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Mitigation of letrozole induced polycystic ovarian syndrome by Melats P: A novel promising combination for reproductive health

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Abstract: **Background**: Polycystic ovarian syndrome (PCOS) is an endocrinal disorder and one of common cause of female infertility. Current treatment options are less effective due to its complex pathophysiology. **Objectives**: The current study aimed to investigate the therapeutic effectiveness of Melats P (MP) formulation in alleviating PCOS induced alterations in female Wistar rats. **Methods**: The polyherbal formulation contains Momordica charantia L., Linum usitatissimum, Symplocos racemosa bark, Allium cepa L., Tribulus terrestris and eggshell. Melats P extract was initially tested for Gas Chromatography-Mass Spectrometry, total phenolic content and in vitro antioxidant DPPH assay. 40 female rats were taken and initially divided into two groups: group I (n=10, control group) and group II (n=30, PCOS group). PCOS was induced by letrozole given orally by gavage. Body weight was recorded weekly and vaginal cytology was analyzed daily. After induction of disease, the PCOS group is further divided into three groups (n = 10): group II (Negative control), group III (metformin 350 mg/kg treated group), group IV (Melats P, 500 mg/kg treated group for 28 days. Blood samples and ovaries were collected for biochemical, histological and PCR analysis. **Results**: Results demonstrated that Melats P effectively restored all the biochemical and hormonal parameters to normal and disappeared cysts in ovaries. Moreover, Melats P restored ovarian mRNA expression significantly upregulating INSR, AR, SOD and GPx and downregulating CytP17A1 compared to PCOS. **Conclusion**: These findings showed that, Melats P treatment significantly restored PCOS-induced alterations in the rats, suggesting that it may have a key role in PCOS management.

Keywords: Endocrinal disorder; Letrozole; Melats p; Metformin; Polycystic ovary syndrome

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INTRODUCTION

Polycystic ovarian syndrome, or PCOS, is a complicated and varied metabolic and hormonal disease affecting women of reproductive age and is a leading cause of infertility (V Gadhave *et al*., 2024). Global prevalence of PCOS, ranges from 4% -20%, with South Asian populations showing even higher rates, up to 50–55% in Pakistan (Rahim R *et al*., 2024). According to the Rotterdam criteria (2003) the PCOS pathogenesis showed three different features anovulation, hyperandrogenism; and polycystic ovaries (Malik-Aslam *et al*., 2010). These features are mainly manifested due to hypersecretion of luteinizing hormone (LH), increased androgen production and insulin resistance (Bries *et al*., 2021, Elmosalamy *et al*., 2022, Khattak *et al*., 2022).

Beyond reproductive effects, PCOS severely impacts mental health and overall quality of life (QoL), causing depression, anxiety, weight gain, acne, alopecia, pain, mood disturbances, irregular menses and infertility (Khomami *et al*., 2015, Teede *et al*., 2010). Considering these challenges, PCOS management needs a holistic approach incorporating life style changes that includes weight control, regular physical activity, dietary modifications, stress management and pharmacological therapy. Numerous studies aimed to investigate and explore underling mechanisms and possible treatment of PCOS, experimental animals are also utilized frequently. Letrozole, an aromatase inhibitor, is commonly used to create a PCOS like condition in animal models by inhibiting testosterone conversion to estradiol, resulting in increased androgen levels and leading to enlargement of ovaries, thickened theca interna and changes in anovulation similar to those seen in human PCOS (Xu *et al*., 2020).

Frequently prescribed therapies for PCOS consist of metformin, estrogen, clomiphene citrate, letrozole, pioglitazone, GnRh agonist, oral contraceptives (OCPSs) and myo-inositol. Although these treatments are effective in reducing symptoms and inducing ovulation, but they are related with side effects such as weight increase, lactic acidosis, cardiac issues, abdominal distress and dysmenorrhea (Hussain *et al*., 2022). Considering the limitation of conventional medicines, herbal treatments are drawing interest for their ability to target multiple pathways, restore hormonal balance naturally, enhance ovulation and reduce symptoms and side effects (Prajapati *et al*., 2022; Hussain *et al*., 2022). Among these polyherbal

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formulations are especially promising because of their synergistic therapeutic potential, where various plant derived compounds work together to increase effectiveness. Their appealing attributes are further reinforced by their safety profile, cost-effectiveness and increased patient compliance (Balasubramanian A *et al*., 2023).With regard to this, the present study aimed to investigate the therapeutic potential of a novel polyherbal formulation that consist of *Momordica charantia L*., *Symplocos racemosa* bark, *Tribulus terrestris, Linum usitatssimum, Allium cepa L. and* eggshell. These particular herbs were combined to formulate a polyherbal treatment and the letrozole-induced PCOS model was used to test the combination's effectiveness. These herbs were chosen on the basis of their distinct pharmacological characteristics that are relevant to PCOS pathophysiology, suggesting that combining these herbs may offer a safe and effective approach for the management of PCOS. Previous researches explained that Polyherbal formulations treat PCOS by synergistically addressing hormonal, metabolic and inflammatory imbalances. They act by reducing hyperandrogenism (lowering testosterone), boosting insulin sensitivity (activating GLUT4 and AMPK pathways), restoring ovarian function (follicular development) and regulating the estrous cycle (Lee *et al*., 2025; Balasubramanian A *et al*., 2023).

Given the complex and heterogeneous nature of PCOS, which involves closely linked metabolic, endocrine and inflammatory disturbances, a single-target therapeutic approach is often insufficient. The rationale for developing the present polyherbal formulation lies in its ability to provide multi-target therapeutic effect through complementary pharmacological actions. By combining botanicals that target distinct yet related mechanisms such as insulin sensitization, anti-androgenic activity, antioxidant protection and restoration of ovarian function, the formulation aims to achieve enhanced efficacy at lower, safe dose. This network pharmacology based approach is relevant for chronic conditions like PCOS where long term safety, tolerability and holistic disease modulation are critical.

Momordica charantia L., also known as bitter melon, enhances insulin sensitivity, an essential factor in managing PCOS and has been conventionally used to address menstrual irregularities and metabolic issues associated with PCOS (Hussain *et al*., 2022). *Symplocos racemosa* bark exhibited effects similar to clomiphene citrate, improving reproductive health by alleviating levels of testosterone, normalizing cholesterol, estrogen and progesterone levels and restoring structure of ovaries (Azeemuddin *et al*., 2019). *Linum usitatissimum* also known as flaxseed, helps regulate menstrual cycle, reduce ovarian volume, improve glycemic control and lower androgen and insulin levels due to its lignans and alpha-linolenic acid (Matar *et al*., 2021). *Allium cepa* or onion, maintains reproductive health by enhancing antioxidant defense system (Ghasemzadeh *et al*., 2013). *Tribulus terrestris* extract has been reported to stimulate ovarian activity in immature rats, helping with the formation of the corpus luteum and showing Leutinizing Hormome like activity, which could be beneficial for ovulatory function (Saiyed *et al*., 2016). Eggshells, rich in bioavailable calcium (≈98% calcium carbonate), along with magnesium, phosphorus and proteins, contribute to overall metabolic and reproductive well-being (Ori, 2011).

MATERIALS AND METHODS

Identification and procurement of the required plant specimens

Plants exhibiting therapeutic potential against PCOS, particularly those demonstrating significant effects on one or more than one of its main pathological pathways, were carefully chosen. The selection criteria were primarily guided by traditional knowledge, the instructions of pharmacognostic and Ayurvedic practitioners, a comprehensive literature review and availability of the plant materials. The plant specimens were verified and deposited in herbarium by department of Botany, University of Karachi, Pakistan. The voucher no. are *Momordica charantia* G.H. No 9112122, *Linum usitatissimum* G.H. No 032004, *Tribulus terrestris* G.H. No 072013, *Alium cepa* G.H. No 9112122 and *Symplocas Racemosa* G.H. No. 956008.

Chemicals and drugs

Analytical grade chemicals and drugs were used. Methanol, ethanol, Sodium Carbonate and formalin solution, were obtained from Sigma Chemicals Company (St Louis, MO, USA). Gallic acid and Folin-Ciocalteu reagent were procured from Merck (Darmstadt, Germany). 0.5% Carboxymethyl cellulose (CMC) was procured from Sigma–Aldrich Co. Ketamine/ Xylazine and DPPH solution, were obtained from MERCK and Sigma–Aldrich Co., USA. Letrozole and Metformin tablets were purchased from the marketed samples of Novartis Pharmaceuticals Pakistan and Martin Dow Pharmaceuticals Pakistan.

Preparation of polyherbal formulation

All selected herbs were shade-dried for three days and then finely ground using a grinder, followed by sieving to obtain a uniform powder. Each powdered herb was separately extracted using a hydroalcoholic solvent system consisting of 70% ethanol and 30% water. After a three-day maceration period, the mixtures were filtered and the solvents were removed using a rotary evaporator (Eyla, Japan). The resulting dried extracts were then combined in a ratio of 2:2:1:1:1:1, based on their therapeutic potential and established clinical dosage relevance.

Determination of phenolic content

The presence of phenolic content in polyherbal extract determined via spectrophotometrical analysis, by the Folin–Ciocalteu assay, in accordance with Chanthasri *et*

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al., 2018. Gallic acid was used as the standard. Initially 1ml of tenfold diluted Folin-Ciocalteu reagent was mixed with 120 µLof the extract (2.5 mg/mL concentration) and left for 5 minutes. The mixture was then added with 1ml of 20% w/v Na_2C0_3 (sodium carbonate) solution and mixed well with a vortex and then kept in dark for 90 minutes at room temperature. Absorbance of sample and standard were taken at wavelength of 725 nm using UV-Vis spectrophotometer (Shimadzu Corporation). The total phenolic contents were calculated on the basis of the calibration curve of Gallic acid and expressed as Gallic acid equivalents (GAE), in milligrams per gram of the sample.

Determination of antioxidant assay

The radical scavenging activity of polyherbal extract was evaluated using DPPH assay, following the method described by Ahonsi *et al*., 2023. Different concentrations of Melats P (200–1000 µg) were prepared in methanol and the volume was adjusted to 100 µL. To each sample, 5 mL of 0.1 mM methanolic DPPH solution was added and incubated at 27 °C for 20 minutes in the dark. The absorbance was then measured at 517 nm. Gallic acid was used as the reference standard. From the inhibition percentage versus concentration curve, the IC_{50} value was calculated. The percentage radical scavenging activity was calculated using the formula:

% DPPH radical scavenging activity=Absorbance control (Absorbance control−Absorbance sample)×100

Determination of GC-MS analysis

For the identification of phytochemicals present in Melats P combination GC-MS analysis were used according to a method previously described by Kashif *et al*., 2025, using a GCMS-QP2020 NX system (Shimadzu Co. Japan), fitted with an autosampler. We injected a 0.3 µL sample into an Rtx-5MS capillary column measuring 30 m × 0.25 mm × 0.25 µm. Helium was used as the carrier gas, flowing steadily at a rate of 1.0 mL/min. All GCMS components, the injection port, ion source and transfer line were maintained at 250°C. We collected mass spectra over a range of 40–1000 m/z. The temperature of oven was kept at 40°C for 15 minutes and then increased gradually at a rate of 3°C/min until it reached 300°C. To identify the compounds, we compared the spectra we obtained with reference libraries.

PCOS model induction

Experimental animals

Healthy female virgin Wistar rats of varying weights (16-18 weeks old and 180-200g body weight) were obtained from the animal house at Dow University of Health Sciences and were housed at the Institute of Pharmaceutical Sciences, Jinnah Sindh Medical University in Karachi. All experimental procedures involving animals were conducted in accordance with institutional ethical guidelines and the ARRIVE guidelines. Throughout the experimental period, the animals were monitored regularly for general health, activity, feeding behavior, body weight, grooming, posture, and other signs of pain, distress, or illness. All rats were acclimated and housed in sterile polypropylene cages, with 3 animals per cage, under controlled environmental conditions, including a temperature of 23 ± 2°C, relative humidity of 60 ± 10%, and a 12h light/dark cycle, one week prior to the experiment. They were provided with a standard pellet diet and water ad libitum to ensure that any observed physiological changes were strictly attributable to letrozole induction (1mg/kg) and the subsequent polyherbal intervention

PCOS induction

Animals (n=40) were divided into two main groups for PCO induction and subsequently randomized into four experimental groups using an online random number generator (GraphPad QuickCalcs Randomize) to minimize selection bias and potential group-related confounding. All investigators were blinded during biochemical and histopathological analysis. Healthy adult female rats meeting the predefined age (16-18weeks), body-weight (180-200g) and health criteria were included priori before the commencement of the experiment, while animals showing illness, injury, or abnormal condition during acclimatization were excluded before randomization. Following allocation, exclusion was limited to death, severe illness requiring euthanasia, or predefined technical/sample-quality failure. All allocated animals completed the study and were included in the final analysis. Control group (n=10), received only 0.5% carboxymethyl cellulose (CMC) as the vehicle. PCOS model group (n=30) administered with letrozole (1 mg/kg) dissolved in 0.5% CMC, orally once a day for 21 days. Through Microscopic evaluation vaginal smears were prepared and observed to ensure that PCOS was successfully induced in rats. Prajapati *et al*., 2022. The letrozole-only model was selected to isolate the formulation's effects on the endocrine-ovarian axis and to avoid the confounding systemic inflammatory variables associated with high-fat dietary interventions.

Study design

After the confirmation of PCOS induction, at 22nd day, rats were allocated to the following groups:

Group I (n=10) the control group, received 1 mL of normal saline for 28 days, while the remaining 30 rats that underwent PCOS induction were further divided randomly into three treatment groups (n = 10 per group):

Group II, the PCOS-induced group, received 1 mL of distilled water for 28 days.

Group III, the standard group, recieved metformin (350 mg/kg/day) orally mixed in 0.5% CMC for 28 days.

Group IV, the treatment group, received the polyherbal formulation Melats P (500 mg/kg body weight) orally mixed in 0.5% CMC for 28 days.

After the 28-day treatment period, the animals (n = 7) were fasted for 12 hours and then anesthetized with an

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intraperitoneal ketamine and xylazine (10/5 mg/kg) injection (Suri *et al*., 2024). Blood samples were collected from the aorta for biochemical and hormonal estimation and afterward, the animals were subjected to necropsy and the ovaries were excised for histopathological evaluation (right ovary) and PCR analysis (left ovary). Remaining (n=3) animals from each group were kept for reproductive studies (Fig. 1).

The primary measured outcome was the change in serum FSH levels following treatment with Melats P. body weight, Fasting glucose, insulin, oxidative-stress biomarkers, and other sex hormones were assessed as secondary outcomes.

Physical measurements: All animals body weights were monitored in the beginning and then after every 7 days interval throughout the experiment.

Examination of vaginal smears: Vaginal smears were taken daily from all rats at 9:00 am to check estrous cycle using vaginal swab method. A cotton-tipped swab soaked in physiological saline was used in the rat's vagina. The swab was gently rotated against vaginal wall and the removed. The collected cells were then evaluated under a light microscope after transferring the cells to a dry glass slide and staining with methylene blue dye (Prajapati *et al*., 2022; Rajan *et al*., 2017).

Biochemical and hormonal analysis: B-Ject gel clot activator vacuum tubes, filled with aseptic blood samples, drawn from the aorta for biochemical and hormonal assessment. An Eppendorf centrifuge was used to centrifuge the samples for ten minutes at 4000 rpm. The separated serum was kept at -80°C until further analysis. Serum biochemical parameters including serum lipid profile, sex hormone-binding globulin (SHBG), Anti-Müllerian Hormone (AMH), FSH, LH test and serum insulin were estimated from the separated serum on day 49 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 standard enzymatic colorimetric assay methods following the manufacturers' protocols. While the HBA1c, sex hormones, AMH, SHBG and serum insulin levels were assayed by using an enzyme-linked immunosorbent assay (ELISA) upon the manufacturer's directions (Roche Diagnostics, Basel, Switzerland) (Suri *et al*., 2024).

Oxidative stress evaluation: SOD (Superoxide Dismutase), Glutathione peroxidase (GPX) and MDA (Malondialdehyde) were measured by the kit obtained from Glory Science Co., Ltd. (Glory Science Co.2022#45) (Kashif *et al*.2025).

Ovarian histopathology: Ovaries from each rat of all groups were excised after study completion and fixed in 10% buffer formalin solution. After fixation, paraffin blocks were made and 5 µm thick slices were cut using microtome. Slides were prepared and stained with eosin and hematoxylin stains (Younas *et al*., 2022).

Ovarian histomorphology: Histomorphological analysis was performed using microphotographs captured with a Scientico STM-50 microscope provided with a Celestron MA411101 camera connected to a computer where the images were transferred and analyzed using Image J software (Haslan, M.A *et al*., 2021).

Quantitative RT-PCR analysis

Total RNA extraction was done from ovarian tissues using TRIzol reagents (Catalog# 15596026, Thermo Fisher Scientific, USA) according to the manufacturer's protocol, followed by RNase-free DNase 1(1U/ µL), to remove any DNA contamination, purified samples were then stored at -80°C. A NanoDrop spectrophotometer (ND-1000, USA), was used to measure RNA purity and concentration. cDNA (complementary DNA) was synthesized from 1 µg of total RNA using a reverse transcription Kit (Catalog# K0221 Thermo Fisher Scientific, USA) according to the manufacturer's guidelines. Targeted genes includes insulin receptor (INSR) androgen receptor (AR) Superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Cytochrome P450 (CytP17A1). mRNA expression level of targeted genes was quantified compared to β-Actin as a housekeeping refrence gene using PCR master mix (Maxima Syber green, Catalog No. K0221 Thermoscientific, USA). Quantitative Real-time (RT) PCR was performed in a 25µL reaction volume. The program was set as follows: initial polymerase activation at 95ºC for 10 min, followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing at 60ºC for 30 seconds and extension at 72ºC for 60 seconds. 2-ΔΔCT method was used to calculate relative gene expression levels between control and treated groups. Reactions were run on an Agilent thermal cycler (Agilent, USA) and data acquisition and analysis were performed using AriaMx System Software version 17.1 (di Masi *et al.*, 2022). The primers sequence for polymerase chain reaction (PCR) are listed below in Table 1.

Reproduction studies

After completing the 28-day drug treatment and then subjected to biochemical and histopathological evaluations, the remaining twelve female rats were utilized for the reproductive study. Females in all the groups were individually housed with a male for mating in a 1:1 mating ratio for up to one week. Vaginal smears were examined for the verification of pregnancy. After the confirmation of conception, the pregnant female rats were separated and housed individually under controlled conditions. The female rats gave birth naturally during 21-23 days gestation period. Litter size (number of pups born), fertility index and survival rate of pups, were included in the fertility and reproductive parameters, recorded for evaluation (Balasubramanian A *et al*., 2023).

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Table 1: Primers sequence for quantitative polymerase chain reaction (PCR) gene expression analysis:

Gene	Forward	Reverse
INSR	5′-TCT TCG AGA ACG GAT CGA GT-3′	5′-CAC AAA CTT CTT GGC GTT CA-3′
SOD	5′-ATGGGGACAATACACAAGGC -3′	5′-TCATCTTGTTTCTCGTGGAC-3′
GPX	5′-GCAATCAGTTCGGACATCAG -3′	5′-CACCGGGTCGGACATACTT-3′
AR	5′-CTAGTCTCAACGAGCTTG -3′	5′-GGGGTTATCTGGAGCC-3′
CytP17AR	5′-ACTGAGGGTATCGTGGATGC-3′	5′-TCGAACTTCTCCCTGCACTT-3′

Fig. 1: Experimental design and timeline

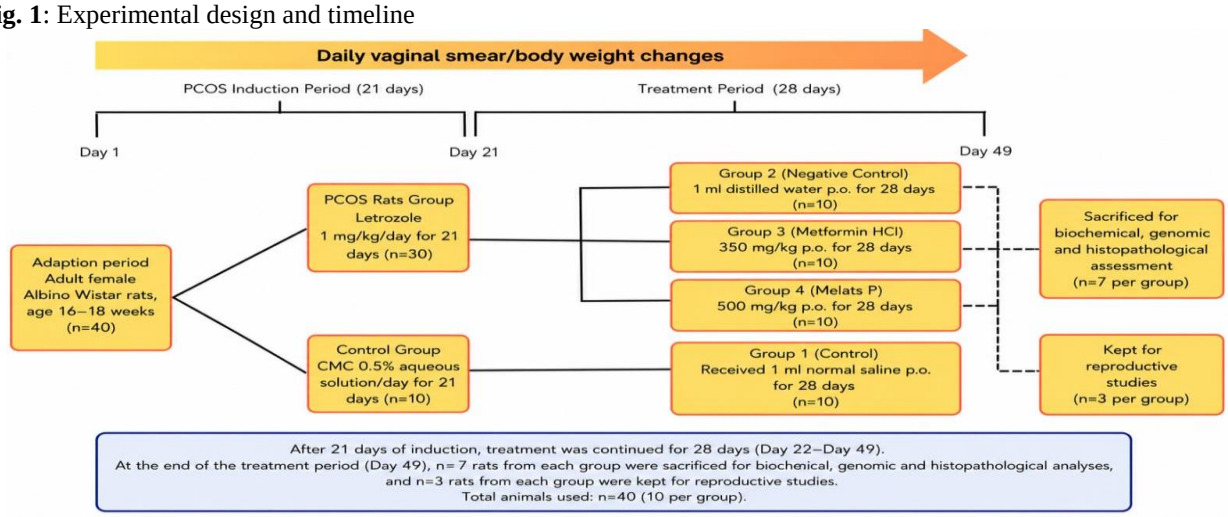

Fig. 1: Experimental design and timeline.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics (Version 20.0). The data are presented as the Mean ± Standard deviation (SD) and analyzed using One-way analysis of variance (ANOVA), followed by Tukey's Post Hoc comparison test. The results were considered statistically significant if the value lies between $P < 0.05$ and highly significant when $P < 0.01$.

RESULTS

Total phenolic content

The total phenolic content in the polyherbal extract using the Folin-Ciocalteu reagent is expressed in terms of Gallic acid equivalent, GAE as mg of GA/g of extract The content of phenolics was calculated from the linear regression equation of Gallic acid standard calibration curve (Y = 1.012x; R2 = 0.996) and was found to be 16.72 mg/g GAE indicating that the polyherbal formulation contain bioactive compounds with antioxidant activity (Table 2).

Table 2: Phenolic content of the polyherbal formulation Melats P:

Sample	Total phenolic content mg/g GAE
Melats P	16.72

Antioxidant assay

The antioxidant activity of the polyherbal formulation Melats P was evaluated using the DPPH radical scavenging assay and compared with, Gallic acid used as standard. The DPPH scavenging activities were recorded in terms of % inhibition and IC_{50} at 0.5 mg/mL concentration, as shown in table 3. The DPPH assay showed that polyherbal formulation, Melats P exhibited 69.28±3.42% free radical inhibition with an IC_{50} value of 25.21±0.023 µM indicating moderate antioxidant activity, compared to the standard.

GC-MS analysis of polyherbal formulation Melats P

The active principles with their names, class, retention time (RT), concentration (peak area %) and molecular formula concentration (peak area %) are presented in table 4 and mass spectra of identified compounds are presented in S1 file. GC-MS analysis of the polyherbal extract resulted in the identification of 31 compounds including flavonoids, phenolics, fatty acids, sterols, terpenoids and saturated and unsaturated fatty acids. The main compounds identified were Alpha-linolenic acid (ALA), an omega 3 PUFA (20.53%), arachidonoyl amide (12.99%) and Docosahexanoic acid (DHA), another omega 3 PUFA (9.02%). Unsaturated fatty acids include Methyl 11-octadecenoate (1.32%), E-9-Tetradecenoic acid (MUFA, 1.17%) and cis-5, 8, 11-Eicosatrienoic acid, methyl ester (PUFA, 0.64%). Whereas, palmitic acid methyl ester (7.65%) and pentadecanoic acid (4.35%) are saturated fatty acids detected in GC-MS. Other fatty acid derivatives such as methyl stearate (3.85%), palmitic amide (2.24%), amd stearic amide (0.99%) were also present. Gallic acid (0.8%), a polyphenol and leuco pelargonidin-3-glucoside (1.54%), a flavonoid was also identified. Detected terpenoids includes Lanosterol (7.8%), 15α-hydroxyculmorin (2.54%) and squalene (0.23%).

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Table 3: DPPH antioxidant assay of the Polyherbal formulation Melats P:

Sample	Conc.(mg/mL)	% Inhibition (Mean ±SD)	IC_{50} ± SEM (µM)
Standard (Gallic Acid)	0.5	95.3±5.21	3.69±0.001
Melats P	0.5	69.28±3.42	25.21±0.023

Table 4: GC-MS analysis of polyherbal formulation Melats P:

Peak#	Name	Class	R. Time	Area%	Molecular formula
1	Methanethiol	Thiols (Mercaptans)	6.921	0.55	CH_3SH
2	Oxalic acid, isobutyl pentyl ester	Oxalate esters	13.305	0.28	$C_{11}H_{20}O_4$
3	Alpha-linolenic acid (ALA)	omega-3 PUFA	15.433	20.53	C_4H_7NO
4	Lanosterol	Triterpenoid	17.65	7.8	$C_{30}H_{50}O$
5	Methyl 8-methyl-nonanoate	Saturated fatty acid methyl ester (FAME)	22.357	0.38	$C_{11}H_{22}O_2$
6	squalene	Triterpenoid	23.276	0.23	$C_{16}H_{34}$
7	2-Bromo-2-phenylacetophenone	Aromatic ketone (acetophenone derivative)	24.816	0.62	$C_{14}H_{11}BrO$
8	Gallic acid	Polyphenol	26.115	0.8	$C_7H_6O_5$
9	Palmitic acid, methyl ester	Saturated fatty acid	26.858	7.65	$C_{17}H_{34}O_2$
10	Pentadecanoic acid	Saturated fatty acid	27.221	4.35	$CH_3(CH_2)_{13}COOH$
11	2-Diphenylacetyl-benzisothiazolinone-1,1-dioxide	Benzisothiazolinone sulfone	27.752	0.5	C21H15NO4S
12	Dihydro-aplotaxene	Diterpenoid alkaloid	28.497	5.38	$C_{17}H_{30}$.
13	Omega-6 Arachidonic acid methyl ester	Polyunsaturated fatty acid (PUFA) methyl ester	28.559	2.67	$C_{21}H_{34}O_2$
14	Methyl 11-octadecenoate	Unsaturated fatty acid methyl ester (FAME)	28.618	1.32	$C_{19}H_{36}O_2$
15	Methyl stearate	Saturated fatty acid methyl ester (FAME)	28.805	3.85	$C_{19}H_{38}O_2$
16	E-9-Tetradecenoic acid	Monounsaturated fatty acid (MUFA)	28.917	1.17	$C_{14}H_{26}O_2$
17	Eicosanoic acid	Saturated fatty acid	29.136	0.79	$C_{20}H_{40}O_2$
18	Palmitic amide	Fatty acid amides	29.301	2.24	$C_{16}H_{33}NO$
19	Palmitic acid chloride	Saturated fatty acid chloride	29.924	0.24	$C_{16}H_{31}ClO$
20	Glycidyl palmitate	Glycidyl fatty acid esters	30.328	0.82	$C_{19}H_{36}O_3$
21	Arachidonoyl amide	Long-chain fatty acid amide	30.9	12.99	$C_{20}H_{33}NO$
22	Stearic amide	fatty acid amide	31.111	0.99	$C_{18}H_{37}NO$
23	1,8,11,14-heptadecatetraene, (Z,Z,Z)-	Polyunsaturated hydrocarbon (alkene)	31.44	3.99	$C_{17}H_{28}$
24	Cis-4,7,10,13,16,19-Docosahexanoic acid	Omega 3 Polyunsaturated fatty acid (PUFA)	31.859 31.838	9.02	$C_{22}H_{32}O_2$
25	cis-5,8,11-Eicosatrienoic acid, methyl ester	Polyunsaturated fatty acid methyl ester	31.953	0.64	$C_{21}H_{36}O_2$
26	1,3-Bis(cinnamoyloxymethyl) adamantane	Adamantine derivative(polycyclic compound)	33.212	1.32	$C_{42}H_{48}O_6$
27	leuco pelargonidin-3 glucoside	Anthocyanin class, a sub-class of flavonoids	33.373	1.54	$C_{21}H_{24}O_{11}$
28	Trichloroacetic acid, undec-2-enyl ester	Trichloroacetate ester	35.584	1.46	$C_{13}H_{21}Cl_3O_2$
29	15.alpha.-Hydroxyculmorin	Sesquiterpenoid (terpenoid)	38.183	2.54	$C_{15}H_{26}O_3$
30	2-Methylphenanthrene	Polycyclic aromatic hydrocarbon	38.865	1.34	$C_{15}H_{12}$
31	Bufogenin	Steroid lactone (cardenolide)	39.067	0.28	$C_{24}H_{32}O_4$

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Table 5: Percentage of rats regaining normal estrous cycle during 28 day treatment

Groups	Day 0-7(%)	Day 7-14 (%)	Day 14-21(%)	Day 21-28 (%)
Control	100.00	100.00	100.00	100.00
PCOS	0.00	0.00	0.00	0.00
Metformin	14.29	28.57	57.14	100.00
MP 500 mg/kg	0.00	28.57	42.86	85.71

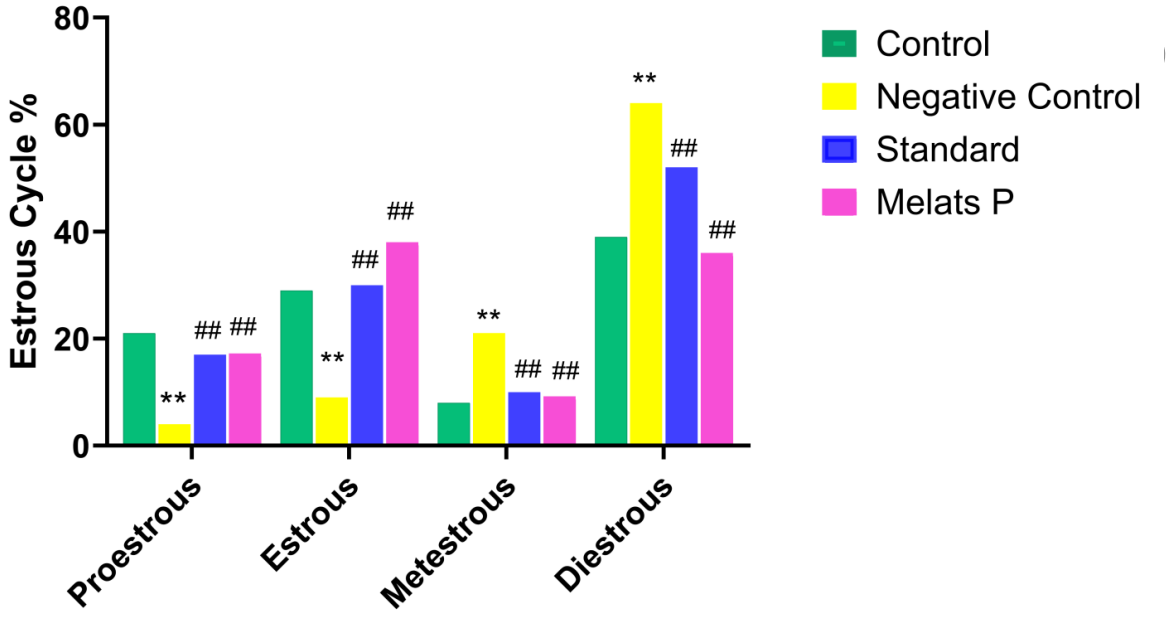

Fig. 2: Percentage of each phase of the estrous cycle after treatment with Melats P and Metformin for 28 days. Data are presented as Mean ± SD (n=10), analyzed with One-way ANOVA, followed by Tukey post-hoc test ***p<0.001 vs control and ##p<0.01, ###p<0.001 vs negative control.

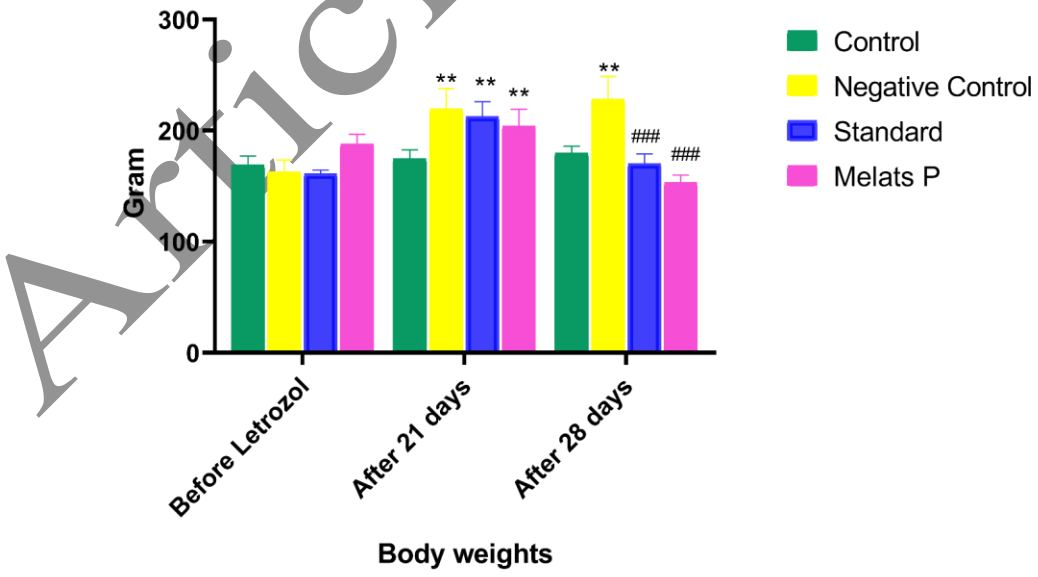

Fig. 3: Effect of Polyherbal formulation Melats P on body weight in the letrozole-induced PCOS rats. Data are presented as Mean ± SD (n=10), analyzed with One-way ANOVA, followed by Tukey post-hoc test. **p<0.01 vs control and ###p<0.001 vs negative control.

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Moreover, many hydrocarbons and ketones such as 2-Methylphenanthrene (1.34%) and 2-Bromo-2-phenylacetophenone (0.62%) were also detected along with a steroidal lactone bufogenin (0.28%) and an oxalic acid ester that trichloroacetic ester (1.46%).

Effect of Melats P on estrous cyclicity

Control rats showed consistent estrous cycles, while those with PCOS experienced an irregular estrous cycle pattern throughout the 28-day period. However, treatment with Melats P and metformin increased the percentage of rats displaying regular estrous cyclicity compared to the PCOS group, with the most significant improvement seen in the fourth week as shown in table 5.

After 28 days of treatment with Melats P and metformin, the regularity of estrous cycle was restored as the percentage of different phases of the estrous cycle of PCOS rats closely resemble those in the control group. Both treatments significantly ($p<0.001$) lowered the amount of time spent in the diestrous phase compared to the negative control (PCOS) group. Additionally, the estrous phase, which had been significantly ($p<0.001$) reduced after the induction of PCOS, was notably increase in both the Melats P and metformin groups when compared to the PCOS group (Fig. 2).

Body weight changes

The impact of letrozole and the following treatments on body weight is outlined in figure 3. Before starting letrozole, all groups had similar body weights. However, after 21 days of treatment with letrozole, body weight of the negative control and treatment groups elevated significantly ($p < 0.01$) compared to control. This indicates that weight gain is linked to PCOS induction. Following 28 days of treatment, both the standard and Melats P led to a significant ($p < 0.01$) weight reduction compared to the PCOS induced rats group, suggesting that Melats P treatment like the standard drug mitigated letrozole induced weight gain.

Metabolic profile

After experiencing hyperglycemia and insulin resistance due to Letrozole, 28 days treatment with the polyherbal formulation Melats P from day 21 to 49 led to significant improvements in metabolic parameters in PCOS rats, as shown in figure. 4. Melats P significantly improved metabolic profile ($p<0.001$) by reducing elevated serum glucose, insulin and HBA1c levels. Melats P effect on metabolic profile was comparable to normal control and metformin (standard control group), indicating its potential to reduce metabolic issues caused by letrozole.

Lipid profile

A significant ($p < 0.001$) increase in total cholesterol, triglyceride and LDL levels accompanied by a significant decrease ($p < 0.05$) in HDL levels was exhibited by PCOS rats compared to the control group, indicating dyslipidemia observed in PCOS. However, the rats co-administered with metformin and Melats P demonstrated a significant reduction in total cholesterol, triglyceride and LDL levels and a significant increase in the HDL levels as compared to that of the PCOS rats, suggesting that Melats P address lipid abnormalities associated with PCOS (Fig. 5).

Hormonal assay

In the negative control group (PCOS induced rats) FSH, estradiol and progesterone levels were significantly decreased ($p<0.001$) whereas LH, testosterone and prolactin levels were significantly increased ($p < 0.001$), however, after treatment with Melats P, the reverse effect was observed as FSH, estradiol and progesterone levels were increased although LH, testosterone and prolactin levels were found to be decreased, it was statistically significant ($p<0.001$) and was comparable to normal control and metformin (standard control group), indicating Melats P effectiveness in restoring hormonal imbalance in PCOS (Fig. 6).

Additionally, in the current study, a significant ($p < 0.001$) decrease in SHBG levels and rise in AMH levels were observed in PCOS induced rats, indicating elevation in free androgens and compromised ovarian function, characteristic pattern of PCOS. Moderate improvement was observed in standard (Metformin) control group. However, in the Melats P treated group a significant ($p < 0.001$) increase in SHBG levels and reduction in AMH levels were observed compared to the PCOS rats (Fig. 7).

Measurement of oxidative stress markers

In comparison, to control group rats, Letrozole-induced PCOS rats exhibited increased oxidative stress, characterized by significantly elevated ($p<0.001$) levels of MDA and reduced ($p<0.01$) antioxidant enzyme activities (SOD and GPx). Melats P treatment exhibit significant antioxidant effect ($p < 0.001$) by increasing the SOD and GPx antioxidant enzyme activity and reducing MDA levels in comparison to PCOS rats. These results highlight the antioxidant potential of Melats P in ameliorating oxidative stress associated with PCOS (Fig. 8).

Ovarian histopathology

In comparison to normal control group that shows normal ovarian structure, histopathological examination of ovarian tissues of letrozole induced PCOS rats revealed morphological alterations showing a considerable increase in the cystic follicles indicating a disruption in the normal folliculogenesis process. Standard control group (metformin treated) showed fewer cystic follicles and intact ovarian stroma. However, Melats P treated group displayed intact ovarian tissue with luteinized cells and cystic follicles appears to be resolved, indicating restoration in the normal folliculogenesis process. (Fig. 9)

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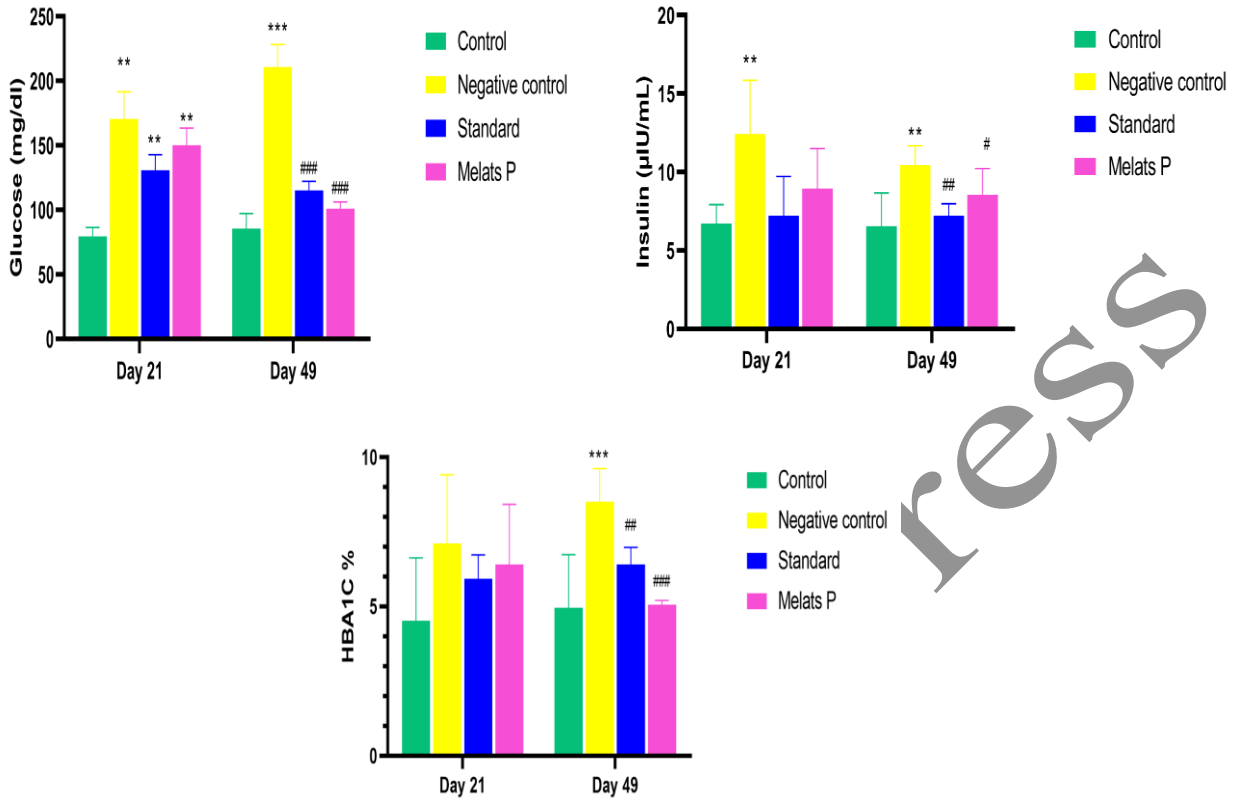

Fig. 4: Effect of Polyherbal formulation Melats P on metabolic profile in the letrozole induced PCOS rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. **p<0.01, ***p<0.001 vs control, and ###p<0.001 vs negative control.

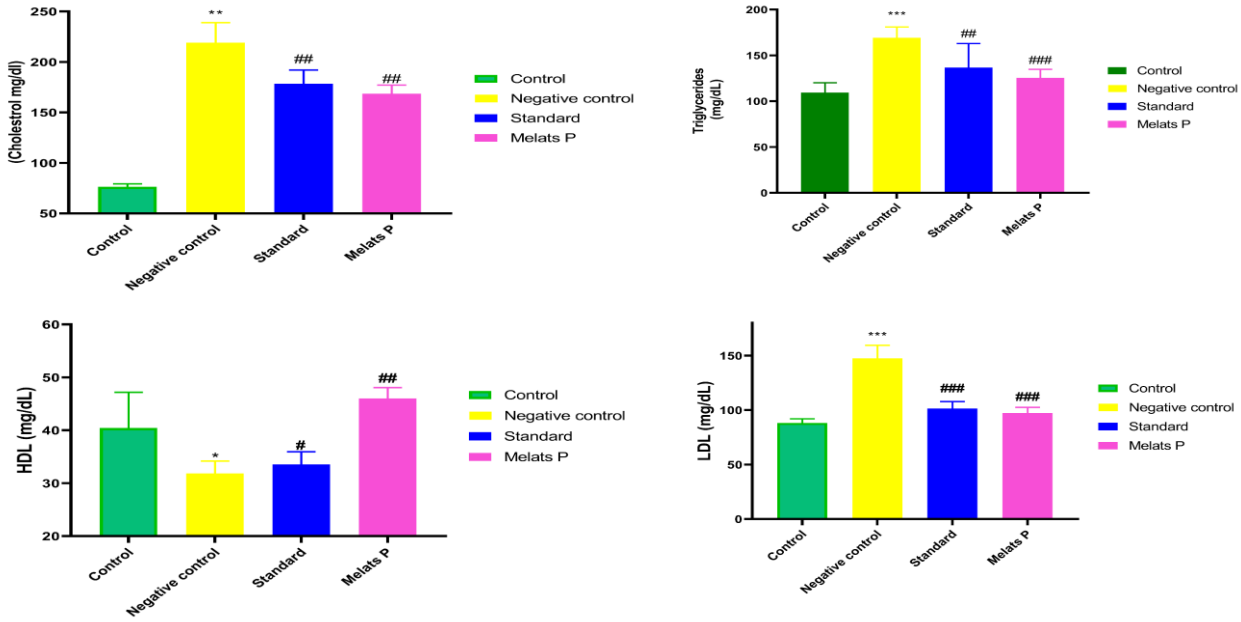

Fig 5: Effect of Polyherbal formulation Melats P on serum lipid profile in the letrozole-induced PCOS rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. ***p<0.001 vs control and #p<0.05, ##p<0.01, ###p<0.001 vs negative control.

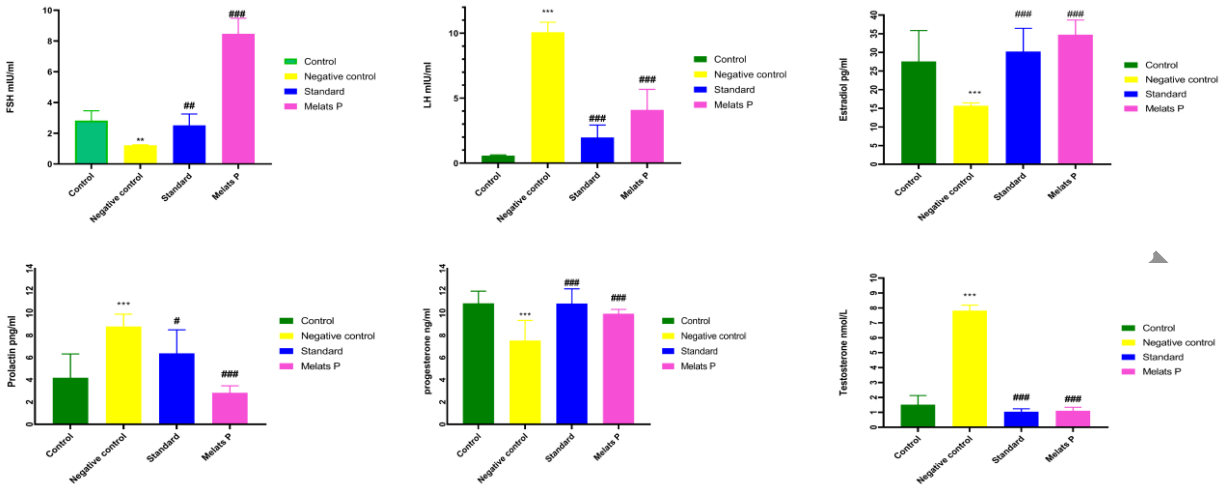

Fig. 6: Effect of Polyherbal formulation Melats P on serum sex hormones level in the letrozole-induced PCOS rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. ***p<0.001 vs control and #p<0.05, ##p<0.01, ###p<0.001 vs negative control.

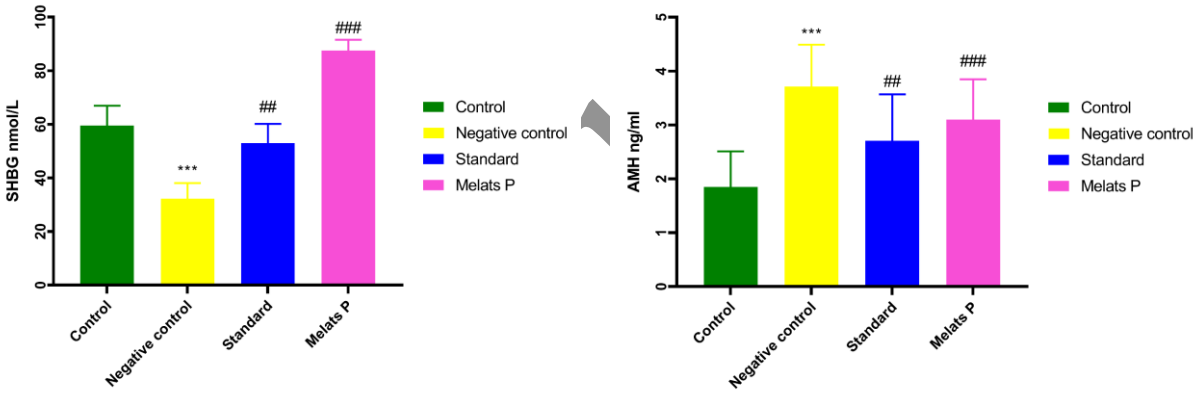

Fig. 7: Effect of Polyherbal formulation Melats P on serum SHBG and AMH levels in the letrozole-induced PCOS rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. ***p<0.001 vs control and ##p<0.01, ###p<0.001 vs negative control.

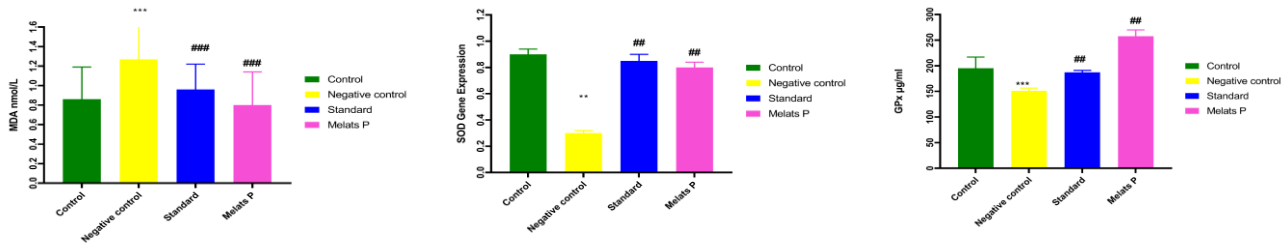

Fig. 8: Effect of Polyherbal formulation Melats P on oxidative stress markers in the letrozole-induced PCOS rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. **p<0.01, ***p<0.001 vs control, and ##p<0.01, ###p<0.001 vs negative control.

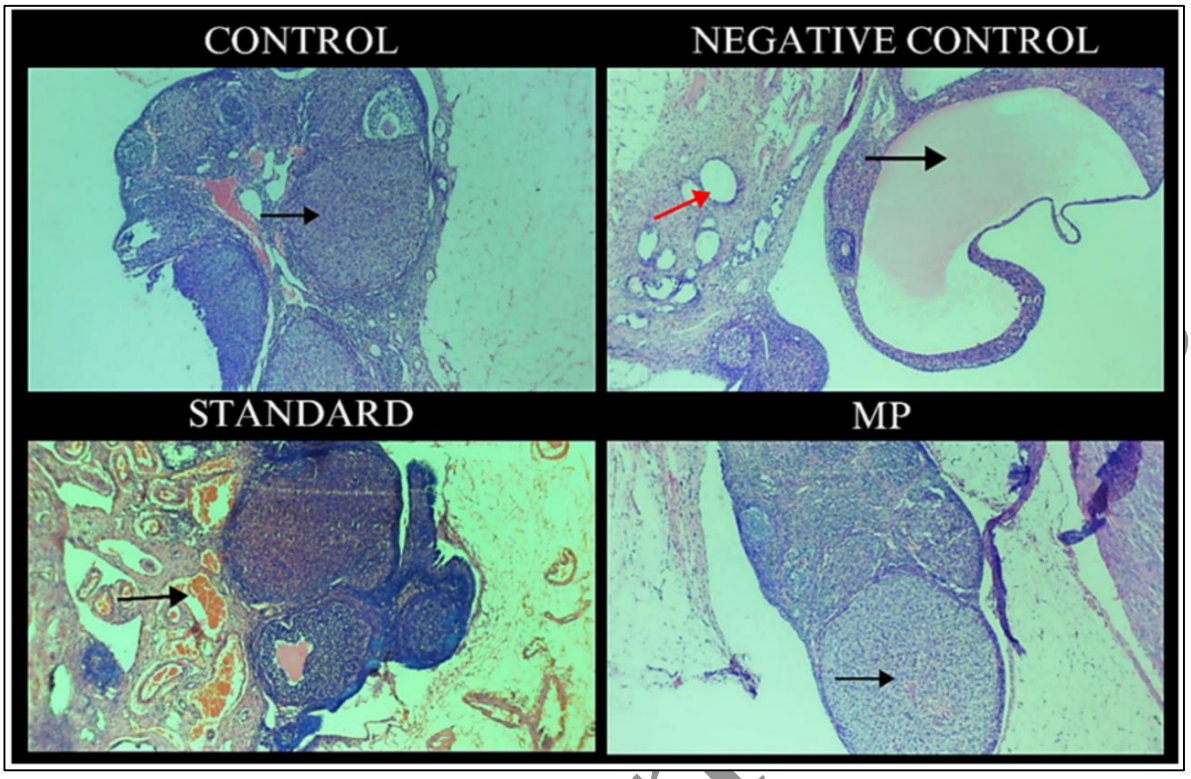

Fig. 9: Effect of the Polyherbal formulation Melats P on ovarian histopathology. A) Section from the normal control group shows normal morphology of ovarian follicles, with intact architecture and luteinization (Black arrow). B) Section from the negative control group shows ovarian stroma with one large cyst (black arrow) and cystic follicles (Red arrow). C) Section from the standard group shows reduced cystic follicles, intact ovarian stroma with some multiple congested blood vessels (Black arrow). D) Section from the Melats P (MP) dose group shows mostly intact ovarian tissue, focus of ovarian tissue shows luteinized cells (Black arrow).

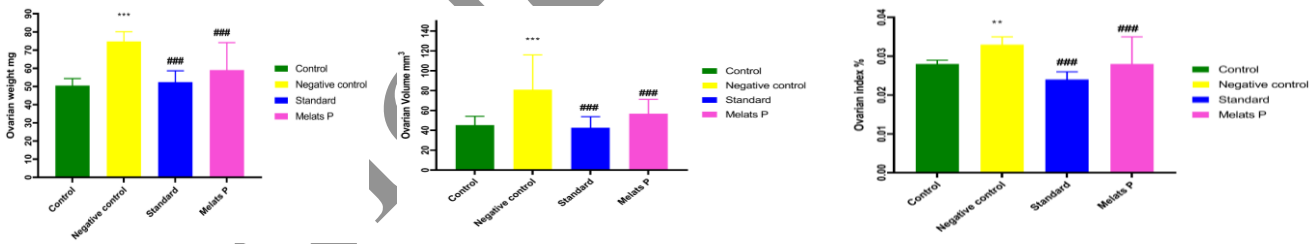

Fig. 10: Effect of Polyherbal formulation Melats P on ovarian parameters in letrozole-induced polycystic rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. **p<0.01, ***p<0.001 vs negative control. control and ###p<0.001 vs negative control.

Measurement of ovarian weight, volume and index

Fig. 10 shows the effect of Melats P on ovarian parameters in letrozole-induced polycystic rats. In the study, rats treated with letrozole as a negative control exhibited notable increases in ovarian volume, weight and index when compared to the control group, indicating changes consistent with polycystic ovaries. However, when these rats were given polyherbal formulation Melats P, there was a significant reduction in ovarian volume, weight and index ($p<0.001$). This suggests that the treatment had a restorative effect on the ovarian morphology.

Ovarian histomorphometry

Histomorphometric analysis, as shown in table 6 revealed disrupted follicular development in PCOS rats, with significant increase in primary, preantral, antral and atretic follicles and reduced pre-ovulatory follicles, corpora lutea and granulosa cells (p values ranging from $p<0.01$ to $p<0.001$). Treatment with Melats P and metformin restored folliculogenesis by reducing atretic follicles and increasing healthy and pre-ovulatory follicles. Both treatment groups also normalized granulosa and theca cell thickness, with Melats P showing the most pronounced improvement ($p<0.001$) in ovarian structure and follicular maturation.

Table 6: Effect of Melats P on ovarian histomorphometry in PCOS rats.

Parameters	Control	Negative control	Standard	MP
No. of primary follicles	3.00 ±2.30	8.14 ± 3.13***	6.00±2.163##	5.11±1.97###
No. of preantral follicles	4.86±2.03	6.43 ±2.99**	2.43 ±2.99###	1.05±1.29###
No. of antral follicles	1.43±0.78	2.43 ±1.61**	1.14 ±0.69###	1.29±1.25###
No. of pre-ovulatory follicles	1.00 ±0.57	0.43 ± 0.53**	1.00 ±0.00##	1.00±0.00##
No. of corpus luteum	4.71 ±1.97	2.86 ±1.34**	5.14 ±3.13###	4.86±1.06###
No. of atretic follicles	1.71 ±0.75	7.29 ±1.38***	4.43±2.44##	1.43±0.53###
No. of healthy follicles	13.57 ±2.50	6.86 ±3.97***	15.29 ±4.34###	16.71±4.07###
Corpus luteum area	0.48 ±0.64	0.20 ±0.21*	0.42 ±0.23##	0.41±0.11##
Thickness of granulosa cells	382.77 ±51.85	316.47 ±168.90**	396.14 ±99.89##	452.91±52.55###
Thickness of theca cells	266.07±40.37	291.05 ±109.19*	217.96 ±55.79###	219.86±36.31###

Footnote: Mean ± SD (n=3), #p<0.05, ##p<0.01, ###p<0.001 vs negative control.

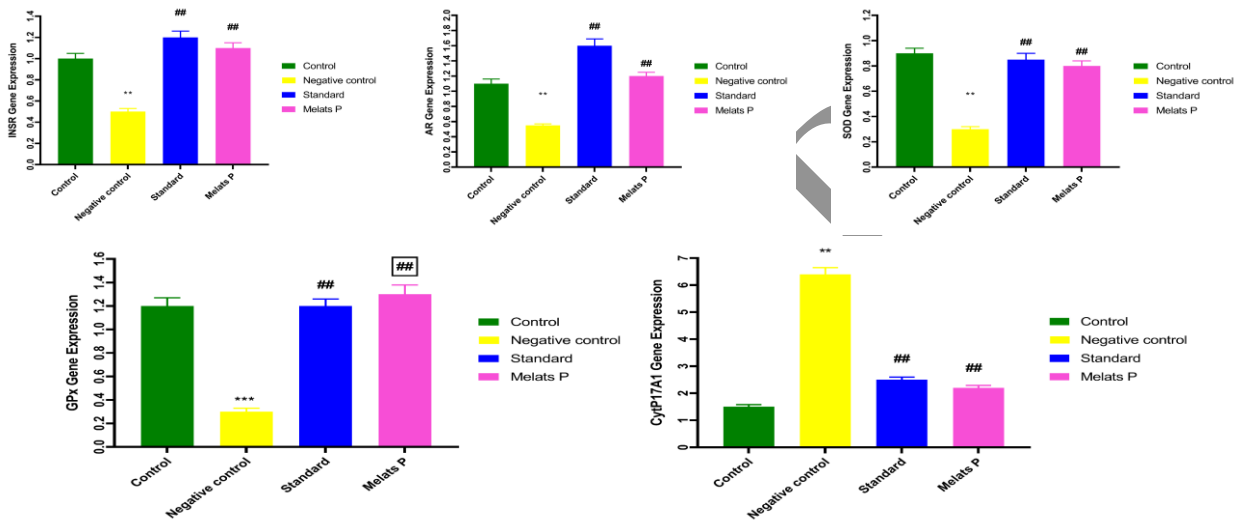

Fig. 11: Effect of Polyherbal formulation Melats P on mRNA expression of ovarian molecular markers in letrozole-induced polycystic rats. Data are presented as Mean ± SD (n=7), analyzed with One-way ANOVA, followed by Tukey post-hoc test. **p<0.01 vs control, and ##p<0.01 vs negative control.

Table 7: Effect of Melats P on reproduction in PCOS rats:

Parameters	Treated No. of pregnant rats	Litter size	Fertility index (%)	No. of pups delivered	No. of pups survived
Control	3	7.33	100	7.33±0.57	7.00±1.00
Negative control	0	0	0	0	0
Standard (Metformin 350 mg/kg/day)	3	4.33	100	4.33±1.52$^{##}$	4.00±1.00$^{##}$
MP	3	8.00	100	8.00±1.00$^{###}$	8.00±1.00$^{###}$
F value				43.48	51.66
P value				<0.001	<0.001

mRNA expression of ovarian molecular markers

To determine the impact of Melats P on mRNA expression of ovarian molecular markers and its potential effect against PCOS, the expressions of INSR, AR, SOD, GPx and CytP17A1 in the ovarian tissue were assessed at the RNA transcript level. The results indicated significant (p<0.01) upregulation of ovarian CytP17A1 and downregulation of INSR, AR, SOD, GPx in the PCOS group following letrozole administration compared to the control ovaries. However, following the administration of Melats *P* and metformin, these aberrations of mRNA expression were reversed, as observed in figure 11, significant (p<0.01) restoration of INSR, AR, SOD and GPx expression levels along with a marked reduction in CytP17A1 expression compared to negative control (PCOS) group were observed.

Evaluation of reproductive performance

Table 7 presents, rats that were given letrozole as a negative control showed complete infertility, with a 0% fertility rate, no pregnancies or pups at all. This confirmed that reproductive dysfunction was effectively induced. However, when treated with the polyherbal formulation Melats P, all the treated rats (3 out of 3) became pregnant, with a litter size of 8.0 ± 1.0 and 100% fertility index and all pups survived. This remarkable reproductive outcome not only showed a significant improvement over the negative control group but also surpassed the results from the standard Metformin group, which had a litter size of 4.33 ± 1.52 and an average of 4.00 ± 1.00 surviving pups. These findings suggest that Melats P is quite effective in reversing letrozole- induced infertility and improving the survival rate of offspring.

DISCUSSION

Polycystic ovarian syndrome is a common metabolic and endocrine disorder in women of reproductive age that can cause insulin resistance, excess androgens (male hormones), chronic anovulation and follicle development issues (Hossain *et al*., 2013). In order to address these challenges associated with PCOS, Melats P polyherbal tablets were prepared based on the ayurvedic relevance, traditional knowledge, and literature survey. Present study demonstrates the novelty of a multi-target Polyherbal formulation Melats P in letrozole induced rat's PCO model. Unlike most polyherbal interventions or PCOS herbal combinations as shown by Lee *et al*., 2025 that primarily focus on metabolic and hormonal modulation, Melats P simultaneously improves hormonal imbalance, metabolic parameters, ovarian morphology and reproductive and gene level outcomes. PCR findings, revealed coordinated modulation ovarian gene expression, including up-regulation of INSR, AR, SOD and GPx and suppression of CYP17A1 expression, insulin sensitivity androgen regulation and improvement of antioxidant defense system. GCMS profiling showed PUFA rich composition that mechanistically support these effects. Together these findings highlight Melats P as a distinct polyherbal formulation capable of holistic restoration of PCOS pathophysiology.

Phenolic component determination revealed Melats P, exhibited 16.72mg/g GAE of total phenolic content, suggesting the presence of bioactive compounds with antioxidant potential. Phenolic compounds belong to a numerous group of antioxidants and act via different modes, e.g., by 'scavenging' free radicals. Phenolic compounds can also enhance the activity of other antioxidants, for example that of fat-soluble vitamins (Salehi *et al.,* 2021). The antioxidant activity of Melats P is evaluated by DPPH assay that showed 69.28 % inhibition at 0.5 mg/mL concentration, this antioxidant effect is likely due to combined benefits of its herbal components such as *Momordia charantia* contains high levels of flavonoids and phenolics which are known for their potential antioxidant properties (Svobodova *et al*., 2017). Similarly, *Tribulus terrestris* exhibits strong free radical scavenging, cardio protective effects and reduce hyperinsulinemia and hyerglycemia (Saeed *et al*., 2024), whereas, *Allium cepa* is rich in flavonoids such as quercetin that enhance antioxidant and anti-inflammatory responses (Marefati *et al*., 2021). *Linum usitatissimum* (flaxseed) contains phenolic acids (p-coumaric, vanillic, sinapic and ferulic acids) and lignans, all of which have reported antioxidant and cardio-protective roles (Kocak, 2024). Likewise, *Symplocos racemose* bark extract is recognized for its free radical scavenging properties, specifically in alleviating lipid peroxidation and boosting the body's natural antioxidant defense (Acharya *et al*., 2016; Akbar 2020; Ghayas *et al.,* 2022). Furthermore, the GC–MS analysis of Melats P revealed 31 bioactive compounds, predominantly polyunsaturated fatty acids (PUFAs). The major components were omega-3 PUFAs, Alpha-linolenic acid (ALA) (20.53%) and docosahexaenoic acid (DHA) (9.02%), followed closely by arachidonoyl amide (12.99%), palmitic acid methyl ester (7.65%), dihydro-aplotaxene (5.38%) and pentadecanoic acid (4.35%). These PUFA are derived from, Flaxseed that contains high amount of α-linolenic acid, which serves as building block for docosahexaenoic acid (DHA) and omega 3 derivatives (Gutte *et al*., 2015) and *Momordica charantia* that contains PUFA and conjugated linolenic acids (Sharma *et al*., 2024). Omega-3 PUFAs, especially DHA, have been linked to improved insulin sensitivity, lower triglyceride levels and anti-inflammatory effects, which can help alleviate metabolic issues in PCOS (Albardan *et al*., 2024; Heskey and Jaceldo-Siegl, 2016). The terpenoid profile in Melats P featured lanosterol (7.8%) and squalene (0.23%) among the triterpenoids, while dihydro-aplotaxene (5.38%) and 15α-hydroxyculmorin (2.54%) were identified as diterpenoids and sesquiterpenoids. These compounds likely come from the bark of *S. racemosa*, which is abundant in pentacyclic triterpenoids (Acharya *et al*., 2016b). Research (Seetharaman B *et al*., 2024) suggests that terpenoids both have beneficial effects in managing PCOS by influencing hormonal and endocrine signaling (like reducing LH), as well as through their antioxidant and anti-inflammatory properties (Yuan *et al*., 2025).

The efficacy of the Melats P were evaluated on letrozole induced PCOS model that showed letrozole effectively replicated the clinical features of the PCO in animals, such as irregular estrous cycles, hyperandrogenism, metabolic issues, oxidative stress, ovarian cysts and fertility challenges (Kafali *et al*., 2004; Khosrowpour *et al*., 2023). As for the confirmation of PCOS, we monitored the estrous cycle which showed that the PCOS rats remained in a persistent diestrous state, which indicates disrupted ovulation. However, Melats P treated rats showed estrous

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cycle patterns (decrease diestrous phase and increase estrous phase), similar to the control group. Both Melats P and Metformin, helped restore regular estrous cycles, indicating normal ovarian function and regular ovulation. These results are consistent with prior research studies that herbal therapies as well as metformin helps in restoring estrous cyclicity in animals by modulating hormones and increasing insulin sensitivity (Zhang *et al*., 2017; Azin and Khazali, 2022; Lakshmi *et al*., 2023). Evaluation of metabolic parameters further underscores the therapeutic potential of Melats P in PCOS. Insulin resistance (IR) and various metabolic issues, are closely related to PCOS, with studies showing that about 55% of women with PCOS experience this, regardless of their body mass index (BMI) (Butt *et al*., 2022). Significantly higher levels of serum glucose, insulin and HbA1c levels were observed in negative control PCO group. Previously Hidayah FN *et al*., 2023 reported after letrozole administration the glucose and HbA1c levels were significantly elevated. However, treatment with Melats P for 28 days, led to notable improvements in glycemic control and insulin sensitivity, showing results similar to those of metformin. This effect highlights the polyherbal formulation potential in addressing IR, which is a crucial metabolic factor in PCOS (Shehab *et al*., 2023). Similarly Hyperinsulinemia linked to obesity which is another major contributor to the clinical manifestations of PCOS and women often increase weight as their most common concern. Insulin resistance and hyperandrogenism leading to obesity that has been observed in letrozole treated negative control animals, also evident by various studies that indicated animals elevates weights after letrozole administration. (Mirseyyed SF *et al*., 2023; Tiboni GM, *et al*., 2009).The primary goal in weight reduction is to control serum glucose, insulin resistance and sex hormonal regulation. We have found Melats P treated rats exhibited reduced weights probably by improving insulin sensitivity reduced serum glucose and hormones. The results are in accordance with Prajapati *et al*., 2022 whose formulation significantly reduce weights and improve insulin sensitivity. Similarly, dyslipidemia, another common feature of PCOS, was evident in the negative control group, with increased cholesterol, LDL and triglycerides and reduced HDL levels. Dyslipidemia in women with PCOS may be consistent with those found in the insulin-resistant state, including reduced HDL cholestrol and elevated triglycerides (Kim and Choi, 2013). Moreover, women with PCOS have been reported to exhibit higher levels of LDL-cholesterol and non-HDL-cholesterol compared to healthy controls, independent of BMI, suggesting that these lipid abnormalities are not solely a consequence of obesity but are strongly associated with the underlying endocrine and metabolic disturbances of PCOS. The results of the current study suggest that after 21 days of letrozole administration animal significantly exhibited dyslipidemia. Similar finding have been reported by Elisaf M *et al*., 2001, showing increased cholesterol and triglycerides levels after letrozole administartion. Melats P administration in PCO's rats corrected these abnormal lipid levels after 28 days of treatment. The herbal ingredients found in Melats P show great promise for addressing metabolic issues related to PCOS. For instance, *Momordica charantia* improves insulin sensitivity and lipid metabolism and is recognized for its antidiabetic, anti-hyperlipidemic and anti-obesity effects (Laczko-Zold *et al*., 2024). Moreover, research studies indicate that *Tribulus terrestris* can result in significant improvements in insulin resistance, lipid levels and body mass index (BMI) in women suffering from PCOS (Malik *et al*., 2024). Likewise, *Allium cepa* demonstrates strong hypoglycemic and hypolipidemic effects in animal and clinical research by reducing fasting glucose, cholesterol and triglycerides levels (Campos *et al*., 2003, Eldin *et al*., 2010). *Symplocos racemosa* bark also exhibits lipid lowering effects and improve metabolic parameters in letrozole induced PCOS model (Jadhav *et al*., 2013; Haidari *et al*., 2020; Arentz *et al*., 2017).

Hormonal imbalance plays essential role in PCOS diagnosis, marked by significant rise in serum testosterone, luteinizing hormone (LH), prolactin and reduced follicle stimulating hormone (FSH), estrogen and progesterone levels (Balasubramanian A *et al*., 2023), as shown in the current study all animals showed disturbed sex hormone profile in PCOS negative control rats. LH/FSH altered ratios and excess androgens levels are key features of PCOS that can result to anovulation and infertility in reproductive age women (Kamble *et al*., 2020). Whereas, decreased Sex hormone binding globulin (SHBG) levels, in PCOS elevate free testosterone, causing issues like oily skin, acne and hirsutism (Khattak *et al*., 2022). Another PCOS indicator is increased serum Anti-Müllerian hormone (AMH) levels, indicating enhance antral follicles activity (Butt *et al*., 2022). However, treatment with Melats P normalized all sex hormonal abnormality and successfully restored LH, FSH, prolactin, SHBG and AMH levels in treated rats. These findings align with earlier studies that found similar hormonal improvements through herbal therapy (Rashidi *et al*., 2021). The positive effects observed can likely be attributed to the combined action of M. charantia (which helps with insulin sensitivity), T. terrestris (which regulates steroid production), L. usitatissimum (a source of phytoestrogens) and S. racemose (known for its anti-androgenic and uterine tonic properties) (Hussain *et al*., 2022; Jadhav, 2013). Another reason for regulating the sex hormones is due to the presence of poly unsaturated fatty acids (PUFAs) in Melats P as PUFAs are abundantly found in flex seeds and other herbs used in the current study. Like Flaxseed (Linum usitatissimum) is a rich source of polyunsaturated fatty acids (PUFAs), particularly alpha-linolenic acid (an omega-3 fatty acid) and phytoestrogenic lignans. These constituents have been shown to influence reproductive physiology through multiple mechanisms (Tahir *et al*., 2026; Mohamed Abd El-Galil and Fathy Mohammed,

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2021). Beyond flaxseed, several of the selected herbs in our formulation have reported reproductive or endocrine modulatory properties. For example, *Symplocos racemosa* has demonstrated anti-androgenic effects and improvements in ovarian histology and hormone levels in PCOS animal models, suggesting potential support for fertility outcomes. *Tribulus terrestris* has been traditionally used for fertility support, with experimental evidence indicating favorable effects on reproductive parameters in both male and female models, potentially attributable to its steroidal saponins (Azeemuddin *et al*., 2019; Ghanbari *et al*., 2021).

Oxidative stress, a recognized factor in PCOS pathogenesis, impairs follicular growth and ovulatory function (Wang *et al*., 2020). Letrozole treated PCOS rats exhibited elevated MDA levels and decreased antioxidant enzyme (SOD, GPx) activity. These results are in accordance with Elmosalamy SH *et al*., 2022 who reported letrozole produces free radicals that produces oxidative stress and showed elevated MDA levels and reduced SOD and GPx levels. In contrast rats treated with Melats P showed significantly reduced MDA levels and increased antioxidant enzymes (SOD and GPx) activity. Previous studies reported that herbal formulations increases the level of antioxidants in the body via reducing oxidative stress and improving oxidative balance Svobodova *et al*., 2017. Melats P antioxidant effect may be derives from its constituent herbs containing high amount of omega 3 fatty acids, phenolic and flavonoid compounds (Svobodova *et al*., 2017; Marefati *et al*., 2021; Koçak, 2024). It has been reported that letrozole significantly altered histo-architectural changes in the ovary (Haslan M.A *et al*., 2021; Wang *et al*., 2020; Adelakun SA *et al*., 2022) as we observed altered ovarian histology. Whereas, treatment with Melats P restored ovarian morphology by significantly reducing all these parameters. These results align with previous studies on herbal treatments in letrozole induced PCOS models (Prajapati *et al*., 2022; Khosrowpour *et al* 2022). The significant reduction in cystic follicles and the restoration of corpus luteum formation following Melats-P treatment suggest normalization of folliculogenesis and resumption of ovulation, likely mediated through suppression of excessive androgen synthesis and rebalancing of gonadotropin signaling. Several components of the formulation, including Allium cep, *Tribulus terrestris* and *Symplocos racemosa*, have been reported to modulate ovarian steroidogenesis and normalize LH and FSH levels, thereby promoting granulosa cell proliferation and follicular maturation. Concurrently, *Momordica charantia* and *Allium cepa* possess insulin-sensitizing and antioxidant properties, which may reduce insulin-driven theca cell androgen production and oxidative damage to ovarian tissue. Additionally, *Linum usitatissimum,* rich in polyunsaturated fatty acids and phytoestrogens, may exert anti-inflammatory and estrogen-modulatory effects, contributing to improved granulosa layer thickness and follicular stability. Collectively, these mechanisms explain the observed decrease in theca cell hyperplasia, increase in granulosa cell layers and restoration of corpus luteum, findings that are consistent with previous reports on herbal interventions in letrozole-induced PCOS models (Prajapati *et al*., 2022; Khosrowpour *et al*., 2022).

In ovarian tissue of negative PCOS rats, PCR findings showed altered gene expression, with increased CytP17A1 expression and reduced INSR, AR, SOD and GPx expression, consistent with earlier reports on PCOS animal model: INSR (Balasubramanian *et al*., 2023), AR (Lim *et al*., 2017), CytP17A1 (Ortega *et al*., 2013; Elmosalamy *et al*., 2022) and SOD and GPx (Ijaz *et al*., 2024). INSR downregulation in PCOS induced rats, indicates insulin resistance (IR) (Balasubramanian *et al*., 2023). In PCOS, AR can be influenced by numerous factors and is important for follicular development and function of granulosa cells (Zeng *et al*., 2023). Further, in PCOS excessive reactive oxygen species (ROS) and mitochondrial dysfunction reduce antioxidant enzyme (SOD and GPx) activity, contributing to oxidative stress and ovarian dysfunction (Liu *et al*., 2025)**.** Moreover**,** in theca cells, elevated androgen levels characteristic of PCOS are associated with increased CYP17A1 gene expression, which encodes enzymes 17-α-hydroxylase and 17, 20-lyase essential for androgen synthesis (Alqadoori *et al*., 2025). PCR results of current study showed ovaries of both treatment groups significantly increased expression of INSR, AR, SOD, GPx and decreased expression of CytP17A1 when compared to PCOS rats. Findings showed Melats P effectively restored the dysregulated ovarian gene, these improvements likely arise from the synergistic and multi-targeted actions of its constituent herbs, described earlier for their insulin-sensitizing, antioxidant and androgen-modulating effects (Laczko-Zold *et al*., 2024; Azeemuddin *et al*., 2019; Malik *et al*., 2023; Hussain *et al*., 2022; Jadhav, 2013; Svobodova *et al*., 2017; Saeed *et al*., 2024 Marefati *et al*., 2021;). Further the restoration of ovarian gene expression observed following Melats-P treatment can be mechanistically correlated with the known pharmacological properties of its constituent herbs. The significant upregulation of insulin receptor (INSR) expression may be attributed primarily to Momordica charantia and Allium cepa, both of which possess well-documented insulin-sensitizing and glucose-lowering effects. Improved insulin signaling reduces insulin-mediated overstimulation of ovarian theca cells, thereby indirectly suppressing androgen overproduction, a central feature of PCOS pathology. Similarly the observed downregulation of CYP17A1 expression, a key enzyme governing ovarian androgen biosynthesis, can be linked to the androgen-modulating and anti-hyperandrogenic properties of *Tribulus terrestris* and *Symplocos racemosa*. These botanicals have been reported to normalize gonadotropin balance and ovarian steroidogenesis, leading to reduced theca cell hyperactivity

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and restoration of normal follicular progression. Suppression of CYP17A1 expression likely contributes directly to decreased androgen synthesis and alleviation of follicular arrest.

The significant increase in antioxidant gene expression (SOD and GPx) may be explained by the potent antioxidant and free radical scavenging activities of Allium cepa, Linum usitatissimum and Momordica charantia. Enhanced antioxidant defense mitigates oxidative stress–induced granulosa cell apoptosis and theca cell hyperplasia, thereby preserving follicular integrity and supporting luteinization. Reduction in oxidative stress is also known to improve ovarian responsiveness to gonadotropins.

The upregulation of androgen receptor (AR) expression observed in treated groups likely reflects restoration of physiological androgen signaling rather than pathological androgen excess. Linum usitatissimum, rich in phytoestrogens and polyunsaturated fatty acids, may contribute to hormonal receptor balance and improved steroid hormone sensitivity within ovarian tissue. Normalized AR expression, in conjunction with reduced CYP17A1-mediated androgen synthesis, facilitates appropriate follicular development and ovulatory competence.

The reproductive efficacy of Melats P was further assessed in letrozole-induced PCOS rats. In the negative PCOS group, both fertility index and delivery rate was absent showing no pregnancy, likely due to anovulation caused by hyperandrogenism and insulin resistance leading to the formation of multiple ovarian cysts (Zeng X *et al*., 2020). In metformin treated PCOS rats, low delivery rate was observed maybe due to presence of antral and atretic follicles, with reduced corpus luteum, as these mechanism relates reduction in the chances of conception (Balasubramanian A *et al*., 2023). However, treatment with polyherbal formulation Melats P significantly improved fertility index and reproductive performance compared to the standard treatment, indicating better reproductive outcomes, possibly through restoration of ovarian morphology and normalization of endocrine imbalance associated with PCOS. Similar results have been reported by other studies on herbal treatment in POCS (Moustafa *et al*., 2025; Radha *et al*., 2014; Srivastava *et al*., 2025).

Strengths and limitations

The present study is strengthened by the use of a letrozole-induced PCOS model that closely recapitulates endogenous hyperandrogenism observed in human PCOS, thereby offering greater translational relevance than exogenous hormone-based models. The polyherbal formulation evaluated in this work adopts a synergistic, multi-target therapeutic approach, addressing key pathogenic features of PCOS including hormonal dysregulation, oxidative stress and impaired ovarian folliculogenesis, which aligns with the multifactorial nature of the disorder and extends beyond the scope of single-agent therapies such as metformin. Despite the significant restorative effects observed in this PCOS model, several preclinical limitations must be acknowledged. First, the pharmacokinetic (PK) profile of the formulation, including the bioavailability and metabolic pathways of its primary PUFAs and phytochemicals, has not yet been elucidated. Second, while no overt adverse effects were observed during the study period, formal toxicological assessments are required to establish safety margins. Finally, although GC-MS provided a chemical fingerprint, further batch-to-batch standardization using validated chemical markers is necessary for clinical translation. These factors define the scope of our current findings as a proof-of-concept for the formulation's therapeutic potential. Furthermore, the analysis was qualitative; future studies employing LC–MS/MS and marker-based quantification are required for full chemical standardization.

CONCLUSION

The results of the current study reveal the effectiveness of the novel polyherbal formulation Melats P against letrozole induced PCOS rats. Melats P restored estrous cyclicity, improved hormonal profile and mitigated weight gain, oxidative stress, insulin resistance and dyslipidemia associated with PCOS induction. In conclusion, the polyherbal formulation demonstrated a significant restorative effect on the endocrine and metabolic profiles of Letrozole-induced PCOS rats, likely mediated by the synergistic action of its identified phytochemicals and PUFAs. However further pharmacokinetic, toxicological and standardization studies are essential to validate its safety and clinical applicability.

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Authors' contributions

Saba Zubair: Conceived and designed the study contributed to the formulation development, laboratory investigations, and data collection; Rabia Parveen Mangi: Supervised, designed and drafted the manuscript and developed the research methodology; Muhammad Ali Ghoto: Contributed to the study design and interpretation of the findings, and critically revised the manuscript to the final approval; Sadaf Naeem: Conceived, developed the research methodology, conducted the experimental work, collected and analyzed the data, interpreted the findings, and drafted the manuscript; Surriyya Sarwat: Performed histopathological analysis and interpreted the findings; Qalab Hussain: Provided administrative support for the study; Mah'd Musa Eid Abu Dhair: Data interpretation and

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Provided technical and logical support for the study; Zeeshan Ali: Provided administrative, technical and logical support for the study

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Data availability statement
All data generated or analyzed during this study are included in this published article and will be made available on request.

Ethical approval
The study protocol was in accordance with guidelines of ethical review committee (Ref # IRB/JSMU/2023/70) Institute of Pharmaceutical Sciences, Jinnah Sindh Medical University Karachi in January 2023 and adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. ((National Research Council, 2011). This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The authors declare no competing interests.

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