
**ADDIS ABABA UNIVERSITY**
**COLLEGE OF HEALTH SCIENCES**
**SCHOOL OF MEDICINE**
**DEPARTMENT OF ANATOMY**

# Teratogenic effects of *Catha edulis* Forsk (Khat) in rat embryos and fetuses.

A thesis submitted to the department of Anatomy, School of Medicine, College of Health Sciences, Addis Ababa University, for the partial fulfillment of the requirement for the degree of Master of Science in Anatomy.

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## Declaration

This is to certify that the thesis prepared by Selamawite Belete, entitled: Teratogenic Effect Of Khat In Rat Embryo And Fetuses at Addis Ababa University on year 2017/2018 and submitted in partial fulfillment of the requirements for the degree of Master of science in Anatomy complies with the regulations of the University and meets the accepted standards with respect to originality and quality. This thesis has not been presented for a degree in any other university, and that all sources of materials used for the thesis have been duly acknowledged.

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## LIST OF ABBREVATIONS AND ACRONAMES

| | |
|---|---|
| $^{O}C$ …….. | Degree Celsius |
| AAU …….. | Addis Ababa University |
| ANOVA …… | Analysis Of Variance |
| BP …….. | Blood Pressure |
| CNS ……... | Central Nervous System |
| CVS ……... | Central Nervous System |
| DS …….. | Digestive System |
| EPHI…… | Ethiopian Public Health Institute |
| g …… | Gram |
| H&E …….. | Hematoxilin and Eosin |
| IUFD ……. | Intra Uterine Fetal Death |
| ICU …… | Intensive Care Unit |
| Kg …….. | Killogram |
| Mg ……. | Milligram |
| OECD …… | Organization for Economic Cooperation and Development |
| PPH ……... | Post Partum Hemorrhage |
| PROM ……... | Premature Rupture Of Membrane |
| SDR ……... | Sprague Dawley Rats |
| SD …….. | Standard Deviation |
| SPSS …….. | Statistical Package For Social Science |

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## ABSTRACT

**Background:** Khat (*Catha edulis* Forsk) is a plant chewed habitually by people in different parts of Ethiopia. It is also commonly chewed in other East African countries and Southern Arabia to attain a state of euphoria and stimulation. There are several reports in the literature concerning its toxic effects on different aspects of health but the teratogenic effects of Khat after exposure during pregnancy have not been elucidated.

**Objective:** The main aim of the present study was to evaluate the teratogenic effects of Khat in Albino Wistar rats.

**Materials and Methods:** Three experimental and two control groups were included in the study. Pregnant Albino Wistar rats were randomly assigned to the experimental and control groups to minimize the effects of subjective bias when allocating animals. The 80% methanolic extract of Khat at doses of 250mg/kg, 500mg/kg and 750mg/kg were orally administered to pregnant albino wistar rats from day 6 through day 12 of gestation. Fetuses and embryos were recovered on gestational day-20 or day-12, respectively, and were quantitatively and qualitatively assessed for developmental anomalies. Histopathological examination was carried out on the placentae from the treatment and control groups.

**Results:** Results of the present study showed that Khat exposure during pregnancy had dose dependent teratogenic effects in rat embryos and fetuses. Prenatal growth retardation such as reduced fetal weight and crown-rump length were observed in near term fetuses of Khat treated animals with the highest dose in excess of those in the pair-fed control and Ad libitum group($p<0.05$). Growth retardation and developmental anomalies were also observed in day-12 embryos of Khat treated rats. Maternal weight gain of Khat treated group was also significantly lower than the control group. In addition cytolysis, decidual hypoplasia and atrophy were observed under microscopic examinations of placenta of Khat treated rats. However in this study, offspring of Khat treated pregnant rats did not show gross external malformations.

**Conclusions:** The findings of the present study revealed that exposure of rats to high dose of crude Khat extract during pregnancy could have teratogenic effects on the rat embryos. Exposure to higher dose of Khat extract (750mg/kg) during pregnancy resulted in decreased maternal weight gain and prenatal growth retardation in albino wistar rats.

**Key words: -** *Catha edulis*, teratogenecity, khat, fetuses, embryo, gestation

## 1. INTRODUCTION

*Catha edulis* Forsk (Celestraceae), which is commonly called Khat, Qat, or Mirra is a plant commonly grown in the countries of the horn of Africa (Ethiopia, Somalia, Somaliland, Kenya, Eritrea, Djibouti and Uganda), and across the Arabian Sea into Yemen and Saudi Arabia. It is an evergreen shrub cultivated as a small tree. People consume the leaves of Khat usually for its psychostimulant action; to improve their performance, to stay alert and to increase their work capacity. Besides chat chewing has a deep rooted socio-cultural tradition and widespread habit in countries around the horn of Africa and Yemen. The traditional way to consume khat is to pick a few leaves of a young shoot and chew them slowly. Once the leaves are pulped, they are kept in the side of the cheek and the mouth is filled with fresh leaves. The user then chews slowly and intermittently to release the active components of khat that are then swallowed with saliva. Sometimes it is ingested by making a drink from dried leaves(1).

Khat chewing usually takes place in group of social setting. The session of chat chewing may last for several hours and during this time chewers drink copious amount of nonalcoholic fluids such as cola, tea, and cold water and usually accompanied by cigarette smoking.

Despite having health and economic consequences, Khat chewing has been practiced as stimulant and social custom for thousands of years in the Horn of Africa and Arabian Peninsula. The number of Khat chewers has significantly increased over the years in Ethiopia. Previously, Khat was mainly cultivated and chewed in the eastern part of the country but these days, Khat is spreading to all Ethiopian geographic regions, religious and ethnic groups because of social mobility, accessibility (affordability and availability of khat leaf in the whole year) and the importance of khat as cash crop. Khat chewing is highly prevalent in Harari regional state where above half of the population chew Khat [53.2%]. The prevalence in Dire Dawa was the second highest followed by Oromiya and Somali regional states. The lowest prevalence was found in Tigray regional state (2). Number of studies showed that the prevalence of khat chewing among adult men was higher as compared to females(3,4) Females were 77% less likely to chew khat as compared to males. Khat chewing for female is more culturally restricted than males. However women as users are often reported as the hidden population either using it in isolation in the home or as a group of women in isolation from others. This might create bias on the exact prevalence of Khat chewing among women.

The main psychoactive ingredients of khat include cathine, cathinone and chemicals that are similar to amphetamines. According to previous studies chemical components of khat have toxic effects on different systems of the body especially on the central nervous system (CNS), cardiovascular system (CVS) and digestive system (DS). However, only few data were found on the effect of Khat on the reproductive system especially on its teratogenic effects following exposure during pregnancy(5). The placenta is also a highly susceptible target organ for drug or chemical induced adverse effects, and many placenta toxic agents have been reported. However, histopathological examination of the placenta is not generally performed, and the placental toxicity index is only the placental weight change in rat reproductive toxicity studies. The detailed histopathological approaches to investigation of the pathogenesis of placental toxicity are considered to provide an important tool for understanding the mechanism of teratogenicity and developmental toxicity with embryo lethality, and could benefit reproductive toxicity studies(6).

Therefore it was decided to conduct this study on the teratogenic effects of Khat in Albino Wistar rats in order to assess the outcome of pregnancy in regard to maternal health and fetal outcomes and embryonic development.

**Figure 1 Photograph of fresh leaves of Khat**

## 1.1 STATEMENT OF THE PROBLEM

The use of Khat (*Catha edulis*) is widespread in Ethiopia. It is also widely used in eastern Africa, including the Horn of Africa. In Ethiopia, Khat is easily obtainable and mostly used by adolescents and young adults. Studies found that there was a significant increase in the number of Ethiopians chewing Khat. Khat, previously known to grow mainly in the eastern part of Ethiopia, is now widely cultivated in all parts of the country. Moreover, Khat consumption, traditionally confined to a certain segment of the population, had become popular among all segments of the population. Khat-chewing also led to the abuse of illicit substances (7).

Surveys and anecdotal data show that Khat chewing has become cosmopolitan, with users now living in Europe and North America as well (8). The major effects of Khat include adverse effects on the nervous system and also on gastro-intestinal system. Constipation, urine retention and acute cardiovascular effects may be regarded as autonomic (peripheral) nervous system effects while increased alertness, dependence, tolerance and psychiatric symptoms are its effects on the central nervous system. Other toxic effects of Khat include increased blood pressure, tachycardia, insomnia, anorexia, constipation, general malaise, irritability, migraine and impaired sexual potency in men (5).

While early reports indicated greater prevalence in men than in women, recent reports have shown sharp increases in Khat use by women in some countries. In Ethiopia, recent estimates of prevalence approach 50%, with 17% self-described as daily users, predominantly men (a reported 5:1 male to female ratio). In addition women where much hidden Khat users from their family (9). Recent reports indicate that 80–90% of East African males use Khat on a daily basis and 10–60% of East African females use it on a daily basis. There is evidence to suggest that gender differences in Khat use disappear in these countries (10).

A study conducted in Haromaya district on Khat chewing and restrictive dietary behaviours showed the association of chewing Khat frequently with higher risk of anemia explained by the loss of appetite. Furthermore, Khat contains a substantial amount of tannin, which reduces the bioavailability of non-heme iron from the maternal diet that is mainly based on foods of plant sources in the population. The interaction between chewing Khat and consuming iron rich foods

in the stratified analysis for the risk of anemia further strengthened the possibility of the hypothesized inhibitory effect of Khat on iron absorption (11).

A large study of 1,141 pregnant mothers in Yemen showed that Khat chewing mothers were older and had a greater number of pregnancies than non-Khat using mothers. The Khat chewing mothers had more low birth weight babies than non-chewers but there was no difference between the groups in stillbirth or congenital abnormalities. There is one report of a pregnant woman developing tachycardia, hypertension and chest pain after a Khat chewing session. However there are no other published data of any adverse incidents associated with Khat chewing during pregnancy (5).

A case-control prospective study conducted on 60 regular Khat-chewer pregnant women attending the antenatal care clinic of AL- Gamhouri Teaching Hospital in Taiz (Republic of Yemen) showed that Khat chewer pregnant women were having a statistically significant increased risk for preterm labor, labor induction and a significant lower mean hemoglobin concentration at delivery when compared with the control. Khat chewer pregnant women showed other risks when compared with the control such as a risk of preeclampsia, blood transfusion, for fetal distress, premature rupture of membrane (PROM), post partum hemorrhage (PPH) and perineal tears and intra uterine fetal death (IUFD). However, these risks were found statistically insignificant. Fetal outcome among the studied groups showed that Khat chewer pregnant women when compared with the control were having a significant risk of deliver fetuses with low birth weight (< 2500 gm); and a significant risk of neonatal admission to the intensive care unit (ICU). They showed other risks of perinatal mortality and congenital malformations. However, these risks were found statistically insignificant (12)

There is very little animal data that have revealed some potentially harmful effects of Khat use during pregnancy. Extracts of Khat given to pregnant rats was associated with reduced maternal food consumption, a reduction in litter size and reduced foetal growth. Some visceral and skeletal malformations also were noticed at the highest dose of Khat suggesting a teratogenic effect (8).

The mammalian embryo cannot develop without the placenta. The placenta is vital for embryonic as well as fetal development. Its specialized cells (trophoblast, endoderm, and

extraembryonic mesoderm) form early in development. They attach the embryo to the uterus (implantation) and form vascular connections necessary for nutrient transport. In addition, the placenta redirects maternal endocrine, immune, and metabolic functions to the embryo's advantage. These complex activities are sensitive to disruption and effects on the developing embryo and fetus will affect the placenta as well (13). Thus this study will have the chance to identify the teratogenic effects of Khat, if any, in rat embryos and fetuses and its histopathologyic effects on the rat placenta.

## 1.2 SIGNIFICANCE OF THE STUDY

Exposure to toxic effects of different substances during pregnancy can result in acute and long term sequels on maternal health and newborns. Serious toxic effects of Khat on different body systems have been reported. Despite the evidence of the negative effect of Khat people still chew it. Although there is some limited data on the adverse effects of Khat, very little is known about its teratogenic effects.

Therefore, it was conducted worthwhile to evaluate the teratogenic effects of Khat. The outcome of the study may serve as a premise for further investigation on this plant and may help develop intervention strategies to control the abuse of Khat.

## 2. LITERATURE REVIEW

Khat is a flowering plant, indigenous to tropical East Africa and the Arabian Peninsula. Even though the origin of the plant is often argued, many believe that its origin is Ethiopia and others state that Khat originated in Yemen before spreading to Ethiopia and the nearby Arab countries, Kenya, Somalia, Uganda, Tanzania, Malawi, Congo, Zambia, Zimbabwe and South Africa (14). However the Harar region of Ethiopia is universally believed to be the origin of Khat use (4). The leaves have an aromatic odor and tastes astringent and slightly sweet. Many different compounds are found in Khat including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins and minerals. The Phenylalkylamines and the cathedulins are the major alkaloids which are structurally related to amphetamine (5).

Chewing the leaves of the plant for their pleasurable stimulant effect is a habit that is widespread in the mentioned geographical areas. It is estimated that about 5–10 million people chew it every day. In Yemen for example, 60% of the males and about 35% of the females were found to be Khat users and had chewed daily for long periods of their lives (14).The psycho-stimulant effect of Khat, the main reason people chew Khat leaves, is due to the alkaloid chemical ingredient cathinone present in the fresh leaves of the plant. Cathinone's chemical structure is similar to amphetamine (4).

Traditionally, Khat has been used as a socializing drug and this is still very much the case. It is mainly a recreational drug in the countries where it grows, though it may also be used by farmers and agricultural and other laborers for reducing physical fatigue and by lorry drivers and high-school students for improving attention(14).

Effects observed following Khat consumption usually involve central nervous system (CNS) and this effect is believed to be induced by cathinone, an active ingredient of Khat leaves. Cathinone has a rapid and intense action due to its higher lipid solubility which facilitates access into the central nervous system. Several studies have shown that the psychostimulant effects induced by chewing Khat leaves include a moderate degree of euphoria and mild excitement resulting in promotion of social interaction. While attaining a state of subjective well-being, the chewers feel an increased alertness and energy together with enhanced depth of perception. These effects were found to be maximum between 1.5–3.5 hours after starting to chew and they were progressively

replaced by mild dysphoria, anxiety, reactive depression insomnia and anorexia. In recent years Khat-induced psychosis has become more common in Europe (15).

Literature surveys and clinical diagnostic studies revealed the presence of a direct relationship between Khat chewing and lifetime prevalence of the psychiatric morbidity in Ethiopia (16,17). A study in Yemen to investigate effects of chronic Khat use on cardiovascular, adrenocortical, and psychological responses to acute stress showed, Khat users exhibited enhanced evening and attenuated morning cortisol levels, reflecting a blunted diurnal pattern of adrenocortical activity compared to nonusers. Khat users reported greater negative effect during the ambulatory period and during the laboratory session. In addition, they exhibited attenuated BP (18,19). According to animal study on neuropsychopharmacological effect of Khat at a dose of 200 mg/kg, it produced significant motor in-coordination and emotional instability; as revealed by impairment in both cliff avoidance and forelimb grip strength, as well as by an increase in stereotyped behavior such as grooming, and in the percent of time spent in open arms. On the other hand, at a dose of 100 mg/kg, Khat had an effect only on grip strength where a decrement was noted. Results from this study also showed increased latency to reach the goal box and the number of wrong decisions in both the learning and the recall tests at the dose of Khat 200 mg/kg. Alterations in the biochemical indices of liver and kidney function were also noted with Khat 200mg/kg. These findings indicate that Khat exposure produces dose-related central and peripheral effects during pregnancy and lactation which might pose a serious impediment to the physical and mental development of the offspring (20).

Studies have demonstrated an association of Khat with increased sympathetic activity and clinical observations suggest increased risk for acute myocardial infarction and stroke. The vasoconstrictor activity of cathinone explains the increase in blood pressure seen in humans and in animals, and might be related to the increased incidence of myocardial infarction occurring during Khat sessions, i.e. during the Khat-effective period, and associated with heavy Khat chewing (5)

A study conducted on habitual Khat users in Royal Free Hospital of London shows, chewing Khat decreases subjective feelings of hunger and increases systemic sympathetic tone and they conclude that the anorexigenic effect of khat may be secondary to central mechanisms mediated via cathinone (21).

According to a study conducted in Yemen, chewing Khat every day and restrictive dietary behaviors during pregnancy were associated with having anemia and therefore, dietary counseling and reducing the habit of chewing Khat during pregnancy were recommended. In addition, the higher risk of anemia associated with higher parity levels and advanced stages of pregnancy implies the need of tailored interventions for these vulnerable groups of women (11).

Khat also produces constipation and brings about antispasmodic action. It was not possible, however, to deduce the exact mechanism of action of Khat extract for its constipating and antispasmodic action (22). A study conducted in1005 Ambo University (Ethiopia) students on the association between Khat chewing and gastrointestinal disorders shows that the prevalence of gastritis was 580 (57.7%); constipation 235 (23.4%); hemorrhoids 54 (5.4%) and that of dental problems (carries, decay, filling and extraction) was 225 (22.4%) of all study participants. Gastrointestinal disorders were found to be higher among Khat chewers, where 64 (36.2%) of them had dental problems; 127 (71.8%) symptoms of gastritis; 86 (48.6%) constipation and 26 (14.7%) hemorrhoids which demonstrated statistically significant association so that Khat chewing could be a predisposing factor to gastrointestinal disorders(23).

Khat chewing may result in a number of changes in the oral mucosa and the dentition. Colour changes of oral mucosa to white were significantly more prevalent in the Khat chewers, identified primarily at the chewing site (83% vs. 16%). The difference in the prevalence of oral mucosal pigmentation between non- chewing nonsmoking (66.7%) and the Khat-chewing (100%) groups was highly significant. The mean gingival index and the depth of periodontal pockets of the Khat-chewing subjects were significantly reduced at the chewing side compared with the non chewing side. Increased gingival recession was recorded on the Khat-chewing side. Discoloration of the teeth adjacent to the site of chewing was recorded. Oral dryness occurring 30 minutes after initiating the Khat-chewing session was reported by its users(24,25).

Animal study on toxicological features of Khat on liver and kidneys shows, administration of Khat to male and female Sprague Dawley (SD)-rats was not safe and resulted in hepatorenal toxicity on short-term exposure assessed by histological lesions. Hepatotoxicity was common in both male and female SD-rats with hepatic hypertrophy, elevated liver enzymes, and zone 3 necrosis on histology. In addition, Khat-induced nephrotoxicity was limited to female and not to

male SD-rats. Hence, hepatorenal toxicity of Khat was established both clinically and experimentally (26).

A study on the effect of Khat on utroplacental blood flow in awake, chronically catheterized, late-pregnant guinea pigs, placental blood flow was reduced by 10% 75 min and by 24% 180 min after Khat feeding. Since the Khat dose used gave urinary concentrations of norpseudoephedrine of the same magnitude as those reported in Khat chewing women, the study conclude that Khat chewing in pregnancy may reduce placental blood flow and impair fetal growth (27). Since one of the active constituent of Khat, norpseudoephedrine, cause vasoconstriction in utroplacental vascular bed, it is possible that Khat chewing could reduce placental blood flow, and as a consequence, impair fetal growth (27).

Another study on the influence of Khat chewing on birth weight in full term infants was found that healthy full-term, singletons, born after uneventful pregnancies and deliveries, had a significantly lower average birth-weight when the mothers were Khat-chewers, either habitually or occasionally so that Khat- chewing appears to be one of several maternal practices adverse to the fetus (28).

Animal study on effect of Khat on maternal food intake, maternal weight gain and fetal growth in the late pregnant guinea pig showed, maternal daily food intake was significantly reduced during the first 10 days of feeding and maternal weight gain was slightly lower in the Khat group. Khat feeding of the mother significantly reduced the mean birth weight of the offspring by 7% without any effect on litter size or length of gestational period. Since low birth weight is a well-established risk factor for both perinatal and young infant death, Khat chewing during pregnancy may be one of the factors contributing to infant mortality (29).

According to Islam *et al*. (30) Khat reduced the food consumption and maternal weight gain and also lowered the food efficiency index, as compared to control mothers. The administration of Khat had no effect on fetal sex ratio. However, at a dose of 125 mg/kg body weight and above, it produced a significant increase in resorptions and fetal wastage. Khat administration in utero also reduced the litter size and caused intrauterine growth retardation. External, visceral and skeletal examination of the fetus of treated dams showed several types of malformations and variations in all the groups of animals. However, a consistent tendency of abnormalities was observed in the

highest dosed (500 mg/kg) group. The data of this study revealed that Khat retarded fetal growth and induced terata. The observations also indicate that Khat possesses both embryotoxic as well as teratogenic properties and he developmental toxicities of Khat are dose-related (30).

A research conducted on the effect of reproductive parameters of Khat in male rats indicated that Khat has biphasic effects on male rat sexual behavior, high dose of the extract diminishing sexual motivation and performance, whereas low dose of the extract enhancing sexual motivation. This could be related to amount of testosterone produced by the rats. Khat also decreased epididymal sperm count without affecting the endocrine glands. Moreover, effect of Khat on reproductive behavior of male rats seems to depend on the level of its cathinone content (31). Another study on the effects of oral administration of high-dose Khat on sperm parameters and male hormonal levels in olive baboons showed that Khat extract induced a significant reduction in sperm motility, sperm count, sperm chromatin integrity, testosterone levels and prolactin levels, but not in cortisol levels and sperm volume. The results suggest that high-dose Khat decreases sperm quality and testosterone and hence may contribute to male infertility (32).

Another study by Mwenda *et. al*. (33) on the potential effect of Khat on fertility, on olive baboon through oral administration, by measuring its effects on circulating hormones showed that Khat administration causes a significant increase in the mean levels of testosterone while prolactin and cortisol levels were reduced. These effects were also evident 1 month post treatment and indicate Khat may exert a transient effect on male fertility by interfering with the hormonal profiles (33).

Genotoxic effect of Khat was studied by Adel Al-Zubairi *et al*. (34) by administering sub chronic crude extract of Khat. Their results showed no DNA damage detected using comet assay in both the Khat treated groups, while the results of chromosomal aberrations assay showed a significant increase in the dose 2000 mg/kg body weight treated group compared to the control group (34). In light of the large body of evidence on the close association between genetic damage and cancer, these results suggested that Khat consumption, especially when accompanied by alcohol and tobacco consumption might be a potential cause of oral malignancy (35).

# 3. OBJECTIVES

## 3.1 GENERAL OBJECTIVS

- To evaluate the teratogenic effects of Khat on rat embryos and fetuses.

## 3.2 SPECIFIC OBJECTIVES

- To evaluate possible teratogenic effects of Khat on 12 days old whole rat embryos.
- To asess possible teratogenic effects of Khat on 20 day old rat foetuses; and
- To study the effect of Khat on the histopathology of placenta of 20 day old rat foetuses.

# 4. MATERIALS AND METHODS

## 4.1 EXPERIMENTAL DESIGN

The experiments were designed to study the teratogenic effects of Khat in nulliparus female Wistar rats. Experiments were carried out in day-20 fetuses (Day-20 Experiment) and in day-12 embryos (Day-12 Experiment). Animals were treated with different doses of Khat for a period of 1 week; from day-6 through day-12 of gestation because this is the period of organogenesis in rats. During the treatment period, the animals were fed on pellets and water. In addition, pregnant animals were treated with Khat extracts. The weight of pregnant animals was recorded on days 1, 6, 12 and 20 of gestation (for day 20 experiment) and on day 1, 6 or 12 of gestation (for day 12 experiment).

## 4.2 PLANT MATERIAL

Bundles of khat shoots and small branches (about 7000 g) were purchased fresh at a local market in Aweday, 515 km east of Addis Ababa, Ethiopia, which is one of the common natural habitats. The fresh bundles were packed in plastic bags and transported in an ice box to the laboratory. The fresh leaves were immediately kept in a deep freezer (-20 ºC). The plant was identified by a taxonomist and a voucher specimen (collection no SB-001) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University for future reference.

## 4.3 PREPARATION OF CRUDE EXTRACT

The leaves were chopped with knife in a dark place, weighed by electronic digital balance and placed in an Erlenmeyer flask containing 80% methanol. Enough volume of the solvent was added so as it cover the crushed plant material in the flask. The flask was wrapped with aluminum foil and contents were continuously stirred using a rotary shaker at 120 rpm for 24 h. It was then filtered using Whatman No. 1 filter paper (90 mm diameter, Whatman Ltd, England) and the marc remacerated 3X for 72h and filtered. The combined filtrates were concentrated using Rota Vapor (Büchi Rota Vapor R-205, Switzerland at a temperature not exceeding 40ºC. the concentrated extract was then transferred into freeze drying flask and lyophilized (OPERAN Lyophilizer, KOREA). The dried extract was kept in a tightly sealed container at -20 ºC until use.

## 4.4 EXPERIMENTAL ANIMALS

Throughout the course of this investigation, nulliparus female albino Wistar rats and sexually matured males of proven fertility were used. The rats were obtained from the animal house of EPHI. The animals were housed in suspended stainless steel cages in an environmentally controlled room (22 - 23°C); relative humidity of 50% ± 10). A cycle of 12 h of light and 12h of dark maintained at all times. During the period of adaptation, all the animals were fed with conventional laboratory diets and unlimited supply of tap water. The handling of animals and all experimental procedures were carried out according to internationally accepted OECD guidelines (OECD/OCDE, 1995; OECD/OCDE, 2008) (36) and the protocol was approved by the Institutional Review Board of EPHI and AAU department of Anatomy.

After 5 days of adaptation period, the animals mate overnight by placing a male albino Wistar rat into a cage containing two nulliparous female rats. The male rat was introduced into the cage at about 17:00 hours. After an overnight mating, female rats were inspected for the presence of copulatory plug the following morning and vaginal smears were taken for microscopic examination. If sperm cells are detected in the vaginal smear it was considered as day-1 of gestation.

## 4.5 GROUPING AND DOSING OF ANIMALS

Pregnant animals were randomly divided into five groups, each comprising 5animals per group. Group I was treated with vehicle and was the control group (CON). Group II-IV were treated with three doses of the crude Khat extract; 250 mg/kg (K250), 500 mg/kg (K500), and 750 mg/kg (K750). Group V was Ad libitum control group. The various doses of the Khat extract were selected based on previous reports. The khat extract was weight and mixed with the vehicle (1.0 ml distille water) and administered orally using oral gavage. Such route is used since the Khat leaves are usually taken orally in humans. In addition, pharmacokinetics studies have shown that Khat is readily absorbed into the blood stream from the stomach and mouth.

### 4.6 DAY- 12 KHAT EXPERIMENT

The day-12 experiment was designed to investigate possible teratogenic effects of Khat in pregnant animals. In this experiment, once pregnancy was confirmed, animals were randomly assigned to control group, Group I (CON), Group II (K250), Group III (K500), Group IV ( K750) and Group V, Ad libitum control.

Each group consisted of 5 pregnant animals. The control group, Group I (CON) received vehicle while Group II, Group III and Group IV were given khat at a dose of 250mg /kg, 500mg/kg and 750mg/kg respectively. Group V animals were untouched or Ad libitum control group.

Each animal in the experimental groups (Group II, III and IV) received the daily diet of equal amount and the control groups were take the same diet and kept in the same environment except the Khat which was given only for the experimental group (group II, III and IV). The treatment period was from day-6 to day-12 of gestation at 2:00am in the morning. The rationale for administration of the treatment from day 6 through day 12 of gestation is because this period represents a period of active embryogenesis and organogenesis. Embryonic days 1 to 12 represent the critical period of development in the rat (37).

On day 12 of gestation, at 12:00 hours, the animals anaesthetized with diethyl ether and the uterine horns were removed, placed in normal saline and then incised along the antimesometrial border to reveal the embryos. With the aid of fine forceps, the membranes surrounding the embryo were removed to reveal the underlying visceral yolk sac and the yolk sac circulation development was evaluated. The embryos were then explanted and the development of the circulatory, nervous, visual, auditory, olfactory, and skeletal systems as well as craniofacial development were assessed quantitatively on the basis of 16 recognizable developmental endpoints (morphological scores)(Annex,9.1), according to the criteria of Brown and Fabro (1981) (38). In addition, the numbers of somites were also counted.

**Table 1: Treatment schedule: day-12 Khat experiment.**

| Treatment group | Number of animal | Treatment |
|---|---|---|
| Group I (CON) | 5 | Distilled water + diet |
| Group II (K250) | 5 | Khat 250 mg/kg + diet |
| Group III (K500) | 5 | Khat 500 mg/kg + diet |
| Group IV (K750) | 5 | Khat 750 mg/kg + diet |
| Group V | 5 | Ad libitum |

## 4.7 DAY- 20 KHAT EXPERIMENT

This experiment was designed to study gross congenital anomalies (teratogenic effects) caused by exposure to Khat of pregnant animals. In this experiment, once pregnancy was confirmed, animals were randomly assigned to control group,Group I (CON), Group II (K250), Group III (K500), Group IV( K750) and Group V, Ad libitum control group.

Each group consisted of 5 pregnant animals. The control group, Group I (CON) received vehicle while Group II, Group III and Group IV were given khat at a dose of 250mg /kg, 500mg/kg and 750mg/kg respectively. Group V animals were untouched or Ad libitum control group.

Each animal in the experimental groups (Group II, III and IV) received the daily diet of equal amount and the control groups were take the same diet and kept in the same environment except the khat which was given only for the experimental group (group II, III and IV). The treatment period was from day-6 to day-12 of gestation

On gestational day 20, gravid females were scarified by an overdose of ether; the uterine horns were exposed and examined intact. The number of implantation sites were determined by counting the metrial glands which are yellowish nodules located along the mesometrial margin of the uterine horns. The metrial nodules which are not occupied by living or recently dead foetuses represented the number of prior resorptions. The number of live or dead fetuses was determined by applying a gentle pressure on fetuses. The uterine horns were incised along the antimesometrial border to reveal the fetuses, fetal membranes and the placenta. Fetuses then recovered and dissected free of the placenta. Weight of each foetus was recorded. The

crownrump length and placental weight were also recorded. Following these measurements the fetuses were examined for gross external malformations.

**Table 2: Treatment schedule: day-20 Khat experiment.**

| Treatment group | Number of animal | Treatment |
|---|---|---|
| Group I (CON) | 5 | Distilled water + diet |
| Group II (K250) | 5 | Khat 250 mg/kg + diet |
| Group III (K500) | 5 | Khat 500 mg/kg + diet |
| Group IV (K750) | 5 | Khat 750 mg/kg + diet |
| Group V | 5 | Ad libitum |

## 4.8 EXTERNAL EVALUATION

Fetuses were examined head to tail for gross external malformations. The parameters to assess were:

- Craniofacial development (exencephaly, anencephaly, microphthalmia and anophthalmia)
- Development of the limbs (syndactyly, adactyly, polydactyly)
- Vertebral column (neural tube defect, kyphosis, scoliosis)
- Tail development (missing tail); and
- External genitalia.

## 4.9 HISTOPATHOLOGICAL STUDIES OF PLACENTA

Three to four placentae from each group were randomly selected and put in 10% formalin. The fixed placentas were transferred into 70% ethylalcohol overnight. Following routine processing for light microscopy, the placentae were blocked in paraffin and cut into sections of 4 µm. The sections were then mounted on glass slides, stain with haematoxyline and eosin (H&E) and covered with cover slip for microscopic examination.

The sections were examined for evidence of structural and vascular alterations using binocular light microscope. The following structures were examined and used as indices of functional as well as structural changes in the placenta

- ✓ Decidual zone of the placenta
- ✓ Labyrinthine zone; and
- ✓ Giant cells and trophoblasts

## 4.10 LIGHT MICROSCOPY AND PHOTOGRAPHY

Stained tissue sections of placenta were carefully examined by a pathologist under binocular compound light microscope. Tissue sections from the experimental groups were examined for any evidence of histopathological changes with respect to those of the controls. After examination, photomicrographs of selected samples of placental sections from both the experimental and control groups were taken under a magnification of x40 objective.

## 4.11 DATA ANALYSIS

The data, regarding weight gain, pregnancy outcome, maternal weight gain, fetal growth, and developmental anomalies were analyzed using one-way analysis of variance (ANOVA) followed by post- hoc test using the SPSS version 20 to identify the possible mean difference between the control and the treatment groups. The data regarding embryonic development and histopathogical investigations were analyzed by using Chi-Square analysis to identify weather there is a significant association between the control and treatment groups at the level of $p<0.05$.

# 5. RESULTS

## 5.1 DAY 12 EXPERIMENT

### 5.1.1 PREGNANCY OUTCOMES

Treatment of pregnant rats with Khat at dose of 250 mg/kg, 500 mg/kg and 750 mg/kg body weight, from days 6 to 12 of gestation produced a dose dependent decrease in the body weight gain of the dams, compared to control groups. Body weight was found significantly altered in the Khat-treated group at the dose 750 mg/kg and 500 mg/kg during the gestation period days 6 up to12 as compared to control values (Table 3).

Maternal daily food intake was also significantly reduced compared to the Ad libitum control group. A significantly higher incidence of resorptions ($p<0.05$) was observed in the 750 mg/kg Khat treated group. The same is true for implantation sites per dams which shows significant decrease in the number of implantation sites, ($p<0.05$) at the doses of k500 mg/kg and k750 mg/kg compared to the pair-fed control and Ad libitum control groups. Results are summarized in Table 4.

**Table 3: Pregnancy outcome of day-12 Khat experiment**

| Treatment group | Maternal weight gain per litter (g) | *Implantation* sites/dam | Resorptions/dam |
|---|---|---|---|
| Group I(pair-fedcontrol) | 4.6±0.01 | 9.8±0.8 | 0.2±0.4 |
| Group II (k250mg/kg) | 4.9±0.4 | 9.8±0.8 | 0 |
| Group III (k500mg/kg | 3.1±4.9* | 9.6±1.1 | 0.2±0.4 |
| Group IV (k750mg/kg | 3.9±4.5** | 7.8±0.8** | 0.6±0.9** |
| Group V (Ad *libitum*) | 14.2±-2.9 | 10.2±0.8 | 0 |

Results are summarized as mean ± SD; significantly different ($p < 0.05$) from Ad *libitum* control and pair-fed control group (ANOVA). *; significantly different ($p<0.05$) from Ad libitum group, **; significantlt different ($p<0.05$) from pair-fed control and untouched Ad *libitum* control group.

### 5.1.2 EMBRYONIC DEVELOPMENT

Developmental status of the primordia of the various systems was studied according to the morphological scoring system of Brown and Fabro (1981) (38). The degree of development of the yolk sac circulation and heart were affected ($p < 0.05$) in embryos of k500 mg/kg and k750 mg/kg groups compared to the pair fed control and to the Ad libitum control groups (Table4).

**Table 4: Embryonic circulatory and auditory systems development following treatment of pregnant rats with Khat: day 12 experiment.**

| Treatment group | % retarded development | | |
|---|---|---|---|
| | Yolk sac | Heart | Otic system |
| Control | 0 | 0 | 2.6 |
| K250 mg/kg | 0 | 1.7 | 2.6 |
| K500 mg/kg | 8.5* | 3.4 | 7.8* |
| K750 mg/kg | 5.6* | 10* | 12.2* |
| Ad libitum | 0 | 0 | 2.2 |

Results are summarized as mean ± SD; significantly different ($p < 0.05$) from *ad libitum* control and pair-fed control group (ANOVA). *; significantly different ($p<0.05$) from untouched Ad libitum group and pair-fed control group.

Flexion of the embryos was affected in embryos of k750 mg/kg group compared to the pair fed control and Ad libitum control groups, but the effect was not statistically significant. The forebrain, midbrain hindbrain and caudal neural tube development were not affected in all the treatment groups as compared to the control ones.

Embryonic development of otic system, optic system olfactory system and brachial bars were delayed in treatment groups of k500mg/kg and k750 mg/kg compared to the pair fed control and the Ad libitum control groups ($p<0.05$) (Table 4 and Table 5).

**Table 5: Embryonic craniofacial development after treatment of pregnant rats with Khat : day 12 experiment**

| Treatment group | % retarded development | | | |
|---|---|---|---|---|
| | Optic system | Olfactory system | Branchial bars | Maxillary process |
| Control | 0 | 1.7 | 4 | 4 |
| K250 mg/kg | 0 | 3 | 5 | 6 |
| K500 mg/kg | 5.6* | 4.1 | 7 | 8 |
| K750 mg/kg | 8.2* | 10* | 10* | 10* |
| Ad *libitum* | 1.3 | 1.5 | 4 | 4 |

Results are summarized as mean ± SD; significantly different ($p < 0.05$) from Ad *libitum* control and pair-fed control group (ANOVA). * ; significantly different ($p<0.05$) from Ad *libitum* control group and pair-fed control group.

The development of maxillary process, fore limb and hind limb were significantly affected in treatment groups of k500mg/kg and k750mg/kg ($p< 0.05$) compared to the pair-fed control and the Ad libitum control groups. However the development of mandibular process was not affected in both the treatment and control groups (Table5 and Table 6).

**Table 6: Embryonic musculoskeletal system development following treatment of pregnant rats with Khat: day-12 experiment.**

| Treatment group | Somite number | Forelimb | Hindlimb |
|---|---|---|---|
| Control | 39.2±0.9 | 0 | 0 |
| K250 mg/kg | 38.9±0.8 | 0 | 1 |
| K500 mg/kg | 37.8±1.1* | 0 | 1 |
| K750 mg/kg | 35.8±1* | 8.3* | 32* |
| Ad libitum | 39.2±1.9 | 0 | 0 |

Results are summarized as mean ± SD; significantly different ($p < 0.05$) from *ad libitum* control and pair-fed control group (ANOVA). * ; significantly different($p<0.05$) from Ad libitum control group and pair-fed control group.

The number of somites, the indices of musculoskeletal system development, was affected in treatment group of k750 mg/kg compared with the control groups, but the effects were not statistically significant (Table 6).

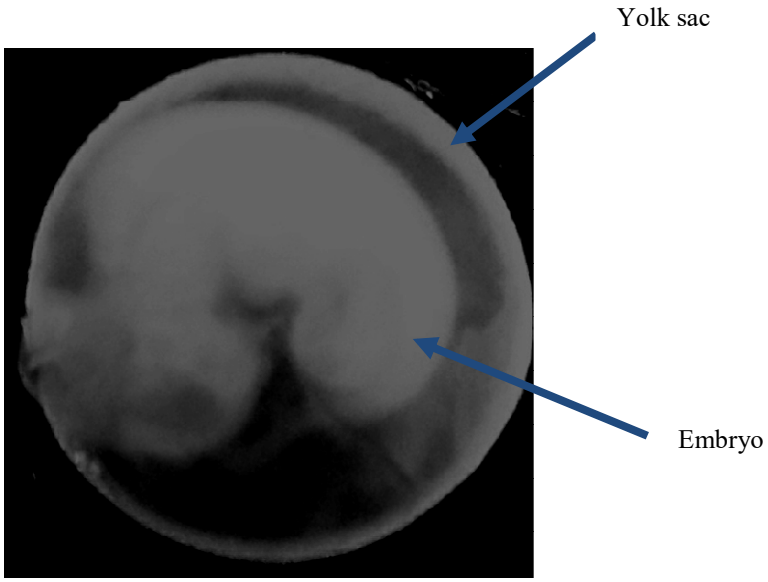


**Figure 2: Day-12 embryo inside the yolk sac**

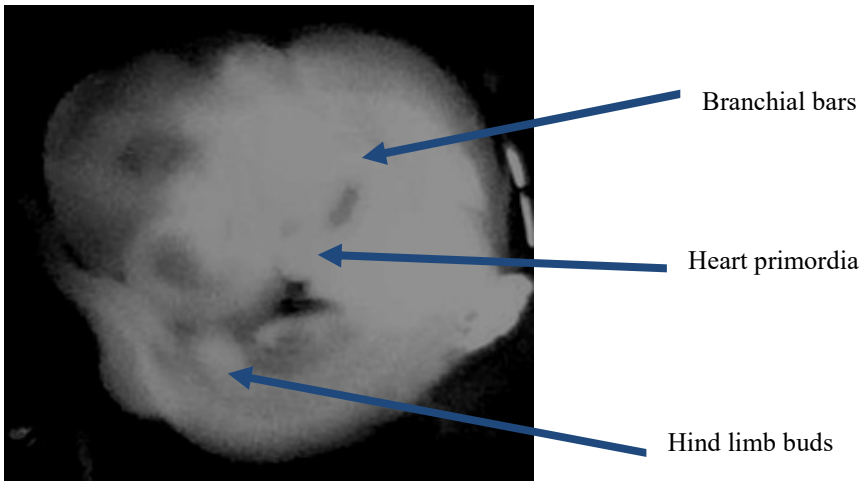


**Figure 3: Day-12 embryo outside the yolk sac showing hind limb buds and heart primordial and branchial bars**

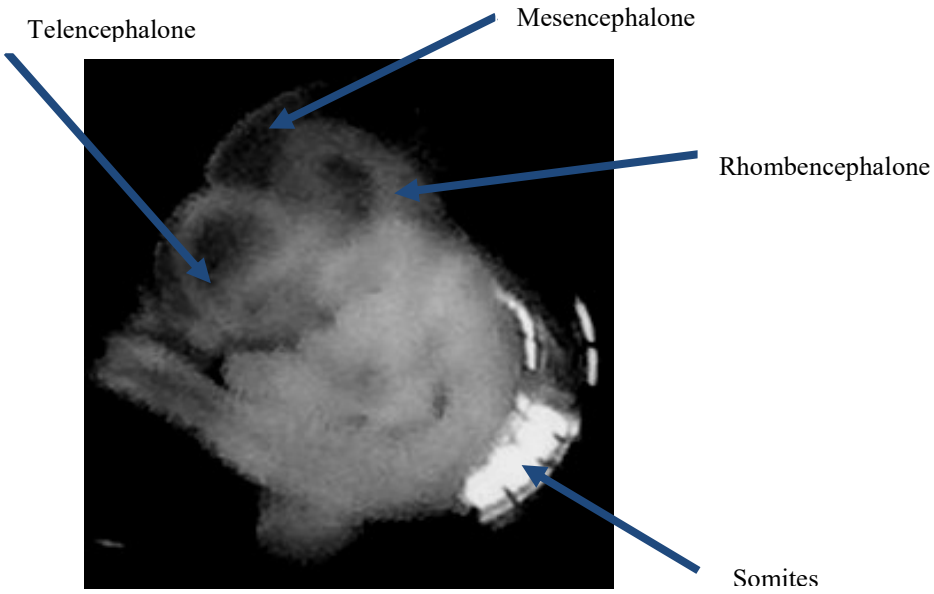


**Figure 4: Day-12 rat embryo showing somites, rhombencephalon, mesencephalone and telencephalone**

## 5.2 DAY-20 EXPERIMENT

### 5.2.1 PREGNANCY OUTCOME

Maternal weight gain in the treatment group was significantly lower ($p<0.05$) compared to the pair-fed control and Ad libitum control groups. Significant differences in weight gain were observed at the doses of 500 mg/kg and 750 mg/kg groups compared to the pair-fed control group and the Ad libitum control group ($p<0.05$).

The number of litter size was significantly decreased ($p<0.05$) in Khat 750mg/kg group compared to that of the pair-fed control and Ad libitum control groups. Decrease in litter size was also observed at the doses of 500 mg/kg but was not statistically significant.

There was also a significance difference in the number of implantation sites at the dose of 750mg/kg compared to both the pair-fed control and the unrestricted Ad libitum control groups. The same is true for the number of live foetuses per dam, which showed significant decrease in the Khat 750 mg/kg dose group ($p<0.05$) (Table 7).

### Table 7: Pregnancy outcomes of day-20 Khat experiment

| | Group I | Group II | Group III | Group IV | Group V |
|---|---|---|---|---|---|
| Maternal weight gain(g)<br>a. Day 6-12 | | | | | |
| b.Day 6-20 | 16.3±-0.3 | 12.4±1.3 | 8.8±0.1* | 9.9±2.1** | 16.5±-2.3 |
| | 63.8±1.5 | 55.26±1.3 | 42.2±11.4** | 41.34±1.6** | 61±-3.3 |
| Litter size | 9.8 | 9.8 | 9.2 | 6.6** | 9.8 |
| Implantation sites | 10±0.7 | 10±1 | 9.8±0.4 | 7.8±0.4** | 10±1.2 |
| Number of live fetus/litter | 9.8±0.8 | 9.6±1.1 | 9.2±0.8 | 6.2±0.8** | 9.8±1.1 |
| Number of resorbtions/litter | 0.2±0.4 | 0.4±0.5 | 0.6±0.8 | 1.2±1.3 | 0.2±0.4 |
| Number of dead fetus/litter | 0 | 0 | 0 | 0.4±0.5 | 0 |

Results are summarized as mean ± SD; *: Significantly different ($p < 0.01$) from Ad *libitum* control group and **significantly different ($p<0.05$) from both Ad libitum control group and pair-fed control group.

The frequencies of fetal resorbtions and dead foetuses per dam were higher at the dose of khat 750 mg/kg compared to the pair-fed control and Ad libitum control groups; however the difference was not statistically significant.

### 5.2.2 FETAL GROWTH

The growth of foetuses at term was affected significantly ($p<0.05$) in all of the treatment groups compared to the pair-fed control and the unrestricted Ad libitum control group. Both CRL and fetal weight were significantly reduced ($p<0.05$) even in the low dose, k250 mg/kg group (Table 8).

**Table 8: Growth of rat foetus following treatment of Khat**

| Treatment group | Fetal weight (gm) | Crown rump length (cm) |
|---|---|---|
| Group I, control pair fed | 4.9±0.2 | 3.6±0.2 |
| Group II, k250 mg/kg | 4.8±0.3 | 3.7±0.2 |
| Group III, k500 mg/kg | 4.7±0.3* | 3.5±0.1 |
| Group IV, k750 mg/kg | 3.9±0.3* | 3±0.2 |
| Group V, Ad libitum | 5±0.1 | 3.8±0.2 |

Results are summarized as mean ± SDM; *: Significantly different ($p < 0.01$) from the ad libitum control and pair-fed control groups, Khat 250 mg/kg, 500 mg/kg and 750 mg/kg groups (ANOVA).

Gross external developmental anomalies were not observed on craniofacial development, development of the limbs, vertebral column (neural tube defect), tail development (missing tail) and development of external genitalia.

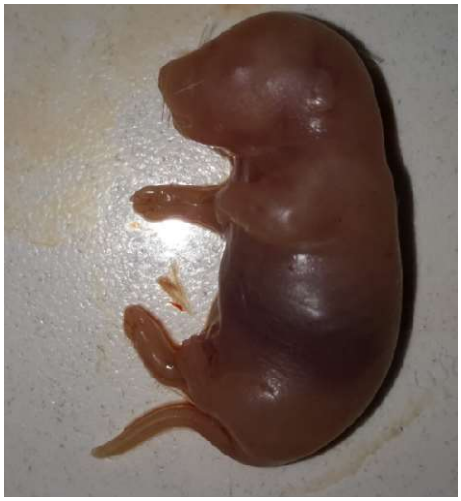

**Figure 5: Near-term rat fetus**

### 5.2.3 HISTOPATHOLOGICAL ANALYSIS OF PLACENTA

Gross placental examination in the treatment group showed neither significant reduction in weight nor gross anomalies as compared to the control group. However, light microscopic examination in the placentas of treatment group showed structural changes and differences as compared with the control groups. The results revealed dose-dependent pathological changes in

the placenta. These structural changes are found in deciduas basalis site, trophoblastic zone and labyrinth zone.

Sections which were obtained from treatment groups showed multiple lesions that include decidual hypoplasia and necrosis, cytolysis, apoptosis and vacular degeneration. In the labyrinth the trophoblasts showed necrosis and disintegration of the barrier that separates the maternal blood from embryonic capillaries, resulting in admixing of maternal and fetal blood. Furthermore, vascular congestion and extensive areas of hemorrhage were observed in labyrinth.

**Table 9: Comparision of histopathological effects of Khat in rat placenta; decidual necrosis**

| | % of decidual necrosis | |
|---|---|---|
| | present | Absent |
| Control | 0 | 100 |
| K250 mg/kg | 50 | 50 |
| K500 mg/kg | 25 | 75 |
| K750 mg/kg | 50 | 50 |
| Ad libitum | 0 | 100 |

**Table 10: Comparision of histopathological effects of Khat in rat placenta; cytolysis**

| | % of cytolysis | |
|---|---|---|
| | present | absent |
| Control | 0 | 100 |
| K250 mg/kg | 25 | 75 |
| K500 mg/kg | 100* | 0 |
| K750 mg/kg | 50 | 50 |
| Ad libitum | 0 | 100 |

*; significance difference seen from the control pair fed and Ad libitum group ($p<0.05$)

**Table 11: Comparision of histopathological effects of Khat in rat placenta; necrosis**

| | % of necrosis | |
|---|---|---|
| | present | Absent |
| Control | 0 | 100 |
| K250 mg/kg | 0 | 100 |
| K500 mg/kg | 0 | 100 |
| K750 mg/kg | 25 | 75 |
| Ad libitum | 0 | 100 |

**Table 12: Comparision of histopathological effects of Khat in rat placenta; apoptosis**

| | % of apoptosis | |
|---|---|---|
| | present | Absent |
| Control | 0 | 100 |
| K250 mg/kg | 0 | 100 |
| K500 mg/kg | 25 | 75 |
| K750 mg/kg | 50 | 50 |
| Ad libitum | 0 | 100 |

**Table 13: Comparision of histopathological effects of Khat in rat placenta; sever decidual hypoplasia and atrophy**

| | % of sever decidual hypoplasia and atrophy | |
|---|---|---|
| | present | Absent |
| Control | 0 | 100 |
| K250 mg/kg | 0 | 100 |
| K500 mg/kg | 0 | 100 |
| K750 mg/kg | 25 | 75 |
| Ad libitum | 0 | 100 |

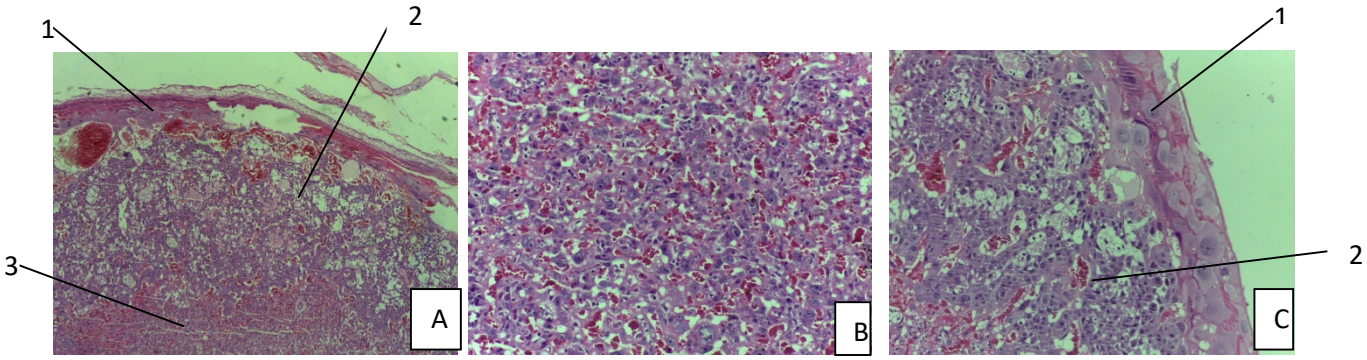


**Figure 6: Photomicrographs of placenta (H&E staining, x40) of control group(A&B ad libitum,C pair-fed) rats showing normal structural architecture; (A) decidual /1/, trophoblast zone /2/ and labyrinth zone/3/;(B) labyrinth zone of rat placenta; (C) decidual and trophoblastic zone of rat placenta**

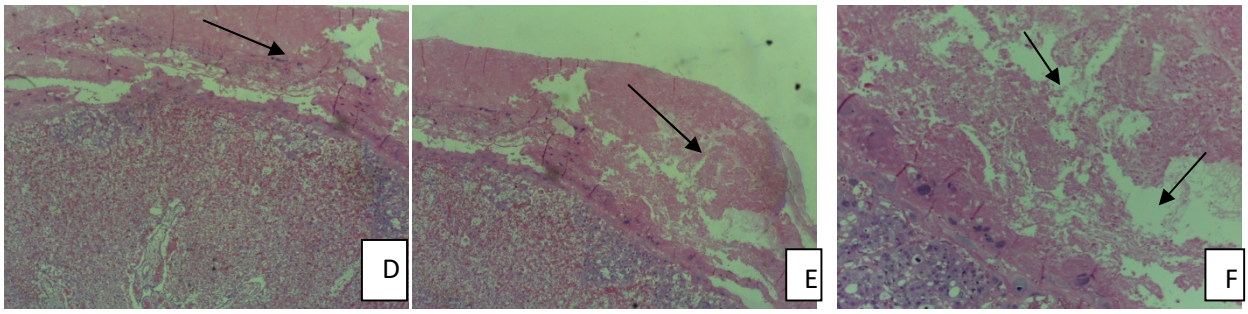


**Figure 7: Photomicrographs of placenta (H&E staining, x40) of Khat treated (500mg/kg)rats showing decidual hypertrophy D & E (arrow) and necrosis F (arrow)**

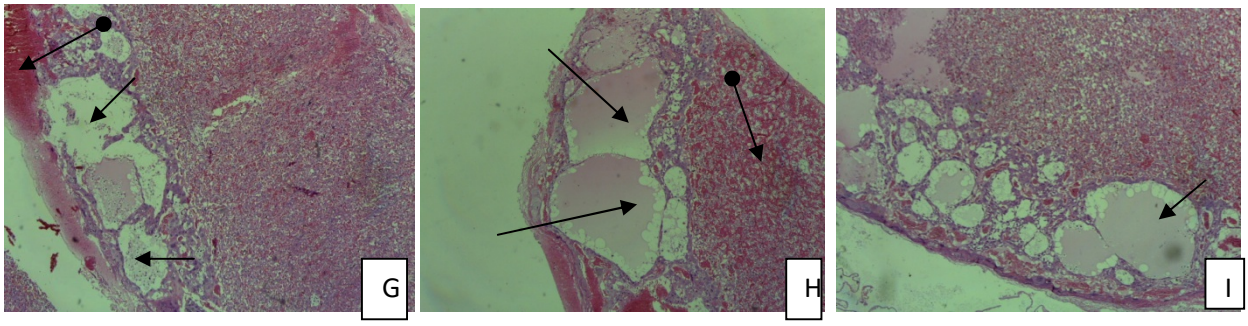


**Figure 8: Photomicrographs of placenta (H&E staining, x100) of Khat treated (750mg/kg)rats showing cystic changes on the deciduas (arrow→) and hemorrhage on the deciduas and labyrinth zones (arrow●→) G, H &I**

## 6. DISCUSSION

The findings of the present study showed that exposures to Khat extract during pregnancy have teratogenic effects: including effects on pregnancy outcomes and embryonic and fetal development in the rat. The observed teratogenic effects of Khat were mostly dose dependent. Embryonic development was assessed according to the morphological scoring system of Brown and Fabro (1981) to investigate the effects of Khat on development of the embryos and foetuses. Results of the present study showed a statistically significant delay in the development of the embryo at the doses of Khat 500 mg/kg and 750 mg/kg. Delayed development of heart, otic system, optic system and olfactory system primordia was observed. Development of primordia of fore-limb, hind-limb and brachial bars were also significantly delayed in the Khat treated groups. The development of musculoskeletal system, as indicated by the number of somites was affected in the treatment groups but yet not statistically significant. According to this study, the development of central nervous system including caudal neural tube, forebrain, mid brain and hind brain were not affected at all. Also development of maxillary process and the morphological score of somites were not significantly affected in all doses of Khat treatment.

Pregnancy outcome is affected in Khat treated pregnant rats. Maternal weight gain was lower in the treatment groups of k500 mg/kg and k750 mg/kg compared to the Ad libitum control group. This finding was supported by other animal and human studies which demonstrated that Khat exposure during pregnancy resulted in decreased maternal weight gain (9,39). Early studies tried to relate the decreased maternal weight gain with the anorexic effects of Khat but in the present study the reduced weight gain was observed throughout the gestational period, since treatment with Khat extract was only up to the $12^{th}$ day of gestation. In relation to this, maternal weight gain was also reduced in Khat treated pregnant rats compared to control rats despite similar intake of foods. Therefore the observed significantly reduced weight gain in the present study could be attributed to Khat treatment.

Prenatal growth retardation was observed in near term fetuses of Khat treated rats in excess of those in the pair-fed control group and Ad libitum control group. A significant reduction in fetal weight and crown-rump length was observed in all doses of khat treated pregnant rats. These adverse effects of Khat were also related to increased frequencies of fetal resorption per dam and number of dead foetuses at the doses of 750mg/kg, but not statistically significant compared with

the pair-fed control and Ad libitum control groups. This result is in line with few human and animal studies on teratogenic effects of Khat. In another study, it was reported that there was a highly significant difference ($p<0.05$) in birth- weight in newborns of non-users and users of Khat (28). Other animal studies involving late pregnant guinea pig have shown that Khat feeding of the mother during gestation significantly reduced the mean birth weight of the offspring (29).

The present study has also revealed a dose dependent decrease in number of foetuses per dam. The decrease was statistically significant at 750 mg/kg dose group. However developmental anomalies and gross external organ malformations were not observed in all foetuses of animals treated with Khat at all dose.

The weight of rat placenta, at term, is approximately 1/10th of the fetal body weight. Placental growth precedes fetal development (40). Proceeding outward from the fetus, the layers include the amnion, the yolk sac, Reichert's membrane, the placental labyrinth, the basal zone (trophospongium), decidua basalis, and metrial gland (Figure 8, A). Normal placental development and vascularisation depend on a tightly orchestrated coordination between the maternal decidua and trophoblast cells. They depend on adequate transformation of the uteroplacental vasculature by trophoblast cells following their proliferation, migration, and invasion into the maternal deciduas. During pregnancy, the depth of invasion by placental trophoblast cells into the uterine wall is critical and finely controlled. Poor invasion of maternal vessels has been correlated with placental pathologies such as fetal growth restriction, whereas an excessive trophoblast invasion is associated with choriocarcinoma (41). The placenta is a highly susceptible target organ for drug or chemical induced adverse effects, and many placenta toxic agents have been reported. However, histopathological examination of the placenta is not generally performed, and the placental toxicity index is only the placental weight change in rat reproductive toxicity studies. The detailed histopathological approaches to investigation of the pathogenesis of placental toxicity are considered to provide an important tool for understanding the mechanism of teratogenicity and developmental toxicity with embryo lethality, and could benefit reproductive toxicity studies.

In the current study study, exposure to Khat during pregnancy caused a disorganization of the vascular tree in the labyrinth layer (the major site of nutrient and gas exchange), with less branching in the treatment group at highest doses that is at 500mg/kg and 750mg/kg (Figure 9 D

and Figure 9, G). Since this region is the major site where nutrient and gas exchange takes place, this finding supports the retardation of fetal growth seen in Khat treated rat foetuses at highest doses and also intra uterine fetal death (IUFD) was observed in 500 mg/kg and 750 mg/kg khat treated groups.

Decidua basalis, thin layer at the base of the placenta is an important site for maternal angiogenesis. The decidua basalis includes newly developed blood vessels, which play essential roles in the development of vascularized decidual-placental interface. The decidua can produce a wide range of hormones, cytokines, growth factors and immunomodulatory molecules involved in the recruitment of the limited but specific immune cell populations and growth of the placenta (6). Findings of the current study show moderate to sever decidual hypertrophy, atrophy, necrosis and cystic formation and cytolysis (Figure 8 and Figure 9). Findings of the current study can be explained according to the possible causes of placental hypertrophy which is induced as a compensatory reaction to intra uterine growth retardation (IUGR) under the conditions of a slightly unfavorable maternal environment, poor capacity of the uteroplacental unit for transferring glucose to fetuses, drug and chemical induced compensatory placental hypertrophy, decreased number of corpora luteum, implantation sites and fetuses and hormone imbalance reaction, such as estrogen deficiency (6).

## 7. CONCLUSION

From the presented data it can be concluded that administration of crude extract of Khat at a higher dose to pregnant albino Wistar rats may not be safe and its toxic effects were evidenced by significant delay in embryonic and fetal developments and decreased maternal gestational weight gain. The study has also demonstrated that Khat has harmful effects on several histopathological features of rat placenta resulting in cytolysis hypertrophy and necrosis.. Eventhough further detailed studies may be required to comfortably determine the teratogenic effects of Khat, it is possible to suggest that Khat consumption during pregnancy should be discouraged.

## 8. RECOMENDATIONS

- Further studies are recommended during early periods of gestation following treatment throughout gestational period
- Detailed histopathological analysis on the different cells of decidual, trophoblastic and labyrinth zones are necessary for further knowledge on the effect of Khat on the placenta.
- Effects of Khat on post natal development at least 2 weeks after birth is recommended.
- In- vitro studies should be conducted to understand more on the mechanisms of action of Khat during pregnancy.
- Higher doses of Khat should be investigated in order to determine any effects on organs such as the brain.
- A further study by increasing number of animals per group is recommended.

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# 10. ANNEX

## 9.1. MORPHOLOGICAL SCORING SYSTEM FOR RAT EMBRYO

| | | 0 | 1 | 2 | 3 | 4 | 5 | SCORE |
|---|---|---|---|---|---|---|---|---|
| A | YOLK SAC CIRCULATORY SYSTEM | no visible, or scattered, blood islands | Corona of blood islands w or w/o anastamoses | Vitelline vessels with few yolk sac vessels | Full yolk sac plexus of vessels | Yolk stalk obliterated; vitelline artery & vein well separated | | |
| B | ALLANTOIS | Allantois free in exocoelome | Allantois fused with chorion | Umbilical vessels | Separate aortic origins of umbilical and vitelline vessels | | | |
| C | FLEXION | Ventrally convex | Turning | Dorsally convex | Dorsally convex with spiral torsion | | | |
| D | HEART | Endocardial rudiment not visible, or visible but not beating | Beating 's' shaped cardiac tube | Convoluted cardiac tube | Bulbus cordis, atrium commune and ventriculus communis | Dividing atrium commune | | |
| E | CAUDAL NEURAL TUBE | Neural plate or neural folds | Closing, but unfused neural folds (groove) | Neural folds fused at level of somites 4/5 | Posterior neuropore formed, but open | Posterior neuropore closed | | |
| F | HIND BRAIN | Neural plate | Rhombomeres A and B | Anterior neuropore formed but open | Anterior neuropore closed, rhombencephalon formed | Pronounced pontine flexure with transparent roof of 4th ventricle | | |
| G | MID BRAIN | Neural plate | Mesencephalic brain folds | Closing or fusing mesencephalic folds | Completely fused mesencephalon | Visible division between mesencephalon & diencephalon | | |
| H | FORE BRAIN | Neural plate or no visible prosencephalon | Prosencephalic brain folds | Completely fused prosencephalon | Visible telencephalic evaginations | Well elevated telencephalic hemispheres | | |
| J | OTIC SYSTEM | No sign of otic development | Flattened or indented otic primordium | Otic pit | Otocyst | Otocyst with dorsal recess | Otocyst with endolymphatic duct | |
| K | OPTIC SYSTEM | No sign of optic development | Sulcus opticus | Elongated optic primordium | Primary optic vesicle with open optic stalk | Indented lens plate | Lens pocket or lens vesicle | |
| L | OLFACTORY SYSTEM | No sign of olfactory development | Olfactory plate | Olfactory plate with rim | Distinct olfactory ridges | Lateral nasal process and medial rim | | |
| M | BRANCHIAL BARS | None visible | I visible | I and II visible | I, II and III visible | II overgrowing and obscuring III | | |
| N | MAXILLARY PROCESS | No sign of maxillary development | Maxillary process demarkated, visible cleft anterior to bar I | Maxillary process fused with nasal process | | | | |
| P | MANDIBULAR PROCESS | No sign of mandibular development from bar I | First brancial bars fused and forming mandibular process | | | | | |
| Q | FORE LIMB | No sign of fore limb development | Distinct evagination of wolfian crest at level of somites 9-13 | Fore limb bud | Paddle shaped fore limb bud | Distinct Apical ridge on fore limb bud | | |
| R | HIND LIMB | No sign of hind limb development | Distinct evagination of wolfian crest at level of somites 26-30 | Hind limb bud | Paddle shaped hind limb bud | | | |
| S | SOMITES | 0 - 6 | 7 - 13 | 14 - 20 | 21 - 27 | 28 - 34 | 35 - 41 | |

Fig. 1. Morphological Scoring System. To use the score sheet, each of the 17 features (A–S) is examined and scored in a fresh sample, and the total of all scores is the morphological score. Flexion should be evaluated before opening the yolk sac, and the heart is easier to examine while still containing blood. The text contains further explanation of some of the stages and Figures 6–8 illustrate many of the features and their appropriate scores. A larger copy of the score sheet for routine laboratory use is available from the authors.

## 9.2. PREPARATION OF WORKING CHEMICALS OR SOLUTIONS

**1. 10% Neutral Buffered formalin**

40%formalin…….100ml

Distilled water……...……900ml

Sodium dihydrogen phosphate, monohydrate…….....4gm

Disodium hydrogen phosphate anhydrous..…….6.5gm

2. **Harris Hematoxylin (H)**

Hematoxylin crystals……. 2.5gm

Potassium alum…… 50gm

Glacial acetic acid……...20ml

Absolute alcohol……...25ml

Distilled water……..500ml

3. **1% Acid alcohol**

70% Alcohol……..…… 500ml

Hydrochloric acid, concentrated…… 0.5ml

4. **Preparation of bluing solution or Sodium bicarbonate**

Sodium bicarbonate……..1gm

Distilled water……100ml

5. **1 % Aqueous Eosin (E)**

Distilled water…… 100ml

Eosin, both water alcohols soluble……. 1gm

Glacial acetic acid……. 0.5ml

## 9.2. TISSUE FIXATION PROTOCOLS

1. 10 % buffered formalin …….overnight
2. Running tape water…….overnight
3. 70% alcohol……...2hrs
4. 90% alcohols …….2hrs
5. Absolute alcohol: I ……1½ hrs
6. Absolute alcohol: II ……1½ hrs
7. Absolute alcohol: III……1½ hrs
8. Absolute alcohol: IV……..……..overnight
9. Xylene: I……1½ hrs
10. Xylene: II……..1½ hrs
11. Wax: I ……...1½ hrs
12. Wax: II ……..2½ hrs
13. Wax: III ……...overnight

## 9.3. TISSUE STAINING PROTOCOL (H & E STAINING PROCEDURE)

1. Xylene-I……..10min
2. Xylene-II…… 10 min
3. Absolute alcohol- I…… 5 min
4. Absolute alcohol- II…….. 5 min
5. 95 % alcohol…….. 3 min
6. 70 % alcohol…….. 3 min
7. 50% alcohol…… 3 min
8. Distilled water……. 5min
9. Harris Hamatoxylin…….. 10min
10. Running tape water…….5 min
11. 1% acid alcohol…….…. 30 sec
12. Running tape water…… 5 min
13. Sodium carbonate solution…….. 30 sec
14. Running tape water…….5 min
15. Eosin…… 1-2min
16. Running tape water……5 min
17. 70 % alcohol……3 min
18. 95 % alcohol…….. 3 min
19. Absolute alcohol- I……1½ hrs
20. Absolute alcohol- II……..1½ hrs
21. Xylene – I…… 5 min
22. Xylene-II……. 5 min