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Cinnamon zeylanicum extract improves some metabolic disorders associated with polycystic ovary syndrome by modulating miR-21/ SIRT-1/GSK-3β pathways

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is a commonly occurring condition, influencing both metabolic and reproductive system-related functions. Hence, the objective of this research was to examine the possibility influence of Cinnamomum zeylanicum (CZ) on hormonal disorders, hyperinsulinemia, inflammation and some gene expressions in rats with PCOS pathogenesis. 50 female albino rats were classified into five groups: Control group: rats were received no medication. Induced PCOS Group: rats were treated daily with Letrozole at dose 1 mg/kg for 28 days. Metformin group: rats were given metformin at dose 150 mg/kg for 30 days after receiving Letrozole. Cinnamomum zeylanicum group: rats received Letrozole like group II and treated for 30 days with 200 mg/kg of the hydroalcoholic extract of CZ. Metformin + CZ group: rats given daily metformin and hydroalcoholic extract of CZ for 30 days after Letrozole administration. PCOS Rats exhibited a marked elevation of serum testosterone and LH hormone with a marked decrease of FSH hormone. A significant increase in insulin and glucose concentrations, augmented in TNF-α and IL-6 concentrations, upregulation of GSK-3β and miRNA-21 genes, and downregulation of SIRT-1 expression. Histopathological examination revealed multiple ovarian cysts with a notable absence of corpora lutea, increased stromal thickness, hyperplasia of the theca cells, and follicular arrest. Management of diseased rats with CZ extract showed noticeable improvement in all parameters. The findings indicate that cinnamonaldehyde in Cinnamomum zeylanicum extract has beneficial effects on hormonal imbalance, insulin sensitivity, and inflammation underscoring its potential as a treatment approach for PCOS.

Introduction

Polycystic Ovary Syndrome (PCOS) is a multifaceted endocrine disorder that poses significant challenges to reproductive health. Although extensively investigated in humans, there is increasing interest in understanding its effects on the reproductive systems of animals. The syndrome is typified by hormonal irregularities, including elevated androgen levels, imbalances in luteinizing hormone (LH), ovarian dysfunction, and the presence of polycystic ovarian structures. These disruptions result in fertility issues, abnormal estrous cycles, and reduced reproductive performance in animals, with notable impacts observed in livestock and laboratory species (Padmanabhan and Veiga-Lopez, 2014).

Understanding PCOS in animals is vital for improving reproductive management in agriculture and for using animal models to advance PCOS research relevant to human health. In livestock, PCOS poses significant challenges for reproductive efficiency, leading to economic losses in industries reliant on breeding. Animal models such as rodents, sheep, and non-human primates have been used extensively to study PCOS-related reproductive problems. Rodent models, particularly rats and mice, are commonly induced with PCOS-like conditions. These models replicate key reproductive traits of PCOS, including prolonged estrous cycles, cystic ovarian morphology, and reduced fertility (Ryu *et al*., 2019).

The study of PCOS in animals improves reproductive outcomes in livestock by identifying and mitigating factors contributing to infertility. Animal models provide a controlled environment to investigate the molecular and physiological underpinnings of PCOS. Findings from animal research have direct translational relevance, offering insights into potential therapies for PCOS in humans, such as ovulation induction protocols, dietary interventions, and novel pharmacological treatments (Stener-Victorin, 2022).This syndrome is also accompanied by many serious metabolic problems, including insulin resistance, diabetes, and heart disease (Rocha *et al*., 2019). According to many previous studies and research, there is no clear cause for the occurrence of polycystic ovary, but there are many factors responsible for pathophysiology of the syndrome, the most important of which are inflammation and Hyperinsulinemia, which are closely related to high androgens and ovarian dysfunction (Duleba and Dokras, 2012).

Previous studies have identified several factors contributing to inflammation, including elevated blood glucose and insulin levels, alongside tissue insulin resistance. Hyperglycemia leads to increased secretion of nuclear factor-κB (NF-κB), which heightens inflammatory responses associated with PCOS (Park *et al*., 2009). Additionally, genetic alterations in pro-inflammatory cytokines, such as C-reactive protein (CRP), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6), are major contributors to Hyperandrogenism and PCO syndrome (Lau *et al*., 2005).

Hyperinsulinemia contributes to various complex health issues, such as type 2 diabetes, dyslipidemia, PCOS, atherosclerosis, and hypertension (Bednarska and Siejka, 2017). In PCOS, hyperinsulinemia enhances androgen production in the ovaries. Insulin may act as a gonadotropin, directly stimulating ovarian recognition sites for insulin and IGF proteins or indirectly increasing the frequency and intensity of LH (luteinizing hormone) pulses (Ranjithkumar *et al*., 2019). Research shows that PCOS increased phosphorylation of serine at insulin recognition sites, leading to insulin resistance. Consequently, any factor that disrupts insulin signaling pathways supports the development of ovarian cyst syndrome.

On the other hand, MicroRNAs (miRNAs) are important mRNA expression regulators that have the proficiency to regulate an extensive range of targets, they may play crucial regulatory responsibilities in the majority of biological processes. MiRNAs investigation has offered a new insight into the post-transcriptional gene expression modulation (Ho *et al*., 2022). Furthermore, miRNAs have undergone extensive investigation due to their potential applications in diagnosing, predicting outcomes,

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and treating various conditions. These include type 2 diabetes, adiposity, cardiopathy, polycystic ovary syndrome. In ovarian cyst syndrome, miRNAs exhibit abnormal expression in granulosa cells, theca cells, follicular fluid, serum, adipose tissue, and peripheral blood leukocytes and when compared to individuals without PCOS. Consequently, miRNAs hold promise as valuable biomarkers for detecting and monitoring this disease (Nasser *et al*., 2024).

Hulsmans *et al*. (2011) indicated that there is a connection between miRNA-21, and some gene expressions as GSK-3β gene, and SIRT-1 gene in the occurrence of inflammatory processes, hormone metabolism disorders, and insulin signaling dysfunction, which supports the PCOS progression, this is one of our goals in this research.

There is no one drug available to treat PCOS. However, insulin sensitizers like metformin and thiazolidinedione derivatives are currently accessible therapy options that are frequently used to manage PCO Syndrome (Renato, 2015). A derivative of biguanide as metformin lowers insulin insufficiency, reduces hepatic capacity for glucose synthesis, and inhibits the manufacture of androgen by ovarian theca cells. Apart from these benefits, metformin can cause lactic acidosis, digestive issues, mood fluctuations, memory impairment, and renal function disorders (Yen *et al*., 2021). As a result, its extended use as a PCO Syndrome treatment may not be the most suitable choice.

However, some plants have been well-known and classified as "medicinal plants" for centuries because of their specific curative efficacy (Yaseen *et al*., 2019). In this regard, Cinnamomum zeylanicum (CZ), is a therapeutic plant with different medical properties that have been prescribed for gynecological, respiratory, and digestive disorders. (Ranasinghe *et al*., 2013). *Cinnamon zeylanicum* extracts contain polyphenols, alkaloids, saponin, tannins, flavonoids, cinnamic acid, cinnamonaldehyde and Hesperidin are considered the most active components according to Khodaeifar *et al*. (2019b). Cinnamaldehyde, the main chemical constituent of CZ, has also a powerful anti-diabetic, antioxidant, antibacterial, and anti-inflammatory characteristics (Dorri *et al*., 2018).

The target of this research was to investigate the possible impact of Hydroalcoholic extract of cinnamon on improving hyperinsulinemia, proinflammation targeting miR-21, SIRT-1 and GSK-3β gene expressions in PCOS rats induced by Letrozole.

Materials and methods

Ethical approval

The ethical approval was obtained from Ethics Committee in the Faculty of Veterinary Medicine at Benha University in Egypt, and adhered to the established ethical standards (Approval no. BUFVTM 23-02-23).

Drugs

Letrozole (Femara®(: Aromatase inhibitors produced by Novartis in Istanbul, Turkey, were utilized in this study. It is available in tablet form with a concentration of 2.5 mg of Letrozole per tablet. The tablets were dissolved in fresh water and taken orally at a dosage of 1 mg/kg for a period of 4 weeks, (Ibrahim *et al*., 2020). Metformin: Marketed as Glucophage® by Merck Santé in Germany, metformin was provided in tablet form at various concentrations. Freshly prepared metformin tablets were dissolved in 0.9% saline water as a vehicle and taken orally at a dose of 150 mg/kg for a period of 30 days, (ul haq Shah *et al*., 2022).

Preparation of Hydroalcoholic extract of Cinnamon zeylanicum

To prepare the *Cinnamon zeylanicum* (CZ) extract, around 0.5 kg of cinnamon bark was acquired. The extraction process followed the method described by Khodaeifar *et al*. (2019b). The final extract was then dissolved in isotonic saline to reach the desired concentration for the study.

HPLC analysis of Hydroalcoholic extract of Cinnamon zeylanicum

HPLC analysis was conducted using an Agilent 1260 series instrument, utilizing a Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm, 5 µm particle size). The mobile phase was composed of water (A) and acetonitrile containing 0.05% trifluoroacetic acid (B), flowing at a rate of 0.9 ml/min. A linear gradient was employed: starting at 82% A for 0–1 min, decreasing to 75% A for 1 to 11 min, then to 60% A for 11 to 18 min, and returning to 82% A for 18 to 24 min. Detection occurred at 280 nm with a multi-wavelength detector, and each sample was injected in a 5 µl volume. The column temperature was maintained at 40 °C during the analysis.

Determination of the Median Lethal Dose (LD50) of Cinnamon zeylanicum

The determination of LD50 of *Cinnamon zeylanicum* administered orally was carried out on Wister Albino rats according to IU *et al*. (2018) . For the determination of acute lethal dose (LD100) and median lethal dose (LD50) of *Cinnamon zeylanicum*, doses that were used are 100, 300, 500, 700, 1000 and 1500 mg/Kg body weight. Mortality was recorded after 24 h.

PCOS induction and confirmation

PCOS was induced in albino rats by using Letrozole at a dosage of 1 mg/kg B.wt. for 4 weeks according to Feyzollahi *et al*. (2021). Letrozole is a non-steroidal aromatase inhibitor, that hinders the conversion of androgens into estrogens within the ovaries. Our study confirmed the PCOS induction by the histopathology of ovary tissue samples from random rats that received Letrozole, stained by H and E stain before starting the treatment period.

Animals and Experimental Design

In our study, we used 50 adults female Wistar albino rats, distinguished by their wide heads, long ears, and tails shorter than their bodies, with an average weight of 200 to 250 grams and approximately 3 months old at the study's onset. These rats were obtained from the National Research Centre in Giza, Egypt. During the experiment, they were housed in separate cages and provided with a nutritious diet in a suitable environment. A two-week acclimatization period was allowed before the study began. The mature rats were segmented into 5 sets of 10 at random, housed in various cages, and then categorized as follows: Control group (C): rats received no medication. Induced PCOS Group (PCOS): rats were treated daily with Letrozole at dose 1 mg/kg B.wt. for 28 days. Metformin group (PCOS+M): rats were given metformin orally at dose 150 mg/kg B.wt. for 30 days after receiving Letrozole for 4 weeks. Cinnamomum zeylanicum group (PCOS+CZ): rats received Letrozole like group II and orally treated for 30 days with 200 mg/kg B.wt. of the Hydroalcoholic extract of CZ according to (Khodaeifar *et al*. (2019b). Metformin + Cinnamomum zeylanicum group (PCOS+M+CZ): rats given daily metformin and Hydroalcoholic extract of CZ orally for 30 days after Letrozole administration.

The Study Samples

Blood samples were collected from the retro-orbital venous plexus of rats after 58 days of experimentation. These samples were placed in plain tubes and incubated at room temperature for 30 minutes to allow clot formation. The sera were then isolated by centrifugation at 3500 RPM for 15 minutes, transferred into Eppendorf tubes, and stored at -20 °C for subsequent biochemical analyses. One ovary from each rat was preserved in a neutral 10% buffered formalin solution for pathological analysis, while the other was washed with distilled water and saline and stored at

-80 °C for gene expression analysis. For ovary tissue homogenate preparation, ovarian tissues were cut into small pieces and homogenized into nine volumes of ice-cold 0.05 mM potassium phosphate buffer (pH 7.4) to create a 10% homogenate. The homogenate was then centrifuged at 6000 RPM for 15 minutes, and the supernatant was used to assess gene expressions in the study (Shoaib *et al*., 2023).

Biochemical analysis

The quantification of serum testosterone was performed using ELISA kits (Demeditec Diagnostics, Germany) in addition to follicle-stimulating hormone (FSH), and luteinizing hormone (LH) in the serum was measured using ELISA kits (Cusabio Kit, China)(Abelson *et al*., 2016). Serum Glucose level was measured by colorimetric method according to Trinder (1969). Also, serum insulin Level was measured by a method based on enzyme-linked immunosorbent assay (ELISA) using the kit of Rat Insulin (Mercodia). (Khodaeifar *et al*., 2019a). Additionally, the level of inflammatory markers including tumor necrosis factor-α (TNF-α) and interlukin-6 (IL-6) were measured using the Sandwich ELISA technique in the sera (PerkinElmer Health Sciences, Inc., USA) according to Yudkin *et al*. (1999).

Molecular Analysis (RT-PCR)

The RNA extraction process followed the instructions provided by the RNeasy Mini Kit. To synthesize cDNAs, the Revert Aid TM First Strand cDNA Synthesis kit from Thermo Fisher Scientific was utilized. Real-Time quantitative PCR (qPCR) was utilized to analyze the molecular expressions of specific genes, including Glycogen Synthase Kinase-3 Beta (GSK3-β), Sirtuin-1 (SIRT-1), and miRNA-21. The qPCR was conducted using SYBR Green and specific primers as listed in Table 1. To determine The disparity in molecular expression across the RNA samples, the "ΔΔCt" method, as described by Yuan *et al*. (2006).

Table 1. Primers designed for reverse transcription-polymerase chain reaction (RT-PCR) of GSK-3β, SIRT-1 and miRNA-21.

Gene	Primers sequence (5'-3')	References
GSK-3β	TCG CCA CTC GAG TAG AAG AAA ACT TTG TGA CTC AGG AGA ACT	Sklepkiewicz *et al*. (2011)
SIRT-1	CAC-CAG-AAA-GAA-CTT-CAC-CAC-CAG ACC-ATC-AAG-CCG-CCT-ACT-AAT-CTG	Braidy *et al*. (2015)
miRNA -21	CGGCGGTAGCTTATCAGACTGATGT GTGCAGGGTCCGAGGT	Y. Wu *et al*. (2017)

Histopathological Examination

Ovaries from both control and experimental groups were collected and fixed in 10% neutral buffered formalin. After fixation, they were dehydrated, cleared with xylol, and embedded in paraffin. The tissues were sectioned at 5-µm thickness, stained with hematoxylin and eosin (H and E), and examined under a Nikon Eclipse E800 microscope (Japan) with an Olympus camera for imaging (Bancroft and Gamble, 2008).

Statistical Analysis

In our study, ANOVA (one-way analysis of variance) was employed to evaluate the differences in variables among the various sets. The data, presented as (Mean ± S.E.), underwent a one-way analysis of variance. Post hoc multiple comparisons between groups were conducted using Duncan's test in SPSS 25. The results of the study indicated that mean values marked with different superscripts are significantly distinct at a significance level of ($P<0.05$).

Results

HPLC report of the chemical compounds of Cinnamon zeylanicum hydroalcoholic extract

HPLC analysis of the Hydroalcoholic cinnamon extract identified cinnamaldehyde as the dominant compound, showing the highest peak. Cinnamaldehyde is crucial for the extract's ability to mitigate oxidative stress, inflammation, hyperinsulinemia, and hyperglycemia. Other compounds found in the extract include cinnamic acid, Gallic acid, Daidzein, Chlorogenic acid, Caffeic acid, methyl gallate, rosmarinic acid, and quercetin. The phytochemical composition is detailed in (Table 2), with the chromatogram shown in (Figure 1).

Table 2. HPLC analysis of the polyphenols of the CZ Hydroalcoholic extract.

	Cinnamon		
	Area	Conc. (µg/ml)	Conc. (µg/g)
Gallic acid	26.7	47.23	2361.4
Chlorogenic acid	7.6	19.72	986.14
Catechin	0	0	0
Methyl gallate	5.84	5.89	294.35
Caffeic acid	8.59	13.3	664.91
Syringic acid	1.72	2.51	125.73
Pyro catechol	0	0	0
Rutin	0	0	0
Ellagic acid	1.26	2.53	126.27
Coumaric acid	2	1.42	71.12
Vanillin	1.72	1.28	63.92
Ferulic acid	4.17	4.84	242.03
Naringenin	0	0	0
Rosmarinic acid	2.68	5.75	287.29
Daidzein	18.3	20.53	1026.27
Querectin	1.62	4.37	218.62
Cinnamic acid	278	99.57	4978.43
Kaempferol	0	0	0
Cinnamaldehyde	5864.98	5767.06	288352.84

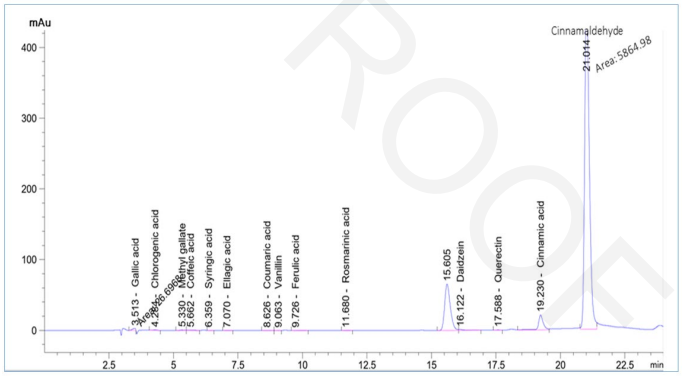

Fig. 1. HPLC chromatograms of the polyphenols of the CZ Hydroalcoholic extract.

Result of the Median Lethal Dose (LD50) of Cinnamon zeylanicum:

Mortality was zero and all doses used in LD50 determination were safe for rats. Our result was consistent with the results of IU *et al*. (2018) who documented that Cinnamon is safe for the dose used in this experiment. We used 200 mg/kg B.wt. of the Hydroalcoholic extract of CZ according to (Khodaeifar *et al*. (2019b).

Biochemical Results

Testosterone, Luteinizing Hormone and Follicle-stimulating Hormone

As showed in (Table 3) that Letrozole-treated rats had significantly elevated Testosterone and LH levels, with a marked drop in FSH compared to the normal group (P < 0.05). Metformin treatment lowered Testosterone and LH levels while increasing FSH. The Hydroalcoholic CZ extract normalized Testosterone, LH, and FSH levels more effectively than metformin alone. The combination of metformin and CZ extract offered the best hormonal regulation overall.

Table 3. Impact of Hydroalcoholic extract of CZ / metformin treatment on Serum Testosterone, LH, and FSH levels in all study groups.

Groups	Testosterone (ng/ml)	LH (mIU/ml)	FSH (mIU/ml)
GI: Control	0.24±0.09^{c}	0.25±0.03^{c}	0.53±0.05^{b}
GII: PCOS	1.13±0.09^{a}	1.21±0.12^{a}	0.13±0.02^{c}
GIII: PCOS + M	0.78±0.06^{b}	0.66±0.05^{b}	0.26±0.05^{c}
GIV: PCOS + CZ extract	0.73±0.10^{b}	0.35±0.05^{c}	0.64±0.04^{b}
GV: PCOS + M + CZ extract	0.61±0.06^{b}	0.30±0.06^{c}	1.19±0.09^{a}

Data are displayed as Mean ± Standard Error (S.E). Average results marked with different superscript letters within the same column are considerably diverse at P < 0.05.
PCOS: Polycystic Ovary Syndrome; M: Metformin; CZ: Cinnamomum zeylanicum

Glucose and insulin levels

PCOS group induced by Letrozole showed a Marked rise in glucose level when compared to control group (P < 0.05). Metformin reduced glucose level compared to PCOS group. CZ extract treatment caused more decrease in glucose level compared to PCOS group and Metformin group. The mix between metformin and CZ extract showed the most reduction in of glucose level compared to PCOS group, Metformin group and CZ group (Figure 2A). PCOS group showed a Noticeable increase (P < 0.05) in insulin level when compared to control group. Metformin treatment decreased insulin level when compared to PCOS group. CZ extract treatment caused more decrease in insulin level when compared to PCOS group and Metformin group. In addition to, the mix between metformin and CZ extract displayed the most reduction in of insulin level compared to other treated groups (Figure 2B).

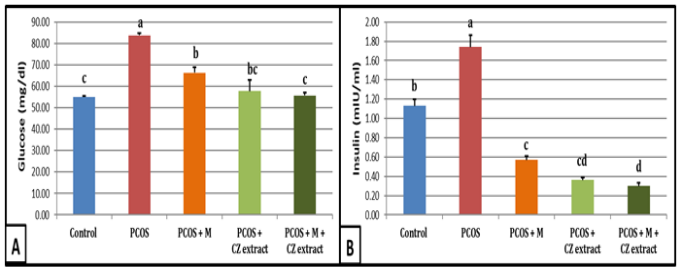

Fig. 2. Impact of hydroalcoholic extract of CZ and metformin treatment on Serum glucose and insulin levels in all study groups.

Tumor necrosis factor and interlukin-6

The PCOS group exhibited a notable rise (P < 0.05) in TNF-α levels when compared to the control set. Metformin reduced TNF-α level when compared to PCOS group. CZ extract treatment caused more decrease in TNF-α level when compared to PCOS group and Metformin group. Finally, the mix between metformin and CZ extract showed the most reduction in of TNF-α level compared to PCOS group, Metformin group and CZ group. The PCOS set demonstrated a noticeable rise in IL-6 level compared to the control set. Metformin treatment decreased IL-6 level when compared to PCOS group. CZ extract treatment caused more decrease in IL-6 level compared to PCOS group and Metformin group. In addition to, the mix between metformin and CZ extract showed the most reduction in of IL-6 level compared to other treated groups as described in (Table 4).

Table 4. Effect of hydroalcoholic extract of CZ and metformin treatment on serum TNF-α and IL-6 in letrozole induced - PCOS.

Groups	TNF-α (pg/mL)	IL-6 (pg/mL)
GI: Control	35.73±3.22^{c}	1.39±0.03^{d}
GII: PCOS	78.33±1.45^{a}	7.02±0.19^{a}
GIII: PCOS +M	62.24±2.37^{b}	4.26±0.17^{b}
GIV: PCOS +CZ extract	42.80±1.93^{c}	2.55±0.06^{c}
GV: PCOS + M+CZ extract	39.49±2.36^{c}	2.17±0.09^{c}

The data is displayed as Mean ± Standard Error (S.E). Average results marked with different superscript letters within the same column are considerably diverse at P < 0.05.
PCOS: Polycystic Ovary Syndrome; M: Metformin; CZ: Cinnamomum zeylanicum

Molecular Analysis

Our findings, as presented in Figure 3, indicate that rats treated with Letrozole demonstrated a substantial upregulation in the molecular expression of GSK-3β and miRNA-21 in ovarian homogenates, alongside a crucial downregulation in the molecular expression of SIRT-1, as opposed to the control group of healthy rats. Conversely, diseased rats treated with Hydroalcoholic extract of CZ and/or metformin displayed a vital downregulation in the molecular expression of GSK-3β and miRNA-21 in ovarian homogenates, accompanied by a significant upregulation in the SIRT-1 expression, in contrast to the PCOS control set. Average results marked with different superscript letters within the same column are considerably diverse at P < 0.05.

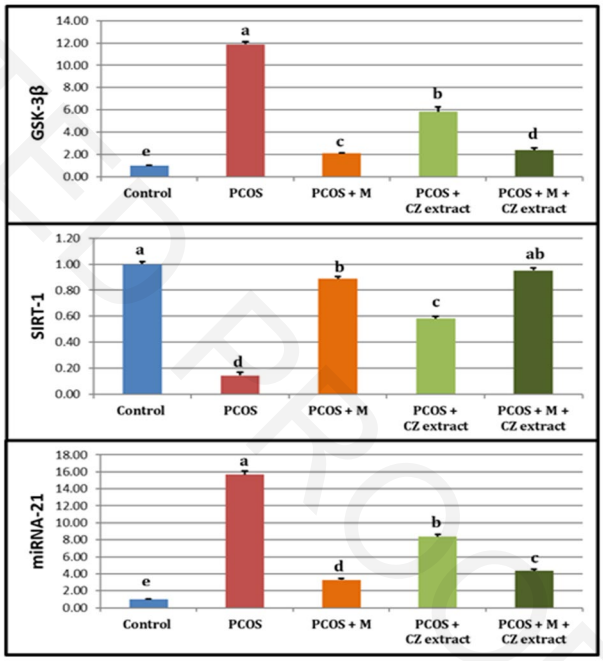

Fig. 3. Influence of hydroalcoholic CZ extract and metformin treatment on serum GSK-3β, SIRT-1 and miRNA-21 levels in all study groups.

Histopathological Examination

Comparison of the numbers of follicular cysts and corpus lutea in ovaries among control, PCO group, and the treated groups was illustrated in (Figure 4 and Figure 5) as followings: The ovaries from the control group showed normal structure, with primordial and growing follicles at various stages and several fresh corpora lutea (Figure 4A). In the polycystic ovary group, there were significant disruptions, including impaired follicle growth, reduced primary, secondary, and Graffian follicles, and notable follicular atresia with cysts (Figure 4B). Metformin treatment improved ovarian structure, increasing follicle growth and reducing cysts

(Figure 4C). Cinnamon treatment also reduced cysts and preserved ovulation (Figure 4D). The combination of metformin and cinnamon provided the best protection, showing complete absence of cysts and normal follicle development with fresh corpora lutea (Figure 4E).

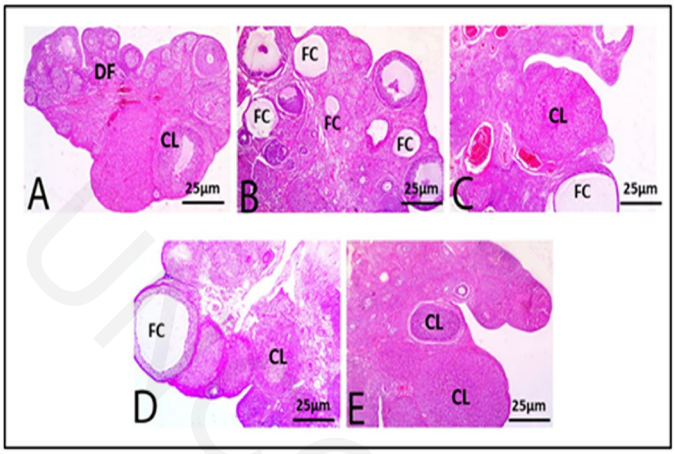

Fig. 4. Representative photomicrographs of ovaries in control (A) Normal ovarian structure with different stage developing follicles (DF) and Corpora lutea (CL), PCOS (B) increased count of follicular cysts (FC) with absence of Corpora lutea, PCOS + metformin (C) reduction in the count of follicular cysts and presence of Corpora lutea with congestion of ovarian blood vessels. PCOS + cinnamon (D) Marked decrease in the count of follicular cysts and presence of Corpora lutea with medullary edema. PCOS+ metformin + cinnamon -treated group (E) Different stage developing follicles and Corpora lutea. H and E stain X100.

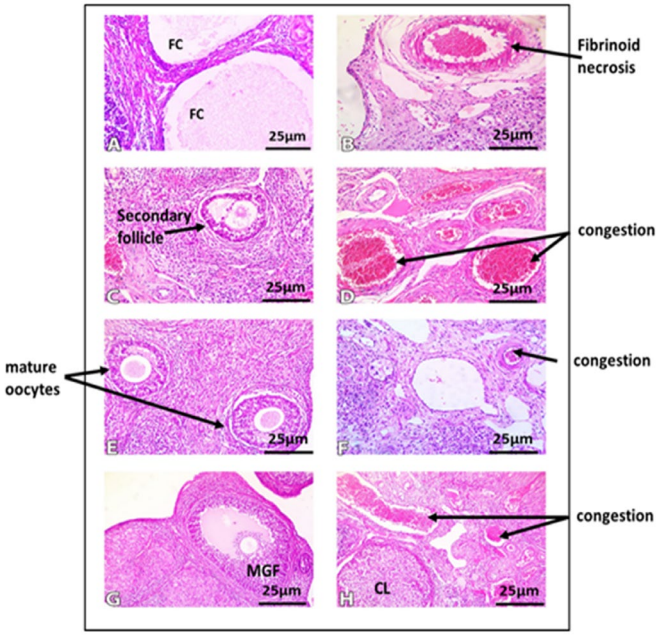

Fig. 5. Representative photomicrographs of ovaries in PCOS (AandB), A- follicular cysts (FC) with a thin granulosa cell layer B- Fibrinoid necrosis in the wall of ovarian blood vessels. PCOS + metformin (CandD), C- Secondary follicle surrounded by flat stromal cells. D- Severe congestion of medullary blood vessels with fibroplasia and focal edema. PCOS + cinnamon (EandF), E- Preovulatory follicle with mature oocyte surrounded by granulosa cells. F- Congestion, fibroplasia, edema in the medulla. PCOS+ metformin + cinnamon -treated group (GandH). G- Mature Graffian follicle (MGF). H-Congestion of blood vessels and thin connective tissue layer surrounded corpus lutea. H and E stain X200

In the polycystic ovary group, cystic follicles had a thin or absent granulosa cell layer with a large antral cavity (Figure 5A), and the absence of corpora lutea indicated anovulation. Some sections showed thicker, fibrotic stroma, fibrinoid necrosis in blood vessel walls, and severe vascular congestion (Figure 5B). Metformin-treated rats showed many growing follicles, secondary follicles with fluid-filled cavities, and an increase in corpora lutea, along with occasional congestion and edema in the medulla (Figure 5C and 5D). Cinnamon treatment revealed follicles at various developmental stages, with preovulatory follicles and medullary congestion (Figure 5E and 5F). The combination of metformin and cinnamon resulted in mature Graffian follicles with a cumulus oophorus, large fluid cavity, and well-structured granulosa layers. Vascular congestion and thin connective tissue around the corpus lutea were also observed (Figure 5G and 5H).

Discussion

This research presents a novel approach to addressing PCOS by exploring the prospective benefits of using Cinnamomum zeylanicum hydroalcoholic extract as a natural plant with Glucophage® for PCOS management.

Briefly, Metformin is a widely used treatment for PCOS. PCOS manifests with menstrual cycle disorders, high levels of androgens, and the formation of small ovarian cysts. Excessive insulin in the body is believed to be a contributing factor to PCOS development (Johnson, 2014). Metformin improves insulin sensitivity, thereby reducing circulating insulin levels and positively impacting adipose (fat) tissue. Although traditionally recommended for PCOS-affected individuals with a higher Body Mass Index (BMI), Metformin has shown efficacy even in non-obese individuals with anovulatory PCOS, potentially aiding in weight reduction and lowering the risk of diabetes (Renato, 2015). However, typical adverse effects involve gastrointestinal symptoms such as bloating, nausea, and diarrhea. Moreover, Metformin may interfere with vitamin B12 absorption in the small intestine, and in rare instances, diabetic patients may experience lactic acidosis (Yen *et al*., 2021).

Based on our research findings, the utilization of Letrozole led to a remarkable rise in the overall contents of testosterone and LH hormones, coupled with a reduction in FSH levels, which were indicative of the emergence of Hyperandrogenism in PCOS-afflicted rats. These outcomes are in accordance with the observations made by Wang *et al.* (2020), who noted that Letrozole effectively mimics the abnormal hormonal profile linked to PCOS in rats. The alterations in sex hormone levels are attributed to Letrozole, a non-steroidal agent that blocks aromatase activity, inhibits the androgen-to-estrogen transformation in the ovaries. This inhibition leads to increased testosterone levels and decreased estrogen production. Ovarian steroidogenesis typically relies on the interplay between the interna theca cells and granulosa cells, which are essential for both follicular growth and ovulation (Maganhin *et al*., 2013). Actually, androgens are produced from cholesterol within the thecal cells in response to LH and then transformed into estrogen hormone in granulosa cells stimulated by FSH. The LH receptor is responsible for converting pregnenolone and progesterone into dehydroepiandrosterone and androstenedione, are predominantly present in the theca interna cells. On the other hand, the FSH receptor and aromatase are expressed in granulosa cells, where the transformation of androgens to estrogens takes place (Akhtar *et al*., 2005). In PCOS conditions occur defects in the steroidogenesis pathway leading to increase the biosynthesis of androgens in both the ovaries and adrenal glands causing Hyperandrogenism. One key factor contributing to androgen overproduction in the ovaries is the heightened pulsatile secretion of LH. This elevated LH secretion influences the theca internal cells, prompting androgen synthesis (Roland and Moenter, 2014).

The administration of cinnamon extract resulted in the restoration of elevated testosterone and LH levels, as well as a decrease in FSH levels, when compared to normal rats. The primary approach to control the hormonal imbalance associated with PCOS is to reduce testosterone levels, leading to the normalization of gonadal hormones. Our study showed that the use of cinnamon extract achieved this goal by controlling factors associated with testosterone synthesis, in accordance with several previous studies. L. Chen *et al.* (2012) explained that Cytochrome P450c-17α, an essential enzyme with increased activity in PCOS, is a key catalyst in androgen production, which is directly stimulated by insulin in this condition. Cinnamomum zeylanicum (CZ) has the potential to lower plasma insulin levels, thereby downregulating the expression of Cytochrome P450c-17α. This downregulation may inhibit androgen synthesis, leading to the regulation of plasma gonadotropin hormone levels. Additionally, cinnamon extract can stimulate the liver to produce sex hormone-bind-

ing globulin, which helps reduce blood androgen levels. This finding aligns with a study by Hajimonfarednejad *et al.* (2018), who demonstrated a reduction in testosterone levels in PCOS-affected women following cinnamon supplementation. Furthermore, Qin *et al.* (2010) suggested that plants possessing antioxidant properties can result in a decrease in serum levels of gonadotropins and insulin. This aligns with the findings of Khodaeifar *et al.* (2019a), who similarly reported that the usage of Cinnamomum zeylanicum extract, owing to its antioxidant properties, had the potential to safeguard ovarian tissue against cellular oxidative injury.

In our study, significant increases in blood glucose and insulin measurements were observed, along with an upregulation of the GSK-3β gene in PCOS rats, indicating the presence of insulin resistance. This aligns with previous research suggesting that Letrozole can lead to insulin resistance and hyperglycemia (Maharjan *et al.* 2010). Hyperglycemia and hyperinsulinemia observed in PCOS may result from reduced protein binding receptor function, impaired glucose transport, or defects in other enzymes engaged in metabolic processes of glucose (Wang *et al.*, 2020). Insulin has two recognition sites for substrates, and when phosphorylation occurs at the serine sites, it inhibits insulin signaling. This phosphorylation also reduces the expression of the insulin-sensitive glucose transporter type 4 (GLUT4). The structure of the insulin recognition sites closely resembles that of the insulin-like growth factor 1 (IGF-1) binding site, enabling interaction between insulin and the IGF-1 binding site. When activated by insulin, the IGF-1 receptor enhances androgen synthesis in theca cells by increasing their responsiveness to LH for androgen production. It has been shown that insulin specifically affects steroidogenesis through receptors on both granulosa and theca cells (Tsilchorozidou *et al.*, 2004). Hyperinsulinemia, in turn, stimulates the ovaries to produce more enzymes responsible for androgen synthesis within theca cells and diminishes the creation of androgen- carrier globulin in the hepatocyte cells (Yao *et al.*, 2017).

The upregulation of the GSK-3β gene in Letrozole-induced PCOS rats may be explained by PCOS increasing the tyrosine phosphorylation of GSK-3β and impairing insulin-stimulated serine phosphorylation of GSK-3β (W. Chang *et al.*, 2008). This phosphorylation finding aligns with presence of a persistently hyper activated GSK-3β, which remains unaffected by insulin suppression, indicating a potential inherent (likely genetic) abnormality in adipocytes associated with PCOS. Considering GSK-3β's role in inhibiting glycogen synthase, these results correspond with previous indications of reduced glycogen synthesis in PCOS-cultured ovarian granulosa cells. Elevated GSK-3β activity, determined genetically, may thereby facilitate the progress of PCOS by fostering insulin unresponsiveness (Goodarzi *et al.*, 2007). GSK-3β could potentially impact PCOS by influencing androgen biosynthesis and/or activity. Studies indicate that in ovarian cells, increased expression of GSK-3β enhances the 17-hydroxylase activity of P450c17, a key enzyme involved in androgen synthesis; moreover, basal GSK-3β activity appears elevated in theca cells derived from PCOS patients (Ranasinghe *et al.*, 2013).

In our study, cinnamon extract showed a marked downregulation of GSK-3β expression and restored normal levels of blood sugar and insulin. Cinnamon boosts glycogenesis process by initiating glycogen synthase and suppressing glycogen synthase kinase 3β (Couturier *et al.*, 2011). Additionally, it reduces glucose uptake from the small intestine by enhancing glucosidase enzymes and inhibiting intestinal ATPase according to Adisakwattana *et al.* (2011). Furthermore, cinnamon contains polyphenolic compounds such as Rosmarinic acid and quercetin that exhibit insulin-like characteristics (Qin *et al.*, 2010), these active component are present in our extract according to HPLC report. Cinnamon-derived Procyanidin polyphenol type-A polymers participate in insulin receptor auto-phosphorylation and the inhibition of protein tyrosine phosphatase I. Consequently, cinnamon reduces insulin resistance and stimulates glucose uptake by enhancing the function of phosphatidylinositol 3-kinase (PI-3K) within the insulin transmission pathway, thus amplifying the effects of insulin (Qin *et al.*, 2003). Additionally, cinnamon plays a crucial role in augmenting tyrosine phosphorylation and minimizing phosphatase performances, which facilitates the activation of insulin receptors. This, in turn, leads to the triggering of insulin receptors. Cinnamon also elevates the levels of GLUT4 protein, insulin receptor β, and the activities associated with glycogen synthesis, resulting in decreased activity of GSK-3β (Mishra and Srivastava, 2022).

Our data revealed a significant elevation in the levels of TNF-α and IL-6 in PCOS rats caused by Letrozole as compared to normal rats. These findings align with the results reported by Yu *et al.* (2021). The administration of Letrozole led to the development of Hyperandrogenism, resulting in elevated serum levels of C-reactive protein, a substantial rise in the counts of lymphocytes plus monocytes, and the presence of these markers in the ovarian tissues of PCOS patients, as noted by Xiong *et al.* (2011). According to research by Li *et al.* (2021), excessive testosterone levels led to reduced macrophage viability, increased macrophage death, and the stimulation of TNF-α and IL-6 release as proinflammatory cytokines. These effects potentially contribute to the increased proliferation of theca-interstitial cells and the suppression of estradiol synthesis, which are associated with the development of PCOS, as suggested by Figueroa *et al.* (2015). Furthermore, studies conducted by Fernández-Real *et al.* (2000) indicated that IL-6 gene polymorphisms may elevate the risk of insulin resistance in PCOS patients. As an inflammatory cytokine, IL-6 has shown a positive correlation with obesity in women with PCOS, based on the existing body of evidence.

Epigenetic changes display a pivotal responsibility in the pathophysiology and development of ailments like PCOS. Furthermore, microRNAs, which are single-stranded RNA molecules without coding functions, contribute to epigenetic changes that impact gene expression by regulating the activity of two enzymes: DNA methyl transferases and histone deacetylases, as highlighted by Y.-H. Chen *et al.* (2013). Research conducted by Sirotkin *et al.* (2014) has identified numerous miRNAs that influence the progress of PCOS and hold potential as biological indicators. Notably, miRNA-21 has a relationship with adipose tissue development and inflammation, aligning with our own study results, as reported by Sathyapalan *et al.* (2015). Our study showed PCOS rats had a significant upregulation of miRNA-21 expression compared to normal rats. That result was aligned with Yu *et al.* (2021) who demonstrated that miRNA-21 expression is significantly elevated in PCOS. miRNA-21 enhances mRNA translation, resulting in increased secretion of TNF-α and IL-12, thus implicating it in the inflammatory processes related to PCOS. In polycystic ovary syndrome, miRNA-21 expression is notably higher in granulosa cells (GCs) as compared to normal cells, highlighting its important role in follicular development and steroidogenesis.

The heightened expression of miRNA-21 in GCs promotes the translation of toll-like receptor 8 (TLR8) mRNA. Activation of TLR8 triggers the output of inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin-12 (IL-12). This upregulation of miRNA-21 and subsequent activation of TLR8 contribute to the inflammatory environment observed in PCOS, as outlined by Yu *et al.* (2021). The disrupted inflammation could potentially affect follicular development, steroid hormone synthesis, and overall ovarian function in individuals with PCOS. miRNA-21 holds promise as a potential biomarker for both diagnosing and managing PCOS.

In comparison to normal rats, PCOS-afflicted animals exhibited a marked drop in the molecular SIRT-1 gene expression. SIRT-1 has an important role in regulating cell metabolic pathways and antioxidant defense mechanisms by acetylation-removing transcription factors, regulatory cofactors, and histone proteins (C. Chen *et al.* 2020). According to M. Wu *et al.* (2022) study, SIRT-1 is involved in the pathophysiological mechanisms of oxidative injury, autophagy, apoptosis, ovulation disruption, and insulin unresponsiveness, making it a crucial factor in the development of PCOS. Cellular oxidative stress can disrupt normal SIRT-1 function. When exposed to oxidative stress, SIRT-1 undergoes chromatin-level redistribution, leading to transcriptional deregulation. A study demonstrated that

H2O2 significantly influenced several SIRT-1-associated genes related to metabolism and apoptosis (Oberdoerffer *et al*., 2008). Moreover, SIRT-1 has a crucial role in DNA repair after degradation induced by H2O2. This observation aligns with a separate study that highlights how oxidative injury can cause the dissociation of SIRT-1 (Abdelmohsen *et al*., 2007). Patients with PCOS showed an overproduction of reactive oxygen metabolites, elevated levels of circulating oxidative injury biomarkers, and a gradual lack in capacity for antioxidant defense (Papalou *et al*., 2016). It was found that methylglyoxal (MG) deposition disrupts the balance of SIRT-1, leading to a reduction in proteins that protect mitochondria and increase oxidative stress which observed in PCOS Di Emidio *et al*. (2020).

The administration of a Hydroalcoholic extract of CZ to rats afflicted with PCOS resulted in the restoration of TNF-α and IL-6 levels when compared to normal rats. This improvement is likely attributable to certain active components in cinnamon including cinnamaldehyde, eugenol, and terpene. Specifically, cinnamaldehyde has been reported to exhibit anti-inflammatory characteristics according to Penckofer *et al*. (2002) study which aligned with our HPLC report. Additionally, various studies have demonstrated the blockage of arachidonic acid pathways and the eugenol's antihistamine effects, contributing to the prevention of inflammation (Hsueh and Law, 1998). Furthermore, the terpene components in cinnamon are reported to inhibit arachidonic acid pathways and the nitric oxide synthase (NOS) (S. K. Lee *et al*., 2002). Moreover, studies have demonstrated that cinnamon extract effectively inhibits the production of TNF-α, and prostaglandin E2. The anti-inflammatory properties of cinnamon are further attributed to the inhibition of nitric oxide synthase (NOS) in inflamed regions (H. J. Lee *et al*., 2006).

Our study results indicate that cinnamon administration in PCOS rats significantly downregulated the miRNA-21 gene compared to untreated rats. The expression of miRNA-21 can be regulated by reducing inflammatory cytokines. Cinnamaldehyde, a bioactive compound in cinnamon, exhibits strong anti-inflammatory properties by minimizing the output of arachidonic acid, which helps reduce inflammation in ovarian tissues. In PCOS, inflammatory cytokines can impair ovarian function, and cinnamon's anti-inflammatory effects may alleviate this by lowering cytokine levels. For example, it could potentially suppress IL-1β, a cytokine known to negatively impact the expression of follicle-stimulating hormone (FSH) and luteinizing hormone receptor (LHR) in granulosa cells (GCs) (Luo *et al*., 2023).

Moreover, Hong *et al*. (2012) found that oral consumption of cinnamon extract at doses of 20, 100, and 500 mg/kg of body weight significantly reduces plasma levels of IL-6 and TNF-α in rats. Additionally, this treatment led to a decrease in testosterone levels, increased macrophage viability, reduced macrophage death, and a reduction in the efflux of pro-inflammatory signaling molecules (cytokines), including TNF-α and IL-6, as documented by (Mishra and Srivastava, 2022). In summary, miRNA-21's involvement in inflammation sheds light on the intricate pathogenesis of PCOS, providing valuable insights for future research and clinical interventions.

Conversely, cinnamon treatment induced the overexpression of the SIRT-1 gene within ovarian tissues when compared to PCOS rats. That result may be possibly due to cinnamon antioxidant properties and its ability to improve mitochondrial functions. Cinnamon comprises antioxidant compounds including limonene and linalool, as well as tannin, coumarin, resin, and phenylpropane compounds such as aldehyde hydroxy cinnamate (Khodaeifar *et al*., 2019b). Numerous studies have demonstrated that the antioxidant benefits of cinnamon, including an increase in antioxidant capacity, enhanced glutathione production, heightened efficiency of free radical scavengers such as glutathione peroxidase and superoxide dismutase, and reduced levels of MDA and lactate dehydrogenase, as highlighted by (Seyed Ahmadi *et al*., 2019). The primary targets for SIRT1-mediated alteration of the redox state include P53 and nuclear factor kappa B (NF-κB) (H.-C. Chang and Guarente, 2014). SIRT1 also contributes to the stabilization of antioxidative processes by upregulating nuclear factor erythroid 2 (NRF2). This is achieved through the deacetylation of nuclear Factor E2-related Factor 2, thereby promoting the expression of antioxidative enzymes like SOD, CAT, and GSH (Ding *et al*., 2016). Recent findings indicate that SIRT-1 can mitigate PCOS by ameliorating mitochondrial abnormalities, diminishing the production of oxidative stress markers, and lowering methylglyoxal (MG) levels, which are linked to glycosylation stress, as described by (Di Emidio *et al*. (2019)). Through our study, we can conclude that SIRT-1 gene expression can be controlled by controlling oxidative stress, and thus we can diminish the impact of oxidative stress in the progress of this syndrome, and this will be confirmed through future studies. The histopathological analysis of ovarian tissues strongly supported our findings.

Conclusion

The findings of this study suggest that administering metformin (Glucophage®) and/or hydroalcoholic *Cinnamon zeylanicum* extract to rats with Letrozole-induced PCOS effectively corrected hormonal imbalances, hyperinsulinemia, hyperglycemia, and inflammation, while also improving gene expression. These treatments showed potential in alleviating ovarian changes linked to PCOS, indicating that *Cinnamon zeylanicum* could be a promising therapeutic option for managing PCOS. Further research is needed to explore the mechanisms behind these benefits, particularly focusing on the roles of miR-21 and GSK-3β in PCOS development.

Conflict of interest

The authors have no conflict of interest to declare.

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