European Review for Medical and Pharmacological Sciences 2022; 26: 2740-2754

Spirulina therapeutic potentiality in polycystic ovarian syndrome management using DHEA-induced rat model

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Abstract. – OBJECTIVE: The most prevalent endocrinopathy in women is polycystic ovarian syndrome (PCOS). Multiple gene abnormalities like *Ar, Cyp19a1, Hsd3b1, Srd5a1, Bcl-2*, and *Bax* genes are associated with PCOS. Herein, the PCOS model was induced by oral administration of dehydroepiandrosterone (DHEA). Metformin (Met) is one of the most common drugs affecting the most relevant genes involved in PCOS development but with unwanted side effects. Natural treatments have been known for their safer effects. Spirulina (SP) is a type of blue-green algae that contains nutrients and compounds that would treat PCOS and lower the possible side effects of Met in combination therapy. We aim to evaluate the clinical effectiveness and safety of SP on PCOS by multi confirmatory tests and to demonstrate its effects on regulating the expression of multiple genes that are responsible for the occurrence of PCOS in comparison to Met.

MATERIALS AND METHODS: Herein, sixty adult female Wistar albino rats were subdivided into equal six groups with 10 rats in each group. All drugs were given orally once daily for one month. The expression of *Ar, Cyp19a1, Hsd3b1, Srd5a1, Bcl-2*, and *Bax* genes, were examined by quantitative real-time PCR (qRT-PCR).

RESULTS: The present study showed that SP has a remarkable effect on the reduction of the development of PCOS by regulating the expression of the examined genes. As a result, it may be a useful therapy alternative for PCOS complications, symptoms, and infertility as well.

CONCLUSIONS: Collectively, SP is considered a promising therapeutic drug in the treatment of PCOS-like symptoms induced by DHEA.

Key Words:
PCOS, DHEA, SP, Met, qRT-PCR, Ovary, Immunohistochemistry.

Introduction

Polycystic ovarian syndrome (PCOS) is the prevalent heterogeneous endocrinopathy condition affecting women[1]. According to PCOS Awareness Month September 2021, PCOS is one of the most likely reasons for infertility affecting as much as 20% of women between the ages of 18 and 44, and at the same time, there is currently no known cure[2]. PCOS is a complex, polygenic syndromic disease. It has been attributed to several genes that have direct or indirect effects on fertility[3]. Thus, it is important to do such studies that detect the clear PCOS genetic association which would be affected by new suggested treatments. Herein, the novelty is addressing the potential effect of SP on the major PCOS-related problems. Like the relationship between apoptosis of granulosa cells and follicle development arrest, and hormonal disturbance abnormality in PCOS. Therefore, SP could be considered as an important treatment suggestion for PCOS.

Many biochemical pathways have been involved in the pathogenesis of PCOS. Several genes have been tested including genes involved in steroidal hormone biosynthesis and metabolism, such as the androgen receptor (*Ar*) gene. Some researchers consider that androgen excess is an essential condition of PCOS. This is accordant with the theory that androgens determine the characteristic features of the syndrome[4]. Moreover, the occurrence of female pattern hair loss in women with PCOS is associated with the hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*Hsd3b1*) gene expres-

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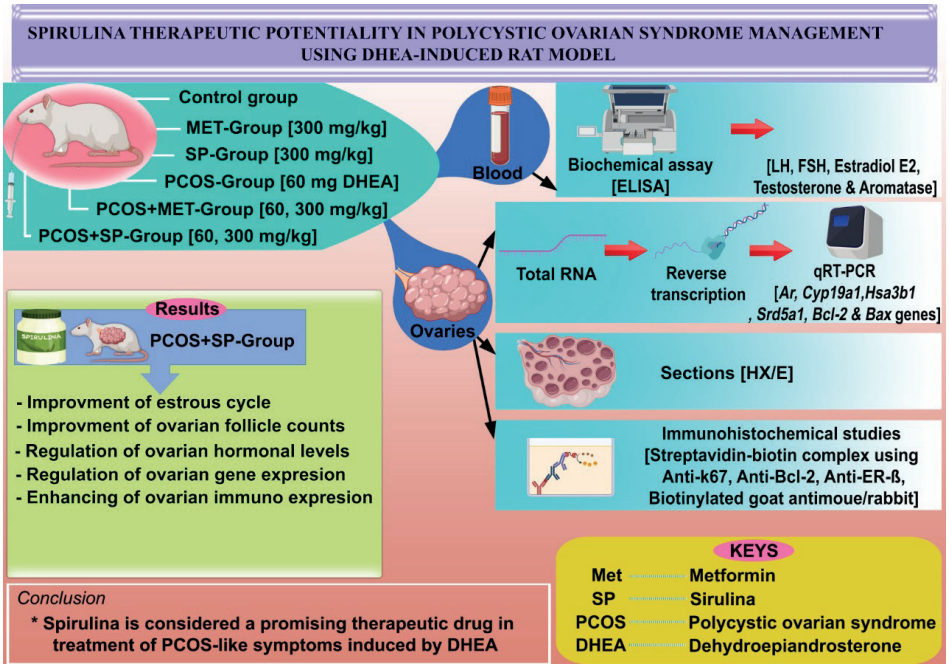

Graphical Abstract. Spirulina therapeutic potentiality in polycystic ovarian syndrome management using DHEA-induced rat model.

sion[5]. Although the genetic etiology of PCOS appears to be unclear the steroid 5 Alpha-Reductase (*Srd5a1*) activity in polycystic ovaries supported the hypothesis that the *Srd5a1* gene produces hyperandrogenism and play an important function in the syndrome pathogenesis[6]. The aromatase cytochrome P450 (*Cyp19a1*) gene (encoding the aromatase enzyme) may have a key role in the PCOS pathogenesis since it has been linked the hyperandrogenism development in PCOS[7].

The ovarian cycle is marked by significant tissue remodeling, in which 99% of mammalian follicles fail to ovulate and become atretic as a result of hormonally induced apoptosis[8]. Several members of the *Bcl-2* gene family may act as intracellular cell survival mediators. The anti- and pro-apoptotic factors produced by the *Bcl-2* and *Bax* genes, respectively, have been described[9-11]. In addition, the BCL-2 family protein members are implicated in the ovarian follicles atresia with significant value in PCOS development[8].

According to the previously mentioned facts, investigators suggested that multidisciplinary therapy should be considered for PCOS complications, metabolic and psychological aspects set of symptoms, including reproductive function and follicles atresia[12].

Initially, in the 1990s, Met was the first-line oral treatment for type 2 diabetes and was indicated to reduce testosterone levels in women with PCOS. Met is used to treat PCOS in women. It can control ovarian functions, raise the blood concentration of sex hormone-binding globulin (SHBG), and decline the androgens levels[13,14]. The trouble associated with Met is the elevation of homocysteine levels in PCOS patients that have been linked to an increased risk of cardiovascular disease[15]. Met has also been attributed to different side effects such as nausea, vomiting, and gastrointestinal disturbance[16]. To prevent adverse side effects of chemical medications, researchers studied another promising treatment for PCOS.

Natural products such as SP extracts contribute to nutritional requirements, stimulate the endocrine system, and intermediate nutrient metabolites[17]. SP is a filamentous, tiny cyanobacterium that gets its name from the spiral or helical shape of its filaments. It has been used for a long time as a safe dietary supplement that has been eaten for centuries due to its high nutritional value and its anticipated health benefits driven by its antioxidant and anti-inflammatory properties. It was found to be enriched with vitamins, nutrients, antioxidants, proteins, pigments, minerals etc., and also was reported to be effective in the treatment of type 2 diabetes[18].

Here we conducted a study to evaluate the clinical effectiveness and safety by comparing SP with Met administrated by DHEA-induced PCOS rats.

Materials and Methods

Drugs and Chemicals

SP® tablets were purchased from Puritan's Pride Company, Inc. (Oakdale, NY, USA) Each tablet contained 500 mg of the active ingredient. The drug was ground and administrated orally as a fresh suspension in 0.9% saline. DHEA® tablets were bought from Puritan's Pride Company, Inc. (Oakdale, NY, USA). Each DHEA pill contained 60 mg of the active ingredient. The drug was ground and administrated orally as a fresh suspension in sesame oil obtained from El Captain Pharmaceutical Company (Cairo, Egypt). Metformin hydrochloride® was purchased from GlaxoSmithKline pharmaceutical company (Cairo, Egypt).

Experimental Animals and Study Design

Sixty adult female Wistar albino rats (70-80 days old and 120 ± 20 g body weight) were attained from the National Cancer Institute (NCI) animal house, Cairo University (CU) (Cairo, Egypt). Also, animals' general care and housing were approved at (NCI) animal house according to the experiment Ethics of living animals' research in association with the recommendation of the Institutional Animal Care and Use Committee (IACUC) (publication No. 85-23 revised 1985). Animals were retained in stainless steel cages at room temperature 25 ± 5 °C. All the rats were kept under a 10/14 h dark/light cycle and fed with standard laboratory food pellets and tap water. Rats were separated into six groups, each of 10 rats, as follows: 1) Control group: rats were given the vehicle. 2) Met-group: rats treated with 300 mg/kg b.wt/day of Met. 3) SP-group: rats treated with 300 mg/kg b.wt/day of SP. 4) PCOS-group: rats treated orally with 60 mg/kg b.wt/day of DHEA. 5) PCOS+Met-group: rats treated with 60 mg/kg b.wt/day of DHEA and 300 mg/kg b.wt/day of Met. 6) PCOS+SP-group: rats treated with 60 mg/kg b.wt/day of DHEA and 300 mg/kg b.wt/day of SP. The drugs were administrated orally for one month. The treatment duration of DHEA was one month because the actual number of follicular cysts is occurring in ovaries in 20-30 days[19]. The route of administration of DHEA was oral according to Kavitha et al[20]. Rats were administrated DHEA followed by SP or Met 1 hour later. The dose of Met was chosen according to Furat Rencber et al[21] and that of SP was chosen according to Karaca and Şimşek[22].

Estrous Cycle Monitoring

According to Mahmoud et al[23], the estrous cycle of all rats was checked daily before treatment using vaginal smears to demonstrate normal cyclicity. Furthermore, according to Westwood[24], the estrous cycle was tiered depending on vaginal cell shape. Daily vaginal smears were also collected during the trial to validate the model's occurrence and assess the effect of SP on PCOS animals.

Samples Collection

When the experiment is finished, animals were sacrificed at the diestrous stage. Blood was drawn and centrifuged for 15 min at 3000 rpm. For the biochemical assay, the serum was kept at -20°C. The ovaries were excised, blotted, and weighed. The left ovaries were left to the histological and immunohistochemical studies, while the right ones were left to the molecular studies. The bodyweight of all rats was recorded at the beginning and end of the experiment for the determination of body weight changes.

Biochemical Assay

To evaluate ovarian function after treatment with DHEA, serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), testosterone, and aromatase were quantified by Elabscience® ELISA Kits (Houston,Texas, USA) according to the manufacturer's instruction on a Cobas® e601 immunoassay analyzer (Roche-Hitachi Diagnostics, Mannheim, Germany).

RNA Isolation and qRT-PCR

The total cellular RNA from ovarian tissue was purified using Invitrogen™ TRIzol™ Reagent (Invitrogen™ 15596018 Corporate entity: Life Technologies Corporation, Carlsbad, CA, USA; Toll-Free in USA 1 800 955 6288) and reversely transcribed to cDNA using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Corporate entity Life Technologies Corporation, Carlsbad, CA, USA; Toll-Free in USA 1 800 955 6288). The qRT-PCR was performed using Applied Biosystems PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer's instruction on Applied Biosystems Step One™ Real-Time PCR System (Foster City, CA, USA). The sequences of the forward and reverse primers for *Ar*, *Hsd3b1*, *Srd5a1*, *Cyp19a1*, *Bcl2*, *Bax,* and *Gapdh* are shown in Table I. The primers were designed using the Primer-BLAST

Table I. The sequences of the forward and reverse primers for *Ar, Hsd3b1, Srd5a1, Cyp19a1, Bcl-2, Bax,* and *Gapdh.*

Gene		Primer sequence
Ar	Primer forward	GGGGCAATTCGACCATATCTG
	Primer reverse	CCCTTTGGCGTAACCTCCCTT
Hsd3b1	Primer forward	CAGGGCCCAACTCCTACAAG
	Primer reverse	AAGGCAAGCCAGTAGAGCAG
Srd5a1	Primer forward	ATGGCCTTCGTGTCCATTGT
	Primer reverse	AAAACCAGCGTCCTTTGCAC
Cyp19a1	Primer forward	ATGGGGATTGGAAGTGCCTG
	Primer reverse	TCATGAAGAAAGGGCGGACC
Bcl-2	Primer forward	AGCATGCGACCTCTGTTTGA
	Primer reverse	TCACTTGTGGCCCAGGTATG
Bax	Primer forward	GGGCCTTTTTGCTACAGGGT
	Primer reverse	TTCTTGGTGGATGCGTCCTG
Gapdh	Primer forward	CTCATGACCACAGTCCATGC
	Primer reverse	TTCAGCTCTGGGATGACCTT

tool available from the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All samples were examined in duplicate. The CT values were obtained for all genes and normalized to *Gapdh*. All of the CT values were in the linear range of detection. The $2^{-\Delta\Delta CT}$ method was used to determine the relative expression of target genes.

Histological and Immunohistochemical Assays

Left ovaries were fixed with 10% buffered formalin solution at room temperature for 24 hours. After that, samples were dehydrated in ethanol ascending ranks, cleared in terpineol, and then embedded in paraffin wax. Hematoxylin and eosin were used to stain paraffin sections (5 µm thick). To evaluate ovarian follicles, 10 serial sections of the rat's ovary from each group were examined for differential follicle numbers at 100 X. Follicles were categorized according to Luo et al[25] into the primordial follicle, in which an oocyte is surrounded by a layer of squamous granulosa cells; primary follicles, with the oocyte surrounded by a single layer of cuboidal granulosa cells; secondary follicles, with more than one layer of cuboidal granulosa cells, but with no visible antrum; antral (tertiary) follicles which have a clearly defined antral space and cumulus granulose cell layer; Graafian follicles with a large antrum, cornora radiate, and cumulus oophorous; corpora lutea which are formed only after ovulation, and more filled with lutein cells; and atretic follicles with abnormal structures such as inspissating follicular fluid, degenerating egg, thickening granulose layers or filling with organizing fibrinous material in the antrum.

Ovarian immunohistochemical analysis was performed on paraffin sections (5 µm thick) using a streptavidin-biotin complex (ABC) method[26]. The following primary antibodies were used: anti-KI-67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-BCL-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-ER-β (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody used in all cases was biotinylated goat anti-mouse/rabbit (Ig, Duet kit Dako, Agilent Technologies, Santa Clara, CA, USA). Brown coloration indicated positive immunoreactions. Sections incubated without primary antibodies were considered as negative controls. The immunohistochemical evaluation of BCL-2 and ER-β was done by semi-quantitative scoring analysis (H) as described by Mahamed et al[27]. In all groups, KI-67 expression was measured as a % of positively stained cells, with at least 500 cells counted per animal in all cases. A camera linked to a Leica DM LS2 microscope (Leica Microsystems, Wetzlar, Germany) was used to photograph all sections.

Statistical Analysis

Mean values and the standard error were reported as numerical data. All statistical analyses were performed using GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA, USA). For a comparative analysis between the groups, data were evaluated statistically using one-way ANOVA followed by post hoc multiple comparisons (Tukey method). p-value < 0.05 was considered significant.

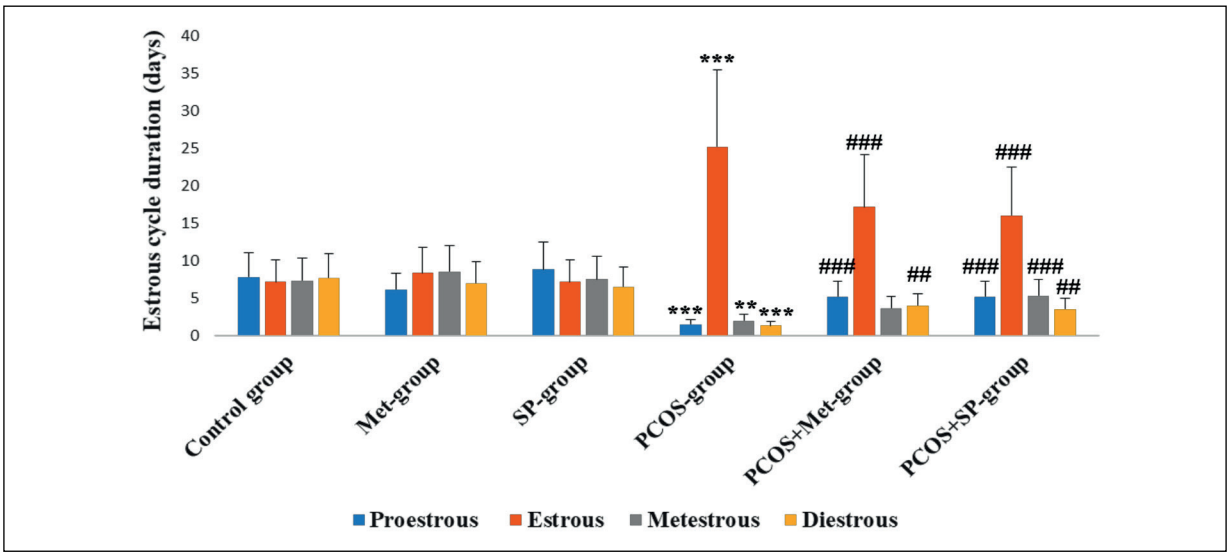

Figure 1. Effect of SP and Met on estrous cycle duration in PCOS rats. Data are represented as mean ± SEM (n = 10). Values are expressed as means ± SEM. *Indicates the significant difference of Met-, SP, and PCOS-groups *vs.* the control group. ** $p < 0.01$, *** $p < 0.001$. ♯Indicates the significant difference between PCOS+Met and PCOS+SP-groups *vs.* the PCOS-group. ♯♯ $p < 0.01$, ♯♯♯ $p < 0.001$.

Results

Effect on Estrous Cycle and Body Weight

The daily vaginal smears showed a regular estrous cycle with a duration of 4-5 days and with all four phases in consecutive order in control rats, while they were significantly interrupted with a dominant estrous stage in PCOS rats. The treated PCOS rats with Met or SP showed moderate restoration of the estrous cycle (Figure 1).

PCOS rats exhibited a significant increase in body weight gain in comparison with that of control rats ($p < 0.01$) at the end of the trial. On the other hand, the treatment of the PCOS group with Met or SP revealed a significant reduction in the body weight gain compared to the PCOS group ($p < 0.001$) (Figure 2A).

Ovarian Gross Morphology and Weight

The ovaries of control, Met, and SP-treated rats exhibited normal mean ovarian weight (Fig-

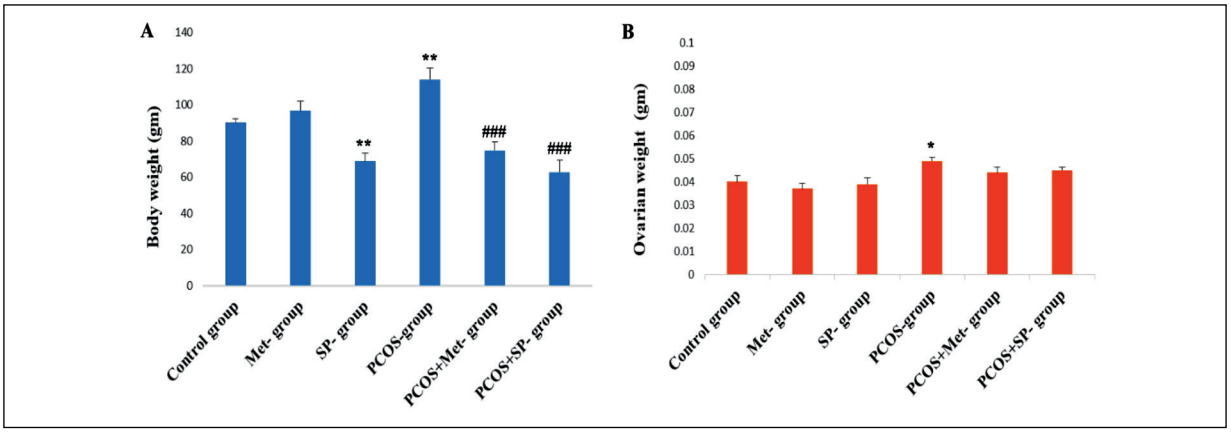

Figure 2. Effect of SP compared to Met on the body and ovarian weights in PCOS-rats. **A,** body weight gain (gm). **B**, Ovarian weight (gm). Data are represented as mean ± SEM (n = 10). Values are expressed as means ± SEM. *Indicates the significant difference of Met-, SP-, and PCOS-groups *vs.* the control group. * $p < 0.05$, ** $p < 0.01$. ♯Indicates the significant difference between PCOS+Met and PCOS+SP-groups *vs.* the PCOS-group. ♯♯♯ $p < 0.001$.

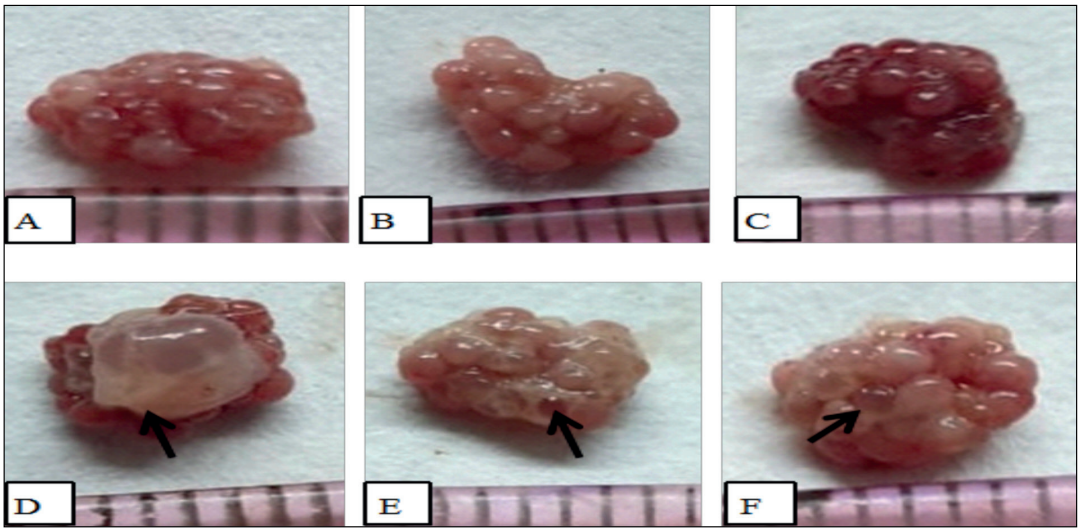

Figure 3. The effect of SP compared to Met on the ovarian gross morphology on PCOS rats. **A,** Control ovary showing normal color and size. **B,** Ovary treated with Met. **C,** Ovary treated with SP. **D,** Ovary of PCOS rats. **E,** Ovary of PCOS rats treated with Met. **F,** Ovary of PCOS rats treated with SP. Note the cystic follicle (*arrows*).

ure 2B) and a grape-like look with normal color, and size (Figures 3A, 3B, and 3C, respectively). On the other side, the ovaries of the PCOS group revealed a significant increase in their weights compared to the control group (Figure 2B), in addition to the appearance of large cystic follicles bulging from the ovarian surface (Figure 3D). The weight of ovaries of the PCOS rats treated with Met or SP was insignificantly decreased ($p > 0.05$) (Figure 2B), however, they had small cystic follicles in size and number on the surface (Figures 3E and 3F, respectively).

Histological Assessment

Ovarian histological examination of the control group displayed developmental various stages of follicles (Figure 4A, Table II). In PCOS rats, the ovarian sections showed a significant reduction in

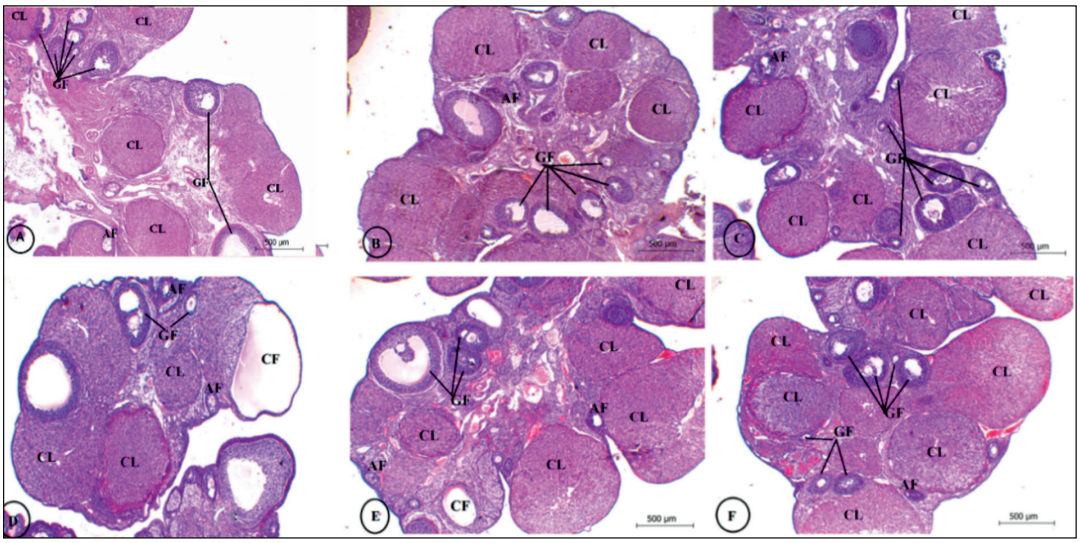

Figure 4. Photomicrograph showing the effect of SP compared to Met on ovarian histology on PCOS rats. **A,** Control ovary. **B,** Ovary treated with Met. **C,** Ovary treated with SP. **D,** Ovary of PCOS rats. **E,** Ovary of PCOS rats treated with Met. **F,** Ovary of PCOS rats treated with SP. GF: growing follicles; AF: Atretic follicles; CL: Corpora lutea; CF: Cystic follicle. Note the increase in the number of growing follicles and corpora lutea and the decrease in the cystic follicles in the number of differently treated PCOS rats compared to those of PCOS rats (magnification 200 ×).

Table II. Effect of SP on the follicular count of PCOS model.

Follicles	Control group	Met- group	SP- group	PCOS group	PCOS+Met- group	PCOS+SP- group
Primordial follicles	4.4 ± 0.25	3.4 ± 0.51	4.2 ± 1.18	2.2 ± 0.2***	4.2 ± 0.38$^{\#\#}$	3.6 ± 0.25$^{\#\#}$
Primary follicles	3.0 ± 0.25	1.8 ± 0.38*	2.8 ±0.58	1.4 ± 0.25**	2.2 ± 0.2$^{\#}$	2.4 ± 0.25$^{\#}$
Secondary follicles	2.8 ± 0.2	3.0 ± 0.54	4.0 ± 0.63	2.6 ± 0.25	3.2 ± 0.38	3.6 ± 0.25$^{\#}$
Tertiary (antral) follicles	4.6 ± 0.25	3.2 ± 0. 2**	3.6 ± 0.4	1.8 ± 0.2***	1.6 ± 0.51	1.4 ± 0.25
Graafian follicles	1.2 ± 0.2	0.6±0.25	0.6 ± 0.25	0.4 ± 0.25*	0.6±0.25	0.6 ± 0.25
Corpus luteum	14.4 ± 0.4	11.0 ± 1.09*	12.0 ± 0.32**	9.2 ± 0.58***	10.2 ± 0.58$^{\#\#}$	11.6 ± 0.68$^{\#\#}$
Atretic follicles	3.0 ± 0.32	4.0 ± 0.77	4.0 ± 0.54	6.2 ±0.38***	4.4 ± 0.25$^{\#\#}$	5.0 ± 0.32$^{\#}$
Cystic follicles	–	–	–	1.6 ± 0.25***	0.6 ± 0.25$^{\#}$	0.58 ± 0.22$^{\#}$

Values are expressed as means ± SEM. *Indicates the significant difference of Met-, SP-, and PCOS-groups *vs*. the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $^{\#}$Indicates the significant difference between PCOS+Met and PCOS+SP-groups *vs*. the PCOS-group $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

most of the growing follicle's number and corpora lutea and a significant elevation in the number of atretic follicles related to the control; numerous large cystic follicles were also observed (Figure 4D, Table II). However, the co-treatment of PCOS rats with Met or SP has a remarkable increase in most of the growing follicle's number and a significant decrease in both atretic and cystic follicles count in comparison with those of PCOS rats (Figure 4E and 4F, respectively, Table II).

Immunohistochemical Assessment

The immunohistochemical results revealed that the cellular proliferation indicator (KI-67) % was significantly higher in granulose cells of the antral follicles in the control groups é [Figure 5 and Figure 6 (a-c)] compared to those of the PCOS group (Figure 5 and Figure 6d). Treatment of PCOS rats with SP showed a significant surge in the KI-67 immunoexpression % in their antral follicle's granulosa cells (Figure 5 and Figure 6f).

The antral follicles of the PCOS group showed a low immunoreactivity score to the anti-apoptotic marker (BCL-2) in their granulosa layer compared to those of the control groups (Figure 5 and Figure 7j). On the other hand, co-administration of the model group with SP exhibited higher im-

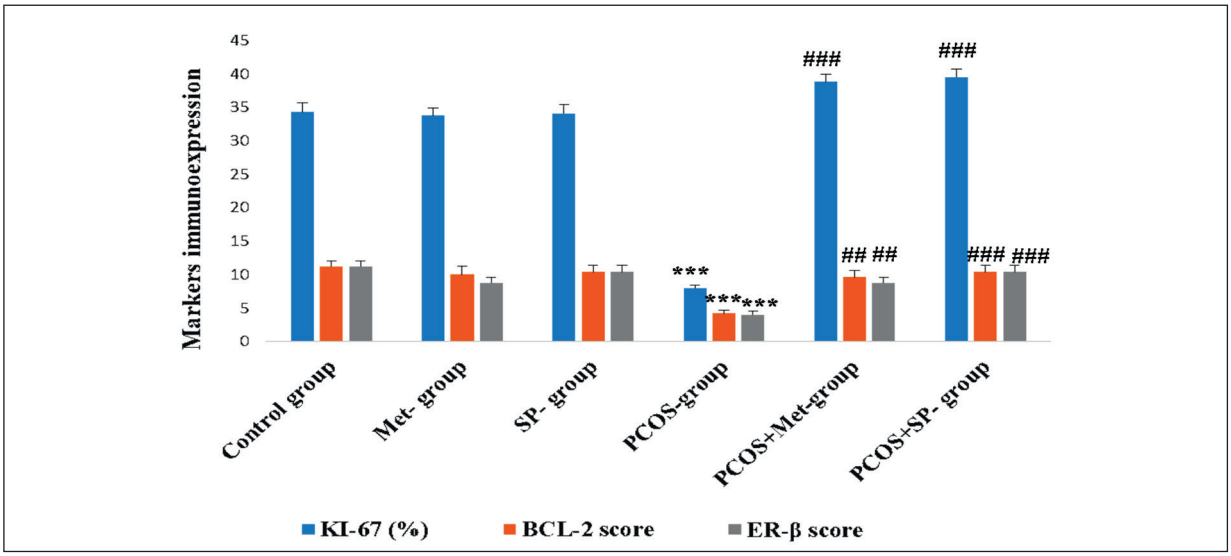

Figure 5. Effect of SP compared to Met on the immunohistochemical staining for KI-67, BCL-2, and ER-β in PCOS rats. Data are represented as mean ± SEM (n = 10). Values are expressed as means ± SEM. *Indicates the significant difference of Met-, SP-, and PCOS-groups *vs*. the control group. ***$p < 0.001$. $^{\#}$Indicates the significant difference between PCOS+Met and PCOS+SP-groups *vs*. the PCOS-group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

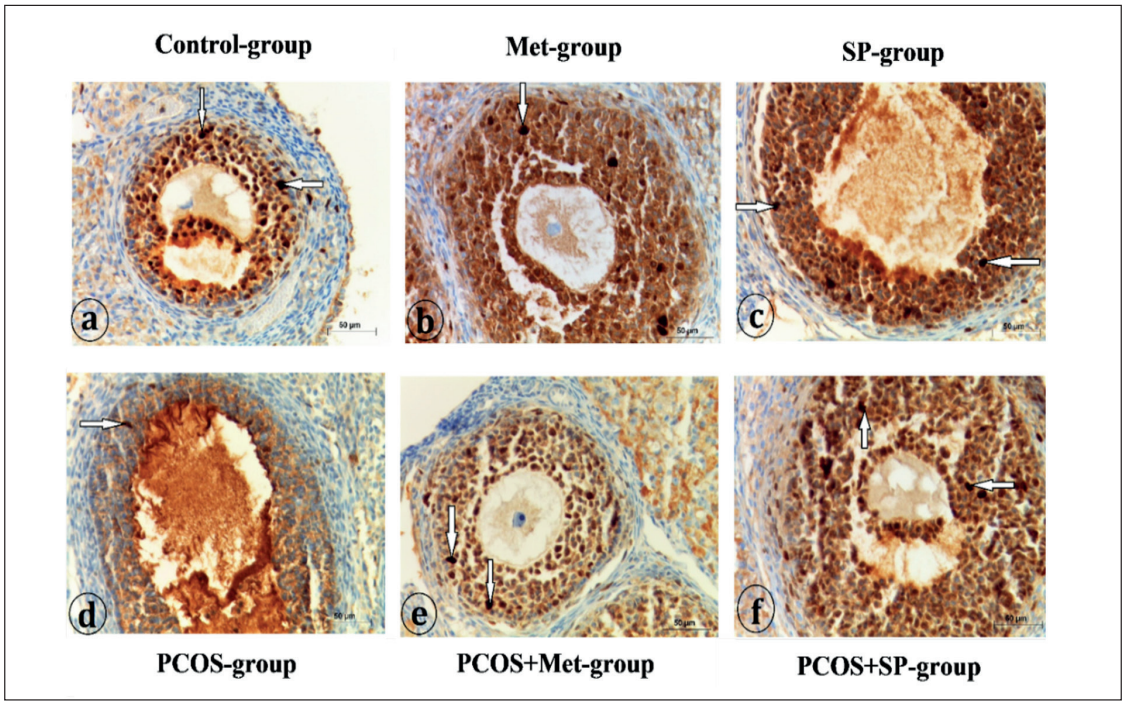

Figure 6. Photomicrograph showing the effect of SP compared to Met on the immunohistochemical staining for KI-67 **(a-f)** in the ovaries of PCOS rats. **a,** Control ovary. **b,** Ovary treated with Met. **c,** Ovary treated with SP. **d,** Ovary of PCOS rats. **e,** Ovary of PCOS rats treated with Met. **f,** Ovary of PCOS rats treated with SP. The KI-67 positive cells (arrows) are indicated as brown color in the nuclei of granulosa cells. Note the decrease in KI-67 % in the PCOS model. The KI-67 % was increased in the PCOS group treated with SP (magnification 400 ×).

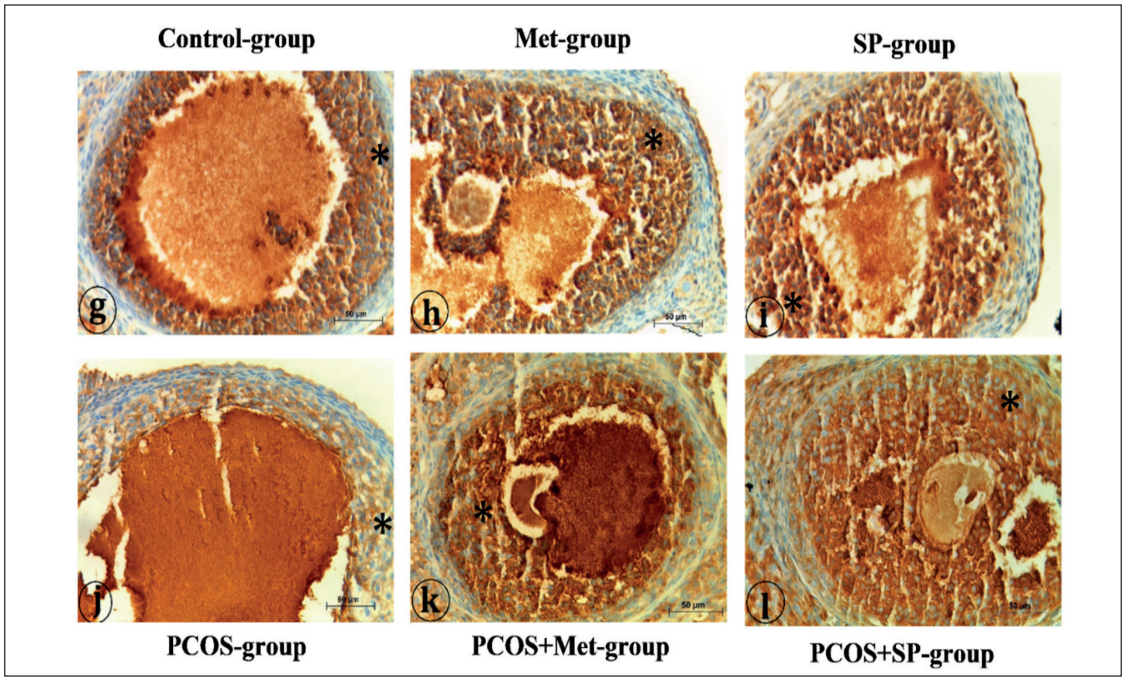

Figure 7. Photomicrograph showing the effect of SP compared to Met on the immunohistochemical staining for BCL-2 **(g-l)** in the ovaries of PCOS rats. **g,** Control ovary. **h,** Ovary treated with Met. **i,** Ovary treated with SP. **j,** Ovary of PCOS rats. **k,** Ovary of PCOS rats treated with Met. **l,** Ovary of PCOS rats treated with SP. The BCL-2 positive cells (*) are indicated as brown color in the cytoplasm of granulosa cells. Note mild BCL-2 immunoreactivity in PCOS-model. The BCL-2 immunoreactivity increased in PCOS-group treated with SP (magnification 400 ×).

munoreactivity scores in their follicle's granulosa cells (Figure 5 and Figure 7l) in comparison with those of PCOS rats.

Examination of ovarian immunohistochemical sections stained for estrogen receptor-β (ER-β) showed a significant decline in immunoreactivity score in antral follicle's granulosa cells of the PCOS group compared to control group follicles (Figure 5 and Figure 8p). The co-treatment of the model group with SP exhibited a significant increase in the ER-β immunoexpression score compared to the model group (Figure 5 and Figure 8r).

Biochemical Assay

The model group revealed a significant decrease in the values of serum FSH, whereas serum LH, E2, and testosterone concentrations were significantly increased in comparison with those of the control rats. The co-treatment of the PCOS group with Met or SP showed a significant improvement in all hormonal values (Figure 9). Moreover, the aromatase value was significantly decreased in the PCOS group in comparison with the control group. On the other hand, the co-treatment of the model group with SP resulted in an improvement in this value in comparison with the model group (Figure 9).

Expression Pattern of Ar, Hsd3b1, Srd5a1, Cyp19a1, Bcl-2, and Bax in the Rat Ovaries of the Different Groups

In the present study, the profiling of targeted genes exhibited differential expression between comparable groups. In addition, the fold change of *Ar*, *Hsd3b1*, *Srd5a1*, *Cyp19a1*, *Bcl-2,* and *Bax* were analyzed as shown in Figure 10. The present study fold change analysis showed that *Ar*, *Hsd3b1*, *Srd5a1* genes expression elevated in the model group in comparison to the control group. However, the co-treated groups showed lower expression of these genes. On the other hand, in the model group, *Cyp19a1* and *Bcl-2* showed significantly lower expression than that of the control group. The co-treated group with SP showed higher *Cyp19a1* and *Bcl-2* expression than the model group. *Bax* expression was statistically significantly higher in the model group than in the control, while co-treated PCOS group with SP exhibited lower *Bax* expression compared to that of the model group (Figure 10).

Discussion

Hyperandrogenism, ovulation dysfunction, and/or polycystic ovarian morphology are all symp-

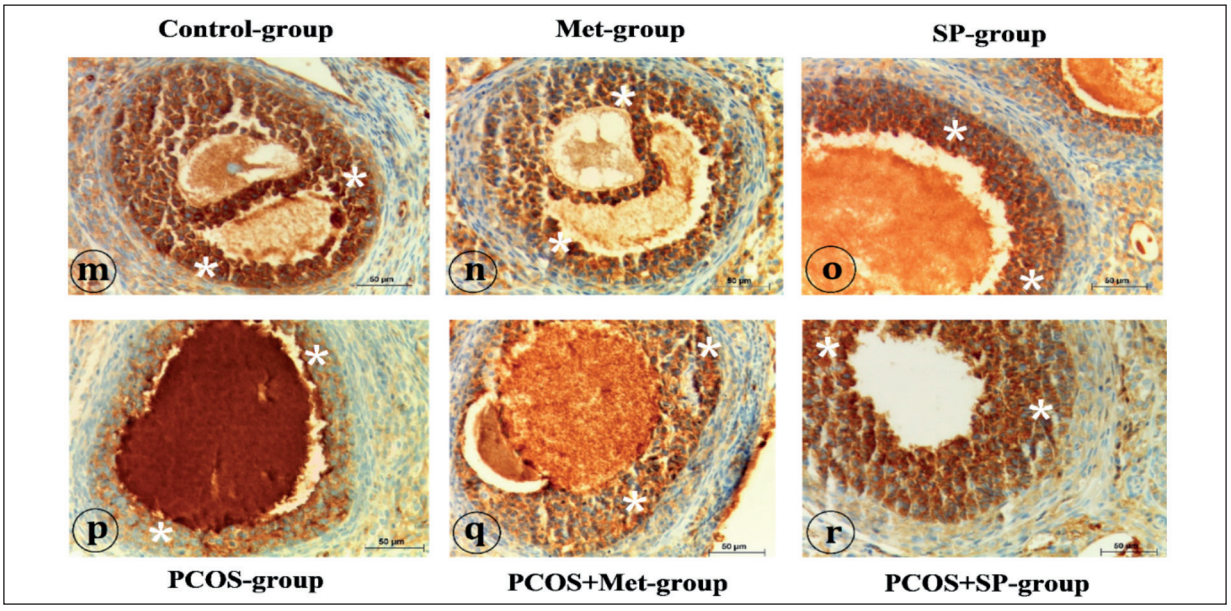

Figure 8. Photomicrograph showing the effect of SP compared to Met on the immunohistochemical staining for ER-β **(m-r)** in the ovaries of PCOS rats. m) Control ovary. **n,** Ovary treated with Met. **o,** Ovary treated with SP. **p,** Ovary of PCOS rats. **q,** Ovary of PCOS rats treated with Met. **r,** Ovary of PCOS rats treated with SP. The ER-β positive cells (*) are indicated as brown color in the cytoplasm of granulosa cells. Note mild immunopositivity in ER-β in PCOS-model. The ER-β immunopositivity increased in PCOS-group treated with SP (magnification 400 ×).

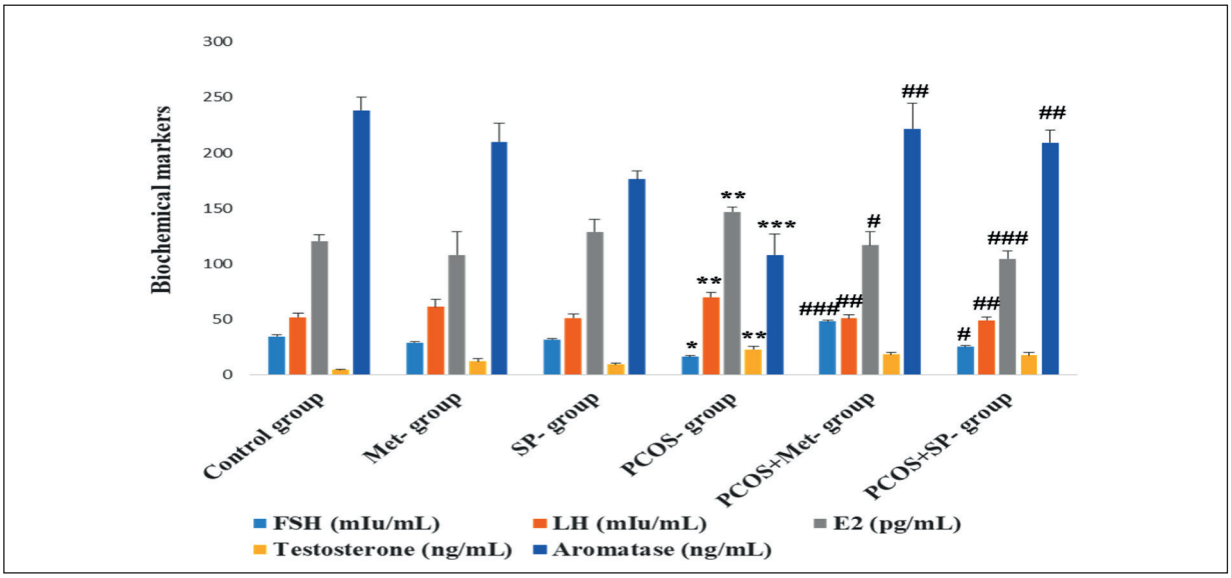

Figure 9. Effect of SP compared to Met on the Biochemical markers (FSH, LH, E2, Testosterone, and aromatase) in PCOS-rats. Data are represented as mean ± SEM (n = 10). Values are expressed as means ± SEM. * indicates the significant difference of Met-, SP-, and PCOS-groups *vs*. the control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. $^{\#}$Indicates the significant difference between PCOS+Met and PCOS+SP-groups *vs.* the PCOS-group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

toms of PCOS. For the development of PCOS, a range of etiological foundations, including genetic and environmental variables, have been postulated[28]. Researchers have shown that SP has various health benefits, therapeutic properties, and several other biological activities. To our knowledge, the present study is the first which aims at evaluating whether SP has a successful effect in modulating reproductive dysfunction in the DHEA-induced PCOS model.

The vaginal smear is the main marker of ovarian physiology. PCOS rats revealed an irregular

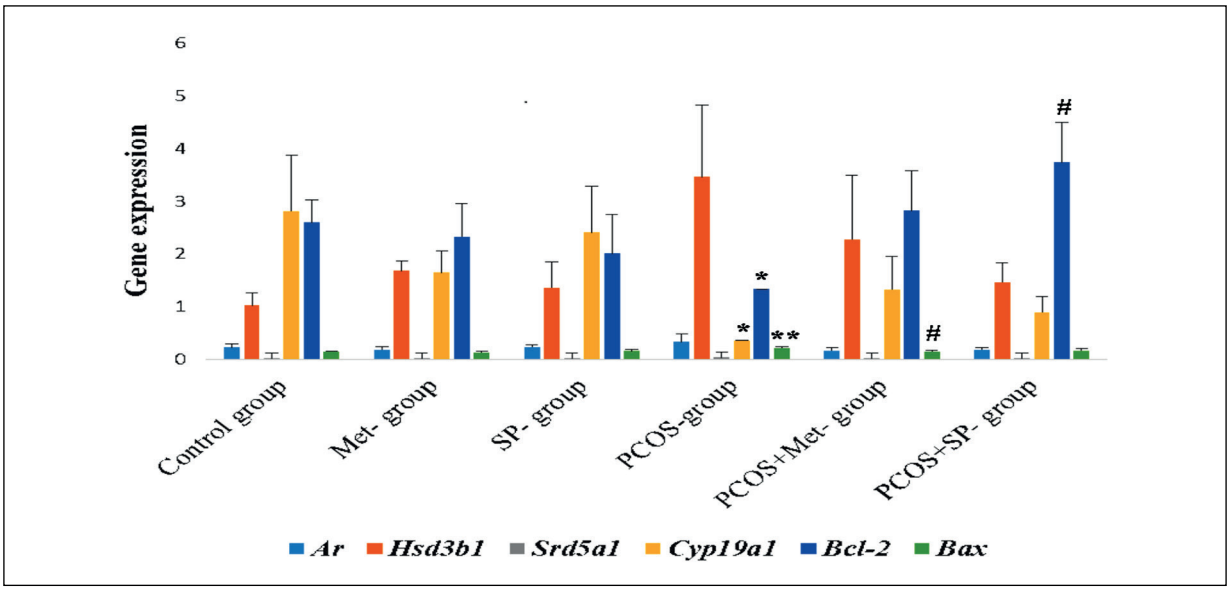

Figure 10. Effect of SP compared to Met on the gene expression profile of *Ar*, *Hsd3b1*, *Srd5a1*, *Cyp19a1*, *Bcl-2,* and *Bax* in PCOS-rats. Data are represented as mean ± SEM (n = 10). Values are expressed as means ± SEM. *Indicates the significant difference of Met-, SP-, and PCOS-groups *vs*. the control group. *$p < 0.05$, **$p < 0.01$. $^{\#}$Indicates the significant difference between PCOS+ Met and PCOS+SP-groups *vs.* the PCOS-group. $^{\#}p < 0.05$.

cycle with a permanent estrous stage indicating the disruption of the ovulatory cycle. DHEA might directly affect reproductive phenotypes, e.g., it might increase the duration of the estrous phase[29]. Nah et al[30] reported that SP enhances spermatogenesis and steroidogenesis in rats. In this study, the treatment of PCOS rats with SP restored the estrous cycle regularity. Furthermore, the PCOS model exhibited a significant increase in body weight and ovarian weight in comparison with the control rat, which is consistent with the findings of Kim et al[31] and Prabhu and Gopalakrishnan[32]. Treatment of PCOS-group with SP revealed a significant drop in the body weight gain and ovarian weight compared to the model. In 2020, DiNicolantonio et al[33] found that the decrease in macrophage infiltration into visceral fat, prevention of hepatic fat formation, and reduction in oxidative stress are all mechanisms of action for SP. Overall, SP provides a variety of health advantages, including weight loss, dyslipidemia, and obesity.

The histological ovarian analysis is a good feature to evaluate ovarian changes. In this study, the ovaries of PCOS rats showed a remarkable decline in all growing follicles and an increase in follicular atresia, in addition to the appearance of numerous large cysts. These findings are well-matched with other previous reports[34,35]. Exogenous increase of DHEA level increases the androgen state in PCOS and leads to irregular follicular growth and elevation in follicular artesia[36]. All these changes are attributed to a failure in the selection of dominant follicles, cessation of folliculogenesis, and inovulation[37].

Herein, SP treated PCOS group exhibited a marked recovery of ovarian tissue with the improvement in the follicular number and corpora lutea and a marked decrease in the number of atretic and cystic follicles. The more corpora lutea presence could be linked to changing of the estrous cycle to normal functioning[38]. Similarly, other authors reported that SP improved the ovarian histopathological alterations and has a protective effect in drugs-induced ovarian toxicity in rats[39].

DHEA-induced PCOS animals showed high levels of LH, LH/FSH ratio, and testosterone which serve as biomarkers to diagnose PCOS in women[40], and these results supported the successful modeling. In the present study, SP supplementation to the PCOS animals showed a decrease in LH/FSH ratio and increment in FSH levels, which is maybe due to enhancing ovarian folliculogenesis. Also, the reduction of serum LH/FSH ratio can delay the progress of the disease by regulating the endocrine disorder[41]. The current DHEA supplementation resulted in a significant increase in testosterone level as reported by many researchers[30,34]. The PCOS rats treated with SP showed a decrease in testosterone level that may reflect diminished androgen biosynthesis in the ovary. In this work, the E2 level was decreased in the DHEA-treated group compared to the control one which was confirmed by some previous studies[40]. Where the treatment of the PCOS group with SP revealed a significant increase in E2 levels. This result is in agreement with the study done by Abdel-Aziem et al[42], which reported that SP elevated the lower serum concentrations of E2 and FSH in ovarian dysfunctions induced by monosodium glutamate in mice. Our study revealed a significant decrease in serum aromatase in the DHEA-treated group compared to the control rats which is consistent with Ashraf et al[43]. They reported that the aromatase activity is lowered in PCOS, which promotes an increase in androgens. Aromatase (CYP19A1) is a key enzyme in the production of estradiol through testosterone aromatization[44]. It is responsible for keeping the homeostatic balance between androgens and estrogens in both sexes[45]. The decline in aromatase activity, which may be resulted from diminished response to FSH stimulation[46] leads to intraovarian disturbance in steroidogenesis, then ovarian androgen production and development of PCOS[40]. The observed increase in testosterone levels probably reflected the androgens accumulation that confirms the decrease in aromatase level in our study.

Individual genes, gene-gene interactions, and gene-environment interactions have all been linked to the development of PCOS[3]. To investigate the effect of SP on the underlying mechanism of increased androgen production, estrogen synthesis, and ovarian-steroidogenesis in the PCOS model, we examined the expression of the key genes involved in these mechanisms using qRT-PCR as an integral to the used tests in the present study. Overall, previous studies reported that analyzing ovarian follicles found higher levels of *Ar*, *Hsd3b1,* and *Srd5a1* mRNA in ovarian tissue of PCOS groups[47-52]. Our observations of increased *Ar*, *Hsd3b1*, *Srd5a1* mRNA levels in the PCOS group than the PCOS group co-treated with SP supported the hopeful effect of SP in PCOS treatment. Furthermore, other researchers reported that the relative expression rate of *Cyp19a1* mRNA in PCOS groups is lower than in non-PCOS groups[53-55]. The present study showed elevated *Cyp19a1* mRNA in the SP-treated group, which supports the beneficial effect of SP in the

elevation of aromatase level after treatment as observed from our biochemical findings.

Regarding the immunohistochemical observations, the current investigation showed that the expression of all the studied markers such as KI-67, BCL-2, and ER-β was significantly lower in the PCOS group in comparison to the control. The significant decrease in KI-67 immunoexpression % in PCOS coincides well with the results of other researchers who reported that proliferation is significantly lower in the granulosa cells of PCOS animals compared to the control specimens[56,57]. Meanwhile, treatment of PCOS-model with SP showed a significant increase in KI-67 immunoreactivity % in their ovarian granulosa cells that reflects the increased number of granulosa cells after treatment.

Follicular atresia is caused by apoptosis, which is crucial for the cyclical development and regression of follicles in the human ovary. BCL-2 is a member of the BCL-2 family and is one of the most important apoptosis regulators (anti-apoptotic protein). In the current work, we investigated an intense BCL-2 immunoexpression in control follicles compared to the PCOS group as previously reported by Chi et al[58]. The immunoexpression of BCL-2 increased in the PCOS group treated with SP. This finding is coincident with El-Atrsh et al[59], who reported that the treatment of Ehrlich solid tumor with SP increased BCL-2 immunoexpression. Moreover, the fact that immunohistochemical investigations identify staining in each follicular stage may explain why varied expressions of BCL-2 were found utilizing immunohistochemical assays. A possible dilution could take place when the total ovarian level of *Bcl-2* was measured. Also, we found the findings supporting our results on the molecular level, as the *Bcl-2* mRNA elevated in the SP-treated group. So, there is a great balance between the results of the multi-tested groups which confirm our study finding. Otherwise, Jahan et al[60] reported that apoptosis is one of the harmful effects of reactive oxygen species (ROS) that resulted from the high oxidative profile and antioxidants imbalance in PCOS patients. Since SP has free radical scavenging activity (antioxidant) and antiapoptotic properties that prevent the cell injury and oxidation process in the body organs, it leads to an increase in BCL-2 immunoexpression.

Based on the ovarian expression of *Bax* (pro-apoptotic protein) and *Bcl-2* (anti-apoptotic protein), these genes are changed in the PCOS model induced by DHEA administration in rats[61]. Accordingly, the present study evaluated *Bax* on the molecular biological level. This gene showed a significantly higher *Bax* mRNA level in the PCOS group. On the other side, co-treatment with SP exhibited a lower *Bax* mRNA level compared to the PCOS group.

Likewise, the current results revealed that the immuno-staining of ER-β in control rats was more intense than in PCOS rats. Our results are in accordance with other researchers, they reported that levels of ER-β protein expression were diminished in the cystic follicles granulosa layers of PCOS from a rodent model[62] and PCOS patients[63]. Because ER-β is needed and essential for ovarian function, alterations in its activation or expression may have clinical consequences, such as infertility[64]. So, it is perhaps not surprising that ER-β is reduced in granulosa cells from the PCOS model. Moreover, there is evidence that suggests that in response to the increase of LH, ER can be down-regulated[65] that confirming our hormonal results. The PCOS rats treated with SP revealed a significant increase in the immunoreactivity of ER-β compared to the PCOS model.

Conclusions

The current research explored the mechanism behind the therapeutic action of SP compared to Met in the PCOS-rat model induced by DHEA. Our results determined that SP is as successful as Met through the enhancement of ovarian structure and function, enhancing of KI-67, BCL-2, ER-β immune-expression, in addition to the regulation of *Ar*, *Hsd3b1*, *Srd5a1*, *Cyp19a1*, *Bcl-2,* and *Bax* genes. Based on these findings, we believe that SP might be an effective therapy for PCOS. More research is needed to determine the SP concentration in the blood and its bioavailability. This study is limited to a single dose of SP; however, a dose-dependent study should be carried out to compare the effects of multiple SP doses in the PCOS treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

The authors extend their appreciation to the research center at Almaarefa University for funding this work. Also, the authors gratefully thank Alaa K. Alnaggar, Hadeer A. Elshemy, Mariam N. Abdelmalak, Nesreen H. Abd El Azeem, Al-Shimaa A. Alsayed, Rodina M. Amer, Nasr S. Alamri, Marina M. Awadalla, Hager M. El Emam, Nada S. El-Hadad, Amal Ghanem and Sahar Tarek (Misr university for science and technology) for their help in the research finalization.

Ethics Approval

Animals' general care and housing were approved at (NCI) animal house according to the experiment Ethics of living animals' research in association with the recommendation of the Institutional Animal Care and Use Committee (IACUC) (publication No. 85-23 revised 1985).

Authors' Contribution

Conceptualization, Asmaa A El Leithy, and Faten Sabra Abo-Zeid; methodology, validation and formal analysis, Asmaa A El Leithy, Omar M. Youssif, Yassen A. Ebrahim, Alaa S. Khalifa, and Faten Sabra Abo-Zeid; writing-original draft preparation, Asmaa A El Leithy, Ahmed A. Al-Karmalawy, and Faten Sabra Abo-Zeid; writing-review and editing, Asmaa A El Leithy, Ahmed A. Al-Karmalawy, Eslam B. Elkaeed, and Faten Sabra Abo-Zeid; supervision and project administration, Asmaa A El Leithy, Ahmed A. Al-Karmalawy, and Faten Sabra Abo-Zeid. All authors have read and agreed to the published version of the manuscript.

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References

1) Bozdag G, Mumusoglu S, Zengin D, Karabulut E, Yildiz BO. The prevalence and phenotypic features of polycystic ovary syndrome: a systematic review and meta-analysis. Hum Reprod 2016; 31: 2841-2855.
2) U.S. Polycystic Ovarian Syndrome Awareness Month – September 2021. 2021; Available from: https://nationaltoday.com/polycystic-ovarian-syndrome-awareness-month/.
3) Khan MJ, Ullah A, Basit S. Genetic basis of polycystic ovary syndrome (PCOS): current perspectives. Appl Clin Genet 2019; 12: 249.
4) Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis, and diagnosis. Nat Rev Endocrinol 2011; 7: 219-231.
5) Tu YA, Lin SJ, Chen PL, Chou CH, Huang CC, Ho HN, Chen MJ. HSD3B1 gene polymorphism and female pattern hair loss in women with polycystic ovary syndrome. JFMA 2019; 118: 1225-1231.
6) Jakubowski L. Genetic aspects of plycystic ovary syndrome. Endokrynol Pol 2005; 56: 285-291.
7) Amato P, Simpson JL. The genetics of polycystic ovary syndrome. Best Pract Res Clin Obstet Gynaecol 2004; 18: 707-718.
8) Hsueh AJ, Eisenhauer K, Chun SY, Hsu SY, Billig H. Gonadal cell apoptosis. Recent Prog Horm Res 1996; 51: 433-455.
9) Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. Blood 1992; 80: 879-886.
10) Boise LH, González-García M, Postema CE, Ding L, Lindsten T, Turka LA, Nuñez G, Thompson, CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74: 597-608.
11) Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Biol 1994; 124: 1-6.
12) Ali A, Guidozzi F. Midlife women's health consequences associated with polycystic ovary syndrome. Climacteric 2020; 23: 116-122.
13) Ghandi S, Aflatoonian A, Tabibnejad N, Moghaddam MHS. The effects of metformin or orlistat on obese women with polycystic ovary syndrome: a prospective randomized open-label study. J Assist Reprod Gen 2011; 28: 591-596.
14) Banaszewska B, Duleba AJ, Spaczynski RZ, Pawelczyk L. Lipids in polycystic ovary syndrome: role of hyperinsulinemia and effects of metformin. Am J Obstet Gynecol 2006; 194: 1266-1272.
15) Kilicdag EB, Bagis T, Zeyneloglu HB, Tarim E, Aslan E, Haydardedeoglu B, Erkanli S. Homocysteine levels in women with polycystic ovary syndrome treated with metformin versus rosiglitazone: a randomized study. Hum Reprod 2005; 20: 894-899.
16) Lord JM, Flight IH, Norman RJ. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. BMJ 2003; 327: 951.
17) Hannan JMA, Ansari P, Azam S, Flatt PR, Wahab YHA. Effects of Spirulina platensis on insulin secretion, dipeptidyl peptidase IV activity and both carbohydrate digestion and absorption indicate potential as an adjunctive therapy for diabetes. Br J Nutr 2020; 124: 1021-1034.
18) Deng R, Chow TJ. Hypolipidemic, antioxidant, and antiinflammatory activities of microalgae Spirulina. Cardiovasc Ther 2010; 28: e33-e45.
19) Anderson E, Lee MT, Lee GY. Cystogenesis of the ovarian antral follicle of the rat: ultrastructural changes and hormonal profile following the administration of dehydroepiandrosterone. Anat Rec 1992; 234: 359-382.
20) Kavitha A, Babu AN, Kumar MS, Kiran SV. Evaluation of effects of Commiphora wightii in dehydroepiandrosterone (DHEA) induced polycystic ovary syndrome (PCOS) in rats. Pharma Tutor 2016; 4: 47-55.
21) Furat Rencber S, Kurnaz Ozbek S, Eraldemır C, Sezer Z, Kum T, Ceylan S, Guzel E. Effect of resveratrol and metformin on ovarian reserve and ultrastructure in PCOS: an experimental study. J Ovarian Res 2018; 11: 1-16.
22) Karaca T, Simşek N. Effects of spirulina on the number of ovary mast cells in lead-induced toxicity in rats. Phytother Res 2007; 21: 44-46.
23) Mahmoud YI, Mahmoud AA, Abo-Zeid FS, Fares NH. Effects of dehydroepiandrosterone on the ovarian reserve and pregnancy outcomes in perimenopausal rats (DHEA and fertility in perimenopausal rats). Life Sci 2018; 199: 131-138.

24) Westwood FR. The female rat reproductive cycle: a practical histological guide to staging. Toxicol Pathol 2008; 36: 375-384.

25) Luo LL, Huang J, Fu YC, Xu JJ, Qian YS. Effects of tea polyphenols on ovarian development in rats. J Endocrinol Invest 2008; 31: 1110-1118.

26) Elkomy A, Abdelhiee EY, Fadl SE, Emam MA, Gad FAM, Sallam A, Alarifi S, Abdel-Daim MM, Aboubakr M. L-carnitine mitigates oxidative stress and disorganization of cytoskeleton intermediate filaments in cisplatin-induced hepato-renal toxicity in rats. Front Pharmacol 2020; 11.

27) Mahamed RR, Maganhin CC, Sasso GRS, de Jesus Simões M, Baracat MCP, Baracat EC. Metformin improves ovarian follicle dynamics by reducing theca cell proliferation and CYP-17 expression in an androgenized rat model. J Ovarian Res 2018; 11: 1-10.

28) Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, Piltonen T, Norman RJ. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Hum Reprod 2018. 33: 1602-1618.

29) Iwasa T, Matsuzaki T, Tungalagsuvd A, Munkhzaya M, Yiliyasi M, Kato T, Kuwahara A, Irahara M. Effects of chronicEffects of chronic DHEA treatment on central and peripheral reproductive parameters, the onset of vaginal opening and the estrous cycle in female rats. Gynecol Endocrinol 2016; 32: 752-755.

30) Nah WH, Koh IK, Ahn HS, Kim MJ, Kang HG, Jun JH, Gye MC. Effect of Spirulina maxima on spermatogenesis and steroidogenesis in streptozotocin-induced type I diabetic male rats. Food Chem 2012; 134: 173-179.

31) Kim EJ, Jang M, Choi JH, Park KS, Cho IH. An improved dehydroepiandrosterone-induced rat model of polycystic ovary syndrome (PCOS): Post-pubertal improve PCOS's features. Front Endocrinol 2018; 9: 735.

32) Prabhu Y, Gopalakrishnan A. γ-Linolenic acid ameliorates DHEA induced pro-inflammatory response in polycystic ovary syndrome via PPAR-γ signaling in rats. Reprod Biol 2020; 20: 348-356.

33) DiNicolantonio JJ, Bhat AG, OKeefe J. Effects of spirulina on weight loss and blood lipids: a review. Open heart 2020; 7: e001003.

34) Motta AB. Dehydroepiandrosterone to induce murine models for the study of polycystic ovary syndrome. J Steroid Biochem Mol Biol 2010; 119: 105-111.

35) Bakhshalizadeh S, Amidi F, Alleyassin A, Soleimani M, Shirazi R, Shabani Nashtaei M. Modulation of steroidogenesis by vitamin D3 in granulosa cells of the mouse model of polycystic ovarian syndrome. Syst Biol Reprod Med 2017; 63: 150-161.

36) Homburg R. Androgen circle of polycystic ovary syndrome. Human Reprod 2009; 24: 1548-1555.

37) Ghafurniyan H, Azarnia M, Nabiuni M, Karimzadeh L. The effect of green tea extract on reproductive improvement in estradiol valerate-induced polycystic ovarian syndrome in rat. Iran J Pharm Res 2015; 14: 1215.

38) Rezvanfar MA, Rezvanfar MA, Ahmadi A, Saadi HS, Baeeri M, Abdollahi M. Mechanistic links between oxidative/nitrosative stress and tumor necrosis factor alpha in letrozole-induced murine polycystic ovary: biochemical and pathological evidences for beneficial effect of pioglitazone. Hum Experim Toxicol 2012; 31: 887-897.

39) Yener NA, Sinanoglu O, Ilter E, Celik A, Sezgin G, Midi A, Deveci U, Aksungar F. Effects of spirulina on cyclophosphamide-induced ovarian toxicity in rats: biochemical and histomorphometric evaluation of the ovary. Biochem Res Int 2013; 2013: 764262.

40) Kafali H, Iriadam M, Ozardali I, Demir N. Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. Arch Med Res 2004; 35: 103-108.

41) Miao M, Peng M, Zhu Z, Yan X, WeiZ, Li M. Effects of dodder total flavone on polycystic ovary syndrome rat models induced by DHEA combined HCG. Saudi J Biol Sci 2019; 26: 821-827.

42) Abdel-Aziem SH, Abd El-Kader HA, Ibrahim FM, Sharaf HA. Evaluation of the alleviative role of Chlorella vulgaris and Spirulina platensis extract against ovarian dysfunctions induced by monosodium glutamate in mice. J Genet Eng Biotechnol 2018; 16: 653-660.

43) Ashraf S, Nabi M, Rashid F, Amin S. Hyperandrogenism in polycystic ovarian syndrome and role of CYP gene variants: a review. Egypt J MedHum Genet 2019; 20: 1-10.

44) Sørensen AE, Wissing ML, Salö S, Englund ALM, Dalgaard LT. MicroRNAs related to polycystic ovary syndrome (PCOS). Genes 2014; 5: 684-708.

45) Wang H, Li Q, Wang T, Yang G, Wang Y, Zhang X, Sang Q, Wang H, Zhao X, Xing Q, Shi J, Lin He, Wang L. Common polymorphism in the human aromatase gene alters the risk for polycystic ovary syndrome and modifies aromatase activity in vitro. Mol Hum Reprod 2011; 17: 386-391.

46) Hurst BS, Zacur HA, Schlaff WD, Berkovitz GD. Use of granulosa-luteal cell culture to evaluate low and high clinical responses to menotropin stimulation. J Endocrinol Invest 1992; 15: 567-572.

47) Goodarzi MO, Shah NA, Antoine HJ, Pall M, Guo X, Azziz R. Variants in the 5α-reductase type 1 and type 2 genes are associated with polycystic ovary syndrome and the severity of hirsutism in affected women. J Clin Endocrinol Met 2006; 91: 4085-4091.

48) Plaza-Parrochia F, Poblete C, Gabler F, Carvajal R, Romero C, Valladares L, Vega M. Expression of steroid sulfated transporters and 3β-HSD activity in endometrium of women having polycystic ovary syndrome. Steroids 2015; 104: 189-195.

49) Walters K, Bertoldo M, Handelsman D. Evidence from animal models on the pathogenesis

of PCOS. Best Pract Res Clin Endocrinol Metab 2018; 32: 271-281.

50) Song S, Tan Y. Expression of FKBP52 in the ovaries of PCOS rats. Int J Mol Med 2019; 43: 868-878.

51) Ortega I, Sokalska A, Villanueva JA, Cress AB, Wong DH, Stener-Victorin E, Stanley SD, Duleba AJ. Letrozole increases ovarian growth and Cyp17a1 gene expression in the rat ovary. Fertil Steril 2013; 99: 889-896.

52) Kayampilly PP, Wanamaker BL, Stewart JA, Wagner CL, Menon KMJ. Stimulatory effect of insulin on 5α-reductase type 1 (SRD5A1) expression through an Akt-dependent pathway in ovarian granulosa cells. Endocrinology, 2010; 151: 5030-5037.

53) Panghiyangani R, Soeharso P, Andrijono, Suryandari DA, Wiweko B, Kurniati M, Pujianto DA. CYP19A1 Gene Expression in Patients with Polycystic Ovarian Syndrome. J Hum Reprod Sci 2020; 13: 100-103.

54) Maliqueo M, Sun M, Johansson J, Benrick A, Labrie F, Svensson H, Lönn M, Duleba AJ, Stener-Victorin E. Continuous administration of a P450 aromatase inhibitor induces polycystic ovary syndrome with a metabolic and endocrine phenotype in female rats at adult age. Endocrinology 2013; 154: 434-445.

55) Suriyakalaa U, Ramachandran R, Doulathunnisa JA, Aseervatham SB, Sankarganesh D, Kamalakkannan S, Angayarkanni J, Akbarsha MA, Achiraman S. Upregulation of Cyp19a1 and PPAR-γ in ovarian steroidogenic pathway by Ficus religiosa: a potential cure for polycystic ovary syndrome. J Ethnopharmacol 2021; 267: 113540.

56) Lombardi LA, Simões RS, Maganhin CC, Baracat MCP, Silva-Sasso GR, Florencio-Silva R, Soares Jr JM. Baracat E. C. Immunohistochemical evaluation of proliferation, apoptosis and steroidogenic enzymes in the ovary of rats with polycystic ovary. Revista da Associação Médica Brasileira 2014; 60: 349-356.

57) Tadros SJ, Mohamed DH, Ahmed MM, Hussein YH. Effect of Adipose-derived stem cells versus clomiphene on treatment of experimental polycystic ovary in rats: Histological and Immunohistochemical Study. Egypt J Histol 2018; 41: 373-385.

58) Chi XX, Zhang T, Chu XL, Zhen JL, Zhang DJ. The regulatory effect of Genistein on granulosa cell in ovary of rat with PCOS through Bcl-2 and Bax signaling pathways. J Vet Med Sci 2018; 80: 1348-1355.

59) El-Atrsh A, Tousson E, Elnahas EE, Massoud A, Al-Zubaidi M. Ameliorative effects of spirulina and chamomile aqueous extract against mice bearing ehrlich solid tumor induced apoptosis. Asian Oncol Res J 2019; 1-17.

60) Jahan S, Munir F, Razak S, Mehboob A, Ain QU, Ullah H, Afsar T, Shaheen G, Almajwal A. Ameliorative effects of rutin against metabolic, biochemical and hormonal disturbances in polycystic ovary syndrome in rats. J Ovarian Res 2016; 9: 1-9.

61) Bas D, Abramovich D, Hernandez F, Tesone M. Altered expression of Bcl-2 and Bax in follicles within dehydroepiandrosterone-induced polycystic ovaries in rats. Cell Biol Int 2011; 35: 423-429.

62) Zurvarra FM, Salvetti NR, Mason JI, Velazquez MM, Alfaro NS, Ortega HH. Disruption in the expression and immunolocalisation of steroid receptors and steroidogenic enzymes in letrozole-induced polycystic ovaries in rat. Reprod Fertil Develop 2009; 21: 827-839.

63) Jakimiuk AJ, Weitsman SR, Yen HW, Bogusiewicz M, Magoffin DA. Estrogen receptor α and β expression in theca and granulosa cells from women with polycystic ovary syndrome. J Clin Endocrinol Metabol 2002; 87: 5532-5538.

64) Simão VA, Chuffa L, Camargo ICC. Ovarian sex steroid receptors and sex hormones in androgenized rats. Reproduction 2016; 152: 545-559.

65) Iwai T, Nanbu Y, Iwai M, Taii S, Fujii S, Mori T. Immunohistochemical localization of oestrogen receptors and progesterone receptors in the human ovary throughout the menstrual cycle. Virchows Arch 1990; 417: 369-375.