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Gonadal histo-morphologies and serum hormonal milieu in female rats treated with *azadirachta indica* leaf extract

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Abstract

Background: *Azadirachta indica* is a tree with most of the parts having various medicinal values. It is however popular because of its high potencies, as antimalarial and anti-fertility agents, which the locals still exploit.

Objective: We investigated the effect of the methanol leaf extract on the serum levels of the pituitary-gonad hormones and the histo-morphology of the ovary and uterus of adult female rats.

Materials and Methods: Eighteen adult female Wistar rats were divided into three groups (A, B and C) of six animals each. Group A was the control group that received distilled water orally, while groups B and C were the experimental groups that received 200mg/kg and 400mg/kg of the extract respectively by oral intubation for fourteen days. The animals were sacrificed on the fifteenth day, and blood was collected from the left ventricles of the hearts and subsequently spurned in heparinized bottles for serum hormonal assay. The ovaries and the uteri were then dissected out and preserved in Bouin's fluid. Routine haematoxylin and eosin method was used to stain them.

Results: There were significant ($p<0.0001$) lower serum levels of luteinizing hormone (LH) in the treatment groups, especially in the 400mg/kg group, while there were significant ($p<0.0001$) higher progesterone (PH) levels in the treatment groups. The follicle stimulating hormone (FSH) levels were however not different ($p=0.0502$) from the control. The histo-morphologic studies revealed no obvious pathological changes in the ovaries and uteri of the treatment groups.

Conclusion: 200mg/kg and 400mg/kg of methanol extract of the leaf of *A. indica* does not have any obvious effect on the histo-morphologies of the ovary and uterus, but showed significant changes in the serum levels of LH and PH of female Wistar rat, implying that the effect of the extract may have been at a level other than these organs of study.

Key words: *Azadirachta indica, Follicle stimulating hormone, Luteinizing hormone, Progesterone, Ovary, Uterus, Wistar rat.*

Introduction

Azadirachta indica (*A. indica*) commonly called neem is a tree whose parts have diverse medicinal values (1-3). The plant is made up of different alkaloids which include; nimbitin, azadirachtin and salanin (1), whose individual effects contribute to the general properties of the plant. It is documented to have insecticidal properties, as well as, pesticidal abilities (1, 3-7). It also has inhibiting action on a wide spectrum of micro-organisms, including malaria parasites (4,8,9). Its use has also been reported to cause mortality in molluscs and

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mosquitoes (3, 10), hence, its use as pesticides (4, 11, 12).

A. indica leaves and its constituents exhibit immune-modulatory, anti-inflammatory, anti-hyperglycemic, anti-diabetic and anti-carcinogenic properties (2, 13, 14), and can induce cellular immune reactions (15). The bark extract of this tree has been reported to be useful in the control of gastric hyper-secretion, and gastro-esophageal and gastro-duodenal ulcers (16).

Some anti-fertility effects of the leaf extracts of *A. indica* have been reported. The crude leaf extract has been reported to cause reduced serum levels of testosterone and luteinizing hormones (17, 18), and reduced sperm counts with abnormal-shaped spermatozoa (19). Immuno-contraceptive activity (15, 20), inhibition of folliculogenesis (21) and reversible alterations in the male reproductive organ by aqueous leaf extract have also been reported (22).

Most reported effects of neem are centred on research on the seed and oil extracts, and where the leaf extracts are researched, it is mostly on the males. On the other hand, the leaf extracts happens to be the most commonly consumed part of *A. indica* in this part of the world. Hence reports on the seed and oil extracts as researched on the females necessitated this study on the effect of the methanol leaf extracts on the female gonads and the pituitary-gonadal hormone levels.

Materials and methods

This is an original experimental research, and eighteen adult female Wistar rats (*Rattus norvegicus*) were assigned equally into three groups of A, B and C. The animals whose weights ranged 110-150g were acclimatized for two weeks before the commencement of the experiment in the animal house of the Department of Anatomy. The Care and Management of Laboratory Animals was followed strictly after ethical approval was sort from the Ethics Committee of the University. Feeds and water were allowed *ad libitum*. Fresh neem leaves were shade-dried and grounded into fine powder with an electric blender, and the powder weighed to be 220g. Soxhlet extractor using absolute methanol was employed in the extraction leaving a stock solution of 40g.

Briefly, 220g of *A. indica* (neem) powder was homogenized by blending it with 220ml of absolute methanol. The mixture was allowed to stand for 48hrs in the refrigerator at 4^{o}C for thorough extraction of the plants' active components. These were then filtered with cheese cloth, and later with Whatman No. 1 filter paper to obtain a homogenous filtrate. These filtrates were then concentrated *in vacuo* at 37- 40^{o}C to about one-tenth the original volume using a rotary evaporator. The concentrates were allowed open in a water bath (40^{o}C) for complete dryness yielding 40g of brown oily *A. indica*. The extracts were then refrigerated at 4^{o}C until use. Distilled water was used to dilute this stock solution to concentrations of 200mg/kg and 400mg/kg.

Group A animals served as the control group and the animals received distilled water, while groups B and C were the experimental groups, and were treated with 200mg/kg and 400mg/kg of the leaf extract respectively for fourteen days. The administration was by oral intubation and was done in the mornings.

Administration began when the animals were on di-estrous 1, while the animals were sacrificed on a pro-estrous after the last dose.

The animals were sacrificed on the fifteenth day of the experiment by chloroform anaesthesia. Blood were collected from the left ventricles of the hearts and stored in heparinized bottles for hormonal assay. Luteinizing hormone (LH), follicle stimulating hormone (FSH) and progesterone (PH) in serum were determined using the Enzyme-Linked Immuno-absorbent Assay (ELISA) method with Microwell's kit. The ovaries and uteri were dissected out. Their morphologies observed, and subsequently preserved in Bouin's fluid, and thereafter routine haematoxylin and eosin staining method was applied to stain them for histo-morphology study.

Statistical analysis

Using analysis of variance (ANOVA) was used to compare results from the experimental groups and the control group. P-values <0.05 were reported to be statistically significant.

Results

Hormonal assay

Luteinizing hormone (LH): The serum LH levels of groups B and C treated with 200mg/kg and 400mg/kg respectively of *A. indica* leaf extract were significantly ($p<0.0001$) lower compared with the control.

Follicle stimulating hormone (FSH): FSH level of groups B and C. treated with 200mg/kg and 400mg/kg respectively of *A. indica* leaf extract were not significantly ($p=0.0502$) different from the control.

Progesterone (PH): The serum progesterone levels were significantly ($p<0.0001$) higher in the groups treated with 200mg and 400mg/kg of *A. indica* leaf extract compared with the control (Table I).

Histo-morphology

Ovary: The control showed normal ovarian morphology. Histological sections showed the covering epithelium (tunica albuginea), cortex and medulla. The stroma was made up of reticular fibres and fusiform cells with follicular cells at different stages of development. Group B treated with 200 mg/kg of *A. indica* leaf extract showed primordial, atretic and matured Graafian follicles, as well as, corpus luteum with no observable adverse effect compared with the control. Group C treated with 400 mg/kg of *A. indica* leaf extract showed a normal section that appeared like group B compared with the control.

Uterus: The control revealed normal uterine morphologic features. Histological section showed the endometrium made up of a single layer of columnar epithelium. Deep to it, was the lamina propria which connected it with the compactly arranged smooth muscle layer, the myometrium. The myometrium was surrounded by the serosa and deep to it a rich network of blood vessels. Groups B and C showed similar morphologic features compared with the control. Histological section showed no obvious pathology compared with the control.

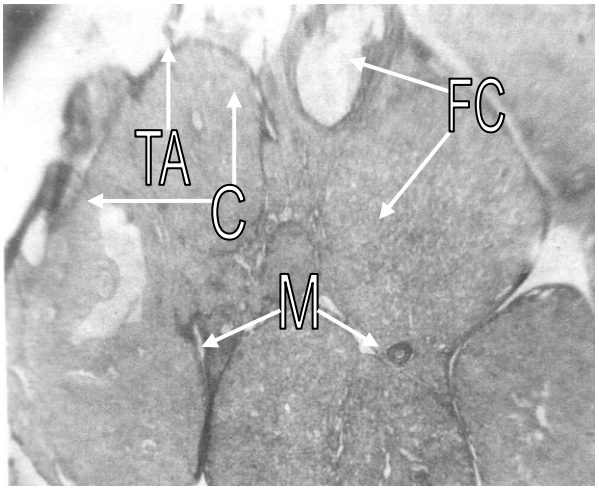

Figure 1. Photomicrograph of the control section shows normal ovarian histology. The covering epithelium (tunica albuginea), TA, cortex, C and medulla, M. The stroma being made up of reticular fibers and fusiform cells with follicular cells, FC at different stages of development. Mag ×400. H & E.

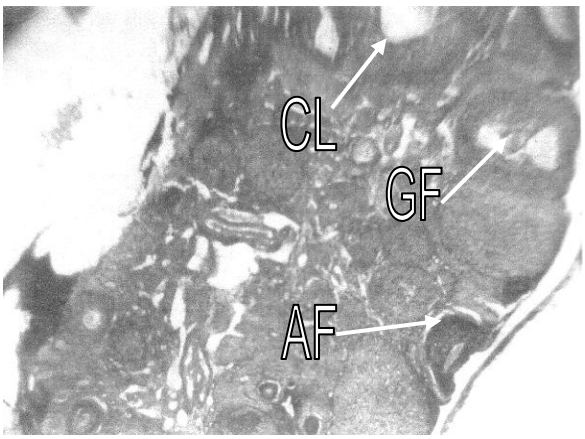

Figure 2. Photomicrograph of group B section treated with 200mg/kg of *A. indica* leaf extract shows primordial, atretic, AF, and matured Graafian follicles, GF, as well as corpus luteum, CL, with no observable adverse effect Mag ×400. H & E.

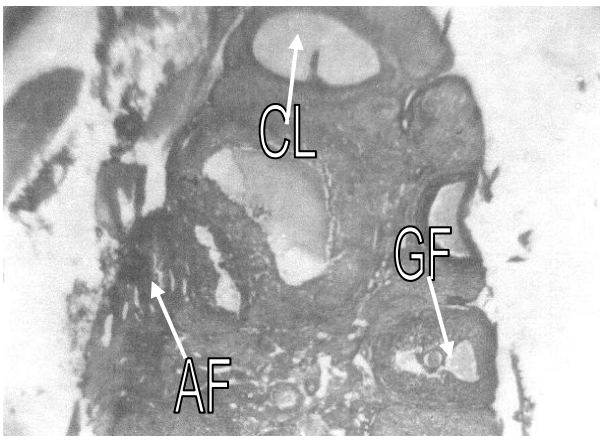

Figure 3. Photomicrograph of group C section treated with 400mg/kg of *A. indica* leaf extract shows primordial, atretic, AF, and matured Graafian follicles, GF, as well as corpus luteum, CL, with no observable adverse effect. Mag ×400. H & E.

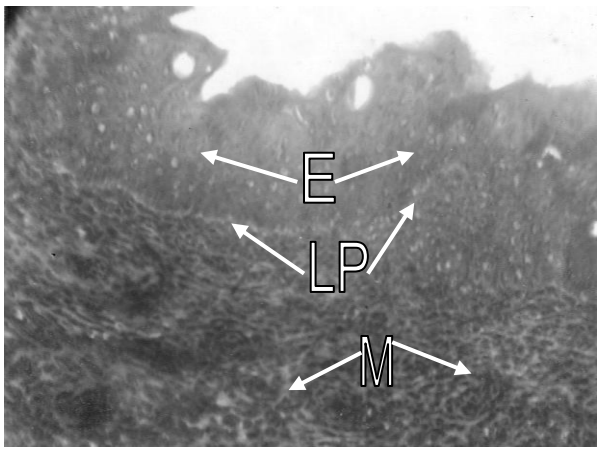

Figure 4. Photomicrograph of the control section shows normal uterine histology, the endometrium, E, was made up of a single layer of columnar epithelium and deep to it, the lamina propria LP, which connects it with the compactly arranged smooth muscle layer, the myometrium, M. Mag ×400. H & E.

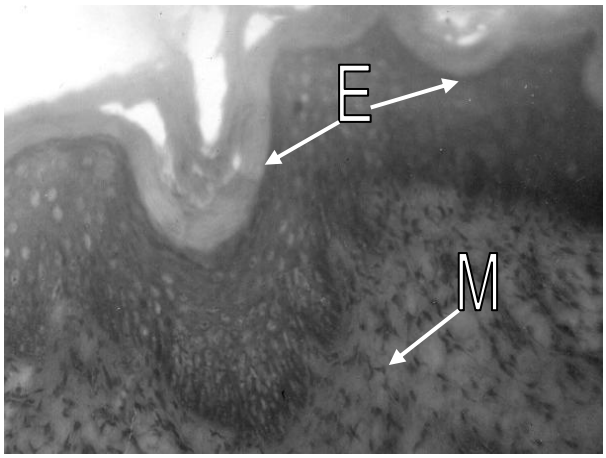

Figure 5. Photomicrograph of group B section treated with 200mg/kg of *A. indica* leaf extract shows endometrium, E, and myometrium, M, with no observable adverse effect. Mag ×400. H & E.

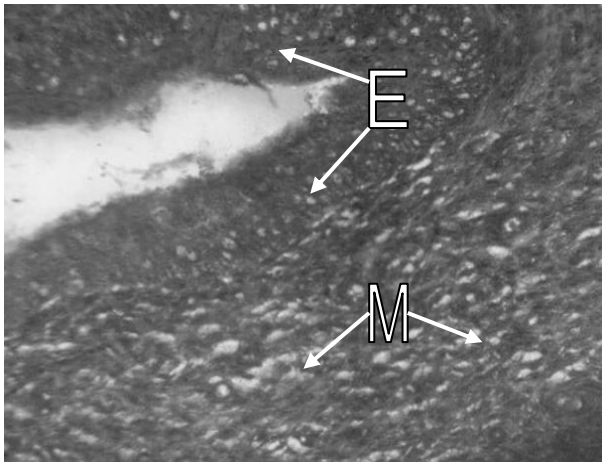

Figure 6. Photomicrograph of group C section treated with 200mg/kg of *A. indica* leaf extract shows normal endometrium, E, and myometrium, M, with no observable adverse effect. Mag ×400. H & E.

Table I. The serum hormonal levels of LH, FSH and PH in the control, 200 mg/kg and 400 mg/kg of *A. indica* groups.

Hormones/ Group	A Control	B 200mg/kg of *A. indica*	C 400mg/kg of *A. indica*
LH	1.24±0.09	0.62±0.18***	0.34±0.29***
FSH	0.34±0.11	0.24±0.003 ^{NS}	0.26±0.04 ^{NS}
PH	30.4±2.48	40.0±0.45***	40.2±1.99***

n=6
Result are presented as mean± SD
^{NS}: Not significantly different from control at p>0.05
***Significantly different from control at p<0.001
LH: Luteinizing hormone
FSH: Follicle stimulating hormone
PH: Progesterone
A. indica: *Azadirachta indica*

Discussion

This study investigated the effect of methanol leaf extract of *Azadirachta indica* on the serum levels of LH, FSH and PH, and the histo-morphology of the ovary and uterus of female Wistar rats. The ovary and uterus are important organs in the female reproductive system. The ovary is the female sex organ and site for oogenesis and estrogen secretion, while the uterus functions in implantation and nourishment of the developing zygote (23).

This process is influenced by FSH and LH of the anterior pituitary, as well as, PH and estrogen produced by the sex organs (24). A normal histo-morphology of the ovary and uterus and normal levels of these hormones is indicative of the normal sexual processes in the female. In this study, the animals were sacrificed on the proestrous phase when the serum PH level is expected to be at its peak (25, 26). The observed higher levels of serum PH in the experimental groups may have resulted from the neem leaf extract which may have fastened the oestrous cycle. This probably may be the reason for the presence of atretic follicles and corpora lutea in the histology of the experimental groups compared with the control.

The result of this study revealed no pathological effects in the ovaries and uteri of the rats treated with 200mg/kg and 400 mg/kg of *A. indica* leaf extract. This is in line with Upadhayay *et al* (27) who reported normal uterine and ovarian morphologies, and functions with the seed oil extract of *A. indica*. This also corroborates the work of Prakasha *et al* (28) who earlier reported normal histo-architecture of the uterus of rats treated with neem oil extract.

FSH indirectly stimulates gametogenesis in both sexes and directly stimulates estrogen synthesis and follicular development. It also maintains the structure of the gonads in conjunction with LH (30). On the other hand, LH is critical to luteinization of the ovarian follicles and post-ovulatory follicular function (24). Ovulation occurs as a result of the "LH surge" which takes place from the onset of oestrous (24-26) and decreases after ovulation due to the increase in the serum level of PH. During post-ovulation, the ruptured follicle is stimulated by LH to become near structure, corpus luteum, which secrets both estrogen and PH (23). When this occurs, there is a negative feedback inhibition of FSH and LH (30).

In this study, LH levels were significantly ($p<0.0001$) lower, while PH levels were significantly ($p<0.0001$) higher in the two treatment groups treated with 200mg/kg and 400mg/kg of *A. indica* leaf extracts. No difference existed in the FSH serum level of the treatment groups compared with the control. This may suggest that 200mg/kg and 400mg/kg of methanol neem leaf extracts stimulated ovulation, which may have resulted in subsequent stimulation of PH synthesis, and a resulting inhibition of the LH surge. This study is in line with Ragi *et al* (18) who reported reduction in serum LH levels after treatment with extract of *A. indica*, while Prakasha *et al* (28) reported that neem oil did not possess any estrogenic, anti-estrogenic or progestational activity, while appearing not to have interfered with the actions of progesterone.

This study is however at variance with reports by Gbotolorun *et al* (31), Roop *et al* (21) and Upadhyay *et al* (27). They reported folliculogenesis inhibition, prolonged diestrous and partial blockage of ovulation, as well as, enhanced antigen-presenting ability of the uterus with seed and oil extracts of *A. indica.* Significant damage to the luminal epithelium of the uterus and to the uterine glands, with decreased glycogen and total protein contents in the ovary and uterus, has also been reported on administration of neem oil to cyclic and ovariectomized rats (32), while Mandal and Dhaliwal (33) reported alterations in morphologies and functions of the uterus in rats treated with the seed extract of neem.

In conclusion, 200mg/kg and 400mg/kg of methanol extract of the leaf of *A. indica* does not have any obvious effect on the histo-morphologies of the ovary and uterus, but showed significant changes in the serum levels of LH and PH of female Wistar rat, implying that the effect of the extract may have been at a level other than these organs of study.

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