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Research article

Therapeutic exploration of polyherbal formulation against letrozole induced PCOS rats: A mechanistic approach

Arul Balasubramanian [a], Sudhakar Pachiappan [b], Surendiran Mohan [c], Harikrishnan Adhikesavan [c], Indira Karuppasamy [d], Kothai Ramalingam [a,*]

[a] *Department of Pharmacology, Vinayaka Mission's College of Pharmacy,Vinayaka Mission's Research Foundation (Deemed to be University), Salem, 636308, Tamil Nadu, India*
[b] *Department of Pharmacology, Swamy Vivekanandha College of Pharmacy, Elayampalayam, Tiruchengode, Tamil Nadu, India*
[c] *Department of Chemistry, School of Arts and Science, AVIT Campus, Vinayaka Mission's Research Foundation (Deemed to be University), Chennai, India*
[d] *Emerging Materials for Energy and Environmental Applications Research Group, School of Engineering and Technology, Van Lang University, Ho Chi Minh City, Viet Nam*

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ABSTRACT

Objective: This study aimed to develop an effective alternative medicine with multi potential herbs against polycystic ovarian syndrome (PCOS) in rats induced by letrozole treatment.
Materials and method: Polyherbal syrup was prepared with a combination of *S. asoca* bark, *G. sylvestre* leaves, *P. daemia* aerial parts, *C. zeylanium* stem bark, *C. bonduc* seeds, and *W. somnifera* roots ethanolic extract. *In vitro* cell viability study, adenosine monophosphate-activated protein kinase (AMPK), and glucose transporter 4 (GLUT4) gene expression assay were carried out on the Chinese Hamster Ovarian (CHO) cell line. For the PCOS induction letrozole (1 mg/kg *p. o.*) was given for 21 consecutive days. The PCOS induction was confirmed by measuring estrus irregularity, insulin resistance by oral glucose tolerance test (OGTT), and hyperandrogenism by measuring serum total testosterone level 21 days after completion of letrozole treatment. After induction of PCOS, metformin (155 mg/kg *p. o.*), and polyherbal syrup (100 mg/kg, 200 mg/kg, and 400 mg/kg *p. o.*) were administered for further 28 days. The treatment efficacy was measured by measuring serum lipid profile, fasting insulin level, sex hormones level, ovarian steroidogenic enzymes, ovarian tissue insulin receptor, AMPK, and GLUT4 protein expression levels, and histomorphological studies. The post-treatment effect was confirmed by reproductive performance studies.
Results: Letrozole-induced PCOS rats showed significant estrus irregularity, abnormal sex hormones levels, and hyperandrogenism indicated by showing increased free androgenic index and decreased sex hormones binding globulin (SHBG) level. The insulin resistance in PCOS rats was indicated by increased fasting glucose levels with impaired glucose clearance in the OGT test. Homeostasis Model Assessment Index of Insulin Resistance (HOMA-IR) increased level, also decreases INSR, GLUT4, and AMPK mRNA expression in ovarian cells confirming the insulin resistance in PCOS rats. Ovarian histology in PCOS rats also showed many follicular cysts, atretic follicles, and the absence of corpus luteum. The administration of polyherbal syrup, in a dose-dependent manner, effectively restored these alterations. The treatment of polyherbal formulation 400 mg/kg possesses highly significant efficacy over the treatment of metformin in PCOS

* Corresponding author.
E-mail address: kothair@vmpha.edu.in (K. Ramalingam).

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rats. It mainly acts by reducing peripheral and ovarian hyperandrogenism and improves insulin sensitivity via activating the insulin receptor and AMP-activated kinase-mediated transcription and translation of GLUT4 from the cytoplasm to the ovarian membrane improves glucose uptake and promotes the follicular development and ovulation. The higher fertility rate, delivery index, and survival of delivered pups confirm the broader and superior efficacy of PCOS. These beneficial actions are mainly attributable to the formulation's inclusion of the key secondary metabolites flavonoids and phytosterols. In conclusion, the prepared polyherbal syrup was found to be the safest and most effective alternative medicine for both endocrinal and metabolic complications of PCOS women.

1. Introduction

Polycystic ovarian syndrome (PCOS) is an intricate endocrinal and metabolic disorder of women at reproductive stages. In adolescent women, it is one of the main reasons for infertility and irregular menstruation, with a highly variable global prevalence from 2.2% to 26%; in India, it ranges from 3.7% to 22.5% [1]. PCOS is characterized by hyperandrogenism, hirsutism, amenorrhea, painful and irregular menstrual cycles, ovarian cysts, and anovulation [2]. The metabolic features are insulin resistance with compensatory hyperinsulinemia, obesity, dyslipidemia, inflammation, hypertension, and endothelial dysfunction resulting in an increased risk of diabetes and cardiovascular diseases [3,4]. In addition, PCOS is associated with reduced quality of life, anxiety, depression, and other mood disorders [5].

The present therapeutic approaches to PCOS depend on the patient's desired outcomes whether the treatment of menstrual irregularity to achieve pregnancy or contraception, the anti-androgenic therapy for hyperandrogenic symptoms like hirsutism, the insulin sensitizer for insulin resistance associated with PCOS, and the ovulation inducer for infertility to induce ovulation [6]. All these therapeutic approaches are short-term symptomatic approaches; consequently, the therapy of PCOS lacks long-term systemic techniques. Even though PCOS is a heterogeneous complex disorder hence multidisciplinary approaches are needed for the effective management of PCOS.

An efficient Ayurvedic strategy for treating chronic sickness is polyherbalism or polyherbal formulation, because of the synergistic effect of combining various potent herbs [7]. Polyherbal formulations have extensive varieties of advantages like wide therapeutic efficacy, high potency, and safety index. It is due to the presence of different phytoconstituents and the combination of different compactable phytoconstituents together in a formulation that produces a potent synergistic effect. Due to the affordability, availability, higher patient compliance, and higher therapeutic efficacy, the global demand for polyherbal formulations is increasing day by day [7].

Besides this study was intended to develop a polyherbal formulation with compactable herbs that possess multiple potent pharmacological actions in relevance to the polycystic ovarian syndrome. The selected plant materials in this study are *Saraca asoca*, *Gymnema sylvestre*, *Pergularia daemia*, *Cinnamomum zeylanicum*, *Caesalpinia bonduc*, and *Withania somnifera*. *Saraca asoca* was reported to possess uterine tonic, blood purifier, anti-estrogenic, and anti-menorrhagic properties [8,9], *Gymnema sylvestre was* reported to possess insulin sensitivity improving property, reduce hyperandrogenism, and hypolipidemic property [10,11], *Pergularia daemia* reported to possess uterine tonic and stimulant property, regularize estrus cycle and hormonal abnormality in PCOS and reduces obesity [12,13], *Cinnamomum zeylanicum* reported to restore ovarian morphology by improving blood circulation and tissue regeneration, and down-regulate testosterone improves insulin sensitivity, and possess hypolipidemic property [14,15], *Caesalpinia bonduc* reported as an ovulation inducer in PCOS by correcting hormonal imbalance, insulin resistance, and hyperandrogenism, also used to manage diabetic complications by improving the lipid profile [16,17], and *Withania somnifera* reported as used to improve FSH, LH level and folliculogenesis, and reduces the stress associated female sexual disorder [18,19]. Hence these selected herbs were formulated as polyherbal syrup and their efficacy was evaluated against letrozole induced PCOS model.

2. Materials and Methods

2.1. Selection of plant materials

The plants which are effective against polycystic ovarian syndrome with prominent action against anyone or more of the major pathological pathways of PCOS were selected. This selection was mainly based on traditional knowledge, Ayurvedic and Siddha practitioner instructions, a literature survey, availability, and affordability of the plant materials.

3. Collection and authentication of plant materials

The selected plant materials were collected from the different geographical locations of the Dharmapuri, Salem, and Namakkal districts in Tamil Nadu, India. These plant materials were identified and authenticated by Dr. P. Radha, Research officer (Botany), Siddha Medicinal Plants Garden (Central Council for Research in Siddha), Ministry of AYUSH, Govt. of India, Mettur Dam, Tamil Nadu, India, where the voucher specimen is preserved for further reference.

3.1. Preparation of polyherbal formulation

The selected plant materials *Saraca asoca* stem bark, *Gymnema sylvestre* leaves, *Pergularia daemia* aerial parts, *Cinnamomum zeylanicum* stem bark, *Withania somnifera* roots, and *Caesalpinia bonducella* seeds were collected and shade dried, coarsely powdered, and sieved through sieve no. 40 to get uniform powder for extraction. Each powdered plant material was individually extracted by cold maceration with 95% ethanol for 72 h at room temperature by occasional shaking. After that, a muslin towel was used to separate the filtrate from the marc and the filtrate was further filtered by Whatman no. 1 filter paper. The same procedure was performed by two consecutive times with the marc material. All three filtrates were combined and evaporated under reduced pressure and controlled temperature at 40 °C in a rotary evaporator until all the solvent was removed. The extracted plant materials extracts were uniformly mixed all together in the ratio of 4:2:1:1:1:1 (ratio based on the potency and individual clinical doses in therapeutic relation to PCOS) [8–19]and prepared polyherbal syrup with honey as syrup base. It was further stored in a clean, dried, and air-tight amber-colored plastic container.

3.2. In-vitro studies

3.2.1. Cell culture and cell viability assay

From the National Center for Cell Science (NCCS), Pune, India, the Chinese Hamster Ovary (CHO) cell line was obtained. In a CO_2 incubator at 37 °C with 5% CO_2, the CHO cell line was individually plated using 96-well plates at a density of 1×10^4 cells/well in RPMI media with 1x Antimycotic antibiotic solution and 10% fetal bovine serum (Himedia, India). The cells were treated with the polyherbal formulation in concentrations of 25, 50, 100, 250, and 500 μg/ml in serum-free medium and incubated for 24 h after being washed with 200 μl of 1 X Phosphate buffer saline (PBS). After the treatment phase, the media was aspirated from the cells. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) produced at 0.5 mg/ml in 1 X PBS was added, and the mixture was then incubated at 37 °C for 4 h in a CO_2 incubator. Following incubation, the MTT-containing media was taken out of the cells and washed with 200 μl of PBS. The created crystals were thoroughly mixed and dissolved in 100 μl of DMSO. A purple-blue color appears as the formazan dye changes. A microplate reader was used to measure the produced color intensity at 570 nm. The test was run in triplicate. The cell viability percentage was calculated using the untreated cells as control by the following formula-1 [20,21].

$$\%\ Cell\ viability = \frac{Mean\ OD_{Sample}}{Mean\ OD_{Control}} x100 \quad (1)$$

Where

OD- Optical density

3.3. GLUT4 and AMPK gene expression study

3.3.1. Cell culture and treatment

In RPMI medium (Himedia, India) supplemented with 10% fetal bovine serum (Himedia, India) and 1 X Antimycotic Antibiotic solution, the CHO cells were seeded in a 12-well culture plate. It was then allowed to incubate overnight at 37 °C under 5% CO_2. The polyherbal syrup dose that produced the least amount of cytotoxicity was applied to the CHO cells (100 μg/ml), and they were then incubated for 24 h at 37 °C in a CO_2 incubator. In order to extract RNA (ribonucleic acid), cells were collected in sterile microcentrifuge tubes, washed with phosphate buffer solution, and utilized.

3.3.2. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

TRIzol reagent (Ambion, USA) was used to extract the total RNA from CHO cells in accordance with the manufacturer's instructions. The PrimeScriptTM 1st strand cDNA (Complementary DNA) synthesis kit (Takara Bio Inc.) was used for reverse transcription in accordance with the manufacturer's instructions for the gene expression investigation. Reverse transcription (RT) reactions contained 3 μg of total RNA, 1 μl of Oligo dT primer (50 μM), 1 μl of dNTP mixture (10 mM each), 4 μl of 5X PrimeScript buffer, 0.5 μl of RNase inhibitor (40 U/μl), 1 μl of PrimeScript RTase (200 U/μl), and 20 μl of diethyl pyrocarbonate (DEPC) treated water as the final component. The reaction was stopped by incubating at 95 °C for 5 min after 60 min of incubation at 42 °C for reverse transcription. The undiluted 1 μl of cDNA was used in the RT-qPCR procedure. The SYBR® chosen master mix was used to perform RT-qPCR (Applied Biosystem, India). A Rotor-Gene Q 2PLEX HRM Real-Time PCR machine was used to conduct this RT-qPCR (Qiagen, Germany). A final volume of 25 μl was used for the qPCR reaction, which also contained 10 μmol of forward and reverses primers

Table 1
Primer sequences for quantitative PCR gene expression analysis.

Gene name	Forward primer	Reverse primer
GLUT4	AAGGTTCGGTCCCCAGACA	TGGAGCCTTAAAGGGTTGGC
AMPK	ACTACATTCTGGGGGACACG	ATGTGAGGGTGCCGGAAAAG
β-actin	CTGTGCTATGTTGCCCTGGA	GCCACAGGATTCCATACCCAG

GLUT4- Glucose transporter 4; AMPK- 5′ AMP-activated protein kinase.

(Table 1), 12.5 μl of 2 X SYBR® master mixture (Applied Biosystem, India), and cDNA samples. Enzyme activation at 50 °C for 2 min, denaturation at 95 °C for 2 min, then 40 cycles at 95 °C for 15 s and 60 °C for 60 s made up the amplification protocol. As a control, β actin was utilized (Housekeeping gene). The 2 (-ΔΔC(T)) technique was used to express the results as relative gene expression [22].

3.4. Acute oral toxicity study

Acute oral toxicity of polyherbal syrup was evaluated by the acute toxic class method as per OECD test guideline 423 [23]. The female non-pregnant rats were selected and overnight fasted and provided water *ad libitum* before the experimentation. The initial dose of 300 mg/kg polyherbal syrup was administered to a rat through a gastric intubation tube because there was no *in vitro* and *in vivo* toxicity information regarding the prepared polyherbal syrup. At this dose level, no toxic symptoms and mortality were observed, further dose was increased to 2000 mg/kg and administered preliminary to a rat, which also showed no toxic symptoms. Then the main test was conducted on the three rats with a single oral dose of 2000 mg/kg of polyherbal syrup administered. After administration, each animal was individually observed first 30 min for clinical signs, toxic symptoms, and mortality, followed by special attention for the first 4 h and periodically for 24 h, and then daily monitoring for the following 14 days.

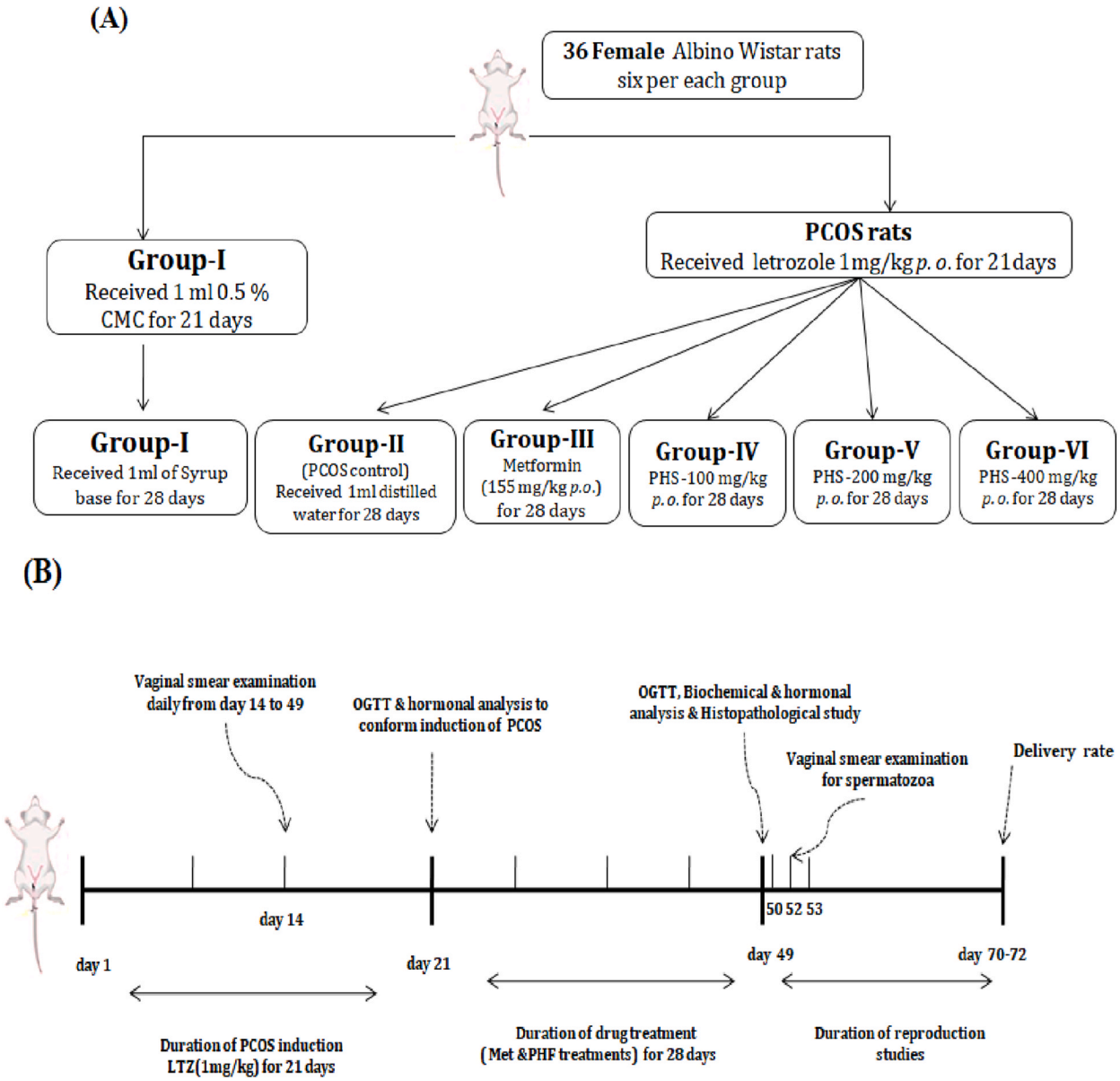

Fig. 1. (A). Experimental design; (B) Experimental timeline.

3.5. In- vivo polycystic ovarian syndrome studies in rats

3.5.1. Animals

The 36 colonies inbreed adult virgin female Albino Wistar rats weighed around 130–150 gm (aged around 8–10 weeks) were obtained from the Tamil Nadu Veterinary and Animal Sciences University, Chennai-51, India, central animal facility. A week before the experiment, all the animals were acclimated and housed in sterile polypropylene cages at standard temperatures (23 ± 2 °C), relative humidity of (60 ± 10%), and 12–12 h of light/dark. They were fed with a standard pellet diet and water *ad libitum*.

3.5.2. Induction of PCOS in rats

Letrozole 1 mg/kg (dissolved in 0.5% Carboxymethyl Cellulose (CMC)) was administered orally once daily for 21 days to induce PCOS in rats [24]. Before administration, the drug solution was freshly prepared daily and administered. Letrozole tablet was purchased from the marketed sample of Wynclark Pharmaceuticals Private Limited, India.

3.5.3. Experimental design

The selected 36 virgin female Albino Wistar rats with regular estrous cycles were randomly divided into six groups each consisting of six animals (Fig. 1(A)). Group I served as normal control rats and received 1 ml of 0.5% CMC orally for 21 days, Group II-VI served as PCOS rats and received letrozole (1 mg/kg body weight orally) for 21 days to the induction of PCOS. After induction of PCOS from day 22 Group I received 1 ml of syrup base *p. o.*, Group II served as PCOS control and received 1 ml of distilled water per oral (*p. o.*) Group III served as the standard drug-treated group and received metformin 155 mg/kg body weight (b. w.) *p. o.* Group IV-VI served as the polyherbal syrup (PHS) treatment group and received 100, 200, and 400 mg/kg b. w. *p. o.* respectively. All the treatments were continued for 28 days after the confirmation of induction of PCOS. After 21 days of letrozole treatment, the induction of PCOS was confirmed by measuring the developed menstrual irregularity (vaginal smear examination), insulin resistance (OGTT), and hormonal abnormality (serum total testosterone analysis) (Fig. 1(B)).

3.5.4. Vaginal smear observation

The estrous cycle regularity in rats was evaluated by the vaginal smear technique. In the early morning (8.00 a.m. to 9.00 a.m.) vaginal fluid was collected from day 14 to the end of the study. In order to collect the vaginal fluid, the tip of a pipette containing 10 μl of normal saline was inserted into the rat vagina at a depth of 5–10 mm and flushed in and out three times; if the solution become cloudy during the first flush, there was no need for additional flushing. The smear was prepared after a drop of the vaginal fluid was placed on the glass slide. The prepared smear was dried and stained with 0.5% methylene blue solution. In order to identify the stages of the estrous cycle, the stained slides were examined under a light microscope (40 X objective lens magnification) (ADELTA OPTEC, Haryana, India) [25].

3.5.5. Oral glucose tolerance test (OGTT)

The Oral Glucose Tolerance Test (OGTT) was carried out on day 21 after the completion of letrozole administration and on day 50 of the study following the post-drug treatments. Before the experiment animals fasted for 12 h, and the glycemia was measured by tail vein blood sampling using Accu Check Active glucometer (Roche Diagnostics Ltd, India). On experiment day blood glucose level was assessed before (baseline) and after the single oral glucose (2 gm/kg b.w.) challenge at 30, 60, and 120 min [26].

3.5.6. Collection of blood samples and separation of serum

Blood samples from each rat were collected on day 21 of the study after the completion of letrozole administration and on day 50 of the study after the completion of the drug treatment. The blood sample was withdrawn from the fasted animal through retro-orbital plexus under ketamine (90 mg/kg b.w. *i. p.*) and xylazine (10 mg/kg *i. p.*) anesthesia [27]. After collecting the blood sample allowed to stand for 10 min to separate serum. Then it was centrifuged at 3000 rpm for 10 min in Remi microcentrifuge RM-03 plus (Remi, India). The separated serum was stored at −20 °C until further biochemical analysis.

3.5.7. Serum biochemical analysis

The serum biochemical parameters like serum lipid profile, sex hormone-binding globulin (SHBG), and serum insulin were estimated from the separated serum on day 50 of the study. The serum lipid profile like low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL) cholesterol, and triglyceride (TG) were analyzed by using the semi-auto analyzer kits techniques following the manufacturer's instruction (ARKRAY Healthcare Pvt. Ltd., Surat, India). The kits code are LDL cholesterol (71LS400-56), HDL cholesterol (71LS300-56), and for triglyceride (72LS100-60). The SHBG and serum insulin levels were assayed by using an enzyme-linked immunosorbent assay (ELISA) kit (#EH421RB & #KAQ1251 respectively) upon the manufacturer's directions (Thermo Fisher Scientific, India).

3.5.8. Serum sex hormonal assay

The induction of PCOS was assessed by estimation of serum testosterone on day 21 of the study, after completion of letrozole treatment. On day 50 the serum sex hormones testosterone, estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone were assayed on Cobas e411 immunoassay analyzers (Roche Diagnostics International Ltd., USA) using the particular ELISA kits technique according to the manufacturer's direction (Roche diagnostics, India). The product codes are testosterone (052022301090), estradiol (06656021500), LH (03561097190), FSH (117758635000), and progesterone (07092539500). The

free androgen index (FAI) was calculated by the formula-2 [28].

$$FAI = \frac{Total\ testosterone}{SHBG} x100 \quad (2)$$

3.6. Homeostasis model assessment index for insulin resistance (HOMA-IR) in rats

The homeostasis model assessment index for insulin resistance (HOMA-IR) was used to assess insulin resistance (IR) in rats. The fasting blood glucose level of all the rats was measured on day 50 of the study by tail vein blood sampling using a glucometer. The following formula-3 was employed to calculate the HOMA-IR [29].

$$HOMA - IR = \frac{Fasting\ insulin\ (mIU/l)\ x\ Fasting\ blood\ glucose\ (mg/dl)}{405} \quad (3)$$

3.7. Ovarian Steroidogenic Enzyme Assay [30]

3.7.1. Ovarian tissue homogenate preparation

After the blood samples collection, three rats in each group were sacrificed by over dose of anesthesia. The abdominal cavity was cut open, the ovaries were removed, and the left ovary was used for protein estimation and steroidogenic enzyme assay while the right ovary was used for histological evaluation. In order to prepare 10% of ovarian tissue homogenate, 0.1 M tris hydrochloride buffer at pH 7.8 was used, and the mixture was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was utilized for further ovarian steroidogenic enzymes 17β-hydroxy steroid dehydrogenase (17β-HSD) and 3β-hydroxy steroid dehydrogenase (3β-HSD).

3.7.2. 3β-hydroxy steroid dehydrogenase activity assay

For the 3β-HSD assay nicotinamide adenine dinucleotide (NAD) (500 μM) in 0.1 M, tris HCl buffer (pH-7.8), and the substrate of dehydroepiandrosterone (100 μM) add and the total volume was made up of 3 ml. The assay reaction was initiated by adding 100 μl of ovarian tissue homogenate with iodonitro tetrazolium (INT) coloring reagent. The combined solution was incubated at 37 °C for 1 h. The reaction was stopped by adding 2 ml of phthalate buffer (pH 3.0). The absorbance was read at 490 nm. From the nicotinamide adenine dinucleotide hydrogen (NADH) standard curve the 3β-HSD enzyme activity was calculated, it was expressed as nano-moles of NADH formed/min/mg protein.

3.7.3. 17β-hydroxy steroid dehydrogenase activity assay

For the 17β-HSD assay, 100 μl of tissue homogenate supernatant was mixed with 200 μl of 0.5 μM NADPH and 100 μl of 0.8 μM androstenedione and the volume was made up to 3 ml with 100 μM phosphate buffer solution (pH-7.4). Adding substrate initiated the test reaction and the decrease in NADPH absorbance was monitored at 340 nm for 5 min at 20-sec intervals. The 17β-HSD enzyme activity was expressed as nano-moles of NADPH oxidized/min/mg protein.

3.8. Western blot analysis

The ovarian protein expression of insulin receptor (INSR), adenosine monophosphate-activated protein kinase (AMPK), and glucose transporter 4 (GLUT4) were estimated by Western blot analysis. The frozen ovarian tissue lysate was prepared by using radioimmunoprecipitation assay buffer and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was separated and used following analysis. The protein concentration in the sample was determined by the Bradford method. For the blotting study sample (30–50 μg protein) was loaded on 10% SDS- polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Then it was blocked in blocking buffer solution (5% nonfat milk in TBST (tris buffer solution with 0.05% tween −20)) at room temperature for 1 h, followed by overnight incubation at 4 °C with specific primary antibodies anti-INSR, anti-AMPK, and anti-GLUT4. After that secondary antibody was added and incubated for 1.5 h at room temperature. β-actin protein was used as a control. After washing ECL chemiluminescence kit (ThermoFisher Scientific, India) was used to visualize the developed protein bands with the help of chemidoc system (Biorad). Protein levels were measured as fold changes calculated by the following formula-4 [31,32].

$$T_{Protein\ level} = \frac{E_{band\ desity} / E_{corresponding\ \beta-actin\ density}}{C_{band\ desity} / C_{corresponding\ \beta-actin\ density}} \quad (4)$$

Where, E-Experimental group; C- Control

3.9. Ovary histopathological and histomorphological study

The right ovary of each rat was fixed in 10% neutral formal saline, dehydrated with the increasing concentration of ethanol, and after that immersed in xylene and embedded in paraffin wax. The blocks were longitudinally sectioned at 5 μm thickness from the center. The section was mounded on a slide and stained by the hematoxylin and eosin (HE) staining procedure and examined under a light microscope with 40 X magnification for histopathological changes [33].

The photographs of stained ovaries were taken in a microscopic photographic unit of LYNX LM521710 (Lawrence and Mayo India

Pvt. Ltd). The ovarian morphological study was carried out at 40 X magnification using image Ultrascope 9.1V software. A larger area section (40 X) was chosen to identify and count the number of preantral, antral (Grafian), cystic, atretic follicles, and corpus luteum (CL). The follicles were identified and counted by the following ovarian morphology. The preantral follicles include primordial, primary, and secondary follicles that were identified as Primordial follicles: oocytes surrounded by single-layer flatted epithelial cells, primary follicles: oocytes surrounded by single or multi-layer cuboidal epithelial cells, secondary follicles: oocytes covered by two or more layers of cuboidal epithelial cells with one or more cavity in which commencement of antrum, Antral follicles: Single large antrum filled with follicular fluid and the oocyte surrounded by granulosa cells. Atretic follicles were identified by degenerated oocytes with pyknosis in granulosa cells and granulosa cell debris in the antral cavity [34]. Corpus luteum is identified by when one of the following observations, such as basophilic cytoplasm, larger size with polygonal and thinly vacuolated luteal cells, apoptosis of luteal cells, and vacuolation, and increased cytoplasmic fibrous tissue and decreased vacuolation [35]. Cystic follicles were identified by cystic follicles with an inner thickened theca cell layer and irregular thickness of granulosa cells [36].

3.9.1. Reproduction studies

After 28 days of drug treatments followed by biochemical and histopathological studies, the remaining 18 female rats were included in reproduction studies. Each female rat from the control and treatment groups was mated with a male in a ratio of 1:1. They were caged overnight and the early morning vaginal smear was collected and examined microscopically to spot the presence of spermatozoa. The female rats with vaginal smear spermatozoa positive were taken as positively mated and the same day was considered as day 0 of gestation. After the successful insemination, the male was separated from the female. If the rat was not inseminated it cohabitated with the male until the vaginal smear was positive for spermatozoa. The gestation period was 21–23 days [37]. The number of pregnancies and a number of pups delivered and survived in all treatments were recorded and the mean pups delivered, percentage of pregnancy per treatment, copulation index, and fertility index were calculated by formula 5 & 6 [38,39].

$$Copulation\ index = \frac{Number\ of\ Sperm\ Positive}{Number\ of\ Cohabitated} x100 \tag{5}$$

$$Fertility\ index = \frac{Number\ of\ Pregnancy}{Number\ of\ Sperm\ Postivie} x100 \tag{6}$$

3.10. Ethics statement

This study was reviewed and approved by the Institutional Animal Ethics Committee of Vinayaka Mission's College of Pharmacy, Salem-636 008, Tamil Nadu, India. CPCSEA guidelines were followed for the care and use of experimental animals. IAEC Approval reference number: P.COL/35/2021/IAEC/VMCP

3.11. Statistical analysis

To determine the statistical significance all the data were expressed as mean ± standard error mean (mean ± SEM). For the *in vitro* gene expression assay paired-sample *t*-test was used. One-way analysis of variance (ANOVA) was used to compare rats over time or within groups, followed by post hoc Dunnett's multiple comparison test using SPSS V.17. The level of P values < 0.05 were judged as statistically significant.

4. Results

4.1. In-vitro cell viability assay

In vitro cell viability assay of the polyherbal syrup was performed on the Chinese Hamster Ovarian cell lines (CHO) by the MTT assay method. The polyherbal syrup produced a dose-dependent cytotoxic effect on the CHO cell lines. The concentration of 100 μg/ml produced a minimal cytotoxic effect. The IC_{50} value of the polyherbal syrup formulation was found to be 561.00 ± 2.7 μg/ml. Table 2 and Fig. 2 illustrate these findings.

Table 2
In- vitro Cell Viability Assay of Polyherbal Syrup Formulation on CHO-Ovarian Cell Line.

S. No.	Test concentration (μg/ml)	% Cell viability	IC_{50}
1.	25	99.84 ± 0.34	561.00 ± 2.7 μg/ml
2.	50	99.21 ± 0.21	
3.	100	97.95 ± 0.55	
4.	250	89.60 ± 0.49	
5.	500	57.45 ± 0.85	
6.	Control	100.00 ± 0.96	

N = 3, results are shown as the mean ± SEM. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

4.2. In- vitro GLUT4 and AMPK expression assay

In Vitro GLUT4 and AMPK expression assay on CHO cell lines study results are represented in Table 3. Treatment of polyherbal syrup formulation 100 μg/ml showed a significant ($P < 0.001$) increased effect on the GLUT4 expression and AMPK expression as compared to the control treatment.

4.3. Acute oral toxicity studies of polyherbal syrup formulation

Acute oral toxicity of the polyherbal syrup formulation was tested at the maximum dose of 2000 mg/kg. There were no toxic symptoms, mortality, observational changes, somatomotor changes, and behavioral changes observed after single oral dose administration of polyherbal syrup, and also there were no significant body weight, feed intake, and water intake changes were observed during the 14 days observation period after acute oral dosing of polyherbal syrup formulation. These results indicated that the prepared polyherbal syrup formulation was found to be safer at the tested dose level of 2000 mg/kg.

Based on the acute oral toxicity results considering the body surface factor 1/10th of the maximum 2000 mg/kg acute oral toxicity dose of 200 mg/kg was selected as the middle dose, twofold higher than the middle dose 400 mg/kg was selected as the high dose, and the 50% of the middle dose 100 mg/kg was selected as low dose for further *in vivo* PCOS studies.

4.4. Effect of polyherbal syrup on the estrus cycle in the letrozole-induced PCOS rats

End of letrozole treatment on day 22, the estrus cycle was completely irregular in the letrozole-treated animals as compared to the normal control rats which displayed an estrus irregularity in PCOS-induced rats. From day 22 to day 50 PCOS control group displayed a

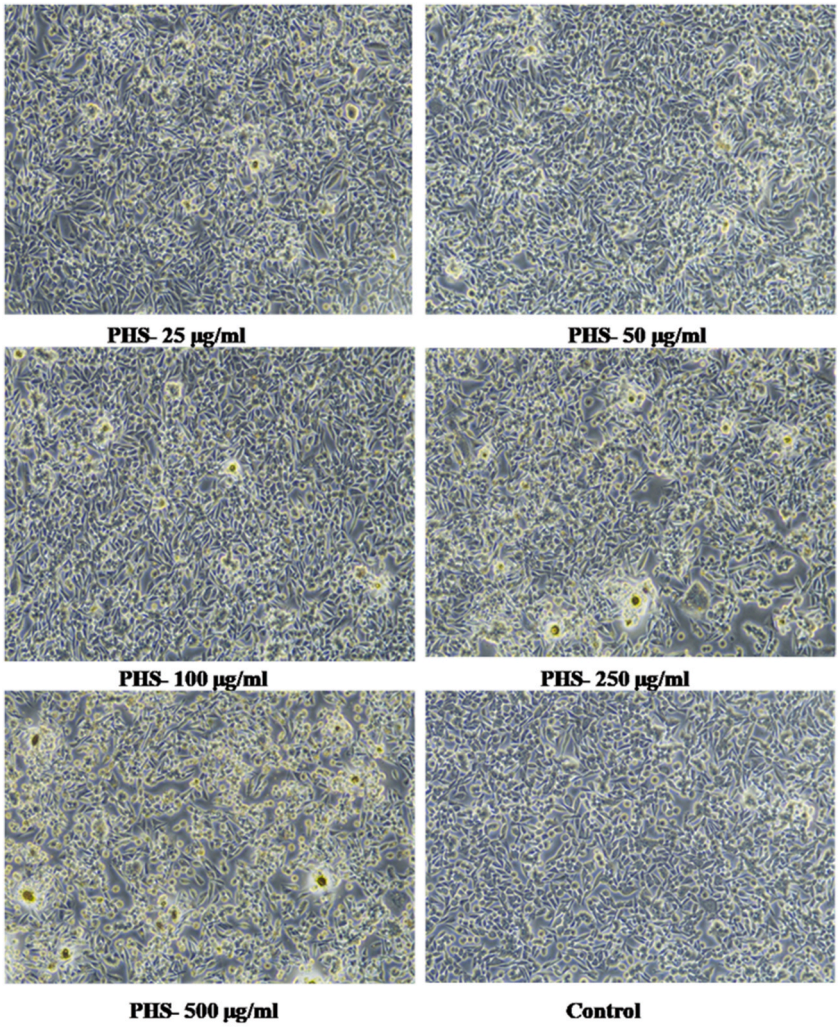

Fig. 2. *In-vitro* Cell Viability MTT assay of Polyherbal Syrup Formulation on CHO-Ovarian Cell Line. PHS- Polyherbal Syrup.

Table 3
In- vitro GLUT4 and AMPK Expression Assay.

S. No.	Treatment	GLUT4 mRNA expression (AU)	AMPK mRNA expression (AU)
1.	Control	1.05 ± 0.04	1.89 ± 0.02
2.	PHS-100 μg/ml	7.80 ± 0.49***	8.60 ± 1.02***

N = 3, values are mentioned as an arbitrary unit (AU) and expressed as mean ± SEM, ***P < 0.001. Paired-samples *t*-test was employed to analyze the data.

constant diestrus phase. After 28 days of metformin and polyherbal syrup treatment, the estrus irregularity was improved and diestrus duration decreased as compared to the PCOS control rats. The results in Table 4 represent the number of estrus cycles during the total 5 weeks of the study period. The number of estrus cycles was significantly (P < 0.001) less in the PCOS control as compared to the normal control. It indicates the development of PCOS in letrozole-treated rats. The metformin treatment, PHS 200 mg/kg, and 400 mg/kg treatment showed high significant (P < 0.001) improvement and PHS 100 mg/kg treatment showed moderate significant improvement (P < 0.01) in the number of estrus cycles as compared to the PCOS control.

4.5. Oral glucose tolerance test (OGTT)

After 21 days of letrozole administration, the rats receiving letrozole had considerably higher fasting blood glucose levels than the normal control group. After the oral glucose challenge, the letrozole-treated rats showed significantly (P < 0.001) higher blood glucose levels when compared to the normal control. It indicates the development of insulin resistance in the letrozole-induced PCOS condition (Table 5).

After 28 days of drug treatments, the fasting blood glucose level in the PCOS control group was significantly (P < 0.001) higher than in the normal control group. After the oral glucose challenge, the blood glucose level was significantly (P < 0.001) higher in the PCOS control as compared to the normal control. The treatment of metformin and polyherbal syrup formulation (100 mg/kg, 200 mg/kg, and 400 mg/kg) showed a significant (P < 0.001) reduction in the blood glucose level after the oral glucose challenge as compared to the PCOS control (Table 6).

4.6. Effect of polyherbal syrup on serum lipid profile in the letrozole-induced PCOS rats

The data in Table 7 represents a significant (P < 0.001) elevation of VLDL, LDL cholesterol, and triglycerides serum levels and a significant (P < 0.001) reduction of HDL level in the PCOS control when compared to the normal rats. These altered lipid profiles were significantly (P < 0.001) improved by the drug treatments as compared to the PCOS control. The treatment of polyherbal syrup formulation illustrated dose-dependent improvement in the lipid profile.

4.7. Effect of polyherbal syrup on serum insulin and SHBG level in the letrozole-induced PCOS rats

The serum fasting insulin level was significantly (P < 0.001) higher and sex hormone-binding globulin (SHBG) levels were significantly (P < 0.001) less in the PCOS control as compared to the normal control rats. The treatment of metformin and polyherbal syrup formulation (100 mg/kg, 200 mg/kg, and 400 mg/kg) showed a significant (P < 0.001) reversal effect on the serum insulin and SHBG levels as compared to the PCOS control (Table 8).

4.8. Effect of polyherbal syrup formulation on serum sex hormones level in the letrozole-induced PCOS rats

End of 21 days of letrozole administration, the serum total testosterone was significantly (P < 0.001) higher in the letrozole-treated groups as compared to the normal control. It confirms the induction of PCOS by displaying the hyperandrogenic character of human PCOS. The 28 days after the drug treatments (On day 50 of the study) the serum total testosterone level was significantly (P < 0.001) higher in the PCOS control as compared to the normal control. The treatment of metformin, polyherbal syrup formulation 200 mg/kg

Table 4
Effect of polyherbal syrup on the number of estrus cycles in the letrozole-induced PCOS rats.

Treatment	Number of Complete Estrus Cycles in 5 Weeks
Group-I (Vehicle Control)	6.50 ± 0.43
Group-II (PCOS Control)	1.33 ± 0.21[c]
Group-III (Metformin-155 mg/kg)	3.17 ± 0.31[cf]
Group-IV (PHS-100 mg/kg)	2.67 ± 0.21[ce]
Group-V (PHS-200 mg/kg)	3.67 ± 0.33[cf]
Group-VI (PHS-400 mg/kg)	4.50 ± 0.43[cf]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] P < 0.05; [b] P < 0.01; [c] P < 0.001 Vs Group I.; [d] P < 0.05; [e] P < 0.01; [f] P < 0.001 Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 5
Oral glucose tolerance test (OGTT) in the letrozole-induced PCOS rats.

Treatment	Blood Glucose Level (mg/dl)			
	Baseline	After the Oral glucose Challenge		
		30 min	60 min	120 min
Group-I (Vehicle Control)	77.67 ± 4.84	153.00 ± 7.00	124.67 ± 5.04	82.33 ± 2.03
Group-II (PCOS Control)	100.30 ± 4.10[b]	186.00 ± 4.16[b]	156.33 ± 7.13[a]	119.33 ± 2.40[c]
Group-III (Metformin-155 mg/kg)	102.00 ± 6.66[b]	182.67 ± 6.98[a]	156.33 ± 7.80[a]	114.33 ± 6.89[c]
Group-IV (PHS-100 mg/kg)	99.67 ± 8.67[a]	193.67 ± 4.06[b]	161.67 ± 8.41[b]	113.67 ± 5.49[c]
Group-V (PHS-200 mg/kg)	97.00 ± 2.65[a]	187.33 ± 7.80[b]	156.33 ± 9.52[a]	114.67 ± 4.06[c]
Group-VI (PHS-400 mg/kg)	95.67 ± 3.71[a]	188.67 ± 12.56[b]	157.67 ± 8.83[a]	113.00 ± 6.03[c]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 6
Effect of polyherbal syrup on the OGTT in the letrozole-induced PCOS rats.

Treatment	Blood glucose level (mg/dl)			
	Baseline	After the oral glucose challenge		
		30 min	60 min	120 min
Group-I (Vehicle Control)	79.67 ± 5.33	162.33 ± 7.62	106.33 ± 4.23	95.33 ± 4.26
Group-II (PCOS Control)	153.00 ± 6.08[c]	222.33 ± 12.47[c]	187.00 ± 5.69[c]	157.67 ± 5.49[c]
Group-III (Metformin-155 mg/kg)	107.33 ± 4.41[bf]	184.67 ± 4.06[d]	112.00 ± 7.57[f]	96.67 ± 5.78[f]
Group-IV (PHS-100 mg/kg)	124.33 ± 8.37[ce]	208.67 ± 16.59[b]	121.67 ± 6.74[f]	114.67 ± 6.57[af]
Group-V (PHS-200 mg/kg)	103.67 ± 3.76[bf]	164.33 ± 8.37[f]	115.67 ± 11.41[f]	98.67 ± 5.04[f]
Group-VI (PHS-400 mg/kg)	93.33 ± 5.17[f]	157.33 ± 4.91[f]	113.33 ± 4.81[f]	96.33 ± 5.78[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 7
Effect of polyherbal syrup on serum lipid profile in the letrozole-induced PCOS rats.

Treatment	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)
Group-I (Vehicle Control)	55.53 ± 4.60	17.07 ± 2.69	41.33 ± 2.91	76.33 ± 3.84
Group-II (PCOS Control)	147.6 ± 7.89[c]	52.73 ± 5.75[c]	21.33 ± 4.06[c]	137.00 ± 8.89[c]
Group-III (Metformin-155 mg/kg)	58.47 ± 12.59[f]	26.20 ± 3.03[f]	33.00 ± 1.53[e]	97.00 ± 11.50[e]
Group-IV (PHS-100 mg/kg)	65.00 ± 6.42[f]	22.47 ± 3.80[f]	33.67 ± 2.19[e]	87.33 ± 6.69[f]
Group-V (PHS-200 mg/kg)	63.13 ± 7.70[f]	21.73 ± 2.74[f]	37.00 ± 1.53[e]	84.67 ± 5.90[f]
Group-VI (PHS-400 mg/kg)	59.40 ± 6.89[f]	19.80 ± 2.72[f]	41.67 ± 3.28[f]	81.00 ± 8.74[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 8
Effect of polyherbal syrup on serum insulin and SHBG level in the letrozole-induced PCOS rats.

Treatment	Fasting Insulin (mIU/l)	SHBG (nmol/l)
Group-I (Vehicle Control)	21.17 ± 0.92	70.00 ± 4.58
Group-II (PCOS Control)	37.30 ± 2.46[c]	39.00 ± 1.53[c]
Group-III (Metformin-155 mg/kg)	21.67 ± 1.86[f]	64.33 ± 7.22[f]
Group-IV (PHS-100 mg/kg)	26.00 ± 0.58[af]	65.33 ± 4.06[f]
Group-V (PHS-200 mg/kg)	24.67 ± 0.67[f]	66.00 ± 0.18[f]
Group-VI (PHS-400 mg/kg)	21.37 ± 1.45[f]	70.00 ± 4.04[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

and 400 mg/kg showed a significant ($P < 0.001$) reversal effect, and PHS 100 mg/kg illustrated a moderate significant ($P < 0.01$) reversal effect on the total testosterone level as compared to the PCOS control (Table 9).

The PCOS control showed a significant ($P < 0.001$) decrease in the serum estradiol, progesterone, and FSH level, and also a significant ($P < 0.001$) increase in LH as compared to the normal control. These altered serum sex hormones level of estradiol, FSH, and LH was high significantly ($P < 0.001$) reversed by metformin and PHS 200 mg/kg and 400 mg/kg treatment, and moderate significant

($P < 0.01$) reversal effect on progesterone when compared to the PCOS control. PHS 100 mg/kg treatment illustrated a mild ($P < 0.05$) to most ($P < 0.01$) significant effect on serum progesterone and LH levels respectively as compared to the PCOS rats (Table 10).

4.9. Effect of polyherbal syrup on free androgen index (FAI) and homeostasis model assessment index of insulin resistance (HOMA-IR) in the letrozole-induced PCOS rats

The free androgen index (FAI) and homeostasis model assessment index of insulin resistance (HOMA-IR) values showed a high significant ($P < 0.001$) increase in PCOS control when compared to the normal control. It confirms hyperandrogenism and augmented insulin resistance in PCOS conditions. All the treatments significantly ($P < 0.001$) reduce the free androgenic index and homeostasis model assessment index of IR when compared to the PCOS control (Table 11).

4.10. Effect of polyherbal syrup on ovarian steroidogenic enzymes level in the letrozole-induced PCOS rats

The PCOS control rats showed a significant ($P < 0.001$) increase in ovarian 3β-Hydroxysteroid dehydrogenases (3β-HSD) and a significant ($P < 0.001$) decrease in ovarian 17β-Hydroxysteroid dehydrogenases (17β-HSD) steroidogenic enzymes when compared to the normal rats. The treatment of metformin and PHS 200 mg/kg and 400 mg/kg treatment showed a significant ($P < 0.001$) reversal effect on these ovarian steroidogenic enzymes when compared to PCOS rats (Table 12).

4.11. Effect of polyherbal syrup on INSR, AMPK, and GLUT4 Protein Expression in Ovarian Tissue of the letrozole-induced PCOS rats

In the PCOS control ovarian tissue, INSR, AMPK, and GLUT4 mRNA levels were significantly ($P < 0.001$) reduced when compared to the normal control. The treatment of metformin and polyherbal syrup formulation (100 mg/kg, 200 mg/kg, and 400 mg/kg) significantly ($P < 0.001$) increases the INSR, AMPK, and GLUT4 protein levels in the PCOS rats as compared to the PCOS control. Fig. 3 (A-C) illustrated this result.

4.12. Effect of polyherbal syrup on ovarian histology in the letrozole-induced PCOS rats

The normal control rats showed the normal histological structure of the ovary like the presence of various stages of follicles including primary, growing antral follicles, mature follicles, and corpus luteum (Fig. 4(A)). In comparison to normal control, letrozole-induced PCOS rats showed altered histoarchitecture of the ovary such as the absence of corpus luteum and increased cystic follicles and undeveloped follicles (Fig. 4(B)). The metformin treatment illustrated the signs of improvement as a reduced number of follicular cysts, numerous different stages of follicles, few atretic follicles, and the existence of corpus luteum (Fig. 4(C)). The treatment of polyherbal syrup formulation of 100 mg/kg also showed few follicular cysts, various stages of follicles, and the presence of corpus luteum (Fig. 4 (D)). The treatment of PHS 200 mg/kg and 400 mg/kg showed better improvement in the histoarchitecture of the ovaries of PCOS rats i.e. improved number of developing follicles and presence of the corpus luteum (Fig. 4 (E & F)).

4.13. Ovarian histomorphology

The results in Table 13 show the histomorphology of ovaries, the PCOS control rats showed a significantly ($P < 0.001$) increased number of cystic follicles, and the number of antral follicles and corpus luteum significantly ($P < 0.001$) decreased when compared to the normal control, which shows the increased number of corpus luteum and antral follicles. It indicates the presence of an ovulatory cycle in the normal control rat ovary and its absence in the PCOS control rat ovary. In the treatment groups, cystic follicles were significantly ($P < 0.001$) decreased and the number of antral follicles and corpus luteum significantly improved when compared to the PCOS control.

Table 9
Effect of polyherbal syrup on serum total testosterone level in the letrozole-induced PCOS rats.

Treatment	Total Testosterone (ng/dl)	
	Day 21	Day 50
Group-I (Vehicle Control)	27.97 ± 3.53	27.13 ± 2.78
Group-II (PCOS Control)	74.57 ± 4.78[c]	80.53 ± 6.00[c]
Group-III (Metformin-155 mg/kg)	76.27 ± 6.47[c]	29.33 ± 2.17[f]
Group-IV (PHS-100 mg/kg)	72.10 ± 2.95[c]	52.10 ± 9.20[be]
Group-V (PHS-200 mg/kg)	71.83 ± 1.58[c]	31.13 ± 4.33[f]
Group-VI (PHS-400 mg/kg)	74.10 ± 3.25[c]	27.33 ± 2.35[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 10
Effect of polyherbal syrup on serum sex hormones level in the letrozole-induced PCOS rats.

Treatment	Estradiol (pg/ml)	Progesterone (ng/ml)	LH (ng/ml)	FSH (ng/ml)
Group-I (Vehicle Control)	33.75 ± 3.43	29.16 ± 3.61	7.20 ± 0.64	25.00 ± 3.46
Group-II (PCOS Control)	9.62 ± 0.19[c]	12.22 ± 1.92[c]	15.67 ± 0.88[c]	11.47 ± 1.30[c]
Group-III (Metformin-155 mg/kg)	29.95 ± 2.06[f]	28.52 ± 1.82[e]	7.30 ± 0.40[f]	23.67 ± 2.03[e]
Group-IV (PHS-100 mg/kg)	12.63 ± 1.73[c]	21.80 ± 2.28[d]	10.60 ± 0.70[bf]	17.00 ± 1.53[a]
Group-V (PHS-200 mg/kg)	28.78 ± 1.12[f]	28.05 ± 4.63[e]	9.20 ± 0.64[af]	25.00 ± 2.08[f]
Group-VI (PHS-400 mg/kg)	30.88 ± 4.16[f]	29.55 ± 3.35[e]	7.27 ± 0.46[f]	25.00 ± 2.65[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 11
Effect of polyherbal syrup on free androgen index (FAI) and homeostasis model assessment index of insulin resistance (HOMA-IR) in the letrozole-induced PCOS rats.

Treatment	FAI	HOMA-IR
Group-I (Vehicle Control)	38.71 ± 2.68	4.14 ± 0.21
Group-II (PCOS Control)	205.99 ± 8.26[c]	14.09 ± 1.09[c]
Group-III (Metformin-155 mg/kg)	46.00 ± 1.78[f]	5.78 ± 0.71[f]
Group-IV (PHS-100 mg/kg)	82.18 ± 9.73[bf]	7.99 ± 0.63[cf]
Group-V (PHS-200 mg/kg)	47.15 ± 6.49[f]	6.32 ± 0.29[af]
Group-VI (PHS-400 mg/kg)	39.67 ± 5.37[f]	4.90 ± 0.21[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

Table 12
Effect of polyherbal syrup on ovarian steroidogenic enzymes level in the letrozole-induced PCOS rats.

Treatment	NADH formed/mg/min/mg protein	
	3β-HSD	17β-HSD
Group-I (Vehicle Control)	1.33 ± 0.15	2.53 ± 0.15
Group-II (PCOS Control)	2.90 ± 0.17[c]	1.23 ± 0.09[c]
Group-III (Metformin-155 mg/kg)	1.40 ± 0.12[f]	2.43 ± 0.09[f]
Group-IV (PHS-100 mg/kg)	2.23 ± 0.20[be]	1.53 ± 0.15[c]
Group-V (PHS-200 mg/kg)	1.53 ± 0.20[f]	2.33 ± 0.20[f]
Group-VI (PHS-400 mg/kg)	1.40 ± 0.17[f]	2.57 ± 0.18[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

4.14. Effect of polyherbal syrup on reproduction performance in the letrozole-induced PCOS rats

Results in Table 14 represent the post-treatment reproductive performance of letrozole in PCOS rats. In the PCOS control copulation index was 100% but the fertility index and the number of pups delivered were nil when compared to the normal control it was significantly very less, indicating the reproductive abnormality in PCOS conditions. In comparison between the treatment groups, the number of pups delivered and survived was significantly ($P < 0.001$) higher in the polyherbal syrup formulation of 200 mg/kg and 400 mg/kg treatment.

5. Discussion

Polycystic Ovarian Syndrome (PCOS) is a heterogeneous endocrinal and metabolic abnormality of women at the reproductive stage [40]. Even though PCOS is a complex heterogeneous condition, current treatments are short-term symptomatic [6]; hence, multidisciplinary systemic approaches are required for the optimal management of PCOS. Besides, this study attempted to prepare a polyherbal formulation as an effective alternative medicine for PCOS.

This polyherbal syrup was prepared based on the ayurvedic relevance, traditional knowledge, and literature survey. The selected herbs possess any one or more potent activities in relevance to PCOS like anti-androgenic activity, hypoglycemic, hypolipidemic, and insulin resistance improving the property and anxiolytic/anti-stress properties. The presence of phytoconstituents such as alkaloids, flavonoids, phytosterols, terpenoids, and phenolic compounds are primarily responsible for these reported activities [41–44].

The selected herbs were formulated as syrup preparation because the insoluble uniformly distributed herbal ingredients in the

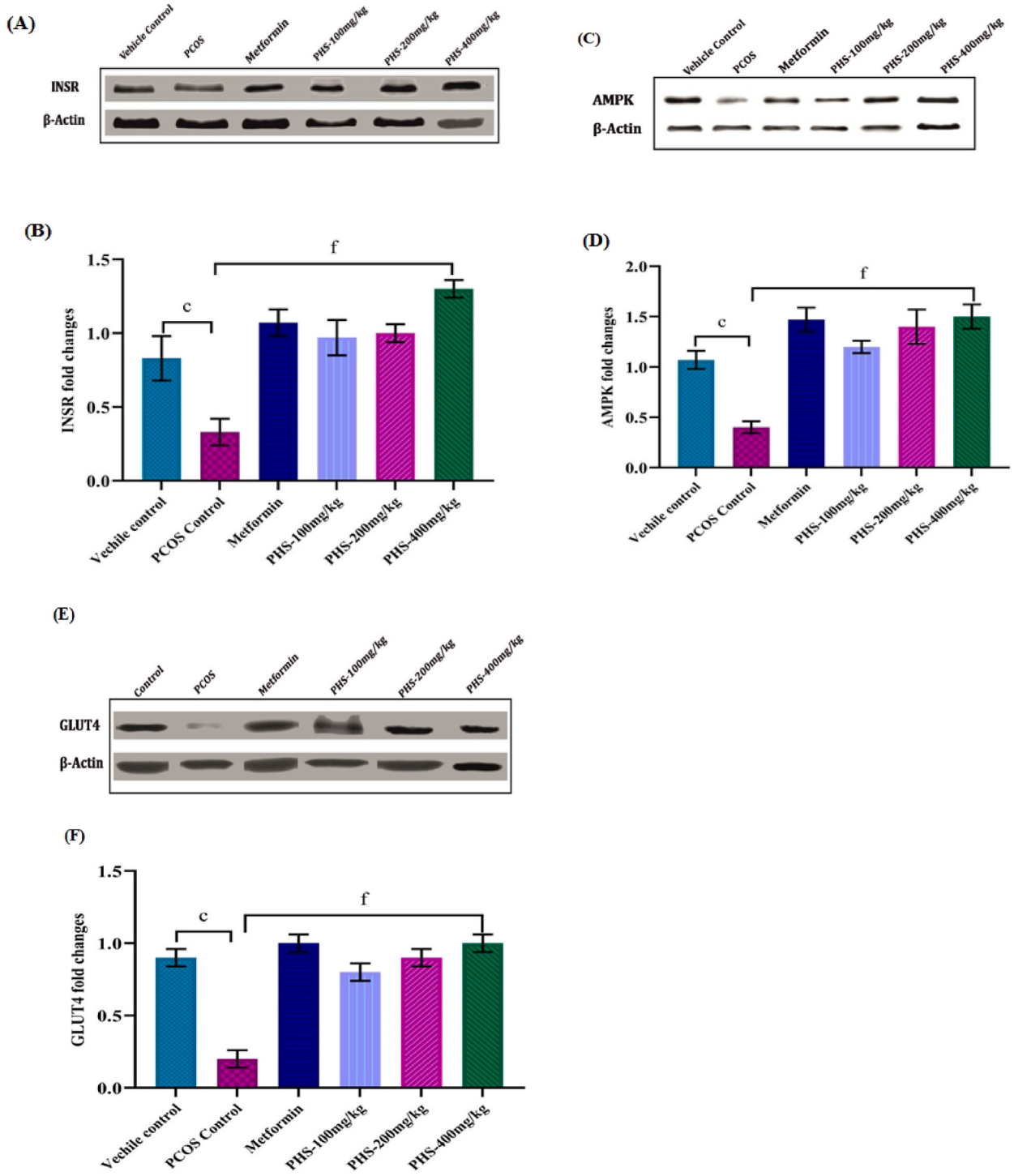

Fig. 3. Effect of Polyherbal Syrup on INSR, AMPK, and GLUT4 Protein Expression in Ovarian Tissue of the Letrozole-induced PCOS Rats. (A& B) INSR expression, (C& D) AMPK expression, (E&F) GLUT4 expression.

liquid medium have a better gastric absorption rate than the other oral preparations. Also, the most preferred and effective route of herbal drug administration is the oral route administration [45,46]. Honey was used as a syrup base in this formulation to mask the bitter taste of the phytoconstituents as well as it has an estrous cycle normalizing capacity and fertility rate improving properties in females [47,48].

In vitro cell viability study in the normal cell lines is a key parameter for the safety evaluation of any herbal formulations [49]. In our

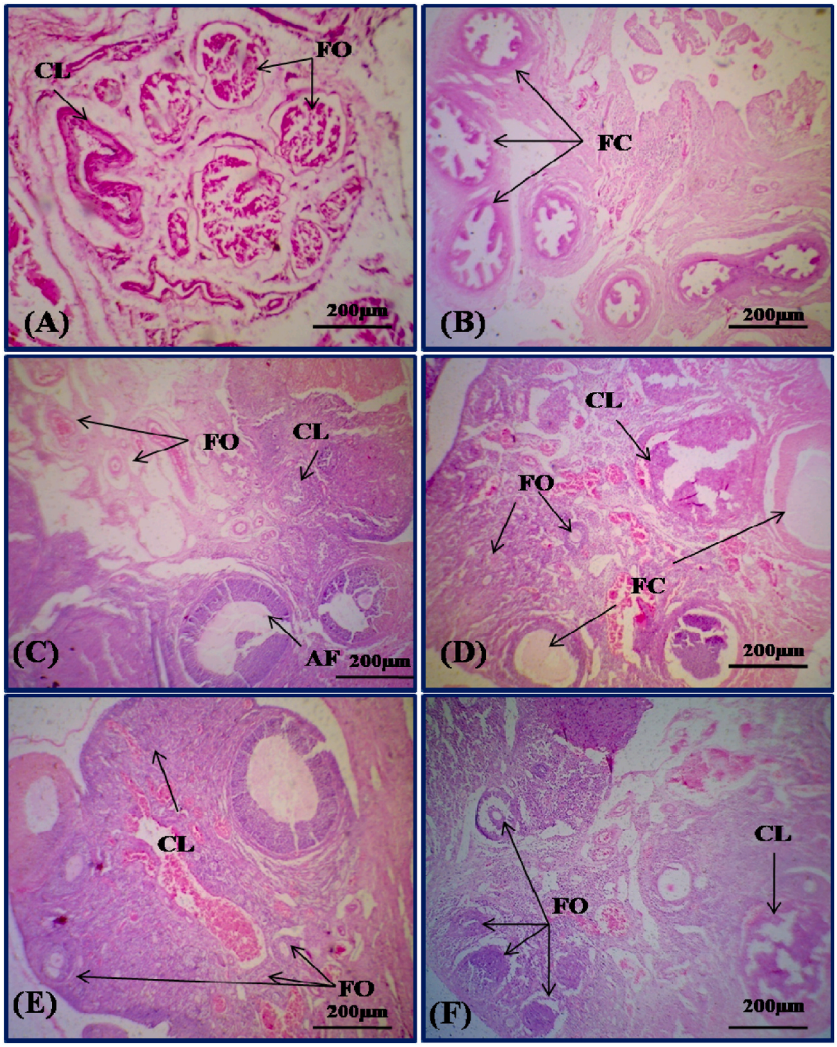

Fig. 4. Histopathological Photo Microscopic Images of Ovary Sections (40 X). (A) Normal control, (B) PCOS control, (C) Letrozole + Metformin, (D) Letrozole + PHS-100 mg/kg, (E) Letrozole + PHS-200 mg/kg, (F) Letrozole + PHS-400 mg/kg, AF- Atretic Follicles; CL-Corpus Luteum; FC-Follicular Cyst; FO-Follicles.

Table 13
the mean number of preantral follicles, antral follicles, cystic follicles, and corpus luteum in treatment groups.

Treatment	Preantral Follicles	Antral Follicles	Cystic Follicles	Corpus Luteum
Group-I (Vehicle Control)	4.00 ± 0.58	6.00 ± 0.58	0	7.00 ± 0.58
Group-II (PCOS Control)	5.67 ± 0.88	2.00 ± 0.58^{c}	9.00 ± 0.58^{c}	0.67 ± 058^{c}
Group-III (Metformin-155 mg/kg)	4.67 ± 0.33	5.67 ± 0.33^{f}	3.00 ± 0.58^{bf}	6.00 ± 0.58^{f}
Group-IV (PHS-100 mg/kg)	5.67 ± 0.33	3.33 ± 0.33^{cd}	3.67 ± 0.88^{cf}	5.00 ± 0.58^{af}
Group-V (PHS-200 mg/kg)	5.00 ± 0.58	5.67 ± 0.33^{f}	2.33 ± 0.67^{bf}	5.67 ± 0.88^{f}
Group-VI (PHS-400 mg/kg)	4.33 ± 0.33	6.33 ± 0.33^{f}	1.67 ± 0.33^{f}	7.33 ± 0.33^{f}

Values are represented as mean ± SEM, N = 3, Statistical significance represented as ^{a} $P < 0.05$; ^{b} $P < 0.01$; ^{c} $P < 0.001$ Vs Group I.; ^{d} $P < 0.05$; ^{e} $P < 0.01$; ^{f} $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

study, the cytotoxic effect of polyherbal syrup was assessed on the Chinese Hamster Ovary (CHO) cell line. CHO cell line is the most accepted mammalian host for the human protein expression study [50]. The percentage of cell viability was increased with the decreasing concentration of polyherbal syrup. The IC50 value of the polyherbal syrup on the CHO cell line was found to be 561.00 ± 2.7 µg/ml. The results of this study showed that polyherbal syrup does not have any significant cytotoxicity on the CHO cell line, which indicates the safety of polyherbal syrup on mammalian tissues.

The CHO cells were treated with the dose of polyherbal syrup which produced minimal cytotoxicity (100 µg/ml) and was tested for

Table 14
Effect of polyherbal syrup on reproduction performance in the letrozole-induced PCOS rats.

Treatment	Copulation index (%)	Fertility index (%)	No. of Pups delivered	No. of Pups survived
Group-I (Vehicle Control)	100	100	7.00 ± 0.58	7.00 ± 0.58
Group-II (PCOS Control)	100	0	0	0
Group-III (Metformin-155 mg/kg)	100	100	4.00 ± 0.58[cf]	4.00 ± 0.58[cf]
Group-IV (PHS-100 mg/kg)	100	67	8.00 ± 0.58[f]	6.67 ± 0.33[f]
Group-V (PHS-200 mg/kg)	100	100	7.33 ± 0.88[f]	7.00 ± 0.58[f]
Group-VI (PHS-400 mg/kg)	100	100	8.00 ± 0.58[f]	8.00 ± 0.58[f]

Values are represented as mean ± SEM, N = 6, Statistical significance represented as [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ Vs Group I.; [d] $P < 0.05$; [e] $P < 0.01$; [f] $P < 0.001$ Vs Group II. After one-way ANOVA the post hoc Dunnett multiple comparison tests were employed to analyze the data.

the AMPK and GLUT4 expression study. The treatment of polyherbal syrup significantly improves the mRNA expression of AMPK and GLUT4 in the CHO cell line. The polyherbal syrup-activated AMPK expression in ovarian cells indicates that it can have the capacity to improve insulin resistance and regulate menstruation and promote ovulation by activating AMPK/PI3K/AKT/FoxO3 signaling pathway [51,52]. Also, the activation of AMPK down-regulates gluconeogenesis gene expression. The increased expression of GLUT4 improves insulin sensitivity by promoting glucose uptake in ovarian cells, it indicates insulin sensitivity improving the property of the prepared polyherbal syrup.

The acute toxicity study results confirm the safety profile of the prepared polyherbal syrup. Acute toxicity study results exposed that the lethal dose of 50% (LD_{50}) value of the polyherbal syrup was found to be > 2000 mg/kg. Hence in line with the Globally Harmonized System (GHS) of toxicity classification, the prepared polyherbal syrup was classified as category 5 or unclassified (2000 mg/kg < LD_{50} < 5000 mg/kg). It indicates that the prepared polyherbal syrup might be safer for use in animals and humans. The efficacy and potency of the prepared polyherbal syrup were evaluated on letrozole induced PCOS model. The letrozole-induced PCOS rats in this study showed that PCOS clinical features like endocrinal features such as peripheral and ovarian hyperandrogenism, sex hormones imbalance, follicular cysts, and absence of corpus luteum in the ovary indicate the irregular and anovulatory estrus cycle and the metabolic features are insulin resistance and hyperlipidemia. The considerable reduction of INSR, AMPK, and GLUT4 expression in the ovaries of PCOS rats verifies the development of hyperandrogenism, insulin resistance, and its associated PCOS clinical features [53].

Insulin resistance (IR) is one of the key pathogenic factors of PCOS [54], IR with compensatory hyperinsulinemia plays a significant role in ovarian function, and it stimulates ovarian theca cells to produce androgen leading to the arrest of follicular maturation and anovulation in PCOS. The result of this study indicates the development of insulin resistance in letrozole PCOS rats by showing increased fasting glucose and insulin levels with impaired glucose clearance in oral glucose tolerance tests following an oral glucose challenge. These results are in line with previous study reports [55]. Further, the increase in the Homeostasis Model Assessment Index of Insulin Resistance (HOMA-IR) value in the letrozole-induced PCOS confirms the development of insulin resistance. All the treatments significantly improve the IR in letrozole-induced PCOS rats by reducing fasting blood glucose and serum insulin levels and promoting glucose clearance rate in the OGGT model. The less HOMA-IR value of treatment groups also indicates the treatment efficacy on insulin resistance. In comparison between the treatment groups, metformin and polyherbal syrup 400 mg/kg treatment show superior results on insulin resistance in PCOS rats. Hence the results of this study indicate the treatment of the prepared polyherbal syrup at 400 mg/kg showed a superior effect on insulin resistance by improving hyperinsulinemia-mediated insulin resistance by increasing glucose uptake and promoting glucose clearance in PCOS rats.

Sex hormone abnormality is the major diagnostic feature of PCOS like elevated levels of serum total testosterone and luteinizing hormone (LH) and decreased estradiol, follicle-stimulating hormone (FSH), and progesterone levels. Hyperandrogenism is the hallmark of PCOS [56]. Administration of letrozole blocks the ovarian conversion of androgen to estrogen leading to hyperandrogenism in the ovary, by decreasing the sex hormone binding globulin (SHBG) increases the peripheral circulatory androgen level in the letrozole-induced PCOS rats. Similar findings were observed in this study. The free androgenic index is the marker of hyperandrogenism in PCOS patients [28]. The induction of PCOS was confirmed in this study by measuring elevated total testosterone levels after 21 days of letrozole treatment. The results of this study indicate the development of hyperandrogenism in PCOS rats by showing the increased concentration of serum total testosterone and free androgenic index. Hyperandrogenism in PCOS rats' feedback to the pituitary gland results in abnormal fluctuation increase in LH and decrease in FSH secretion. Also, a significant reduction in estradiol and progesterone was observed in the letrozole-induced PCOS rats. All the treatments significantly normalize the hormonal abnormality induced by the administration of PCOS. In comparison between the treatments, the treatment of polyherbal syrup at 400 mg/kg showed a superior effect on normalizing the sex hormonal abnormality in PCOS rats. This impact could be attributed to its ability to help PCOS patients normalize their sex hormone imbalance.

Hyperinsulinemia and hyperandrogenism in PCOS conditions stimulate lipolysis in adipocytes resulting in increased free fatty acid leading to dyslipidemia [57]. The results of this study also confirmed that PCOS rats showed dyslipidemia like increased serum concentration of LDL, VLDL cholesterol, and triglycerides level and decreased HDL cholesterol levels. It might be due to letrozole-induced hyperinsulinemia and hyperandrogenism-produced dyslipidemia. All the treatments significantly reverse the lipid abnormality in the letrozole-induced PCOS rats. In comparison between the treatments, polyherbal syrup showed a better dose-dependent beneficial effect on lipid abnormality in letrozole PCOS rats as compared to the metformin treatment. Polyherbal syrup 400 mg/kg treatment possesses a superior hypolipidemic effect in all lipoprotein abnormalities. It might be due to its potent hypoglycemic and anti-androgenic properties.

The increased ovarian concentrations of steroidogenic enzyme 3β-HSD in PCOS rats showed that increased ovarian androgen

production and decreased concentration of 17β-HSD indicate the reduced interconversion of estradiol in the ovary [58]. The results of this study are in line with the previous findings, it confirms the steroidal hormonal abnormality in the ovaries of PCOS rats. The treatment of metformin, polyherbal syrup 200 mg/kg, and 400 mg/kg significantly normalize the ovarian steroidogenic enzyme abnormality in letrozole-induced PCOS rats. Hence these findings indicate that the treatment of polyherbal syrup 200 mg/kg and 400 mg/kg improves the ovarian morphology, particularly the follicular development, and regularizes the estrus irregularity by correcting the letrozole-induced ovarian hyperandrogenism in PCOS in rats.

The Western blot analysis of ovarian tissue showed decreased expression of AMP-activated protein kinase (AMPK), glucose transporter 4 (GLUT4), and insulin receptor (INSR) in letrozole-induced PCOS rats. It indicates insulin resistance development in PCOS rats. The results of this study agree with the clinical feature of decreased expression of AMPK and GLUT4 in the endometrium of PCOS patients. AMPK acts as a major energy sensor in the endometrium of the ovary and regulates the expression of GLUT4 protein [59]. All the treatment groups showed significantly increased expression of INSR, AMPK, and GLUT4 when compared to PCOS rats. The reduced GLUT4 protein levels in PCOS rats were restored by the AMPK-dependent insulin-sensitizing mechanism. The activation of AMPK is involved in the regulation of reproduction through multiple mechanisms. Intimately related to GLUT4 transcription and translation processes to the membrane and thus increases the glucose uptake in ovarian cells and promotes follicular development, maturation, and ovulation. AMPK activation also affects the secretion of GnRH and gonadotropins in the hypothalamus and pituitary and suppresses the androgen receptor levels [60]. The activation of insulin receptor (INSR) phosphorylates the intercellular insulin receptor substrate (IRS), it binds with PI3K protein and activates PKC and Akt signaling molecules, which results in the translocation of GLUT4 from intracellular vesicles to the plasma membrane and permits the cells to absorb more glucose essential for the development and ovulation of the oocyte. Thus the alterations of INSR/IRS/PI3K/PKC/Akt pathways are closely related to insulin resistance [55].

In comparison between the treatments, polyherbal syrup 400 mg/kg showed superior action on increasing the ovarian expression of INSR, AMPK, and GLUT4, which indicates that the treatment of prepared polyherbal syrup promotes follicular development and ovulation, and normalizes the steroidal hormone abnormality provoked by the letrozole induced PCOS. It might be mediated through its insulin sensitivity-improving mechanism.

The histopathological and histomorphological results of this study confirmed the alteration of ovarian function in PCOS rats. Whereas the ovaries of PCOS rats showed an increased number of follicular cysts and very few/or absence of corpus luteum, and the presence of fewer antral follicles with hyperplasia of theca cells and thickened ovarian capsules. Additionally, the cystic follicle sizes were larger than other follicles, which is correlated to the rise in intraovarian androgen levels [61]. The treatment of metformin showed different phases of follicles with the presence of corpus luteum indicating the recovery of ovary morphology in PCOS rats, but the presence of atretic follicles indicates an anovulatory cycle. The treatment of polyherbal syrup showed normal histology of the ovary with different stages of developing follicles, and an increase in the number of corpus luteum. The presence of corpus luteum indicates the occurrence of ovulation and the different stages of follicles indicate the regular estrus cycle. In comparisons between the treatments, polyherbal syrup 400 mg/kg treatment showed superior action in normalizing ovarian morphology in PCOS rats. The results of this study confirm that the treatment of prepared polyherbal formulation by reducing follicular cysts promotes follicular maturation and ovulation through improving insulin sensitivity and reversal of androgen-mediated alteration of ovarian morphology of PCOS rats.

The treatment effectiveness of prepared polyherbal syrup was evaluated by measuring its reproduction rate in the letrozole-induced PCOS rats. The fertility index and delivery rate were completely absent in the PCOS rats, it might be due to hyperandrogenism and insulin resistance-induced anovulatory cycle and the multiple cysts in the ovary. The treatment of metformin and polyherbal syrup improves the fertility index and reproduction rate. It might be due to the improved ovarian morphology in PCOS rats by altering hyperandrogenism and insulin resistance. The delivery rate was less in the metformin-treated group, it might be due to the presence of atretic follicles. In comparison between the polyherbal syrup treatments, the treatment of polyherbal syrup at 400 mg/kg showed the highest fertility index, delivery rate, and survival rate of delivered pups. It confirms the treatment efficacy and potency of the prepared polyherbal syrup on the letrozole-induced PCOS rats.

The standard metformin treatment in PCOS rats reverses the pathological changes induced by letrozole. It regularizes the estrus cycle by improving ovarian morphology through increasing insulin sensitivity and reducing the hyperandrogenism in the ovary mediated by activation of AMPK and INSR in the ovary to increase the expression of GLUT4. The reproductive rate and fertility index were comparatively less in the metformin treatment; it might be due to the presence of few atretic follicles indicating an anovulatory cycle and partial recovery in ovarian morphology.

The treatment of polyherbal syrup showed a dose-dependent effect on PCOS. It regularizes the estrus irregularity by correcting sex hormonal abnormalities and normalizes the ovarian histology by reducing the ovarian cysts and promoting folliculogenesis in PCOS. Additionally, the treatment of polyherbal syrup is effective in the metabolic features of PCOS like improving lipid abnormality and improving insulin sensitivity by decreasing fasting blood glucose levels and improving glucose clearance via the activation of INSR, AMPK, and GLUT4 mediated ovarian pathways. In Fig. 5 clearly explained the possible mechanisms of prepared polyherbal syrup on POCS mediated through improving the insulin sensitivity via activation of insulin receptor INSR/IRS/PI3K/PKC/Akt pathway to translocate GLUT4 from intracellular vesicles to the plasma membrane and permits the ovary cells to absorb more glucose essential for the follicle development and ovulation [54,62]. Also, the activation of AMPK in ovarian cells promotes transcription and translation processes of GLUT4 to the membrane and thus increases glucose uptake and promotes follicular development, maturation, and ovulation. The activation of AMPK also reduces the androgenic receptor level in the ovary [31,60]. *In vitro* AMPK and GLUT4 expression study also confirms the AMPK and GLUT4 activation properties of the prepared polyherbal syrup. This potent hypoglycemic activity, insulin sensitivity improving capacity, and anti-androgenic activity of prepared polyherbal syrup are principally due to the existence of key phytoconstituents like quercetin and β-sitosterol [44,63]. The treatment of polyherbal syrup at 400 mg/kg showed superior fertility and reproductive rate, it confirms the potent effectiveness of prepared polyherbal syrup on PCOS.

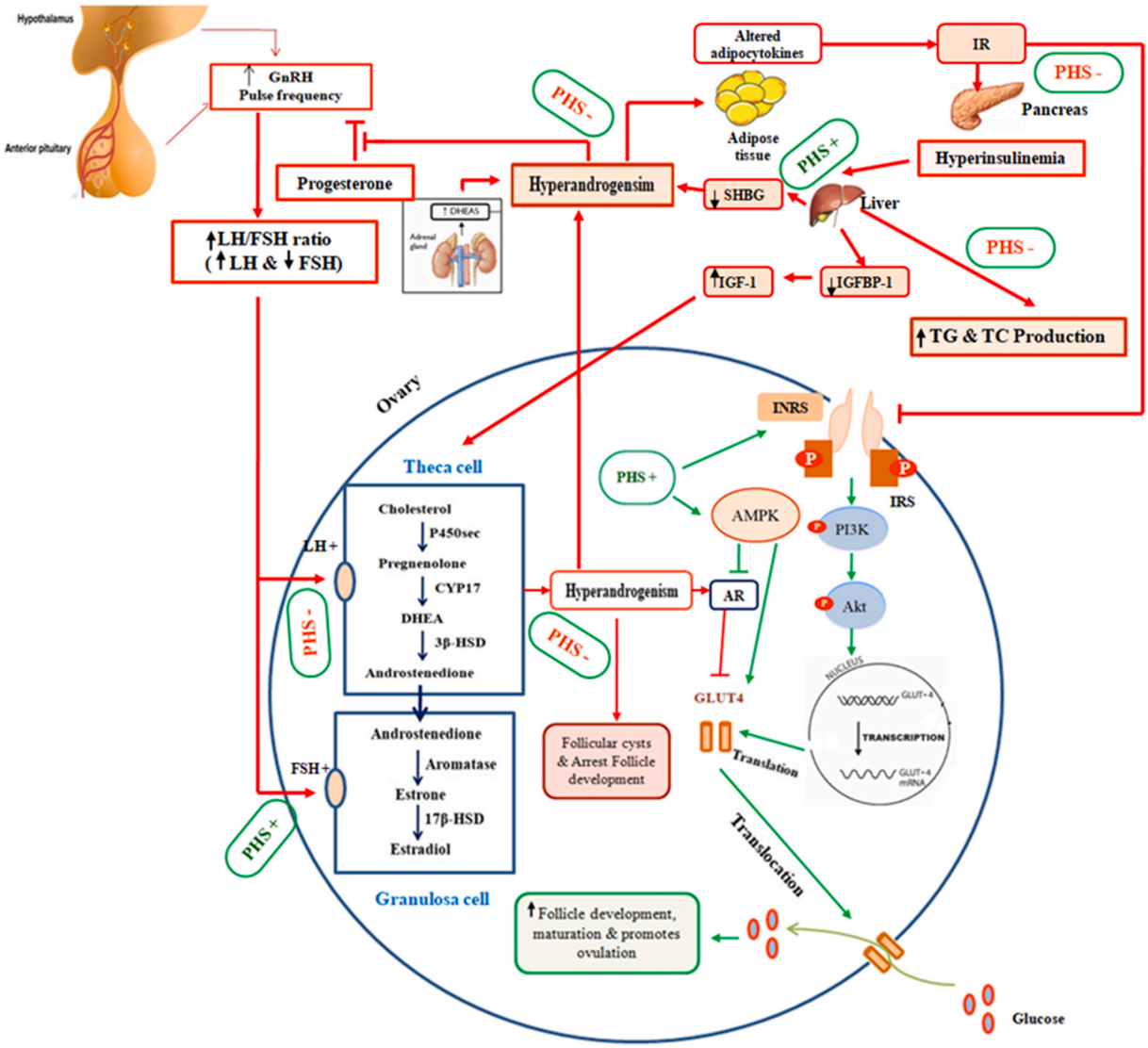

Fig. 5. The Proposed Mechanism of the Prepared Polyherbal Syrup on PCOS. AMPK- adenosine5′monophosphate-activated protein kinase, AR-androgen receptor, Akt-serine/threonine-specific protein kinase, DHEA-dehydroepiandrosterone, FSH- follicle-stimulating hormone, GLUT4-glucose transporter 4, GnRH-gonadotropin releasing hormone, IGF-1- insulin-like growth factor-1, IGFBP-1- insulin-like growth factor binding protein-1, INSR-insulin receptor, IR-insulin resistance, IRS-insulin receptor substrate, LH- luteinizing hormone, PHS- polyherbal syrup, PI3K- phosphatidylinositol 3-kinase, SHBG-sex hormone-binding globulin, TC- total cholesterol, TG-triglyceride, 3β-HSD - 3β-hydroxyl steroid dehydrogenase, 17β-HSD-173β-hydroxyl steroid dehydrogenase. **PHS +** = stimulation/increase, **PHS -** = inhibition/decrease, → = positive influence, ⊣ = inhibition influence on pathogenic pathway, → = positive influence, ⊣ = inhibition influence on therapy pathway.

6. Conclusion

In conclusion, the prepared polyherbal syrup at 400 mg/kg was found to be the safest and most effective alternative medicine for PCOS. It is effective in both endocrinal and metabolic complications of PCOS. It mainly acts by reducing peripheral and ovarian hyperandrogenism and improves insulin sensitivity via activating insulin receptor (INSR) and AMP-activated kinase (AMPK) mediated transcription and translation of GLUT4 from the cytoplasm to the ovarian membrane which improves glucose uptake and promotes the follicular development and ovulation. These beneficial and broader therapeutic effects of the prepared polyherbal syrup are due to the combination of multiple potent herbs that may act on multiple targets at the same time and produce complete relief of PCOS. This potential positive interaction of the selected herbs in the formulation might be due to its synergistic or additive effects. However, further clinical studies are needed to explore this medication as an effective alternative to treat both reproductive and metabolic complications of PCOS.

Author contribution statement

Arul Balasubramanian: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sudhakar Pachiappan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kothai Ramalingam: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Surendiran Mohan, Indira Karuppusamy: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

References

[1] M.A. Ganie, V. Vasudevan, I.A. Wani, M.S. Baba, T. Arif, A. Rashid, Epidemiology, pathogenesis, genetics & management of polycystic ovary syndrome in India, Indian J. Med. Res. 150 (2019) 333–344, https://doi.org/10.4103/ijmr.IJMR_1937_17.

[2] R.J. Norman, D. Dewailly, R.S. Legro, T.E. Hickey, Polycystic ovary syndrome, Lancet (London, England) 370 (2007) 685–697, https://doi.org/10.1016/S0140-6736(07)61345-2.

[3] R.-M. Ni, Y. Mo, X. Chen, J. Zhong, W. Liu, D. Yang, Low prevalence of the metabolic syndrome but high occurrence of various metabolic disorders in Chinese women with polycystic ovary syndrome, Eur. J. Endocrinol. 161 (2009) 411–418, https://doi.org/10.1530/EJE-09-0298.

[4] R.A. Wild, E. Carmina, E. Diamanti-Kandarakis, A. Dokras, H.F. Escobar-Morreale, W. Futterweit, R. Lobo, R.J. Norman, E. Talbott, D.A. Dumesic, Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society, J. Clin. Endocrinol. Metab. 95 (2010) 2038–2049, https://doi.org/10.1210/jc.2009-2724.

[5] A.P. Chaudhari, K. Mazumdar, P.D. Mehta, Anxiety, depression, and quality of life in women with polycystic ovarian syndrome, Indian J. Psychol. Med. 40 (2018) 239–246, https://doi.org/10.4103/IJPSYM.IJPSYM_561_17.

[6] A. Hywood, Phytotherapy for polycystic ovarian syndrome, Aust. J. Herb. Med. 24 (2012) 81–86. https://search.informit.org/doi/10.3316/informit.723768744321611.

[7] S. Parasuraman, G.S. Thing, S.A. Dhanaraj, Polyherbal formulation: concept of ayurveda, Pharm. Rev. 8 (2014) 73–80, https://doi.org/10.4103/0973-7847.134229.

[8] A. Mishra, A. Kumar, N. Rajbhar, A. Kumar, Phytochemical and pharmacological importance of Saraca indica, Int. J. Pharm. Chem. Sci. 2 (2013) 1009–1013.

[9] A.P. Shahid, S. Salini, N. Sasidharan, J. Padikkala, A.C. Raghavamenon, T.D. Babu, Effect of Saraca asoca (Asoka) on estradiol-induced keratinizing metaplasia in rat uterus, J. Basic Clin. Physiol. Pharmacol. 26 (2015) 509–515, https://doi.org/10.1515/jbcpp-2014-0124.

[10] P.P. Joy, J. Thomas, S. Mathew, B.P. Skaria, Medicinal Plants, Aromatic and Medicinal Plants Research Station, Kerala agricultural university, 1998.

[11] M. Anis, M.P. Sharma, M. Iqbal, Herbal ethnomedicine of the gwalior forest division in Madhya Pradesh, India, Pharm. Biol. 38 (2000) 241–253, https://doi.org/10.1076/1388-0209(200009)3841-AFT241.

[12] K. Karthishwaran, S. Mirunalini, Therapeutic potential of Pergularia daemia (forsk.): the ayurvedic wonder, Int. J. Pharmacol. 6 (2010) 836–843.

[13] K. Kirtikar, B. Basu, Indian Medicinal Plants, second ed., International Book Distributors, Dehradun, 1999.

[14] L. Dou, Y. Zheng, L. Li, X. Gui, Y. Chen, M. Yu, Y. Guo, The effect of cinnamon on polycystic ovary syndrome in a mouse model, Reprod. Biol. Endocrinol. 16 (2018) 99, https://doi.org/10.1186/s12958-018-0418-y.

[15] X. Sheng, Y. Zhang, Z. Gong, C. Huang, Y.Q. Zang, Improved insulin resistance and lipid metabolism by cinnamon extract through activation of peroxisome proliferator-activated receptors, PPAR Res. 2008 (2008), 581348, https://doi.org/10.1155/2008/581348.

[16] B.M. Murugesan, P. Muralidharan, R. Hari, Effect of ethanolic seed extract of Caesalpinia bonducella on hormones in mifepristone induced PCOS rats, J. Appl. Pharmaceut. Sci. 10 (2020) 72–76.

[17] S. Parameshwar, K.K. Srinivasan, C.M. Rao, Oral antidiabetic activities of different extracts of Caesalpinia bonducella seed kernels, Pharm. Biol. 40 (2002) 590–595, https://doi.org/10.1076/phbi.40.8.590.14656.

[18] A.A. Al-Qarawi, H.A. Abdel-Rahman, A.A. El-Badry, F. Harraz, N.A. Razig, E.M. Abdel-Magied, The effect of extracts of Cynomorium coccineum and Withania somnifera on gonadotrophins and ovarian follicles of immature Wistar rats, Phytother Res. 14 (2000) 288–290, https://doi.org/10.1002/1099-1573(200006)14, 4<288::aid-ptr603>3.0.co;2-9.

[19] S. Dongre, D. Langade, S. Bhattacharyya, Efficacy and safety of ashwagandha (Withania somnifera) root extract in improving sexual function in women: a pilot study, BioMed Res. Int. 2015 (2015), 284154, https://doi.org/10.1155/2015/284154.

[20] S. Kamiloglu, G. Sari, T. Ozdal, E. Capanoglu, Guidelines for cell viability assays, Food Front. 1 (2020) 332–349, https://doi.org/10.1002/fft2.44.

[21] L. Florento, R. Matias, E. Tuaño, K. Santiago, F. Dela Cruz, A. Tuazon, Comparison of cytotoxic activity of anticancer drugs against various human tumor cell lines using in vitro cell-based approach, Int. J. Biomed. Sci. 8 (2012) 76–80.

[22] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[23] OECD, OECD Guideline for the Testing of Chemicals, Acute Oral Toxicity, 2001. https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd_gl423.pdf.

[24] S. Pachiappan, K. Ramalingam, A. Balasubramanian, Combined Effects of Gymnema Sylvestre and Pergularia Daemia on Letrozole-Induced Polycystic Ovarian Syndrome in Rats, 2021, p. 68, https://doi.org/10.4103/2305-0500.311610, 10.

[25] M.C. Cora, L. Kooistra, G. Travlos, Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears, Toxicol. Pathol. 43 (2015) 776–793, https://doi.org/10.1177/0192623315570339.

[26] C.K. Fofié, E.P. Nguelefack-Mbuyo, N. Tsabang, A. Kamanyi, T.B. Nguelefack, Hypoglycemic properties of the aqueous extract from the stem bark of *Ceiba pentandra* in dexamethasone-induced insulin resistant rats, evidence-based complement, Alternative Med. 2018 (2018), 4234981, https://doi.org/10.1155/2018/4234981.

[27] L.F. Van Pelt, Ketamine and xylazine for surgical anesthesia in rats, J. Am. Vet. Med. Assoc. 171 (1977) 842–844.
[28] E. Sağsak, M. Keskin, S. Cetinkaya, S. Erdeve, Z. Aycan, The diagnostic value of free androgen index in obese adolescent females with idiopathic hirsutism and polycystic ovary syndrome, J. Acad. Res. Med. 11 (2021) 81–85, https://doi.org/10.4274/jarem.galenos.2021.22932.
[29] M.M. Atef, R.N. Abd-Ellatif, M.N. Emam, R.E. Abo El Gheit, A.I. Amer, Y.M. Hafez, Therapeutic potential of sodium selenite in letrozole induced polycystic ovary syndrome rat model: targeting mitochondrial approach (selenium in PCOS), Arch. Biochem. Biophys. 671 (2019) 245–254, https://doi.org/10.1016/j.abb.2019.06.009.
[30] R.K. Rajan, , S.S.K.M., B. Balaji, Soy isoflavones exert beneficial effects on letrozole-induced rat polycystic ovary syndrome (PCOS) model through anti-androgenic mechanism, Pharm. Biol. 55 (2017) 242–251, https://doi.org/10.1080/13880209.2016.1258425.
[31] A. Mihanfar, M. Nouri, L. Roshangar, M.H. Khadem-Ansari, Therapeutic potential of quercetin in an animal model of PCOS: possible involvement of AMPK/SIRT-1 axis, Eur. J. Pharmacol. 900 (2021), 174062, https://doi.org/10.1016/j.ejphar.2021.174062.
[32] J. Xu, J. Dun, J. Yang, J. Zhang, Q. Lin, M. Huang, F. Ji, L. Huang, X. You, Y. Lin, Letrozole rat model mimics human polycystic ovarian syndrome and changes in insulin signal pathways, Med. Sci. Monit. Int. Med. J. Exp. Clin. Res. 26 (2020), e923073, https://doi.org/10.12659/MSM.923073.
[33] A. Dăneasă, C. Cucolaş, L.M. Lenghel, D. Olteanu, R. Orăsan, G.A. Filip, Letrozole vs estradiol valerate induced PCOS in rats: glycemic, oxidative and inflammatory status assessment, Reproduction 151 (2016) 401–409, https://doi.org/10.1530/REP-15-0352.
[34] S. Furat Rencber, S. Kurnaz Ozbek, C. Eraldemır, Z. Sezer, T. Kum, S. Ceylan, E. Guzel, Effect of resveratrol and metformin on ovarian reserve and ultrastructure in PCOS: an experimental study, J. Ovarian Res. 11 (2018) 55, https://doi.org/10.1186/s13048-018-0427-7.
[35] Y. Taketa, Luteal toxicity evaluation in rats, J. Toxicol. Pathol. 35 (2022) 7–17, https://doi.org/10.1293/tox.2021-0058.
[36] L. Mannerås, S. Cajander, A. Holmäng, Z. Seleskovic, T. Lystig, M. Lönn, E. Stener-Victorin, A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome, Endocrinology 148 (2007) 3781–3791, https://doi.org/10.1210/en.2007-0168.
[37] G.C. Jain, H. Pareek, S. Sharma, M. Bhardwaj, B.S. Khajja, Reproductive toxicity of vanadyl sulphate in male rats, J. Health Sci. 53 (2007) 137–141.
[38] L.A. Dostal, L.R. Whitfield, J.A. Anderson, Fertility and general reproduction studies in rats with the HMG-CoA reductase inhibitor, atorvastatin, Fundam. Appl. Toxicol. Off. J. Soc. Toxicol. 32 (1996) 285–292, https://doi.org/10.1006/faat.1996.0132.
[39] T. Tsilchorozidou, C. Overton, G.S. Conway, The pathophysiology of polycystic ovary syndrome, Clin. Endocrinol. 60 (2004) 1–17, https://doi.org/10.1046/j.1365-2265.2003.01842.x.
[40] E.D. Emamuzo, S.I. Miniakiri, E.J.O. Tedwin, K.H. Delesi, A. Precious, Effects of ethanol extract of leaves of Helianthus annus on the fecundity of Wistar rats, Asian Pac. J. Tropical Med. 3 (2010) 435–438, https://doi.org/10.1016/S1995-7645(10)60104-6.
[41] R. Rani, Y.A. Hajam, R. Kumar, R.A. Bhat, S. Rai, M.A. Rather, A landscape analysis of the potential role of polyphenols for the treatment of Polycystic Ovarian Syndrome (PCOS), Phytomedicine 2 (2022), 100161, https://doi.org/10.1016/j.phyplu.2021.100161.
[42] S. Jahan, A. Abid, S. Khalid, T. Afsar, Qurat-Ul-Ain, G. Shaheen, A. Almajwal, S. Razak, Therapeutic potentials of Quercetin in management of polycystic ovarian syndrome using Letrozole induced rat model: a histological and a biochemical study, J. Ovarian Res. 11 (2018) 26, https://doi.org/10.1186/s13048-018-0400-5.
[43] F. Ashkar, S. Rezaei, S. Salahshoornezhad, F. Vahid, M. Gholamalizadeh, S.M. Dahka, S. Doaei, The Role of medicinal herbs in treatment of insulin resistance in patients with Polycystic Ovary Syndrome: a literature review, Biomol. Concepts 11 (2020) 57–75, https://doi.org/10.1515/bmc-2020-0005.
[44] Y. Yu, Y. Cao, W. Huang, Y. Liu, Y. Lu, J. Zhao, β-Sitosterol ameliorates endometrium receptivity in PCOS-like mice: the mediation of gut microbiota, Front. Nutr. 8 (2021). https://www.frontiersin.org/articles/10.3389/fnut.2021.667130.
[45] G.B. Nitin, G.G. Surendra, S.S. Shailesh, Design, development and evaluation of oral herbal formulations of Piper nigrum and Nyctanthes arbortristis, Int. J. PharmTech Res. 2 (2010) 171–176.
[46] H.C. Ansel, L. V Allen, N.G. Popovich, Pharmaceutical Dosage Forms and Drug Delivery Systems, seventh ed., Lippincott-Williams & Wilkins, Philadelphia, 1999.
[47] S.S.M. Zaid, S.S. Ruslee, M.H. Mokhtar, Protective roles of honey in reproductive health: a review, Molecules 26 (2021), https://doi.org/10.3390/molecules26113322.
[48] S.S.M. Zaid, S. Othman, N.M. Kassim, Potential protective effect of Tualang honey on BPA-induced ovarian toxicity in prepubertal rat, BMC Compl. Alternative Med. 14 (2014) 509, https://doi.org/10.1186/1472-6882-14-509.
[49] S. Selvakumar, B. Sarkar, In-vitro cytotoxicity analysis of chloroform extract of novel poly herbal formulation, Int. J. Pharmacogn. Phytochem. Res. 9 (2017) 193–196.
[50] S.M. Sharker, A. Rahman, A review on the current methods of Chinese hamster ovary (CHO) cells cultivation for the production of therapeutic protein, Curr. Drug Discov. Technol. 18 (2021) 354–364, https://doi.org/10.2174/1570163817666200312102137.
[51] W. Zhang, M. Zuo, J. Lu, Y. Wang, Adiponectin reduces embryonic loss rate and ameliorates trophoblast apoptosis in early pregnancy of mice with polycystic ovary syndrome by affecting the AMPK/PI3K/Akt/FoxO3a signaling pathway, Reprod. Sci. 27 (2020) 2232–2241, https://doi.org/10.1007/s43032-020-00237-z.
[52] A. Abdelrahman, A.A. Mahmoud, Y. Lamie Fanous, N.G. Abd Elhaliem, H. Elalaf, Impact of erythropoietin and myoinositol versus metformin on insulin resistance in a rat model of polycystic ovary syndrome, Arch. Physiol. Biochem. (2021) 1–12, https://doi.org/10.1080/13813455.2021.1949023.
[53] T. Strowitzki, E. Capp, H. von Eye Corleta, The degree of cycle irregularity correlates with the grade of endocrine and metabolic disorders in PCOS patients, Eur. J. Obstet. Gynecol. Reprod. Biol. 149 (2010) 178–181, https://doi.org/10.1016/j.ejogrb.2009.12.024.
[54] U. Ezeh, I.Y.-D. Chen, Y.-H. Chen, R. Azziz, Adipocyte insulin resistance in PCOS: relationship with GLUT-4 expression and whole-body glucose disposal and β-cell function, J. Clin. Endocrinol. Metab. 105 (2020) e2408–e2420, https://doi.org/10.1210/clinem/dgaa235.
[55] K. Unluhizarci, Z. Karaca, F. Kelestimur, Role of insulin and insulin resistance in androgen excess disorders, World J. Diabetes 12 (2021) 616–629, https://doi.org/10.4239/wjd.v12.i5.616.
[56] S. Bednarska, A. Siejka, The pathogenesis and treatment of polycystic ovary syndrome: what's new? Adv. Clin. Exp. Med. Off. Organ Wroclaw Med. Univ. 26 (2017) 359–367, https://doi.org/10.17219/acem/59380.
[57] G.D. Kolovou, K. Anagnostopoulou, N.D. Pilatis, K.D. Salpea, I.S. Hoursalas, I. Petropoulos, H.I. Bilianou, D. V Cokkinos, Fasting serum triglyceride and high-density lipoprotein cholesterol levels in patients intended to be treated for dyslipidemia, Vasc. Health Risk Manag. 1 (2005) 155–161, https://doi.org/10.2147/vhrm.1.2.155.64079.
[58] I. Ortega, J.A. Villanueva, D.H. Wong, A.B. Cress, A. Sokalska, S.D. Stanley, A.J. Duleba, Resveratrol reduces steroidogenesis in rat ovarian theca-interstitial cells: the role of inhibition of Akt/PKB signaling pathway, Endocrinology 153 (2012) 4019–4029, https://doi.org/10.1210/en.2012-1385.
[59] U.A. Ndefo, A. Eaton, M.R. Green, Polycystic ovary syndrome: a review of treatment options with a focus on pharmacological approaches, P T 38 (2013) 336–355.
[60] W. Yang, L. Wang, F. Wang, S. Yuan, Roles of AMP-activated protein kinase (AMPK) in mammalian reproduction, Front. Cell Dev. Biol. 8 (2020), 593005, https://doi.org/10.3389/fcell.2020.593005.
[61] V.B. Mahesh, T.M. Mills, C.A. Bagnell, B.A. Conway, Animal models for study of polycystic ovaries and ovarian atresia, Regul. Ovarian Testic. Funct. (1987) 237–257.
[62] C. Wu, F. Jiang, K. Wei, Z. Jiang, Exercise activates the PI3K-AKT signal pathway by decreasing the expression of 5α-reductase type 1 in PCOS rats, Sci. Rep. 8 (2018) 7982, https://doi.org/10.1038/s41598-018-26210-0.
[63] K. Bogovich, J.S. Richards, Androgen biosynthesis in developing ovarian follicles: evidence that luteinizing hormone regulates thecal 17 alpha-hydroxylase and C17-20-lyase activities, Endocrinology 111 (1982) 1201–1208, https://doi.org/10.1210/endo-111-4-1201.