A comprehensive investigation on female Wistar rats examining the therapeutic potential of *Pueraria tuberosa* on letrozole-induced Polycystic ovarian syndrome

Ujjval P. Vaghela[a], Devang B. Sheth[b], Bhavik Jani[c], Ravi Ajudia[c], Anjana Bera[c], Pratik Vediya[c], Sejal Pandya[c], Vishvesh B. Kanabar[c], Ghanshyam Parmar[a]

[a]Department of Pharmacy, Sumandeep Vidyapeeth Deemed to be University, Vadodara, [b]Department of Pharmacology, L. M. College of Pharmacy Ahmedabad, [c]School of Pharmacy, RK University, Rajkot, Gujarat, India

Correspondence to Ujjval P. Vaghela, Assistant Professor, M. Pharm (Pharmacology), Department of Pharmacy, Sumandeep Vidyapeeth Deemed to be University, Vadodara, Gujarat 391760, India.
Tel: +91 6351715533;
E-mail: ujjvalvaghela08@gmail.com

Received: 22 March 2024
Revised: 24 April 2024
Accepted: 30 April 2024
Published: 25 July 2024

Egyptian Pharmaceutical Journal 2024, 0:0–0

Background
Polycystic ovarian syndrome (PCOS) stands out as the most prevalent endocrine disorder affecting women of reproductive age with 5–10% of women experiencing its effects. Historically known as Stein-Leventhal syndrome, its pathology involves various irregularities including heightened androgen levels, insulin resistance, diminished estrogen and progesterone levels, and irregular gonadotropin levels. However, the drugs commonly used to treat PCOS come with multiple side effects and limited efficacy in targeting the disorder's underlying pathology. Indian kudzu tubers, specifically *Pueraria tuberosa* (Willd.) DC. tubers are a valuable source of phytoestrogens such as puerarin, daidzein, biochanin-A, and formononetin. Phytoestrogens are natural compounds derived from plants that stimulate estrogenic activity through estrogen receptors (ERα and ERβ), thereby increasing estrogen levels. Hence, the objective of this research was to assess the impact of the ethanolic extract obtained from *Pueraria tuberosa* (Willd.) DC. on the ovarian steroidogenesis pathway in a rat model with induced PCOS using letrozole.

Objective
To determine the hormonal parameters i.e. estrogen, testosterone, progesterone, follicle-stimulating hormone and luteinizing hormone level, as well as to evaluate body weight, ovarian weight, and histopathology of the ovary in female rats, we investigated a comprehensive investigation on female Wistar rats examining the therapeutic potential of *Pueraria tuberosa* on letrozole-induced PCOS.

Materials and methods
For this aim, animals were divided into six groups (n=6). Control group, untreated letrozole-induced PCOS group (1 mg/kg bwt) for 21 days, PCOS group treated with tuber extract of *Pueraria tuberosa* (Willd.) DC (100 200 and 400 mg/kg bwt) for 14 days, and PCOS group treated with clomiphene citrate (1 mg/kg bwt) for 14 days. Finally, body and ovarian weight, and hormonal assays (estrogen, testosterone, progesterone, follicle-stimulating hormone, and luteinizing hormone levels) were conducted. Histomorphometric ovarian evaluation of cystic follicles was determined.

Results and conclusion
The ethanolic extract of *Pueraria tuberosa* (Willd.) DC. tuber exhibited a significant enhancement in both body weight and ovarian weight when compared with the PCOS-induced group. It positively influenced hormonal levels by increasing estrogen and progesterone while decreasing testosterone levels. In addition, the extract normalized the ratio of follicle-stimulating hormone and luteinizing hormone levels and assessed histomorphometric changes, leading to a reduction in cystic follicles. In summary, the ethanolic extract derived from the tuber of *Pueraria tuberosa* (Willd.) DC. demonstrates the potential to alleviate certain symptoms associated with polycystic ovary syndrome. This positive impact is attributed to its components, including puerarin, daidzein, biochanin-A, and formononetin, which exhibit estrogenic and antiandrogenic effects. Notably, in this study, doses of 200–400 mg/kg of the extract were identified as the most effective, suggesting their promise as a potential therapeutic intervention for PCOS.

Keywords:
clomiphene citrate, letrozole, polycystic ovary syndrome, Pueraria tuberosa (Willd) DC

Abbreviations:
CMC, Carboxymethyl Cellulose, ELISA, Enzyme linked Immunosorbent Assay, EPT, Ethanolic extract of Pueraria tuberosa, FSH, follicle stimulating hormone, GABA, Gamma aminobutyric Acid, GnRH, Gonadotropin releasing Hormone, H and E, Hematoxylin and Eosin, LCMS, Liquid Chromatography Mass Spectrometry, LH, Luteinizing Hormone, PCOS, Polycystic ovarian syndrome, SHBG, Sex Hormone binding Globulin, T2DM, Type 2 Diabetes Mellitus

Egypt Pharmaceut J 0:0–0

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Introduction

A complicated hormonal condition known as polycystic ovarian syndrome (PCOS) impacts 5–10% of women worldwide [1]. Those with PCOS frequently encounter prolonged or irregular menstrual cycles and heightened levels of male hormones, diminishing their chances of conceiving. Acne, greasy skin, increased body and facial hair, and hair loss are all signs of PCOS [2]. Furthermore, PCOS is associated with metabolic imbalances such as diabetes, abnormal blood lipid profiles, cardiac complications, and gynecological malignancies [3].

Studies conducted recently have demonstrated that PCOS is a serious metabolic illness., amplifying the susceptibility to both type 2 diabetes mellitus and metabolic syndrome [4]. It has been suggested that women with mild hyperandrogenism and specific ultrasonic indications of polycystic ovaries, yet maintaining normal ovulatory function, may manifest a milder form of PCOS and are at risk of developing the syndrome [5]. The primary exacerbating factors for the underlying abnormality in steroidogenesis are elevated levels of luteinizing hormone (LH) and insulin. Excessive androgen activity in PCOS disrupts the synthesis of estrogen and progesterone induced by gonadotropins in the follicles [6]. Normally, testosterone converts to estradiol and androstenedione converts to estrone, facilitated by the crucial enzyme P450 aromatase, maintaining hormonal balance in the ovaries. However, reduced activity of this enzyme leads to heightened ovarian androgen production, contributing to the development of polycystic ovary (PCO) condition [7]. Extended hyperandrogenism is associated with inefficient hypothalamic–pituitary response, elevated secretion of LH, and cessation of antral follicle progress [4]. The LH/follicle-stimulating hormone (LH/FSH) ratio is three times greater than the usual range in PCOS because androgen levels rise while estrogen and progesterone levels fall.

Pueraria tuberosa (Willd.) DC., commonly known as Indian Kudzu or Vidarikanda, belongs to the Fabaceae family and is native to India, spanning regions from Gujarat and the western Himalayas to Sikkim, Nepal, and Pakistan [8]. *P. tuberosa* contains a variety of compounds, including phytosterols (e.g., β-sitosterol, α-sitosterol, and stigmasterol), flavonoids (such as Puerarin, Daidzein, Genistein, Puerarone, Tuberosin, Quercetin, Biochanin A, Biochanin B, and Formononetin), phenolic acids, and terpenoids [9,10]. *P. Tuberosa* displays a range of biological activities, including antioxidant, nootropic, antidiabetic, hepatoprotective, anti-inflammatory, immunomodulatory, antihypertensive, neuroprotective, cardiovascular, and nephroprotective activities. With the increasing use of herbal remedies for treating various conditions, we aimed to assess the efficacy of an ethanolic extract obtained from *P. tuberosa* tubers in treating PCOS in rats brought on by letrozole.

Materials and methods

Gathering and identification of plants

Pueraria tuberosa tubers were gathered in Ayavej village, situated near Bhavnagar in Gujarat, India. The identification and verification of these tubers were conducted by the Department of Botany at Christ College in Rajkot.

Extraction of *P. tuberosa*

The Soxhlet apparatus method was used to extract the tubers, utilizing 95% ethanol. Subsequently, the extract was subjected to drying under reduced pressure with the assistance of a rotary vacuum evaporator and then stored in an airtight container. This extract was later administered to rats with induced PCOS for experimental purposes [10,11].

Plant characterization

Preliminary phytochemical screening was determined through a different chemical test like tests for alkaloids, flavonoids, phenolic compounds, sterols, and triterpenoids as per the previous studies. LCMS analysis was conducted to determine the presence and quantity of flavonoids in the ethanol extract of *P. tuberosa* [12,13].

Reagents

Analytical grade chemicals and reagents including letrozole (Letroz Tablet, manufactured by Sun Pharmaceutical Industries Ltd), clomiphene citrate (Siphene Tablet, manufacturer Serum Institute of India Ltd), carboxy methyl cellulose and Hematoxylin and Eosin stain, and ethanol are procured from a central chemical store. Serum estrogen and testosterone, progesterone, follicle-stimulating hormone, and luteinizing hormone levels were determined using an enzyme-linked immunosorbent test (ELISA) kit.

Animals and treatment

Female Albino adult Wistar rats, weighing an average of 200 g when they were 6–8 weeks old, were produced and kept in the animal housing facility. All rats were housed in well-ventilated conditions at a controlled temperature (22°C), with humidity maintained between 45 and 65%, and subjected to a 12-hour light–dark cycle. Every week, their weights were noted. Approved by the Institutional Animal Ethics Committee (IAEC), the experimental plan adhered to the regulations established by the Ministry of Social Justice and Empowerment, Government of India's Committee for Control and Supervision of Experiments on Animals (CCSEA). (Number of protocol: BKMGPC/IAEC22/RP52/2018)

The experimental model was structured as follows: Each rat was given a daily vaginal swab, and the study only included the rats that showed two consecutive regular estrous cycles. Subsequently, the rats were divided into six groups at random (n=6). As the control group, the first one received 1 ml of 0.5% CMC (vehicle). In the remaining groups, letrozole was given once daily for 21 days at a dose of 1 mg/kg p.o., dissolved in 0.5% CMC (see Table 1). Earlier research that produced cystic follicle formation led to the selection of this model [14]. Vaginal smears were taken every day to verify the PCOS induction. One rat per group was killed after receiving letrozole for 21 days, and tests (biochemical and histological) were performed to confirm that the rats had PCOS. Beginning on day 22, the fourth through fifth groups received oral doses of *P. tuberosa* ethanolic extract at 100, 200, and 400 mg/kg (*P. tuberosa* groups), the third group received treatment with Clomiphene citrate at 1 mg/kg (Clomiphene citrate group), and the second group received distilled water through gavage (PCOS group) [15]. For 15 days, the test materials were administered. After the treatment session, ketamine/xylazine (5/1 mg/kg) was used to anesthetize every rodent. All rats had blood drawn from the retroorbital vein, and serum was separated using centrifugation for 10 min at 3000 rpm. Table 1 Design of experimentation

Table 1 Design of experimentation

Animal Clades	Treatments
Group I	0.5% carboxymethyl cellulose (CMC)
Group II	1.0 mg/kg b.w. of letrozole dissolved in 0.5% carboxymethyl cellulose for 21 days (P.O.)
Group III	Rats with PCOS produced by letrozole were given clomiphene citrate (1.0 mg/kg b.w.) for 15 days.
Group IV	For 15 days, rats with PCOS caused by letrozole were given 100 mg/kg of *P. tuberosa* tuber extract (P.O.).
Group V	For 15 days, rats with PCOS caused by letrozole were given 200 mg/kg of *P. tuberosa* tuber extract (P.O.).
Group VI	For 15 days, rats with PCOS caused by letrozole were given 400 mg/kg of *P. tuberosa* tuber extract (P.O.).

Body weight and hormonal assay

After obtaining serum samples from the female rats, the levels of follicle-stimulating hormone and luteinizing hormone, estrogen, progesterone, and androgen were determined using an ELISA kit.

Histopathological studies

Soon after the last blood sample, the rats' ovaries were removed. Each ovary was weighed and subsequently preserved in 10% neutral-buffered formalin. Hematoxylin and eosin staining of the ovaries was done according to standard histological protocols.

Analytical statistics

Presenting the data as mean±SEM was the intended format. A Tukey-Kramer post hoc test was to be performed using the computer-based fitting program (Graph Pad Prism 8.0) after a one-way analysis of variance (ANOVA) was used for statistical analysis to find significant differences. To determine statistical significance, a significance level of P less than 0.05 was established.

Results and discussion

% yield of extract

Through the use of an extraction device and ethanol, *Pueraria tuberosa* powder was extracted dry and powdered for 12 h. The extracted material had a deep brown hue. The percentage yield of ethanol extract of 450 gm of powder tubers of *Pueraria tuberosa* was found to be 4%w/w.

Screening of preliminary phytoconstituents

The presence of several phytoconstituents, including flavonoids, phytosterol, carbohydrates, alkaloid, and phenolic compounds, was discovered during the

preliminary phytochemical screening of the ethanolic extracts of *Pueraria tuberosa* (EPT) (Willd.) DC. tubers. Sterols, alkaloids, phenolic compounds, and flavonoids were detected by EPT. Previous scientific papers are validated and confirmed by the phytochemical analysis outcomes.

Analysis of phytoconstituents of *P. tuberosa* using LC-MS

Using LC-MS, the impact of *Pueraria tuberosa* (Willd.) DC. ethanolic extract in the presence of phytoestrogen (Puerarin, Daidzein, and Biochanin A) was examined. The qualitative results confirmed that ethanolic extract of *Pueraria tuberosa* (Willd.) DC. tubers contain phytoestrogens such as Puerarin, Daidzein, and Biochanin A (Figs 1 and 2).

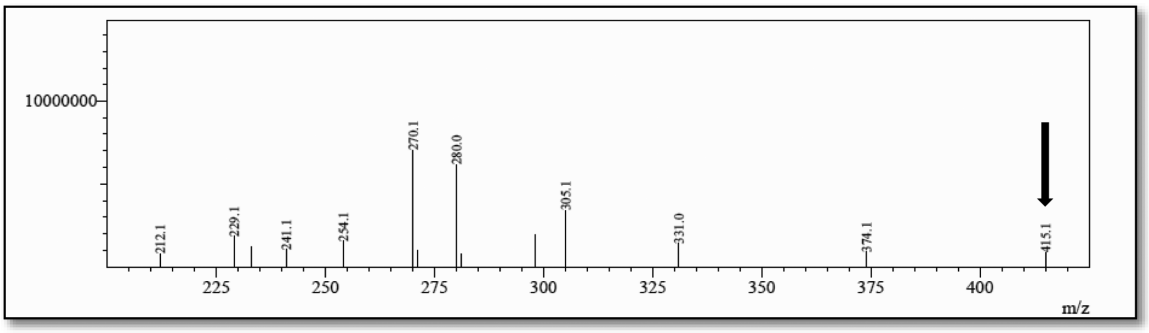
Effect of ethanolic extracts of *Pueraria tuberosa* impact on the body weight of female rats

Administration of letrozole 1 mg/kg, p.o., 21 days significantly increased weight when compared with the weight of 0 days to 36 days. The disease control group of animals also showed increased weight (35.00 ±3.41$^{\#}$) and was found to be statistically significant compared with the normal control group (15.83±1.53). (Fig. 3) Treatment with clomiphene citrate (1 mg/kg, p.o., 15 days) in letrozole-treated rats significantly decreased weight (15.83±2.71*) as compared with the disease control group of weight of animals on day 36 day (35.00±3.41$^{\#}$). Treatment groups (100 mg/kg, p.o., 15 days), (200 mg/kg, p.o., 15 days) and higher (400 mg/kg, p.o., 15 day) of EPT led to a decrease in body weight (16.66±2.47*), (15.00±2.23*), and (15.83±3.00*) respectively as compared with the disease control

Effect of ethanolic extracts of *Pueraria tuberosa* on serum estrogen, testosterone, and progesterone levels of female rats

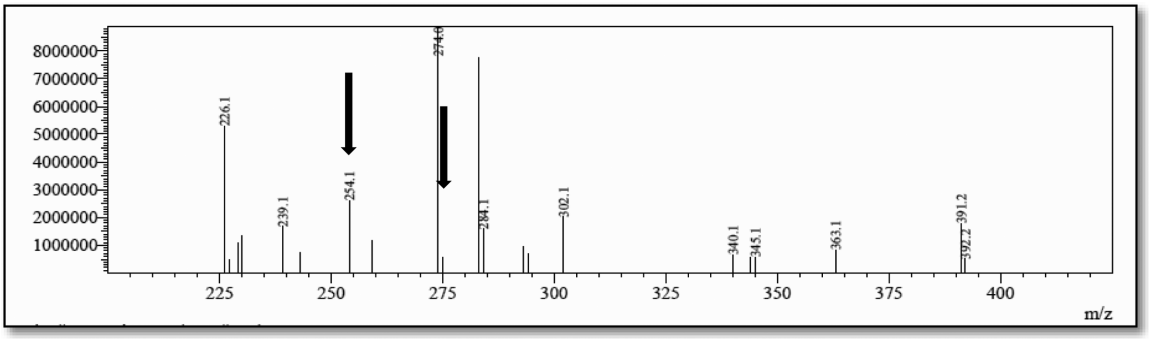
Letrozole (1 mg/kg, p.o.) was given intraperitoneally for 21 days. The serum estrogen levels in albino Wistar female rats were substantially lower (70.72±6.76# pg/ml) than in the animals in the normal control group (110.09±10.64 pg/ml). (P<0.05). (Fig. 4) Treatment with standard clomiphene citrate resulted in a significant increase in serum estrogen levels (136.50 ±12.24* pg/ml) compared with the disease group (70.72±6.76 pg/ml). In the treatment groups with EPT (administered at 100 mg/kg, p.o., for 15 days), (200 mg/kg, p.o., for 15 days), and (400 mg/kg, p.o.,

Figure 1



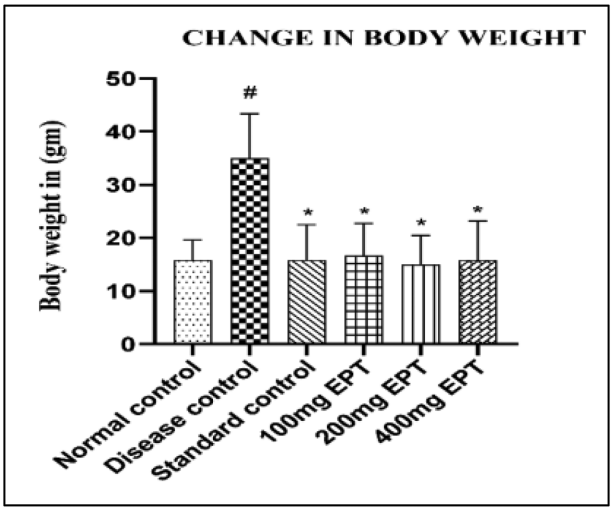
Negative ion mass spectra show major ion product at m/z (415.1 for Puerarin).

Figure 2



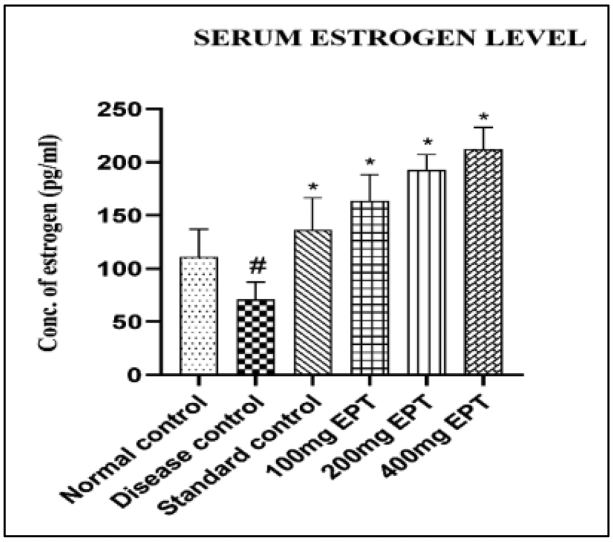
Negative ion mass spectra show major ion product at m/z (254.1 for Daidzein, and 284.1 for Biochanin A).

Figure 3



Effect of ethanolic extract of *Pueraria tuberosa* impact on the body weight of female rats (Group II) showed a significant increased as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group –III), and *P. tuberosa* tuber extract treated (Group –IV, V, VI) was significantly decreased as compared to (Group –II) body weight of female rats. (P <0.05) Mean and SEM are used to represent all values (n=6). * significantly different from disease control, # significantly different from normal control.

Figure 4



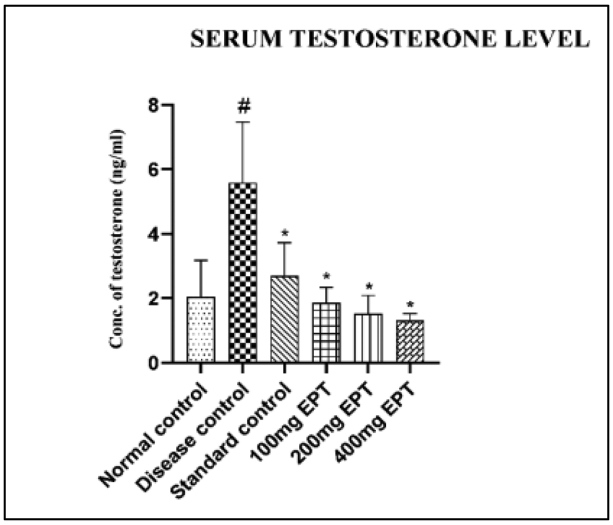
Effect of ethanolic extract of *Pueraria tuberosa* on serum estrogen level of female rats (Group II) showed a significant decrease as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group –III), and *P. tuberosa* tuber extract treated (Group IV, V, VI) was significantly increased as compared with Group II) body weight of female rats (P <0.05). Mean and SEM are used to represent all values (n=6). * significantly different from disease control, # significantly different from normal control.

for 15 days), there was a significant increase in serum estrogen levels (163.60±10.04* pg/ml), (192.60 ±5.86* pg/ml), and (212.30±8.27* pg/ml), respectively (P<0.05), in a dose-dependent manner.

Regarding testosterone levels, the serum testosterone level in the disease group of female rats (5.58±0.76# ng/ml) was significantly higher compared with the normal control group of animals (2.05±0.45* ng/ml) (P<0.05). (Fig. 5) Serum testosterone levels were significantly lower (2.69±0.41* ng/ml) following treatment with conventional clomiphene citrate (1 mg/kg, p.o., for 15 days) than in the disease control group (5.58 ±0.76 ng/ml) (P<0.05). The treatment groups administered EPT at doses of 100 mg/kg, p.o., for 15 days; 200 mg/kg, p.o., for 15 days; and 400 mg/kg, p.o., for 15 days exhibited a significant decrease in serum testosterone levels (1.86±0.19* ng/ml), (1.52 ±0.23* ng/ml), and (1.31±0.08* ng/ml), respectively (P<0.05).

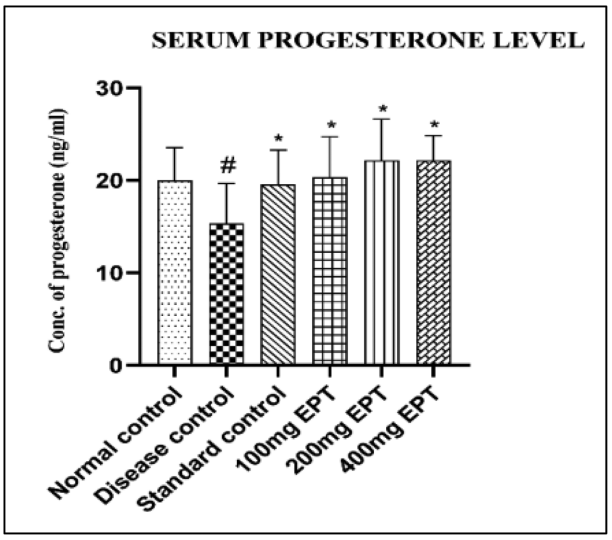
In terms of progesterone levels, there was a reduction in the serum progesterone level of female rats in the disease group (15.37±1.75# ng/ml) compared with the normal control group of animals (20.02±1.44 ng/ml) (Fig. 6). In the treatment with standard clomiphene citrate, there were no significant changes in serum progesterone level (19.56±1.52* ng/ml) compared with the disease control group of animals. The treatment groups administered EPT at doses of 100 mg/kg, p.o., for 15 days; 200 mg/kg, p.o., for 15 days; and 400 mg/kg, p.o., for 15 days showed a slight increase in serum progesterone levels: (20.37±1.77* ng/

Figure 5



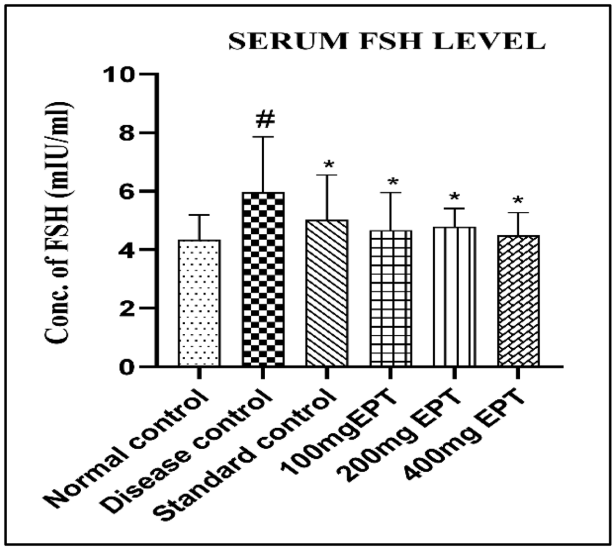
Effect of ethanolic extract of *Pueraria tuberosa* on serum testosterone level of female rats (Group II) showed a significant increase as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group III) and *P. tuberosa* tuber extract treated (Group IV, V, VI) was significantly decreased as compared with Group –II body weight of female rats. (P <0.05). Mean and SEM are used to represent all values (n=6). * significantly different from disease control, # significantly different from normal control.

Figure 6



Effect of ethanolic extract of *Pueraria tuberosa* on serum progesterone level of female rats (Group – II) showed a significant decrease as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group –III) and *P. tuberosa* tuber extract treated (Group –IV, V, VI) was significantly increased as compared with Group II body weight of female rats. (P <0.05) Mean and SEM are used to represent all values (n=6). *-significantly different from disease control and # -significantly different from normal control.

Figure 7



Effect of ethanolic extract of *Pueraria tuberosa* on serum FSH level of female rats (Group II) showed a significant increase as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group III) and *P. tuberosa* tuber extract treated (Group IV, V, VI) was significantly decreased as compared with Group II body weight of female rats. (P <0.05) Mean and SEM are used to represent all values (n=6). *significantly different from disease control, # significantly different from normal control.

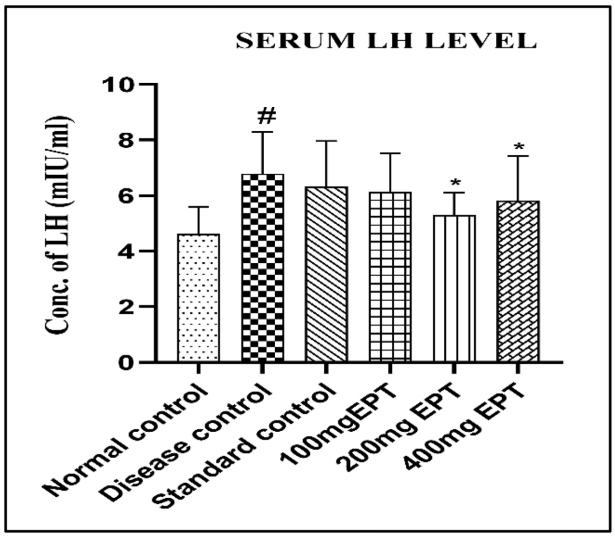
ml), (22.18±1.80* ng/ml), and (22.20±1.05* ng/ml), respectively.

Effect of ethanolic extracts of *Pueraria tuberosa* on serum follicle-stimulating hormone and luteinizing hormone level

Regarding FSH levels, the illness group's female rats had a higher serum FSH level (5.96±0.77# mIU/ml) than the animals in the normal control group (4.34 ±0.34 mIU/ml) (P<0.05). (Fig. 7) After treatment with standard clomiphene citrate (1 mg/kg, p.o., for 15 days), there was a slight decrease in serum FSH level (5.02±0.62* mIU/ml) compared with the disease group. Similarly, oral administration of treatment groups with EPT at doses of 100 mg/kg, p.o., for 15 days; 200 mg/kg, p.o., for 15 days; and 400 mg/kg, p.o., for 15 days, resulted in a decrease in serum FSH levels (4.67±0.52* mIU/ml), (4.78±0.25* mIU/ml), and (4.49±0.31* mIU/ml) (P<0.05), respectively

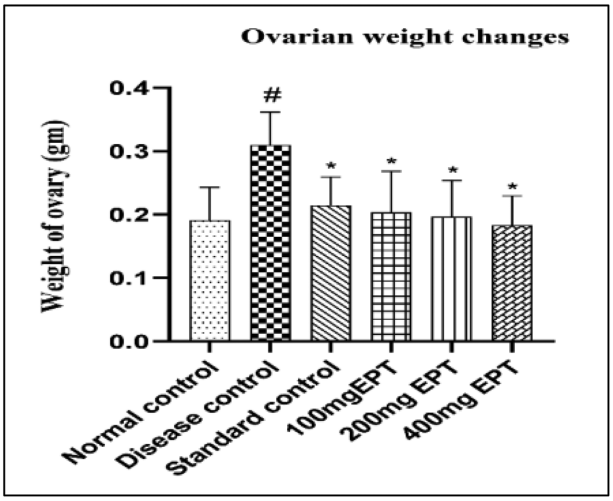
To LH levels, there was an elevation in serum LH level among female rats in the disease group (7.15±0.53# mIU/ml) compared with the normal control group of animals (4.62±0.39 mIU/ml) (P<0.05). (Fig. 8) After the treatment with standard clomiphene citrate (1 mg/kg, p.o., for 15 days), there was a reduction in serum LH level (6.32±0.67* mIU/ml) compared with the disease control group. The treatment groups receiving EPT at doses of 100 mg/kg, p.o., for 15 days; 200 mg/kg, p.o., for 15 days; and 400 mg/kg, p.o. exhibited a decrease in serum LH levels of (6.14 ±0.56* mIU/ml), (5.30±0.32* mIU/ml), and (5.81 ±0.65* mIU/ml) (P<0.05), respectively.

Figure 8



Effect of ethanolic extract of *Pueraria tuberosa* on serum LH level of female rats (Group – II) showed a significant increase as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group III) and *P. tuberosa* tuber extract treated (Group IV, V, VI) was significantly decreased as compared with Group II body weight of female rats (P <0.05). Mean and SEM are used to represent all values (n=6). *significantly different from disease control, # significantly different from normal control.

Figure 9



Effect of ethanolic extract of *Pueraria tuberosa* on ovarian weight of female rats (Group II) showed a significant increase as compared with Group I, whereas that treated with clomiphene citrate 1.0 mg/kg (Group III) and *P. tuberosa* tuber extract treated (Group IV, V, VI) was significantly decreased as compared with Group II) body weight of female rats ($P < 0.05$). Mean and SEM are used to represent all values (n=6). * significantly different from disease control, # significantly different from normal control.

Effect of ethanolic extracts of *Pueraria tuberosa* on ovarian weight of female rats

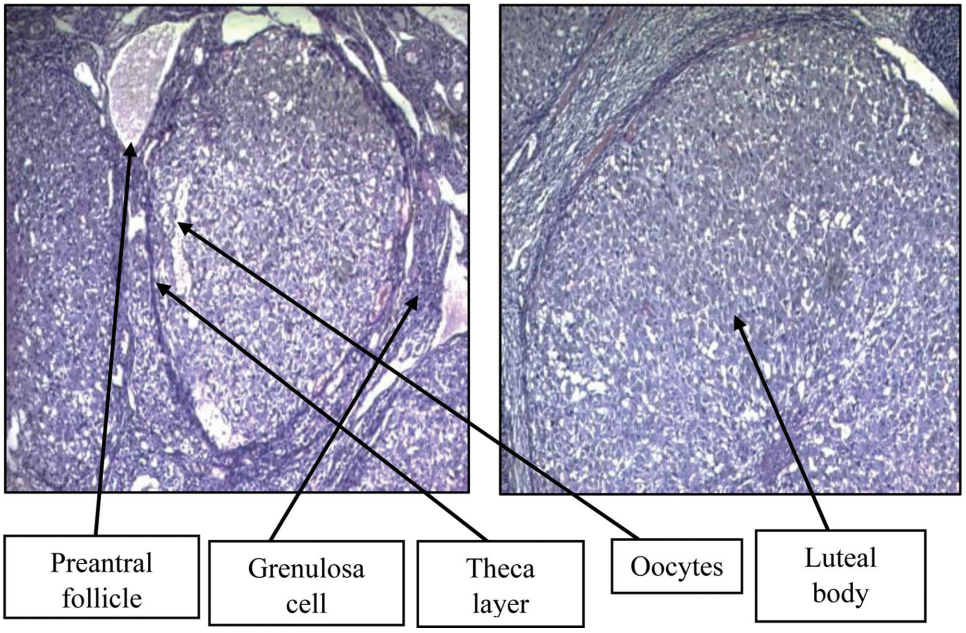
Following letrozole medication, there was a statistically significant rise in ovarian weight from the normal control group (0.19±0.02) mg to the illness group (0.31±0.02#) mg ($P<0.05$). (Fig. 9) After the treatment of clomiphene citrate (1 mg/kg, p.o., 15 days), there was a decrease in ovarian weight in the standard group of animals (0.21±0.01*) mg as compared with the disease control. The ovarian weight of the treatment groups (100 mg/kg, p.o., 15 days), (200 mg/kg, p.o., 15 days), and (400 mg/kg, p.o., 15 days) significantly decreased (0.20±0.02*) mg and (0.19±0.02*) mg and (0.18±0.01*) ($P<0.05$), respectively.

Ovarian histology of polycystic ovarian syndrome-induced female rats

Histopathology of letrozole-induced PCOS ovaries exhibited many similarities to human polycystic ovaries. Many subcapsular cysts were found in Letrozole-induced disease control group. Destruction of granulosa cells and hyperplasia of theca interna were found in PCOS-induced groups (Fig. 10) as compared with the normal control group (Fig. 11) that had antral follicles with oocytes surrounded by granulose cells and theca cell layer and also the presence of corpus luteum [15–17] and the standard clomiphene citrate group showed the presence of developing atretic follicles/ antral follicles and corpora lutea (Fig. 12). Treatment with EPT groups 100mg/kg, 200 mg/kg, and 400 mg/kg (Fig. 13) showed the presence of developing preantral follicles, granulosa cell, theca layer, atretic follicles/antral follicles, and corpora lutea.

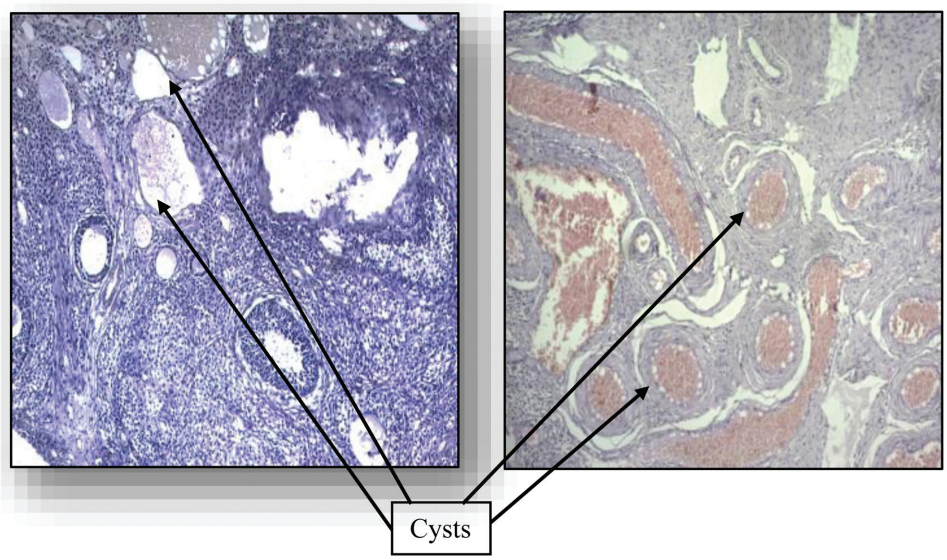
A frequent metabolic illness affecting women in their reproductive years, PCOS has an impact on every element of a person's life—physiological, social, and

Figure 10



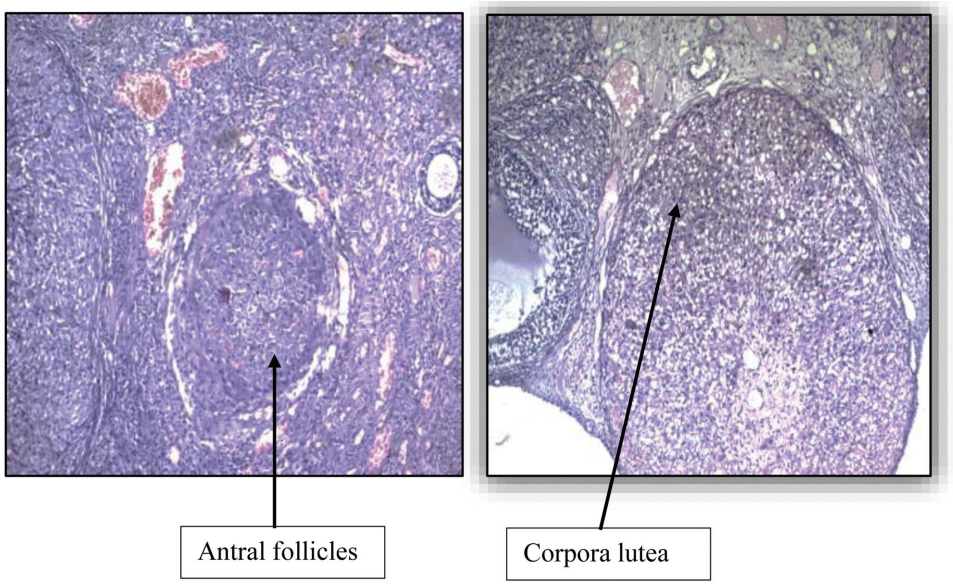
Cross section of rat ovary in the Polycystic ovarian syndrome group II (letrozole 1.0 mg/kg p.o., 21day) (x 100, H and E). Cystic follicles are visibly delayed in the ovarian sections.

Figure 11



Cross section of the rat ovary in the control group I (x 100, H and E), preantral follicle, Granulosa cell, theca layer, oocytes, corpora lutea.

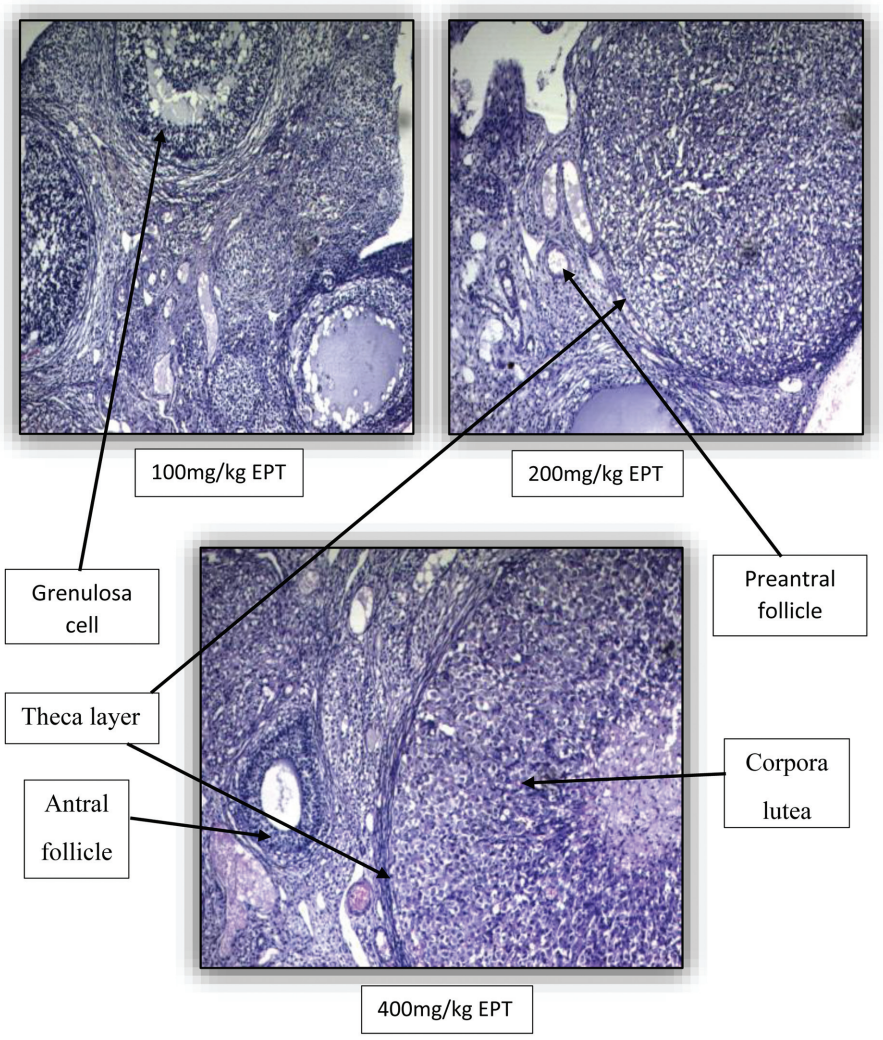
Figure 12



Cross section of rat ovary in the standard clomiphene citrate group (1 mg/kg, p.o. 15 day) (x 100, H and E), showing the presence of developing atretic follicles / antral follicles and corpora lutea

economic. This illness lacks specific diagnostic criteria due to its varied nature [18,19]. However, to verify (1) hyperandrogenism and (2) polycystic ovary, these two symptoms are necessary. As a result, ovarian biopsies and biochemical markers are typically used to diagnose PCOS. Because of this, in the current investigation, we consider both histological and biochemical evaluations. Gonadotropins (FSH and LH), ovarian histology, and blood sexual hormone (estrogen) were our two primary areas of interest. One of the signs of PCOS that is seen in the rats in this study is obesity. The rise in body weight observed in the letrozole-induced PCOS rat could potentially be attributed to alterations in hormone levels, such as increased lipid levels, insulin resistance, decreased estrogen and progesterone, and raised testosterone [20]. Insulin plays a direct and indirect role in the genesis of androgen excess in PCOS. Despite peripheral insulin resistance in PCOS-affected women, ovarian steroidogenesis seems to be insulin-responsive. When women through PCOS produce more hepatic sex hormone-binding globulin (SHBG), insulin has an inhibitory

Figure 13



Cross section of rat ovary in the treatment group of 100, 200, and 400 mg/kg EPT (p.o., 15day) (x 100, H and E), showing the presence of developing preantral follicle, grenulosa cell, theca layer, atretic follicles / antral follicles, and corpora lutea.

impact that increases hyperandrogenism [21]. Disease progression and insulin receptor polymorphism are closely related. PCOS symptoms, including obesity, are linked to PPARγ polymorphism, which raises fasting insulin levels. (Fig. 3) Treatment groups (100 mg/kg, 200 mg/kg, and 400 mg/kg) of EPT extract could prevent weight gain in rats.

The decrease in serum level of estrogen in letrozole-treated animals can be explained by the inhibition of the aromatase enzyme. With the help of the enzyme P450 aromatase, androgens are converted in granulosa cells to estrogens, which are crucial for female fertility and healthy ovarian and uterine function [22,23]. By competitively and reversibly attaching to the heme of its cytochrome P450 unit, letrozole prevents the generation of estrogens in this manner. The administration of 100 mg/kg, 200 mg/kg, and 400 mg/kg of EPT resulted in a noteworthy rise in blood estrogen levels. (Fig. 4) Estrogen level was increased which is due to the presence of phytoestrogen present in the Pueraria tuberosa extract. This phytoestrogen-like (puerarin, daidzein, and biochanin A) estrogen receptor (ERα and ERβ) possesses significant oestrogenic activity.

The increase in serum level of testosterone in the letrozole-induced group was a result of a deficiency of activity of aromatase enzyme, which ultimately leads to impaired steroidogenesis [24]. Aromatase enzyme catalyzes the biosynthesis of estrogen from androgens. Thus, blocking this enzyme will increase androgen (testosterone) levels.

Increased levels of androgen are also a major feature of pathological changes in PCOS. This increase primarily disturbs follicular development and thus oligo or anovulation may result. In addition,

hyperandrogenism may represent acne and hirsutism. Repetitive administration of EPT (100 mg/kg, 200 mg/kg, and 400 mg/kg) led to a significant decrease in testosterone levels in a dose-dependent manner. (Fig. 5) This finding is a result of the presence of phytoestrogen in the *P. tuberosa* extract.

The increase in progesterone levels may be due to the presence of a prolonged luteal phase in animals. It is well established that the corpus luteum secretes progesterone. This finding suggests treatment with the EPT (100 mg/kg, 200 mg/kg, and 400 mg/kg) drug can produce the normal estrous cycle of PCOS-induced rats. (Fig. 6).

The increase in the concentration of serum FSH level may be due to the negative feedback mechanism of estrogen. Various distinct morphological changes in the uterus and vagina are brought on by an increase in estrogen. Along with directly preventing the pituitary's production of both LH and FSH, this increase in estrogen also inhibits the hypothalamus's release of LHRH. (Fig. 7) So, this decrease in serum FSH level after treatment with EPT (100 mg/kg), (200 mg/kg), and (400 mg/kg) might be due to the negative feedback mechanism of estrogen.

The increase in serum level of LH in the letrozole-induced group was a result of a deficiency of activity of aromatase, which is responsible for impaired steroidogenesis because of the aromatase enzyme catalyzed biosynthesis of estrogen from androgens increasing androgen (testosterone) and a decrease in estrogen level and enhanced LH releasing on negative feedback of progesterone. The 15 days of administration of EPT led to a significant decrease in LH level [22,24]. This finding is a result of the estrogenic activity of the *P. tuberosa* extract because of the presence of phytoestrogens, which increase the serum concentration of estrogen and decrease the LH secretion and EPT (100 mg/kg, 200 mg/kg, and 400 mg/kg) treatment. It was found that there was a decrease in serum LH level as the result of the following cycle (Fig. 8). The cycle starts with an increase in LH hormone, which causes the formation of the corpus luteum, and then the prolongation of the corpus luteum causes the secretion of progesterone. Now the increase in progesterone causes negative feedback on the LH hormone, which ultimately decreases the serum LH concentration

The increase in ovarian weight in letrozole-treated animals may be a result of the increase in androgen (testosterone) level and production of immature cysts in the place of mature follicles. Increased size and weight may be due to hypertrophy of the theca interna, which is due to high levels of androgen in letrozole-treated animals. (Fig. 9) Treatment with EPT (100 mg/kg, 200 mg/kg, and 400 mg/kg) was also found to decrease substantial weight gain in ovaries produced by letrozole and normalize the weight of ovaries in a dose-dependent manner.

The LH/FSH ratio is nearly 1 : 1 under healthy circumstances, but it might be twice or three times greater (2 : 1 or 3 : 1) in PCOS women. Our experiment's outcomes also showed that PCOS rats had a 2.5-fold higher LH/FSH ratio than the control group. Comparing the letrozole-induced PCOS group's ovarian sections to the control group, the study showed a statistically significant rise in the number of cystic follicles. (Figs. 10 and 11) Destruction of granulosa cells and hyperplasia of theca interna were found in PCOS-induced groups as compared with the normal control group that had antral follicles with oocytes surrounded by granulose cells and theca cell layer and also the presence of corpus luteum [16]. Our study's data showed that the PCOS group had significantly thicker theca and granulosa layers, respectively, with a substantial drop in respectively. In contrast to the PCOS group, the standard control group and EPT extract significantly increased estrogen and decreased FSH and LH, resulting in a near 1:1 (6.48/5.59) LH/FSH ratio at extract doses (100, 200, and 400 mg/kg). (Figs 12 and 13) As a result, there were substantially more follicles in the various phases and corpora lutea. These results concur with those of another helpful herbal extract used to treat PCOS.

One such potential herb is Indian kudzu tubers *Pueraria tuberosa* (Willd.) DC. tubers are rich sources of phytoestrogens such as puerarin, daidzein, biochanin-A, and formononetin. Phytoestrogen is a plant-derived compound that promotes estrogenic action through estrogen receptors (ERα and ER) [25].

Flavonoids can bind to the GABA (gamma-aminobutyric acid) receptor's benzodiazepine region and function as GABA receptor agonists [26,27]. Numerous studies indicate that changes in LH surge are related to GABAergic control of gonadotropin-releasing hormone (GnRH) neurons. It has been observed that the LH surge is caused by GABA's inhibitory impact on GnRH neurons. This may explain why the PCOS groups that receive high doses of *Pueraria tuberosa* (Willd.) DC. tubers

extract saw a drop in the LH/FSH ratio. Phytoestrogen is found to show beneficial effects in increasing estrogen levels.

An oral nonsteroidal aromatase inhibitor is letrozole. P450 aromatase, an enzyme expressed in the placenta, ovary, and testis as well as in a wide range of human tissues, converted testosterone and androstenedione into estradiol and estrone, respectively. A decrease in this enzyme's activity could be expected to lead to increased ovarian androgen production and the development of PCOS [17]. Through competitive, reversible binding to its cytochrome P450 unit's heme, letrozole inhibits the production of estrogens in this manner. Letrozole has a particular mechanism of action and does not affect corticosteroid or mineralocorticoid activity. Letrozole had dose-dependent effects on blood levels of progesterone and estradiol, but it also increased the levels of testosterone and LH. Therefore, letrozole use for an extended time raises testosterone and causes diseases similar to PCOS. The increase in estrogen and antiandrogenic effects associated with the extract therapy of Pueraria tuberosa (Willd.) DC. tubers and its positive impact on PCOS, as observed in our studies, seems to be caused by the flavonoid content of the plant.

Conclusion

The findings of this investigation suggest that the ethanolic extract of *Pueraria tuberosa* (Willd.) DC. tubers is a useful treatment for female Albino Wistar rats with letrozole-induced polycystic ovarian syndrome including the restoration of ovulation. Ethanolic extract of *Pueraria tuberosa* demonstrates a variety of pharmacological actions, including antiandrogenic, estrogenic, and normalizing the follicle-stimulating hormone and luteinizing hormone ratio, which is associated with its advantageous role in polycystic ovarian syndrome. All of these modifications prevented ovarian cell malfunction and restored the estrus cycle to normal. The phytoestrogens (Puerarin, Diadzein, and Biochanin A) have a beneficial role in the pharmacological effect. This research implies that ethanolic extract of *Pueraria tuberosa* may be useful in addressing the associated pathological problems and therapy for better management of polycystic ovarian syndrome.

Acknowledgments

This study was performed in the Laboratory of Pharmacology at B. K. Mody Government Pharmcy College, Rajkot, Gujarat, India.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or nonprofit sectors.

Authors' contributions: UV and DS conceived and designed the experiments, performed hormonal profiles and analyzed the data, provided reagents/materials/analysis tools, and wrote the paper. BJ, RA, AB, PV, SP, VK, and GP performed the histomorphometric analysis and provided the reagents/materials/analysis tools required. All authors read and approved the final manuscript.

Ethical considerations: Approved by the Institutional Animal Ethics Committee (IAEC), the experimental plan adhered to the regulations established by the Ministry of Social Justice and Empowerment, Government of India's Committee for Control and Supervision of Experiments on Animals (CCSEA). (Number of protocol: BKMGPC/IAEC22/RP52/2018)

Financial support and sponsorship

Nil.

Conflicts of interest

The authors state that they have no conflicts of interest.

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