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Chronic Consumption of Neem Leaves Extract Affects Female Reproductive System of Adult Wistar Rats

ONAYIGA OI[1], FAPOHUNDA DO[1], BAMIRO AS[1], AJISEGIRI SB[1], MAKANJUOLA SBL[2,6], OKOYE II[3], UMOREN GA[1], AJUONUMA UJ[4], AJUONUMA MU[4], CHUKU CL[5], OFFIONG J[6], AJONUMA LC[1, 6*]

[1]Department of Physiology, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria.
[2]Department of Pharmacology, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria.
[3]Department of Oral Pathology, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria.
[4] St. Mary's Maternity Hospital, Amakohia, Ihitte/Uboma, Imo State, Nigeria.
[5] Civil Servants Clinics, Port Harcourt, Rivers State, Nigeria.
[6]Molecular Biology Laboratory, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria.

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***Corresponding Author:** AJONUMA LC

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Abstract **Original Research Article**

Neem (Azadirachta indica L.) have been used traditionally as treatment for some animal and human diseases in many African countries, most especially Nigeria. More so, due to the environmental compatibility of Neem products; lack of resistance development to them and their harmless nature on non-target organisms, Neem have been integrated in folkloric medicine. Although, studies have shown that Neem possess anti-fungal, anti-inflammatory, insecticidal, bactericidal and immuno-modulating potentials. However, there have been recent suspicions that the plant may possess anti-reproductive properties. To address this gap, this study was carried out to evaluate the effects of Neem leaves extract on female reproductive system in adult Wistar rats. Fifteen (15) female Wistar rats were randomly divided into three (3) groups (1,2,3) each. Group 1 were administered 0.5mls of normal saline daily, group 2 were administered with 0.6mls of Neem oil extract while group 3 were administered with 1.2mls of Neem oil extract, via oral gavage for six weeks. Rats in each group were sacrificed after six (6) weeks of prolonged ingestion of Neem leaves extract and tissue samples of ovaries and uteri were excised for histological processing. Sera obtained from the rats were assayed for gonadotropins (Follicle stimulating hormone, FSH and Luteinizing hormone, LH) and sex steroid hormones (Estradiol, E2 and progesterone), using Enzyme-linked Immunosorbent Assay (ELISA). Vagina smear was done for six weeks. Serum hormonal level changes of FSH, LH, E2 and progesterone for the test groups were not significant when compared to the control. There were histopathological changes in the tissues of the ovaries and uteri of the test groups compared to the control. There was also irregular pattern of oestrus cycle (prolonged diestrus, reduced oestrus and proestrus, rare metestrus). Its prolonged consumption may severely interfere with female fertility.

Keywords: Female Reproductive System, Neem Leaves Extract, Oestrus Cycle, Reproductive Hormones.

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INTRODUCTION

Neem, *Azadirachta indica A. Juss* is widely prevalent and highly esteemed wonder tree of Indian subcontinent, belongs to family Meliacae. The environmental compatibility of Neem products, lack of resistance development to them, their harm less nature against non target organisms and lack of toxicity, all have significantly enhanced the integrated use of Neem in medicine. It is a fast growing plant of tropical and sub tropical countries, usually reaches a height of 15-20 m. It has been shown to posses anti-inflammatory (Talwar et al., 1998) insecticidal, (Schumutterer and Ascher, 1987), Jacobson (1989), Randhawa and Parmar, bactericidal (Ara et al., 1989), Antifertility (Jacobson, 1995) and immuno stimulating (Van Dijk et al., 1987), immuno modulating potential (Bose et al., 2007).

Medicinal plants have increasingly become an integral part of the human society in combating various diseases, ranging from skin infection to gastrointestinal problems, since the dawn of civilization. The Neem tree is one of such medicinal plants, and symbolizes all that is wondrous in nature: for every part of the tree has been used as traditional medicine for household remedy against various human ailments from antiquity. In fact, it is considered to be the "village pharmacy" in many parts of India and has played a key role in Ayurvedic medicine and agriculture since time immemorial.

Antifertility potential of Neem has been reported by various researchers but most of the work done has been done in the scientific laboratories of India referring the history and importance of this plant has mentioned that Sadhus of India used to chew Neem leaves to suppress their libido during their meditation (Naqvi, 1998).

In traditional Ayurveda medicine a decoction made from the bark, leaf, root, fruits and flowers is used in the treatment of blood morbidity, biliary afflictions, itching, skin and peptic ulcers. The bitter, astringent bark is applied as a decoction for haemorrhoids. The leaves are steeped for malaria. Neem juice (expressed from the leaves), infusion, or ointment is applied externally to wounds and carbuncles. The twigs are used to clean teeth, firming up gums and preventing gum disease. Neem oil, expressed from the seeds and leaves is commonly used for hair dressing and is believed to be a strong antifungal and antiviral. Neem oil has been used to treat leprosy. The primary objective of this study was to investigate the effects of Neem leaves extract, on female reproductive system of Wistar female rats.

MATERIAL AND METHODS

Animals

Fifteen female Wistar rats were obtained from the Animal House of Lagos State University College of Medicine (LASUCOM), Ikeja, Lagos State, Nigeria. The rats were fed with standard rat chow obtained from Agege Livestock Feed Mills, Agege, Lagos, Nigeria and water ad libertum. The animals were kept in an environment of 12 hours' dark and 12 hours' light cycle and at room temperature. They were allowed to acclimatize for 2 weeks prior to the study. Ethics approval was obtained from Animal Research Ethics Committee of Lagos State University College of Medicine and its rules and regulations for animal experimentation were strictly adhered to.

Preparation of Neem leaves extract

Neem leaves were used for this study. They were gotten from Neem trees at the Lagos State University, Lagos, Nigeria. Upon identification, they were authenticated at the Department of Botany, Lagos state university. Voucher specimen was deposited (accession no LSH 001201) in the Lagos State University Herbarium (LSH). The leaves obtained were air-dried and grinded with few drops of water. Afterwards, it was cooked in olive oil.

Experimental design

The rats were randomly divided into 3 groups. Group A was the control group while groups B and C served as the test groups. Each group consists of 5 rats. Group A received distilled water and while group B (Low dose group) received 0.6 ml of the Neem leaves extract. Group C (High dose group) received 1.2 ml of the aqueous extract of Neem leaves via oral gavage for 6 weeks. After 6 weeks of administration, the rats were anaesthetized using ketamine HCL. Blood samples collected via retroorbital method and cardiac puncture were stored in plain sample bottles for hormonal assay in -20oC until used. Uterus and ovaries removed from each rat were weighed, fixed and stored in 10% buffered formalin for Hematoxilin and Eosin (HE) staining.

Serum Preparation

The collected blood samples in the plain bottles were placed in a tabletop centrifuge (Surgifriend centrifuge, Model SMBO-2, England) and were centrifuged for 20mins at 2500rpm; the separated supernatant was aliquoted by use of micropipettes and placed in a clean Eppendorf tube which was stored at -20°C until used.

Hormonal Assay

The reproductive hormones; Follicle stimulating hormones (FSH), Luteinizing hormones (LH), Estradiol and Progesterone concentrations were determined quantitatively using enzyme linked immune-sorbent assay (ELISA) kits as outlined in the manufacturer's manual as previously described (Ajonuma *et al*. 2017a, Ajonuma *et al*. 2017b). In brief, serum hormone concentrations were then determined from their respective calibration curves. The microwells were formatted for each serum reference. 25ul of enzyme reagent was added to the wells. 100ul of enzyme conjugate was added. The micro wells were swirled gently for 20secs to enable mixing. The micro wells were covered to incubate for 60 minutes at room temperature. After incubation, the content of the micro wells was discarded. 350ul of wash buffer was added and washing was repeated for a minimum of five times and blotted on an adsorbent paper. 100ul of substrate solution was added to each well. The microwells were incubated at room temperature for 20 minutes. 50ul of stop solution was added to each well and was gently mixed for 20 seconds. The solution was read within 30minutes, and each well was read at 450nm using a reference wavelength of 630nm on a STAT Fax 4700 ELISA microwell strip reader (Stat Fax Awareness Technologies, USA).

Haematoxylin and eosin (HE) staining

This was carried out as described by (Ajonuma *et al*. 2005, Ajonuma *et al*. 2017a, Ajonuma *et al*. 2017b). In this procedure, the collected organs previously stored in 10% formalin were cut in slabs of about 0.5cm thick transversely and fixed in 10% formalin for a day after which it was transferred to 70% alcohol to cause the transversely cut tissues to become dehydrated. The tissues were passed through 90% of alcohol and chloroform for different durations before they were

eventually transferred into two changes of molten paraffin wax for 20 minutes each in an oven of 570C. Serial sections were cut using rotary microtome at 5microns. Slides were prepared from these tissues. The slides were then de-waxed and passed through absolute alcohol (2 changes); 70% alcohol and then to water for five minutes. The slides were then stained with haematoxylin and eosin. Photomicrographs of stained slides were captured using a camera attached to a microscope.

Determination of Estrus Cycles of rats

Estrus cycle was determined as earlier described by Ajonuma *et al*. (2018). Briefly, cotton bud swab wetted with normal saline was carefully inserted into the vagina of the restrained rats. The swab was then gently turned and rolled against the vaginal wall and removed. Cells obtained were transferred to a dry glass slide by rolling the swab across the slide. The slides were air dried, and fixed using methanol. It was allowed to dry and then dipped into Field stain A for 15 times and Field stains B for 8 times. The slide was then dipped into distilled water to wash off excess stain and allowed to dry. Each slide was examined under microscope to determine the estrous cycle stages. Vaginal smear of the animals was taken daily.

Statistical Analysis

Data are presented as mean and standard error of mean (SEM). Analysis of variance was used for data assessment and differences between groups were compared using Turkey comparison test. A p-value of ≤ 0.05 was considered significant. Statistical analysis was carried out using Graph Pad Prism Software Version 8.01, Graph Pad Inc. San Diego, CA, USA.

RESULTS

The effect of Neem leaves extract on the body weights of female rats

There was statistical significant difference (P<0.0001) in the weights of rats in test groups compared to the control group.

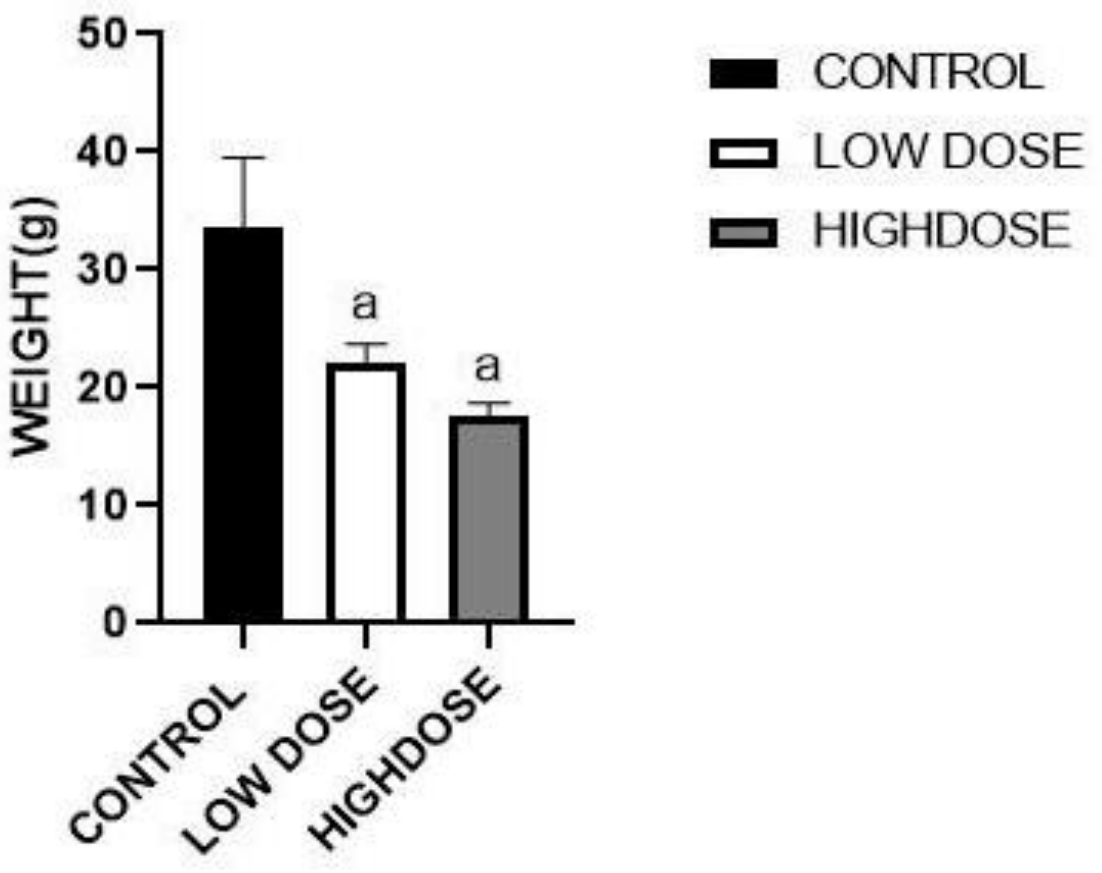

Fig. 1: The effect of Neem leaves extract on the body weights of female rats. [a]P<0.0001, n=5.

The effect of Neem leaves extract on the relative organ body weight ratio of ovary

There was statistical significant increase in the relative ovary weight of rats that were administered Neem leaves extract when compared to control.

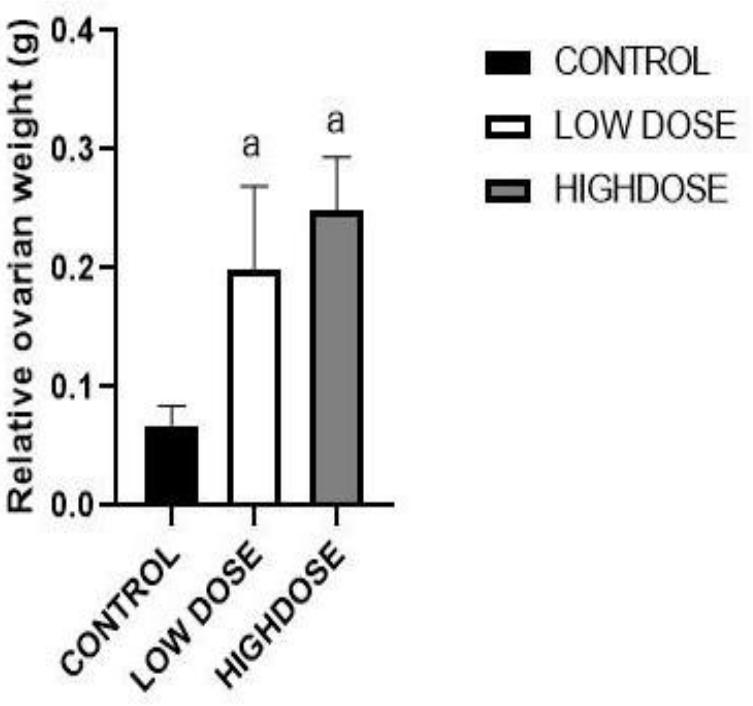

Fig. 2: The effect of Neem leaves extract on the relative ovary weights of female rats. [a]P=0.0002, n=5.

The effect of Neem leaves extract on the relative organ body weight ratio of uterus

There was statistical significant decrease in the relative uterus weight of rats that were administered neem leaves extract when compared to control.

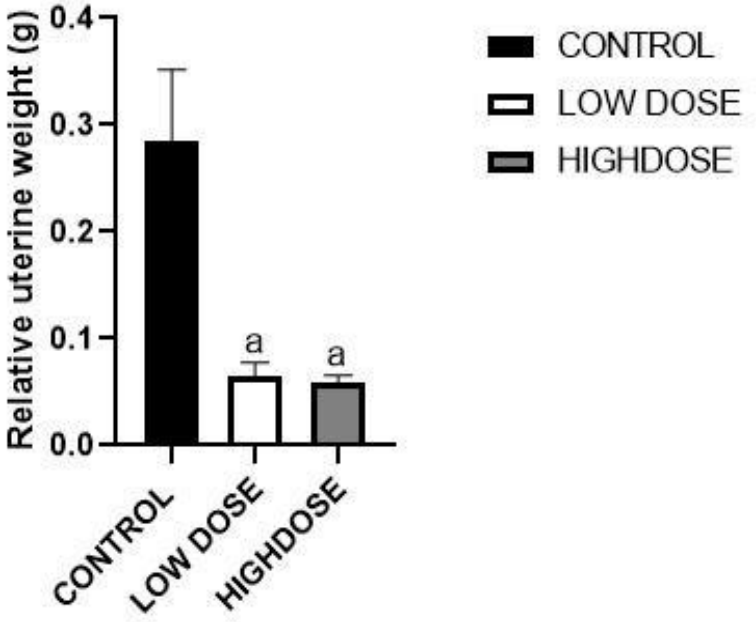

Fig. 3: The effect of Neem leaves extract on the relative uterus weights of female rats. [a]P=0.0001, n=5.

The effect of Neem leaves extract on the serum luteinizing hormone levels in female rats.
There is no statistically significant difference in the serum luteinizing hormone of the test groups when compared to the control group.

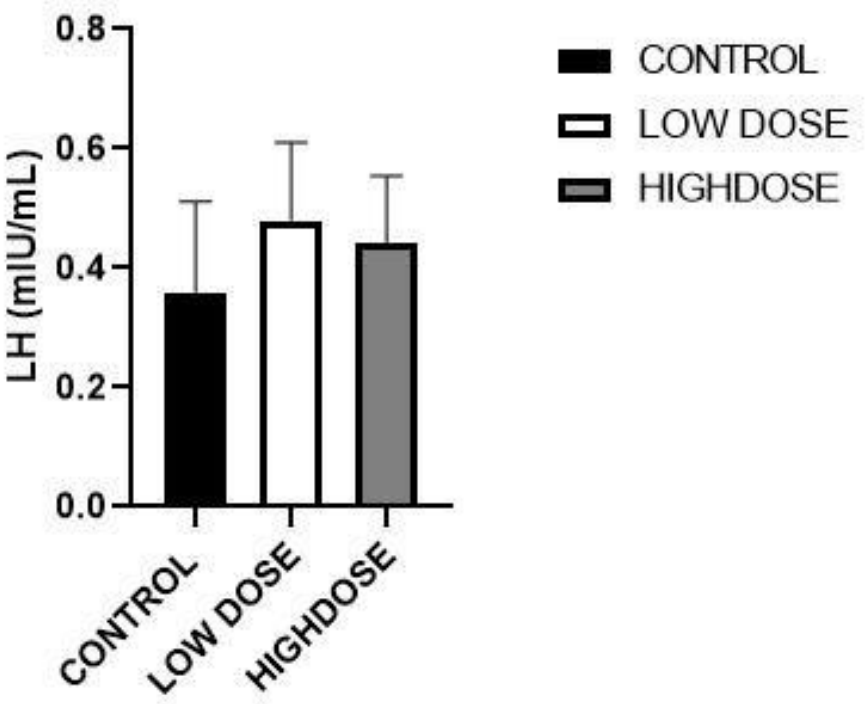

Fig. 4: The effect of Neem leaves extract on the serum luteinizing hormone levels in female rats. p= 0.3799, n=5.

The effect of Neem leaves extract on the serum follicle stimulating hormone levels in female rats.
There is no statistically significant difference in the serum follicle stimulating hormone of the test groups when compared to the control group.

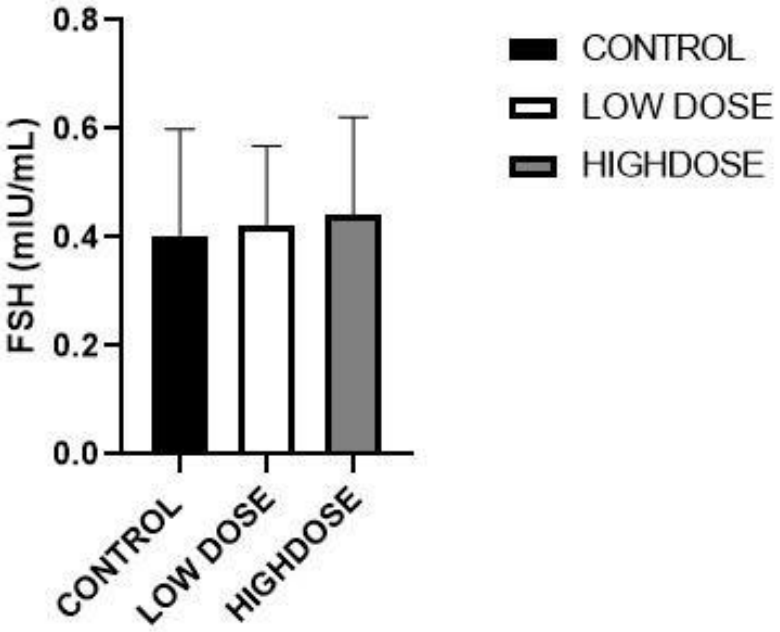

Fig. 5: The effect of Neem leaves extract on the serum follicle stimulating hormone levels in female rats. p= 0.9391, n=5.

The effect of Neem leaves extract on the serum progesterone levels in female rats.
There is no statistically significant difference in the serum progesterone and estradiol levels of the test groups when compared to the control group.

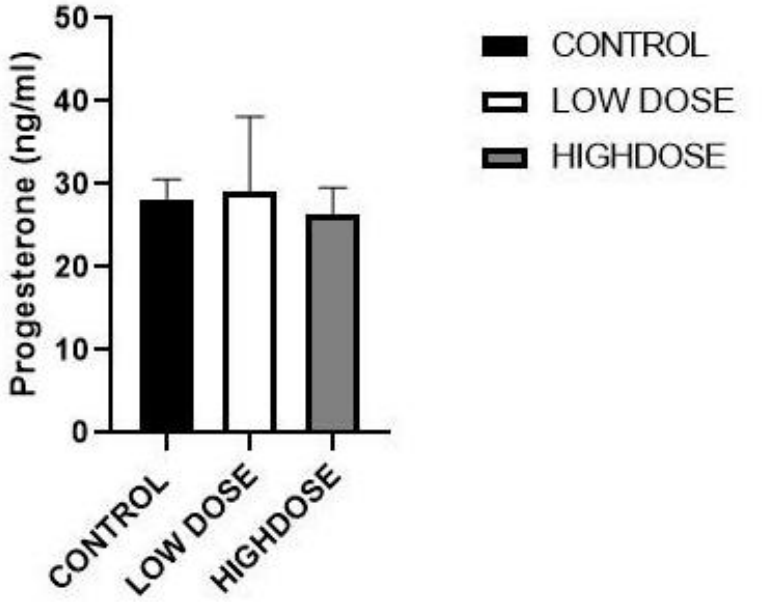

Fig. 6: The effect of Neem leaves extract on the serum progesterone levels in female rats. p= 0.7432, n=5.

The effect of Neem leaves extract on the serum estradiol levels in female rats.
There is no statistically significant difference in the serum progesterone and estradiol levels of the test groups when compared to the control group.

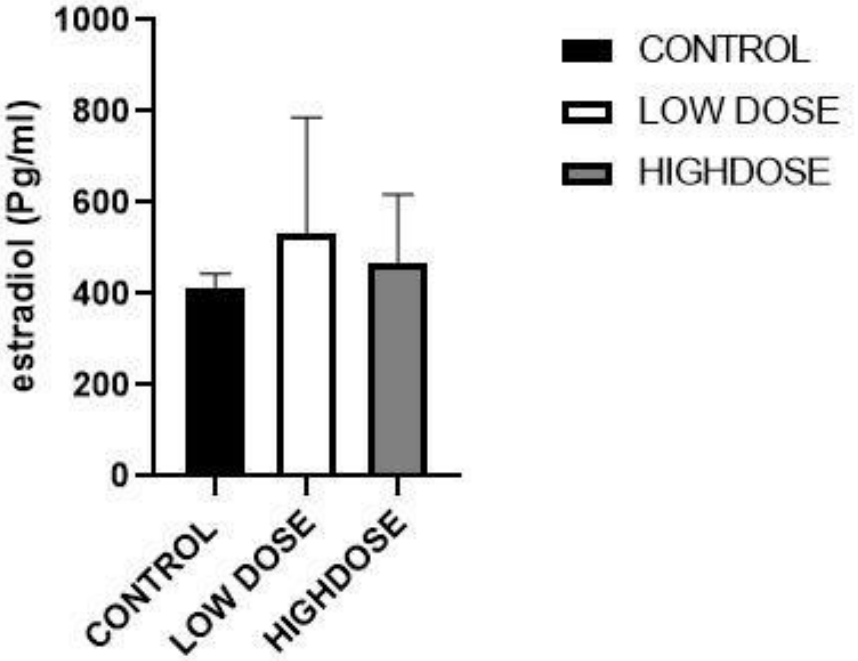

Fig. 7: The effect of Neem leaves extract on the serum estradiol levels in female rats. p= 0.5612, n=5.

The effect of Neem leaves extract on the progesterone-estradiol ratio in female rats.
There is no statistically significant difference on the progesterone-estradiol ratio of the test groups when compared to the control group.

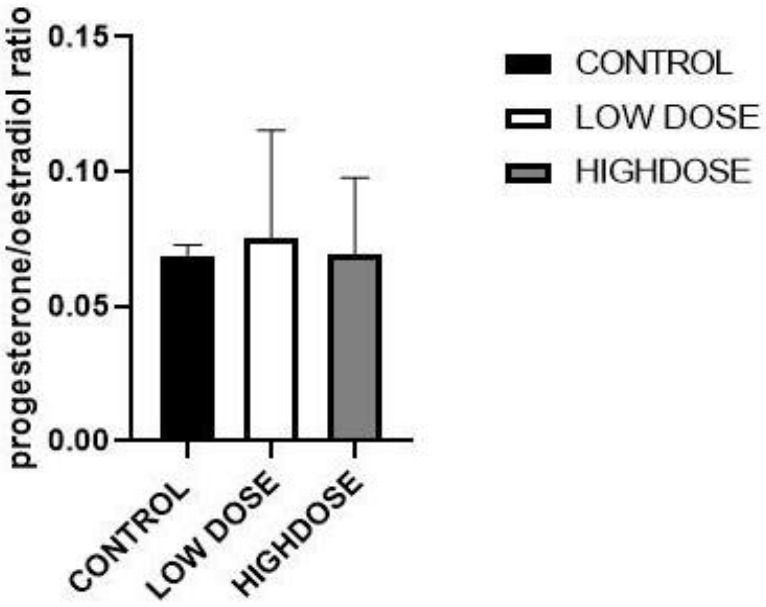

Fig. 8: The effect of Neem leaves extract on the progesterone/estradiol ratio in female rats. p= 0.9255, n=5.

Fig. 9: The effect of Neem leaves extract on the H and E-stained section of the ovary of female rats of Groups A-C
The histology showed loss of ovarian follicle with marked degenerative follicle, the granulosa cells are loss while some have their whole ovarian tissue gone in the test groups. There was mild ovaria toxiciy in the test group.

A B C

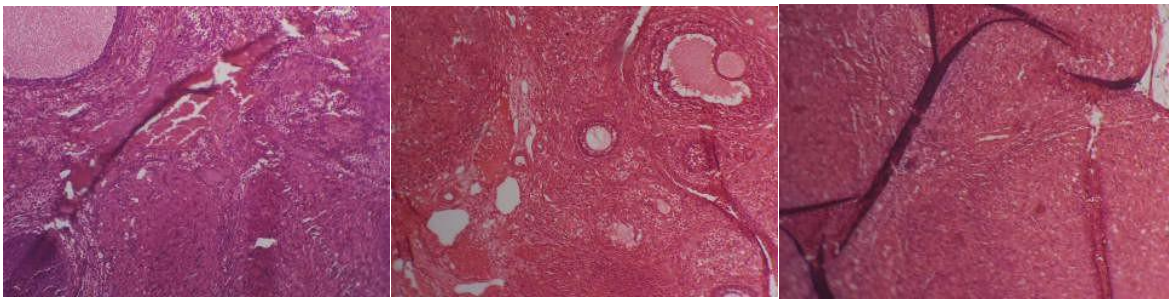

Fig. 10: The effect of Neem leaves extract on the H and E-stained section of the uterus of female rats of Groups 1-3
The uteri were overly dilated with cystic formation, smooth muscle disarray and interface spaces within it. Some have cytoplasmic clearing of the endometrial gland and vacuolation of the glandular epithelium.

A B C

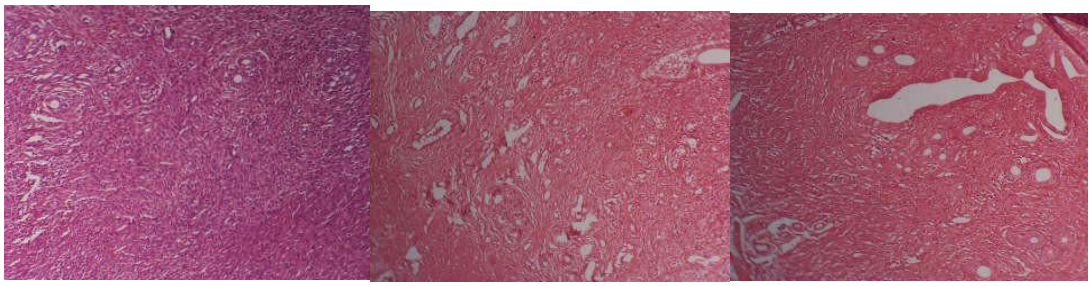

Table 1: The effect of Neem leaves extract on estrus cycle of adult female rats

Administration of Neem leaves extract produced an irregular pattern in the rats' estrus cycle while disrupting cycle. These rats showed a prolonged diestrus pattern in each cycle and the metestrus was rare to that of proestrus. The diestrus was more prominent than the estrus in the cycle. The control followed the normal pattern of the cycle.

	CYCLE 1				CYCLE 2				CYCLE 3				CYCLLE 4				CYCLE 5			
DAY / ANIMAL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CONTROL 1	D	P	E	M	M	M	D	E	M	M	M	D	P	E	E	M	M	D	P	E
CONTROL 2	P	E	M	M	M	D	P	E	M	M	D	P	E	E	M	M	D	P	E	E
CONTROL 3	P	E	M	M	D	P	E	M	M	M	D	P	E	E	M	M	D	P	E	E
CONTROL 4	E	M	M	M	D	D	P	E	M	M	D	P	E	E	M	M	D	P	E	E
CONTROL 5	M	M	M	D	P	E	E	M	M	D	P	E	M	M	D	P	E	M	M	D
LOW DOSE 1	M	E	D	P	E	D	E	E	D	D	D	E	D	D	M	D	D	P	E	E
LOW DOSE 2	P	D	M	E	P	E	E	M	D	D	D	M	D	P	E	D	D	D	E	P
LOW DOSE 3	P	D	E	M	D	D	D	P	E	E	E	D	D	E	D	D	P	D	D	P
LOW DOSE 4	M	D	D	D	P	P	E	E	E	D	D	D	P	E	P	P	D	D	E	E
LOW DOSE 5	D	E	P	E	E	D	E	P	D	D	P	E	E	E	D	D	P	E	E	D
HIGH DOSE 1	M	P	D	E	E	D	D	P	M	D	D	P	D	D	P	D	M	D	P	E
HIGH DOSE 2	D	M	D	M	D	D	D	P	E	D	E	P	E	E	D	E	E	E	E	P
HIGH DOSE 3	D	E	D	D	E	D	M	D	M	P	D	E	E	D	M	P	E	D	M	E
HIGH DOSE 4	D	E	D	D	D	M	P	D	D	E	D	E	M	E	E	E	D	D	P	M
HIGH DOSE 5	M	D	D	P	D	D	M	D	D	E	E	D	D	P	D	D	E	D	E	E

DISCUSSION

The result demonstrates reduction in body weight of rats administered with neem leaves extract. The possible explanation for the decrease in body weight may be due to consistent diarrhoea (watery stool) observed in the animals administered with the extract.

Interestingly, relative ovarian weight was significantly increased in the test groups when compared to control while the relative uterine weight was significantly reduced in test groups when compared to control. The mechanisms involved are not known at the moment and requires further research to elucidate. However, administration of neem leaves extract produced irregular pattern in the rats' estrus cycle. These rats showed a prolonged diestrus pattern in each cycle and the metestrus was rare to that of proestrus. More so, the diestrus was more prominent than the estrus in the cycles it is also not known if the observed non significant increase in estradiol of the test groupsis responsible for the increase ovarian wet weights.

The histology showed marked degenerative ovarian follicles. The granulosa cells were loss while some have their whole ovarian tissue gone in the test groups. There demonstrates ovarian toxicity in the test groups. No viable follicles were noted and they may can no longer give birth to many pups although it was not demonstrated in this study. However, our observations are not the same as those of Upadhyay *et al.*,1992 who reported that Neem leaves extract had no effect on ovarian function.

The uterus was overly dilated with cystic formation, smooth muscle disarray and interface spaces within it. Some have cytoplasmic clearing of the endometrial gland and vacuolation of the glandular epithelium.

In summary, prolonged Neem leaves extract ingestion affects female reproductive organs and alters oestrus cycle. Its prolonged consumption may severely interfere with female fertility.

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