Al-Qadisiyah Journal of Pure Science
ISSN(Printed): 1997-2490 ISSN(Online): 2411-3514
DOI: 10.29350/jops
Al-Qadisiyah Journal of Pure Science

Modulatory Role of *Ilex paraguariensis* on the Cytokine–microRNA Axis in Polycystic Ovary Syndrome (PCOS) Induced in Albino Female Rats

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Received: 14/09/2024; Accepted: 29/09/2024; Published Online: 29/11/2024.

Abstract

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders associated with chronic inflammation in women of reproductive age, adversely affecting reproductive health, metabolism, cardiovascular function, and mental health.

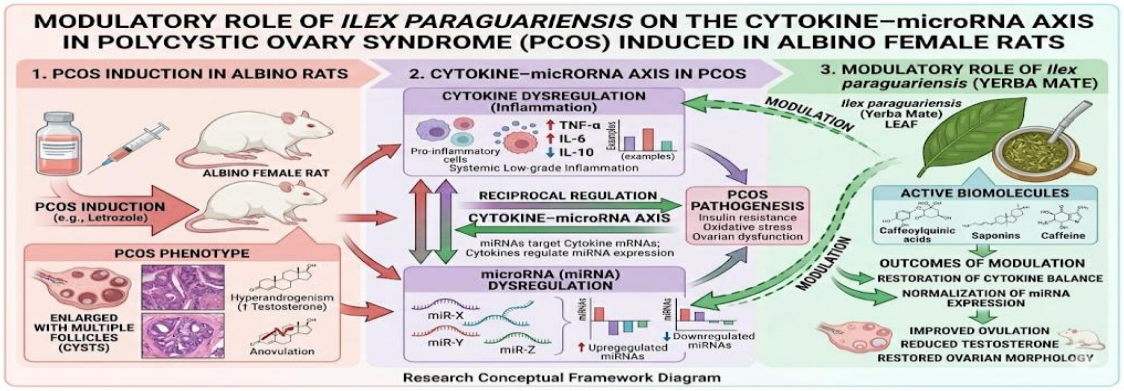
The aim of the study was to evaluate the effect of *Ilex paraguariensis* extract on the gene expression of miR-21 and levels of pro-inflammatory cytokines in rats in which PCOS was induced by the drug letrozole (1 mg/kg) for 21 days.

The experiment included 30 female rats divided into three groups: the control group (C) comprising ten rats; the PCOS group (T1), in which polycystic ovary syndrome was induced; and the group (T2), in which polycystic ovary syndrome was induced and then treated with a dose of 500 mg/kg of mate plant extract for 30 days. At the end of the experiment, the animals were sacrificed, and levels of the hormones FSH and LH, the inflammatory cytokines TNF-α and IL-6, as well as miR-21 expression. The results in the PCOS group showed a statistically significant increase ($p<0.05$) in the levels of FSH, LH and inflammatory cytokines (TNF-α, IL-6) along with an abnormality in miR-21 levels, whilst

treatment with *Ilex paraguariensis* extract resulted in a reduction in hormone and cytokine levels, as well as an improvement in miR-21 expression.

Conclusion: *Ilex paraguariensis* extract acts as an effective cytokine-miRNA regulator, reducing inflammation and improving histological and hormonal parameters in polycystic ovary syndrome.

Keywords*: Ilex paraguariensis*, PCOS, miR-21



Introduction:

Polycystic ovary syndrome (PCOS) is defined as a group of endocrine disorders characterised by androgen excess and associated with impaired ovarian function. PCOS is associated with obesity and metabolic disorders, as well as insulin resistance, with a global prevalence ranging from 6–20% (Correa, de Sá-Nakanishi, & A. B., 2019).

Recent studies have highlighted the key role of inflammation in the development of PCOS, which is associated with elevated levels of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), which contribute significantly to the hormonal imbalance associated with ovarian dysfunction (Zhang et al., 2020).

The regulatory mechanisms linking the inflammatory response and gene regulation have encouraged studies focusing on the cytokine–miRNA axis,

whereby microRNAs (miRNAs) control post-transcriptional gene expression, which in turn influences inflammatory and metabolic processes. Recent studies have confirmed that polycystic ovary syndrome is associated with changes in the expression levels of miRNAs, particularly in the ovaries, as dozens of molecules show significant changes in their levels, suggesting a role in regulating pathways such as PI3K/Akt and MAPK, which are linked to granulosa cell function and hormonal balance, thereby reinforcing the notion of a dynamic interaction between cytokines and miRNAs in disease progression (Bhingardeve et al., 2025). On the other hand, there is growing interest in the use of natural plant compounds as potential therapeutic agents to modulate these complex molecular pathways. Among these plants, (*Ilex paraguariensis*) is a subtropical plant native to South America and belonging to the Aquifoliaceae family; it is used in the production of yerba mate tea, which is consumed as a traditional beverage in South America. Matte possesses therapeutic properties in lowering cholesterol levels, as well as improving liver and digestive functions, owing to its active role as an antioxidant and anti-inflammatory agent (Cassotta et al., 2025).

Studies have examined the properties of its biologically active compounds, which mainly belong to three categories: phenolic acids, methylxanthines and flavonoids. Among the phenolic acids, caffeoylquinic acid derivatives (chlorogenic acids) are the most abundant, accounting for approximately one-third of the total polyphenol content, and include the isomers 3 -caffeoylquinic acid (CQA), 4-CQA, 5-CQA, and di-caffeoylquinic acid. As for flavonoids, although they are present in lower concentrations than phenolic acids, they are consistently found, with rutin being the most abundant (up to 7–8 mg/ g of dry leaves). Together with quercetin and kaempferol, these compounds confer potent antioxidant and anti-inflammatory properties, which biologically justifies investigating their significance in inflammatory and antioxidant pathways (Cassotta et al., 2025)

Previous research has indicated that dietary polyphenols may exert immunomodulatory and antioxidant effects relevant to the pathophysiology of inflammatory diseases, attributed to plant-derived phenolic compounds characterised by their ability to modulate key cellular pathways involved in rheumatoid arthritis, including NF-κB, JAK/STAT and MAPKs (Ruskovska et al., 2023).

Materials and Methods

The experiment was conducted at the Animal Laboratory of the Faculty of Science, University of Al-Qadisiyah. The experiment involved 30 adult female white rats weighing between 180–200 g and aged between 3 and 4 months. They were housed in special cages in a laboratory environment with a temperature of 22 ± 2°C and a humidity level of 55 ± 5%, with light/dark cycles ranging from 14 to 10 hours. The animals were provided with water and feed throughout the experiment and were allowed to acclimatise to the new environmental conditions one month prior to the start of the experiment. The animals' weights were monitored and measured twice daily throughout the experiment, with oestrus stages determined using daily vaginal swabs (Elmosalamy et al., 2022).

Extraction of the *Ilex paraguariensis*

The *Ilex paraguariensis* was purchased from a commercial market and its components were then extracted using the traditional method for preparing tea. 85 g of yerba mate was used, to which 1.5 litres of water at a temperature of 80°C was added, and then filtered using a vacuum pump. After 5 minutes, the extract was frozen, dried and stored at -20°C until use. Doses of the extract amounting to 400 and 800 mg/kg were administered daily (Correa et al., 2019).

Induced polycystic ovary syndrome model

PCOS was induced using the drug letrozole at a dose of 1 mg/kg for 21 days (Elmosalamy et al., 2022) of body weight, administered daily over three weeks,

with the addition of 1% sodium chloride and 0.2% potassium chloride daily to the drinking water in all treatment groups except the control group. The presence of polycystic ovary syndrome was successfully verified by vaginal smear examination and plasma hormone analysis.

Experimental design:

This experiment used 30 female white rats, which were randomly divided into three groups of 10 each, and then subdivided as follows:

1-Group 1: Control group (C): animals administered a physiological saline solution (NaCl) at a concentration of 0.9% throughout the experiment.

2-Group 2 (T1): This group comprised animals in which ovarian cysts were induced using the drug letrozole at a dose of 1 mg/kg body weight, administered orally daily for 21 days.

3.Group 3 (T2): This group comprised animals in which ovarian cysts were induced, and to which the extract was added to drinking water at doses of 400 and 800 mg/kg daily for 30 days.

Sample collection:

On the 30th day of the experiment, the animals were anaesthetised with chloroform and blood was drawn directly from the heart via a cardiac puncture using a sterile 5 ml disposable syringe. 2 ml of the collected blood was placed in EDTA-free glass tubes and left for 15–20 minutes at laboratory temperature. The samples were then placed in a Heraeus-Christ Gumby centrifuge at 3000 rpm for 15 minutes to separate the serum. The serum was isolated using a micropipette, and the samples were placed in plastic tubes for hormonal and biochemical testing. The serum was stored at 20°C Measurement of miRNA-21.

Gene expression using RT-qPCR

RNA extraction

Ovarian tissue samples were collected and total RNA was extracted from them using a miRNA extraction kit, in accordance with the manufacturer's instructions (miRNA easy Mini Kit, Qiagen, Germany)

The concentration and purity of the RNA were measured using a spectrophotometer, and then cDNA was synthesised from the RNA using a reverse transcription kit . Quantitative polymerase chain reaction (RT-qPCR) was used to analyse miR-21-5p, LncMEG3, Bcl-2, Bax, Caspase-3 and β-actin. For the other genes, specific primers were used as shown in Table 1. β-actin was used as an internal reference gene (housekeeping gene), and relative expression was calculated using the 2^-ΔΔCt method.

Relative gene expression was calculated using the 2^-ΔΔCt method according to the following steps:

1-The difference in cycle threshold (ΔCt) between the target gene and the reference gene was calculated as follows:

$\Delta Ct = Ct_{(target)} - Ct_{(reference)}$

2-The ΔCt difference between the experimental sample and the control sample (ΔΔCt) was calculated:

$\Delta\Delta Ct = \Delta Ct_{(sample)} - \Delta Ct_{(control)}$

3-The relative gene expression level (Fold Change) was calculated:

Fold Change = $2^{(-\Delta\Delta)}$.

Specific primers were used for the target genes, as shown in Table 1, whilst β-actin was used as an internal reference gene (housekeeping gene) to normalise gene expression levels until use.

Table 1: PCR primer sequences

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Source
miR-21-5p	AGC AGT GAT TTA TTG GCG TG	Universal reverse primer (commercial miRNA RT-qPCR kit)	QIAGEN miScript RT-PCR system, Hilden, Germany https://www.qiagen.com
LncMEG3	GGA TGT GGT GAA GGA GAA GC	TGC TGT TGT TGT CCT TCT CC	NCBI Primer-BLAST (in silico design) https://www.ncbi.nlm.nih.gov/tools/primer-blast/
Bcl-2	GGTGAACTGGGGGAGG ATTG	GCATGCTGGGGCCATATAG T	Validated RT-qPCR (Liu et al., 2015)

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Source
Bax	GGCGATGAACTGGACAACAA	CAAAGTAGAAAAGGGCAACC	RT-qPCR (Liu et al., 2015)
Caspase-3	GGACCTGTGGACCTGAAAAA	GCATGCCATATCATCGTCAG	RT-qPCR (Liu et al., 2015)
β-actin	CACGATGGAGGGGCCGGACTCATC	TAAAGACCTCTATGCCAACACAGT	Housekeeping gene validated in RT-PCR studies

F forward, R reverse, LncMEG3 long noncoding RNA maternally expressed gene3, miR-21-5p icroRNA-21-5p, Bcl-2 BCL2 mRNA, Bax BAX mRNA, caspase-3 Casp3 mRNA

Measurement of hormone levels:

An ELISA kit was used to measure hormone levels; testosterone, FSH and LH were measured in accordance with the manufacturer's instructions (Ela Science, Wuhan, China).

Measurement of immune markers:

An ELISA kit was used to measure immune markers, with levels of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) determined in accordance with the manufacturer's instructions (Ela Science, Wuhan, China).

Measurement of malondialdehyde (MDA) and glutathione (GSH) levels

The MDA and GSH contents were measured. The samples were placed in a centrifuge at 8000 rpm at 4 °C for 10 minutes, and the supernatants were collected to measure MDA levels at wavelengths of 450, 532 and 600 nm. GSH levels were then measured at wavelengths of 525 and 412 nm, respectively (Li et al., 2022).

Statistical analysis

SPSS version 25.0, developed in the United States of America, was used. Analysis of variance (ANOVA) was performed, and the mean ± standard deviation (SD) was used to present the data.

Results

Gene expression (RT-qPCR)

The results indicated significant changes in the miR-21-5p/LncMEG3 axis and the Bcl-2, Bax, and Caspase-3 genes associated with apoptosis between the control group and the T1 and T2 groups.

The results showed a significant increase in miR-21-5p in the T1 group compared with the C group, and conversely, a relative decrease in the T2 group treated with the extract. Meanwhile, LncMEG3 levels decreased in group T1, with a marked improvement in group T2 compared to the control group, confirming the inverse relationship between the (miR-21-5p / LncMEG3) axis.

As for the apoptosis genes, there was a significant increase in group T1 compared to group C, confirming the activation of the programmed cell death pathway, Conversely, the results showed a relative increase in Bcl-2 levels in the T1 group, though at a lower level than Bax, indicating an imbalance between anti-apoptotic and pro-apoptotic factors. The results also showed improvement in the T2 group compared to the control group, indicating an improvement in cellular response.

Table (2). Relative gene expression of studied genes in control, miR-21-5p stimulated group(PCOS) , and *Ilex paraguariensis* –treated PCOS group

Gene	C	T1	T2	p-value	$LSD_{0.05}$
miR-21-5p	1.00 ± 0.13^{c}	2.09 ± 0.20^{a}	1.52 ± 0.18^{b}	<0.05	0.24
LncMEG3	1.00 ± 0.11^{a}	0.41 ± 0.05^{c}	0.86 ± 0.07^{b}	<0.05	0.14
Bcl-2	1.00 ± 0.12^{c}	1.55 ± 0.17^{a}	1.25 ± 0.13^{b}	<0.05	0.21
Bax	1.00 ± 0.14^{c}	2.08 ± 0.21^{a}	1.37 ± 0.14^{b}	<0.05	0.25
Caspase-3	1.00 ± 0.16^{c}	2.20 ± 0.24^{a}	1.57 ± 0.16^{b}	<0.05	0.28

Values are expressed as mean ± standard deviation (n=6). Means with different superscript letters within the same row are significantly different ($p < 0.05$). C: Control group; T1: PCOS induced group; T2: PCOS-induced + *Ilex paraguariensis* treatment group.

Hormonal parameters

The results showed varying levels of sex hormones across the study groups. Testosterone levels were significantly higher in the T1 group, reaching 2.35 ± 0.28 ng/ml compared with the control group (0.55 ± 0.08 ng/ml), whilst the results in the T2 group showed a statistically significant increase to 1.60 ± 0.22 ng/mL, though this remained lower than that of the T1 group.

Luteinising hormone (LH) levels also increased significantly in the T1 group (13.8 ± 1.4 mIU/mL) compared to the control group (5.2 ± 0.5 mIU/mL), whilst the T2 group showed a level of 8.6 ± 0.9 mIU/mL.

In contrast, follicle-stimulating hormone (FSH) showed opposite results, decreasing significantly in the T1 group (3.0 ± 0.4 ng/mL) compared to the control group (6.4 ± 0.6 ng/mL). The T2 group (5.2 ± 0. 5 mIU/mL) recorded a relative decrease compared with the control group.

Table 3: Effect of *Ilex paraguariensis* on (Testosterone,LH,FSH) in femal rats with (PCOS) -Induced

Parameter	C	T1	T2	p-value	$LSD_{0.05}$
Testosterone (ng/mL)	0.55 ± 0.08^c	2.35 ± 0.28^a	1.60 ± 0.22^b	<0.001	0.42
LH (mIU/mL)	5.2 ± 0.5^c	13.8 ± 1.4^a	8.6 ± 0.9^b	<0.001	1.25
FSH (mIU/mL)	6.4 ± 0.6^a	3.0 ± 0.4^c	5.2 ± 0.5^b	<0.001	0.88

Values are expressed as mean ± standard deviation (n=6). Means with different superscript letters within the same row are significantly different (p < 0.05). C: Control group; T1:PCOS induced group; T2: PCOS-induced + *Ilex paraguariensis* treatment group

Immunological parameters

The results showed a marked difference in the levels of the inflammatory cytokine TNF-α between groups T2 and T1 compared with the control group (C). Group T1 recorded a statistically significant increase to (44.3 ± 3.9 pg/mL) compared with the control group, reflecting the inflammatory response of group T1 compared with the control group, whilst group T2 recorded a statistically significant decrease to (28.6 ± 3.2 pg/mL) compared with C.

As for IL-6, the control group showed a value of (17.6 ± 1.3 pg/mL), whilst it increased significantly in the T1 group to (55.2 ± 4.1 pg/mL). Meanwhile, the IL-6 level in the T2 group decreased to (27.6 ± 3.5 pg/mL), which supports the therapeutic response to the metha extract.

Table 4: Effect of *Ilex paraguariensis* on Serum Inflammatory Cytokines on femal rat with (PCOS) -Induced

Parameter	C	T1	T2	p-value	$LSD_{0.05}$
TNF-α (pg/mL)	16.2 ± 1.2^c	44. 3± 3.9^a	28± 3.2^b	<0.01	3.25

Parameter	C	T1	T2	p-value	$LSD_{0.05}$
IL-6 (pg/mL)	17. 6± 1.3^c	55. 2± 4.1^a	27.6 ± 3.5^b	<0.01	3.60

Values are expressed as mean ± standard deviation (n=6). Means with different superscript letters within the same row are significantly different (p < 0.05). C: Control group; T1:PCOS induced group; T2: PCOS-induced + *Ilex paraguariensis* treatment group.

Malondialdehyde (MDA) and glutathione (GSH) levels

The results showed clear evidence of oxidative stress in the T1 group, manifested by a marked increase in malondialdehyde levels compared to the control group. Conversely, GSH levels were significantly reduced in the same group, reflecting a weakened antioxidant defence system and increased consumption due to oxidative stress. In the T2 group, the results indicated an improvement in the state of oxidative stress, as evidenced by a lower MDA level compared to the T1 group and a higher GSH level, which did not reach the level of the control group, suggesting a therapeutic response despite the presence of an oxidative imbalance.

Table 5: Effect of *Ilex paraguariensis* on Lipid Peroxidation and Antioxidant Status in femal rats with (PCOS) -Induced

Parameter	C	T1	T2	p-value	$LSD_{0.05}$
MDA (nmol/mL)	1. 5± 0.2^c	7.6± 0.7^a	3.1 ± 0.4^b	<0.01	0.85
GSH (nmol/mL)	41.5 ± 3.5^a	21.7 ± 2.3^c	32.6 ± 3.2^b	<0.01	5.20

Values are expressed as mean ± standard deviation (n=6). Means with different superscript letters within the same row are significantly different (p < 0.05). C: Control group; T1: PCOS induced group; T2: PCOS-induced + *Ilex paraguariensis* treatment group.

Discussion

The results indicated statistically significant changes in the miR-21-5p/LncMEG3 axis and the genes Bcl-2, Bax, and Caspase-3—which are associated with apoptosis—between the control group and the PCOS group. This statistically significant increase in miR-21 -5p and the decreased level of LncMEG3 in the T1 group compared to the control group explains the role of miR-21 in granulosa cell proliferation and apoptosis, alongside the disruption in follicular maturation associated with increased oxidative stress, which was consistent with the results of our study showing elevated levels of (MDA) alongside a decrease in (GSH), as well as the effect of reduced LncMEG3 on increased cell death in granulosa cells (Yu et al., 2021).

The significant increase in Bax and Caspase-3 in the PCOS group is evidence of the activation of programmed cell death pathways, whilst the rise in Bcl-2 levels is attributed to a compensatory response that determines the cell's fate as a result of an imbalance between Bcl-2 and Bax (Ibrahim et al., 2026)

Conversely, there was a marked improvement in the miR-21-5p/LncMEG3 axis and the Bcl-2, Bax, and Caspase-3 genes, following treatment with *Ilex paraguariensis*. This is attributed to the role of the extract as an antioxidant and anti-inflammatory agent associated with polycystic ovary syndrome, along with a reduction in oxidative stress levels, which helped to improve the gene expression of the parameters studied. This was confirmed by the results of our study, which showed a decrease in the level of (MDA) and an increase in the level of (GSH) (Palavicini et al., 2025).

The results also revealed disturbances in sex hormones (testosterone, LH, FSH) in the PCOS group, characterised by a significant increase in testosterone and LH levels alongside a decrease in FSH levels, compared with the control group, This is attributed to the increase in androgens associated with PCOS, resulting from disruption of the hypothalamic–pituitary–ovarian axis, which leads to increased activity and enhanced production of male hormones (Wang et al., 2025).

Meanwhile, treatment with *Ilex paraguariensis* extract showed an improvement in the normal values of sex hormones compared to the control group on the one hand and the PCOS group on the other; this is explained by the active extract's effect due to its antioxidant and anti-inflammatory properties, as confirmed by the results of our study, which showed a decrease in the level of (MDA) levels and an increase in (GSH) levels, which play an active role in improving both insulin sensitivity and oxidative stress, which are considered the main causes of PCOS (Feihrmann et al., 2022).

The results also showed elevated levels of TNF-α and IL-6 in the polycystic ovary syndrome (PCOS) group compared with the control group, whilst levels were reduced in the *Ilex paraguariensis* group. The results demonstrate the activity

of inflammatory cytokines, which are directly associated with polycystic ovary syndrome and insulin resistance, and consequently with ovarian dysfunction in the PCOS group (Chen et al., 2023).

In contrast, treatment with *Ilex paraguariensis* showed a reduction in TNF-α and IL-6 levels, which is attributed to active compounds such as polyphenols and flavonoids that inhibit inflammatory cytokines, thereby reducing oxidative stress, which has a positive effect on hormonal function and ovarian function (Rząsa-Duran et al., 2022).

Conclusion

The results of our study showed that the induction of polycystic ovary syndrome (PCOS) in female white rats was accompanied by a clear disruption of the inflammatory-molecular axis, resulting in a significant increase in inflammatory cytokines such as TNF-α and IL-6, as well as increased gene expression of miR-21-5p and decreased expression of LncMEG3. This contributed to the development of the syndrome, which was associated with chronic inflammation and cellular stress .Conversely, administration of Ilex paraguariensis extract to the animals contributed to a reduction in levels of inflammatory cytokines, alongside a modification in gene expression characterised by a decrease in miR-21-5p and an increase in LncMEG3, thereby confirming its regulatory role, which supports the properties of its active compounds and their role as antioxidants and anti-inflammatory agents.

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