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Antifertility Effect of Alcoholic Extract of *Moringa oleifera* Stem Bark on Estrous Cycle and Estrogenic Activity of Female Albino Rat

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ABSTRACT

The present work deals with antifertility effect of the alcoholic extract of *Moringa oleifera* stem bark in female albino rats. Pregnant rats weighing 130 to 200 gm were randomized into 4 groups. Rats were laprotomised on 10th day of pregnancy and live fetuses were observed in both the horns of the uterus. Rats in group 1 (control) were orally administered, with 0.5 ml of distilled water once daily while those in group 2 to 4 (experimental groups) were administered 25, 50 and 100 mg/kg body weight doses of alcoholic extract of *M. oleifera* stem bark respectively. The doses were administered from day 11th to 15th of pregnancy of rats then the animals were allowed to go full term. The effect of alcoholic extract of *M. oleifera* stem bark on estrogenic activity and estrous cycle was observed to confirm the antifertility activity. Clinical toxicity symptoms such as respiratory distress, salivation, weight loss, dull eyes, diarrhea, change in the appearance of fur and mortality were not observed in the animals, at any period of the experiment. The alcoholic extract of *M. oleifera* stem bark exhibited significant antifertility activity (26.26 to 100%). It was found that the extract significantly reduced the number of live fetuses, whereas the resorption index and post implantation losses increased significantly. The % of abortion was found to be highest (100%) with 100 mg/kg dose of alcoholic extract of *M. oleifera* stem bark. In ovariectomized immature young rats, the extract showed significant estrogenic effect (vaginal opening, vaginal cornification and increased uterine weight) and also prolonged the estrous cycle and particularly diestrous phase in the experimental animals at the dose 100 mg/kg body weight of *M. oleifera* stem bark.

Keywords: Antifertility activity, Estraogenic activity, Estrous cycle,

Female albino rat *M. oleifera* stem bark.

INTRODUCTION

Numerous herbs have been reportedly used historically by women to aid child delivery, stimulate menstrual flow or reduce fertility[1,2]. Throughout the history women have tried to control or enhance their fertility with various levels of societal support. Many herbal remedies are traditionally used as contraceptive (to prevent the ovulation or fertilization), abortifacients (to prevent implantation) and emmenagogues (to prevent the uterine flow) or oxytocics (to stimulate uterine contractions, particularly to promote labour)[3]. Herbal contraceptive offer alternative for women who have problems with or lack access to modern contraceptive options particularly women living in the rural areas in developing nations with very high population like India, Africa and Bangladesh[4]. Studying the potency and toxicity of local plants that are reputed for birth control in the folklore medicine of these countries may generate greater confidence in and wider acceptability of herbal contraceptive.

Moringa oleifera (Linn) is a medicinally important plant, belonging to family *Moringaceae*. The plant is also well recognized in India, Pakistan, Bangladesh and Afghanistan as a folkloric medicine[5]. *Moringa oleifera* is a small or medium sized tree up to 10 m tall, with thick, soft, corky, deeply fissured bark, growing mainly in semiarid, tropical and subtropical areas[5]. Different parts of the tree have been used in the traditional system of medicine. Survey in the tribal belt of Melghat region (20° 51′ to 21° 46′ N and to 76° 38′ to 77° 33′ E) of Amravati district of Maharashtra state of India revealed that *Moringa oleifera* stem bark is being used traditionally as an abortifacint. The stem bark has been used in indigenous medicine for over many decades as traditional medicine. The seeds are also known to exert its protective effect by decreasing liver lipid peroxides and, as an antimicrobial agent[6]. The stem bark of *Moringa oleifera* are used as purgative, are applied as poultice to sores, rubbed on the temples for headaches, used for piles, fevers, sore throat, bronchitis, eye and ear infections, scurvy and cataract; leaf juice is also believed to control glucose levels and applied to reduce glandular swelling[7,8,9]. The stem bark is used as an antioxidant[10,11]. The root of *Moringa oleifera* were shown to possess antihelmithic, rubefacient, carminative, antifertility, anti-inflammatory, stimulant in paralytic afflictions; as a cardiac/circulatory tonic, used as a laxative, abortifacient, in treatment of rheumatism, inflammations, articular pains, lower back or kidney pain and constipation[12,13].

However, there is no information to substantiate or refute the abortifacient claims of *Moringa oleifera* stem bark in the scientific literature. Therefore, the present work has been undertaken to validate scientifically the abortifacient role of *Moringa oleifera* stem bark as acclaimed by the traditional tribal users of Melghat region.

MATERIALS AND METHODS

Collection of Plant Material

The stem of *Moringa oleifera* plant (Family: *Moringaceae*) were collected from Melghat region of Amravati district during the period of September to December 2012, identified and authenticated by experts from Botanical Survey of India, Pune (Accession No. VZ- 1).

Procurement and Rearing of Experimental Animal

Healthy wistar strain female albino rats about two month old and weighing 150-250 g were procured from Sudhakarrao Naik Institute of Pharmacy, Pusad (Maharashtra). The rats were housed in polypropylene cages and maintained under environmentally controlled room provided with a 12:12 hr light and dark cycle approximately at 25°C. They were fed on pellets (Trimurti Lab Feeds, Nagpur) and tap water *ad libitum*. The rats were allowed to acclimatize to laboratory environment for 15 days before experimentation.

All experimental protocols were subjected to the scrutinization and approval of Institutional Animal Ethics Committee [registration number 1060/ac/07/ CPCSEA (IAEC/1/2012)].

Preparation of Extract

The stem bark of *Moringa oleifera* were collected, shade dried, powdered and subjected to soxhlet extraction with distilled water. The extract was evaporated to near dryness on a water bath, weighed and kept at 4°C in refrigerator until further use.

Phytochemical Screening

The presence of various plant constituents in the plant extract were determined by preliminary phytochemical screening as per Thimmaiah[14].

Acute Toxicity Study

Healthy female albino rats were starved for 3- 4 hrs and subjected to acute toxicity studies as per Organization of Economic Co-operation and Development (OECD) guidelines No: 423[15]. They were divided into 4 groups of 6 animals each and kept singly in separate cages during the experiment. Group 1 represented the control group, which received 10 ml/kg of distilled water orally. Groups 2- 5 received suspension of different extract (aqueous, alcohol, benzene and diethyl ether) of *Moringa oleifera* stem bark orally at the doses of 250, 500 and 1000 mg/kg daily for 7 days respectively. The rats were observed continuously for 2 hrs for behavioral, neurological and autonomic profile, and for next 24 and 72 hrs for any lethality or death.

Abortifacient Activity

The plant extracts were tested in female albino rats for abortifacient activity as per Khanna *et al*[16]. The female rats in proestrous phase were caged with males of proven fertility in the ratio of 2:1, in the evening and examined the following day for the evidence of copulation. Rats exhibiting thick clump of spermatozoa in their vaginal smear were separated and that day was designated as day 1 of pregnancy. These rats were randomly distributed into 4 groups, 1 control group and 3 experimental groups of 6 animals each. On the day 10 of pregnancy animals were laprotomised under light ether anesthesia using sterile conditions. The two horns of uteri were examined to determine the implantation sites. Thereafter the abdominal wound was sutured in layers. The extract to be tested were then fed to operated pregnant rats i.e. alcoholic extracts of *Moringa oleifera* (stem bark) at doses of 25, 50, 100 mg/kg body weight (one tenth of the highest tolerable dose) once daily by an intragastric (i. g.) soft rubber catheter from day 11 up to the day 15th of pregnancy. The animals were allowed to go full term. After delivery the pups were counted and the abortifacient activity of extract was evaluated.

Estrogenic Activity

The alcoholic extract of *Moringa oleifera* at 100mg/kg was found to be most active amongst the three doses in the anti-fertility testing. Hence it was subjected to a detailed investigation for potential estrogenic activity. The uterine weight and vaginal

cornification method was employed for the estimation of estrogenic activity[17,18]. Immature ovariectimized female albino rats, 21-23 days old, weighing between 35-45 gm were used. The animals were divided into four groups, consisting of six rats each.

Group-I: Control, received 0.2 ml of distilled water orally.

Group-II: Treated, received 0.02 mg ethinyl estradiol/ kg/ rat per day in olive oil orally.

Group-III: Treated, received 100 mg alcoholic extract of *Moringa oleifera* (stem bark)/ kg body weight in 0.2 ml of distilled water orally.

Group-IV: Treated, received 100 mg alcoholic extract of *Moringa oleifera* (stem bark)/ kg body weight in 0.2 ml of distilled water orally +0.02 mg ethinyl estradiol / kg /rat per day in olive oil orally.

All the above treatments were given for 7 days. On the 8th day of experiment, the animal were sacrificed by decapitation and uteri dissected out and surrounding tissues removed .The uteri were blotted on filter papers and weighed quickly on a sensitive balance and fixed in Bouin's fluid for 24 hrs. The tissue were dehydrated and embedded in paraffin. The paraffin section were cut at 5 µm and stained with hematoxylin-eosin for histological observation. The diameter of the uteri and thickness of the endometrium were measured in 16 randomly selected sections using an ocular micrometer.

Effect on Estrous Cycle

The alcoholic stem bark extract of *Moringa oleifera* at 100 mg/kg was found to be most active amongst the three doses in the anti-fertility testing. Hence it was subjected to a detailed investigation for study of estrous cycle. The studies were conducted on adult female rats (150- 200 gm) for 30 days. To study the estrous cycle pattern, animal showing regularity in the normal cycle were separated and chosen for further studies. Those animals showing normal estrus cycle were divided in 2 groups of 6 animals each;

Group I- control, received distilled water (Vehicle)

Group II- treated, received alcoholic stem bark extract at dose of *Moringa oleifera* 100 mg/kg body weight.

Vaginal smear using saline solution were taken twice daily during the entire treatment period, observation of the vaginal opening and the cell type obtained in a vaginal smear was also done. The duration of estrous cycle together with that of various phases was determined[19,20].

Statistical Analysis

All the data are expressed as mean ± S.E. Statistical analysis was done by Student's t-test and one way ANOVA[21].

RESULTS AND DISCUSSION

Preliminary phytochemical screening of *Moringa oleifera* stem bark revealed the presence of alkaloid, steroids, flavanoids, phenolics compound and saponins respectively. Similar finding was reported by Uboh *et al*.,[22] while studying the abortifacient activity of the aqueous extract of *Psidium guajava* stem bark in rats. Phytochemical screening has revealed that many bioactive agents of plant extract coexist and can thus serve as precursors in the manufacture of drugs. For example, alkaloid is known to have adverse effect on pregnancy and is being used by physicians either alone or in combination with oxytocics to induce abortion[23]. Furthermore, antifertility and abortifacient activities of phenolics, phytosteroids and saponins have also been sufficiently confirmed in animal models[24]. Studies on the phytochemical investigation of the various extract of the stem bark of *Alangium salvifolium* used as an abortifacient, showed the presence of alkaloids, steroids, saponin, tannin and flavonoids[25]. Therefore, presence

of alkaloids, phenolics, steroids and saponins in the extract of *Moringa oleifera* stem bark which may act either alone or in combination may be partly responsible for the observed pregnancy-terminating effects in the present study.

The highest dose 1000 mg/kg body weight was used for acute toxicity activity. Clinical toxicity symptoms such as respiratory distress, salivation, weight loss and change in appearance of hair as well as maternal mortality were not observed at any period of the experiment. Similarly no mortality and changes in the behavioural, neurological and autonomic profile were observed in treated groups. This suggested that short term use for this purpose is apparently safe. Similar finding was also observed by Wikhe *et al*[26], while studying the effect of *Cicer arietinum* and by Dabhadkar *et al*,[27] of *Plumeria rubra* in female rats.

Moringa oleifera stem bark extract has a folklore reputation as abortive. In the present study the extracts when tested for abortifacient effect in laboratory animals, exhibited abortive activitites in accordance. The oral administration of alcoholic extract of *Moringa oleifera* stem bark at the doses of 25, 50 and 100 mg/kg body weight produced a dose dependent adverse effect on fertility index and number of implantation in the uterine horns of the female rats by virtue of an increase in the percentage of the post-implantation embryonic loss. All the experimental extract when evaluated for their abortifacient activity, were found to exhibit pregnancy interceptive activity. Administration of 100 mg/kg body weight of the alcoholic extract resulted in 100% abortion, while doses of 25 and 50 mg/ kg body weight of the alcoholic extract resulted in 28.50% and 44.45% abortion (Table 1). This was evident from decrease in the percentage of live fetuses. The percent resorption index increased from zero in the control animals to 100 % in 100 mg/kg body weight alcoholic extract treated animals. Our results are also in agreement of Zade *et al.*,[28] while working on abortifacient effect of *Plumeria rubra* pods on female albino rats. Aqueous and 90% ethanol leaf extract of *Moringa oleifera* was found to be 100 % abortive at doses equivalent to 175 mg/kg in rats[29]. The present work also corroborates 100% abortive effect of ethanol extract of stem bark of *Moringa oleifera* at a dose of 100mg/kg body weight. The antifertility activity of 50 % ethanolic extract of *Moringa oleifera* excluding root was demonstrated in hamstar[30]. The antifertility activity of 50 % ethanolic root extract of *Moringa oleifera* was investigated and it was found that a dose of 200 mg/kg led to foetal resorption in 60 % female pregnant rats[31]. All the treatment reduced significantly the number of litters born, confirming the abortifacient activity of the plant used. No vaginal bleeding was observed. The litter born to the experimental animal did not show any morphological defects hence, it can be stated that the treatment does not exhibit any teratogenic effect.

The ethanolic extract of *Moringa oleifera* stem bark at the dose of 100 mg/kg body weight exhibited significant abortifacient activity hence it was further selected for confirmation of the antifertility activity of the plant. In the estrogenic study, the effect of alcohol extract of *Moringa oleifera* stem bark revealed that none of the control group none of the rats exhibited vaginal opening during the period of study. The alcohol extract at the dose of 100 mg/kg when administered orally for 7 days, showed vaginal cornification in all the animals and also increased the uterine weight ($P<0.001$) of immature rats significantly when compared with control (Table-2). The effect of alcohol extract of seed of *Moringa oleifera* when administered conjointly with ethinyl estradiol caused significant increase in the uterine weight ($P< 0.01$) when compared with

control, but the extent of the uterotropic response was less than that produced by ethinyl estradiol alone (P<0.001). The number of cornified cells in the vaginal smears were considerably higher in alcohol extract treated group (+ to ++) than those of the control (0 to +), but notably less than ethinyl estradiol treated rats (+++) (Table- 3). The test drug significantly increases the diameter of the uterus and thickness of the endometrium (P< 0.01; P<0.001) when compared to control group, but notably less than ethinyl estradiol (P<0.01) treated rats. In histopathological study, the control rats uterine endometrium shows epithelial cell with elongated nuclei, numerous endometrial glands and edematous stroma. The uterus shows numerous spots and folds in luminal epithelium cells. Stroma in control rats was oedematous with fibroblast type of cells (Fig. 1a). However the histological evidences of the uterus treated with 100 mg/kg body weight of alcohol extract of *Moringa oleifera* stem bark clearly supports an unfavourable uterine milieu, showing obliterated lumen with loose stroma, increased height of luminal epithelium and stimulated uterine gland (Fig. 1b). Similar picture was observed in histological section of uterus of ethinyl estradiol treated (Fig. 1c) and ethinyl estradiol plus extract treated group of rats (Fig. 1d). Therefore from the present finding it can safely be said that the extract possesses estrogenic activity. Thus the alcoholic extract of *Moringa oleifera* stem bark which shows estrogenic activity in immature rats seems to be responsible to cause abortifacient activity. It is expected that due to the estrogenic activity, the alcoholic extract may disturb the normal estrogenic titre in the uterus in order to insult the egg to implant. The estrogenic activity of the extract may also affect the rate of ovum transport or may create non receptive uterine milieu.

It has been reported that abortifacient activity may be due to estrogenic activity which is causing the expulsion of ova from the tube and disturbing the luteotropic activity of the blastocyst[32,33]. The estrogen also promotes cornification of the vaginal epithelial cells. Safranski *et al*.,[34] found that vaginal smear characterized by full cornification of vaginal epithelial cells require a higher surge of estrogen level. Jacob *et al*.,[35] demonstrated the uterotophic effects of estrogen when administered to rats. Ljungkvist[36] associated these effects with vaginal opening and cornification, endometrial growth and proliferation. Our results also corroborates with the finding of Keshari *et al.,*[37] who reported that hexane extract of the stem bark of *Nigella sativa* L. when given orally possess estrogenic activity in immature rats. Similar finding was recorded by Dabhadkar and Zade[38], while working on abortifacient and estrogenic activity of *Plumeria rubra* pods.

In the present study, the duration of the diestrous phase was significantly increased while those of proestrous and estrous phases were decreased (Table- 4). This is suggestive of negative influences on the estrous cycle as this reduces the number of days/ ova ovulated during the proestrous and esrous phases. The reason for this could be due to the presence of high level of phytoestrogens like saponins and essential oils[39,40]. This disruption of the estrous cycle may be due to the effect of this extract on the ovary which disrupts ovarian functions and estrous cycle via ovarian and extra ovarian hormones[41]. Cyclic changes in the vaginal smear observed in the estrous cycle gives a reasonable index of the ovarian activity and its hormonal synthesis of estrogen and progesterone. The levels of these hormones are controlled by hypothalamic releasing hormones and pituitary gonadotrophins[42]. A feedback mechanism also operates where the pituitary gonadotrophins secretion in turn is controlled by estrogen and progesterone. The cornification in the vaginal epithelial cells is mainly due to high levels of estrogens

secreted by the ovarian mature follicles. It is also known that exogenous administration of estrogen consistently stimulates the proliferation of the vaginal epithelium in adult female rats[43,44]. Similar observation was recorded by Yadav and Agrawal[45], while working on *Nigella sativa* and Amah *et al.,*[46] on *Momoedica charantia* on rats.

CONCLUSION

The abortifacient activity lends support to the claims for its traditional usage of *Moringa oleifera* as an abortive medicine. Thus, this study may prove to be an effective and safe alternative remedy for contraception. Further studies to identify the bioactive principle of abortifacient and estrogenic activity of the extract are in progress.

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Table 1. Effect of alcoholic extract of *Moringa oleifera* (stem bark) on fertility of rats when fed orally from day 11 to 15 of pregnancy

Treatment groups (dose, mg/kg body wt)		No. of foetus individual rats on	No. of rats delivered	No. of resorption in individual rats	No. of resorption	Abortifacient activity
Control	Group- I Vehicle	8,8,9,8,6,6	6(8,8,9,8,6,6)	0,0,0,0,0,0	0	Nil
Alcoholic extract of *M. oleifera*	Group- II 25	10,6,8,7,9,8	6(9,4,5,4,7,6)	1,2,3,3,2,2	2.16±0.18**	26.26
	Group- III 50	9,9, 8,10,8,10	6(6,6,4,5,3,6)	3,3,4,5,5,4	4.00±0.58***	44.86
	Group- IV 100	3,6,2,8,6,4	6(0,0,0,0,0,0)	3,6,2,8,6,4	4.83±0.80***	100

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared between group, ns= non significant

Table 2. Estrogenic and anti-estrogenic potentials of the alcoholic extract of *Moringa oleifera* stem bark in rats

Groups	Treatment dose (mg/ kg body wt.)	Uterine weight (mg/ 100 gm body wt.)	Vaginal status	Vaginal cornification
I	Control (distilled water)	72.83±2.28	Not opened	0 to +
II	Ethinyl estradiol (0.02mg/kg)	179±2.97***	Opened	+++
III	Alcoholic extract of *M. oleifera* (100 mg/kg)	104±1.45*** ^{c}	Opened	+ to ++
IV	Alcoholic extract of *M. oleifera* (100 mg/kg) + Ethinyl estradiol (0.02mg/kg)	130±0.76*** ^{b}	Opened	+++

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared with control, P values: a <0.05, b < 0.01, c <0.001, when compared with ethinyl estradiol group, ns= non significant.
+ -nucleated epithelial cells, ++ -nucleated and cornified cells, +++ -cornified cells.

Table 3. Histological changes in the uterus and endometrium after treatment with the alcoholic extract of *Moringa oleifera* stem bark in rats

Groups	Treatment dose (mg/kg body weight)	Diameter of uterus (µm)	Thickness of endometrium (µm)
I	Control (distilled water)	292.00±7.27	131.70±3.63
II	Ethinyl estradiol (0.02mg/kg)	514.29±6.62**	345.50±5.67*
III	Alcoholic extract of *M. oleifera* (100 mg/kg)	398±3.68*** ^{b}	310±4.76*** ^{b}
IV	Alcoholic extract of *M. oleifera* (100 mg/kg) + Ethinyl estradiol (0.02mg/kg)	479.4±2.14*** ^{c}	298±1.18** ^{c}

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared with control, P values: a <0.05, b < 0.01, c <0.001, when compared with ethinyl estradiol group, ns= non significant.

Table 4. Histological changes in the uterus and endometrium after treatment with the alcoholic extract of *Moringa oleifera* stem bark in rats

Phases (Days)	Group-II Control group	Group-II Alcoholic extract of *M. oleifera* (100 mg/kg)	Vaginal opening/ cell type obtained in a vaginal smear
Proestrous phase	0.63±0.09	0.43±0.03**	25% to 40% / Epithelial cells only
Estrous phase	0.60±0.15	0.53±0.01*	Above 70% / Few cornified cells
Metaestrous phase	0.87±0.31	1.00±0.08**	50% to 70% / Cornified cells plus many leukocyte
Diestrous phase	2.37±0.13	4.29±0.68**	50% to 70% / Leukocytes plus epithelial cells
Complete estrous	4.47±0.68	6.25±0.29***	-

Values are mean ± SE from 6 animals in each group, P values: *<0.05, **<0.01, ***<0.001, When compared with control, ns= non significant

Figure 1. Histopathological change in immature ovariectomized uterus of rat when treated with alcoholic extract of *Moringa oleifera* stem bark (Photomicrograph at a Magnification of 100X)

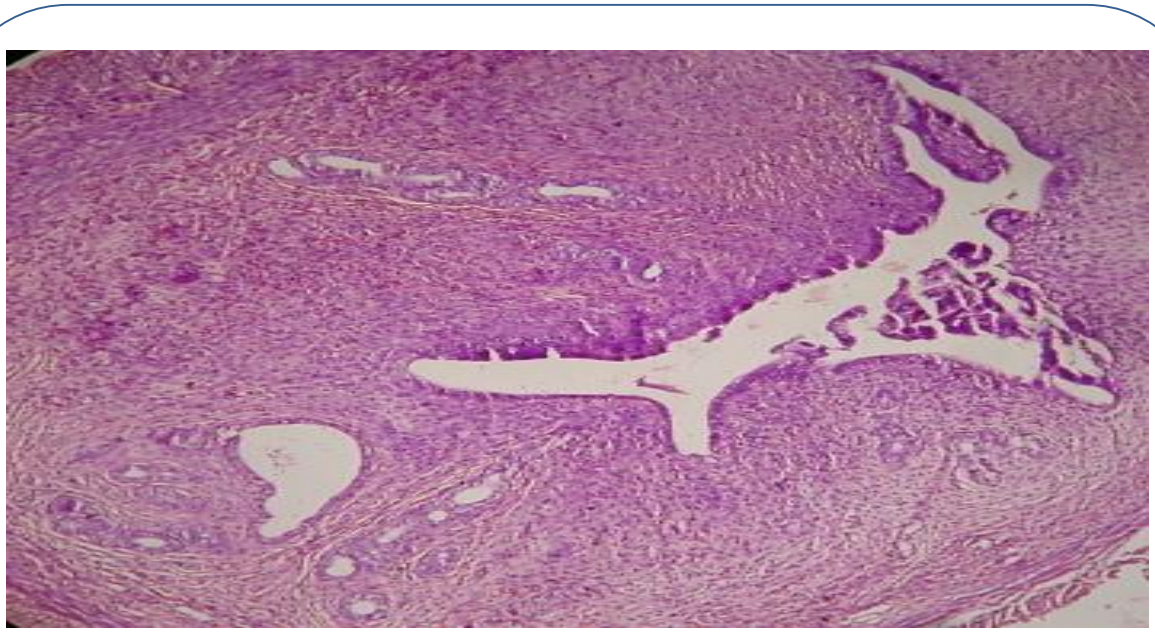

Figure 1a. T. S. of immature ovariectomized control rat uterus

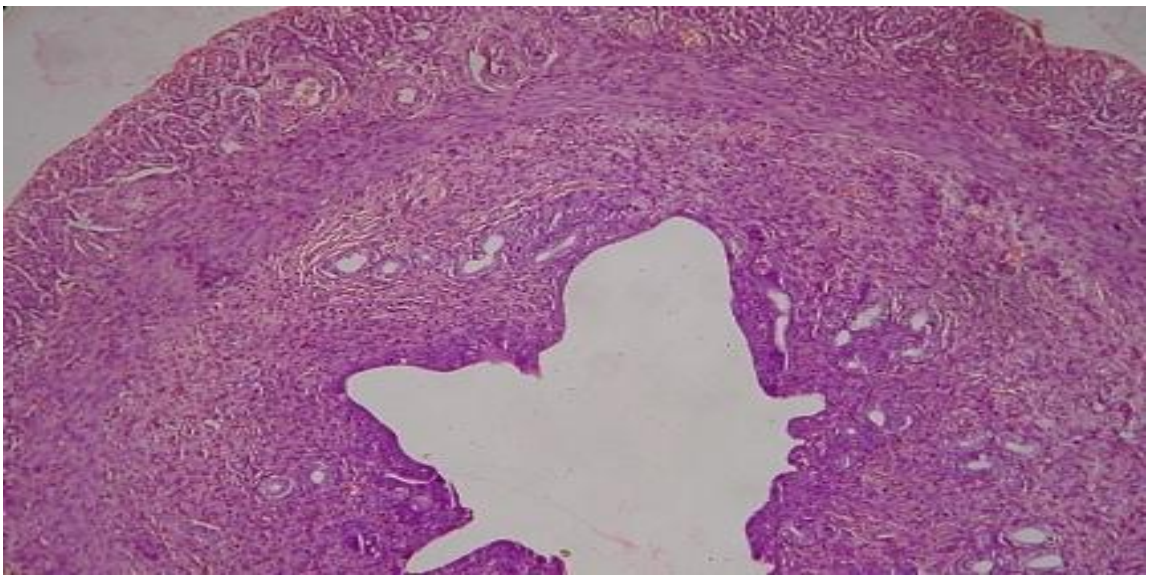

Figure 1b. T. S. of uterus of immature ovariectomized rat treated with ethinyl estradiol

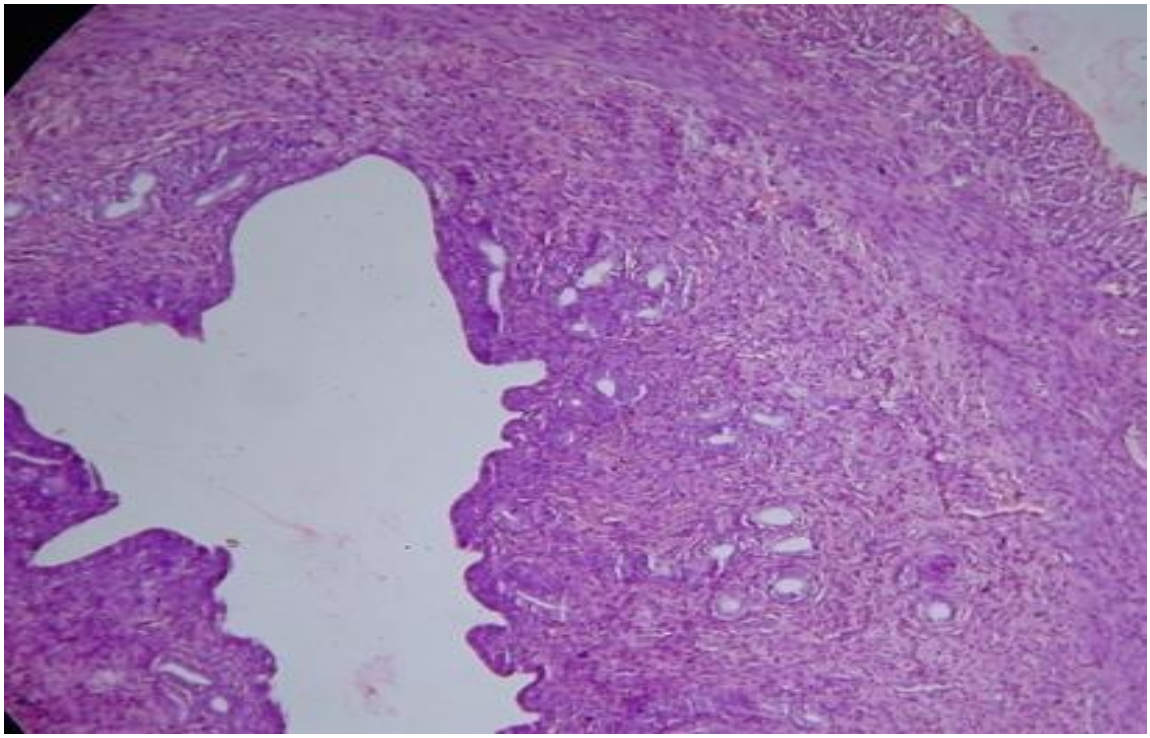

Figure 1c. T.S. of uterus of ovariectomized rat treated with 100 mg/kg b. w. alcoholic extract of *Moringa oleifera* stem bark

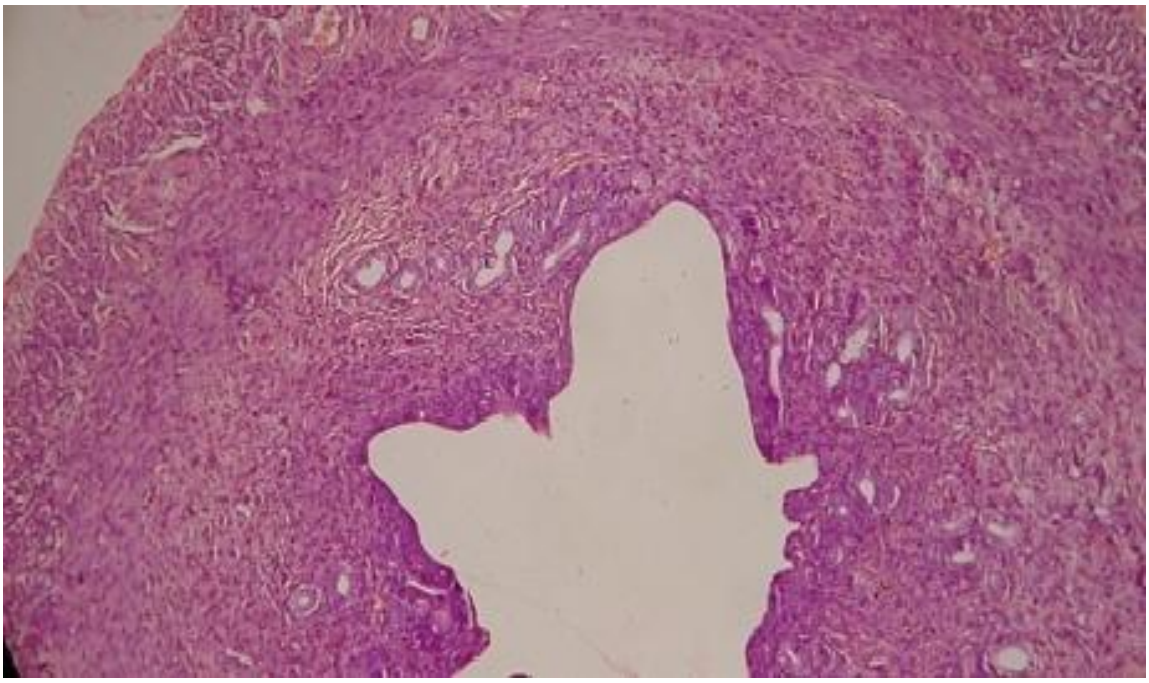

Figure 1d. T.S. of uterus of ovariectomized rat treated with ethanyl estradiol + 100 mg/kg b. w. alcoholic extract of *Moringa oleifera* stem bark