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Phytochemical Profiling, Antioxidant Activity, and Therapeutic Potential of Iris ensata Methanolic Extract in Letrozole-Induced PCOS Rat Models

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A B S T R A C T

Introduction: Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age, causing irregular menstrual cycles, infertility, and metabolic issues like insulin resistance and obesity. This study explores the potential of Iris ensata (IE) methanolic extract in managing PCOS, focusing on its antioxidant effects and impact on ovarian health.

Objectives: This study aims to evaluate the therapeutic potential of Iris ensata (IE) methanolic extract in managing PCOS, focusing on its antioxidant properties, effects on ovarian morphology and function, and modulation of key gene expressions related to ovarian health.

Methods: Iris ensata was analyzed via GC-MS to identify bioactive compounds such as alkaloids, flavonoids, and terpenoids. Antioxidant activity was assessed using DPPH, FRAP, H2O2 scavenging, lipid peroxidation inhibition, and SOD assays. PCOS was induced in female Wistar rats using letrozole, followed by treatment with IE extract or metformin. Histopathological and gene expression analyses were conducted to assess ovarian changes.

Results: GC-MS revealed a diverse phytochemical profile of IE, likely contributing to its antioxidant properties. Antioxidant assays showed significant, dose-dependent activity. In vivo, IE improved ovarian morphology and function, as seen in histopathological analysis. Gene expression studies supported these findings, showing modulation of genes critical to ovarian health.

Conclusions: Iris ensata methanolic extract shows potential in managing oxidative stress-related reproductive disorders like PCOS. Improvements in ovarian function, morphology, and gene regulation highlight its promise as a natural treatment. Further studies are recommended to confirm clinical relevance.

KeywordsAntioxidants, oxidative stress, Wnt pathway, phytochemicals, reproductive health

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine disorders affecting women of reproductive age, with an estimated incidence of 5-10% globally [1]. It is characterized by a combination of symptoms, including irregular menstrual cycles,

hyperandrogenism, and polycystic ovaries [2]. PCOS is often associated with metabolic disturbances such as insulin resistance, obesity, and dyslipidemia, and can lead to infertility, which makes it a major concern for women's health [3]. The pathophysiology of PCOS is multifactorial, involving genetic, hormonal, and environmental factors. The condition is frequently managed with pharmacological interventions, such as metformin and hormonal treatments like oral contraceptives or anti-androgens [4]. However, these treatments often come with side effects, prompting an increasing interest in exploring alternative therapeutic options, particularly from natural sources.

Among the numerous plants investigated for their potential therapeutic properties, *Iris ensata* (IE), commonly known as Japanese iris, has emerged as a promising herb due to its bioactive compounds. Iris species have a long history of medicinal use in traditional medicine systems, particularly in treating inflammatory conditions, infections, and metabolic disorders [5–7]. IE, in particular, is known to possess a range of pharmacological properties, including anti-inflammatory, antioxidant, and antimicrobial effects [8]. However, its potential as a treatment for PCOS has yet to be fully explored. In this study, we aim to evaluate the therapeutic effects of methanolic extract of IE in a PCOS-induced rat model, focusing on its impact on ovarian morphology, and gene expression profiles associated with ovarian dysfunction. Furthermore, gene expression studies focusing on key signaling pathways such as the Wnt/β-catenin pathway, which plays a crucial role in ovarian development and function, could provide valuable insights into the molecular mechanisms underlying the therapeutic effects of IE. The regulation of genes such as Wnt7a and Wnt3a, which are involved in follicular development and ovarian steroidogenesis [9], may be crucial in understanding how this plant extract influences ovarian health in PCOS. This study, therefore, aims to investigate the potential of IE as a natural therapeutic agent for PCOS, evaluating its antioxidant properties, its effects on ovarian tissue morphology, and its influence on gene expression. The results of this research could contribute to the development of alternative treatments for PCOS that are both effective and have fewer side effects compared to conventional therapies.

METHODS

Plant Sample Collection

IE was collected from high-altitude regions of the Sonamarg area, located in the Ganderbal district of Jammu and Kashmir, India. The plant specimen was identified by Dr. Tariq Ahmad, a senior taxonomist at the Department of Botany, University of Kashmir, through organoleptic and microscopic examination. Subsequent confirmation of the plant's identity was obtained by cross-referencing the collected specimen with data available on the online database, www.theplantlist.org.

Preparation of Methanolic Extract

The rhizomes of *Iris ensata* were first thoroughly cleaned and air-dried under shade. Afterward, they were mechanically minced using a grinder. The powdered plant material was extracted via cold maceration using absolute methanol as the solvent [10]. Specifically, the powdered IE was immersed in absolute methanol and subjected to continuous shaking for 72 hours at room temperature. This extraction process was repeated twice to ensure maximum yield of the bioactive compounds. Following the extraction periods, the plant material was filtered through Whatman No. 1 filter paper to separate the solid residue. The combined filtrates from each extraction were then concentrated under reduced pressure at 45–50°C to obtain a crude extract. The final extract was stored at 4°C for future use [5,6].

Phytochemical Screening

Phytochemical screening of IE extract was conducted to evaluate the presence of various bioactive compounds, including alkaloids, glycosides, terpenoids, tannins, flavonoids, carbohydrates, proteins, and glycosides. The extract was subjected to GC-MS analysis using helium as the carrier gas to identify the specific phytochemicals. The ion source temperature was set at 230°C, and the oven temperature was initially held at 80°C for 2 minutes, before being gradually increased to 280°C. The MS transfer line temperature was maintained at 250°C. Chromatographic data were acquired using the XCALIBER software, which facilitated peak identification and data analysis [8]. The resulting mass spectra of the components were compared against reference data from the National Institute of Standards and Technology (NIST) library to identify and characterize the individual compounds present in the extract.

Evaluating Antioxidant Activity

The antioxidant activity of the methanolic extract of IE was evaluated using a combination of assays, including DPPH radical scavenging assay, FRAP assay, H_2O_2 scavenging assay, Lipid Peroxidation inhibition assay, and SOD assay [5]. These assays assess the extract's potential to neutralize oxidative stress through different mechanisms, providing a comprehensive understanding of its antioxidant properties.

DPPH- free radical scavenging activity

A 0.025 g/L DPPH solution is prepared in methanol. The extract is mixed with varying concentrations (50, 100, 150, 200, and 400 µg/mL) of this solution. The reaction mixture is incubated at room temperature in the dark for 30 minutes to allow for the radical scavenging to occur. Absorbance is measured at 517 nm using a UV-Vis spectrophotometer [11]. The percentage inhibition of the DPPH radicals is calculated using the formula:

Radical Scavenging Activity (%) = ($Abs_{control} - Abs_{sample}$ / $Abs_{control}$) × 100

Abs control is the absorbance of the control sample (without the extract) and Abs sample is the absorbance of the extract solution.

Ferric Reducing Antioxidant Power (FRAP assay)

The FRAP reagent is prepared by mixing acetate buffer (pH 3.6), TPTZ (2, 4,6-tripyridyl-s-triazine), and ferric chloride. The FRAP reagent is mixed with different concentrations (50, 100, 150, 200, and 400 µg/mL) of the extract and incubated at room temperature for 30 minutes in the dark. Absorbance is measured at 593 nm, and the reducing power is quantified by comparing the sample's absorbance with that of a standard antioxidant (ascorbic acid) [5]. The FRAP value can be determined using the formula:

FRAP Value = (Abs $_{sample}$ – Abs $_{blank}$) / molar absorptivity coefficient of FRAP reagent

This provides the ability of the sample to reduce Fe^{3+} to Fe^{2+}.

Hydrogen peroxide scavenging activity

A 43 mM solution of hydrogen peroxide is prepared in phosphate buffer (0.1 M, pH 7.4). Varying concentrations of the extract are added to the hydrogen peroxide solution and incubated for 10 minutes at room temperature. Absorbance is measured at 230 nm, and the percentage scavenging activity is calculated by comparing the absorbance values of the test and control (without extract) samples [12].

Scavenging Activity (%) = (Abs $_{control}$ – Abs $_{sample}$ / Abs $_{control}$) × 100

Lipid per-oxidation Assay

Egg yolk homogenate is used as a lipid-rich medium, and lipid peroxidation is induced by adding ferrous sulfate (0.1 M). The extract is added at varying concentrations and incubated at 37°C for 30 minutes. The reaction is terminated by adding acetic acid (20%) and thiobarbituric acid (0.8%), followed by heating at 95°C for 1 hour. The absorbance of the resulting pink chromogen is measured at 532 nm [13,14]. Calculation of Inhibition:

Inhibition of Lipid Peroxidation (%) = (Abs $_{control}$ – Abs $_{sample}$ / Abs $_{control}$) × 100

Superoxide Dismutase (SOD) Assay

The assay utilizes pyrogallol auto-oxidation, with varying concentrations of the extract (50, 100, 150, 200, and 400 µg/mL) in 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM EDTA. The reaction is initiated by adding pyrogallol (0.2 mM), and the increase in absorbance at 420 nm is measured for 5 minutes [15]. The percentage inhibition of superoxide production is calculated using the formula:

SOD activity (% inhibition) = (1−Rate with extract/Rate of control) × 100

The IC50 value, the concentration of the extract required for 50% inhibition, was determined from the dose-response curve. Ascorbic acid served as a positive control. All assays were performed in triplicate, and the results were expressed as mean ± standard deviation. The IC50 values were determined using non-linear regression analysis. The data of antioxidant activities for each assay will be plotted with points and associated error bars. This methodology ensured a robust evaluation of the antioxidant potential of Iris ensata methanolic extract.

In Vivo Experimental Design

Female Wistar rats (aged 6-8 weeks, weighing approximately 190 ± 10 g) were obtained from the animal facility at IIIM, Jammu. The animals were housed in standard polypropylene cages under controlled environmental conditions, with temperature maintained between 20-25°C, relative humidity at 70-75%, and a 12-hour light/dark cycle [16]. The rats were provided ad-libitum access to a standard laboratory diet and water throughout the study. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) under certificate number FVSc/VCC-9/19/286/-87, and were conducted following institutional and national guidelines for the ethical use of animals in research.

PCOS Induction and Therapeutic Intervention Protocols

A total of 24 adult female Wistar rats were used in this study, with the animals randomly divided into four experimental groups, each consisting of six rats. The groups were treated with different interventions, including letrozole (LET), metformin (MET), and IE extract, to investigate their effects on PCOS and subsequent therapeutic outcomes. Group I (Control group), received normal saline (0.9% NaCl) and was provided a standard laboratory diet and water ad-libitum for the entire 42-day study period. PCOS induction or therapy was not administered in this group, serving as a baseline for comparison with the other experimental groups. Group II (PCOS control group), was administered letrozole orally at a dose of 6 mg/kg body weight for 21 consecutive days to induce PCOS. Following the induction phase, the animals were left untreated for an additional 30 days, serving as the positive control group to observe the natural progression of PCOS without therapeutic intervention. Group III (LET + IE extract), was administered letrozole orally at a dose of 1 mg/kg body weight for 21 days to induce PCOS. Subsequently, the animals were treated with an aqueous extract of IE at a dose of 125 mg/kg body weight for 30 days, to evaluate the therapeutic potential of IE in alleviating PCOS symptoms. Group IV (LET + MET), following letrozole-induced PCOS at a dose of 1 mg/kg body weight for 21 days, rats in this group received MET at a dose of 150 mg/kg body weight for 21 days. This group served as the standard treatment control, allowing for comparison between the effects of IE and a widely used pharmaceutical treatment for PCOS. Some animals from each group were sacrificed by cervical dislocation on the 21st day (Induction phase) and on the 42nd day (Treatment phase) of the experiment. Various parameters were then estimated to evaluate the effects of the treatments on PCOS [17].

Collection of Ovarian Tissue Samples

After the experimental period, animals from both the treatment and control groups were euthanized. Immediately following euthanasia, the ovaries were excised, rinsed in chilled 0.9% NaCl to remove any residual blood, and cleared of any surrounding connective tissue and fat. The cleaned ovaries were then blotted dry and sectioned into small pieces. These tissue fragments were placed in separate vials containing Bouin's fixative

for preservation. Each vial was labelled with the corresponding animal's identification number, treatment group, and date of fixation.

Histopathology

The fixed ovarian tissues were processed, embedded, and sectioned into 5 µm thick slices. The tissue sections were stained using Ehrlich's Haematoxylin and Eosin (Ehrlich, 1886) for histopathological examination. The stained sections were analysed for histological alterations using a compound microscope at magnifications of 100x and 400x. Microphotographs of representative tissue sections were captured using a digital imaging system.

RNA Extraction

Total RNA was extracted from ovarian, uterine, and hepatic tissues using the Trizol reagent (Life Technologies, Invitrogen, California, USA), following the manufacturer's protocol. RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). To ensure the removal of genomic DNA, RNA samples were treated with DNase I (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. Following DNase treatment, the RNA was reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit (Promega, India) and a thermal cycler (Bio-Rad, California, USA). Primers for the genes *Wnt*7a, *Wnt*3a, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer3 Plus software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi), with the primer sequences mentioned in Table 1.

Table 1: Primer Sequences of *Wnt3a, Wnt7a* and *GAPDH*

S. No.	Gene ID	Forward (3'-5')	Reverse (5'- 3')
1	*Wnt3a*	AGTCTCGTGGCTGGGTGGA	TTGGGCTCGCAGAAGTTAGG
2	*Wnt7a*	GCCCACCTTTCTGAAGATCAAG	TGGGTCCTCTTCACAGTAATTGG
3	*GAPDH*	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA

Quantitative PCR (qPCR) Analysis

Gene expression levels of *Wnt*7a, *Wnt*3a, and GAPDH were quantified by real-time quantitative PCR (qPCR) using the SYBR Green system (Promega, India) as per the manufacturer's instructions. qPCR was performed on a Rotor-Gene Q real-time cycler (Qiagen). The amplification protocol consisted of an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of amplification: denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds. The relative expression levels of *Wnt*7a and *Wnt*3a were normalized to GAPDH, providing insights into the regulation of these genes in the ovarian, uterine, and hepatic tissues under experimental conditions.

RESULTS

Phytochemical Analysis

The GC-MS analysis of Iris ensata methanolic extract identified 148 distinct peaks, indicating a diverse range of bioactive compounds such as alkaloids, flavonoids, terpenoids, saponins, tannins, phenols, and steroids. Major compounds included eucalyptol (6.7%), phthalic acid esters (6.9%), and bicyclo [2.2.1] heptane derivatives (7.3%). These bioactives were identified by matching mass spectral patterns with the NIST database and further characterized using CAS numbers, peak areas, and retention times. The chemical diversity suggests pharmacological potential, especially for conditions like PCOS.

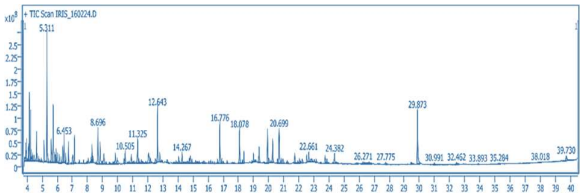

Figure 1: GC-MS Chromatogram of methanolic extract of Iris ensata

Table 2: Major Phyto molecules Identified in the Methanolic Extract of Iris ensata via GC-MS Analysis.

Molecule	Molecular Formula	Retention Time (Min)	CAS	Area (%)
Eucalyptol	$C_{10}H_{18}O$	4.153	470-82-6	6.7
2-Azido-2,4,4,6-Pentamethyl heptane	$C_{12}H_{25}N_3$	4.628	100029-29-0	6.6
Bicyclo[2.2.1]heptane-2-one, 1,7,7-trimethyl-, (1S)-	$C_{10}H_{16}O$	5.311	464-48-2	7.3
Benzene, 1,3-bis(1,1-dimethylethyl)-	$C_{14}H_{22}$	6.453	1014-60-4	6.6
Phthalic acid, isobutyl 4-octyl ester	$C_{20}H_{30}O_4$	20.23	100031 4-84-7	6.9
Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	20.69	628-97-7	6.0

Antioxidant Activity

The methanolic extract demonstrated strong, dose-dependent antioxidant activity in DPPH, H_2O_2 scavenging, lipid peroxidation inhibition, SOD, and FRAP assays. Higher concentrations (400 µg/mL) showed significant free radical scavenging and oxidative stress reduction. These results underline the extract's potent antioxidant capacity, crucial for managing oxidative stress-related conditions like PCOS.

Table 3: Mean ± SD values of antioxidant activity of the methanolic extract of IE evaluated using DPPH radical scavenging, H_2O_2 scavenging, lipid peroxidation inhibition, SOD activity, and FRAP assays.

Conc. (µg/mL)	DPPH Scavenging Activity (% ± SD)	H_2O_2 Scavenging Activity (% ± SD)	Lipid Peroxidation Inhibition (% ± SD)	SOD Activity (U/mL ± SD)	FRAP Value (µmol Fe^{2+}/mL ± SD)
50	21.5 ± 0.9	18.3 ± 1.2	16.7 ± 0.8	1.2 ± 0.1	5.2 ± 0.4
100	35.2 ± 1.1	31.1 ± 1.4	28.3 ± 1.2	1.9 ± 0.2	8.7 ± 0.5
150	50.4 ± 1.7	47.4 ± 1.8	40.2 ± 1.6	3.4 ± 0.3	11.3 ± 0.7
200	64.1 ± 2.3	63.2 ± 2.1	55.9 ± 1.9	4.6 ± 0.4	13.9 ± 0.8
400	85.3 ± 2.7	78.9 ± 2.5	74.4 ± 2.2	6.3 ± 0.5	2.6 ± 1.0

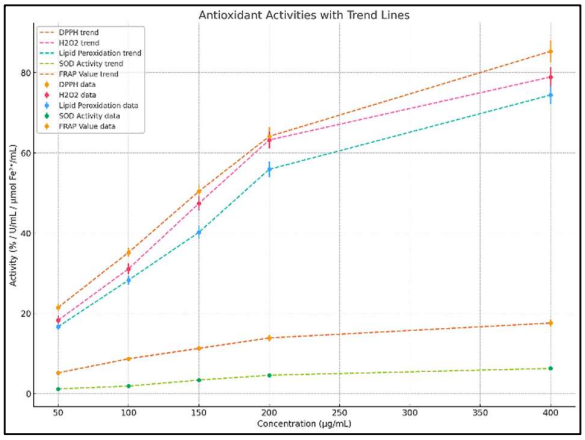

Figure 2: Graph illustrating a dose-dependent increase in DPPH scavenging, H_2O_2 scavenging, lipid peroxidation inhibition, SOD activity, and FRAP value, indicating stronger antioxidant effects of IE at higher concentrations.

Histopathological Analysis

Ovarian histology in untreated PCOS rats revealed cystic follicles, atretic structures, and disorganized granulosa layers, indicative of anovulation. Treatment with *Iris ensata* or metformin significantly restored normal ovarian morphology, with reduced cystic follicles and the presence of healthy developing follicles and corpus luteum. These findings suggest that IE ameliorates PCOS-induced ovarian dysfunction.

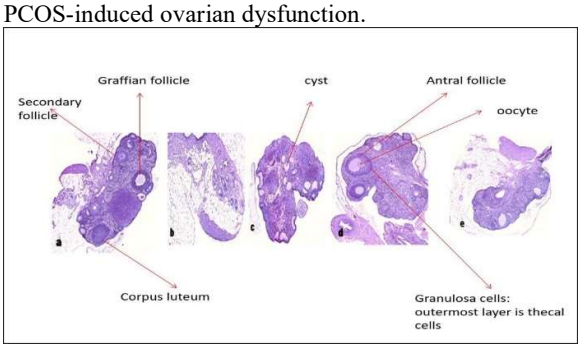

Figure 3: Histopathology of ovarian section (a) (Group I-Day 21); (b & c) (Group II-Day 21 and 42); (d) (Group III-Day 42); (e) (Group IV-Day 42).

Gene Expression Analysis

Expression levels of Wnt3a and Wnt7a, critical regulators of ovarian function, were significantly altered in PCOS rats. Treatment with IE or metformin normalized these expression levels, indicating the modulation of Wnt signaling pathways. This suggests a mechanism by which IE supports folliculogenesis and ovarian health.

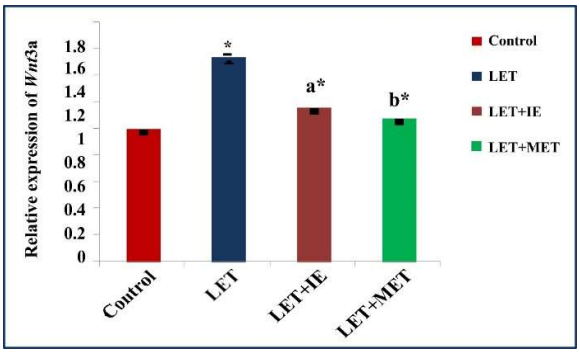

Figure 4: Wnt3a expression levels of control, LET, LET+IE and LET+MET treated female Wistar rats. ±SEM of six animals, *Significance difference ($p \leq 0.05$) compared to the PCOS control by one-way ANOVA, ** More significant difference (≤ 0.01) compared to the PCOS control by one-way ANOVA, *** Highly significant difference ($p \leq 0.001$) Compared to the PCOS control by one-way ANOVA.

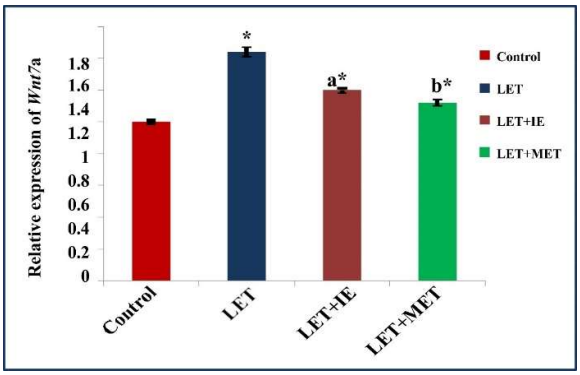

Figure 5: *Wnt7*a expression levels of control, LET, LET+IE and LET+MET treated female Wistar rats. ±SEM of six animals, *Significance difference ($p \leq 0.05$) compared to the PCOS control by one-way ANOVA, ** More significant difference (≤ 0.01) compared to the PCOS control by one-way ANOVA, *** Highly significant difference ($p \leq 0.001$) Compared to the PCOS control by one-way ANOVA

DISCUSSION

The phytochemical richness of Iris ensata, highlighted by the presence of flavonoids, terpenoids, and alkaloids, provides a basis for its pharmacological activity. Compounds like eucalyptol and phthalic acid esters are known for their anti-inflammatory, antioxidant, and potential antidiabetic effects, making the extract a promising candidate for PCOS management. Antioxidant assays confirmed IE's ability to neutralize free radicals and reduce lipid peroxidation, critical for mitigating oxidative stress—a key factor in PCOS pathogenesis. These findings align with previous studies emphasizing the role of oxidative stress in follicular dysfunction.

Histopathological improvements in the IE-treated group underscore its potential to reverse PCOS-induced ovarian damage. The observed changes in Wnt3a and Wnt7a expression levels further highlight IE's role in modulating molecular pathways critical for ovarian function, suggesting a dual mechanism of antioxidant activity and signaling pathway regulation. While metformin remains the standard treatment for PCOS, this study demonstrates that Iris ensata offers comparable efficacy with additional antioxidant benefits. This positions IE as a natural, multi-functional alternative or complementary therapy for PCOS. Future clinical studies are needed to validate these findings and explore its broader therapeutic applications.

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