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Full Length Research Paper

Evaluation of the reversibility and possible mechanisms of antifertility of *Catha edulis F.* (khat) extract following subacute administration in rodents

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The study of antifertility mechanisms of khat can be used to counteract the possible toxicities during pregnancy as abuse of khat is common in all segments of female population. The present study was initiated to assess the reversibility of antifertility effect and to evaluate the possible mechanisms of antifertility of the chloroform diethyl ether extract of *Catha edulis.* K300 exhibited significant decrease in the mean number of litters and mean birth weight of live litters compared to K100 and control group (CON) ($p<0.01$ in both cases). The anti-implantation and antifertility effects were reversible after a 21 day washout period. Post-implantation losses for K200 and K400 were 27% ($p<0.01$) and 30% ($p<0.01$), respectively. Maternal mortality in pregnant mice and morphological changes like massive leukocyte infiltration, necrosis and haemorrhage in rat uterus were observed in K400. No morphological changes were shown in ovary at all doses of the extract. Khat has antifertility effect with possibility of complete sterility in higher dose treated groups. The possible mechanisms include decreased serum estradiol concentration and massive leukocyte infiltration in uterus stroma cells. Khat use should be discouraged in female population especially during pregnancy.

Key words: *Catha edulis*, anti-implantation, reversibility, mechanism, rodents.

INTRODUCTION

Catha edulis Forsk (Celastraceae), commonly called 'khat', is an evergreen perennial plant and mostly refers to the leaves and young shoots (Figure 1). The plant is widely distributed throughout the horn of Africa and Arabian Peninsula and chewed fresh daily by millions of people as a recreational drug. Once harvested, khat is a perishable commodity. Hence, its distribution was limited to these areas (Lemessa, 2001). Owing to the possibility of air transport, however, khat has now made its appearance in Europe and North America (Nezar et al., 2005).

Khat buds and leaves contain alkaloids; cathinone, cathine, and norephedrine, and are chewed by high proportion of the adult population for the pleasant mild stimulant effect (Hassan et al., 2007). Khat also has flavonoids, tannins, oils, vitamins, amino acids and minerals. Moreover, khat consists of terpenoids, alkane hydrocarbons, β-sitosterol (Al-Motarreb et al., 2002; Chappell and Lee, 2010). Cathinone is transformed mainly to cathine in khat leaves and to norephedrine by human metabolism (Rizk et al., 1989).

Recent studies demonstrated that cathinone persists in dried khat for a time frame of several years and simple drying techniques might be used as an effective means to

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Figure 1. Photograph of *Catha edulis* leaves purchased from 'Belechie' market.

preserve seized khat evidence for long term storage (Chappell and Lee, 2010).

Even though khat use is a common practice in men, nowadays it is also common in women, even during pregnancy. Fresh khat is thought to increase sexual motivation or libido which is more frequently observed in females than males (Aziz et al., 2009).

Methanolic khat extract showed dose dependent anti-implantation, aborifacient and anti-estrogenic property (Tariq et al., 1987). Other studies indicated that khat possesses both dose dependent embryotoxic as well as teratogenic properties (Islam et al., 1994). Reports also show that 41.6% of mice did not give any litters upon administration of khat extract (Bedada and Engidawork, 2010). Khat alkaloid (+) norpseudoephedrine causes vasoconstriction in the utero-placental vascular bed which may in turn impair fetal growth through reduction of placental blood flow, which is one of the proposed mechanisms for anti-fertility effect of khat (Jansson et al., 1988).

Previous studies showed that the methanolic extract of khat extract had antifertility effect in experimental animals. However, no study had been done whether the antifertility effect of khat extract is reversible or not. In addition to Khat alkaloid (+) norpseudoephedrine induced vasoconstriction mechanism, other possible mechanisms for antifertility and anti-implantation effect of khat extract need to be understood. This work also tries to address the extent of anti-implantation and antifertility effect of khat extract using different animal models.

Intrauterine growth retardation, low fetal birth weight and infant mortality are some of the most important reproductive health problems affecting most developing countries. In addition to this, khat chewing during pregnancy is on the increase among women of reproductive age, and questions have been raised on the potential effects of khat on maternal fertility and fetal development. Therefore, this study tries to address anti-implantation and antifertility effect, the possible mechanisms and reversibility of these effects of khat extract.

MATERIALS AND METHODS

Chemicals and solvents

Diethyl ether and chloroform were obtained from Fischer scientific, United Kingdom through a locally available distributor. Buffered formalin and phosphate buffer saline (PBS) were prepared in the laboratory.

Experimental animals

Wistar female rats aged 3 to 4 months and Swiss albino mice of 2 to 3 months were maintained at laboratory conditions of controlled environment provided with a 12:12 h light and dark cycle at approximate temperature of 25°C and relative humidity of 50%. Each animal was fed with laboratory diet and water *ad libitum*. Animals with regular estrous cycle were used throughout the experimental period (Marcondes et al., 2002). The animals were cared and treated humanely according to the principles of laboratory animal care (ILAR, 1996) and the protocol was approved by the School of Pharmacy, Addis Ababa University ethics committee.

Plant collection and extraction

Bundles of fresh *C. edulis* shoots and small branches (about 3000 g) were purchased in late December 2010 at local market in 'Beletche', one of the common natural habitats, 290 km South of Addis Ababa, Ethiopia. The fresh bundles were packed in plastic bags and transported in an ice box to the laboratory. The fresh leaves were immediately kept at a deep freezer. The plant was identified by a taxonomist and a voucher specimen was deposited in the National Herbarium, Addis Ababa University (specimen number AS 001). The chopped leaves and buds (1800 g) of *C. edulis* were prepared and extracted as described elsewhere (Banjaw and Schmidt, 2004). The yield was 1.83% and the solid residue was kept in an amber bottle covered with aluminium foil and kept refrigerated for the whole experiment period.

Grouping and dosing of animals

The experimental animals were randomly assigned to four groups. Group I served as control (CON) group and received vehicle (2% V/V Tween 80 in water). Group II were treated with 100 mg/kg khat extract (K100); Group III with 200 mg/kg khat extract (K200) and Group IV with 400 mg/kg khat extract (K400). The doses used were selected based on previous studies (Bedada and Engidawork, 2010; Admassie and Engidawork, 2011).

Reversibility study

Female mice (7 to 8) at stage of proestrus and estrus were left overnight with mature male mice of proven fertility at a ratio of 2:1. The vaginal smear was examined for presence of spermatozoa and/or vaginal plug in the next morning. The day on which the spermatozoa were found in the smear was considered as day 1 of pregnancy. The pregnant mice were then randomly divided into four groups. However, mice in group IV which received K400 showed signs of toxicity and died. So the dose was reduced to 300 mg/kg (K300). The mice received treatments throughout the term and the number and weight of litters delivered (if any) and the gestation

Table 1. Reproductive effects of *Catha edulis* extract administration throughout gestation period and after a 21 day washout period.

Dose	Fertility index (%)		Fetal mortality index (%)		Mean number of live litters		Mean birth weight of litters	
	Before	After	Before	After	Before	After	Before	After
CON	100.0	100.0	0.00	0.00	8.50 ± 0.72	8.88 ± 1.06	1.49 ± 0.05	1.53 ± 0.04
K100	87.5	100.0	0.00	0.00	7.00 ± 1.55	8.88 ± 0.85	1.47 ± 0.92	1.52 ± 0.05
K200	42.85	100.0	11.11	0.00	3.86 ± 1.55^{a}*	8.00 ± 0.85	1.32 ± 0.01	1.60 ± 0.03
K300	25.0	75.0	27.27	0.00	$1.00 \pm 0.7^{a,b}$**	7.37 ± 1.71	$1.10 \pm 0.14^{a,b}$**	1.51 ± 0.08

Data are mean ± SEM, *p<0.05, **p<0.01; n=7-8 mice per group, a as compared to CON, b as compared to K100; CON: Control; K100: 100 mg/kg; K200: 200 mg/kg; K300: 300 mg/kg.

period of dams were recorded. The reversibility of antifertility effect was studied based on previous studies with slight modification (Salhab et al., 1997). After 21 days of extract withdrawal, animals were allowed to mate with male mice in the ratio of 4:1. Pregnant mouse was taken from the group and kept in a separate cage to prevent cannibalism during delivery. The number of litters and birth weight were determined at the end of the term.

Anti-implantation and antifertility study

The anti-implantation and antifertility activities of *C. edulis* in 6 rats per group were determined as described elsewhere with slight modification (Costa-Silva et al., 2007). Pregnancy date was determined as described earlier in reversibility study. The animals were then treated with vehicle or extract for two weeks: pre-implantation period (1st to 6th day) and organogenesis period (7 to 14th day). On the 20th day of pregnancy, the rats were euthanized by cervical dislocation, laparatomized and the uterine horns removed. At day of laparatomy, the number of implants, resorption, live and dead fetuses was recorded. The ovaries were fixed in 10% phosphate-buffered formalin immediately after weighing, then processed for routine hematoxylin and eosin (H&E) staining and the numbers of corpora lutea were counted under light microscope (Armanda-Dias et al., 2001).

Hormonal levels and lipid profile determination

Cycling non-pregnant Wistar rats of 5 per group were treated with either vehicle or extract for 15 days. On day 16th, the animals were anesthetised using diethyl ether and blood was drawn from the orbital sinus, and the ovaries and uteri were dissected out. The blood was then allowed to coagulate for an hour. The serum was separated by centrifuging the coagulated blood at 3000 rpm for 20 min and stored at -20°C. Progesterone, estrogen and cortisol levels were analysed by electro chemiluminescence immunoassay (ECLIA) method using Elecsys 2010 Immunoassay kit (Roche diagnostics, Indianapolis, USA). One ovary from each animal was homogenized with phosphate buffered saline (PBS) (pH 7.4). The serum and homogenized ovary tissue were used for measurement of cholesterol, high density lipoprotein (HDL) (using HDL spin reactor), low density lipoprotein (LDL) and triglyceride contents by enzymatic colorimetric methods.

Histopathological studies of ovary and uterus

The remaining ovary and contralateral uterus were immediately fixed in 10% phosphate buffered formalin, and then routine H&E staining was done (Shivalingappa et al., 2002). The morphologic features including the presence of inflammation, edema, hemorrhage and necrosis were assessed.

Statistical analysis

All data are expressed as mean ± standard error of the mean. Mean from different groups were compared using one way analysis of variance (ANOVA) followed by post hoc tukey test. paired T-test was also used in some parameters. The data were analysed using SPSS version 19.01 and significance was set at $p< 0.05$.

RESULTS

Reversibility study

Administration of K400 resulted in death of 85.7% of mice within a week after showing signs of toxicity like decreased mobility, decreased food intake, seizure, erected hair, and decrease in body weight. Because of these adverse reactions, the dose was decreased to 300 mg/kg.

Although dams exposed to khat extract during pregnancy exhibited decrease in fertility index and increase in mortality of litters (Table 1); dams which gave birth were within the usual expected period of gestation (20 to 22 days). The fertility index of K100 and K200 after washout period was 100% which was comparable to CON. About 25% of K300 were unable to give birth despite efforts of long period of stay (around 2 months) by keeping them with males of proven fertility. The K100, K200 and K300 showed an increase in fertility index by 12.5, 57.2 and 66.7%, respectively, after 21 days of washout period. In case of fetal mortality index, no dead litter was found both in CON and K100. However, K200 and K300 showed 11 and 27% fetal mortality, respectively, which was decreased by 100% after washout period.

Female mice in khat extract treated groups delivered decreased number of litters per dam as compared to CON. K300 decreased mean number of litters compared to K100 and CON ($p<0.01$ in both cases). The K200 also decreased mean number of litters as compared to CON ($p<0.05$). Withdrawal of treatment for 21 days increased mean number of live litters in K100 ($p<0.05$), K200 ($p<0.01$) and K300 ($p<0.01$) by 21.1, 51.8 and 86.4%, respectively compared to treatment values. No apparent difference was, however, noted between controls in pre- and post-withdrawal values (Figure 2A).

K300 decreased mean birth weight of live litters as

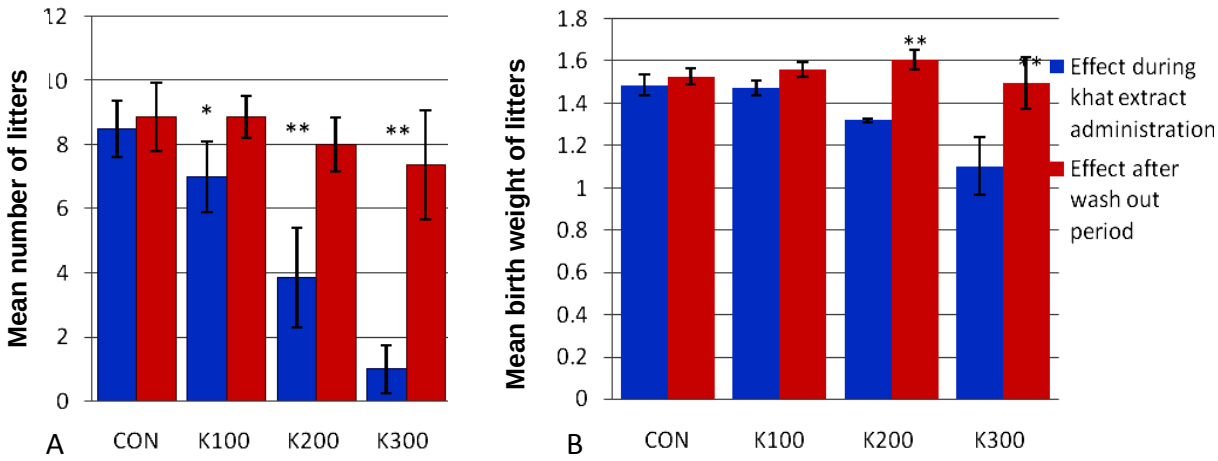

Figure 2. Comparison of mean number of litters (A) and mean birth weight of litters (B) during khat extract administration and after washout period. Data are mean ± SEM, n=7-8 mice per group, * p<0.05, **p<0.01.

Table 2. Reproductive parameters of female rats treated with *Catha edulis* crude extract from 1st to 14th day (pre-implantation and organogenesis period) of pregnancy.

Reproductive parameter	CON	Khat extract	
		K200	K400
Number of corpora lutea	9.67 ± 0.80	9.83 ± 0.60	12.50 ± 1.80
Number of implantation sites	9.50 ± 0.76	8.33 ± 0.42	6.00 ± 1.97
Number of live fetuses	9.33 ± 0.88	6.17 ± 0.75	4.17 ± 1.35[a]**
Number of resorptions	0.17 ± 0.17	1.67 ± 0.33[a]**	1.75 ± 0.25[a]**
Implantation index	98.48 ± 1.52	85.89 ± 5.46	61.25 ± 19.45
Resorption index	2.38 ± 2.38	20.31 ± 4.03[a]**	19.23 ± 1.90 [a]**
Fetal wastage index	2.38 ± 2.38	24.24 ± 6.00[a]**	30.12 ± 3.08[a]**
Ovary per body weight/100g	0.091 ± 0.011	0.057 ± 0.002[a]**	0.057 ± 0.003[a]**
Mean fetal weight (g)	5.27 ± 0.19	5.21 ± 0.26	4.10 ± 0.34[a,b]*
Pre-implantation loss (%)	1.52 ± 1.52	15.44 ± 4.90	38.75 ± 19.45
Post-implantation loss (%)	2.38 ± 2.38	26.63 ± 6.77[a]**	30.12 ± 3.08[a]**
Anti-implantation loss (%)	3.89 ± 2.55	37.89 ± 5.23[a]*	57.31 ± 13.57[a]**

Data are mean ± SEM, n=6 rats per group, *p<0.05, **p<0.01; [a] as compared to CON, [b] as compared to K200; CON: Control; K200: khat extract 200

compared to CON and K100 (p<0.01 for both cases). Although K200 group displayed a decrease in mean birth weight of live litters compared to CON, it failed to reach statistical significance. Litters of extract treated groups regained the mean birth weight after a 21 day washout period. The increase in weight was significant with K200 (17.7%, p<0.01) and K300 (26.9%, p<0.01) groups. Although there was an increase in weight gain in CON and K100 groups by 2.8 and 2.9%, respectively, the difference was not statistically significant (Figure 2B).

Anti-implantation and antifertility study

During the treatment period with *C. edulis*, a death of 16.7% pregnant rat treated with K400 was registered and some clinical signs of maternal toxicity were observed before death. Only two doses of khat extract were used as K100 had no significant antifertility effect during reversibility study in mice. There was no difference in the number of corpora lutea and implantation sites between khat extract treated groups and CON. Likewise, no difference was noted in percentage of implantation in K200 and K400.

As shown in Table 2, K400 decreased the number of live fetuses per dam by 55.3% as compared to CON (p<0.01). Though K200 was able to reduce the number of live fetuses per dam by 33.9%, the difference failed to reach statistical significance. However, K200 and K400 increased fetal wastage by 24 and 30% and percentage

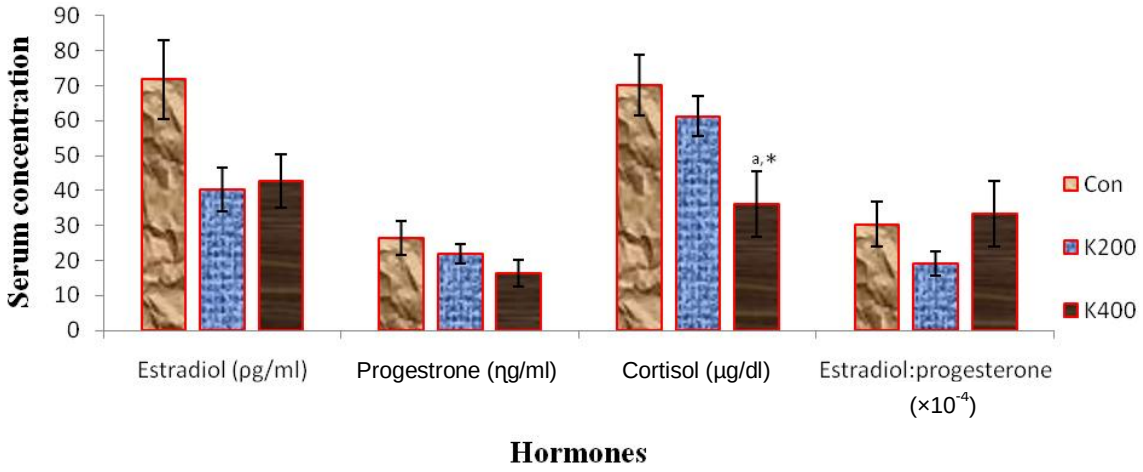

Figure 3. The effects of subacute administration of *Catha edulis* crude extract on serum concentration of hormones in non pregnant rats. Data are mean ± SEM, n=5 rats per group, *p<0.05, [a]as compared to control, CON: control; K200: 200 mg/kg; K400:400 mg/kg of khat extract.

Table 3. The effects of subacute administration of *Catha edulis* crude extract on lipid profile of non pregnant rats.

Parameter	CON	*Catha edulis*	
		K200	K400
Total cholesterol(mg/dl)	100.64 ± 8.34	88.48 ± 2.88	63.05 ± 2.84*a
Triglyceride(mg/dl)	271.36 ± 152.72	82.79 ± 5.48	70.87 ± 9.86
LDL (mg/dl)	8.27 ± 0.57	6.51 ± 0.76	5.38 ± 0.69*a
HDL (mg/dl)	37.20 ± 1.77	38.91 ± 8.68	47.20 ± 6.39
Ovary cholesterol (mg/dl)	22.60 ± 6.46	57.20 ± 13.65	47.03 ± 19.95
Ovary triglyceride (mg/dl)	529.82 ± 102.16	820.37 ± 140.18	710.75 ± 87.34
Ovary weight (mg) /100g	0.048 ± 0.0047	0.048 ± 0.0029	0.043 ± 0.0031
Uterus weight (mg) /100g	0.242 ± 0.052	0.158 ± 0.014	0.194 ± 0.039

Data are expressed mean± SEM; n= 5 rats per group; [a] against CON, *p<0.05, CON: Control; K200: 200 mg/kg; K400: 400 mg/kg.

of resorption by 20.3 and 19.2%, respectively, compared to CON (2.4%). The mean fetal weight was decreased in K400 (p<0.05) compared to K200 and CON. The percentage of pre-implantation loss in K200 and K400 were 15.4 and 38.75%, respectively. The percentage of post-implantation loss for K200 and K400 were 26.6 and 30.1%, respectively. Thus, K200 and K400 showed significant increase in post-implantation loss rate (p<0.05) than pre-implantation loss rate. K400 (p<0.01) and K200 (p<0.05) group had significant anti-implantation loss as compared to CON.

Hormonal and lipid profile

As shown in Figure 3, K200 and K400 decreased serum estradiol levels by 43.7 and 40.6%, respectively, the difference, however, did not reach statistical significance. Similarly, K200 and K400 tended to decrease serum progesterone levels, though once again the difference was not statistically significant. No detectable change was noted in serum cortisol with K200 though levels were decreased by 12.5%. Cortisol levels, however, significantly decreased (49%, p<0.05) with K400 compared to CON.

As shown in Table 3, no apparent difference was observed between CON and K200 in serum cholesterol. By contrast, K400 demonstrated a significant reduction (37%, p<0.05) in total serum cholesterol. Similarly, no detectable change was observed in serum LDL cholesterol between CON and K200, though K200 tended to decrease levels by 21%. K400, however, was capable of significantly reducing LDL levels (35%, p<0.05) compared to CON. Khat failed to increase serum HDL and decrease serum triglyceride levels significantly, although K400 tended to increase HDL by 21.2% and decrease triglyceride levels by 73.9%. Obviously, ovary cholesterol and triglyceride levels were increased by khat treatment.

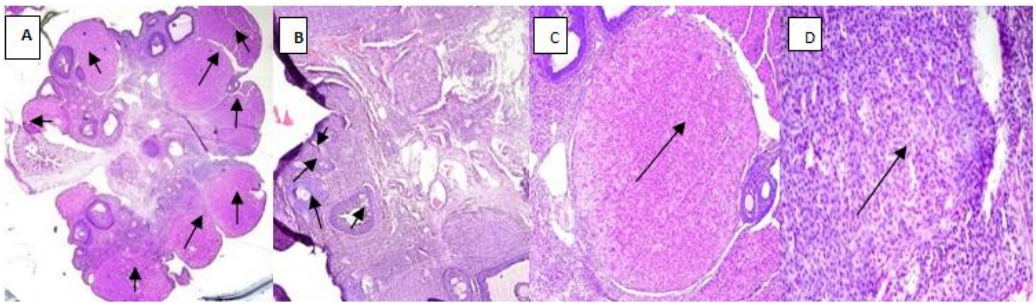

Figure 4. Photomicrograph of hematoxylin and eosin stained ovarian sections for control and *catha edulis* treated rats. CON showed normal ovary morphology with corpora lutea, 2.5× (A); K100, 10× showed ovary components with primary, secondary and tertiary follicles (B); normal structure of ovary with corpora lutea in K200 group, 10×(C) and normal structure of stromal cells in corpora lutea of K400 group, 40×(D).

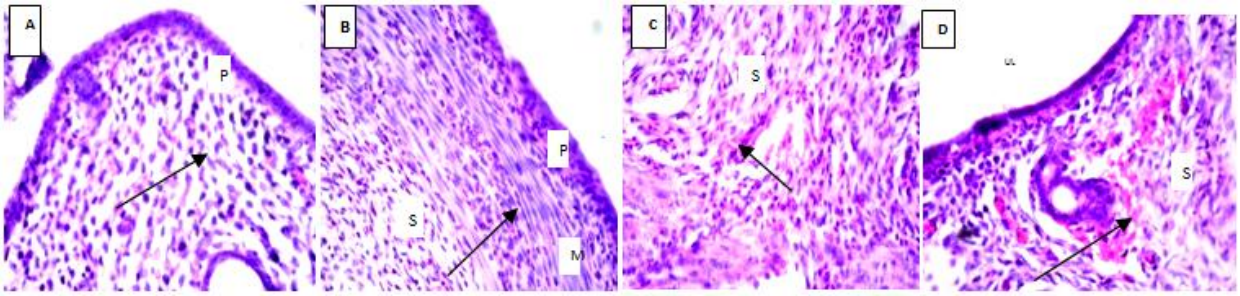

Figure 5. Photomicrograph of hematoxylin and eosin stained uterine sections for control and *Catha edulis* treated rats: CON showed normal stroma cells, 40× (A); K200 rats show evidence of inflammation that migrate towards myometrium, 40× (B); those treated with K400 show inflammatory infiltration, 40× (C) and haemorrhage, 40× (D). M-Myometrium; S-Endometrial Stroma; P-Perimetrium; UL-Uterine Lumen.

Morphometric analysis

The weights of ovary and uterus were decreased in both khat extract treated groups but failed to reach statistical significance. The histological sections of CON, K200 and K400 treated animals showed normal ovarian morphology and no morphological change was observed between the control and khat treated groups (Figure 4). However, extensive inflammation was observed in myometrium in K200 group. K400 also showed extensive inflammatory infiltrates within the endometrial stroma cells with edema, hemorrhage and necrosis (Figure 5).

DISCUSSION

The present study was designed to further evaluate the antifertility effects of *C. edulis*, which is popularly used for its euphoric effects. All the necessary precautions were taken to prevent degradation of cathinone throughout the extraction procedure. The reduction in fertility index, mean number of live litters and increment in fetal mortality index in cyclic rodents after subacute treatment with khat extract reinforces the notion that khat is endowed with antifertility activities in rodents. Methanolic extract of khat at a dose of 500 mg/kg is reported to have around 40% anti-implantation activity (Tariq et al., 1987), which is lower than the present finding (57%) at a dose of 400 mg/kg. The decrease in anti-implantation effect in the previous work might be due to the following reasons: (1) use of dried leaves instead of fresh leaves; (2) safe extraction procedure employed in the present study that used easily volatile solvents and lyophilizer, which enabled to maintain an increasing yield of the highly potent and easily degradable component, cathinone (Banjaw and Schmidt, 2004). It has been demonstrated that drying the green khat leaves with heat decreases the cathinone content by chemical reduction (Chappell and Lee, 2010).

The decrease in the number of live litters per dam at higher dose might be due to increase in the number of resorptions and dead fetuses as *in utero* treatment of khat extract has been reported to increase resorptions during pregnancy (Islam et al., 1994).

The observed decrease in mean birth weight of litters in rodents was also reported in humans (Al Harazi, 2009), and was a contributing risk factor for both fetal and litter mortality. When testing possible fetal toxicity of a specific

substance, it is necessary to establish if these effects are due to direct action on the fetus or an indirect action through the maternal toxicity that could secondarily interfere with the fetus. Clinical signs of maternal toxicity that were observed in rodents indicated that indirect action is also implicated in fetal toxicity. From the evidence that severe toxicity was observed in gavage administration of mice, and it is also possible to conclude that pregnant mice are more sensitive to gavage administration of *C. edulis* than non-pregnant rats like berberine (Jahnke et al., 2006). Fetal mortality might also be due to placental insufficiency that resulted from vasoconstriction caused by khat extract (Jansson et al., 1988). In addition, anorexia is a well known side effect of khat (Zelger and Carlini, 1980) and the possible role of anorexia for intrauterine growth retardation cannot be excluded.

The present finding is perhaps the first report on the reversibility of anti-fertility activity of khat in mice at lower doses (100 and 200 mg/kg) with possibility of sterility in large dose (400 mg/kg) treated mice. The observation that the absence of significant change in the number of litters between control and the post-extract groups and recovery of all induced antifertility effects after withdrawal for 3 weeks shows that the anti-fertility and anti-implantation effects were transient and reversible. The present observations are in agreement with previous studies on the reversible antifertility effect of *Ricinus communis* (castor beans) in female rabbits (Salhab et al., 1997).

The decreased implantation in the present investigation might be due to massive leukocyte infiltration in stroma cells that resulted in degeneration of the early embryos and post-implantation embryonic resorptions in the uteri. This massive leukocyte infiltration was observed in K200 and K400 group. Such infiltration was also reported in khat extract treated hepatic portal tract and the infiltration was reported to increase with dose (Al-Habori et al., 2002). The lack of antifertility effect in K100 could be explained by the pharmacokinetic characteristics of khat components. The main alkaloid, cathinone, has a rate of absorption similar to the rate of inactivation, which limits the cathinone blood levels attainable by gavage administration. This characteristic is important for absence of many adverse effects observed due to cathinone accumulation. However, with increasing dose treatment results in cathinone accumulation and thereby results in anti-implantation/anti-fertility effects (Cox and Rampes, 2003).

Any imbalance in levels of estrogen and progesterone is implicated in infertility. As a result, one possible mechanism of antifertility effect of khat may be hormonal imbalance, as demonstrated by a decrease in serum estrogen and alteration of the estrogen: progesterone ratio. The decreased serum estradiol along with massive leukocyte infiltration might affect the estrogen responsive epidermal growth factor receptor (EGFR) localization in the stroma cells. Overall uterine and vaginal organ growth, in response to estrogen, require EGFR signaling for DNA synthesis in the fibro-muscular stroma and a decrease in estradiol might results in degeneration of the early embryos and post-implantation embryonic resorptions in the uteri (Juneja et al., 1996; Hom et al., 1998). Another hormone which might be implicated for antifertility effect of khat was cortisol. The decrease in cortisol level after khat extract treatment suggests that cortisol is unlikely to play a role in the antifertility mechanisms of khat.

The decreased LDL cholesterol could also be considered as another mechanism for antifertility effect of *C. edulis,* as LDL and HDL cholesterol are the major transporters of fertility factors. But this is not true in rodents as the main transporter for cholesterol to ovarian steroidogenesis is HDL unlike that of other mammals and humans which uses LDL as major transporter (Cedars, 2007). The lack of change in HDL thus excludes the role of lipids as a possible mechanism for anti-fertility effects of khat.

Conclusions

The antifertility and anti-implantation activity of fresh khat extract with possibility of complete sterility was demonstrated. The possible mechanisms include decreased serum estradiol concentration, altered estrogen to progesterone ratio, and massive leukocyte infiltration in stromal cells that prevent the sequential effects of estrogen in uterus by different components of khat. Further researches are recommended on how all these mechanisms work together.

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