International Journal of Biology Sciences

ISSN Print: 2664-9926
ISSN Online: 2664-9934
IJBS 2024; 6(1): 216-223
www.biologyjournal.net
Received: 09-02-2024
Accepted: 02-03-2024

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Impact of lemon peels phenolic extract on the oestrus cycle and uterine histoarchitecture in female Wistar albino rats

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DOI: https://dx.doi.org/10.33545/26649926.2024.v6.i1c.209

Abstract
Infertility is a significant clinical concern, affecting a good percentage of females of reproductive age. Obesity and overweight have been identified as contributing factors to infertility in most cases. Lemon polyphenols have been reported to suppress body weight gain and body fat accumulation, making lemon a commonly consumed fruit among women of all ages for body weight management. While the effects of lemon on body weight are well-studied, the impact of continuous lemon intake on different body systems and organs is still being explored. In this study, we aimed to investigate the effects of the phenolic extract of lemon peels on the oestrus cycle and the histological features of the uterus following the consumption of varied dosages of lemon peel phenolic extract. A total of 24 female Wistar albino rats weighing between 180-250 g were randomly split into four groups (n = 6 per group). Group 1 served as the control group and was given only distilled water and rat pellets for a period of 14 days, while groups 2, 3, and 4 were administered 200 mg/kg, 400 mg/kg, and 600 mg/kg body weight of phenolic extract of lemon peels, respectively, for the same period. The effect of the extract on the body weight, pattern of oestrus cycle, and histological morphological differences between the uteri of the control and treatment groups were evaluated. The results showed that there were no significant differences in weight reduction recorded across the experimental groups ($p>0.05$). However, the oestrus cycle pattern was significantly ($p<0.05$) altered in a dose-dependent manner in groups administered 200 mg/kg, 400 mg/kg, and 600 mg/kg of the extract compared to the control group. The histological analysis of the uteri from the experimental groups revealed several changes compared to the control group. These changes included the presence of inflammatory cells in the treated groups, focal ulcerations, lining epithelial thickening, and, endometrial hyperplasia. These findings suggest that the phenolic extract of lemon peels has an impact on the normal duration of the oestrus cycle and its phases in a dose-dependent manner. Additionally, the extract has shown some potentially harmful effects on uterine histology, which may have implications for reproductive health.

Keywords: Uterine histology, phenolics, oestrus cycle, Wistar rats, medicinal plants

1. Introduction
Phenolic compounds, which are natural antioxidants, can be found in various plants and plant parts, such as herbs fruits, and vegetables. Lemon peels, in particular, have a high concentration of phenolic compounds, especially phenolic acids and flavonoids and these compounds have been extensively researched for their potential health benefits, which include anti-inflammatory, anti-cancer as well as antioxidant properties. The phenolic extract of lemon peels has demonstrated various biological activities in both *in vitro* and *in vivo* investigations [1].

Lemon fruit is primarily consumed for its uniquely flavored juice and exceptional nutritional value. The extract from lemon juice has also been shown to be beneficial for maintaining good health [2]. Several experiments on lemon juice have revealed several health-enhancing effects on the different systems of the human body [3]. These benefits include improving digestive health by alleviating constipation, detoxification of the urinary system, thus averting the possibility of kidney stone formation, reducing the appearance of wrinkles on the face, aiding in the treatment of skin blemishes including pimples, eczema, possessing anti-cancer potentials as well as the ability to reduce heart-related diseases [4].

Citrus fruits are renowned for their abundance of bioactive compounds and essential oils, boasting diverse biological activities, including hormonal activities [5]. Lemon peels, in particular, contain a phenolic principle enriched with d-limonene, a key component of *Citrus* fruit peel oils that is typically found in lesser amounts in other fruits and vegetables. The effects of d-limonene on tumors can vary, exhibiting both tumor-promoting and tumor-inhibiting qualities depending on the dosage and duration of exposure [6]. Furthermore, beyond their rich array of bioactive compounds, *Citrus* fruits have a longstanding relevance in ethno medicine, being utilized for various purposes, including as a contraceptive method [7]. Research exploring the impacts of lemon peel extract on reproductive organs and overall reproductive health remains scarce. Delving into the potential effects of the phenolic extract of lemon peels on the estrus cycle and uterine tissues could yield valuable insights into its safety and effectiveness concerning female reproductive health [8, 9]. The female rat's reproductive system operates through a complex interplay of hormones and factors, driving a dynamic process. Central to this system is the estrus cycle, a pivotal component whose normal duration and phases are crucial for maintaining reproductive health. Any disruptions to this cycle can exert significant effects on overall reproductive well-being [10]. The estrus cycle in female Wistar rats represents a meticulously regulated process, characterized by intricate hormonal changes and physiological events. This phenomenon stands as a well-documented and extensively researched aspect of rat physiology, serving as a cornerstone for comprehending reproductive mechanisms and their regulation. In rats, the estrus cycle is paramount in determining the mating behavior of the animals, their fertility potentials, and their general reproductive well-being. Its orchestration primarily hinges on hormonal fluctuations orchestrated by the hypothalamic-pituitary-gonadal (HPG) axis. Initiated by gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus, this cascade prompts the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland, thereby governing ovarian function and follicular development. The feedback mechanism involving estradiol, progesterone, and other steroid hormones produced by the ovaries intricately regulates the timing and progression of the estrus cycle phases [11]. The estrus cycle unfolds in four distinct stages: Proestrus, estrus, metestrus, and diestrus. Proestrus initiates with a surge in estrogen levels, fostering the development of follicles within the ovaries. This phase transitions into estrus, the pinnacle of sexual receptivity marked by ovulation [12, 13]. Metestrus serves as the intermediary between estrus and diestrus, characterized by a decline in estrogen levels and a rise in progesterone levels. Finally, diestrus denotes a period of subdued hormonal activity, during which the uterus readies itself for potential embryo implantation.

Environmental factors wield considerable influence over the regulation of the estrus cycle in rats, with factors like photoperiod, temperature, social interactions, and nutritional status playing significant roles. Of these, photoperiod stands out, as changes in light-dark cycles have been found to affect both the timing of estrus onset and the duration of estrus phases, consequently impacting reproductive hormone secretion and ovarian function [14]. Furthermore, social dynamics, including aspects such as social hierarchy and exposure to male pheromones, contribute to the synchronization of estrus cycles among female rats housed in groups [15]. Furthermore, dietary elements, encompassing factors like dietary composition and caloric intake, have been implicated in regulating the estrus cycle via their influence on energy balance and metabolic signaling pathways [16]. In both experimental and clinical contexts, pharmacological substances are frequently utilized to manipulate the estrus cycle in rats for research endeavors or therapeutic applications. Hormonal contraceptives, including combined estrogen-progestin formulations or progestin-only implants, are commonly employed to suppress estrus and induce anestrus in female rats [17]. Selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, influence estrus cycle dynamics by selectively targeting estrogen receptors in reproductive tissues [18]. Furthermore, investigations have delved into the potential of herbal supplements and phytoestrogens to regulate the estrus cycle and alleviate reproductive disorders in rats, though comprehensive assessments of their efficacy and safety profiles remain necessary [19]. Numerous substances and plant extracts have been documented to detrimentally impact the reproductive system in rats by disrupting the histological morphology of the uterus. Studies have highlighted the adverse effects of monosodium glutamate (MSG) on the female reproductive system, including alterations in the estrus cycle and histological changes in the uterus [20]. Additionally, several medicinal plants have undergone investigation for their uterotonic properties, promoting uterine contractility and smooth muscle relaxation. Extracts from plants such as *Achillea millefolium*, *Cinnamomum zeylanicum*, and *Pimpinella anisum* have exhibited uterotonic effects in rat models, likely attributed to the presence of bioactive compounds such as flavonoids, alkaloids, and essential oils [21]. The exposure of female Wistar rats to the aqueous flower extract of *Aspilia africana* resulted in distortive changes in the ovaries and disruptive effects on uterine tissues [22]. Similarly, the methanolic extract of *Aspilia africana* leaves negatively impacted the estrous cycle and histo-architecture of the uterus in female rats [23]. Estrogen, pivotal in regulating uterine function and menstrual cycle dynamics, plays a crucial role in these processes. Medicinal plants with estrogenic or antiestrogenic properties have been studied for their effects on the uterus in rat models. Phytoestrogens from plants like Glycine max, *Trifolium pratense*, and *Cimicifuga racemosa* have demonstrated estrogenic effects in the uterus, potentially influencing endometrial thickness and uterine histology [24]. Conversely, certain plants, such as Vitex agnus-castus and Tamoxifen, exhibit antiestrogenic effects, modulating estrogen receptor signaling and the uterine response to estrogen stimulation [25, 26].

This study aims to underscore the importance of comprehending the nuances of the estrous cycle, which serves as a cornerstone in the study of reproductive physiology. By delving into the effects of substances such as the phenolic extract of lemon peels on uterine tissues in female Wistar rats, this research seeks to shed light on the intricate interplay between external agents and reproductive health.

2. Materials and Methods

2.1 Reagents/Chemicals

All reagents utilized in this study were sourced from reputable suppliers and met analytical grade standards. These included products from British Drug House (BDH) in England, E. Merck in Darmstadt, Germany, and the Aldrich Chemical Company.

2.2 Processing of plant material

The collection, identification, and extraction of plant materials began with the harvesting of fresh lemon fruits from the Igbinedion University farms located in Edo State. These fruits underwent authentication and identification at the Department of Plant Science and Biotechnology, Igbinedion University, Okada. Subsequently, the fruits were manually peeled, with the removed tissue comprising the pericarp region, encompassing the epicarp and mesocarp. The peels were then subjected to drying using the vacuum drying method at 60 °C for a duration of 20 hours. Once dried, the lemon peels were finely ground into a powder using a manual blender. The resulting grounded peels underwent sieving through a 1 mm metal sieve, with the process repeated until all material passed through the sieve.

Fig 1: Photo showing lemon peels (Self-taken image)

2.3 Extraction of Polyphenol

The process began with macerating 2 grams of plant leaves powder in 85% ethanol, followed by N-hexane. This mixture was then transferred to a 50 ml centrifuge tube and allowed to stand for 48 hours at room temperature. Subsequently, the mixtures underwent centrifugation using a Mistral 1000 centrifuge at 500 × g for 10 minutes at room temperature. After centrifugation, the supernatants were filtered through Whatman No. 42 filter paper. Following filtration, a 10 ml aliquot of the filtrate was concentrated by evaporating the solvent using a rotary evaporator under partial vacuum at 40 °C until less than 1 ml of filtrate remained.

2.4 Animal Study

For the study, twenty-four adult female albino Wistar rats weighing between 180 and 250 grams were procured from the Faculty of Basic Medical Sciences Animal House, University of Nigeria. These animals were then housed in cages and allowed to acclimatize for a period of two weeks at the Animal House, Department of Anatomy, Igbinedion University Okada. Throughout the acclimatization and experimental periods, the rats were handled by the guidelines outlined by the National Institute for Health, USA (National Institute of Health, 2011). They were provided with free access to standard livestock pellets and water ad-libitum to ensure their well-being.

2.5 Oestrus Cycle Study

The method employed in this study closely followed the protocol outlined by Marcondes *et al.* (2002) [12]. To determine the phase of the estrus cycle, vaginal smears were collected daily between 8 am and 10 am over a period of 14 days.

Procedure: A short pasture pipette containing 10µl of normal saline (NaCl 0.9%) was gently inserted into the vaginal orifice and then released. After a 5-second interval, the pipette was lightly inserted into the vagina of the rats, and a sample of normal saline solution mixed with vaginal secretions and cells was aspirated. This mixture was then smeared onto glass slides. The resulting smears were immediately examined under a light microscope. The phase of the estrus cycle was determined based on the proportion and types of cells observed in the microscope view.

2.6 Histological Studies

The uteri of the experimental animals were harvested and preserved in Bouin's fluid fixative. Subsequently, the samples underwent processing for paraffin wax embedding, where they were carefully sectioned using a Rotatory microtome. These sections were then stained using H&E staining techniques, allowing for the visualization of tissue structures. Photomicrographs of the stained sections were captured for further analysis.

2.7 Statistical Analysis

All values derived from this study were presented as Mean ± Standard Error of Mean (SEM). Statistical differences between groups were assessed using one-way ANOVA (Analysis of Variance), followed by t-test comparisons across all groups. A p-value of less than 0.05 was considered statistically significant

3. Results

3.1 Effects of phenolic extract of lemon peels on the body weight of the female wistar rats

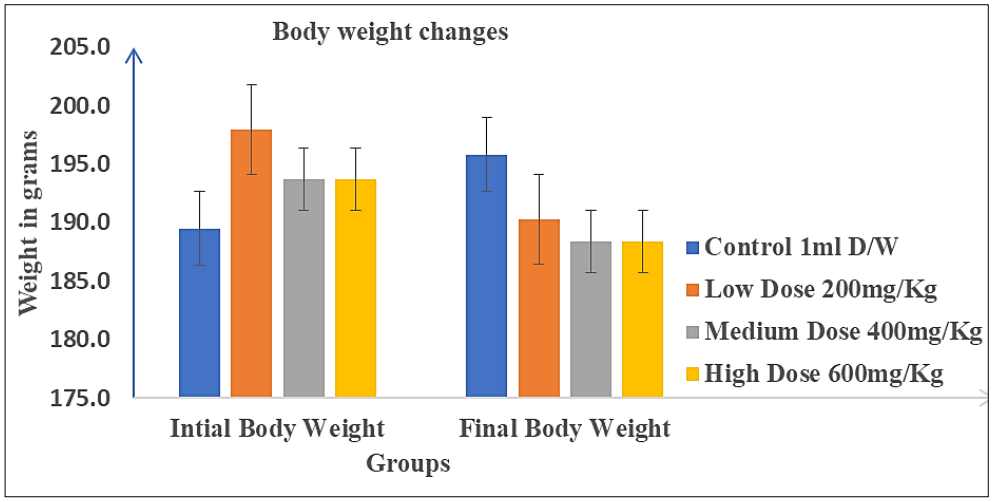

Fig 2: Mean body weight changes of female Wistar rats (Means ± SEM)

3.2: Effects of phenolic extract of lemon peels on the duration and pattern of the oestrus cycle of the female wistar rats

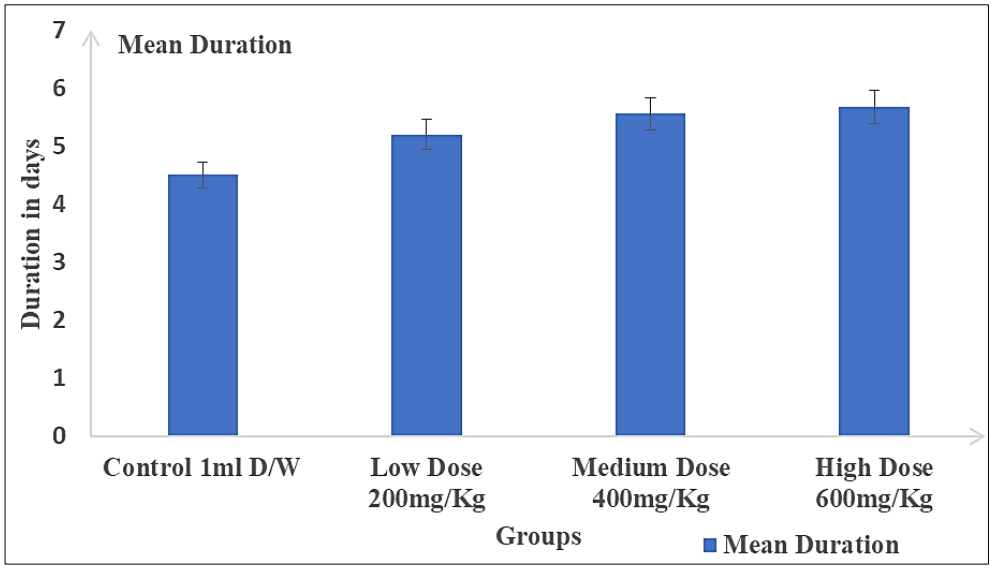

Fig 3a: Mean duration of the oestrus cycle of female Wistar rats (Means ± SEM)

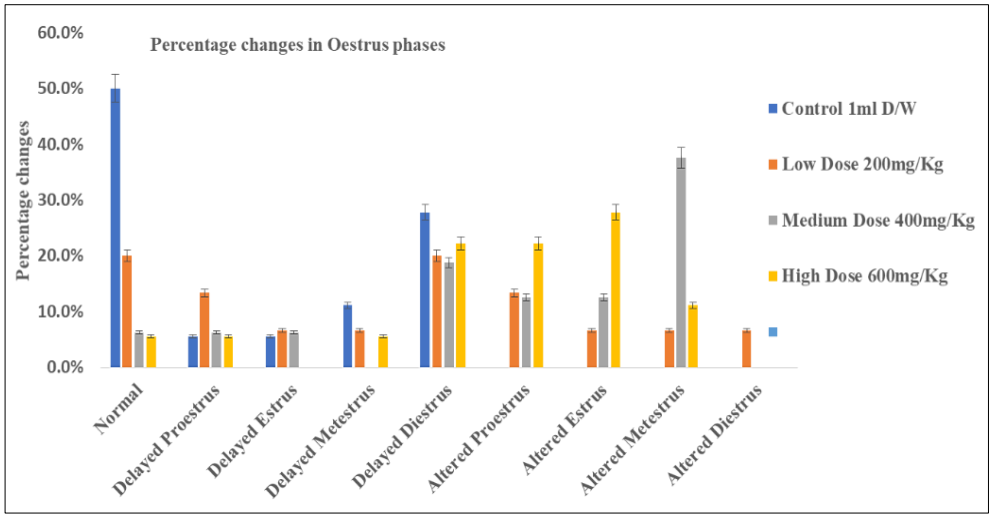

Fig 3b: Percentage of the oestrus cycle phases of the female Wistar rats.

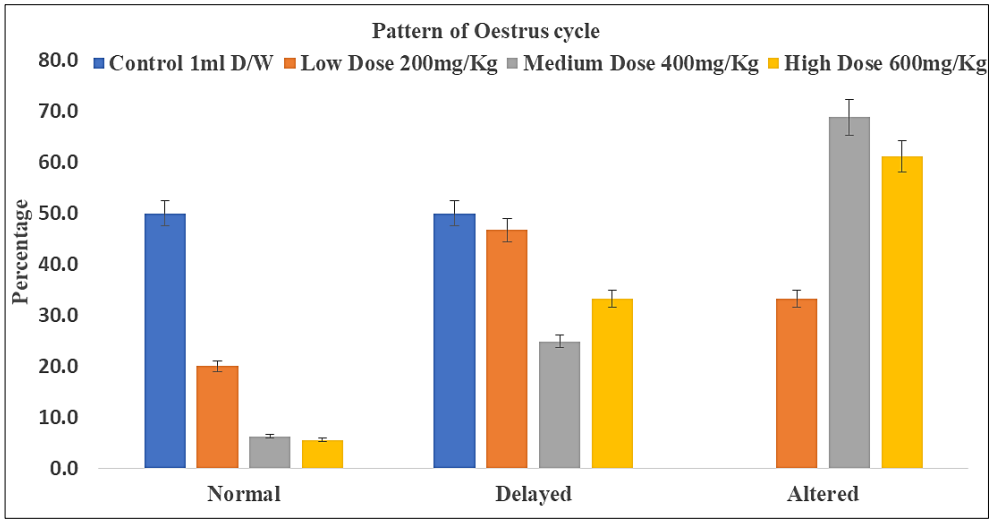

Fig 4: Proportion of the pattern of the oestrus cycle of the female Wistar rats.

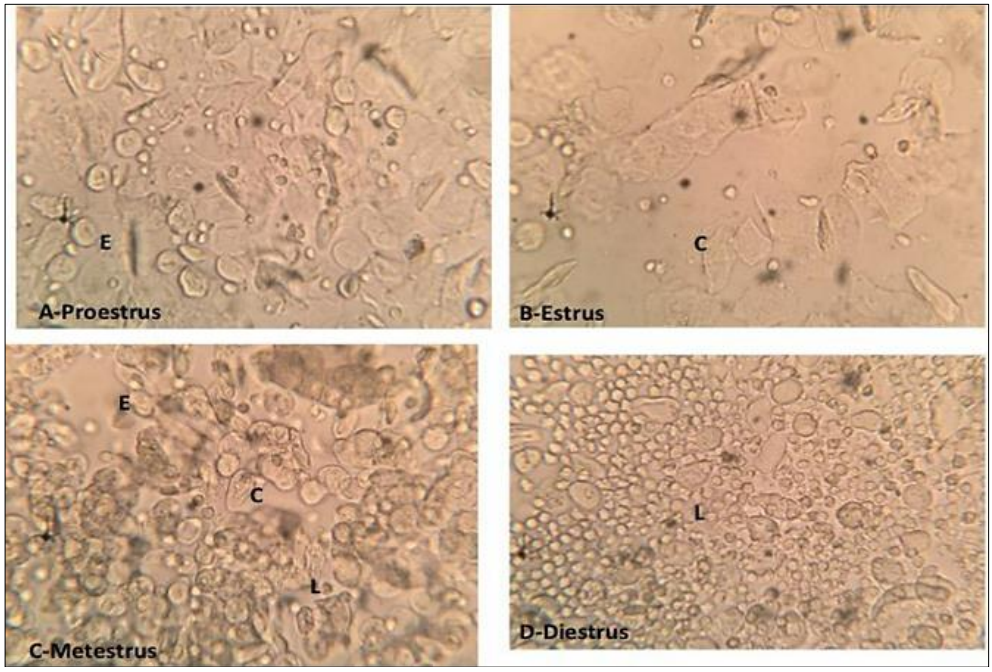

Fig 5: Pictomicrographs of unstained vaginal smears from rats ate different phases of the oestrus cycle.

Histological studies

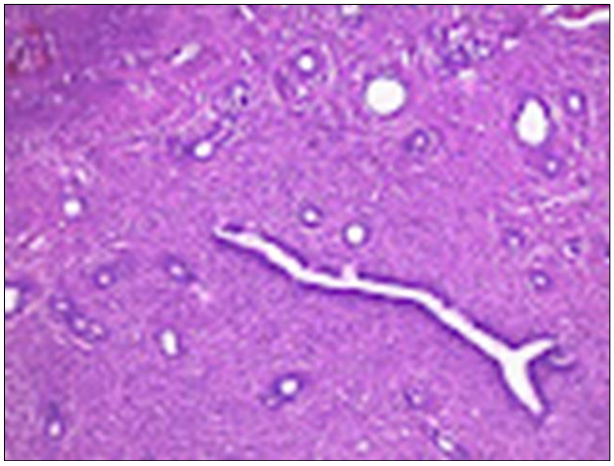

Plate 1: Control uterus showing normal uterine histology endometrial lining- A, endometrial glands- B, and endometrial stroma (H&E X 40)

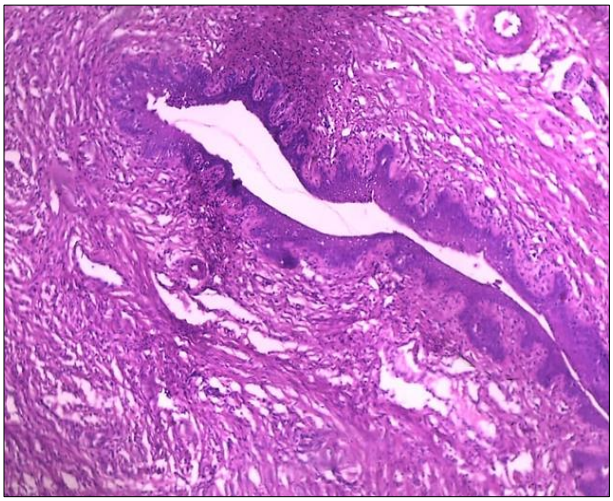

Plate 2: Rat uterus given 200 mg/kg of phenolic lemon peel extract, showing moderate thickening (hyperplasia) of the epithelia lining - A, patchy endometrial ulceration - B, mild distortion of the myometrium - C (H&E X 40

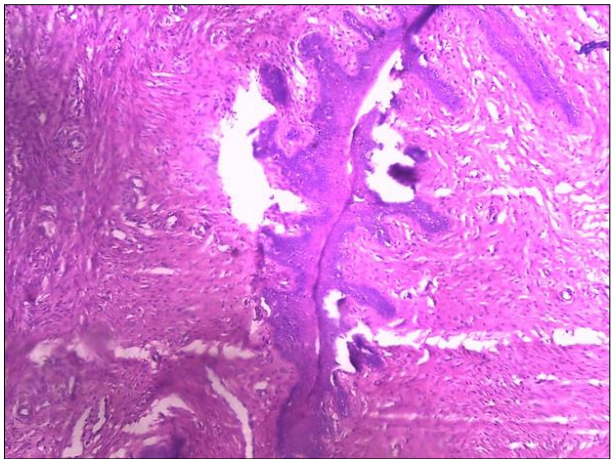

Plate 3: Rat uterus given 400 mg/kg of phenolic lemon peel extract, showing mild endometrial lining hyperplasia - A, mild stroma infiltrates of inflammatory cells - B and focal area of ulceration - C (H&E X 40)

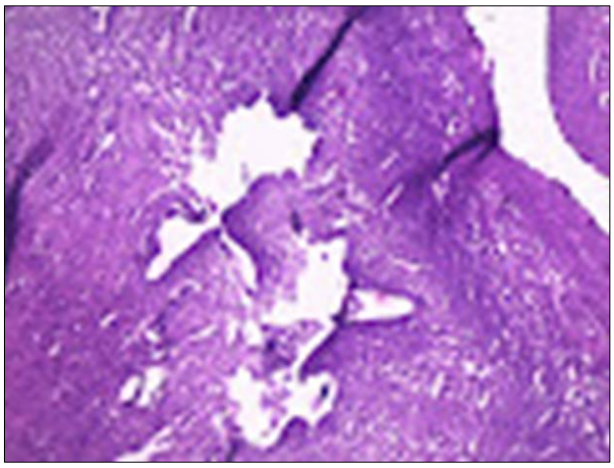

Plate 4: Rat uterus given 600 mg/kg of phenolic lemon peel extract, showing focal endometrial lining hyperplasia - A, and ulceration - B (H&E X 40)

4. Discussion

The study of body weight serves as a valuable indicator for assessing the potential toxicity of substances, including medicinal plants. Changes in body weight can signify disruptions in normal physiological functioning [27]. The findings from this study revealed a non-significant (p>0.05) reduction in weight across the entire experimental group. However, this reduction in body weight did not exhibit a dose-dependent relationship relative to the control group. This observation could be attributed in part to the weight-reducing properties inherent in *Citrus* species [28].

The female groups exposed to graded doses of the phenolic extract of lemon peels were also utilized to evaluate the effects of the extract on the estrus cycle. The decrease in weight among the females could be associated with the stress induced by daily handling of vaginal smear collection as part of the estrus cycle study, as well as potential loss of appetite, which may be linked to the former.

The estrus cycle in rats typically spans 4-5 days, making them highly suitable for studies pertaining to reproductive cycles. Different phases of the cycle are characterized by specific cell types: nucleated cells during proestrus, cornified cells during estrus, leukocytes during diestrus, and an equal combination of these cell types during metestrus [29, 30, 31].

The results of this study revealed a highly significant (p < 0.05) increase in the mean duration of the estrus cycle among the treatment groups compared to the control group. Specifically, the mean duration of the estrus cycle in the control group was found to be 4.50 days, consistent with the normal duration reported by Marcondes *et al.* [32]. However, the groups administered with 200 mg/kg, 400 mg/kg, and 600 mg/kg of the phenolic extract of lemon peels exhibited a dose-dependent increase in the duration of the estrus cycle, with durations of 5.20, 5.56, and 5.67 days, respectively.

The analysis of the estrus cycle pattern revealed notable alterations in the phases among groups administered 200 mg/kg, 400 mg/kg, and 600 mg/kg of the phenolic extract of lemon peels, exhibiting highly significant (p<0.05) dose-dependent differences relative to the control, as suggested by Goldman *et al.* [33]. This observation aligns with findings by Soni *et al.* [34], where the administration of ethanol extract of stem of *Musa paradisiaca* L., Musaceae, induced a prolonged metestrus stage in rats at dosages of 250 and 500 mg/kg body weight for five days.

These alterations in the estrus cycle phases could be attributed to the presence of steroid-like secondary metabolites in the extract capable of inhibiting the hypothalamic-pituitary axis and follicular development. Additionally, the extract's inhibitory properties on pituitary gonadotropins, and potential direct toxic effects on thecal and follicular cells, as suggested by previous studies [35], [36], may have contributed to delays and alterations in the estrus cycles observed in the animals used for this study.

The histological examination of the uteri from Wistar rats in the control group revealed a normal histoarchitecture, characterized by a well-oriented endometrial lining, prominent endometrial glands, and large stromal cells within the stroma, as depicted in Plate 1. These observations align with previous findings on the uteri of normal rats reported by Young & Heath [37, 38].

However, the uterine endometrium of the extract-treated groups exhibited notable alterations, including thickening of the lining epithelium, mild to moderate endometrial hyperplasia, focal areas of ulceration, and mild infiltration of inflammatory cells. The presence of inflammatory cells within the endometrial tissue suggests potential injury to the endometrium and/or the uterus as a whole, triggering activation of the local immune system. Such inflammation may result from interactions between the phytoconstituents of the extract and the body's immune vascular system.

These histological findings, particularly the presence of inflammatory cells, are indicative of endometritis, characterized by inflammation of the endometrium. Gross examination of the uterus from rats administered 600 mg/kg of the extract revealed signs of endometritis, such as abdominal distention or swelling. These observations are consistent with the findings of Emtenan and colleagues [40], who confirmed endometritis through the presence of inflammatory cells in the endometrium.

The disturbances observed in the endometrium of rats administered the extract may underlie the affected normal physiology of the estrus cycle. Furthermore, endometrial hyperplasia, characterized by abnormal or excessive proliferation of the endometrium/endometrial lining, was noted in the uteri of rats treated with the extract. Endometrial hyperplasia can be either physiological or pathological, particularly when it arises from hormonal imbalances, such as excessive estrogen production or

secretion in the absence of progesterone, and is known to precede uterine neoplasms in some cases. This finding aligns with the work of Udoh [41], who reported endometrial hyperplasia and dilation of endometrial glands in rats administered *Gnetum africanum*. Additionally, focal areas of ulceration were observed in the endometrial glands, suggesting epithelial tissue erosion. Overall, these observations suggest that the phenolic extract of lemon peels exerted injurious effects on uterine tissue, potentially leading to reproductive dysfunction. These effects may be attributed to the presence of flavonoids in the extract, as suggested by previous researchers [42].

Conclusion

There was a clear demonstration in this study that the phenolic extract of lemon peels affected the normal duration of oestrus cycle as well as the phases of the cycle in a dose-dependent manner. The study also revealed some deleterious effects on the histology of the uterus. Hence, it is advised that fertile females should not take lemons excessively without recourse to proper dosage. There is also the need for investigative studies on lemon peel extract to be carried out to compare the antifertility effects of higher and varied doses on females as well as males.

Acknowledgement

The authors gratefully acknowledge the technical support provided by the entire technical team of the Department of Anatomy, College of Health Sciences, Igbinedion University Okada. Their assistance was invaluable in the successful completion of this study.

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