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved at a meeting of 29th May 2013 and you are bound by the IPR Intellectual Property Policy.

Signed .......................................................... Chairman IRC: DR. HASTINGI OZWARA

Signed .......................................................... Secretary IRC: DR. ATUNGA NYACHIEO

Date: ..........................................................

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED: 29/5/2013
APPENDIX II: Post graduate studies approval letter, Kenyatta University

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

FROM: Dean, Graduate School
TO: Wambua Philomena Nduku
C/o Zoological Sciences Department.

DATE: 11th August, 2015
REF: 156/24064/13

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting of 29th July 2015, approved your Research Proposal for the M.Sc. Degree Entitled, “Effects of Crude Khat (Catha edulis) extract on Selected Cytokine Profiles and Foetal Growth among Pregnant Baboons (papio Anubis)”.

You may now proceed with data collection, subject to clearance with the Director General, National Commission for Science, Technology and Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking forms per semester. The form has been developed to replace the progress report forms. The supervision Tracking Forms are available at the University’s website under Graduate School webpage downloads.

Thank you,

EDWIN OBUNGU
FOR DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Zoological Sciences Department

Supervisors:

1. Dr. Michael Gicheru
   C/o Department of Zoological Sciences
   Kenyatta University

2. Dr. Atunga Nyachiro
   Department of Reproductive Biology
   Institute of Primate Research
   Karen, Nairobi
   C/o Department of Zoological Sciences
   Kenyatta University
APPENDIX III: Preparation of ELISA Reagents/ Buffers

Preparation of Blocking Buffer (2% BSA/PBS)

- Obtain Albumin Bovine from 4°C refrigerator, weigh out 2g, and place in 100ml beaker.
- Add 100ml of 1X PBS in a beaker.
- Place magnetic rod in beaker, cover with aluminium foil, and stir to dissolve

Preparation of 1M Stop Solution:

- Add 27.8ml of 18M Sulfuric Acid to 500ml glass bottle.
- Using graduated cylinder, measure out 472.2ml distilled water and pour into the 500ml bottle containing sulfuric acid.
- Label bottle and store at room temperature.

Preparation of Second Antibody Diluent (1% BSA/PBS, 0.05% Tween)

- Weigh out 5g BSA and place in a 500ml beaker
- Add 495ml 1X PBS.
- Add 0.25ml Tween.
- Place magnetic rod in beaker, cover top with foil, and place on hot plate until all contents are dissolved.
- Sterilise filter contents in beaker through 0.45um sterile filter system. Be sure to add a 60MM pre-filter to filter system before adding contents of beaker.

Preparation of normal sterile saline

- Weigh out 8.5g sodium chloride and place in a 1000ml beaker
- Add 1litre double distilled water
- Dissolve and filter, sterilize