Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2014, Volume 2, Issue 10
ISSN: 2321–3647(online)

Effects of the Methanol Root Extract of *Cissampelos Mucronata* A. Rich on the Ovaries and Uterus in Rats: A Histological and Hormonal Study

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ABSTRACT

This study was designed to investigate the effect of the methanol root extract of *Cissampelos mucronata on* the histology of the ovaries and uterus as well as the oestrus cycle. A total of 25 female rats were used for this study. The rats were randomly divided into groups of 5 rats per dosage group (I-V). Group I served as the control group and were administered normal saline equivalent to the volume administered to the highest dosed experimental rats. Rats in Groups II, III and IV were administered with 100 $mgkg^{-1}$, 200 $mgkg^{-1}$ and 300 $mgkg^{-1}$ doses of the extract respectively while rats in Group V were administered the highest dose (300 $mgkg^{-1}$) of the extract for 20 days and allowed to stay for at least 14 days post treatment to observe for reversibility, persistence or delayed occurrence of toxic effects. The result of this study showed that administration of the methanolic extract of the root of *Cissampelos mucronata* in rats caused loss in body weight, degenerative changes in Graafian follicles, inflammatory cells, degenerating follicles, proliferation of granulosa cells and atretic follicles with uterine tissues characterized by mild to moderate stromoglandular dissociation, focal areas of necrosis, proliferation of connective tissue stroma and alteration in estrous cycle length, characterized by prolonged metesterus and diestrus phases with no effect on progesterone, Luteinizing Hormone(LH) and Follicle Stimulating Hormone (FSH) levels. The study revealed significant alterations in histological and hormonal profile of female rats. It is recommended that full stereology studies be carried out.

Keywords: Oestrous Cycle, Luteinizing Hormone, Follicle Stimulating Hormone, Graafian Follicle, Myometrium

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Received 08 September 2014, Accepted 10 October 2014

Please cite this article as: Garba SH *et al.*, Effects of the Methanol Root Extract of *Cissampelos Mucronata* A. Rich on the Ovaries and Uterus in Rats: A Histological and Hormonal Study. American Journal of Pharmacy & Health Research 2014.

INTRODUCTION

Medicinal plants are considered as rich sources of ingredients which can be used in drug development and synthesis, thus playing a critical role in the development of human cultures around the whole world [1]. The plant *Cissampelos mucronata* (A. Rich) belongs to the family Menispermaceae and is used worldwide in traditional medicine to treat varieties of ailments and conditions. Its use as an emmenagogue and diuretic have been reported[2]. The plant is also used for the treatment of abdominal pains, swollen stomach and gastro-intestinal upset[3, 4]. It's hepatotoxic, nephrotoxic effect and its ability to decrease foetal weight, placental weight, crown rump length and cause resorptions in pregnancy in a dose dependent manner has also been reported[5,6] . Its indigenous names in Nigeria include Jibdar Kasa or Damarji (Hausa) and abakenwo in Igbo, Jokoje (Yoruba), Barwada (Kanuri), Magirahi (Fulfulde), Zagaduwa (Marghi) and Kwahara or Kwahirka (Babur/Bura). Traditionally *Cissampelos mucronata* is used to facilitate childbirth, as an abortifacient and to regulate menstruation [7-10]. It is also used to prevent or to arrest uterine haemorrhage, painful uterus, to treat infertility, to prevent abortion [3,4,11-14]. The extract is also taken postnatally to stimulate uterine contractions [15,16], menorrhagia and to treat dysmenorrhagia and also used as an aphrodisiac for sexual stimulation [3,4] . Despite the widespread use of *Cissampelos mucronata* in traditional medicine for the treatment of various ailments, its histological and hormonal effects on the ovary and uterus has not been elaborated thus, the objectives of the study was to investigate the effect of the methanol root extract of *Cissampelos mucronata on* the histology of the ovaries and uterus, study the oestrus cycle and levels of FSH, LH and progesterone of female adult Wistar rats.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The plant, *Cissampelos mucronata* A. Rich used in this study was collected around Giwa Military Barracks in Maiduguri metropolis latitude 11° 50' 42" North and longitude 13° 9' 36" East. The plant was identified and authenticated as *Cissampelos mucronata* A. Rich . A specimen voucher (CM.01) of the plant was prepared and deposited at the herbarium of the Department of Veterinary Physiology and Pharmacology, University of Maiduguri, Borno state. The collection, identification and storage of the plant material was carried out according to the World Health Organization's guidelines on quality control methods for medicinal plant materials[17] and quality specifications of plant materials and preparations[18]. The root was then sun-dried, pulverised into powdered form using a pestle and mortar and then stored in cellophane bags at room temperature.

Extraction Procedures

The World Health Organization's research guidelines for evaluating the safety and efficacy of herbal medicines[18] was adopted for the extraction of the root of *Cissampelos mucronata*. A total of one hundred grams (100 g) of the pulverised root was subjected to exhaustive soxhlet extraction in methanol (500 ml) for 72 h at 60^{o}C. The extract obtained was then concentrated in a water bath until a constant dark sticky residue was obtained. The total mean extract yield thus obtained was 11.34 g w/w. The extract was further oven dried , maintained in a desiccator until a constant weight was obtained and stored in a stoppered container in a refrigerator at - 4^{o}C until required. The stock solution of the extract was prepared by dissolving 2 g of the extract in 50 ml of normal saline in the presence of 1 drop (0.05 ml) of dimethylsulfoxide (DMSO) and allowed to stand for about 45-60 minutes. The water soluble portion was separated off using a fine needle syringe and the dry weight of the marc determined after drying on a hot plate. The actual concentration of the water soluble portion of the extract was thus determined and required concentrations prepared from the stock solution by serial dilutions.

Animal Selection and Housing

For the entire study segments young adult nulliparous female Wistar albino rats were obtained from the animal facility centre of the National Veterinary Research Institute (NVRI), Vom, Plateau State. The rats were individually identified by colour tattoo and weighed. They were then quarantined for two weeks and evaluated for weight gain and any gross sign of diseases or injury. The rats were then released from quarantine on the basis of body weight gain and freedom from clinical signs of disease or injury. The rats were then divided by stratified randomisation into groups to eliminate any statistically significant differences among group body weight means within each sex and assigned to study segments. The rats were kept in plastic cages under standard laboratory conditions at room temperature of 32 ± 4^{0}C and provided with standard laboratory diet (Sanders Nigeria Limited, Kaduna) and drinking water *ad libitum.*

Design of Experiment

The rats were randomly divided into five groups of 5 rats per dosage group (I-V). Group I served as the control group and were administered normal saline equivalent to the volume administered to the highest dosed experimental rats. Rats in Groups II, III and IV were administered with 100 mgkg^{-1}, 200 mgkg^{-1} and 300 mgkg^{-1} doses of the extract respectively while rats in Group V served as the satellite group and were administered the highest dose (300 mgkg^{-1}) of the extract for 20 days and allowed to stay for at least 14 days post treatment to observe for reversibility, persistence or delayed occurrence of toxic effects. Dose levels were

selected based on the LD_{50} of >2000 $mgkg^{-1}$ obtained from the acute toxicity study[5]. The extract was administered daily in divided doses and the oral route was adopted because it is the intended human exposure route.

Parameters under Study

General clinical observations and Body weights.

General clinical observations were carried out daily for signs of clinical toxicity while body weights were measured weekly. At the end of the experimental period body weights of all rats were recorded and blood samples were obtained.

Oestrous Cycle Length and Duration of Each Phase.

Vaginal smears were assessed once each day between 9.00 and 10.00 a.m. as described by the pipette smear technique of Marcondes *et al*[19]. This method consisted of drawing approximately 0.2 ml of normal saline (NaCl 0.9 %) into the pipette and the tip gently pushed into the entrance of the vagina to a depth of 2-5 mm and the fluid flushed into the vagina and back up into the pipette until a'cloudy' fluid was seen . Unstained material was observed under a light microscope and classified as 1. Cornified (keratinised) cells - large, angular and irregularly shaped, mostly non-nucleated when mature (as seen at oestrus). 2. Epithelial cells - not quite as large as the cornified cells and much more rounded in shape. 3. Leucocytes - very small, round cells. The proportion among them was used for the determination and classification of the estrous cycle phases into the four basic stages of the cycle i.e. oestrus, metoestrus, di-oestrus and pro-oestrus, abbreviated to E, M, D & P respectively[20,21] and the duration of each phase of the cycle were recorded as described by Makonnen *et al*[22].

Hormonal Assay

Blood samples were collected during necropsy for the analysis of the following hormones follicle stimulating hormone (FSH), luteinizing hormone (LH) and and progesterone at the Department of Chemical Pathology, University of Maiduguri Teaching Hospital, Maiduguri using commercially bought ELISA kits (BioCheck, Inc., 323 Vintage Park Dr., Foster City, CA 94404 U.S.A).

Histopathology

All the sacrificed rats were then subjected to a full, detailed gross necropsy, which included careful examination of the external surface of the body, all orifices, cranial, thoracic and abdominal cavities and their contents. The ovaries and the uterus of all the animals were trimmed of any adherent tissue, as appropriate and their wet weights taken. The tissues were then

preserved in 10% formal saline and processed for routine paraffin sections and stained with Haematoxylin and Eosin for subsequent histopathological examination.

RESULTS AND DISCUSSION

Effects of the Extract on Mean Body Weight

The effects of the administration of the methanolic extract of the root of *Cissampelos mucronata* on mean body weight of female rats are presented in Table 1. Female rats in the control group also had a significant ($p<0.01$) increase in their body weights while rats that were administered with 100, 200 and 300 $mgkg^{-1}$ of the extract presented with a significant ($p<0.01$- 0.001) decrease in their body weights. Withdrawal of the extract caused a recovery in the body weight lost due to the administration of 300 $mgkg^{-1}$ of the extract though the gain was not significant (Table 1).

Table 1. Effects of 28 Days Administration of the Methanolic Extract of the root of *Cissampelos Mucronata* on Mean Body Weights in Female Rats

Doses Administered ($mgkg^{-1}$)	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Difference (g)
0	239.65 ± 2.49	291.89 ±2.22***	52.24
100	245.86 ±2.77	225.55 ±2.27***	20.31
200	233.28 ±1.57	222.06 ±0.77**	11.23
300	245.79 ±2.83	225.10 ±4.12***	20.70
300^{PRG}	228.37 ±0.67	234.88 ±1.06	6.51

PRG= Post Recovery Group: Sacrificed 14 days after the last administration of the extract.

Results are presented as Means ± SEM. Significance relative to initial body weights ***p<0.001,**p<0.01,*p<0.05, N=5.

Effects of the Extract on Mean Ovarian and uterine Weights

Administration of the methanolic extract of the root of *Cissampelos mucronata* did not elicit a significant change on uterine weights following administration of 100, 200 and 300 $mgkg^{-1}$ of the extract but a significant ($p<0.001$) increase in the uterine weight was noticed following withdrawal of the extract while administration of 300 $mgkg^{-1}$ of the extract caused a significant ($p<0.001$) increase in both weights of the ovaries with no post withdrawal effect (Table 2).

Table 2. Effects of 28 days Administration of the Methanolic Extract of the root of *Cissampelos mucronata* on Mean Organ Weights of Female Rats

	Doses administered($mgkg^{-1}$)				
Parameter (g)	0	100	200	300	300^{PRG}
Uterus	0.11±0.02	0.11±0.02	0.10±0.02	0.10±0.01	$0.21±0.01^{aaa}$
Lt. Ovary	0.06±0.01	0.08±0.01	0.07±0.01	0.12±0.02**	0.12±0.02
Rt. Ovary	0.06±0.01	0.07±0.01	0.06±0.01	0.11±0.02*	0.16±0.02

PRG= Post Recovery Group: Sacrificed 14 days after the last administration of the extract.

Significance relative to control (Group I) ***p<0.001, **p<0.01,*p<0.05, [a]p<0.05 Significance between 300mgkg^{-1} and 300^{PRG} groups**.** N=5, Results are presented as Means ± SEM. Lt = Left and Rt= Right

Histopathological Findings

Light photomicrographs of the paraffin section obtained from the ovary of control rats showed a normal ovarian tissue characterized by follicles at various stages of maturation. The mature graafian follicles contained oocytes that were surrounded by an intact theca interna and externa surrounding the zona granulosa and follicular antrum (Figures 1).

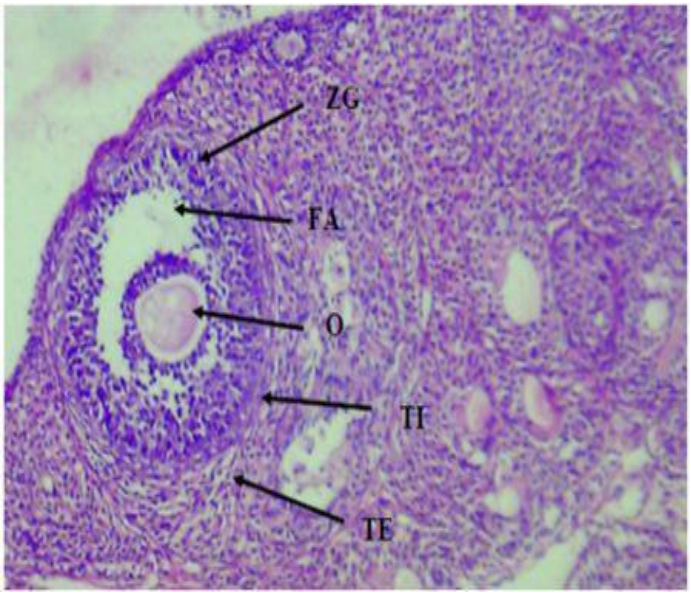

Figure 1 : Photomicrograph of the paraffin section of the ovary of a control rat showing a normal and mature Graafian follicle with an oocyte (O) characterized by zona granulosa (ZG) and follicular antrum (FA) surrounded by an intact theca interna (TI) and theca externa (TE). H and E stain. x 100

Administration of 100, 200 and 300 mgkg^{-1}of the extract caused degenerative changes in Graafian follicles undergoing normal maturation processes. There was also the presence of inflammatory cells, degenerating follicles and atretic follicles with the severity increasing with the doses administered (Figure 2). Withdrawal of the extract for 14 days after the administration of 300 mgkg^{-1}of the extract did not show any remarkable recovery from the injury caused by the extract because the ovarian tissues were still characterized by the presence of inflammatory cells, degenerating follicles and atretic follicles (Figure 3). Photomicrograph of the paraffin section obtained from the uterus of the control rats showed a uterine tissue composed of predominantly tubular endometrial glands lined by tall columnar epithelium in a loose connective tissue stroma (Figures 4). Histopathological findings observed following the administration of 100, 200 and

300 mgkg^{-1} of the extract were mild to moderate stromoglandular dissociation, focal areas of necrosis, proliferation of connective tissue stroma and glandular atrophy (Figure 5) with the severity increasing with dose. Withdrawal of the extract for 14 days after the administration of 300 mgkg^{-1}of the extract did not show any remarkable recovery from the injury caused by the extract because the uterine tissues were still characterised by stromoglandular dissociation, focal areas of necrosis, proliferation of connective tissue stroma and glandular atrophy (Figure 6).

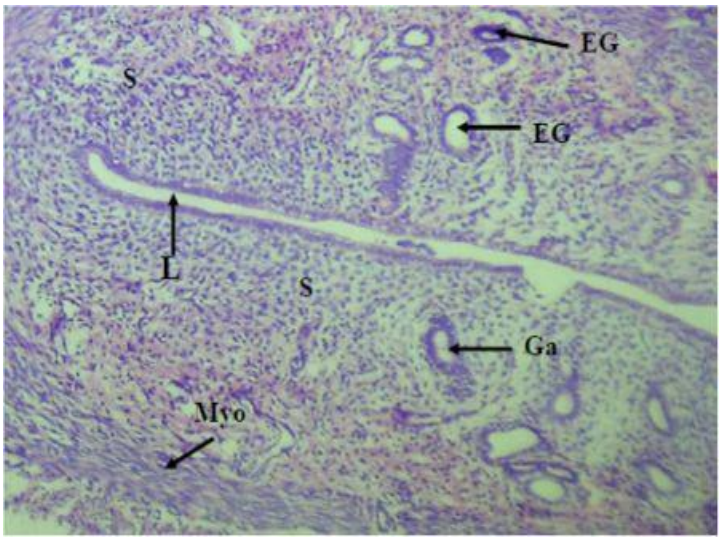

Figure 2: Photomicrograph of the paraffin section of the uterus of a rat treated with 300 mgkg^{-1} of the extract and allowed a recovery period of 14 days showing no sign of recovery because tissue was still characterized by few cystically dilated endometrial glands (EG), glandular atrophy (Ga) ,abundant stroma(S) and the myometrium(Myo) and Lumen (L). H and E stain.x 100.

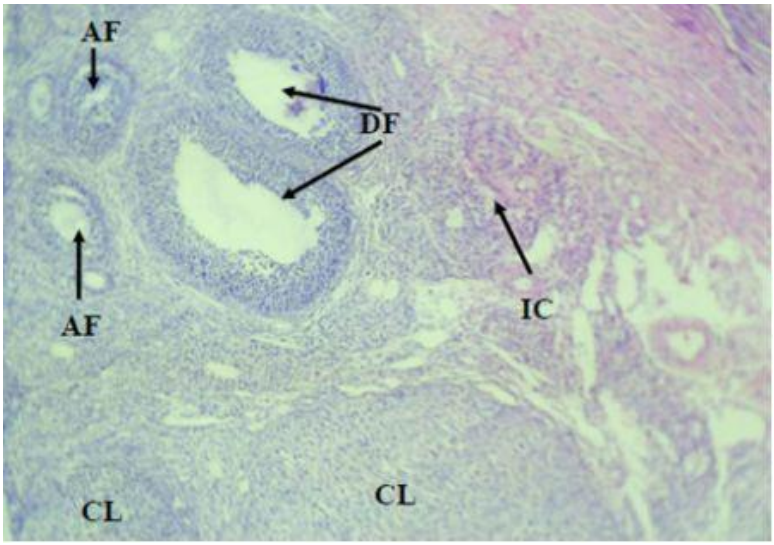

Figure 3 : Photomicrograph of the paraffin section of the ovary of a rat treated with 300 mgkg^{-1} of the extract showing an ovarian tissue characterized by inflammatory cells (IC),

degenerating follicles (DF), atretic follicles (AF) and Corpora lutea (CL). H and E stain. x 100

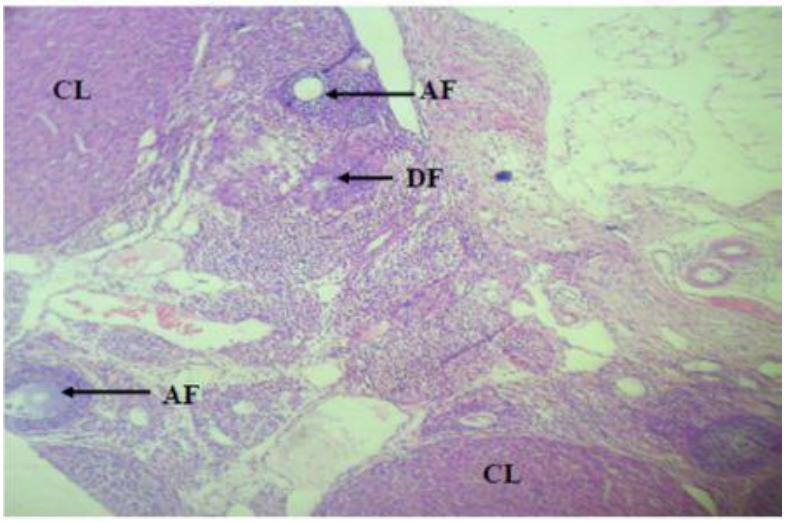

Figure 4:Photomicrograph of the paraffin section of the ovary of a rat treated with 300 mgkg^{-1} of the extract and allowed a recovery period of 14 days showing no sign of recovery because tissue was still characterized by degenerating follicles (DF), atretic follicles (AF) and degenerating Corpora lutea (CL) . H and E stain. x 100

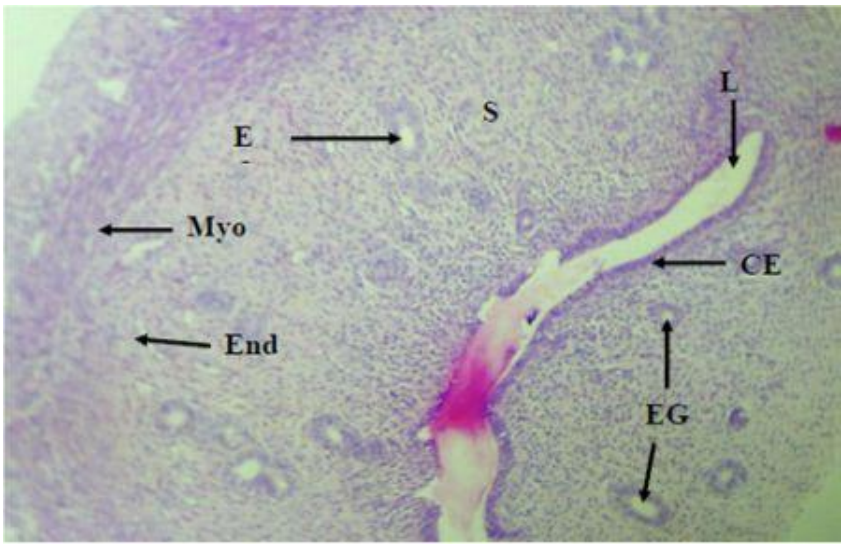

Figure 5 : Photomicrograph of the paraffin section of the uterus of a control rat showing a uterine tissue composed of normal myometrium(Myo) , endometrium (End), lumen (L), tubular endometrial glands (EG) lined by tall columnar epithelium(CE) in a loose stroma (S). H and E stain. x 100.

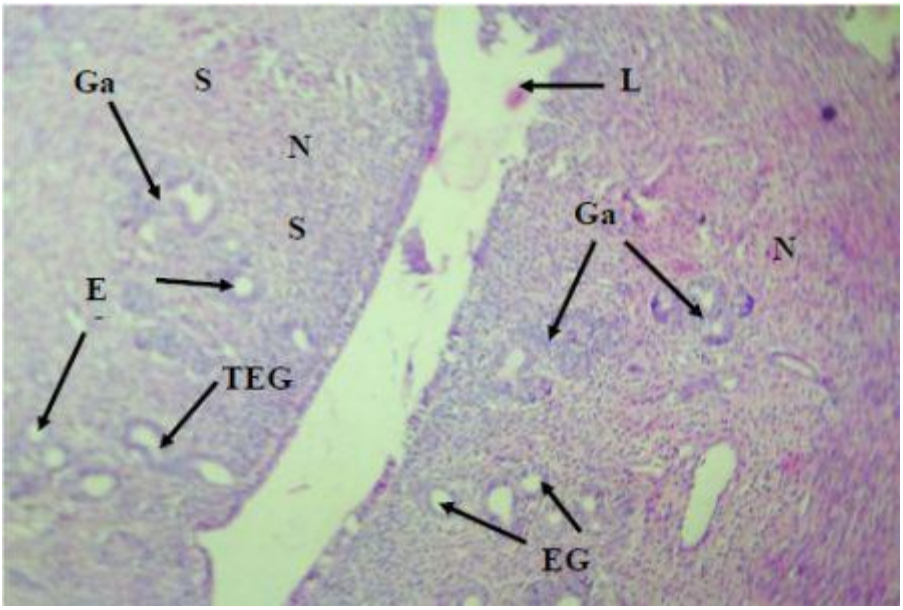

Figure 6: Photomicrograph of the paraffin section of the uterus of a rat treated with 300 mgkg^{-1} of the extract showing a uterine tissue composed of few cystically dilated tubular endometrial glands(EG) and more tortuous endometrial glands(TEG) , focal areas of necrosis (N) , abundant stroma(S) , glandular atrophy (Ga) and the lumen (L). H and E stain. x 100

Oestrous Cycle Length and Hormonal Levels

The effects of administration of the methanolic extract of the root of *Cissampelos mucronata* on oestrous cyclicity and hormonal levels in female rats are presented in Tables 3 and 4.

Table 3. Effects of the Administration of the Methanolic Extract of the Root of *Cissampelos mucronata* on Duration of the Oestrous Cycle in Female Rats.

	Doses administered(mgkg^{-1})			
Oestrous Phases in days	**0**	**100**	**200**	**300**
No. of rats examined	10	10	10	10
Oestrus frequency/20 days	4.33±0.14	4.68±0.08	5.54±0.03**	6.14±0.12**
Proestrus	0.89±0.16	0.89±0.14	0.68±0.08	0.54±0.07
Oestrous	0.91±0.13	0.94±0.17	1.03±0.11	1.07±0.08
Metestetrus	0.74±0.07	0.69±0.10	1.04±0.07*	0.86±0.04
Diestrus	1.79±0.16	2.14±0.09	2.79±0.23**	3.68±0.06**

Significance relative to control (0 mgkg^{-1}) ***p<0.001,**p<0.01,*p<0.05

Table 4 . Effects of the Administration of the Methanolic Extract of the Root of *Cissampelos mucronata* on Duration of the Oestrous Cycle and Hormone Levels in Female Rats through Mating and Implantation (GD6).

	Doses administered(mgkg^{-1})			
Hormones	**0**	**100**	**200**	**300**
No. of rats examined	05	05	05	05
FSH(mIU/ml)	2.79±0.43	2.71±0.34	2.16±0.29	1.98±0.13
LH(mIU/ml)	2.69±0.51	2.23±0.24	2.01±0.32	1.88±0.25
Progesterone (ng/ml)	51.82±1.67	51.14±2.57	44.95±3.85	40.28±4.55

Significance relative to control (0 mgkg^{-1}) ***p<0.001,**p<0.01,*p<0.05

LH= luteinizing hormone and FSH= follicle Stimulating Hormone

The rats in the control group that were administered with only normal saline equivalent in volume to the highest dosed group exhibited regular 4 to 5 day cycle while the length of the cycles in the extract treated groups were significantly ($P < 0.01$) prolonged. The metesterus and diestrus phases of the cycle were increased in the extract treated rats. At a dose of 200 and 300 mgkg^{-1} an irregular oestrous cycle characterized by a prolonged diestrus phase was observed. Hormonal analysis revealed that the administration of the extract caused a non significant decrease in the serum levels of luteinizing hormone (LH), follicle stimulating hormone and progesterone. The body weight loss observed in the treated groups might be attributed to the loss of appetite or the effect of the phytochemical constituents such as polyphenols that have been linked with antiobesity effects[24-29]. It is pertinent to note that withdrawal of the extract caused slight recovery in body weight lost. The decrease in ovarian weight noticed though not significant could probably be due to the saponin and glycoside contents in the plant which is in line with similar works[30,31] that demonstrated that plants rich in saponins and glycosides cause reduction in the wet weights of the ovaries of rats since significant decrease in ovarian weight is an indication of female reproductive toxicity this was corriborated histologically as seen in the degenerative changes induced on the tertiary follicles with deranged granulosa cells following exposure to the extract of *Cissampelos mucronata*. Other phytochemicals that are present in this extract like tannins, steroids, alkaloids, glycosides and terpenes have been linked with anti-ovulatory activities[31]. An alteration in the weight of the uterus may be considered an indication of female reproductive organ toxicity. Compounds that inhibit steroidogenesis and cyclicity can dramatically reduce the weight of the uterus so that it appears atrophic and small. However, uterine weight fluctuates three- to fourfold throughout the oestrous cycle, peaking at proestrus when, in response to increased estrogen secretion, the uterus is fluid filled and distended. This

increase in uterine weight has been used as a basis for comparing relative potency of estrogenic compounds in bioassays[32]. This study demonstrated that the methanolic extract of the root of *Cissampelos mucronata* alters the estrous cycle, by significantly prolonging the duration of the diestrus and estrus phases. The fact that diestrus is especially prolonged, coupled with a significant reduction in the duration of proestrus will most likely reduce the frequency of ovulation. Prolonged estrus phase is suggestive of negative influences of the extract on the estrous cycle as this reduces the number of days/ova ovulated during the proestrus and estrus phases[33]. The methanolic extract of the root of *Cissampelos mucronata* administered between 8.00 and 10.00 a.m. on the morning of proestrus caused irregular changes in the phases of the estrous cycles studied and similarly blocked ovulation partially as observed by these previous authors, suggesting a similar mechanism of blocking and the rise in luteinizing hormone during early proestrus. A possible mechanism of the anti-ovulatory effect of the extract of *Cissampelos mucronata* is through its anti-inflammatory property. Ovulation has been likened to an inflammatory process[34] and is therefore blocked by anti-inflammatory agents[35]. The anti-inflammatory property of *Cissampelos mucronata* may be responsible for its observed effect in partially blocking ovulation when administered to the rats before the expected upsurge of leutenising hormone (which causes follicular rupture and release of ova). Phytochemical screening has revealed many bioactive as well as toxic agents of plant extracts that can affect the regulation of oestrous cycle, conception and reproduction [36,37]. Alkaloids and flavonoids have been shown to reduce plasma concentrations of LH, estradiol and FSH . Therefore, the presence of these phytochemicals may account for the alterations in the levels of the circulating hormone observed in this study. This study agreed with the work of Benie *et al* [36] where administration of *Afrormosia laxiflora*, *Pterocarpus erinaceus* and *Cola nitida* stem bark decreased the release of the gonadotropins (LH and FSH). Therefore, the reduction in the serum LH levels may be explained by an inhibitory effect of the extract on the release of LH which may trigger disruption of ovulation. This may result in impairment of oestrous cycle; hamper conception and normal reproduction in the females. Several studies have demonstrated that flowers of *Malvaviscus conzatii* as well as *Cynomorium coccineum* and *Withania somnifera* can hinder gonadotropin release and induce similar effect on the oestrous cycle [38]. Our findings agreed with that of Jarry *et al* [39] where triterpenenoid glycoside in methanolic and lipophilic extracts of *Cimicifua racemosa* was responsible for the reduction in LH concentration. The reduction in the levels of serum progesterone by methanolic extract of the root of *Cissampelos mucronata* may have consequential effect on conception in females; impede ovulation which may result in

annovulation. Alkaloids have equally been reported to inhibit the synthesis of cellular progesterone [40]. Therefore, the reduced level of progesterone as a result of the administration of the methanolic extract of the root of *Cissampelos mucronata* may not be unconnected with the alkaloidal or saponin component of the extract[41].

CONCLUSION

The result of this study showed that administration of the methanolic extract of the root of *Cissampelos mucronata* in rats were characterized by degenerative changes in graafian follicles, inflammatory cells, degenerating follicles, proliferation of granulosa cells and atretic follicles with uterine tissues characterized by mild to moderate stromoglandular dissociation, focal areas of necrosis, proliferation of connective tissue stroma and alteration in estrous cycle length, characterized by prolonged metesterus and diestrus phases with no effect on progesterone, LH and FSH levels.

ACKNOWLEDGMENT

We wish to acknowledge the technical assistance of Ibrahim Wiam and Ephraim Ayuba of the Departments of Veterinary Anatomy and Human Anatomy, University of Maiduguri, Nigeria

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