ORIGINAL ARTICLE

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The possible protective effect of Bougainvillea spectabilis leaves extract on estradiol valerate-induced polycystic ovary syndrome in rats (biochemical and histological study)

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SUMMARY

Polycystic ovary syndrome (PCOS) is a reproductive and metabolic disorder in which the level of oxidative elements in blood rises. *Bougainvillea spectabilis* is a plant with a potent antioxidant effect, since it contains flavonoids. The aim of this work is to explore the possible protective potency of *Bougainvillea spectabilis* leaves (BSL) extract on ovarian folliculogenesis using a rat model of estradiol valerate (EV) - induced PCOS. Thirty six mature female rats were divided into four groups: 1. Control group (six rats). 2. EV group (ten rats), singly injected with 2mg/kg EV subcutaneously. 3. BSL group (ten rats), given 100mg/kg BSL extract orally for 30 days. 4. EV + BSL group (ten rats), singly injected with EV subcutaneously and given 100mg/kg BSL extract orally for 30 days. Biochemical measurements of serum levels of estrogen, testosterone, LH, FSH, glucose, totals lipids, and total antioxidants were done using ELISA method. Histological (using hematoxylin & eosin and Masson's trichrome stains) and immunohistochemical (using anti cyclooxygenase 2 {COX 2}) studies were performed. This was followed by morphometric measurements and statistical analysis.

PCOS caused massive primordial follicle loss and development of cystic follicles with increase in fibrosis, COX 2 immunoexpression. There was a significant reduction in LH, estrogen and glucose serum levels and increase in FSH and the antioxidant serum levels between the EV + BSL-extract-treated group compared to EV-treated group. Histomorphometric studies also showed the significant changes in the number of follicles and decreased cyst formation in the combined therapy group. There was a significant role of BSL extract in restoration of ovarian folliculogenesis and reduction of biochemical, histological and immunohistochemical changes in EV treated group.

Key words: PCOS – Estradiol valerate – *Bougainvillea spectabilis* leaves – Anti COX 2

INTRODUCTION

Polycystic ovary syndrome (PCOS), affecting women in the reproductive age, causes infertility or subfertility due to anovulation and hyperandrogenism (Goodarzi et al., 2011; Welt and Carmina, 2013; Sirmans and Pate, 2014).

The name of PCOS came from observation of ovarian morphological changes in women who suffer from menstrual disturbances and hyperandrogenism symptoms. These morphological changes include follicular cysts, multiple immature follicles, many atretic follicles and others indicating failure of folliculogenesis (Dewailly et al., 2014).

Estradiol valerate (EV)-induced PCOS models were associated with morphological, endocrinolog-

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ical and/or metabolic changes. Estradiol valerate is an estrogen with a long-acting effect. It could activate the peripheral sympathetic neurons innervating the ovary. This activation may increase the content of ovarian norepinephrine, and decrease the number of ovarian beta-adrenergic receptors. This subsequently down regulates the ovary receiving catecholamine innervation (Zangeneh et al., 2012; Maliqueo et al., 2014).

Bougainvillea spectabilis (family Nyctaginaceae) is a thorny woody plant with flowers. It contains many components. One of these compounds is phytochemicals. Phytocemicals include quinones, saponins, triterpenoids, flavonoids, phenols, sterols, glycosides, tannins, furanoids and small amounts of sugars. *Bougainvillea spectabilis* also contains amylase inhibitor, oxidase and pinitol (hypoglycemic element). Previous studies have shown antimicrobial, antidiabetic, anti-inflammatory, amylase inhibitory, antihyperlipidemic, radical scavenging, anti-atherogenic, thrombolytic, analgesic, antipyretic, anthelmintic, antiulcer properties (Chauhan et al., 2016; Do et al., 2016).

The aim of this study was to explore the possible protective potency of Bougainvillea spectabilis leaves (BSL) extract on ovarian folliculogenesis using a rat model of polycystic ovary syndrome induced by estradiol valerate.

MATERIALS AND METHODS

Animals

Forty adult female albino rats, locally bred at the Animal House of Nodcar, with an average weight of 200-250 g, were used in the present study. The animals were housed at ordinary room temperature and exposed to natural daily light-dark cycles, and had access to food and water *ad libitum* according to the ethics of animal researches.

Drugs

1) Estradiol Valerate (EV) was purchased in the form of 5mg ampoule (folone- misr pharma Company, Egypt) that was dissolved in 0.5 ml of corn oil.

2) Bougainvillea spectabilis leaves (BSL) extract

Bougainvillea spectabilis leaves were collected from public garden Mokkatam, Cairo, Egypt in September 2016. The identification of the plant species was verified from Flora and phytotaxonomy researches department of the Agriculture Museum Dokki Giza Egypt. The leaves were air-dried and milled into powder. 200 g of the powder were percolated in 500 ml of absolute ethanol for two weeks (Bhat et al., 2011). The percolated mixture was filtered and evaporated at room temperature according to the method of Fatope et al. (1996). The aqueous suspension extract was made in 0.25% carboxymethylcellulose just before use.

Vaginal smear

Estrous cycle was detected by vaginal smears obtained between 8:00 and 12:00 am. The rats with three consecutive 4–5 days regular estrous cycles were used in this experiment. Injections were begun on the same day of the estrous cycle of all rats. A vaginal smear was taken daily to monitor the phase of the estrous cycle throughout the entire treatment up to the day of sacrifice. After injection, the test was stopped when disturbance in estrus cycle started, where animals reached the stage of persistent vaginal cornification, which is usually 30 days after injection of EV (Ghafurniyan et al., 2015; Rezvanfar et al., 2016).

Experimental design

The animals were divided into:

Group I (*control group*) included ten animals that were further divided into untreated ones, rats that received 0.2 ml corn oil (solvent of EV) by single subcutaneous injection (SC), and others that received daily 0.5 ml CMC suspension (solvent of BSL) by oral route. Animals in the same experimental group were sacrificed at the same time.

Group II *(EV only group*) included ten rats that were administered 2 mg/kg (0.2 ml) of EV by single SC injection (Brawer et al., 1986, Nabiuni et al., 2015, Mohammadi et al., 2017).

Group III *(BSL group*) included ten rats that were given BSL extract in a daily dose of 100 mg/kg orally by gastric tube for 30 days (Ghauhan et al., 2016).

Group IV *(EV+BSL group*) included ten rats that were administered single SC injection of 2 mg/kg of EV plus BSL extract in a daily oral dose of 100mg/kg for 30 days.

Sample collection

On the 31st day, the animals were weighed and anesthetized using intraperitoneal injection of thiopental sodium 40 mg/kg. The blood samples were directly taken from retino-orbital puncture. The abdomen was then rapidly dissected, and ovaries were removed, cleansed gently with normal saline and weighed. Then, they were immediately fixed in 10% formol saline and processed for paraffin blocks.

The blood samples were centrifuged at 3000 rpm for 15min. The collected serum was stored at −70°C. Serum was analyzed for blood glucose, total antioxidant content and total lipids, according to the methods mentioned by Trinder (1969), Koracevic et al. (2001) and Frings et al. (1972) respectively. Sex hormones were estimated using accu bind ELIZA Microwells kit. Five µm sections were cut and stained with hematoxylin & eosin and Masson's trichrome stains. Immunohistochemical staining was done for detection of expression of cyclooxygenase 2 (COX 2) (Labvision, Thermoscientific, USA) rabbit polyclonal antibody, (catalog no. RM-9121-R7). The reaction is cytoplasmic and the +ve control was the lung. An additional slide of ovary specimen was treated with buffer solution instead of the same concentration of primary antibody in every run as the negative control.

Morphometric study

The measurements were done using the image

Table 1. Mean ± SD of serum levels of LH, FSH, Estrogen, Testosterone, Glucose, Total lipids, and Total antioxidants.

Groups	Control (I)	EV (II)	BSL (III)	BSL + EV (IV)
Mean±SD of LH (mIU/ml)	1.4±0.18	4.6±0.1*	1.37±0.1	3.1±0.4*#
Mean±SD of FSH (mIU/ml)	32.54±1.5	15.72±1.05*	29.7±2.5	20.7±1.68*#
Mean±SD of Estrogen (pg/ul)	27.4±1.4	41.83±2.7*	28.3±1.04	35.8±2.6*#
Mean±SD of Testosterone (mIU/ml)	1.76±0.12	1.86±0.14	1.75±0.13	1.79±0.1
Mean±SD of glucose (mg/dl)	114±15.2	141.8±13.4*	111.6±7.4	119.4±4.7
Mean±SD of total lipids (mg/dl)	906.5±179.7	1315.8±233.03*	928.3±26.8	963.8±152.4
Mean±SD of total antioxidants (mμ/l)	.666±0.13	.444±0.02*	.875±0.14*#	.706±0.16

*P ≤ 0.05, significant difference compared to control
P ≤ 0.05, significant difference compared to EV

analyzer computer system (Leica Qwin 500C) (Leica, England). The mean number of different types of ovarian follicles was counted in 10 non-overlapping low power fields (x10). The mean area percent of collagen fibers was measured in 10 non -overlapping low power fields (x10). The mean area percent of COX 2 immunoexpression was measured in 10 non-overlapping high power fields (x40) for each animal.

Statistical Analysis

Data were expressed as group's mean ± standard deviation (SD) after checking for normality of distribution. The statistical analysis was carried out using Kruskal–Wallis test followed by individual Mann–Whitney tests for comparisons between specific groups, with "SPSS version 10" software (SPSS, Chicago, USA). A value of $P \leq 0.05$ was accepted as statistically significant (Petrie and Sabin, 2005).

RESULTS

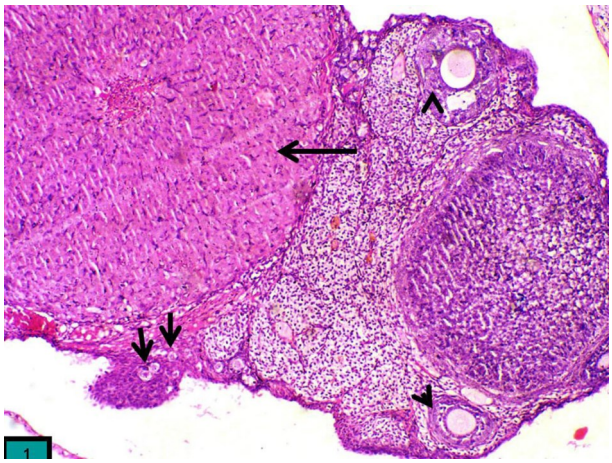

Fig 1. Section of rat ovary in control group showing different types of ovarian follicles which were classified as primordial (surrounded by one layer of flattened granulosa cells) (short arrows), pre-antral (surrounded by 2–5 layers of granulosa cells) (arrowheads), and corpus luteum (long arrow). H & E, x100.

Biochemical results

Hormonal assay

Levels of LH and estrogen in EV group were increased while the level of FSH was decreased compared with the control group. Increase in the level of LH and estrogen and decrease in FSH were significant in EV group compared with other groups. There was non-significant difference in testosterone level between different groups (Table 1).

Total lipids and glucose level

Levels of total lipids and glucose in EV group were increased. This increase was significant when compared with other groups (Table 1).

Total antioxidant status

A level of total antioxidants in EV group was decreased. This decrease was significant when compared with other groups (Table 1).

Histological and immunohistochemical results

Hematoxylin & eosin stain

In the control group (group I), the cortex of the ovary showed different types of ovarian follicles. They were classified as primordial (surrounded by one layer of flattened granulosa cells), pre-antral (surrounded by 2-5 layers of granulosa cells), and corpus luteum (Fig. 1).

In the EV-treated group, it showed massive primordial follicular loss with evident follicular cysts. They also exhibited many degenerative (atretic) follicles (Figs. 2a, b, 3).

In BSL-treated group, it showed a morphology comparable to that of the control group where primordial and antral follicles were noticed. Corpus luteum was also revealed (Fig. 4). While, in EV + BSL treated group the ovarian cortex contained pre-antral, antral follicles as well as corpora lutea. Some atretic and degenerating follicles were also observed (Figs. 5a, b).

Masson's trichrome stain

The control group showed fine collagen fibers in the stroma and around the follicles (Fig. 6). Group

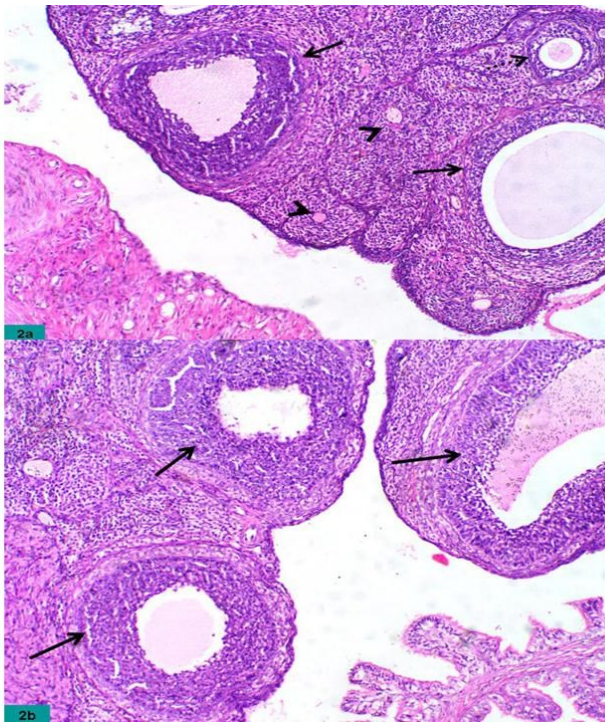

Fig 2a,b. Sections of rat ovary in group II (EV group), showing massive primordial follicular loss with evident follicular cysts (thin arrows). They also exhibited many degenerative (atretic) follicles (arrow heads). Preantral follicle is noticed (dotted arrow). H & E, x100.

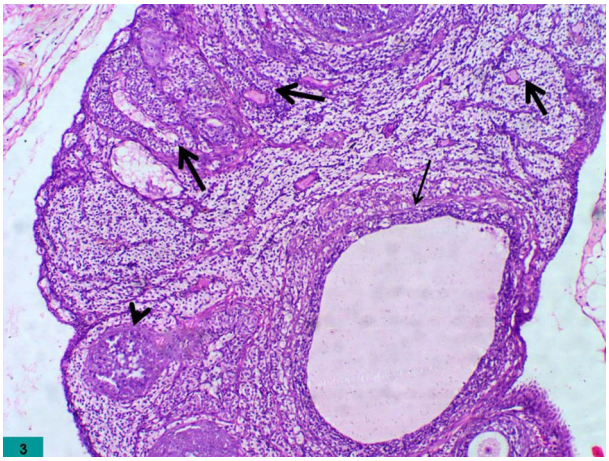

Fig 3. Section of rat ovary in group II (EV group), showing atretic follicles (thick arrows), prominent cystic follicle with thin wall (thin arrow) and a degenerating follicle (arrow head). H & E, x100.

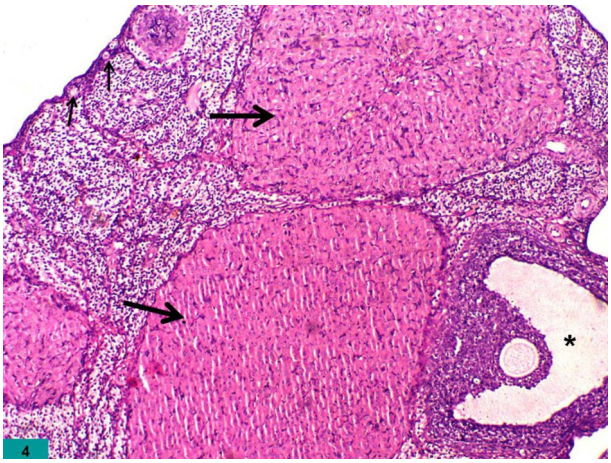

Fig 4. Section of rat ovary in subgroup III (BSL group), showing primordial (thin arrows) and mature Graafian follicle (*). Corpora lutea are also observed (thick arrows). H & E, x100.

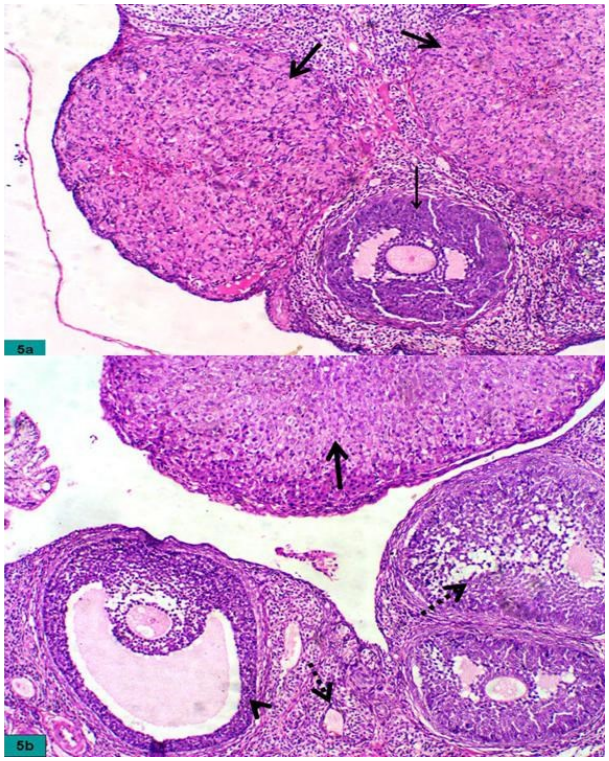

Fig 5a,b. Sections of rat ovary in subgroup IV (BSL+EV group), showing pre-antral (thin arrow), antral follicles (arrow head) as well as corpora lutea (thick arrows). Some atretic and degenerating follicles (dotted arrows) are also observed. H & E, x100.

II demonstrated marked accumulation of dense collagen fibers in the stroma and fine fibers surrounding the cystic follicles (Figs. 7a, b). Groups III and IV recruited fine collagen fibers around the follicles (Figs. 8, 9a, b).

Cox2 immunoexpression

In the control group there was negative immunoreaction in the cytoplasm of granulosa cells, theca cells and stromal cells (Fig. 10). However, in PCO group positive cytoplasmic immunoexpression in the granulosa cells, cells of degenerated follicles and endothelial cell lining of blood vessels were observed (Fig. 11a, b). Group III showed negative immunoreaction while group IV demonstrated very weak immunoexpression in the cytoplasm of degenerated follicles cells (Figs. 12, 13).

Morphometric results

Ovarian weight

Non significant ($P \geq 0.05$) mean ovarian weight change was detected in EV group compared with the other groups (Table 2).

The number of different follicles in different groups

Estradiol valerate administration reduced the

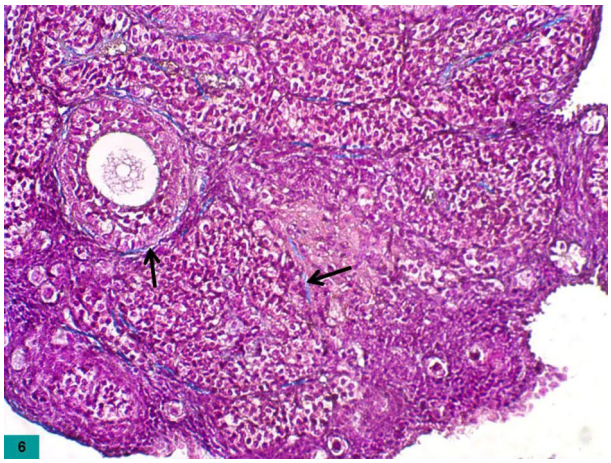

Fig 6. Section of rat ovary in control group, showing fine collagen fibers in the stroma and around the follicles (arrows). MT, x200.

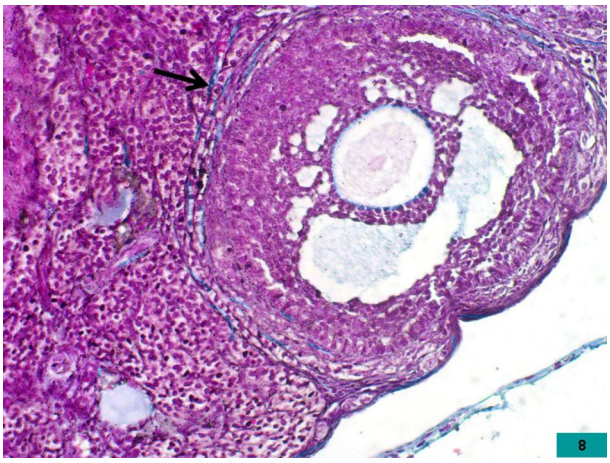

Fig 8. Section of rat ovary in subgroup III, showing fine collagen fibers around the follicles (arrow). MT, x200.

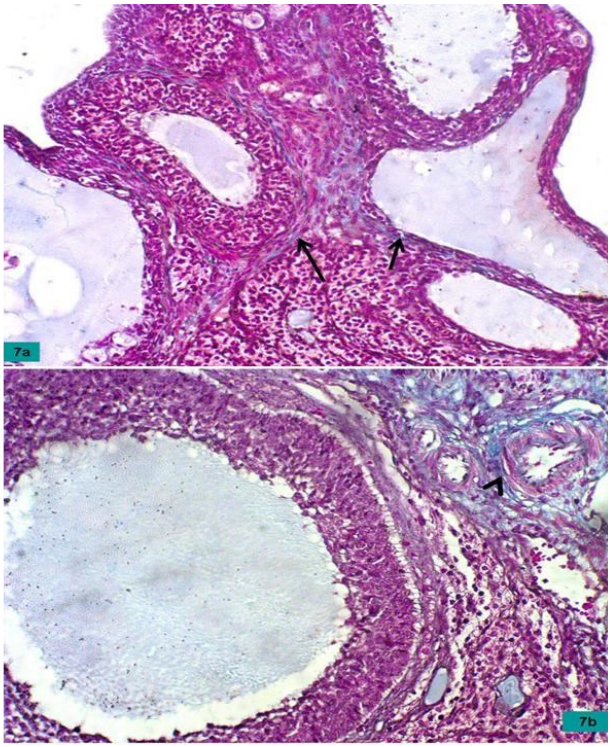

Fig 7a,b. Sections of rat ovary in subgroup II, showing marked accumulation of dense collagen fibers in the stroma (arrowhead) and fine fibers surrounding the cystic follicles (arrows). MT, x200.

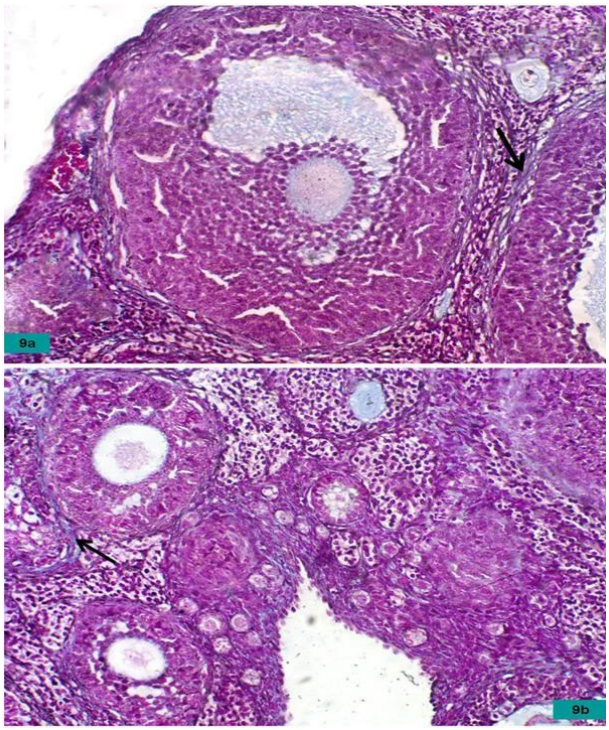

Fig 9a,b. Sections of rat ovary in subgroup IV, showing fine collagen fibers around the follicles (arrows). MT, x200.

number of primordial, primary, preantral, antral follicles and corpus luteum. While, the number of cystic follicles was increased as compared with control group. This reduction was statistically significant. However, in the groups that received the extract (BSL + EV group), the number of these follicles significantly increased while the number of cystic follicles significantly decreased (Table 3).

Mean area % of collagen fibers

The mean area percent of collagen fibers was significantly increased in EV-treated group as compared with the control and with both groups III and IV as well (Table 4).

Mean area % of COX 2 immunoreactivity

There was a significant increase in the mean area percent of COX 2 immunoreactivity in EV-treated group compared to the control as well as to both BSL and combined therapy groups (Table 4).

DISCUSSION

Polycystic ovary syndrome is an endocrine disorder which is characterized by follicular arrest, anovulation, and hyperandrogenism. Its treatment is difficult, because of its complex etiology and pathogenesis. The treatment strategy suggests that regulating abnormal androgen and/or estrogen

Table 2. Mean±SD of ovarian weight.

Groups	Control (I)	EV (II)	BSL (III)	BSL + EV (IV)
Mean±SD of ovarian weight (mg)	9.7±0.12	10±0.13	9.8±0.2	10.1±0.22

*P ≤ 0.05, significant difference compared to all other groups

Table 3. Mean ± SD of number of different follicles in different groups.

Groups	Control (I)	EV (II)	BSL (III)	BSL + EV (IV)
Mean±SD of primordial follicles	21±0.33	7±0.42*	20±0.25	17±0.12*#
Mean±SD of primary follicles	7±0.2	2±0.01*	8±0.23	5±0.05*#
Mean±SD of preantral follicles	8±0.12	3±0.09*	8±0.5	7±0.44*#
Mean±SD of antral follicles	5±0.3	1±0.09*	5±0.23	4±0.31*#
Mean±SD of atretic follicles	8±0.1	20±1.2*	8±0.5	10±0.8*#
Mean±SD of corpus luteum	5±0.7	1±0.002*	6±0.04	3±0.02*#
Mean±SD of cystic follicles	0	6±0.09*	0	2±0.6*#

*P ≤ 0.05, significant difference compared to control
P ≤ 0.05, significant difference compared to EV

Table 4. The mean area percent of collagen, COX 2 ± standard deviation in all groups

Groups	Control (I)	EV (II)	BSL (III)	BSL + EV (IV)
Mean±SD area % of collagen fibers	0.6±0.04	1.08±0.3*	0.7±0.12	0.64±0.07
Mean±SD area % of COX 2	0.3 ± .12	8 ± 1.3*	0.3 ± .02	0.4 ± .07

*P ≤ 0.05, significant difference compared to all other groups

is an effective method of treating PCOS (Sirmans and Pate, 2014).

Human studies are faced with many limitations. Thus animal models are a good and affordable choice for this purpose. Numerous experimental models for induction of PCOS have been developed using EV. A previous study has revealed cessation of ovulatory cycles by 16-20 days after EV-treatment with persistent vaginal cornification. Serum levels of LH and FSH decreased at 11 days after EV administration (Brawer et al., 1986). Thus, EV can produce a PCOS model in rats with interruption in metabolic and physiologic processes (Amini et al., 2016).

Oxidative stress is characterized by production of the free radicals of oxygen and decrease in the antioxidant defense capacity of the body. Reactive oxygen species are formed by the body through different metabolic reactions. They can react with important different molecules of the body such as lipids, proteins and nucleic acids. This may result to oxidative stress and pathological changes in different cells and tissues. It can cause diseases such as cancer, cardiovascular diseases, diabetic nephropathy, PCOS and aging (Zuo et al., 2016).

In this study, significant decrease in total antioxidant levels in the EV-treated group was compared with control and combined therapy groups. This could indicate the role of reactive oxygen species in induction of PCOS. There was also a significant increase in total antioxidants in BSL-only-treated group compared to other groups. This indicated that the protective effect of BSL extract may be due to its antioxidant activities (Ghauhan et al., 2016).

There was also significant increase in the level of glucose and total lipids in EV treated group compared to control and combined therapy groups. This might be due to insulin resistance and obesity, which are common features of PCOS. This can be attributed to the oxidative stress process (Zuo et al., 2016). These results were correlated with those of Ghafurniyan et al. (2015), Rezvanfar et al. (2016) and Wu et al. (2018) that showed similar results in PCOS model in rats.

Previous studies have revealed the antihyperglycemic and antihyperlipidemic effects of BSL (Geethan and Prince, 2008; Mahajan et al., 2015; Ghauhan et al., 2016).

Regarding hormonal assay, Levels of LH, testosterone and estrogen in the EV group were increased while the level of FSH was decreased compared to the control group. Increase in the level of LH and estrogen and decrease in FSH were significant in the EV group compared with other groups. There was a non-significant increase in testosterone level in the EV group compared with other groups, although a slight increase in testosterone can suppress normal menstruation and ovulation (Sterling, 2011). The effect of BSL extract on hormonal levels showed that LH and estrogen levels decreased significantly while FSH increased significantly compared with EV-treated group. Although BSL extract has regulated the secretion of hormones, there was a significant difference between control and combined therapy groups. This

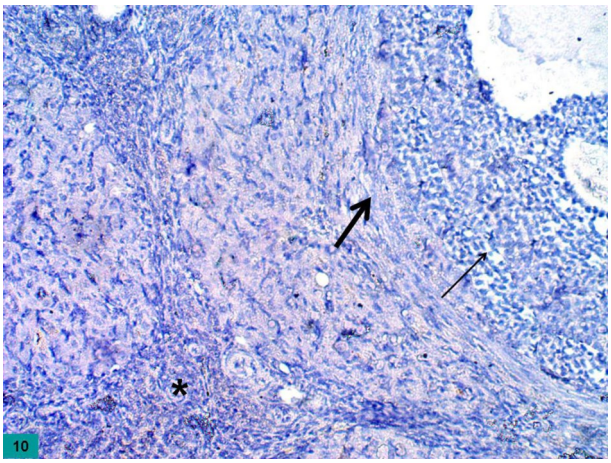

Fig 10. Section of rat ovary in control group, showing negative immunoreaction in the cytoplasm of granulosa cells (thin arrow), theca cells (thick arrow), stromal cells (*). COX 2, x200.

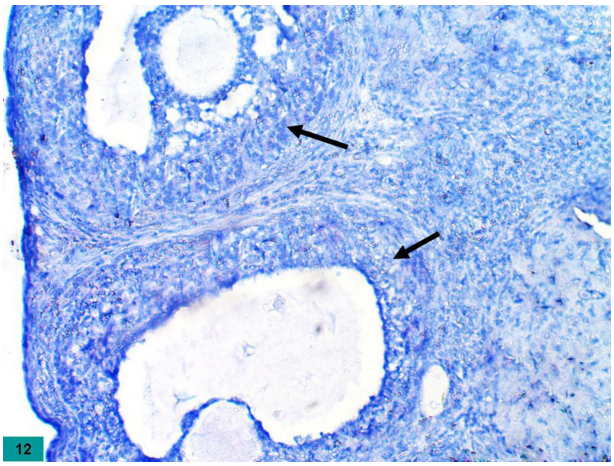

Fig 12. Section of rat ovary in group III, showing negative immunoreaction in granulosa cells of antral follicles (arrows). COX 2, x200.

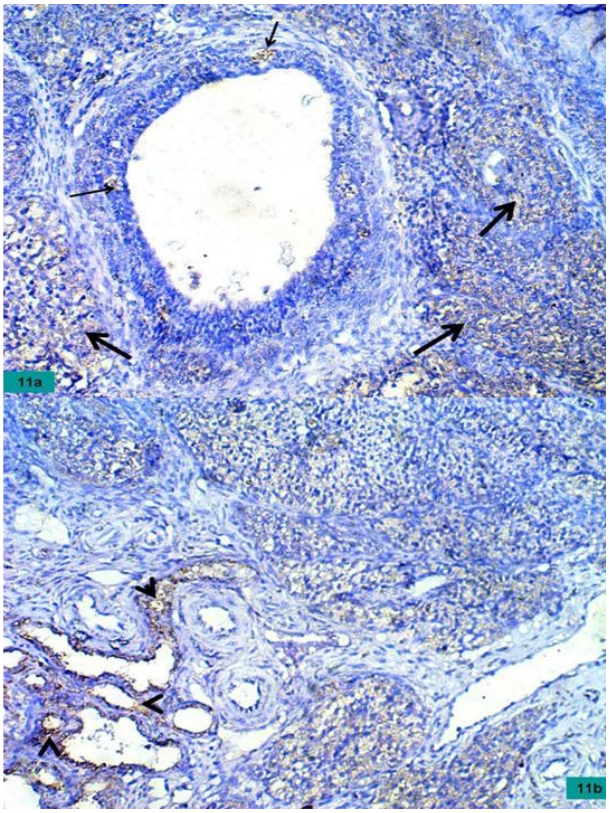

Fig 11a,b. Sections of rat ovary in group II, showing positive cytoplasmic immunoexpression in the granulosa cells (thin arrows), cells of degenerated follicles (thick arrows) and endothelial cell lining of blood vessels (arrowheads). COX 2, x200.

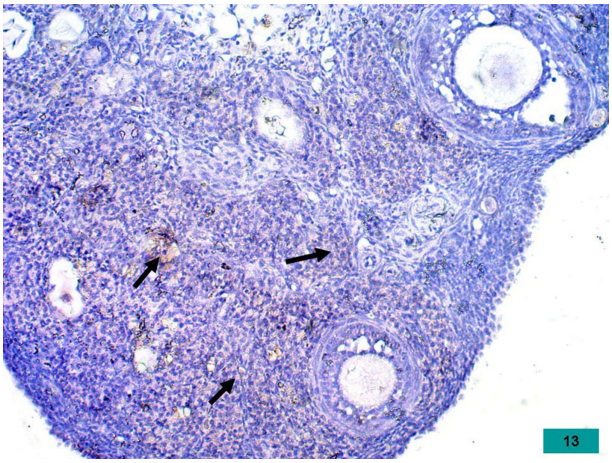

Fig 13. Section of rat ovary in group IV, showing very weak cytoplasmic immunoreaction in cells of degenerated follicles (arrow). COX 2, x200.

may be an indication to change the dose or prolong the treatment duration in future studies.

The previous results were in accordance with Ghafurniyan et al. (2015) and Barzegar et al. (2017), who showed similar results regarding LH, FSH, estrogen levels in EV induced PCOS in rats. However, results regarding testosterone hormone were inconsistent with Ghafurniyan et al. (2015), Rezvanfar et al. (2016), Barzegar et al. (2017) and Wu et al. (2018), who revealed that it increased its level in EV-induced PCOS in rats.

In the present study, marked improvement of EV-induced morphological changes was detected in the ovaries of rats treated with BSL extract. These changes were supported by histological, immunohistochemical and morphometric results.

Examination of hematoxylin & eosin-stained sections of the EV-treated group revealed massive primordial follicular loss in the cortex of the ovary. This was in agreement with Karimzadeh et al. (2013) and Barzegar et al. (2017), who observed decrease in number in all follicular types including primordial follicles. However, this result was inconsistent with Ghafurniyan et al. (2015), who showed no difference in number of primordial follicles in EV induced PCOS and normal rats. Other researchers revealed that here was a greater number of growing follicles in PCOS ovaries compared with normal. In spite of this, there was no difference in number of nongrowing primordial follicles between normal and PCOS ovaries. So, explanation of the increase in growing follicles in PCOS could not be attributed to recruitment of more primordial follicles

into activation pool. They attributed this to abnormally slow primary follicle growth and not to atresia (Maciel et al., 2004).

There was also a reduction in number of primary, preantral, antral follicles and corpus luteum. While, the number of cystic follicles was increased as compared with control group. This was in accordance with Ghafurniyan et al. (2015), Rezvanfar et al. (2016) and Wu et al. (2018), who showed same results.

Endocrine and metabolic interactions are needed for activation of primordial follicles into the growing pool selection of dominant follicle. Coordination of granulosa cell proliferation, theca cell differentiation and oocyte maturation needs also intraovarian paracrine signal. In PCOS, ovarian hyperandrogenism, insulin resistance, and altered intrafollicular paracrine signaling disturb this activation, growth, and selection of follicles. This leads to accumulation of small antral follicles giving it polycystic morphology (Dumesic and Richard, 2013).

After induction of PCOS, follicle growth and development in the experimental group decreased if compared with the control group. The origin of these cysts was most probably atretic follicles. Granulosa cells were degenerated; and the outer layer of the theca cells was thickened. There was an increase in number of corpora lutea, ovulatory, in the combined therapy group. This increase was significant. While number of cystic follicles was significantly decreased. These results indicated the role of BSL extract in regulating the process of folliculogenesis. It was reported that BSL extract may cause a decrease in the thickness of the follicular theca layer in PCOS rats. This could be attributed to increased lipolysis and decreased hypertrophy of this layer. Androgens and steroids produced by this layer would also decrease as consequences of decreased thickness of theca layer (Ghafurniyan et al., 2015).

The present work also demonstrated significantly increased density of collagen fibers in the stroma of ovary in EV-treated group as compared to the control. This is in agreement with Bulut et al. (2015), who similarly showed fibrosis in the ovaries of the EV-treated rats. Meanwhile, in combined therapy group, there was a decrease in the mean area percent of collagen fibers as compared to the EV-treated group. This may indicate that BSL extract has antifibrotic properties, which were attributed to its paracrine effects on fibroblasts. The antifibrosis effect may be due to the regulation of synthesis by the matrix metalloproteinase (MMPs) and the degradation by the tissue inhibitor of metalloproteinase (TIMP) production by fibroblasts (Zhou et al., 2017).

In the current study, positive immunoexpression of COX 2 in the granulosa cells of the ovarian follicles, as well as in the stromal blood vessels, was significantly increased in EV-treated rats. COX 2 causes a decrease in prostanoid biosynthesis. It is involved in inflammation, cell growth and specialization. The importance of COX 2 in PCOS is coming from its proliferative effect on the theca layer cells (Huang et al., 2018).

This was consistent with Karimzadeh et al. (2013), who revealed COX 2 immunoreactivity in theca layers, granulosa layer and weak expression in stroma. However, in the combined therapy group, COX 2 immunoreactivity was significantly decreased. This might be due to antiinflammatory effect of BSL extract (Mandal et al., 2015).

In conclusion, the findings of the present study reinforce the significant role of BSL extract in restoration of ovarian folliculogenesis in EV-induced PCOS through its antioxidant, antihyperglycemic, antihyperlipdemic, and hormonal regulatory effects.

Finally, further studies are required on BSL extract before clinical application of this treatment on humans. These studies are required to evaluate fertility after BSL extract administration using different methods like mating followed through until occurrence of pregnancy, and could also involve new reliable markers of fertility like anti-mullerian hormone.

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