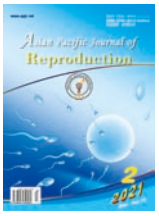

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305-0500.311610

Combined effects of *Gymnema sylvestre* and *Pergularia daemia* on letrozole-induced polycystic ovarian syndrome in rats

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ABSTRACT

Objective: To investigate the combined therapeutic potential of *Gymnema* (*G.*) *sylvestre* and *Pergularia* (*P.*) *daemia* on letrozole-induced polycystic ovarian syndrome (PCOS) in rats.

Methods: Thirty six healthy female Wistar rats with regular estrus cycles were randomly divided into six groups each of 6. Group Ⅰ received 1 mL of 0.5% carboxyl methyl cellulose orally and served as the vehicle control group, while groups Ⅱ to Ⅵ were treated with letrozole (1 mg/kg body weight *p. o.*) for 21 days to induce PCOS. After induction of PCOS, group Ⅱ served as the PCOS control group, without treatment; group Ⅲ received metformin (20 mg/kg body weight *p. o.*) as the standard group, and groups Ⅳ to Ⅵ received *G. sylvestre* (100 mg/kg body weight *p. o.*), *P. daemia* (300 mg/kg body weight *p. o.*), and the combination of *G. sylvestre* and *P. daemia*, respectively, for 28 days. Vaginal smears were collected from all rats daily throughout the study to determine the phases of the estrus cycle. After completing the treatment schedule, oral glucose tolerance test, serum lipid profile and reproductive hormonal analysis were carried out. Subsequently, the rats were sacrificed to collect ovary and uterus for histopathological examination.

Results: The PCOS control rats showed a significant irregularity in the estrus cycle, hyperglycemia, and the altered serum lipid profile such as the increased low and very low density lipoprotein, triglyceride, and decreased high density lipoproteins. In addition, the PCOS control rats showed a significant increase in serum luteinizing hormone, testosterone, and estrogen, and decrease in follicle stimulating hormone and progesterone. These changes were significantly revoked in all the treatment groups. The test drugs also significantly reduced the gained ovary weight (P<0.001), and histopathology of the ovary showed almost normal ovary. Among the treatment groups, the group of combination treatment of *G. sylvestre* and *P. daemia* showed superior ameliorative results in PCOS parameters.

Conclusions: Combination of *G. sylvestre* and *P. daemia* presents potent synergistic activity against hyperandrogenism, hyperinsulinemia, anovulation and follicular cysts in letrozole-induced PCOS rats.

KEYWORDS: Polycystic ovarian syndrome; Metformin; Letrozole; *Gymnema sylvestre*; *Pergularia daemia*; Reproductive hormone; Estrus cycle

1. Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common female reproductive disorders, associated with both endocrinal and metabolic abnormalities. The global prevalence of PCOS is highly variable from a minimum of 2.2% to a maximum of 26.0%[1]; in India, it ranges from 3.7% to 22.5%[2]. Hyperandrogenism, insulin resistance, and disturbed folliculogenesis are the primary pathophysiological factors of PCOS. Insulin resistance and the compensatory hyperinsulinemia result in altered ovarian function and lead to excessive androgen production and anovulation in PCOS[3].

Various drug treatments are used to manage PCOS based on the individuals, including insulin sensitizer, hormonal therapies (clomiphene citrate, progesterone), anti-androgens (spironolactone), and gonadotropin. Metformin is the commonly prescribing insulin-sensitizing agent used for the treatment of type 2 diabetes mellitus and PCOS worldwide. Apart from hypoglycemic action, it also normalizes the menstrual cycle, improves ovulation, and reduces the excessive androgen levels[4,5]. The course of metformin therapy has side effects like joint pain, arthritis, mood swings, hot flushes,

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How to cite this article: Pachiappan S, Ramalingam K, Balasubramanian A. Combined effects of *Gymnema sylvestre* and *Pergularia daemia* on letrozole-induced polycystic ovarian syndrome in rats. *Asian Pac J Reprod* 2021; 10(2): 68-74.

Article history: Received: 9 September 2020; Revision: 15 December 2020; Accepted: 26 December 2020; Available online: 29 March 2021

and bloating[6]. Due to the limitation, contraindication, and side effects of allopathic medication, PCOS women are intense to use the alternative medication in association with traditional medicine to improve the PCOS condition[7].

Gymnema (*G.*) *sylvestre* (Asclepiadaceae), one of the effective ayurvedic medicines, is widely used in the management of diabetes as a sugar destroyer. Various reported activities are anti-inflammatory, antipyretic, antioxidant, liver tonic, astringent, digestive, stomachic, diuretic, laxative, cardiotonic, and uterine tonic[8,9]. The herbal extract of *G. sylvestre* is used as a dietary supplement to reduce body weight, blood cholesterol, and triglyceride. *G. sylvestre* is also useful in the management of PCOS, due to its insulin modulating activity and the added benefits of reducing elevated triglyceride in PCOS[10]. This wide range of therapeutic effects of *G. sylvestre* is mainly due to its secondary metabolites referred to as gymnemic acids which is a mixture of at least 17 different saponins, acidic glycosides, and anthraquinones[11].

Pergularia (*P.*) *daemia* (Asclepiadaceae) is traditionally used to treat jaundice and infantile diarrhea. It also acts as an expectorant, laxative, and antipyretic. The fresh leaf juice of *P. daemia* is used for dysmenorrhea, amenorrhea, infantile diarrhea, catarrhal infection, and analgesic, and the aerial part of the *P. daemia* was reported to possess antidiabetic, antioxidant, hepatoprotective, antibacterial, central nervous system depressant, antipyretic and inflammatory activity. *P. daemia* also potently normalizes the menstrual irregularity and estrous cycle, by restoring the estrous cycle. Besides, it reduces the development of the follicular cyst and normalizes the altered level of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in PCOS. These reported pharmacological activities are mainly due to the presence of secondary metabolites like alkaloids, triterpenes, quercetin, saponins, and steroidal compounds[12-14].

Hence, administration of *G. sylvestre* improves insulin sensitivity and reduces insulin resistance, and also interferes with the androgen synthesis. Supplementation of *P. daemia* normalizes the menstrual irregularity and hormonal imbalance and also reduces the incidence of follicular cysts development. Based on these previous reports, this study was designed to evaluate the combined therapeutic potential of *G. sylvestre* and *P. daemia* on letrozole-induced PCOS rats.

2. Materials and methods

2.1. Animals

Thirty six colony inbred virgin female Albino Wistar rats, weighing 180-230 g (12 weeks), were obtained from Tamil Nadu Veterinary and Animal Sciences University, Chennai-51, India. The animals were acclimated and housed in sterilized polypropylene cages at (23±2) ℃, relative humidity of (60±10)%, and 12/12h light/dark rhythm week before the experimentation. They were fed with a standard pellet diet and water *ad libitum*. All the selected animals had at least two sequential estrous cycles that continued for four days. The animals were transferred to the laboratory 1 h before the experiment. Estrous cycles were monitored by early morning vaginal smear sampling.

2.2. Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade. Letrozole was purchased from Sun Pharmaceutical Industry Limited, India. Metformin was purchased from Cipla Ltd., India. Carboxymethyl cellulose (CMC) sodium salt was from HIMEDIA, India. Biochemical and enzyme immunoassay kits were purchased from Thermo Fisher Scientific, India.

2.3. Plant materials and preparation of extract

The authenticated *G. sylvestre* dried leaf powder was collected from Genius Nature Herbs Pvt. Ltd., Coimbatore, Tamil Nadu, India. The aerial parts of *P. daemia* were collected in October 2019 from in and around Tiruchengode, Namakkal (DT) Tamil Nadu, India. The specimen sample was identified and authenticated by Dr. M.U. Sharief, Scientist 'E' & Head of Office, Botanical Survey of India, Southern Regional Centre, Coimbatore, Tamil Nadu, India (Specimen voucher: BSI/SRC/5/23/2019/Tech/310).

The powdered materials of *G. sylvestre* (100 g) and *P. daemia* (100 g) were extracted separately with 95% ethanol in the Soxhlet apparatus at 75 ℃ for 16 h. The liquid extracts were then evaporated under reduced pressure and controlled temperature (40 ℃) in a rotary evaporator until all solvents were evaporated to obtain a greenish-black jelly residue. The percentage yield of ethanolic extract was 18.2% w/w for *G. sylvestre* and 20.7%w/w for *P. daemia*. The extracts were then stored in airtight containers below 10 ℃ until further use.

2.4. Dosage and administration

The human therapeutic dose of *G. sylvestre* was 600 mg/day[15], which was converted into rat dose as 100 mg/kg/day based on the body surface area of the animal[16]. The effective and non-toxic therapeutic dose of *P. daemia* was 300mg/kg selected as an individual dose[17]. The combined dose of extracts was 400 mg/kg. The extracts and metformin suspensions were freshly prepared daily (less than 1 mL in 0.5% CMC before administration.

2.5. Experimental design and induction of PCOS

PCOS was induced by the administration of letrozole 1 mg/kg body weight (b.w.) (dissolved in 0.5% CMC) once daily for 21 days through gavage to the rats[16,18]. The selected, weighed and regular estrus cycle monitored rats were randomly divided into six groups each of six. Group Ⅰ served as the vehicle control group and received 1 mL of 0.5% CMC orally. Groups Ⅱ-Ⅵ were treated with letrozole (1 mg/kg b.w. *p. o.*) for 21 days. In addition, Group Ⅱ served as the PCOS control group, without treatment, Group Ⅲ received metformin (20 mg/kg b.w. *p.o.*), Group Ⅳ received *G. sylvestre* (100 mg/kg b.w. *p. o.*), Group Ⅴ received *P. daemia* (300 mg/kg b.w. *p. o.*) and Group Ⅵ received the combination of *G. sylvestre* (100 mg/kg b.w. *p. o.*) and *P. daemia* (300 mg/kg b.w. *p. o.*). After induction of PCOS from the 22nd day, the extracts and metformin treatments were continued for further 28 days. The induction of PCOS was primarily confirmed by examining the vaginal smear and measuring the menstrual irregularity on 21st day after letrozole treatment.

2.6. Vaginal smear observation

During the study period, every morning between 8.00 to 9.00 a.m., vaginal fluid was collected by inserting the tip of the pipette with 10 μL of normal saline into the rat vagina. After collecting the vaginal fluid, the smear was prepared on a glass slide and stained with methylene blue (0.5% aqueous solution). The stained slides were observed in the light microscope (40× objective lens magnification) (Manufacturer of ADELTA OPTEC, Haryana, India) to determine the different stages of the estrus cycle based on cell morphology. The presence of small rounded and nucleated epithelial cells indicates the proestrusestrus; anucleated keratinized epithelial cells indicate estrus; the combination of anucleated keratinized epithelial cells and neutrophils indicates metestrus; decrease in the number of anucleated keratinized epithelial cells indicates the diestrus[19].

2.7. Oral glucose tolerance test (OGTT)

At the end of the study period, all the rats fasted for 12 h, and OGTT was performed using an Accu Check Active glucometer (Roche Diagnostics Ltd). After 12 h fasting, an initial blood glucose level was measured using the tail vein blood sample. Each rat received 0.5 mL of 400 mg of glucose solution orally; 2 h after administration of glucose solution, blood glucose level was monitored again[20].

2.8. Serum biochemical and hormonal analysis

After 28 days of drug treatment, the blood glucose level was monitored by glucometer. After that, rats were anesthetized with 90 mg/kg b. w. ketamine and 10 mg/kg b. w. xylazine *i. p.*[21]. The blood sample was drawn through retro-orbital plexus and the serum was separated after centrifugation of total blood without anticoagulants, at 1 100 ×*g*, for 10 min. The serum lipid profile like high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL) cholesterol, and triglyceride were analyzed by auto analyzer kits standard techniques following the manufacturer's instructions (Thermo Fisher Scintific, India). The serum LH, FSH, testosterone, estradiol, and progesterone were measured using enzyme-linked immunosorbent assay standard kit according to the manufacturer's instructions (Thermo Fisher Scientific, India)[22].

2.9. Histopathological examination of ovary

After collection of the blood sample, animals were sacrificed by an overdose of anesthesia. The ovaries and uterus were dissected out, cleaned from fats, and weighed on an electronic weighing balance. The left ovary from one rat of each group was fixed in 10% neutral formal saline, embedded in paraffin wax, and then sectioned at 5-μm thickness. Sections were mounted and stained by the hematoxylin and eosin procedure. Some sections were stained by Masson's trichrome and examined under a light microscope with 100× magnification for histopathological changes[19].

2.10. Statistical analysis

Data were expressed as mean±standard deviation (mean±SD) to determine significance. When rats were compared over time or within multiple groups, one-way analysis of variance was performed, followed by *post hoc* Dunnett's test using SPSS V.17. The values were considered significant when $P<0.05$.

2.11. Ethics statement

The study was approved by the Institutional Animal Ethics Committee (IAEC) of Swamy Vivekanandha College of Pharmacy, Namakkal-637205, India. Care and use of laboratory animals conformed to CPCSEA guidelines (IAEC Reference No: SVCP/IAEC/PG/1/03/2017).

3. Results

3.1. Effect on the duration of the estrus cycle

After 21 days of letrozole treatment, there was a significant reduction in the estrus period ($P<0.001$), this was concomitant to the increase in the duration of diestrus phases in all the treatment groups than the normal control group. The increased diestrus duration was significantly reduced in all the treatment groups after 28 days of drug treatments (namely, on day 50 of the study) when compared with PCOS rats ($P<0.001$). In comparison, no significant differences could be observed between the treatments. But numerically the combination of *G. sylvestre* and *P. daemia* treatment (group Ⅵ) showed better regularization of estrus cycle duration than the individual treatments which was closer to the normal control value (Table 1).

3.2. Outcomes of OGTT

The data in Table 2 indicated that both levels of fasting blood glucose and those after 2 h oral glucose challenge were significantly increased in the PCOS control group as compared with the normal control group ($P<0.001$). All the drug treatments (groups Ⅲ-Ⅵ) significantly decreased hyperglycemia in both cases when compared to the PCOS control group ($P<0.001$). There was no significant changes between the treatments when compared to the standard metformin treatment (group Ⅲ). In comparison between the extracts treatment the fasting blood glucose level was significantly higher in *P. daemia* alone treatment (group Ⅴ) ($P<0.05$), but the combination of *G. sylvestre* and *P. daemia* treatment (group Ⅵ) showed numerically better reduction of hyperglycemic in both the condition when compared to the treatment group Ⅳ and Ⅴ.

3.3. Lipid profile

A significant elevation of serum LDL, VLDL cholesterol, and triglyceride level was accompanied by a significant decrease in HDL

cholesterol in the PCOS control group when compared with the normal control group (P<0.001). All the treatment groups (groups Ⅲ-Ⅵ) showed significant improvement in the altered lipid profile when compared to the PCOS control group. The reduction rate of serum LDH and triglyceride level was significantly higher in the combination of *G. sylvestre* and *P. daemia* treatment when compared to the standard metformin treatment (group Ⅲ) (P<0.01). Among the extract treatment groups (group Ⅳ-Ⅵ), the reduction rate of serum LDL, VLDL and triglyceride were significantly less in *P. daemia* alone treatment, but numerically the combination of *G. sylvestre* and *P. daemia* treatment (group Ⅵ) showed the better improvement in altered lipid profile which was almost closer to the normal control group lipid profile (Table 3).

3.4. Reproductive hormone levels

The PCOS control group showed significant increases in serum LH, testosterone, estradiol, and decreases in FSH and progesterone levels compared to the normal control group (P<0.001). However, the entire drug treated groups (groups Ⅲ-Ⅵ) showed a significant reversal effect on reproductive hormone levels as increases in serum FSH and progesterone and decreases in serum LH, testosterone, and estradiol levels when compared to the PCOS control group. There were significant decreases in the serum LH and estradiol level and increases in the progesterone levels in the combination of *G. sylvestre* and *P. daemia* treatment (group Ⅵ) compared to the standard metformin treatment (group Ⅲ). Among the extract treatment groups Ⅳ to Ⅵ, the increasing serum progesterone level was significantly lower in *G. sylvestre* alone treated group (group Ⅳ) (P<0.05), but the reversal of hormonal irregularity was numerically better in the combination of *G. sylvestre* and *P. daemia* treatment (Table 4).

3.5. Weights of ovary and uterus

A significant increase in ovarian weights was observed in the PCOS control group as compared with the normal control group (P<0.001). The tested treatments (groups Ⅲ-Ⅵ) significantly decreased the ovarian weights as compared to the PCOS control group (P<0.001). However, the mean values of ovarian weights and uterine weights were comparable among all groups (P>0.05) (Table 5).

3.6. Histopathology of ovaries

Histopathological changes of the ovary in the normal control group showed normal histological structure like congested vascular space with spindle shaped cells in the medulla. The cortex showed a few healthy primary and secondary follicles, and the presented corpus luteum contained eosinophilic cytoplasm. The PCOS control group showed few follicles with varying sizes, the presented atretic follicles contained fluid filled antrum, and the corpus luteum was absent. All the treatment groups (groups Ⅲ-Ⅵ) showed the minimal number of cysts, normal healthy follicles, and decreased fluid filled antrum, and also showed the presence of corpus luteum. The results of *G. sylvestre* and *P. daemia* combination treated group (group Ⅵ) showed improved histological changes when compared to other treatment groups, and the changes reached almost nearer to those in the normal control group (Figure 1).

Table 1. Effects of *Gymnema sylvestre* and *Pergularia daemia* on the duration (days) of phases of estrus cycle in letrozole-induced polycystic ovarian syndrome in rats.

Groups	Proestrus		Estrus		Metestrus		Diestrus	
	21st day	50th day	21st day	50th day	21st day	50th day	21st day	50th day
Group Ⅰ	2.0±0.5	1.8±0.5	3.0±0.9	2.6±0.5	2.0±0.5	1.8±0.5	2.0±0.6	2.0±0.7
Group Ⅱ	2.4±0.4	1.5±0.6	1.2±0.5^{a**}	1.2±0.5^{a*}	2.0±0.6	1.6±0.8	3.6±0.5^{a}	5.2±0.8^{a**}
Group Ⅲ	1.8±0.6	1.6±0.5	1.4±0.6^{a**}	2.0±0.7	2.4±0.9	2.0±0.6	4.4±0.7^{a*}	2.8±0.8^{b**}
Group Ⅳ	1.8±0.5	1.6±0.6	1.6±0.8^{a*}	2.0±0.7	2.6±0.7	2.4±0.7	4.4±0.7^{a*}	3.0±0.7^{b**}
Group Ⅴ	2.2±0.7	2.1±0.5	1.4±0.6^{a**}	2.0±0.7	2.0±0.6	1.9±0.5	4.2±0.8^{a*}	2.7±0.8^{b**}
Group Ⅵ	2.0±0.9	1.8±0.7	1.2±0.2^{a**}	1.8±0.8	2.2±0.2	1.8±0.7	3.6±0.7^{a}	2.6±0.9^{b**}

Values are expressed as mean±SD; n=6 in each group. a, a*, a**: Compared to group Ⅰ: P<0.05, P<0.01, P<0.001, respectively; b**: Compared to group Ⅱ; P<0.001. Group Ⅰ receives 1 mL of 0.5% carboxymethyl cellulose orally and serves as the vehicle control group; Groups Ⅱ-Ⅵ are treated with letrozole (1 mg/kg b.w. *p. o.*) for 21 days to induce polycystic ovarian syndrome (PCOS). After induction of PCOS, Group Ⅱ serves as the PCOS control group, without treatment, Group Ⅲ receives metformin (20 mg/kg b.w. *p. o)*, Group Ⅳ receives *Gymnema sylvestre* (100 mg/kg b.w. *p. o.*), Group Ⅴ receives *Pergularia daemia* (300 mg/kg b.w. *p. o.*) and Group Ⅵ receives the combination of *Gymnema sylvestre* (100 mg/kg b.w. *p. o.*) and *Pergularia daemia* (300 mg/kg b.w. *p. o.*).

Table 2. Effects of *Gymnema sylvestre* and *Pergularia daemia* on oral glucose tolerance test in letrozole-induced polycystic ovarian syndrome in rats.

Altered glucose metabolism (mg/dL)	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ	Group Ⅵ
0 h (fasting blood glucose)	103.17±5.67	183.00±12.38^{a**}	109.17±7.13^{b**}	106.17±6.70^{b**}	115.83±12.01^{ab**d}	103.67±5.75^{b**}
2 h	137.67±14.18	275.50±49.04^{a**}	163.67±27.35^{b**}	165.50±38.79^{b**}	178.67±19.94^{ab**}	153.33±12.71^{b**}

a, a**: Compared to group Ⅰ: P<0.05, P<0.001, respectively; b**: Compared to group Ⅱ; P<0.001. d: Compared to group Ⅵ: P<0.05.

Table 3. Effects of *Gymnema sylvestre* and *Pergularia daemia* on lipid profile in letrozole-induced polycystic ovarian syndrome in rats.

Parameters (mg/dL)	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ	Group Ⅵ
HDL	34.33±4.68	15.67±4.41^{a**}	24.00±4.34^{a*b}	28.50±7.34^{b**}	27.67±7.31^{b**}	31.17±5.57^{b**c}
LDL	90.67±8.94	148.17±25.47^{a**}	112.17±17.11^{ab**}	98.50±7.74^{b**}	101.17±10.25^{b**d}	83.17±6.52^{b**c*}
VLDL	46.67±10.39	90.33±9.31^{a**}	53.50±12.05^{b**}	54.67±9.16^{b**}	62.50±9.55$^{a**\ b**d*}$	44.50±7.45^{b**}
Triglyceride	74.86±9.97	165.33±16.8^{a**}	106.00±19.87^{a**b**}	85.00±8.87^{b**c}	106.50±16.12$^{a**\ b**d*}$	75.50±7.56^{b**c*}

a, a*, a**: Compared to group Ⅰ: P<0.05, P<0.01; P<0.001, respectively; b, b**: Compared to group Ⅱ; P<0.05, P<0.001, respectively; c, c*: Compared to group Ⅲ; P<0.05, P<0.01, respectively; d, d*: Compared to group Ⅵ: P<0.05, P<0.01, respectively. HDL: high density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein.

Table 4. Effects of *Gymnema sylvestre* and *Pergularia daemia* on reproductive hormone levels in letrozole-induced polycystic ovarian syndrome in rats.

Parameters	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ	Group Ⅵ
LH (ng/mL)	2.22±0.50	8.68±0.62^{a**}	3.48±0.55^{a*b**}	3.00±0.76^{b**}	2.82±0.69^{b**}	2.62±0.60^{b**c}
FSH (ng/mL)	81.20±9.01	43.80±5.31^{a**}	75.20±7.80^{b**}	74.20±5.17^{b**}	75.40±6.39^{b**}	81.40±5.86^{b**}
Testosterone (ng/mL)	72.20±11.19	109.40±11.31^{a**}	85.80±10.87^{ab**}	79.40±6.43^{b**}	74.60±8.36^{b**}	74.20±9.55^{b**}
Estradiol (pg/mL)	31.60±7.13	68.00±5.79^{a**}	48.40±5.37^{a**b**}	41.60±3.65^{a*b**}	38.60±4.39^{b**c*}	36.00±5.61^{b**c**}
Progesterone (ng/dL)	41.20±6.91	14.40±4.51^{a**}	30.80±3.27^{a**b**}	32.80±4.66^{ab**d}	35.40±4.67^{b**}	40.20±8.08^{b**c}

a, a*, a**: Compared to group Ⅰ: P<0.05, P<0.01; P<0.001, respectively; b**: Compared to group Ⅱ: P<0.001; c, c*, c**: Compared to group Ⅲ; P<0.05, P<0.01, P<0.001, respectively; d: Compared to group Ⅵ: P<0.05. LH: luteinizing hormone; FSH: follicle-stimulating hormone.

Table 5. Effects of *Gymnema sylvestre* and *Pergularia daemia* on ovary and uterus weight in letrozole-induced polycystic ovarian syndrome in rats.

Weight of ovary and uterus (mg/100g body weight)	Group Ⅰ	Group Ⅱ	Group Ⅲ	Group Ⅳ	Group Ⅴ	Group Ⅵ
Ovary	48.80±3.96	75.00±4.47^{a**}	53.20±4.97^{b**}	51.60±5.03^{b**}	49.60±5.55^{b**}	47.80±5.89^{b**}
Uterus	117.60±7.02	113.40±7.99	115.60±7.57	114.00±7.18	117.00±5.39	117.20±8.87

a**: Compared to group Ⅰ: P<0.001; b**: Compared to group Ⅱ: P<0.001.

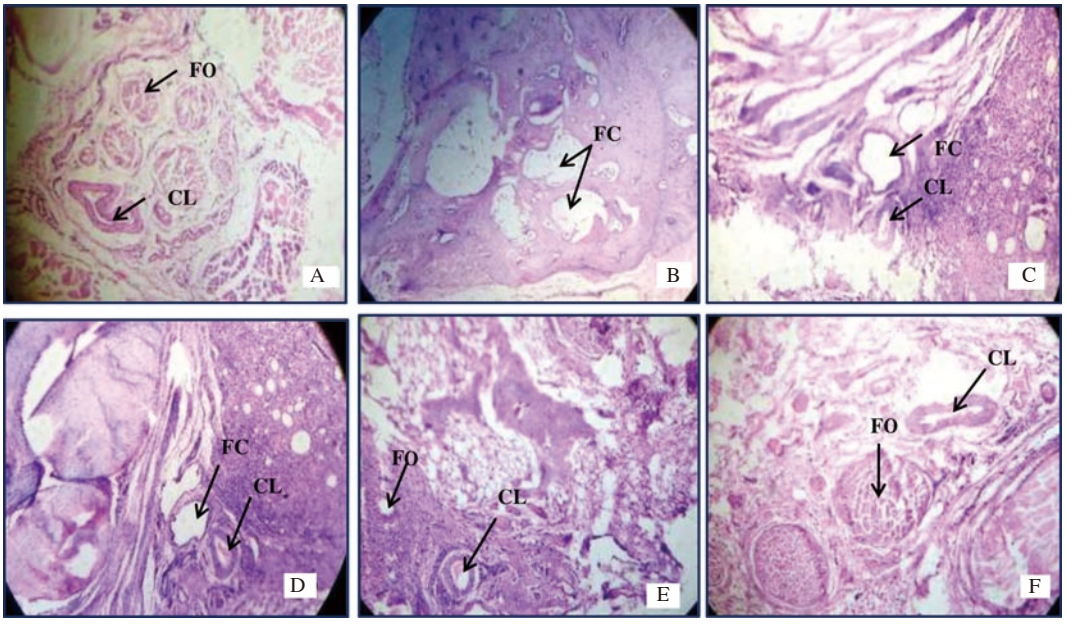

Figure 1. Microscopic images of ovary sections (hematoxylin and eosin stain; magnification: 100×). A: The normal control group shows normal histological structure like congested vascular space with spindle shaped cells in the medulla. The cortex shows few healthy primary and secondary follicles and the presented corpus luteum contains eosinophilic cytoplasm. B: The PCOS control group shows few follicles with varying sizes and the atretic follicles contain fluid filled antrum. Besides, the corpus luteum is absent. C: The metformin-treated group. D: *Gymnema sylvestre*-treated group. E: *Pergularia daemia*–treated group. F: The combination treatment group of *Gymnema sylvestre* and *Pergularia daemia*. All the treatment groups (D-F) show the minimal number of cysts, normal healthy follicles, and decreased fluid filled antrum, and also show the presence of corpus luteum. However, the combination treatment group (F) shows better histological changes than other treatment groups and its changes reach almost nearer to the normal control group. CL: corpus luteum; FO: follicles; FC: follicular cyst.

4. Discussion

Letrozole-induced PCOS model is one of the most widely used animal model, which mimics many clinical features of PCOS women[23]. The clinical manifestation of PCOS includes hyperandrogenism, anovulation, and follicular cysts associated with metabolic complications like insulin insensitivity, hyperinsulinemia, dyslipidemia, and cardiovascular diseases[24]. In this study, the PCOS was induced in rats using letrozole (1 mg/kg b.w. for 21 days) an aromatase inhibitor.

Letrozole-induced PCOS rats showed a decrease in the estrus and increase in the diestrus duration. This irregularity of the estrus cycle might be due to the letrozole-induced sex hormonal imbalance and hyperandrogenism, which is similar to those women associated with PCOS[25]. Our study results showed that the combination of *G. sylvestre* and *P. daemia* produces a better action on normalizing the menstrual irregularity than the individual treatment. This potential effect could be due to its beneficial action on regularizing the altered sex hormones in PCOS[10,13].

Reproductive hormonal imbalance is the key factor in PCOS, mainly hyperandrogenism. Administration of letrozole inhibits the conversion of androgen to estrogen, which leads to hyperandrogenism in the ovary[24]. This excess androgen feedback to the pituitary leads to increased LH and decreased FSH secretion.

Even the increased levels of estrogen also lead to the negative feedback effect on FSH secretion. The excess estrogen and reduced production of progesterone are associated with anovulation[26,27]. Similar hormonal abnormalities were produced in our study by the administration of letrozole. The treatment of *G. sylvestre* and *P. daemia* significantly normalize these hormonal changes in PCOS. Especially, the combination treatment showed significantly better action. This might be due to their antiandrogenic potential[28,29], where the increased serum androgen is reduced and normalizes the hormonal abnormality. Hence, the administration of *G. sylvestre* and *P. daemia* promotes ovulation and reduces the cysts by altering the serum estrogen and progesterone.

Impaired glucose tolerance is a major risk factor in PCOS women[30]. In our study oral glucose tolerance test results also showed that significant hyperglycaemia in both the fasting and oral glucose challenged conditions in the PCOS control group. It confirms the impaired glucose tolerance in letrozole-induced PCOS condition, which mimics the PCOS clinical condition. All the treatments significantly reduce the hyperglycemia and improve the glucose sensitivity in PCOS. Due to the synergistic action, the combination of *G. sylvestre* and *P. daemia* produces superior hypoglycaemic and insulin sensitivity improvement effect, as both the plants individually reported being possessed significant antihyperglycaemic potential. Besides, this finding is also correlated with antidiabetic constituent gymnemic acid in *G. sylvestre* and quercetin in *P. daemia*[8,17].

PCOS is closely associated with dyslipidemia due to the altered lipids and lipoprotein metabolism. It plays a crucial role in the pathophysiology of hyperandrogenism and chronic anovulation in PCOS[31]. Similar results were observed in the PCOS control group, in which the serum levels of LDL, VLDL, and TG were elevated and HDL level was decreased. The treatment groups significantly reversed the altered lipid profile caused by the letrozole-induced PCOS condition. This study results also coincide with the previous study reports of the individual hypolipidemic potential of *G. sylvestre*[32] and *P. daemia*[28]. This effect was highly significant in the combination-treated group of *G. sylvestre* and *P. daemia* as compared with other treatment groups. This might be due to the synergistic action of *G. sylvestre* and *P. daemia*. Hence, it is indicated that the combination of *G. sylvestre* and *P. daemia* has a potent effect on the metabolic complication (dyslipidemia) of PCOS.

Ovary weight was significantly increased in the PCOS control group; it might be due to hyperandrogenism induced by letrozole, which may lead to the development of ovarian cysts with hyperplasia of theca cells and thickened ovarian capsules[33]. The administration of *G. sylvestre*, *P. daemia*, and their combination significantly reduce the ovary weight, which shows the positive impact of *G. sylvestre* and *P. daemia* on ovarian cysts. This might be due to their abilities to reduce the elevated testosterone levels. However, no significant effects were observed in uterine weight.

Histopathological findings also support the results of the reproductive hormonal analysis of this study. The treatment of *G. sylvestre*, *P. daemia*, and their combination reduces the number of follicular cysts and improve the histoarchitecture of the ovary. *G. sylvestre* and *P. daemia* combination showed better action with normal healthy primary and secondary follicles, which is nearer to normal ovary morphology.

Individually, *G. sylvestre* possesses a significant anti-hyperglycemic action with diverse mechanisms. Also, it acts as an insulin sensitivity improving agent, due to the presence of secondary metabolites like gymnemic acid, gymnemagenin, and gymnestrogenin. *G. sylvestre* also significantly reduces the serum androgen level by improving the insulin sensitivity in PCOS. *P. daemia* has a potent effect on normalizing menstrual irregularities and correcting hormonal imbalances thereby reducing the development of follicular cysts in PCOS. Besides, *P. daemia* has a significant antidiabetic action. These beneficial effects of *P. daemia* on PCOS might be due to the presence of secondary metabolite pituitrin, alkaloids, triterpenes, saponins, and steroidal compounds.

This study highlighted the combined effect of *G. sylvestre* and *P. daemia* on letrozole-induced PCOS condition. Our study fails to show the statistical significant different among the extracts treatments due to the limited number of animals, but the results of our study shows that combination produce better improvement on letrozole-induced androgen up-regulation related PCOS complications, even the clinical manifestations of hyperandrogenism in PCOS relies on multiple causes. Hence, this study limits the translational studies, which needs to be evaluated on multiple PCOS model with maximum number of animals.

In conclusion, individual administration of *G. sylvestre* reduces insulin resistance and decreases androgen production, while *P. daemia* is found to have a profound beneficial effect on anovulation and menstrual irregularity. Hence, the combination of *G. sylvestre* and *P. daemia* showed potent synergistic activity against the hyperandrogenism, hyperinsulinemia, anovulation, and follicular cysts in PCOS. For further scope, clinical studies are needed to be conducted to initiate this combination of *G. sylvestre* and *P. daemia* for the better treatment and management of PCOS.

Conflict of interest statement

All authors declare that there is no conflict of interest.

Acknowledgments

The authors are acknowledged to the Chancellor, Vinayaka Mission's Research Foundation (Deemed to be University), Salem for providing necessary support for writing this article.

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