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Neem Leaves Extract Reduces Sex Steroids and Gonadal Function in Female Wistar Albino Rats

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The neem plant (Azadirachta indica) is one of the most important medicinal plants in Asia and Africa. This study aimed to investigate the possible anti-fertility potential of dietary neem leaves' extract in female Wistar albino rats as. This model could be further applied to stray dogs. In this experiment 24 adult female albino Wister rats were divided into two groups: control and treated with neem leave extract, they were tested for estrous regularity by vaginal smears, progesterone, and estrogen hormone were measured. Ovarian caspase-3 expression and histopathological inspection were examined. The results have revealed reducing sex steroids, increment in ovarian caspase-3 expression, and histological deteriorations in the ovary and uterus. The study concluded that neem flower extract can be used as an antifertility agent in animals.

Keywords: Antifertility; Female; Neem; Reproduction.

One of the most serious global problems is stray dogs overpopulation, which has an adverse impact on public health, the environment, and communities, various zoonoses have developed and become endemic as a result of this problem [1]. Thus, it is important to search for a new effective method with low side effects to solve this problem, such as medicinal plants with antifertility effects. Moreover, the human population explosion is considered a major cause of human beings' suffering and environmental squalor all over the world [2]. Thus, it is important to search for a new effective method with low side effects to solve this problem. Synthetic contraceptives possess an effective role in solving such problems however, they are not safe as they may cause allergies or heart attack [3].

Since ancient times, herbs have been reported for their useful remedial influences against various disease conditions owing to their pharmacological belongings[4]. Therefore, plants have provided humans with new medicinal solutions for thousands of years, acting as a basis for traditional medical systems worldwide[5].

Herbs could be used as an alternative to drugs of synthetic origin and they have gained a recent popularity among developing countries due to their affordability, availability and abridged side/ toxicity influences [6,7]. A variety of medicinal plants that were previously proved to have antifertility action could be industrialized into contraceptives for both females and males[8]. They can cause estrous cycle disruption, anti-estrogenic effects, anti-implantation influence, or even abortion [9].

One of the most significant medicinal plants found in Asia and Africa is neem (*Azadirachta indica*), which is rich in proteins and trace minerals, has Anti-inflammatory and Antioxidant effects, and can be used to treat a variety of animal parasites, bacteria, and viruses [10]. Additionally, fresh neem leaves contain polyphenolic flavonoids with antibacterial and antifungal effects, Furthermore, neem seeds contain beneficial components like azadirachtin and gedunin [11]. Gbotolorun, Osinubi, Noronha and Okanlawon [12] detected that the antifertility effects of the neem flower extract on adult female rats, causing disruption of the estrous cycle and a partial blockage in the ovulation. Patil Patil, Shirahatti, VB, Ramu and Prasad [13] detected that the consumption of *A. indica* does not cause any harm to the entire reproductive system, nevertheless, has the ability to be used as a temporary or reversible contraceptive. This study aimed to investigate the possible anti-fertility potential of dietary neem leaves' extract in female Wister albino rats as a model to be further applied to stray dogs.

MATERIAL AND METHODS

Plant extract

Creation of crude aqueous neem extract

According to Mamoon-ur-Rashid, Abdullah and Hussain. [14], the *Azadirachta indica* tree leaves were dried, then grinding and homogenized with distilled water in an electric blender, and triple-folded gauze was used to filtrate the homogenate. Before usage, a rotary vacuum evaporator was used to evaporate the solvent, then 70% dilation of the extracts was prepared.

Experimental animals

A total of 24 mature female rats (5.5–6 months; 180–200 g) of Wistar strain were castoff in this study. Animals were acquired from Laboratory Animal House, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. They were housed in polyethylene cages; four females per cage at room temperature (25°C ± 1°C) and natural daylight cycles. Water and feed were offered ad libitum. Rats were kept 1 week to adapt. The experimental producers adhered to the ethical rules for the utilization of animals in laboratory settings at the Faculty of Veterinary Medicine, Suez Canal University, Egypt (SCU-VET 2024032).

Reproductive procedures

After 1 week of adaptation, daily cytological inspection of vaginal smears was done to detect the estrous cyclicity progression and regularity. Females that revealed two successive regular cycles were designated for the current study and others were excluded.

Design of the experiment

Twenty-four regular cyclic females were split equally into two groups; G (I): control group (n = 12) was fed a basal diet misted with 53 mL distilled water and G (II): neem extract group (n = 12) that was fed basal diet misted with 3 mL neem leaves extract mixed with 50 mL distilled water with a dose 10 mL neem leaves extract / kg diet. A total of 200 g diet was offered / cage. The experimental diet was offered daily for 30 days.

Estrous regularity

After 15 days from the start of the experiment, vaginal smears were smeared from individual rats every 12 hours to detect the average duration/hours for each estrous cycle phase for two consecutive cycles.

Sampling

At the end of 30 days of treatment, 3 females representing each stage of the cycle were sacrificed. Blood was drawn from retro-orbital venous vessels allowed to clot then centrifugated at 3000 rpm to obtain serum. Serum was kept at -70^{o}C. The uterus and ovaries of each female were dissected and weighed.

Feed intake and relative sex organs weights

Final body weights were recorded. The feed intake for rat each experimental animal was calculated. The food remaining was subtracted from the offered food then the obtained value was divided by the number of rats per cage. The dissected ovaries and uteri were weighed, and their relative weights were obtained as follows (organ weight/body weightX100).

Sex steroids levels

Serum levels of 17-â estradiol of females in the follicular phase of the cycle (proestrus and estrus phases) and serum progesterone levels in the luteal phase of the cycle (metestrus and diestrus) were determined using Kamiya Biomedical Company, ELISA kits (USA). Both analyses were performed according to the enclosed pamphlet instructions.

Histopathology

Part of the dissected uteri and ovaries were put in a 10% formalin solution. Afterward, they were immersed into paraffin wax and stained by hematoxylin and eosin[15].

Ovarian Caspase-3 expression

One ovary/ animal was kept at -80^{o}C until RNA extraction has proceeded. The frozen ovaries were subjected to RNA extraction using a total RNA extraction kit (QIAGEN, Maryland, USA), as mentioned in pamphlet of the manufacturer. The complementary DNA was synthesized using (Thermo Fisher Scientific Inc., Lithuania) kit according to manufacturer's instruction. Gene expression of ovarian caspase-3 against the housekeeping gene â-actin following He, Sun and Huang [16] primer sequence and methodology. Primer for caspase-3 was Forward: 52 -GTGGAACTGACGATGATATGGC-32 and reverse: 52 -CGCAAAGTGACTGGAT GAACC-32 . Primers for â-actin were Forward: 52 -AAGATCCTGACCGAGCGTGG-32 and reverse: 52 -CAGCACTGTGTTGGCATAGAGG-32 . Fold change 2-ÄÄCt was implemented to estimate the levels of gene expression of caspase-3.

Statistical Analysis

The results obtained in the present work were calculated by student t-test. Then the data were expressed as a mean ± standard error (mean ± SE), where p d" 0.05 is considered statistically significant. All of the analyses were carried out using the R programming language[17].

RESULTS

The Shapiro-Wilk test for univariate normality showed that the data were normally distributed (p>0.05).

Estrous regularity

Experimental rats had significantly (p<0.05) longer diestrus phases in the neem leaves extract administered group compared to controls. While these rats had no significant differences in the duration of proestrus, estrous, and metestrus phases (Table 1).

Data was expressed as a mean ± standard error (mean ± SE) then data was analyzed by student t-test using analyses carried out using the R programming language. NS means that neem leaves extract group was non significantly varied (p>0.05) than the control group.* means there was a significant difference at p<0.05. Control group (n = 12) was fed a basal diet misted with 53 mL distilled water. Neem extract group (n = 12) was fed a basal diet misted with 3 mL neem leaves extract mixed with 50 mL distilled water with a dose 10 mL neem leaves extract / kg diet.

Feed intake and relative sex organs weights

Female rats treated with neem extract had a mean rat feed intake of 24.42 (g/day),

Table 1. Effect of neem leaves extract on estrous cycle duration of female albino rats

	Proestrus (h)	Estrous (h)	Metestrus (h)	Diestrus (h)
Neem extract treated group	11^{NS} ± 0.93	20.33^{NS} ± 0.95	10.50NS ± 0.50	75.17* ±3.43
Control group	11 ±0.52	19.67 1.20	10.33 0.33	63.67 3.88

Table 2. Influence of neem leaves extract on rats' relative ovarian and uterine weights

	Ovarian relative weight (%)		Uterine relative weight (%)	
	Follicular phase	Luteal phase	Follicular phase	Luteal phase
Neem Extract treated group	0.063^{NS} ± 0.003	0.068^{NS} ± 0.004	0.493 ^{NS} ± 0.018	0.490^{NS} ± 0.011
Control group	0.066 ± 0 0.02	0.077 ± 0.004	0.518 0.026	0.485 0.006

compared to 24.23 (g/day) in the control group. The level of rat feed intake in treated female rats is approximately the same and non-significantly (p0.05) altered as matched to controls (Figure 1). Concerning ovarian and uterine relative weights, there were non-significant (p>0.05) differences observed between neem leaves extract group and the control one either in follicular or luteal phases of the cycle (Table 2).

Data is expressed as a mean ± standard error (mean ± SE) then data was analyzed by student t-test using analyses were carried out using the R programming language NS means that Neem leaves extract group was non significantly varied (p>0.05) than control group. Control group (n = 12) was fed a basal diet misted with 53 mL distilled water. Neem extract group (n = 12) was fed a basal diet misted with 3 mL neem leaves extract mixed with 50 mL distilled water with a dose 10 mL Neem leaves extract / kg diet.

Sex steroids levels

Female rats treated with neem leaves extract had a mean serum estradiol content of 16.45 (pg/mL), compared to 25.26 (pg/mL) in the control group. The level of estradiol in treated female rats is approximately 34.88% lower and significant (p<0.05) as compared to controls (Table 3).

Female rats treated with neem leaves extract had a mean serum progesterone content of 7.83 (ng/mL), compared to 12.70 (ng/mL) in the control females. In comparison to controls, the level of progesterone in treated female rats is approximately 38.35% lower and significant (p<0.05) (Table 2).

Data is expressed as a mean ± standard error (mean ± SE) then data was analyzed by student t-test using analyses carried out using the R programming language. * means there was significant difference at p<0.05. Control group (n = 12) was fed a basal diet misted with 53 mL of distilled water. Neem extract group (n = 12) was fed a basal diet misted with 3 mL neem leaves extract mixed with 50 mL distilled water with a dose 10 mL of neem leaves extract / kg diet.

Histopathology

Microscopically, the ovary of control rats in estrus (CE) was covered externally by a single cuboidal cells layer, germinal epithelium. Additionally, a thick layer of connective tissue rich in fibroblasts; tunica albuginea, was observed beneath the covering epithelium. The cortical region revealed various follicular stages along with stromal cells interspersed in between. The most numerous types of ovarian follicles were primordial follicles that mainly formed by a primary oocyte covered by a monolayer of squamous granulosa cells. Within the ovarian cortical tissue, the primary follicles were recorded. It was larger in

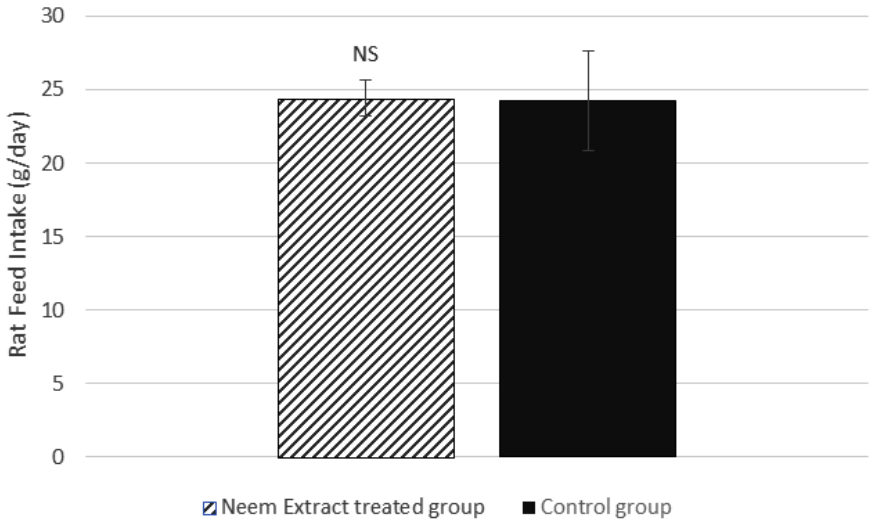

Fig. 1. Effect of neem leaves extract on rats' feed intake. Data is expressed as a mean ± standard error (mean ± SE) then data was analyzed by student t-test using analyses carried out using the R programming language. NS means that neem leaves extract group was non significantly varied (p>0.05) than the control group.

size than the primordial one. It was formed by a larger primary oocyte covered by a monolayer of cuboidal granulosa cells. Late and early stages of secondary follicles were noted, whereas the largest primary oocytes were bounded by numerous layers of granulosa cells that are polyhedral in shape with the various fluid-filled places among the granulosa cells that are covered outwardly by theca cells. Those multiple spaces were merged with each other founding a single big antrum (Figure 3). On the opposite, the ovarian tissue of females fed neem extract in estrus (NE) displayed the same histoarchitecture as that of CE with some differences; the cortical tissue was composed mainly of dense irregular connective tissue rich in fibroblasts and other connective tissue cells that surrounds the ovarian follicles, the follicular developmental stages were declined than that of CE, and finally, great numbers of blood capillaries were evident within the ovarian medulla (Figure 3).

Uterine tissue sections that stained with H&E of CE showed inner folded endometrial mucosa (simple columnal epithelium and lamina propria-submucosa contained uterine glands), myometrial middle (outer longitudinal bundles and inner circular of smooth muscles along with stratum vascular separating) and outermost perimetrium (contained simple squamous epithelium surrounding a connective tissue layer) (Figure 3). The uterus of NE displayed the same histological structures as CE, meanwhile the most prominent uterine findings in NE were the uterine epithelial stratification, few or no uterine glands were recorded in the lamina propria-submucosa and proliferation of blood capillaries was observed in perimetrium (Figure 3).

During the luteal phase, the ovarian sections of control females during metestrus (CM) demonstrated highly active and well-developed corpora lutea with abundant granulosa lutein cells enclosed by a well-vascularized connective tissue

Table 3. Effect of neem leaves extract on serum levels of sex hormones in female albino rats

Group	Serum estradiol (pg/mL)	Serum progesterone (ng/mL)
Neem Extract treated group	16.45* ± 1.39	7.83* ± 1.09
Control group	25.26 ± 2.30	12.70 1.43

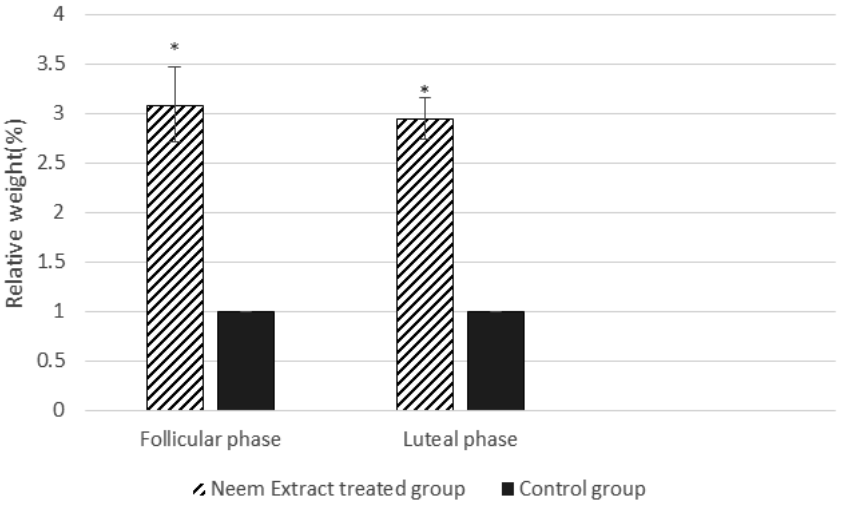

Fig. 2. Effect of neem leaves extract on fold change expression of ovarian caspase-3 in female albino rats. Data is expressed as a mean ± standard error (mean ± SE) then data was analyzed by student t-test using analyses were carried out using the R programming language. * means there was significant difference at $p<0.05$

capsule (Figure 4). However, the ovarian tissue of the neem group (NM was intermingled and characterized by rudimentary ovarian follicles, a huge amount of connective tissue with connective tissue cells and the proliferation of blood capillaries (Figure 4). It was interesting to note that the ovary of both CM and NM groups was covered externally by a single layer of flat cells, germinal epithelium.

The uterine tissue sections of CM and NM showed the same histo-architecture as follows; inner endometrium (simple columnal epithelium and lamina propria-submucosa contained uterine glands), middle myometrial layer (longitudinal bundles and inner circular outer of smooth muscles along with stratum vascular separating) and outer perimetrium (contained simple squamous epithelium surrounding a connective tissue layer) (Figure 4).

Caspase-3-fold change expression

Figure (2) revealed a significant increase ($p<0.05$) in the fold change mRNA of ovarian caspase-3 in the neem leaves extract group as compared to control during the luteal and follicular phases of the estrous cycle.

DISCUSSION

Neem (*Azadirachta indica*) is recognized as a highly potent natural contraceptive [18]. Employing A. indica as a contraceptive offers several benefits due to its safety, effectiveness, and reversibility in both genders [19]. The contraceptive properties of A. indica are linked to its capacity to interfere with various aspects of the female reproductive system, such as altering hormonal balance [20], causing irregularities in the estrous cycle [21], and inducing apoptosis in ovarian cells [7], while having no adversative properties on productive performance [22-24].

The usage of chemical or synthetic contraceptives could also alter the hormonal

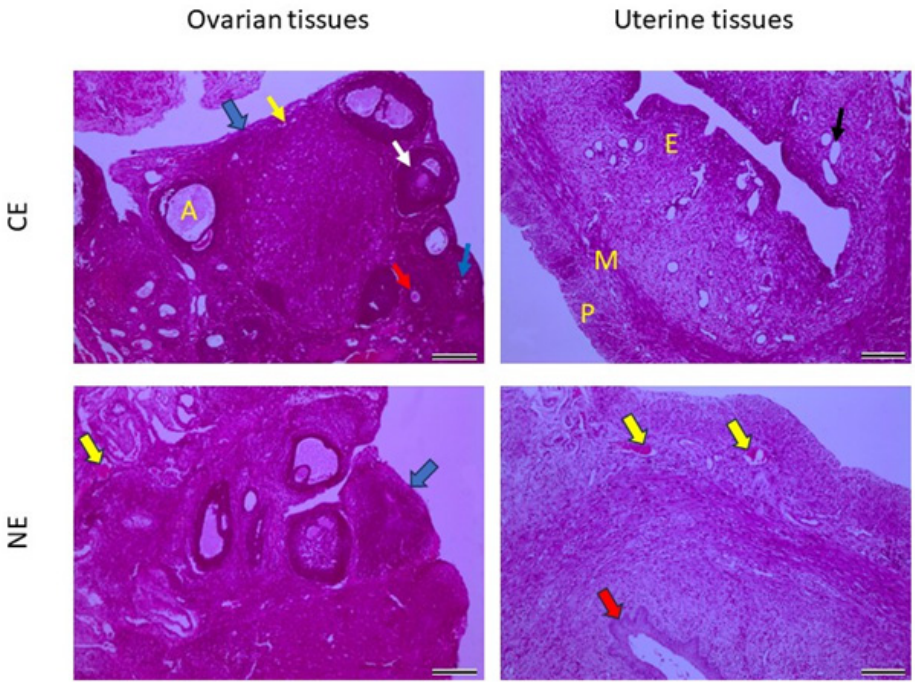

Fig. 3. Representative photomicrographs of H&E-stained ovarian and uterine tissues sections during estrus phase of follicular stage of the estrous cycle. Germinal epithelium (thick blue arrow), tunica albuginea (thin yellow arrow), primordial follicle (thin blue arrow), primary follicle (thin red arrow), secondary follicles (thin white arrow), single large antrum (A), proliferation of blood capillaries (thick yellow arrows), inner endometrium (E), uterine glands (thin black arrows), middle myometrium (M), outer perimetrium (P), epithelial stratification (thick red arrow). Scale bars=50µm

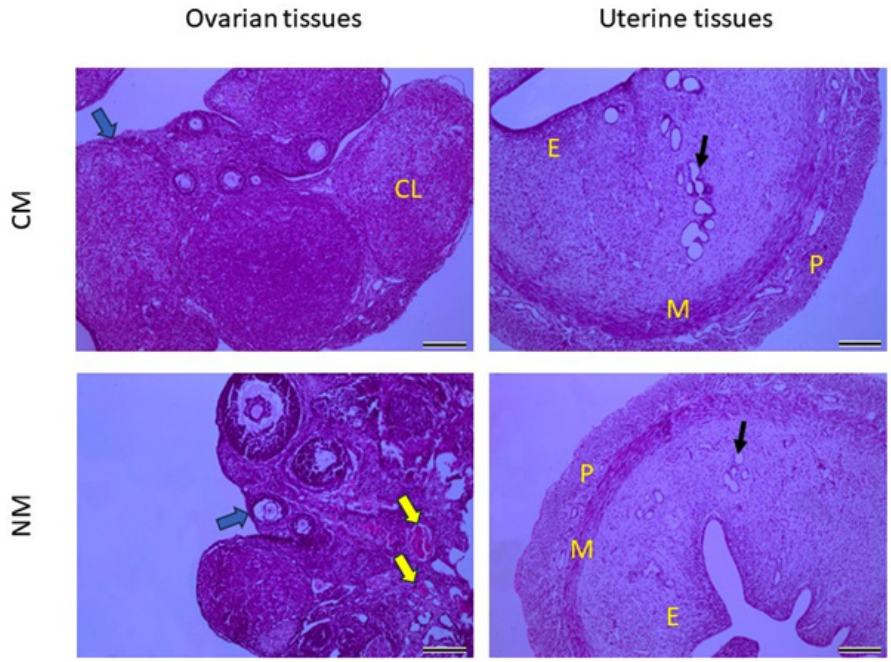

Fig. 4. Representative photomicrographs of H&E-stained ovarian and uterine tissues sections during metestrus phase of luteal stage of the estrus cycle. Corpora lutae (CL), proliferation of blood capillaries (thick yellow arrows), inner endometrium (E), uterine glands (thin black arrows), middle myometrium (M), outer perimetrium (P). Scale bars=50µm.

balance and reproductive cycle that impede ovarian and uterine functions [25]. Among these effects; are the suppression of folliculogenesis, follicle development and suppression of ovulation. Moreover, most of them are hormones that interact with hypothalamic endocrine neurons to suppress pituitary gonadotropins [26]. The usage of these contraceptives could produce myriads of side effects such as hypertension, breast cancer susceptibility, dyslipidemia and susceptibility to thrombosis [27] and cardiovascular diseases [28] that made herbal contraceptives a safe alternative.

Our treated rats showed that A. indica extract significantly prolongs the diestrus phase without altering the durations of the proestrus, estrus, and metestrus phases. Such a result aligned with that of Mamoon-ur-Rashid, Abdullah and Hussain [12] who declared prolonged diestrus in female rats that were given 1 g/kg body weight of alcoholic neem flower extract with a total 80% estrous irregularity percentage. Auta and Hassan [29] found that neem extract administration at doses 5, 50 and 100 mg/kg neem wood aqueous extract for 20 days in mice produced a significant prolongation in the diestrus phase in a dose dependent pattern. Sitasiwi, Isdadiyanto and Mardiati [19], also reported that ethanolic A. indica leaves at levels 8.4, 11.2, and 14 mg/mice/day for 21 days can disrupt the regularity of the estrous cycle and prolong diestrus in Swiss Webster mice. Despite the significant physiological disruptions within the estrous cycle, *A. indica* extract did not significantly affect mean daily feed intake, or the relative weights of the ovaries and uterus as shown in herein study and in parallel with El-Zaiat, Elshafie, Al-Marzooqi and Dughaishi [30] as well as Biswas, Chattopadhyay, Banerjee and Bandyopadhyay [31]. This implies that the contraceptive effects of *A. indica* extract are not due to changes in overall nutrition, the weight of reproductive organs or body conditions [19,21,32]. So, the extension of the diestrus phase in neem-treated rats suggests that A. indica extract disrupts the hormonal feedback mechanisms essential for the progression of the estrous cycle. Where the estrous cyclicity is completely governed by neuroendocrine feedback that is completely

controlled by anterior pituitary gonadotropins secretion and gonadal steroids production [33-35] that seemed to be reduced in the current study (noticed by 17-â estradiol and progesterone reductions).

Normally, an increase in 17-â estradiol signals the end of the diestrus phase and the start of a new cycle [36]. However, in neem-treated rats, 17-â estradiol levels were about 34.88% lesser in females those in the control group during the follicular phase. The former result was aligning with observations mentioned by Sitasiwi, Isdadiyanto and Mardiati who reported that ethanolic A. indica in leaves 8.4, 11.2, and 14 mg/mice/day for 21 days can reduce 17-â estradiol in mice [19]. Also, Shaikh, Naqvi and Khani [37] demonstrated that neem oil administration to female albino rats at doses 0.6 and 1.2 mL/animal significantly reduced 17-â estradiol in the blood. Moreover, Tripathi, Shrivastav and Chaube [38] detected lower 17-â estradiol in follicular lysate after administration 50 mg/day aqueous neem extract to immature female rats for 10 days. This reduction suggests that the hormonal signal, needed to end diestrus and trigger follicular development and maturation (proestrus) [39], is impaired. As a result, the hormonal threshold for transitioning from diestrus might not be reached, leading to an extended diestrus phase. These decreased 17-â estradiol levels imply that A. indica extract exhibits strong anti-estrogenic effects[19].

Our findings show that serum progesterone levels were significantly reduced, approximately 38.35% lower than in controls, which in line with the study by Moravati, Mahmoudi, Ghazi-Khansari, Aria and Jabbari [40] who found that gavage of neem methanolic extract at a dose of 15 mg/ kg for 6 days significantly abridged blood progesterone level in female rats that support the strong anti-progestational effects exerted by A. indica extract. Moreover, Physiologically, progesterone plays a crucial role in maintaining the diestrus phase, with its decline signalling the end of this phase [39]. Progesterone upregulation signals neuroendocrine reflex through the hypothalamus to promote pituitary gonadotropin production to establish a new cycle. The reduction of progesterone level in the herein study led to prolongation of diestrus due to delay of its feedback on hypothalamic releasing hormones that delay pituitary gonadotropins secretion thus hindering initiation of new cycle [41]. This interference might involve altering progesterone receptor sensitivity, acting as a progesterone receptor blocker, or disrupting the signalling pathways that process these hormone levels, resulting in a delayed transition despite low progesterone [42]. The signalling pathways that process progesterone involve dimerization to receptors where there are different progesterone receptor (PR) subtypes related to reproductive function (PR-A, PR-B and PR-C) as well as G-protein coupled membrane receptors. PR-A limits transcription of target genes referring to the active inhibitory domain that prohibits further translation into new proteins. However, PR-B receptors stimulate DNA transcription and further translation to new proteins specifically nuclear receptor coactivator that has a close association with maintenance of wet uterine weight [43]. PR-C lacks DNA binding affinity as PR-A or PR-B but it can dimerize receptors as a heterodimer that is not as effective as a homodimer[43]. The G protein coupled PR exerts its regularity function in ovaries and hypothalamus via inhibition of cAMP [44]. The ablation of PR function by neem could contribute to altering of neuroendocrine regulation exerted by such receptors in reproductive cycle regulation.

Beyond their role in the progression of the estrous cycle, 17-â estradiol and progesterone have protective, anti-apoptotic effects on ovarian cells, supporting the proper development and maintenance of follicles and the corpus luteum [45]. When these hormone levels decline, ovarian caspase-3 activity increases, as demonstrated in our results. The increase in ovarian caspase-3 expression may be due to neem suppression to catalase activity that promotes H_2O_2 induction to BAX and P53 that hasten caspase-3 expression[46]. Caspase-3 is a key executioner of apoptosis [47]. Its activation triggers the cleavage of cellular proteins, leading to programmed cell death. This process constitutes a crucial part in the follicular atresia of non-dominant follicles during the follicular phase and the relapse of the corpus luteum during the luteal phase if pregnancy does not occur [48]. Consequently, increased caspase-3 activity leads to heightened apoptosis, disrupted ovarian function, and increased follicular atresia. This results in fewer follicles maturing and ovulating, accompanied by increased connective tissue in the ovary compared to control groups, as observed

in the present histological examination. These findings are consistent with studies by Akpantah, Ekong, Obeten, Akpaso and Ekanem [49] in female rats and Swamy and Mohan [50] in freshwater fish.

Additionally, insufficient endometrial proliferation and glandular development are consequences of reduced levels of 17-â estradiol in A. indica-treated rats [51]. This explains the observed epithelial stratification and the scarcity of uterine glands in our histological sections, which align with the finding of Auta and Hassan [29] who found that neem extract administration doses of 5, 50, and 100 mg/kg neem wood extract for 20 days to mice produced uterine epithelial stratification especially in 50 mg and 100 mg doses . Furthermore, the low progesterone levels in these treated rats impair secretory functions, leading to diminished glandular activity and compromised structural integrity of the endometrium [29]. The absence of proper glandular structures in the endometrium reflects impaired uterine preparation for potential implantation, which is consistent with the contraceptive properties of neem extract [29,51].

CONCLUSION

Neem leaves extract produced antifertility potential in female Albino rats through

Reducing sex steroids; estrogen in the follicular phase and progesterone in the luteal phase. That led to the prolongation of estrous cycle duration especially the diestrus phase. Moreover, the reduced sex steroids by neem leaves extract promoted an increment in the ovarian caspase-3 mRNA expression that reflected follicular apoptosis and atrasia. The previous events provoked histological deteriorations in the ovary such as a reduced number of developing follicles and connective tissue proliferation beside uterine epithelial stratification with diminished uterine glands that reflect their incompetence for ovulation and implantation.

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Conflict of interest

The authors do not have any conflict of interest

Data Availability

The manuscript incorporates all datasets produced or examined throughout this research study.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required

Authors' Contribution

Conceptualization, E.M.A., Heba M.A. Abdelrazek; Methodology, Heba M.A. Abdelrazek, Asem A. Awad, Nayrouz A. Attia, Mohamed R. Saad, Marwa S. Kamel; Formal analysis, Rana M. Al-awadhi., Heba M.A. Abdelrazek., Eman M. Abouelhassan, Investigation, Rana M. Al-awadhi., Heba M.A. Abdelrazek., Nayrouz A. Attia., Marwa S. Kamel.; Resources, Rana M. Al-awadhi, Heba M.A. Abdelrazek., Mohamed R. Saad, Asem A. Awad.; Writing- original draft preparation, Nayrouz A. Attia, Mohamed R. Saad., Asem A. Awad; Writing-review and Editing, Rana M. Al-awadhi., Heba M.A. Abdelrazek., Eman M. Abouelhassan., Marwa S. Kamel All authors have read and agreed to the published version of the manuscript.

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