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Original Research Article

Ethanol Extract from Yellow Pumpkin Modulate Hormonal Levels and Ovarian Follicle Dynamics in Hypoestrogenic Wistar Rats

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ABSTRACT

Depo Medroxyprogesterone Acetate (DMPA) is a progesterone analog that induces a hypoestrogenic response by inhibiting gonadotropin-releasing hormone (GnRH) needed for the release of follicle-stimulating hormone (FSH) and Luteinizing Hormone (LH), thereby preventing ovulation, decreasing the number of antral follicles, and suppressing stromal and epithelial cell proliferation. Side effects of DMPA can be reduced by using natural ingredients such as yellow pumpkin seeds (*Cucurbita pepo L.).* This study aimed to determine the effect of pumpkin seed ethanol extract on FSH levels and activation of Ki-67 expression in endometrial stromal cells, beta estrogen receptors, antral follicles, stromal cells, and endometrial epithelial cells in hypoestrogen type Wistar rats with DMPA injection. This *in vivo* study used 25 female rats (*Rattus norvegicus*) that were randomly divided into five groups: a control group and four treatment groups. The control group, without DMPA (sterile aquadest injection), as well as the treatment groups, were injected with a dose of 2.7 mg/kg bw of DMPA every 3 days for 21 days. Ovarian FSH levels were evaluated by ELISA, Ki-67, and ER*β* expression by immunohistochemistry (IHC), antral follicle count, stromal cells, and endometrial epithelium by Hematoxylin Eosin (HE). The findings of the study revealed significant differences and correlations between extract dose and FSH levels, Ki-67 expression, ER-beta, antral follicle count, stromal cells, and endometrial epithelial cells. The result of the study showed that yellow pumpkin seed ethanol extract in various doses enhanced FSH levels, Ki-67 expression, ER-beta, antral follicle count, stromal cell count, and endometrial epithelial cell count compared to a positive control group exposed only to DMPA.

Keywords: DMPA, FSH, Ki-67 expression of stromal cells, ER*β* epithelial cells, Ovarian Antral Follicles, yellow pumpkin seed extract

Introduction

Depo medroxyprogesterone acetate (DMPA) is one of the agents that is used as hormonal interference for pregnancy control or as contraception that is administered by injection. In Indonesia, 42.4% of couples of childbearing age use 3-month injectable contraceptives, with more than 50% of East Java province accepting 3-month injectable contraceptives in 2018. [1] DMPA is injected at 150 mg/kg into the muscle once every three months. Progestin, a naturally occurring progesterone hormone in females, is also a component of DMPA. DMPA can respond quickly by blocking the increase in FSH, LH, and ovulation after 20 min of injection.[2]

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The adverse effect of DMPA contraception include irregular bleeding in 70% of women in the first year of use, weight gain (48 percent), joint pain (24 percent), vaginal dryness (10.4%), and a reversible reduction in bone density (5 - 7%). [3] DMPA exposure can increase progesterone levels, thereby inhibiting estradiol production. Long-term use disrupts the menstrual cycle due to impaired ovarian function and inhibition growth of endometrial cells. Antral Follicles produce estrogen during the pre-ovulatory phase to significantly affect the growth of endometrial cells. The endometrium undergoes modification due to DMPA, including a failed secretion transition due to atrophy of the endometrium can interfere with the implantation process.[4 5] Therapy to accelerate fertile recovery includes hormone replacement therapy (HRT), synthetic estrogens, amino acids, and vitamins in the form of antioxidants. HRT is widely used and has been shown to accelerate fertility return through endometrial cell repair. However, long-term use can increase the risk of breast, endometrial, and ovarian cancer. With this risk, alternative therapies from natural ingredients such as phytoestrogens are needed. [6] Phytoestrogens have polyphenolic compounds with a functionally similar molecular structure to 17*β*-estradiol, one of which is yellow pumpkin seeds (*Cucurbita pepo L.*). Isoflavone compounds in yellow pumpkin seeds can bind to the ER and produce an estrogenic effect under hypoestrogenic conditions.

Phytoestrogens can restore fertility, as evidenced by the interaction of lignan and flavonoid compounds with the estrogen receptors on the ovaries. This active compound can stimulate follicle growth, and estrogen secretion increases in mature follicles (graafian). Activation of estrogen receptors and increased estrogen hormone can accelerate fertile recovery by increasing endometrial cell proliferation. [7 8] This study aimed to ascertain how phytoestrogens in ethanol extract from yellow pumpkin seeds (*Cucurbita pepo L.*) might affect FSH levels, Ki-67 expression, ER*β* expression, and amount of ovarian antral follicles, stromal, and endometrial epithelial cells in a hypoestrogen rat model.

Materials and Methods

Experimental Animals

In this study 25 female white Wistar rats (*Rattus norvegicus*), aged 8 to 10 weeks with a body weight of 180-200 g used. An acclimatisation period of 7 days was observed before the study began. The samples were divided into five groups (n=5): a negative control group, a positive control group (DMPA dose of 2.7 mg/kg only was administered every 3 days for 21 days), and treatment groups (DMPA treated with 50 mg/kg BW, 100 mg/kg BW, and 200 mg/kg BW of *Cucurbita pepo L* extract was administered every day for 7 days). The experiments and maintenance of the rats in this study were carried out at the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya. This study was approved by the Ethical Committee of Universitas Brawijaya number 176-KEP-UB-2023.

Extractions

A local plant, yellow pumpkin seeds (*Cucurbita pepo L.*) from the Tegaldlimo District, Banyuwangi Regency, East Java Province was used for this study. The seed of the plant was identified through plant determination tests conducted by herbal management institutions (UPT. Materia Medika Batu, East Java, Indonesia) with letter number 000.9.3/2867/102.20/2023 as a species of *Cucurbita pepo L*. The extraction procedure started by making simplisia until a simple powder of 200 g was obtained and macerated, three times in 24 h with 96% ethanol solvent, in a ratio of 5:1. The extract was evaporated using a rotary evaporator at a temperature of 40-60^{o}C at a speed of 50 rpm until the crude extract of yellow pumpkin seed was obtained.

Treatment and drug dosing

DMPA injection was administered intramuscularly to the quadriceps muscles in each treatment group (T1, T2, and T3) at a dose of 2.7 mg/kg. Injections were given every three days and 7 injections were administered for 21 days. After exposure to DMPA injection, serum estrogen levels and vaginal smears were analyzed to determine the condition of hypoestrogenism. If estrogen levels dropped, then three treatment groups (T1, T2, and T3) were administered yellow pumpkin seed ethanol extract at various doses, T1 dose 50 mg/kg BW, T2 dose 100 mg/kg BW, and T3 200 mg/kg BW. The extract was administered daily for 7 days after orally. After the administration of the extract therapy, a vaginal smear was performed to determine the proestrus phase for the rat surgical phase. The vaginal smears were prepared in 3 stages. Vaginal smear I (8th Day) (At the beginning before the study to determine the phase of estrus), Vaginal smear II (28th Day) (the second swab was done after DMPA injection to establish a clinical diagnosis of hypoestrogen support), and Vaginal smear III (36th Day) (the 3rd vaginal swab was done after the last pumpkin seed extract round). Rats were dissected in the proestrus phase; heart blood was taken from the right ventricle for analysis using an ELISA kit with the brand catalog number Elabscience Cat. No.: E-EL-R0391; the ovaries and uterine were taken to make histopathology slides with hematoxyIin-eosin (HE) staining and immunohistochemistry (IHC).

The uterus was observed for the expression of markers of Ki-67 on endometrial stromal cell proliferation and expression of estrogen receptor beta, in the functionalist layer of the endometrium. Stromal cells and endometrial epithelial cells were cut with transverse sections. Immunohistochemical observations using a Raxvision b550 microscope with a Panasonic G9 camera at 400 x magnification, in 5 fields of view were obtained. Ki-67 and ER*β* expression were calculated based on the total percentage of positive cells compared with the total amount of cells in each field of view.

The number of antral follicles, stromal cells, and endometrial epithelial cells was observed using an Amscope T340B-LED Slide Dot Microscope at 400 x magnification with 5 fields of view. The antral follicles were 2200 assessed by observing their constituent structures, namely the external theca cells, internal theca, granulosa, antral fluid, zona pellucida, and oocytes. Assessed stromal cells have a small oval-shaped nucleus, coils, and cytoplasm. Epithelial cells are assessed as the number of surface epithelial cells in the functional layer of the endometrium.

Data Analysis

Data normality was tested using the Shapiro-Wilk test, test and data homogeneity was tested using the Levene Test. Furthermore, the hypothesis test used a one-way ANOVA test. The results of the study found significant differences, and the analysis continued with a difference test using Tukey's height significant difference (HSD) test. A correlation test between the extract dose and independent variables was performed using Pearson's correlation test.

Result and discussion

FSH levels were measured using an ELISA kit (Elabscience) and 3 mL of blood serum extracted from the right ventricle of the heart. The Shapiro-Wilk normality test (p-value>0.05) and Levene's homogeneity test (p-value>0.05) were then performed. Data distribution was normal and homogeneous. Then, a one-way ANOVA test was carried out to determine the effect of the ethanol extract of pumpkin seeds (*Cucurbita pepo L.*) at various doses on FSH levels in the hypoestrogen *Rattus norvegicus* model that was injected with DMPA. Table 1. indicates significant differences among all groups. The Tukey test results showed that the highest mean was in the negative control group, with a mean value of 198.41±6.78 ng/mL, and the lowest mean was in the positive control group with a mean value of 156.75±24.78 ng/mL and a value of $0.00 < \alpha$. As shown in Table 1, the results of the one-way ANOVA test on the mean FSH levels showed a statistically significant increase in the average. The results of the average FSH levels are shown in Figure 1. Figure 1 shows the histogram of the average FSH levels in each group, the negative control group of rats that were not injected with DMPA, the positive control group that was injected with DMPA, and three groups of rats that were injected with DMPA, and administered pumpkin seed extract at a dose of 50 mg/kg BW, 100 mg/kg BW, and 200 mg/kg BW. At a dose of 50 mg/kg BW, the average FSH level was 187.14±2.59 ng/ml p-value <0.007, at a dose of 100 mg/kg BW, the average FSH level was 194.85±5.53 ng/ml p-value (<0.01), and the highest increase was at a dose of 200 mg/kg BW with a mean of 195.81±7.96 ng/ml p-value (<0.01), which could mean that the first dose of ethanol extract from pumpkin seeds made a significant difference in the levels of DMPA. FSH levels of *Rattus norvegicus* were significantly reduced by a 2.7 mg/kg DMPA injection administered every three days and repeated seven times. Based on theoretical research examining the effects of progestin medroxyprogesterone acetate (MPA), which resembles natural progesterone functions by attaching itself to progesterone receptors in the pituitary, brain, and female reproductive system to inhibit the release of gonadotropin-releasing hormone (GnRH). Moreover, MPA stops the mid-cycle increase in LH ovulation and follicular development, which lowers FSH levels. [9] In comparison to the PC group, the mean FSH levels in groups T1, T2, and T3 increased significantly. Table 1 shows that as the dose of pumpkin seed ethanol extract increased, the mean FSH levels also increased. The findings of Zhang *et al*. (2022) support the current study, demonstrating that pumpkin seed extract can elevate estrogen levels in ovariectomised rats, thereby stimulating oogenesis and promoting follicular development. Additionally, the present research indicates that pumpkin seed extract can increase FSH and estrogen levels, suggesting it has the potential as a natural alternative for addressing hormonal imbalances in reproductive health. This is consistent with the work of Domínguez-López *et al*. (2020), who found that phytoestrogens, by mimicking natural estrogen, bind to estrogen receptors and trigger a positive feedback loop involving GnRH and FSH, essential for ovarian

function and folliculogenesis. [10] [11] Furthermore, the Ki-67 of Endometrial Stromal Cell Proliferation was investigated from immunohistochemistry studies. An overview of Ki-67 proliferative expression was assessed based on the total number of ki-67 antibody expressions compared to the total number of cells in one field of view.

Table 1: Comparison of FSH Levels between Research Groups

Treatment Group	Means ± SD (ng/ml)	p-value
NC	198.41 ± 6.78[b]	
PC	156.75 ± 24.78[a]	0.000<α
T1	187.14 ± 2.59[b]	
T2	194.85 ± 5.53[b]	
T3	195.81 ± 7.96[b]	

Description: In the mean ± SD results, the letters ([a] and [b]) indicate a significant difference (p-value < 0.05); if the letters are the same, it indicates the opposite.

Table 2: One-Way ANOVA Test Results on Ki-67 Expression

Treatment Group	(n)	Mean ±SD (Percentage)	·value
NC	5	63.55±10.34[b]	
PC	5	29.10±13.34[a]	
T1	5	37.94±7.37[ab]	0.006
T2	5	43.35±16.98[ab]	
T3	5	56.88±19.21[b]	

Description: If the mean SD contains different letters ([a] and [b]), there is a significant difference ($p < 0.05$), whereas if it contains the same letters ([a], [ab], [b], and [ab]), then there is no significant difference ($p > 0.05$).

Table 3: One Way Anova test results on ERβ expression

Treatment Group	(n)	Mean ± SD (Percentage)	p-value
NC	5	44.13 ±12.85[c]	
PC	5	3.60 ±2.98[a]	
T1	5	20.14 ±12.60[ab]	0.000
T2	5	23.29 ±10.45[ab]	
T3	5	37.14 ±10.48[bc]	

Description: The mean SD ± contains different letters ([a] and [b]) showing a significant difference (p < 0.05); if it contains the same letters ([a], [ab], [b], and [ab]), indicating that there is no significant difference (p > 0.05).

Table 4: Difference Test for Antral Follicles Between Research Groups

Treatment Group	(n)	Means ± SD	p-value
NC	5	5.80 ± 1.79[b]	
PC	5	1.40 ± 0.55[a]	0.000
T1	5	3.40 ± 1.52[ab]	
T2	5	4.20 ± 0.45[b]	
T3	5	4.40 ± 1.52[b]	

Description: The mean ± SD shows the results of the descriptive analysis, and the p-value ($p<0.05$) indicates that there are significant differences in all the data.

The increase in expression can be seen from the increasing number of brownish stromal cells, describing the cells positively expressing Ki-67. The expression was indicated by brownish discoloration on DAB staining. The results of IHC observations on the percentage of Ki67 expression in endometrial stromal cell proliferation found that the percentage of Ki-67 expression in the PC group was fewer cells with Ki-67 expression compared to the NC group. In contrast, the T1, T2, and T3 treatment groups showed an increase in ki-67 expression after the administration of yellow pumpkin seed ethanol extract at three different doses compared to the PC group that was not administered the extract. The T3 group extract at a dose of 200 mg/kg BW after DMPA exposure showed a greater increase in Ki-67 expression than the T1 and

Table 5: One-Way ANOVA Test Results on Amount of Endometrial Stromal Cells

Treatment Group	(n)	Mean ±SD (Percentage)	›-value
NC	5	344.09 ± 31.99[b]	
PC	5	254.62 ± 23.93[a]	
T1	5	315.85 ± 44.08[ab]	0.008
T2	5	321.08 ± 31.19[ab]	
T3	5	330.44 ± 43.23[b]	

Description: If the average SD contains different letters ([a] and [b]), there is a significant difference ($p < 0.05$), whereas if it contains the same letters ([a], [ab], [b], and [ab]), then there is no significant difference ($p > 0.05$).

Table 6: One-way ANOVA test results number of endometrial epithelial cells

Treatment Group	(n)	Mean ±SD (Percentage)	p-value
NC	5	189.73 ± 18.25[b]	
PC	5	89.40 ± 9.47[a]	
T1	5	106.33 ± 13.31[a]	0.000
T2	5	109.26 ± 5.53[a]	
T3	5	167.86 ± 12.64[b]	

Description: The mean SD ± contains different letters ([a] and [b]) showing a significant difference ($p < 0.05$); if it contains the same letters ([a], [ab], [b], and [ab]), indicating that there is no significant difference ($p > 0.05$).

T2 groups, and the increase in Ki-67 expression was almost similar to that in the NC group. These results showed that the dose with the greatest effect on fertility restoration through increased expression of Ki-67 stromal cell proliferation after exposure to DMPA was 200 mg/kg BW. An overview of the percentage of expression of Ki-67 endometrial stromal cell proliferation was subjected to normality test with Shapiro Wilk p-value >0.05, and homogeneity test with Levene Statistic test p-value 0.275>0.05, with normally distributed and homogeneous data. The results of the percentage of expression of Ki-67 in endometrial stromal cell proliferation were then carried out by one-way ANOVA test to determine the effect of ethanol extract of yellow pumpkin seeds (*Cucurbita pepo L.*) at various doses against Ki-67 expression in endometrial stromal cell proliferation in the uterus of female white rats Wistar strain hypoestrogenic model DMPA-injected (Table 2). The mean expression of Ki-67 stromal cell proliferation in endometrial tissue of female white rat hypoestrogenic model showed significant differences in the five treatment groups. Based on Table 2 of the one-way ANOVA test results on the mean expression of ki-67 endometrial stromal cell proliferation, there was a statistical increase in the mean, which can be seen from the average expression of Ki-67 endometrial stromal cell proliferation in hypoestrogenic model rats in the form of a histogram in Figure 3. The mean value of Ki-67 expression of endometrial stromal cell proliferation in the positive control group (PC) (29.10±13.34) was lower than the negative control group (NC) (63.55±10.34), statistically using the Tukey HSD test showed a significant difference (p=0.008). The mean value of ki-67 expression in groups T1 (37.94±7.37), T2 (43.35±16.98), and T3 (56.88±19.21) was Iower than that in the negative control (NC) group with Tukey HSD test showing no statistically significant difference with a p-vaIue of 0.064>α (T1); p = 0.199> α (T2); and p-value 0.943>α (T3). When compared with the positive control group (PC) (29.10±13.34), the mean value of Ki-67 expression in the T1 (37.94±7.37), T2 (43.35±16.98), and T3 (56.88±19.21) groups was higher. However, the Tukey HSD test showed a significant difference only in the positive control group (KP) against the T3 group, with a p-value = 0.039. DMPA is an acetate ester resulting from the formal condensation of a 17 alpha-hydroxy

medroxyprogesterone group with a carboxyl group of acetic acid. DMPA is known as methylprogesterone acetate (MPA), a derivative of its parent compound medroxyprogesterone (MP), the contraceptive form of progesterone.[12] Progestin compounds in DMPA androgen activity with the mechanism of action diffusing freely into target cells

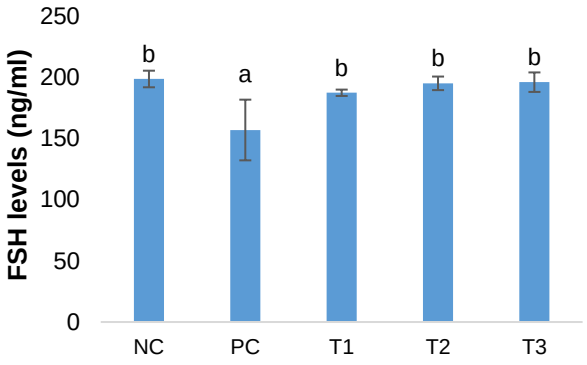

Figure 1: FSH Levels

Description: *Rattus norvegicus* rats had mean FSH levels. Negative control (NC); Positive control (PC) with DMPA only; Treatment 1 (T1) with extract of Pumpkin seeds 50 mg/kg BW; Treatment 2 (T2) with extract of Pumpkin seeds 100 mg/kg BW); Treatment 3 (T3) with extract of Pumpkin seeds 200 mg/kg BW.

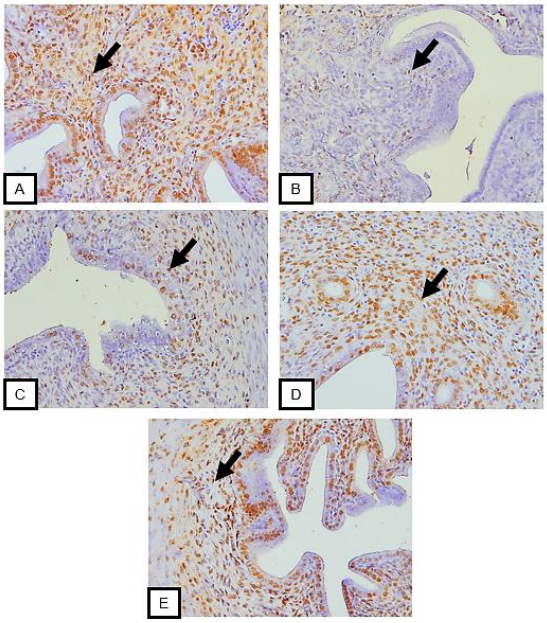

Figure 2: Results of Immunohistochemistry (IHK) staining of monoclonal antibody Ki-67 endometrial stromal cell proliferation

Description: Figure (A) is a picture of the Negative Control IHK (NC). (B) Positive Control (PC), (C) treatment group 1 (T1), (D) treatment group 2 (T2), and (E) treatment group 3 (T3). Brown immunohistochemistry features showed ki-67 expression in the target cells with DAB staining. The expression of ki-67 in endometrial stromal cells is characterized by brown cells in the cell nucleus and cell membrane. The red arrow indicates one of the cells positive for Ki-67. The observation was performed using a Raxvision b550 microscope with Panasonic G9 at 400 × magnification.

and binding to progesterone receptors. After binding to progesterone receptors, progestins will slow down the release of GnRH from the hypothalamus and will suppress the preovulatory LH surge, thus preventing follicular maturation and inhibiting ovulation.[13 8 14] The results of this study showed that the average percentage of ki-67 expression in the positive control group (PC) was lower than in the negative control group (NC). This occurs because of the effect of DMPA administration in rats, which reduces the expression of ki-67

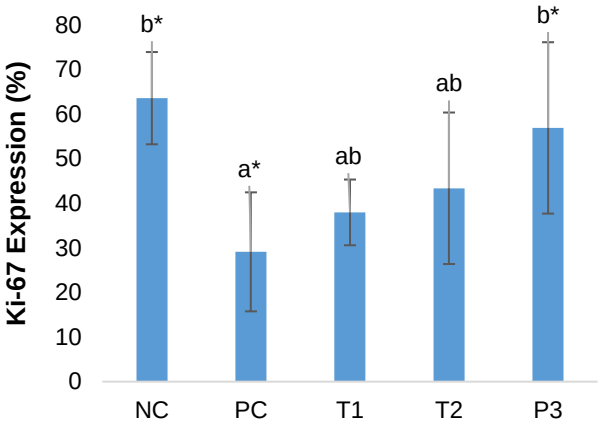

Figure 3: Histogram of mean Ki-67 expression of endometrial stromal cell proliferation

Description: If the average SD contains different letters ([a] and [b]) then there is a significant difference (p-value < 0.05), while if it contains the same letters ([a] and [ab] or [b] and [ab]) then there is no significant difference (p-value > 0.05). The (*) sign indicates the most significant p-value. Negative control (NC); Positive control (PC) with DMPA only; Treatment 1 (T1) with extract of Pumpkin seeds 50mg/kg BW; Treatment 2 (T2) with extract of Pumpkin seeds 100 mg/kg BW); Treatment 3 (T3) with extract of Pumpkin seeds 200 mg/kg BW.

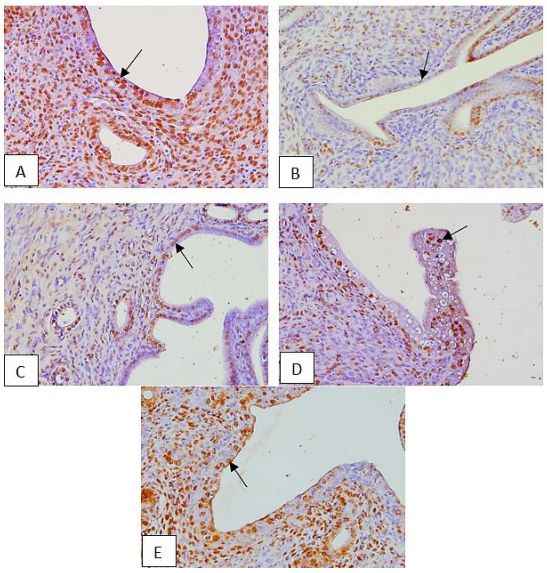

Figure 4: Results of immunohistochemical examination of ERβ expression in endometrium

The following are described: A. Negative control; B. Positive control; and C. Treatment 1 (T1), D. Treatment 2 (T2), and E. Treatment 3 (T3). Brown cells in the cell membrane and nucleus of the endometrial surface epithelium were indicative of ERβ expression in this tissue. Raxvision b550 microscope with Panasonic G9 displays cells expressing ERβ in groups A, B, C, D, and E (400 × magnification).

endometrial stromal cell proliferation. DMPA inhibits endometrial cell proliferation by inhibiting cyclin D1 and cyclin E expression, as well as

inhibiting the expression of vegetative endothelial growth factor (VEGF) and epidermal growth factor (EGF), so the endometrium experiences atrophy, affecting menstrual bleeding disorders until it becomes difficult to recover fertility again. [15] [16] The marker of cell proliferation is the Ki-67 antibody. When stromal cell proliferation does

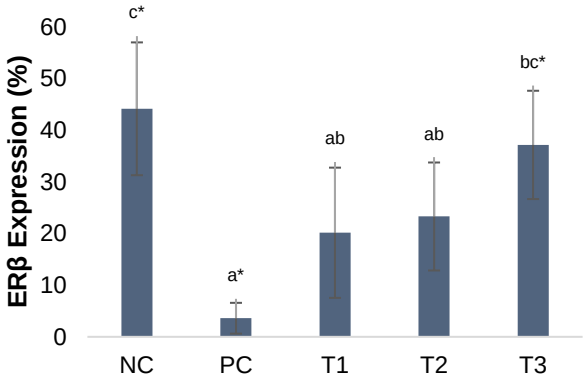

Figure 5: Histogram of mean ERβ expression
Description: On average, SD ± contains different letters (a and b) showing a significant difference ($p < 0.05$); if it contains the same letters (a, ab, b, and ab), there is no significant difference ($p > 0.05$). The (*) sign indicates a significant p-value. Negative control (NC); Positive control (PC) with DMPA only; Treatment 1 (T1) with extract of Pumpkin seeds 50 mg/kg BW; Treatment 2 (T2) with extract of Pumpkin seeds 100 mg/kg BW); Treatment 3 (T3) with extract of Pumpkin seeds 200 mg/kg BW.

not occur, ki-67 is not expressed in the stromal cells which is marked by decreased ki-67 expression. In the groups T1 and T2, there was a more pronounced increase in Ki-67 expression than in the PC group. The estrogenic activity of phytoestrogens is highly dependent on the concentration of a given phytoestrogen, estrogen receptors, the location of estrogen receptors, and competing concentrations of endogenous estrogens. This can occur because phytoestrogens affect several signal transduction pathways and the activity of protein tyrosine kinases, which may affect periodic or continuous cell growth.[17] The dose of the extract that was considered capable of increasing Ki-67 expression close to normal conditions was the T3 group, with a dose of yellow pumpkin seed ethanol extract of 200 mg/kg BW. This is in line with the research of Monteseirin *et al.* (2019), where the lignan content in phytoestrogens activates ERα and ER*β* through genomic signalling pathways, which then bind to DNA response elements. Activation of ERα and ER*β* by lignans can alter the function of transcription factors that can affect the expression of genes involved in proliferation, thereby increasing the proliferation of Ki-67 endometrial cells. [1819] Similarly, the percentage of ER*β* expression was determined by dividing the total number of cells in the field of view by the total number of positive cells expressing ER*β*. The surface of the endometrium becomes increasingly covered with brownish-colored epithelial cells, which are cells that positively express ER*β*. This indicated an increase in expression. An overview of ER*β* expression using IHC is shown in Figure 4. The immunohistochemical data indicated that the positive group had a lower percentage of ER*β* expression than the negative group. The treatment groups (T1, T2, and T3) exhibited an increase in ER*β* expression, as indicated by the cells that positively expressed ER*β* becoming brownish. Brownish discoloration indicated by DAB staining indicated expression. A 200 mg/kg BW/day dose of pumpkin seed ethanol extract was administered to the T3 group, which resulted in a greater number of cells being expressed on the endometrial surface epithelium. Additionally, the Levene Statistic test was used to perform a homogeneity test, and homogenous findings were achieved for the ER*β* variable with a significance level of 0.161. Subsequently, a One-Way ANOVA test was employed to ascertain the impact of diverse concentrations of yellow pumpkin seed ethanol extract on the expression of ER*β* in the endometrium of hypoestrogenic rats. The results are presented in Table 3 which displays significant variations, with p-values of <0.05, in the mean expression of ER*β* among the five groups. The T3 and negative control groups had the highest mean ER*β* expression, whereas the T1 and T2 groups had the lowest average ER*β* expression. Figure 5 shows a typical image of ER*β* expression in the endometrium of rats treated with hypoestrogen. The ER*β* test in the positive control group (3.60±2.98) had a lower value than that in the negative control group (44.13±12.85), according to the Tukey HSD test results. A p-value of 0.000 was obtained when comparing the two control groups. The average ER*β* expression level in the negative control group was higher than that in the positive control group. ER*β* expression in the positive control group was $0.133>\alpha$, indicating no significant difference, whereas the T1 group's mean value (20.14±12.60) was higher. With a mean ER*β* expression value of 23.29±10.45, the T2 group outperformed the positive control group, with a significant difference not exceeding $0.053>\alpha$. A significant difference of $0.001<\alpha$ was seen in the mean value of ER*β* expression in the T3 group (37.14±10.48) as compared to the positive control group. In this study, the positive control group that received DMPA injections had substantially lower levels of ER*β* expression than the negative group. The growth of stromal and epithelial cells requires estrogen binding to *α* and *β* estrogen receptors, which function in a similar mechanism. Administering GnRH antagonists reduces estrogen levels, decreasing estrogen binding to these receptors and subsequently lowering their expression. This suppression inhibits the transcription process, reducing granulosa cell proliferation in ovarian cells by downregulating BMP and GDF-9 gene expression. As a result, folliculogenesis in the ovaries is impaired. Therefore, estrogen binding to ER*α* and ER*β* is essential for endometrial proliferation. [20] [21] The T3 group received an extract dose of 200 mg/kg BW, which was thought to be the most effective in increasing ER*β* expression. The phytoestrogen in pumpkin seeds is called secoisolariciresinol diglucoside (SDG) and has been shown to help treat postmenopausal osteoporosis (PMOP), which is brought on by an estrogen shortage. The pharmacological tests demonstrated that SDG might raise serum E2 levels and have an impact on hormonal markers. Avenant *et al*. (2023) also found that OVX reduced the amount of E2 and the expressions of ER*α* and ER*β,* suggesting that PMOP developed because of a drop in serum estrogen levels and ER expressions following OVX surgery. A significant rise in E2 concentration and a decrease in OVX-induced ER*α* and ER*β* expression was observed following SDG administration, indicating that SDG may prevent PMOP by upregulating ER expression. [22] Phytoestrogens, such as lignans in pumpkin seeds, activate estrogen receptors (ERs) by binding to them, enabling the receptors to interact with transcription factors or EREs in the nucleus to regulate estrogen-responsive genes. Experimental studies show that some phytoestrogens stimulate uterine growth. These compounds mimic estrogen by penetrating cell membranes and binding to ER*α* and ER*β*, though with lower affinity compared to estrogen. ER*β* exhibits significantly higher affinity and activity than ER*α,* making phytoestrogens particularly effective in tissues rich in ER*β*. However, certain phytoestrogens can bind more strongly to ER*β* than estrogen, suggesting unique mechanisms of action. The effects of phytoestrogens vary depending on the tissue and receptor type, with ER*α* predominant in the uterus, ER*β* in the prostate, and GPER1 in blood vessels, where it regulates cell proliferation.[23] [24] [25]Haematoxylin-Eosin (HE) staining is used in the histological evaluation of antral follicles to count the number of antral follicles. The results of microscopic observations of rat ovarian follicles in each treatment group in this study began with the use of a microscope at 30 x magnification to view an image of the ovaries. Observations were made using 100 x magnification to count the number of antral follicles and analyze the type of follicles. The findings from the observations of antral follicles are as follows. Based on the description of the average number of stromal cells, the normality test using the Shapiro-Wilk test resulted in a p-value > 0.05, and the homogeneity test using the Levene Statistics test resulted in a p-value > 0.05, indicating that the data were normally distributed and homogeneous. This was followed by a one-way ANOVA test carried out to determine the effect of ethanol extract of pumpkin seeds (*Cucurbita pepo L.*) at various doses on the number of antral follicles in hypoestrogenic female Wistar rats injected with DMPA (Table 4). The mean number of antral follicles showed significant differences among the five treatment groups, with a p-value of $0.000<\alpha$. Figure 7 shows

that when a dose of pumpkin seed extract was administered, the number of antral follicles increased. An increase in the dose caused an increase in the number of an th f antral follicles in rats administered DMPA was in group T3, at a dose of 200 mg/kg BW. However, the effective dose was T2 (100 mg/kg

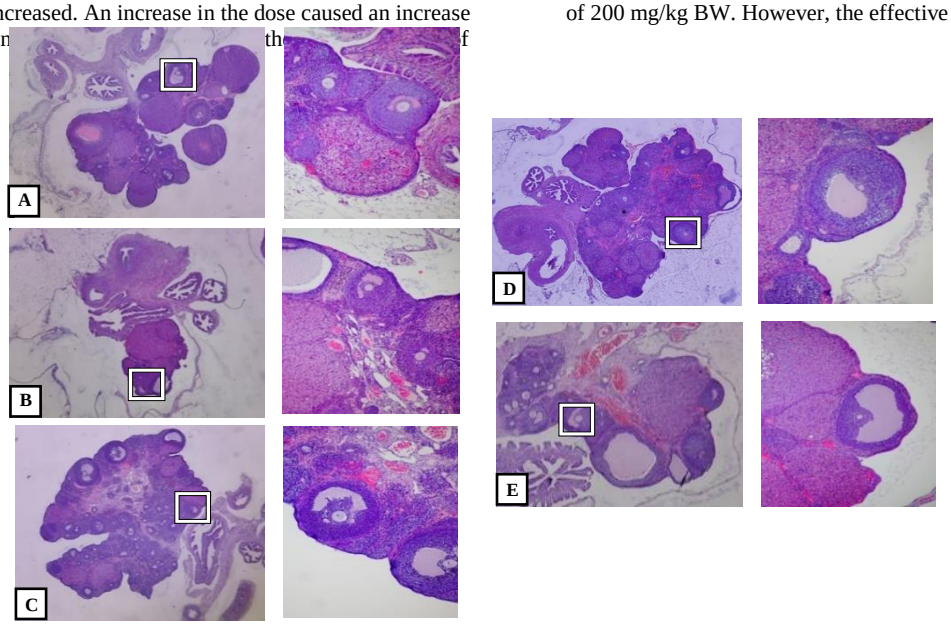

Figure 6. Observation of antral follicles with Hematoxylin Eosin (HE) staining

Description: HE staining of mouse ovaries to count the number of antral follicles in images (1) A, B, C, D, and E at 30 × magnification with an Olympus SZ51 microscope and (2) A, B, C, D, and E at 100 × magnification with the Amscope Microscope series T340B-LED. (A) negative control; (B) positive control + DMPA; (C) DMPA treatment + 50 mg/kg BW; (D) DMPA treatment + 100 mg/kg BW; and (E) DMPA treatment + 200 mg/kg BW. In pictures (2) A, B, C, and D, the arrows show antral follicles with an already-formed antrum and two or more layers of granulosa cells surrounding the oocyte. In image E, the cell nucleus begins to shift to the edge, the antrum becomes larger, and granulosa cells become thinner.

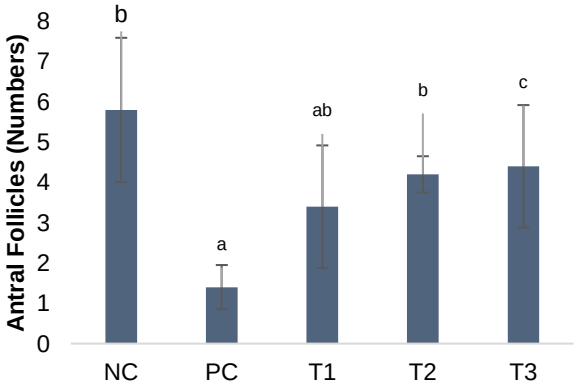

Figure 7. Mean Number of Antral Follicles

Description: Number of antral follicles in a group of *Rattus norvegicus* rats. Negative control (NC); Positive control (PC) with DMPA only; Treatment 1 (T1) with extract of Pumpkin seeds 50 mg/kg BW; Treatment 2 (T2) with extract of Pumpkin seeds 100 mg/kg BW); Treatment 3 (T3) with extract of Pumpkin seeds 200 mg/kg BW.

BW) because this dose increased the number of follicles. After all, it was significantly different from the positive control rather than the negative control. Progesterone's function in controlling reproductive hormones has been better clarified by recent research, especially with depot medroxyprogesterone acetate (DMPA). The progestin, glucocorticoid, androgen, and mineralocorticoid receptors are among the receptors that progesterone binds to. It then diffuses to target cells, especially those in the pituitary, mammary, and brain. The hypothalamus's production of gonadotropin-releasing hormone (GnRH) is suppressed by this interaction.[5] Excessive progesterone may suppress the release of GnRH (gonadotropin-releasing hormone) by affecting neurons in the central nervous system and inducing the release of neurotransmitters such as opioids, dopamine, and GABA. This reduced GnRH secretion limits the stimulation of the anterior pituitary, leading to lower levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). As a result, the decreased levels of FSH prevent the maturation and development of ovarian follicles, inhibiting ovulation.[26] Long-term DMPA exposure causes ovarian follicle reduction, which leads to ovarian dysfunction via follicular atresia and apoptosis, which exacerbates hypoestrogenic conditions. Follicle growth and development are significantly stimulated by FSH. The ovary is surrounded by granulosa and theca cells, which cooperate to enlarge the follicle and eventually produce the antrum. Studies show that granulosa cell mitochondrial and metabolic dysfunctions may lead to follicular atresia and apoptosis, affecting the general health and function of follicles. [27] Phytoestrogens, which are compounds derived from plants, might impact the functions of estrogen-regulated tissues such as the gonads, reproductive tract, and central nervous system. They can potentially handle estrogen deficiency, especially in women who use hormonal contraceptives like DMPA. Phytoestrogen supplements may lessen hypoestrogenic symptoms and promote hormonal balance by interacting with estrogen receptors and regulating processes including folliculogenesis and bone metabolism.[28] This is consistent with studies that have shown phytoestrogens obtained from pumpkin seed ethanol extract to enhance the number of antral follicles. Histological examination of endometrial stromal cells with HematoxyIin Eosin (HE) staining of 1% was observed in the endometrial stromal cells of rats after treatment with yellow pumpkin seed extract at various doses. Microscopic images of endometrial stromal cells in each treatment group showed results that varied according to treatment. The microscopic observations of endometrial stromal cells in each group are shown in Figure 8. Figure 8 shows that the number of stromal cells in the positive control (PC) was less and less dense than in the negative control (NC). DMPA affected the number of endometrial stromal cells in the positive control group; stromal cells appeared less frequently and in fewer numbers. Administration of the ethanol extract of yellow pumpkin seeds (*Cucurbita pepo L.*) at various doses resulted in changes in the density of endometrial stromal cells. The T1, T2, and T3 treatment groups showed an increase in the density of endometrial

stromal cells. In T3, given the DMPA injection and yellow pumpkin seed ethanol extract at a dose of 200 mg/kg BW, the number of stromal cells appeared more and denser compared to T1 and T2. In T3, stromal cells appeared close to conditions similar to NC. This shows that the ethanol extract of yellow pumpkin seeds can increase the proliferation of endometrial stromal cells, characterised by an increase in the number

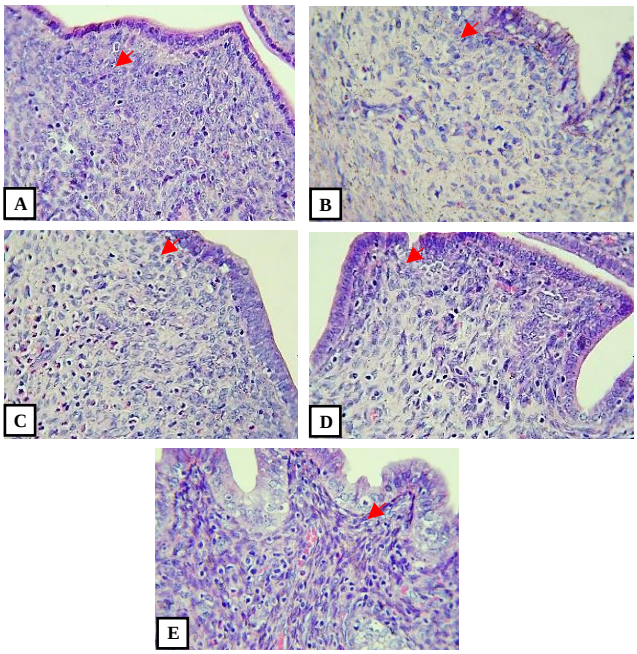

Figure 8. Observation of endometrial stromal cells in rats.

Description: Figure (A) shows stromal cells of the negative control (NC). Figure (B) Positive Control (PC), (C) treatment group 1 (T1), (D) treatment group 2 (T2), and (E) treatment group 3 (T3). The red arrow indicates one example of stromal cells in a single field of view. Endometrial stromal cells are dark blue in the cell nucleus. Observation using microscope dot Slide Amscope T340B-LED at 400 × magnification.

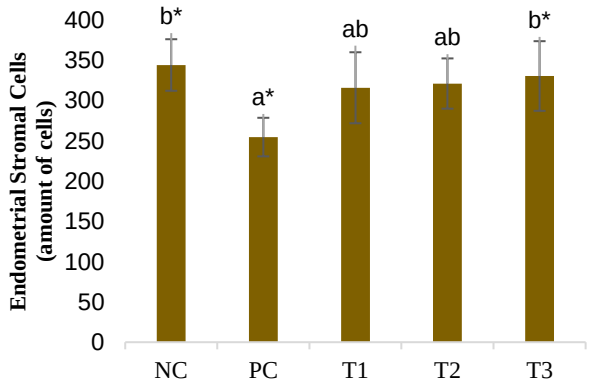

Figure 9. Histogram of mean endometrial stromal cells

Description: If the mean SD contains different letters (^{a} and ^{b}) then there is a significant difference (p-value<0.05), while if it contains the same letters (^{a} and ^{ab} or ^{b} and ^{ab}) then there is no significant difference (p-value>0.05). The sign (*) indicates the most significant p-value. Negative control (NC); Positive control (PC) with DMPA only; Treatment 1 (T1) with extract of Pumpkin seeds 50 mg/kg BW; Treatment 2 (T2) with extract of Pumpkin seeds 100 mg/kg BW); Treatment 3 (T3) with extract of Pumpkin seeds 200 mg/kg BW

of stromal cells and a density closer to normal conditions. Based on the description of the average number of stromal cells, the normality test with Shapiro Wilk results was p-value > 0.05, and the homogeneity test with the Levene Statistic test p-value 0.661>0.05, the data were normally distributed and homogeneous. Furthermore, a one-way ANOVA test was carried out to determine the effect of ethanol extract of yellow pumpkin seeds (*Cucurbita pepo L.*) at varying doses on endometrial stromal cell counts in female white rats of the hypoestrogenic model injected with DMPA is presented in Table 5. Regarding the average number of endometrial stromal cells in female rats, the hypoestrogenic model showed significant differences in the five treatment groups, with a p-value of 0.008<α. The average value of stromal cells can be seen in the form of a histogram in Figure 9. The average number of stromal cells in the positive control group (PC) was lower than that in the negative control group (NC). The positive control group (PC) also had lower average values than treatment 1 (T1), treatment 2 (T2), and treatment 3 (T3) after treatment with various doses of yellow pumpkin seed ethanol extract. In the treatment groups (T1, T2, and T3), the number of stromal cells increased with an increase in the dose of ethanol extract of yellow pumpkin seeds (*Cucurbita pepo L.*). The T3 group showed a statistically significant increase in the number of endometrial stromal cells compared to the positive control group (PC). DMPA administration in the PC led to a significant decrease in stromal cell count after exposure to the 7th DMPA injection. Research by Irina A. Zalenskaya et al (2018) also proved that progesterone content in DMPA directly affects the number of endometrial stromal cells by inhibiting their proliferation of endometrial stromal cells. This is because progesterone has a dual function, namely stimulating stromal paracrine factors and inhibiting estrogen and progesterone receptors. [29] A decrease in the number of endometrial stromal cells is associated with atrophy in the endometrial wall which can inhibit the implantation of fertilization and changes in bleeding patterns. [30] DMPA lowers endogenous estrogen levels, which cause hypoestrogenic conditions characterized decrease and inhibition of endometrial cell proliferation to endometrium experiences atrophy.[31] The mechanism of lignan signaling pathways is similar to that of natural estrogen (E2) in stimulating cell growth and proliferation in the endometrium. ERβ can directly bind to the estrogen response element (ERE) on the promoter of the target gene, thereby activating its transcription of the target gene, modulating gene expression, and triggering cell proliferation. [27] Cell proliferation triggers an increase in the number of cells, one of which is endometrial stromal cells. The pumpkin seed extract dose in the group (T1 and T2) can increase the number of stromal cells. Previous research has shown that yellow pumpkin seed ethanol extract at a dose of 100 mg/kg BW does not significantly increase estrogen levels in hypoestrogenic model rats.[33] Therefore, the doses of yellow pumpkin seed ethanol extract at T1 and T2 were not optimal for increasing the number of stromal cells. The dose considered most effective in increasing the number of stroma cells was T3 group at a dose of 200 mg/kg BW/day was significantly different from that of PC. This happens because isoflavone group compounds can bind to estrogen receptors and work both genomically and non-genomically locally in response to cell targets, thus affecting cell proliferation and regulation of steroid sex hormone receptors. [23]

Cell proliferation is characterised by an increase in the number of cells, as observed in this study, where there was an increase in the number of endometrial stromal cells by administering yellow pumpkin seed extract at various doses. A yellow pumpkin seed extract has a positive influence on endometrial wall thickness since phytoestrogens have a role resembling that of endogenous estrogen. [19] Phytoestrogens bind to estrogen receptors on target cells to increase endometrial thickness, both by increasing cell proliferation and endometrial cell count.[25] EstradioI (E2) significantly enhances products that stimulate endometrial proliferation thereby influencing the interaction between the endometrial epithelium and stroma and their spread. Primiani's research (2016) also proved that the administration of phytoestrogens can increase cell proliferation in the endometrial layer. [35]

The number of surface epithelial cells in the rat endometrium following surgery was counted using histological examination of endometrial epithelial cells. Up to five fields of view were used to observe each uterine sample before the data were averaged and

combined. There was variation in the number of endometrial surface epithelia in the NC, PC, T1, T2, and T3 groups (Figure 10). The NC group exhibited a surface epithelium that was stratified, tight, spherical, and longitudinally organized between each epithelium. The surface epithelium in the PC group appeared to be more dispersed between each cell than that in the NC group, and it was structured unevenly.

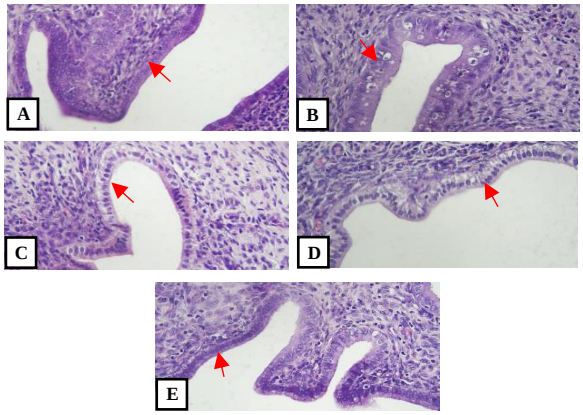

Figure 10. Observation of endometrial epithelial cells with Haematoxylin Eosin staining

Description: A. Negative control; B. Positive control (DMPA); C. Treatment 1: extract of pumpkin seeds 50 mg/kg BW; D. Treatment 2: extract of pumpkin seeds 100 mg/kg BW; E. Treatment 3: extract of pumpkin seeds 200 mg/kg BW. Endometrial surface epithelial cells were characterized by a spherical and nucleated cell shape indicated by a red arrow using the Amscope T340B-LED Slide Dot Microscope (400 x magnification).

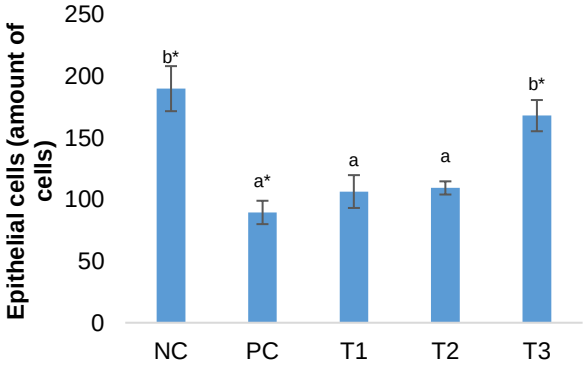

Figure 11. Histogram of the mean number of endometrial epithelial cells

Description: On average SD ± contains different letters ([a] and [b]) showing a significant difference ($p < 0.05$); if it contains the same letters ([a], [ab], [b], and [ab]), there is no significant difference ($p > 0.05$). The sign (*) indicates a significant p-value. Negative control (NC); Positive control (PC) with DMPA only; Treatment 1 (T1) with extract of Pumpkin seeds 50 mg/kg BW; Treatment 2 (T2) with extract of Pumpkin seeds 100 mg/kg BW); Treatment 3 (T3) with extract of Pumpkin seeds 200 mg/kg BW.

The T1 group had a more tenuous surface epithelial arrangement lengthwise than the NC group. Comparing the T2 group to the NC and T3 groups, it is evident that the epithelial cells are denser, and the surface epithelium was nicely structured and dense but still appears tenuous. Additionally, the Levene Statistic test was used to perform homogeneity tests, and homogenous findings on the variable number of epithelial cells were achieved, with a significance level of 0.179. Table 6 shows that there were significant variations ($p = 0.000$) in the average number of epithelial cells among the five test groups. The positive control groups, T1 and T2, had the lowest average number of epithelial cells, whereas the negative control and T3 groups had the highest average number. The bar chart below shows the average number of epithelial cells in the endometrium of the hypoestrogen model rats. The Tukey HSD test revealed a significant difference of $0.000<\alpha$ in the number of epithelial cells between the PC (89.40±9.47) and the NC (189.73±18.25). In the Tukey HSD test, group T1's mean epithelial cell count (106.33±13.31) was larger than that of the PC, with no significant difference of $0.246>\alpha$. A significant difference of $0.130<\alpha$ was observed in the mean value of the number of epithelial cells T2 (109.26±5.53) greater than that of PC, while a significant difference of $0.000>\alpha$ was observed in the case of T3 (167.86±12.64) higher than that of PC. The findings of this study indicated a significant difference in the average number of endometrial epithelial cells between NC and PC. DMPA can induce apoptosis in endometrial tissues, inhibiting its growth. Oxidative stress, caused by an imbalance between growth factors and damaging signals like DNA damage and oxidants, triggers apoptosis, which is further increased by reduced eNOS activity, leading to endometrial artery damage. Research also shows that DMPA elevates immune-regulation-related genes, including those involved in T-cell and B-cell receptor signalling, cytokine-mediated signalling, and neutrophil immunity. However, pathways related to epithelial barriers, such as skin development and keratinocyte differentiation, are significantly downregulated. Overall, DMPA use is linked to immune modulation and changes in epithelial barrier function. [36 37 38] The number of endometrial epithelial cells was found to be higher in rats treated with yellow pumpkin seed extract at varying doses than in the positive control group, which did not receive therapy, according to the study's findings. Compared to the positive control group, which had fewer and less frequent epithelial cells, the epithelial cells in the treatment group were denser and more frequent after treatment with yellow pumpkin seed extract. Although it was not statistically significant in the PC group, administration of pumpkin seed extract at doses of T1 (50 mg/kg BW) and T2 (100 mg/kg BW) increased the number of epithelial cells. For the T3 group, an average dose of 200 mg/kg BW/day was thought to be the most effective in increasing the number of endometrial epithelial cells near normal levels. Yellow pumpkin seeds contain phytoestrogens that resemble endogenous estrogens and thicken the endometrial wall in areas of the endometrium, where there is an increase in the stroma and epithelium. This is because phytoestrogens can attach to target cells' estrogen receptors, increasing the endometrium's cell count and thickness. [39]

These findings are consistent with Hadiningsih *et al* (2020) study, which showed that administering phytoestrogens at different dosages can increase the quantity of stromal and endometrial epithelial cells. In the endometrial layer, phytoestrogens can promote cell proliferation, and their function may be similar to that of endogenous estrogen. The effects of DMPA can be enhanced by administering pumpkin seed extract at the proper dosage, as the phytoestrogens in pumpkin seeds can bind to ERβ and promote cell proliferation. As a result, administering ethanol extract from pumpkin seeds can enhance hypoestrogen levels, which should enhance reproductive function. [40]

Conclusion

The results of this study showed that administration of yellow pumpkin seed ethanol extract at a dose of 200 mg/kg BW increased FSH levels, Ki-67, and ERβ expression and increased the number of antral follicles, stromal cells, and endometrial epithelial cells in hypoestrogenic model rats injected with depo medroxyprogesterone acetate (DMPA). Phytoestrogens from pumpkin seeds in the form of secoisolariciresinol and lariciresinol compounds, isoflavones, and flavonoids can increase fertility through increased hormones and endometrial repair due to long-term DMPA use. There is need therefore for further research into the use of phytoestrogens in the management of female fertility-related issues.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims related to the content of this article will be borne by them.

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