

# Microstructural evidence of reversal of PCOS by steroidal saponins of asparagus racemosus in PCOS induced rats

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## SUMMARY

The purpose of this study is to investigate the histological and hormonal observations in fructose-fed, letrozole-induced polycystic-ovarian-syndrome (PCOS) rats treated with various doses of extract of asparagus racemosus (EAR) and Steroidal saponin (SAPO). 48 female Wistar albino rats were divided into 8 groups, including Vehicle Control (VC); PCOS; EAR 400 mg/kg; SAPO 40 mg/kg; PCOS + EAR 200 mg/kg; PCOS + EAR 400 mg/kg; PCOS + SAPO 20 mg/kg; PCOS + SAPO 40 mg/kg. PCOS group was administered letrozole at a concentration of 1 mg/kg dissolved in 1% CMC per oral(p.o.) once daily for 28 days. Along with these, rats were allowed free access of 10% fructose solution daily. Calculated dosages of EAR and SAPO were given with oral gavage for 30 days. During experimental period, vaginal smears were collected daily for estrus cycle determination. Rats were sacrificed and blood samples were collected for hormonal assay. Ovaries were removed to proceed with histopathological study. Slides were stained using hematoxylin and eosin (H&E) stains. When compared to the vehicle control group, PCOS ovaries had a higher incidence of ovarian cysts,

incomplete luteinization, and a lower number of corpus lutea. Although serum estradiol, progesterone, and Follicle-stimulating hormone (FSH) levels were lower in the PCOS group, testosterone and luteinizing hormone (LH) levels were higher. The findings of this study indicated that taking EAR 400 mg/kg and SAPO 40mg/kg orally could alleviate PCOS-related symptoms. It appears that consuming SAPO 40mg/kg reduces LH and testosterone levels while increasing FSH, estrogen, and progesterone hormone levels. Because of the hormonal balancing nature of these drugs, EAR 400mg/kg- and SAPO 40mg/kg-treated rats had a lower number of cystic follicles and a higher number of corpora lutea. In PCOS rats, this results in a normal process of folliculogenesis and ovulation. In the current study, we observed that SAPO 40mg/kg is better compared to EAR 400mg/kg treatment.

**Keywords:** PCOS – Asparagus racemosus – Saponin – Hormones

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## INTRODUCTION

PCOS has a complex etiology that includes a number of reproductive and metabolic factors. There is no single etiology that can explain the prevalence of PCOS. Furthermore, no single etiological gene or inheritance pattern for PCOS has been identified. The pituitary gland secretes an abnormally large amount of LH into the bloodstream in PCOS, disrupting the normal menstrual cycle. As a result, follicle maturation and ovulation are delayed, which can result in anovulation. The immature follicles that do not mature remain as fluid-filled sacs or cysts. Because of an increased amount of testosterone, these cysts cause hormonal imbalances. In addition, there is an increased level of insulin hormone produced by the pancreas (Anadu et al., 2013).

Polycystic ovaries are many times the size of a normal ovary. There are a lot of immature follicles (subcortical cysts) that cause the ovary to change shape. The ovary turns whitish in color, with multiple cystic follicles surrounded by a dense fibrous capsule. There is hyperthecosis as well as thickening of the tunica albuginea (Krishnamurthy et al., 2009). PCOS women's ovarian and adrenal glands are typically sites of increased androgen production. It is also suggested that PCOS women have an increase in the production of the CYP17 enzyme, which is responsible for the formation of androgens in the ovaries and adrenal glands (Haywood et al., 2004). The ovaries produce a variety of androgens, the most prominent of which is testosterone. Other androgens produced by the ovaries include androstenedione and DHA. The stroma and theca cells produce an excess of testosterone, which is a common and significant feature of polycystic ovary (Nagarthna et al., 2014).

*Asparagus racemosus* (AR) is a tropical and subtropical Indian plant with medicinal properties. This plant was traditionally used as a fertility tonic. AR's primary active constituents are steroidal saponins (Shatavarins–IV) found in the roots. Shatavarin IV is a sarsasapogenin glycoside composed of two rhamnose molecules and one glucose molecule. RP-HPLC methods were used to isolate five steroidal saponins, shatavarins

VI–X, as well as five known saponins, from AR roots (Hayes et al., 2008). AR is best known for its phytestrogenic properties. As people become more aware of the dangers of synthetic estrogens, there has been a surge in interest in plant-derived estrogens, making AR especially important. The molecular structures of the major phytestrogens, isoflavones and coumestans, are compared to 17-estradiol, the most powerful naturally occurring estrogen. The structural similarities between phytestrogens and 17-estradiol are sufficient to allow phytestrogens to occupy the ER, but affinity for the receptor is significantly lower when compared to 17-estradiol (Sharma et al., 2013). Its beneficial uses in correcting menstrual irregularities are mentioned in ancient literature, and they are still prescribed by ayurvedic physicians to correct menstrual irregularities with products available in the market. Clearly, more research is required to define the effect of phytestrogens from AR while also standardizing and characterizing formulations and/or isolated phytestrogens. Furthermore, developing an understanding of the effects of phytestrogens derived from AR versus human estrogens holds great promise for future research (Verma et al., 2014).

Many studies have found that saponin reduces the harmful effects of chemotherapy on reproductive organs. Saponins also possess antioxidant properties (Zhang et al., 2008). The current study was conducted to assess the effect of ethanolic extract of AR roots and steroidal saponins on hypothalamic-pituitary-gonadal axis hormones and histological observations in PCOS rats due to the presence of many compounds affecting gonadotropic and ovarian hormones in asparagus extract, such as steroidal saponins, vitamins, and amino acids.

## MATERIALS AND METHODS

### Animals

6-week-old Adult female Wistar albino rats (180–225 g) with regular estrus cyclicity were obtained from TANUVAS, Chennai. They were housed in polypropylene cages with paddy husk bedding, standard rat pellets, and ad libitum drinking

water, and acclimatized on a 12-hour light and 12-hour dark schedule in temperatures ranging from 23 to 25°C. The cages are categorized with the group, the animal's weight, and the drug dosage. Before beginning the study, each group of animals was given an average of one week to acclimatize. The experimental study protocol was approved by Tagore Medical College and Hospitals Institutional Animal Ethics Committee (TMC/IAEC/02/003, dated 12.01.2015; TMC/IAEC/01/002, dated 03.12.2016).

### Collection and preparation of plant material

AR fresh roots were obtained from Kolapakkam, Vandalur, Chennai, Tamil Nadu, India between July and September and authenticated by the Department of Botany, National Institute of Siddha, Chennai (Voucher no. NISMB1492014, dated December 29, 2014) The specimen was stored at the National Institute of Siddha in Chennai, India, for future use. For a week, AR roots were shade dried. The dried roots were powdered using an electric blender before being subjected to a 48-hour Soxhlet extraction with 99 % ethanol. A rotary flash evaporator was employed to dry the mixture, and the condensed extract was stored in the refrigerator before being used for preliminary phytochemical screening (Harborne, 1973).

### Chemicals

Letrozole was purchased from sun pharmaceutical, (Mumbai). Carboxmethylcellulose (CMC) and fructose were purchased from southern India scientific corporation, (Chennai).

### Experimental design

The animals were divided into eight groups with each group having 6 animals. Group 1 - 1% CMC administered p.o., for 28 days (Vehicle control). Group 2 -Letrozole 1 mg/kg in 1% of CMC (2 mL/kg) administered p.o., with free access of 10% solution of fructose for 28 days (PCOS). Group 3 - 400 mg/kg of EAR treated p.o., for 30 days (EAR 400). Group 4 - 40 mg/kg of SAPO treated p.o., for 30 days (SAPO 40). Group 5 - PCOS rats +200 mg/kg of EAR treated p.o., for 30days (PCOS + EAR 200). Group 6 - PCOS rats + 400 mg/kg of EAR treated p.o., for 30 days (PCOS

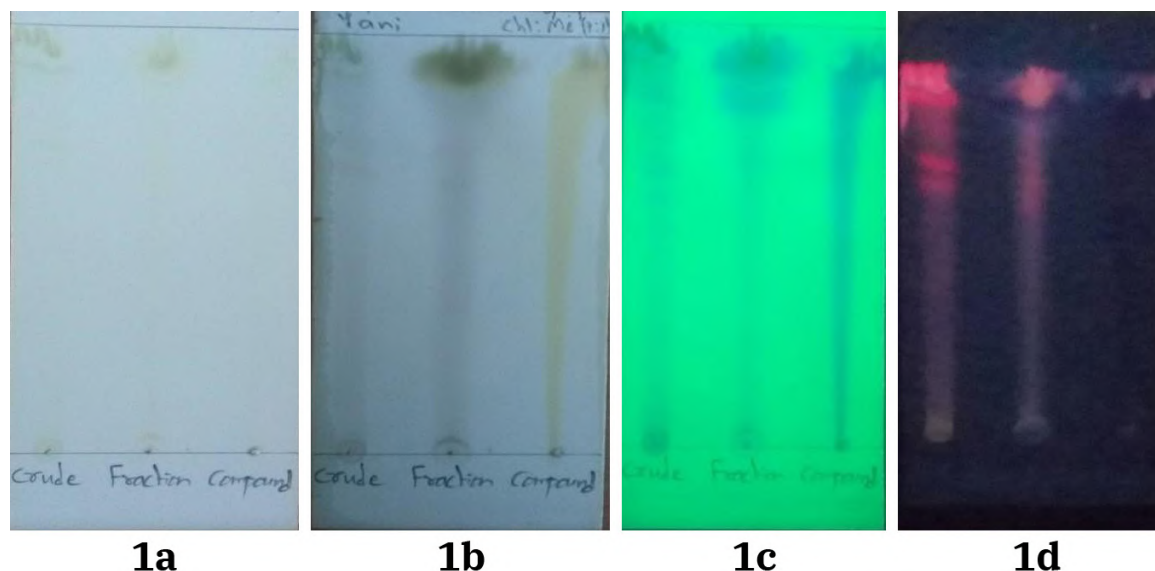
+ EAR400). Group 7 - PCOS rats +20 mg/kg of SAPO treated p.o., for 30 days (PCOS + SAPO20). Group 8 - PCOS rats+ 40 mg/kg of SAPO treated p.o., for 30 days (PCOS +SAPO40). Throughout the entire study period vaginal smears were taken daily and evaluated microscopically for estrous cycle determination.

### Column and thin layer chromatography

A small plug of cotton was pushed at the bottom of the glass column after it had been cleaned and dried vertically. The fractions were eluted with different ratios of solvents such as hexane (low polar), chloroform (middle polar), ethyl acetate (polar), and ethanol (highly polar) in the order of increasing polarity. The fractions collected were then concentrated for use in thin-layer chromatography. TLC plates 20 x 20 cm, 1 mm thick, were taken and cut into appropriate sizes so that samples could be loaded. The column chromatography fractions were used as samples, and individual spots were placed in the TLC plates using a capillary tube. After that, the TLC plate was placed in the solvent chamber for elution. When the sample reached the desired height, the TLC plate was removed from the chamber and stored for drying. The vanillin sulphuric acid solution was prepared, and the TLC plate containing the sample was dipped in it and allowed to dry. The spots obtained were the AR's active compounds known as steroidal saponins (SAPO) (Marston et al., 1997) (Fig. 1 a,b,c,d).

### Administration of drugs

Oral gavage was performed using an 18-gauge cannula (feeding tube) with a bulb tip attached to a syringe. The drugs were loaded into a syringe and administered orally. To avoid volume-induced changes, all animals received the same volume of fluid. The cannula was designed to be slightly longer than the distance between the animal's mouth and the last rib. Following the roof of the mouth, the flexible cannula was slowly advanced into the esophagus and then to the stomach, with no resistance. This was accomplished by gently restraining the rats by grasping the loose skin of the neck and back and immobilizing the head.



**Fig. 1.- a, b, c and d** show the visible light, short UV, long UV and vanillin dyed TLC sheet of the crude, fraction and compound AR respectively.

### Vaginal smears

Throughout the study, vaginal secretions were collected with a plastic pipette every morning between 9:00 and 10:00 a.m. by inserting the tip into the rat's vagina and filling it with 10  $\mu$ l of normal saline. On each glass slide, one drop of collected vaginal fluid was placed. Each animal received its own glass slide and pipette tips. The collected vaginal fluid was fixed on slides using a slide warming table and examined under a light microscope (40x). There were three types of cells identified: round and nucleated cells were epithelial cells, irregular cells without a nucleus were cornified cells, and small round cells were leucocytes; their mutual proportion was used for determining different phases of the estrus cycle (Fig. 2).

### Blood collection and hormonal assay

After CO<sub>2</sub> inhalation, blood samples are taken from the heart via cardiac puncture, which can be accessed via the left side of the chest, through the diaphragm, from the top of the sternum, or by performing a thoracotomy. By inhaling CO<sub>2</sub>, blood is slowly withdrawn from the heart to prevent it from collapsing while under deep anesthesia. Blood samples were quickly collected into plain sample bottles, allowed to clot, and then centrifuged at 3,000 RPM for 15 minutes to obtain clear serum samples, which were then frozen

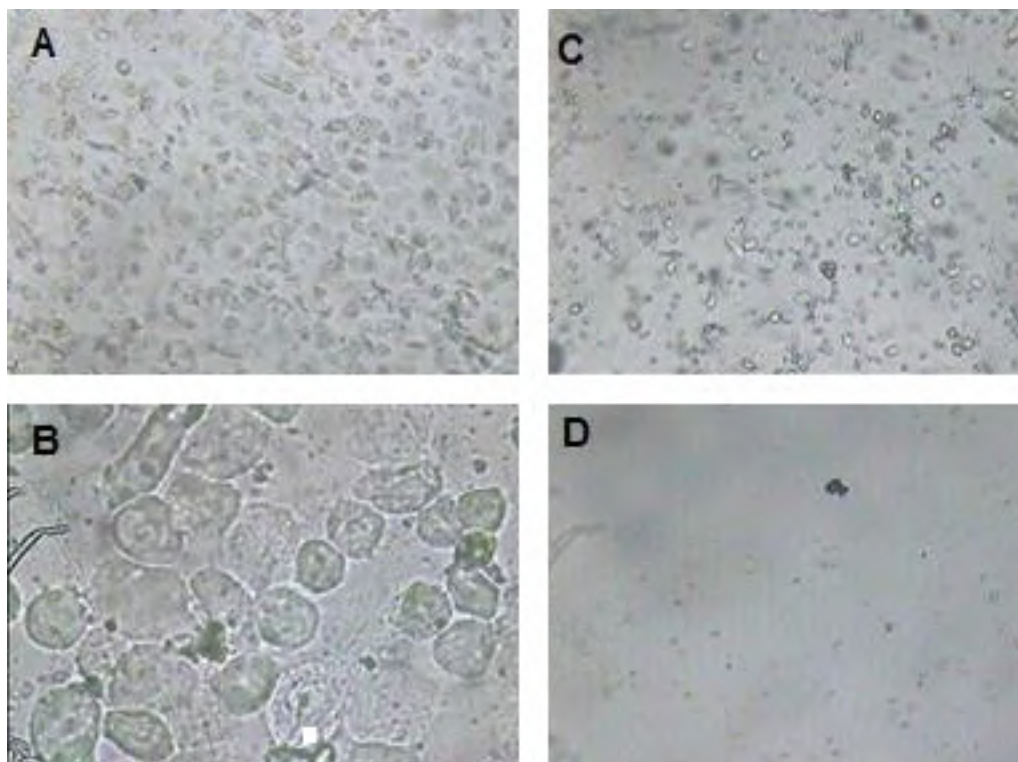
(-20°C) until hormonal assays were performed. The serum was tested for LH, FSH, testosterone, progesterone, and estrogen levels, among other things. ELISA Kit was used for hormone analysis (Elabscience).

### Perfusion of animals

Following CO<sub>2</sub> deep inhalation, rats were cut open through the midline thoraco-abdominal incision and transcatheterial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffered saline on the left ventricle and the right atrium. When limbs flicker, tissues blanch, and the animal's entire body hardens, this indicates that perfusion has been completed. The ovaries and uterus were carefully dissected and collected as per standard procedure (Gandhare et al., 2013). The organs were washed with cold saline solution and weighed immediately before being fixed in formalin (10% paraformaldehyde solution) for 48 hours. Tissue samples were collected from each organ, processed and stained according to the standard procedure outlined below.

### Histological techniques

The ovaries were cut in the equatorial plane and fixed for 48 hours in Gendre's fluid. The fixed ovaries were dehydrated for 2-4 hours using 70% alcohol, 90% alcohol, absolute alcohol, and absolute alcohol II. The dehydrated ovaries were



**Fig. 2.-** Stages of estrous cycles in rats pipette smears unstained with original microscope (x100). **A.** Estrous: large cornified cells inclumps. **B.** Metaestrous: large numbers of leucocytes with smaller numbers of non- nucleated epithelial cells. **C.** Diestrous: mainly leucocytes but with small number of epithelial and cornified cells. **D.** Proestrous: epithelial cells - mostly rounded but some cells showing early stages of cornification of approaching estrous.

then cleared twice in xylene for 3 hours before being impregnated in molten paraffin wax at 60°C for 3 hours with two changes of wax. The ovaries that had been impregnated with wax were now embedded in paraffin wax. The ovaries were embedded by inserting one half from above and the other half from below. Three 5 µm thick sections were taken at different levels in each half of the ovaries using a rotary microtome, then mounted on slides and dried in a hot plate at 40°C. The ovaries section slides were dewaxed with xylene, then removed with absolute alcohol I and II, 95% alcohol, 70% alcohol, and finally water for staining (Culling, 1975).

#### Haematoxylin and Eosin staining

After hydration, ovarian sections were transferred to haematoxylin and held for 10 minutes, then slides were transferred to a slide washing tray and washed until blue, then placed in flowing tap water for around 10 minutes at pH 8. After bluing the sections, they were dipped in acid alcohol for a few seconds, agitated, and then returned to the slide washing tray until they turned

blue again. The slides were counter-stained for 2 minutes in 1 percent eosin and then washed in water to separate the eosin stain. The stained slides are now dehydrated for 10 to 15 seconds in various grades of alcohol, then cleaned for 10 to 15 seconds in xylene, then mounted with DPX and a cover slip (Culling, 1975). Following that, the slides were examined under a light microscope and photomicrographs (Olympus BX51, Japan) were obtained at various magnifications for study.

#### Statistical analysis

All the results were expressed as Mean ± SEM. The statistical analysis was carried by one-way ANOVA followed by Tukeys multiple comparison tests using SPSS 20.0 version,  $P < 0.05$  was considered as significant.

## RESULTS

In the present study the values of LH, FSH, estradiol, testosterone, progesterone and histological observations of all the experimental groups were compared with vehicle control group and PCOS group animals.

### Follicle stimulating hormone (mIU/mL)

The mean ± SEM of serum concentration of FSH level was noted and shown in the Fig. 3, PCOS group rats shown significant (p<0.001) reduction in the level of FSH when compared to vehicle control group. EAR 400 mg/kg and SAPO 40mg/kg alone treated group rats' FSH level was found to be similar to that of the vehicle control group. EAR 200 mg/kg treated to PCOS rats had shown that FSH level was increased but not significant, compared with PCOS group. Treatment with EAR 400 mg/kg, SAPO 20 mg/kg and SAPO 40 mg/kg to PCOS rats showed significant (p<0.001) increased FSH level when compared to the PCOS group rats. One-way ANOVA showed that there was a statistically significant difference between the groups (F=16.60, p<0.001).

### Luteinizing hormone (mIU/mL)

The LH serum level mean ± SEM are shown in Fig. 4; in PCOS group rats there was a significant increase (p<0.001) in the level of LH compared to vehicle control group. EAR 400 mg/kg and SAPO 40 mg/kg alone treated rats LH level was found to be similar that of vehicle control group.

Treatment with EAR 400 mg/kg (p<0.001), SAPO 20 mg/kg (p<0.01) and SAPO 40 mg/kg (p<0.01) in PCOS-induced rats showed significant decrease in the level of LH when compared to the PCOS group. EAR 200 mg/kg treated to PCOS-induced rats had shown that the LH level was altered but not significant compared to the PCOS group and vehicle control group. One-way ANOVA showed that there was a statistically significant difference between the groups. (F=5.717, p<0.001).

### Estrogen (pg/mL)

It can be seen from the Fig. 3, mean ± SEM of estrogen levels. It can be noticed that in PCOS group there is significant (p<0.001) reduction in the level of estrogen when compared to vehicle control group. EAR 400 mg/kg and SAPO 40 mg/kg alone treated group rats' estrogen was found to be similar compared to the vehicle control group. Treatment with EAR 400 mg/kg, SAPO 20 mg/kg and SAPO 40 mg/kg in PCOS rats showed significant (p<0.01) increase in the level of estrogen when compared to the PCOS group. One-way ANOVA showed that there was a statistically significant difference between the groups (F=12.99, p<0.001).

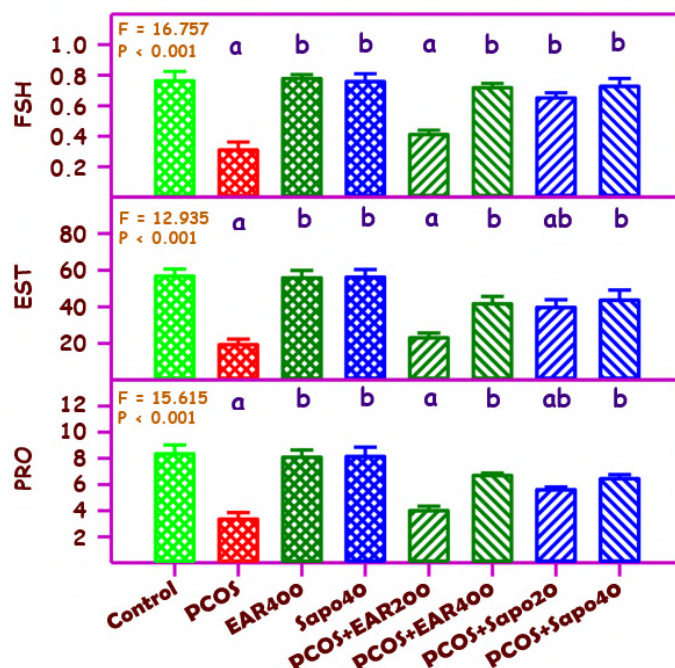


Fig. 3.- The effect of ethanolic extract of Asparagus racemosus (EAR) and its active component saponin (SAPO) in fructose and letrozole induced poly cystic ovary syndrome (PCOS). 20, 40, 200 and 400 are doses per kilogram body weight. Values are mean ± SEM (n = 6 each). Follicle stimulating hormone (FSH) (IU/mL); Estrogen (ngl/mL); Progesterone (ngl/mL). The 'F' and 'P' values are by one way ANOVA with Tukey multiple comparison test. <sup>a</sup> significantly different from vehicle control, <sup>b</sup> significantly different from PCOS.

### Testosterone (g/dL)

Fig. 4 shows the mean values of testosterone across the study groups. The serum testosterone level increased significantly ( $p < 0.001$ ) in PCOS group compared to vehicle control group. EAR 400 mg/kg and SAPO 40 mg/kg alone treated group rat's testosterone were found to be similar compared to the vehicle control group. EAR 200 mg/kg treatment to PCOS rats has not significantly decrease the level of testosterone compared to the PCOS group. Treatment with EAR 400 mg/kg, SAPO 20 mg/kg and SAPO 40 mg/kg to PCOS rats showed significant ( $p < 0.001$ ) decrease in the level of testosterone when compared to the PCOS group. One-way ANOVA showed that there was a statistically significant difference between the groups ( $F = 9.086$ ,  $p < 0.001$ ).

### Progesterone (ng/mL)

The mean values of progesterone were shown in Fig. 3. In PCOS group there was a significant ( $p < 0.001$ ) reduction in the level of progesterone compared with vehicle control group. Treatment with EAR 200 mg/kg was not statistically significant with PCOS group. Treatment with EAR

400 mg/kg, SAPO 20 mg/kg and SAPO 40 mg/kg in PCOS rats showed significant ( $F = 15.32$ ,  $p < 0.001$ ) increase in the level of progesterone when compared to the PCOS group.

### Effect of ethanolic extract of AR and SAPO on histology of ovaries

Light microscopic images (40 $\times$  and 10 $\times$ ) of ovary sections from the vehicle control group, EAR 400 and SAPO 40 (Fig. 5) showed congested vascular spaces with spindle-shaped cells in the medulla. The cortex showed primary and secondary follicles with aggregation of granulosa cells and scanty follicular antrum; few follicles showed intact oocyte; the corpus luteum contains uniform round cells with abundant eosinophilic cytoplasm. In PCOS group (Fig. 6) the medulla and cortex showed multiple follicular cysts of varying sizes with diminished granulosa cells and increased follicular antrum, some atretic follicles and few follicles contain degenerated oocytes. The corpus luteum also showed atrophic changes. After-treatment with EAR and SAPO leads to disappearance of cysts and appearance of corpus luteum and healthy follicles. Sections of EAR 200 mg/kg treatment in PCOS rats exhibited follicles

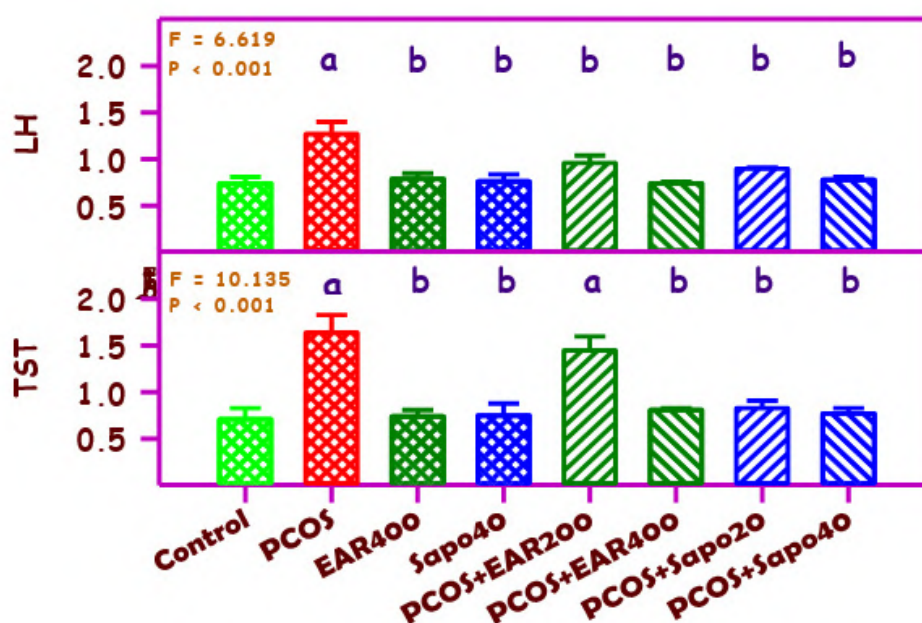
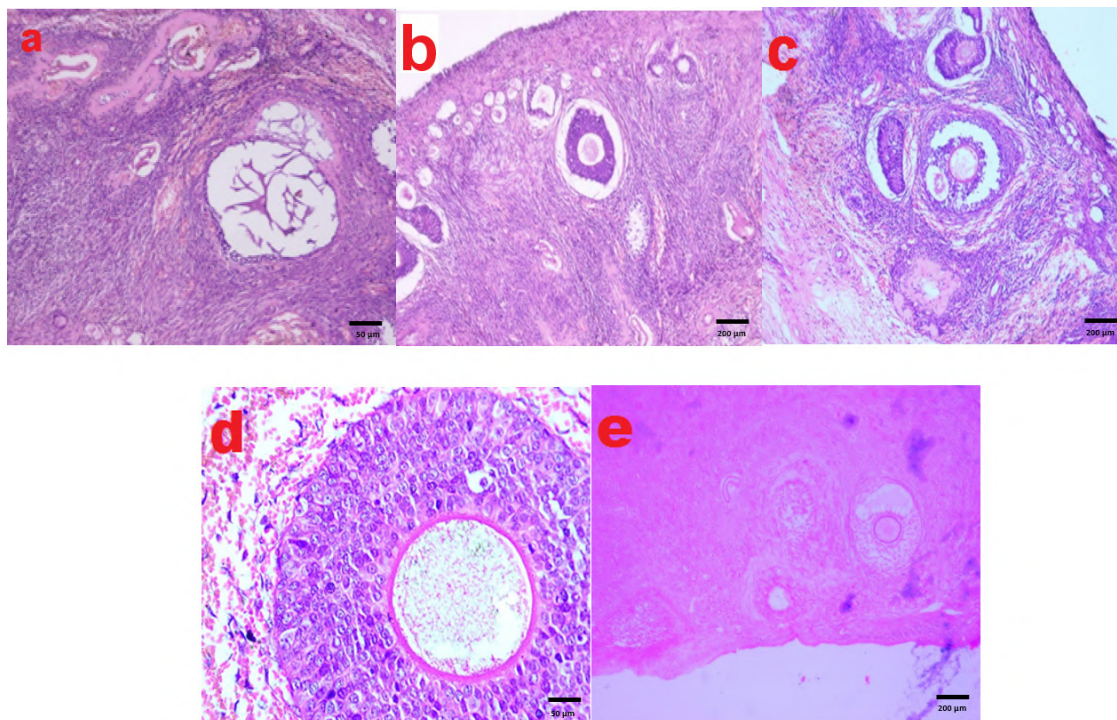


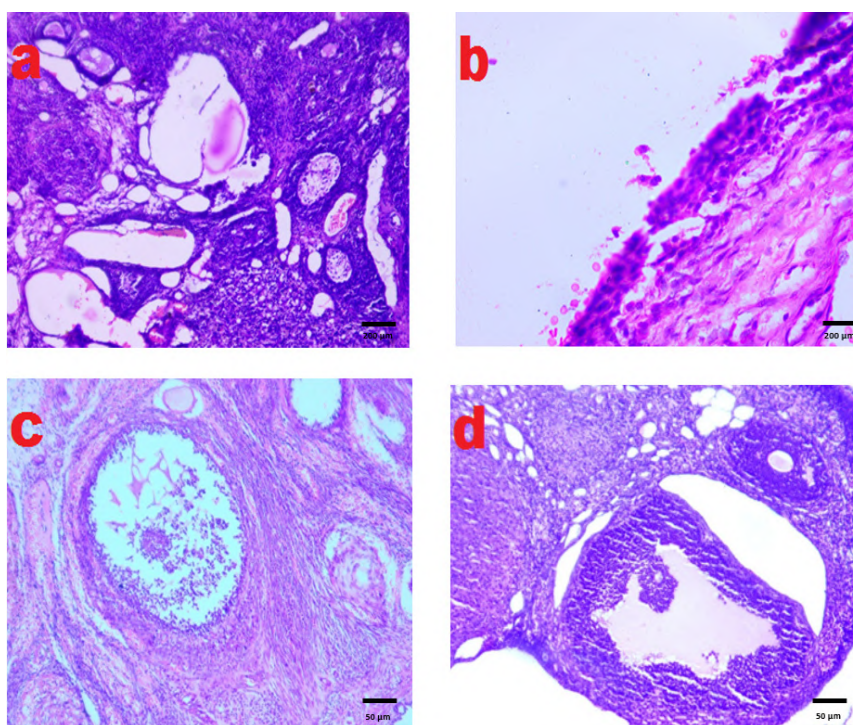
Fig. 4.- The effect of ethanolic extract of *Asparagus racemosus* (EAR) and its active component saponin (SAPO) in fructose and letrozole induced polycystic ovary syndrome (PCOS). 20, 40, 200 and 400 are doses per kilogram body weight. Values are mean  $\pm$  SEM ( $n = 6$  each). Luteinizing Hormone (LH) (IU/mL); Testosterone (ng/dL). The 'F' and 'P' values are by one way ANOVA with Tukey multiple comparison test. <sup>a</sup> significantly different from vehicle control, <sup>b</sup> significantly different from PCOS.



**Fig. 5.** - Hematoxylin and Eosin-stained microscopic images of vehicle control and drug control group. **a.** congested vascular spaces with spindle shaped cells in medulla. The cortex showed **b.** primary and **c.** secondary follicles with **d.** aggregation of granulosa cells and **e.** scant follicular antrum few follicles showed intact oocyte. Scale bars: a, d = 50 µm.; b, c, e = 200 µm.

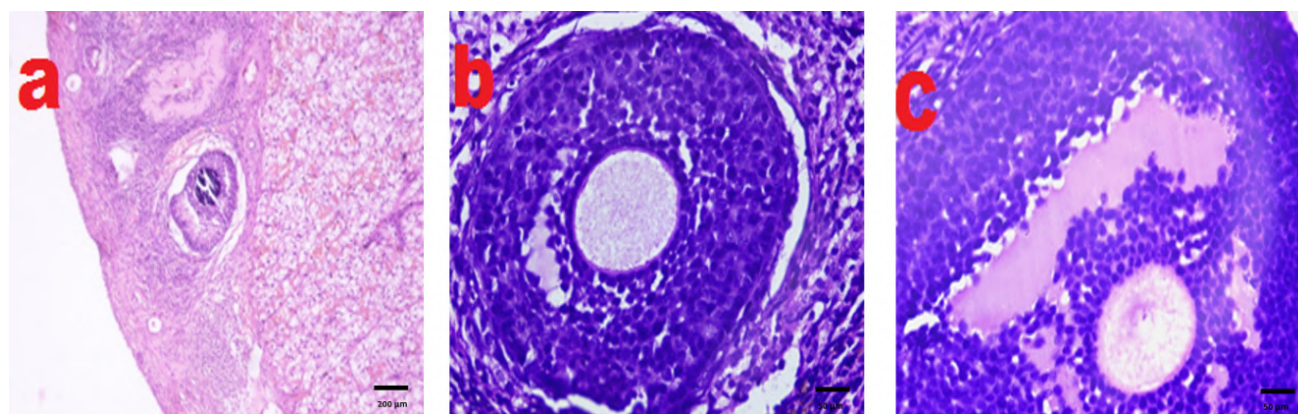
larger in size and few corpora lutea (Fig. 7). Cysts were absent and normal-sized healthy follicles at different developmental stages with oocytes were found in section from EAR 400 mg/kg treated PCOS rats (Fig. 7). Sections from SAPO 20 mg/

kg and SAPO 40 mg/kg administered PCOS rats show many corpora lutea and antral follicles with clearly differentiated oocyte, granulosa cell layer, corona radiata, cumulus oophorus and thecal cells were observed (Fig. 8).

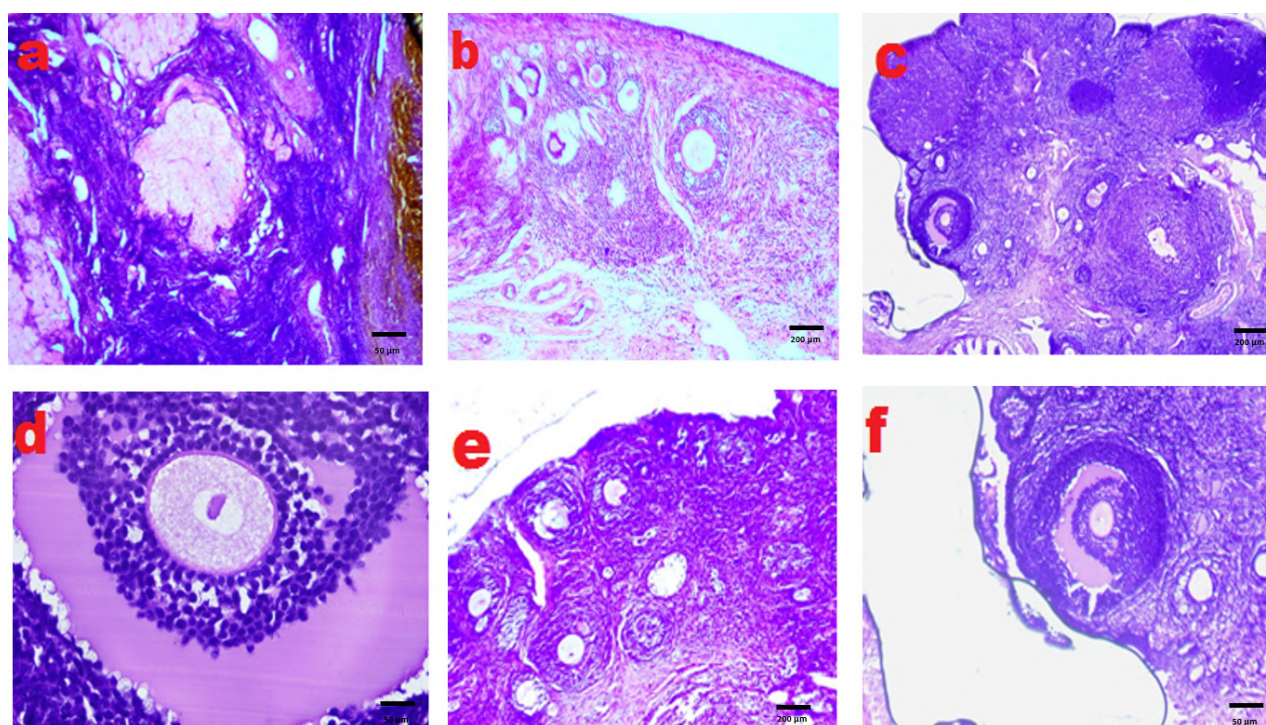


**Fig. 6.** - Hematoxylin and Eosin-stained microscopic images in PCOS group. **a.** medulla and cortex showed multiple follicular cysts of varying sizes, **b.** with diminished granulosa cells, **c.** some atretic follicles and few follicles contain degenerate oocyte and **d.** increased follicular antrum. Scale bars: a, b = 200 µm.; c, d = 50 µm.





**Fig. 7.-** Histopathological changes of ovary after treatment with EAR200 and EAR400 in PCOS rats. **a.** appearance of corpora lutea, **b.** appearance of new follicle, **c.** decreasing follicular cyst. Scale bars: a, b = 200 µm.; c = 50 µm.



**Fig. 8.-** Histopathological changes of ovary after treatment with SAPO20 and SAPO40 in PCOS rats. **a.** appearance of numerous corpus luteum, **b.** cortex with many new follicles, **c.** cortex with varying no of corpora lutea, **d.** decreasing of pyknotic granulosa cells, **e.** disappearance of cyst, and **f.** visible granulosa cell layer. Scale bars: a, d, f = 50 µm.; b, c, e = 200 µm.

## DISCUSSION AND CONCLUSIONS

PCOS has a wide range of clinical manifestations, including oligomenorrhea and hyperandrogenism, which can lead to metabolic dysfunction (Dickerson et al., 2010). The phenotype PCOS was created in this study, and the effects of EAR roots on the development of follicular growth and the improvement of this disease were investigated using hormonal and histological observations. The current study measured five hormonal parameters: FSH, LH, estradiol, progesterone, and testosterone. The current study found that PCOS increased testosterone and LH levels while de-

creasing FSH, estrogen, and progesterone levels in PCOS rats. Treatment with EAR and SAPO can increase serum levels of FSH, estrogen, and progesterone while decreasing serum levels of LH and testosterone. In addition, the number of ovarian follicles was found to be lower in the PCOS group of rats. According to the findings, dose-dependent EAR and SAPO treatment restores the hypothalamic-pituitary-gonadal axis hormone imbalance in PCOS rats. Among the various drug doses, the most effective is SAPO 40 mg/kg, which causes an increase in FSH, estrogen, and progesterone while decreasing LH and testosterone levels.

One of the most important diagnostic criteria for PCOS is a change in hormone levels. In some cases, the serum FSH level in PCOS does not change (Durant et al., 2009), and hyperandrogenism and increased serum LH levels are very common (Ari-fet et al., 2016). In the case of PCOS, it could be an increase in testosterone and LH secretion and a decrease in FSH hormone secretion (Karampoor et al., 2014). One possible explanation for sex hormone changes in PCOS is a lack of aromatase enzyme in the ovary, which could increase androgen concentration (Requena et al., 2008).

We used fructose and letrozole to create a PCOS model in rats for 28 days in this study. Letrozole inhibits aromatase activity, resulting in an increase in ovarian androgens by preventing androgens from being converted to estrogens, resulting in hyperandrogenism, a defining feature of PCOS with clinical manifestations of elevated levels of testosterone, increased levels of LH, and decreased levels of FSH. It should also be noted that serum progesterone and estradiol levels were reduced nearly two-thirds of the time in the PCOS group in the current study. Letrozole inhibits testosterone aromatization to estradiol, as previously reported (Gnanadesigan et al., 2014). Letrozole-induced hormonal imbalance resulted in an irregular and/or prolonged estrus cycle in our study. Other studies have found similar results, which support our findings (Maharajan et al., 2010). In light of these findings, female rats were given letrozole, a non-steroidal aromatase inhibitor, along with fructose, which causes increased insulin resistance and hyperinsulinemia. Compensatory hyperinsulinemia is a major contributor to the development of metabolic abnormalities and high androgen levels in women with PCOS (Legro et al., 2013). As a result, our PCOS rat model exhibited all hormonal imbalances that occur in PCOS women, and it is the first model that mimics the real-life situation.

Anovulation is also characterized by low progesterone and estrogen levels, which results in irregular menstrual cycles (Srivastava et al., 2008). In our study, progesterone levels decreased, but treatment with EAR and SAPO increased progesterone and estradiol levels in a dose-dependent manner. Both SAPO 40 mg/kg and EAR

400 mg/kg are extremely effective at increasing progesterone and estradiol levels. AR's previously reported phytestrogenic activity was confirmed by this discovery (Gopumadhavan et al., 2005). Phytestrogens are plant-derived compounds that have estrogen-like properties. The presence of phytestrogens such as phytosterol, saponins, phenols, and flavonoids was discovered in the phytochemical analysis of EAR, demonstrating the efficacy of AR in the treatment of PCOS. This phytestrogenic activity is shared by the active components of AR of steroidal saponins such as diosgenin, gitogenin, chlorogenin, and ruscogenin; thus, steroidal saponins with hormone-like actions in the body (Anderson et al., 1997). These results indicated that EAR and SAPO had phytestrogenic activity.

Phytestrogens have been shown to bind to two types of estrogen receptors ER $\alpha$  and ER $\beta$ , and acts as a pure estrogen antagonist by stimulating Gonadotropin releasing hormone secretion (Osaki et al., 2003). Steroid saponins such as sarsaponin, protodioscin, and diosgenin are the most likely estrogenic components extracted from asparagus roots (Shao et al., 1997). The mechanism of action of the EAR and SAPO may be due to the presence of phytoestrogen. It is thought to be similar to the standard drug clomiphene citrate in normalization of the hormonal level and induction of ovulation. Thus, the current study suggested that the SAPO and EAR both promote regularity of ovarian cycle by correcting hypothalamus-pituitary axis function, which in turn may reduce ovarian cyst in rats having PCOS. This finding was well supported by (Barton, 2013). Studies on plants with similar compositions to EAR found that plants containing flavonoids and phenolic compounds could help to maintain a natural balance of estrogen and progesterone during the ovarian cycle, and that these plants have specific pharmacological-physiological effects on rebalancing increased or decreased levels of sex hormones. As a result, it is possible that EAR and SAPO could restore estrogen and progesterone hormone balance in PCOS-induced rats via flavonoids and antioxidant activity of AR, which contradicts previous research findings (Liu et al., 2004). As a result, there was a dose-

dependent increase in progesterone and estrogen levels in the groups that received EAR and SAPO.

The positive effects of AR root extract on the folliculogenesis process were reported in a study performed in young females with a dose of 100 mg/kg; it may increase ovarian weight, increase FSH hormones, and may enhance folliculogenesis, as evidenced by a histological study of immature female rats' ovaries (Kalia et al., 2003). Researchers have linked the stimulatory effects of EAR on the ovary to the presence of compounds such as glycosidal flavonoids, saponins, alkaloids, and steroidal compounds (Sharma et al., 2013). Previous research on LH and FSH levels changes in PCOS found a link between an increased level of LH and a decreased level of FSH. Increased LH hormone levels stimulate ovarian theca cells, which in turn stimulates androgen production. (Loffler et al., 2000). Furthermore, androgens may increase the number of FSH receptors in PCOS, resulting in a decrease in the concentration of this hormone and an increase in the level of LH (Requena et al., 2008). In the current study, increased LH levels and a significant decrease in FSH levels were observed in PCOS group rats due to excess antigen production, which causes LH levels to rise and FSH levels to fall. Furthermore, these changes are most likely the result of a mutation in the aromatase enzyme (Requena et al., 2008). Treatment with SAPO 40 mg/kg improved the level of LH and FSH, resulting in an increase in weight and the number of ovary follicles. This study found that SAPO 40 mg/kg improved folliculogenesis when compared to the PCOS group. SAPO 20 mg/kg and EAR 400 mg/kg treatment of PCOS rats was also significant in restoring LH, FSH levels and improving folliculogenesis. EAR and SAPO were found to have a positive and dose-dependent effect on serum LH and FSH levels in PCOS.

AR are high in amino acids and aspartic acid and arginine derivatives. Aspartic acid stimulates gonadotropin-releasing hormone and LH secretion. Tests revealed that this amino acid regulates LH synthesis via looped guanosine monophosphate as a second messenger in the pituitary gland (Pinilla et al., 2001). Asparagus arginine is also converted to nitric oxide, which is

one of the most important factors controlling LH and FSH release (Sato et al., 2000). These findings were robustly supported. AR may also act through its amino acid content, eventually resolving the hormonal imbalance.

There was a significant increase in testosterone levels in the PCOS group in the current study. Letrozole inhibited the conversion of androgen substrates into estrogen, resulting in androgen accumulation. This increased testosterone concentration in peripheral blood may be the cause of the rats' prolonged diestrous phase and increased body weight in the study (Abdulghani et al., 2012). Serum testosterone levels were normalized after treatment with EAR 400 mg/kg, SAPO 20 mg/kg, and SAPO 40 mg/kg. The decrease in testosterone concentration in SAPO 40 mg/kg treated rats reflects decreased androgen biosynthesis by the ovary and resulted in a lower percentage of vaginal diestrous days and body weight in PCOS rats, which could be attributed to their ability to lower testosterone concentration in the peripheral blood. Reduced estrogen production caused by aromatase inhibition can result in increased LH secretion in the hypothalamus and pituitary, most likely due to estrogen negative feedback. Previous research has shown that AR reduces testosterone levels in male rats by interfering with steroid production in the adrenal glands (Mishra et al., 2013). SAPO 40 mg/kg treatment resulted in significant testosterone recovery, a good anti-androgenic effect by lowering elevated androgen levels, and prevention of ovarian cell dysfunction in PCOS to improve fertility.

Aside from the positive effects of restoring hormonal balance between gonadotropin and ovarian hormones in alleviating PCOS symptoms, the administration of various antioxidants such as vitamin E and selenium is also regarded as a common treatment approach for this syndrome (Amini et al., 2015). EAR is high in natural antioxidants, including vitamins and minerals like zinc and selenium (Karmakar et al., 2012). According to research findings, polycystic ovary syndrome causes an increase in reactive oxygen species (ROS) in ovarian tissue, and the balance between the oxidant and antioxidant systems is

disrupted. Natural growth of theca interstitial layer is required for normal ovarian function, and oxidative substances and free radicals interfere with regular growth and apoptosis in this layer. It has been established that there is a direct link between reduced oxidative stress and increased oocyte maturation in PCOS and infertile women (Shirsath et al., 2015).

Histopathological examination of ovaries from the PCOS group revealed sub-capsular cysts lined with a thin layer of granulosa cells and hyperplasia of theca cells, which was strikingly similar to human PCOS. Increased intra-ovarian androgen levels have been linked to abnormal follicular growth and an increase in follicular atresia. The PCOS group had a lower number of corpus luteum and developing follicles. Cystic follicles were significantly larger in size than other ovarian follicles with a clear antrum but no oocyte. When comparing PCOS follicles to control follicles, the thickness of the peripheral granulosa layer was reduced while the thickness of the theca layer was increased. Cysts were absent, and normal sized healthy follicles with oocytes were found in sections from the EAR 400 mg/kg, SAPO 20 mg/kg, and SAPO 40 mg/kg treatments, which resulted in the disappearance of cysts and a decrease in the incidence of pyknotic granulosa cells. Ovulation and normal estrous cyclicity were indicated by the presence of a variable number of corpora lutea. Follicles with oocytes and clear, visible granulosa cell layers at various stages of development were observed. The ovarian cortex appeared to be normal, with numerous follicles. These histological findings pointed to the presence of biologically active levels of FSH and LH in the PCOS group, as well as a lack of interplay between granulosa and theca cells, which would otherwise result in ovulation. Another study on the effects of AR extract on ovary tissue found that it increases the number of primary, secondary, and graafian follicles while decreasing the number of atretic follicles in rats (Verma et al., 2014).

Asparagus roots are high in calcium, magnesium, phosphorus, and zinc (Joham et al., 2015). Minerals found in follicular fluid regulate follicle growth and steroidogenesis. Minerals not only act as cofactors in various enzymatic

activation systems for oocyte growth and maturation, but also influence ovarian function and fertility. Histological analysis in this study revealed an increase in the number of ovarian follicles and corpus luteum, as well as an increase in the number of atretic follicles in the SAPO-treated groups. This finding corresponds to an increase in hypothalamic-pituitary-gonadal axial hormones.

FSH influences the growth and development of ovarian follicles through the proliferation and differentiation of granulosa cells. Granulosa cells proliferate slowly in the early stages of follicular development, but granulosa cells in preantral follicles respond to FSH stimulation and secrete large amounts of estradiol. As a result, an increase in estrogen due to an increase in follicle number was not unexpected in the current study. The greatest increase in Graafian follicles was seen in the experimental group that received 40 mg/kg of SAPO. This group also had the highest estrogen level. LH affects theca corpus luteum cells, which increases progesterone hormone synthesis (Krishnamurthy et al., 2009). As a result of the increased number of corpora lutea, an increase in progesterone levels was not unexpected according to increased number of corpora lutea in this study. The findings of this study indicated that oral administration of EAR 400 mg/kg and SAPO 40 mg/kg could alleviate PCOS-related symptoms. It appears that consumption of SAPO at a dose of 40 mg/kg reduces levels of LH and testosterone while increasing levels of FSH, estrogen, and progesterone hormones. Because of the hormonal balancing nature of these drugs, EAR 400 mg/kg and SAPO 40 mg/kg treated rats had a lower number of cystic follicles and a higher number of corpus luteum. In PCOS rats, this results in a normal process of folliculogenesis and ovulation. In the current study, we discovered that SAPO 40 mg/kg is superior to EAR 400 mg/kg treatment.

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