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RESEARCH ARTICLE

Therapeutic Potential of *Phyllanthus niruri* (*Bhumi Amla*) in Ameliorating Polycystic Ovarian Syndrome Induced with Letrozole

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Abstract

Background: Polycystic Ovary Syndrome (PCOS) is the most common endocrine disease, often characterised by hyperandrogenism, Insulin Resistance (IR), and Oxidative Stress (OS). Conventional treatment with medicine has side effects, which has created interest in using herbal remedies. *Phyllanthus niruri* belongs to the family *Euphorbiaceae*. Several studies have reported that this plant has rich antioxidant and anti-inflammatory properties. This has generated interest in exploring the efficacy of *P. niruri* on PCOS. **Aim:** The present study was conducted to assess the therapeutic potential of *Phyllanthus niruri* on PCOS, through hormonal, oxidative stress, histopathological, and immunohistochemical (IHC) analysis. **Methods:** To induce PCOS, rats were treated with Letrozole (1 mg/kg) for 21 days. Following induction, rats received *P. niruri* at 200 and 500 mg/kg for 28 days. After the treatment period, hormonal, histopathological, and immunohistochemical analysis was performed at assesses anti PCOS effect. **Results:** Treatment with *P. niruri*, particularly at high dose, resulted in restoration of oestrous cyclicity, normalisation of hormonal levels, and enhancement of antioxidant enzyme activity. Histopathological examination showed considerable improvement in ovarian structure. In addition, IHC analysis revealed a decreased expression of IHC markers in treated animals. **Conclusion:** *P. niruri* showed dose-dependent therapeutic efficacy in alleviating PCOS by *r*egulating hormonal imbalance and oxidative stress. These results highlight its potential as a plant-based therapeutic agent for PCOS management.

Major Findings: The experimental findings demonstrate that treatment with *Phyllanthus niruri* showed dose-dependent therapeutic efficacy in PCOS rats with restoration of oestrous cyclicity and normalisation of hormonal imbalance. These findings highlight the multi-dimensional therapeutic potential of *Phyllanthus niruri.*

Keywords: *Phyllanthus niruri,* Oxidative Stress (OS), Anti Mullerian Hormone (AMH), Insulin Resistance (IR)

1. Introduction

Polycystic Ovarian Syndrome (PCOS) most common hormonal disorder affecting 5-10% of women during their reproductive age[1]. Androgen Excess Society (AES) in the year 2006, reported some diagnostic criteria for PCOS, including biochemical and clinical hyperandrogenism, with ovarian cysts in ultrasound[2]. Hyperandrogenism is caused by the overproduction of Luteinizing Hormone (LH) by the anterior pituitary that activates thecal cells to secrete more male androgens such as testosterone. PCOS is a multifactorial disease that may occur due to various factors, such as hormonal, genetic, and metabolic. However, the specific aetiology of PCOS remains unclear.

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Several studies have indicated that the presence of low-grade inflammation and oxidative stress (OS) are major factor in the aetiology of PCOS[3,4]. OS develops when there is an imbalance between reactive oxygen species (ROS) and antioxidants. ROS are extremely unstable molecules that can cause damage to cells and DNA, resulting in OS[5]. OS enhances lipid peroxidation and protein damage, which results in the manifestation of diseases such as cancer, diabetes, cardiovascular diseases[6] and chronic inflammatory diseases.

Phyllanthus niruri, popularly known as a stone breaker, belongs to the family of *Euphorbiaceae*[7]. This plant traditionally has been used to cure renal calculi, hepatitis, diabetes, and various inflammatory diseases[8,9]. Various studies have reported that this plant dissolves renal stones by inhibiting calcium oxalate crystals[10,11]. Additionally, several studies have found that *P. niruri* possesses powerful antioxidant properties. The therapeutic properties of this plant are attributed to the presence of numerous active bio compounds like alkaloids, flavonoids, terpenoids, and anthraquinones[12]. Flavonoids that are members of polyphenols have antioxidants[13], anti-inflammatory[14], and antiviral effects. This study aims to assess the therapeutic potential of *Phyllanthus niruri* in mitigating PCOS induced by letrozole.

2. Materials and Methods

2.1 Drugs and Chemicals

Carboxymethyl cellulose sodium salt (CMC sodium) Sisco Research Laboratories Pvt Ltd, Dimethyl sulfoxide (DMSO) Merck Life Science Private Limited, Letrozole from CIPLA LTD, Enzyme-Linked Immunosorbent Assay (ELISA) kits for Testosterone (KLR4465), Fasting Insulin (KLR0707), Anti Mullerian Hormone (AMH) (KLR3479), Superoxide dismutase (SOD) (KLR0168) purchased from KRISHGEN Biosystems, INDIA. NF-kB2 Rabbit mAb for IHC (A19605) from ABclonal company, TNF-alpha (Tumour Necrosis Factor-alpha) (DS-0544-C) from Diagnostic Biosystems.

2.2 Preparation of Plant Extract

The aerial parts of the plant were harvested from local fields in Kadapa district, Andhra Pradesh, cleaned, dried, and ground into coarse powder. The plant powder of *P. niruri* (1100 g) was extracted with 99% ethanol by using a Soxhlet apparatus and concentrated using a rotary evaporator and stored in air tight container. The percentage yield was 5.57±0.91. Later, phytochemical analysis was performed to observe bioactive compounds.

2.3 Experimental Design

This study has been approved by the Institutional Animal Ethics Committee (IAEC) with registration number TBPL/PCOS/008/24. The present experiment was conducted on 24 healthy female Wistar rats with regular oestrous cyclicity, weighing 180 -200 grams, and aged about 10-12 weeks. Animals were acclimatised for 2 weeks before the experiment under controlled environmental conditions (22 ± 3°C), with 12 hours light/dark cycles. Animals were provided with standard pellets of feed and distilled water ad libitum. During the experiment, estrous cycle was examined daily to check the stages.

Animals with regular oestrous cyclicity were randomly divided into the following 4 groups.

Group-1(- ve control): Administered with 0.5% Na CMC for 21days.

Group-2(+ve control): with Letrozole (1mg/kg/day) for 21 days.

Group-3(low dose): Administered with Letrozole for 21 days(1mg/kg/day) and treated

with *P. niruri* (200mg/kg/day) for 28 days.

Group -4(High dose): Administered with Letrozole for 21 days(1mg/kg/day) and treated with *P. niruri* (500mg/kg/day) for 28 days.

2.3.1 Induction of PCOS

Animals in Groups 2 to 4 were administered Letrozole at a dose of 1 mg/kg/day for 21 days for PCOS induction. Group 1 administered with vehicle (0.5% Na CMC).

2.3.2 Treatment Phase

After the induction period, animals in Groups 3 and 4 were treated with plant extract at low and high doses for 28 days.

Group 3: *P. niruri* (200mg/kg/day)

Group 4: *P. niruri* (500mg/kg/day)

Groups 1 and 2 do not receive treatment, as they serve as control and induction groups, respectively.

Doses of positive control for PCOS induction are selected based on the available literature. Doses for *P. niruri* were selected based on the available literature (which showed LD 50 at >5000mg/kg and NOAEL at 1000 mg/kg/day) and by expecting the recovery from PCOS either at a lower dose or higher dose, which were selected[11,15,16].

2.4 Oestrous Cyclicity Observation

Vaginal secretions were collected daily between 9 and 10 am by using a Pasteur pipette. Slides were examined under a microscope to check oestrous cyclicity by observing relative percentages of cells[17,18].

2.4.1 Stages of Oestrous Cycle

- Proestrus- predominant nucleated epithelial cells
- Oestrous- more cornified epithelial cells
- Metestrus- all 3 types of cells
- Diestrus- predominant leucocytes

2.5 Euthanasia and Evaluation

After 28 days of treatment period, animals from Groups 1 to 4 were fasted overnight. On day 50th, the animals were anaesthetised, and blood was collected from the retro-orbital plexus. Animals were euthanised by carbon dioxide asphyxiation, and necropsy was performed to collect the ovaries, which were then fixed in 10% neutral buffered formalin (NBF) for histopathological and immunohistochemical (IHC) analysis.

2.6 Hormonal Analysis

Testosterone (Catlog#: KLR4465), Fasting Insulin (Catlog#: KLR0707), Anti Mullerian Hormone (AMH) (Catlog#: KLR3479), Superoxide dismutase (SOD) (Catlog#: KLR0168) were analysed by ELISA technique using kits supplied by KRISHGEN Biosystems, INDIA, according to the manufacturer's protocol by using SpectraMax iD3 Multi-Mode Microplate Reader.

2.7 Histopathology Analysis

The ovaries were fixed in 10% NFB for 24 hours, and a routine histological procedure was performed. According to standard procedures, paraffin blocks were prepared and sectioned at a thickness of 5µm using a rotary microtome. Sections were stained with Hematoxylin and Eosin (H and E) and examined under a microscope[19].

2.8 Immunohistochemistry

Ovarian sections were deparaffinized using xylene, followed by rehydration through graded alcohols. in a series of graded alcohols. Slides were pretreated in a tris EDTA buffer, PH 9.0, for 20 minutes at high temperature. Pretreat the slides for antigen retrieval in tris EDTA buffer, PH 9.0, for 20 minutes and cool at room temperature for 20 minutes. Slides were pretreated for antigen retrieval in Tris-EDTA buffer (pH 9.0) by heating in a microwave at high temperature for 20 minutes, followed by cooling at room temperature for 20 minutes. Slides were kept in 2 changes of distilled water for 5 minutes. Specimens were kept in 2 changes of 3% hydrogen peroxidase for 5-10min to prevent endogenous peroxidase activity. Slides were then incubated in TNF-alpha for 45 minutes in a moisture chamber. Then slides were washed with 2 changes of phosphate buffer saline (PBS) for 5 minutes in each. Cleared the buffer and added HRP and kept it in the moisture chamber for 20 minutes. Again, washed within 2 changes of PBS for 5 min in each. Cleared buffer solution and covered the slides with 2-3 drops of DAB solution for 3-5minutes and then washed in running tap water for 5-8 min. Stained with hematoxylin for 30-60sec. Slides were then dehydrated through graded alcohols, cleared in xylene, mounted, and covered with coverslips.

2.9 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism (version 10.4.2). An unpaired *t*-test was conducted to compare the -ve control (G1) with the +ve control (G2). ANOVA was employed for comparisons among multiple groups, followed by Dunnett's post hoc test to evaluate differences between the treatment groups and the positive control group. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Phytochemical Analysis

The percentage of yield obtained after the extraction of powdered *P. niruri* was 5.57±0.91. Qualitative

phytochemical analysis revealed the presence of phytochemicals including alkaloids, Phenols, Flavonoids, glycosides, steroids, saponins, triterpenoids, tannins and carbohydrates.

3.2 Effect on Oestrous Cyclicity

Irregular oestrous cyclicity was observed in Group 2(PCOS induced), confirming successful PCOS induction, whereas regular cyclicity was maintained in the control and treatment groups (Groups 1 and 4), indicating restoration of regular oestrous cyclicity.

3.3 Hormone Analysis

The administration of Letrozole caused significant hormonal changes in all groups (Groups 2 to 4), indicating that Polycystic Ovary Syndrome (PCOS) was successfully induced in them. After 28 days of treatment with plant extract, significant changes in hormonal levels were noted in Group 4(high dose), indicating successful restoration of hormones and OS (Table 1).

3.4 Effect on Hormonal Levels

3.4.1 Testosterone

One of the main characteristics of PCOS is hyperandrogenism (excess androgen production), mainly testosterone, which leads to anovulation. In this study, testosterone levels were measured among control, PCOS and treatment groups. Significant increase in serum testosterone levels was noted in Group 2 (2.216 ± 0.102 ng/mL) and Group 3(2.08 ± 0.094 ng/mL), compared with Group1 (0.821 ± 0.049 ng/mL). Testosterone levels decreased in Group 4(1.194 ± 0.047 ng/mL) after treatment. The plant extract showed a promising dose-dependent effect in reducing testosterone levels. Notably, the high dose was more effective in lowering testosterone than the low dose, bringing the levels closer to the control. This indicates that higher doses of the plant extract have a stronger impact on reducing elevated testosterone levels associated with PCOS.

3.4.2 Insulin

Insulin resistance (IR) is mainly observed in PCOS. Fasting insulin levels were increased among Group 2 (3.301 ± 0.155 ng/mL) and Group 3 (3.161 ± 0.163 ng/mL), whereas in Group 1(1.812 ± 0.14ng/mL). In Group 4, there is a notable decrease in insulin levels (2.245 ± 0.105 ng/mL) when compared with Group 2.

3.4.3 Anti-Mullerian Hormone (AMH)

Under the influence of FSH, estrogen produces more follicles. This increased number of follicles will produce more AMH by granulosa cells, which results in high AMH levels in the blood. AMH levels were elevated in Group 2 (18.357 ± 0.214 ng/mL) and Group 3(18.163 ± 0.242 ng/mL). A significant decrease in AMH levels was observed in Group 4(16.225 ± 0.45 ng/mL) when compared with the control (15.99 ± 0.327 ng/mL). Results suggest that plant extract at a high dose reduces AMH levels, indicating improved ovarian function in PCOS rats.

3.4.4 Effects on SOD Levels

SOD is an antioxidant enzyme that fights against Reactive Oxygen Species (ROS) by converting superoxide anion (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2)[20,21]. In the present study, there is a decrease in SOD levels in Groups 2(0.040 ± 0.005 ng/mL) and 3(0.041 ± 0.004 ng/mL) when compared with Group 1(0.056 ± 0.002 ng/mL). However, SOD levels were elevated in group 4(0.056 ± 0.002 ng/mL) after treatment.

Table 1. Showing comparison of hormonal and antioxidant parameters in control, PCOS and treatment groups

Parameter	Group 1 (control)	Group 2 (PCOS induced)	Group 3 (200mg/kg/day)	Group 4 (500mg/kg/day)
Testosterone (ng/mL)	0.821 ± 0.049	2.216 ± 0.102 ↑	2.08 ± 0.094↑	1.194 ± 0.047 ↓
Insulin (ng/mL)	1.812 ± 0.14	3.301 ± 0.155 ↑	3.161 ± 0.163↑	2.245 ± 0.105 ↓
AMH (ng/mL)	15.99 ± 0.327	18.357 ± 0.214 ↑	18.163 ± 0.242↑	16.225 ± 0.45 ↓
SOD (ng/mL)	0.056 ± 0.002	0.040 ± 0.005 ↓	0.041 ± 0.004↓	0.056 ± 0.002↑

Note: Values are represented as Mean ± SD; Results are considered significantly different when $p < 0.05$.

A significant reversal of these hormonal levels was observed in Group 4. Providing strong evidence of the therapeutic potential of high-dose *P. niruri* in alleviating PCOS-associated metabolic and oxidative dysfunction. This group reached a level of hormones between that of the control (Group1) and the PCOS Groups (Group 2), indicating successful recovery. On the other hand, plant extract of low dose (200 mg/kg/day) did not bring about any statistically significant change in the hormone profile compared to Group 1, suggesting that a lower dose may be insufficient to elicit a therapeutic response against Letrozole-induced PCOS.

3.5 Histopathology

In Group 1, ovaries showed normal histology with intact corpus luteum, healthy polyhedral cells, and all stages of follicular development (Figure 1a). In Group 2, severe ovarian damage was observed, including degeneration of corpus luteum, cystic follicles, follicular atresia, mononuclear cell infiltration, necrosis, and interstitial fibrosis, which is indicative of PCOS (Figure 1b). In Group 3, there is partial improvement noted but not sufficient to reverse letrozole-induced damage (Figure 1c). Histopathological analysis of Group 4 showed significant improvement in ovarian damage, with mild follicular atresia and little mononuclear cell infiltration, indicating a strong protective effect of the treatment (Figure 1d).

3.6 Immunohistochemistry

3.6.1 TNF alpha

In Group 1, normal TNF alpha (Figure 2a) expressions were observed in the cytoplasm of polyhedral cells of CL and granulosa cells of ovarian tissue, indicating the absence of inflammatory activity. In Group 2, ovaries showed markedly enhanced TNF alpha (Figure 2b) expression in the cytoplasm and nucleus of granulosa cells of follicles. These changes can be attributed to letrozole induced active inflammation and cell death in ovarian tissue. In Group 3, moderately enhanced TNF alpha (Figure 2c) expression was observed in the cytoplasm of granulosa follicle cells. This indicates poor efficacy of plant extract at a low dose (200mg/kg) in minimising inflammation induced by letrozole. In Group 4, minimal TNF alpha (Figure 2d) expressed in cytoplasm of granulosa follicles and polyhedral cells of CL. This indicates prominent efficacy of plant extract at high dose(500mg/kg) on minimizing inflammation induced by letrozole.

3.6.2 NF-kB

In Group 1, immunohistochemically, normal NF-kB expression was observed in the cytoplasm of polyhedral

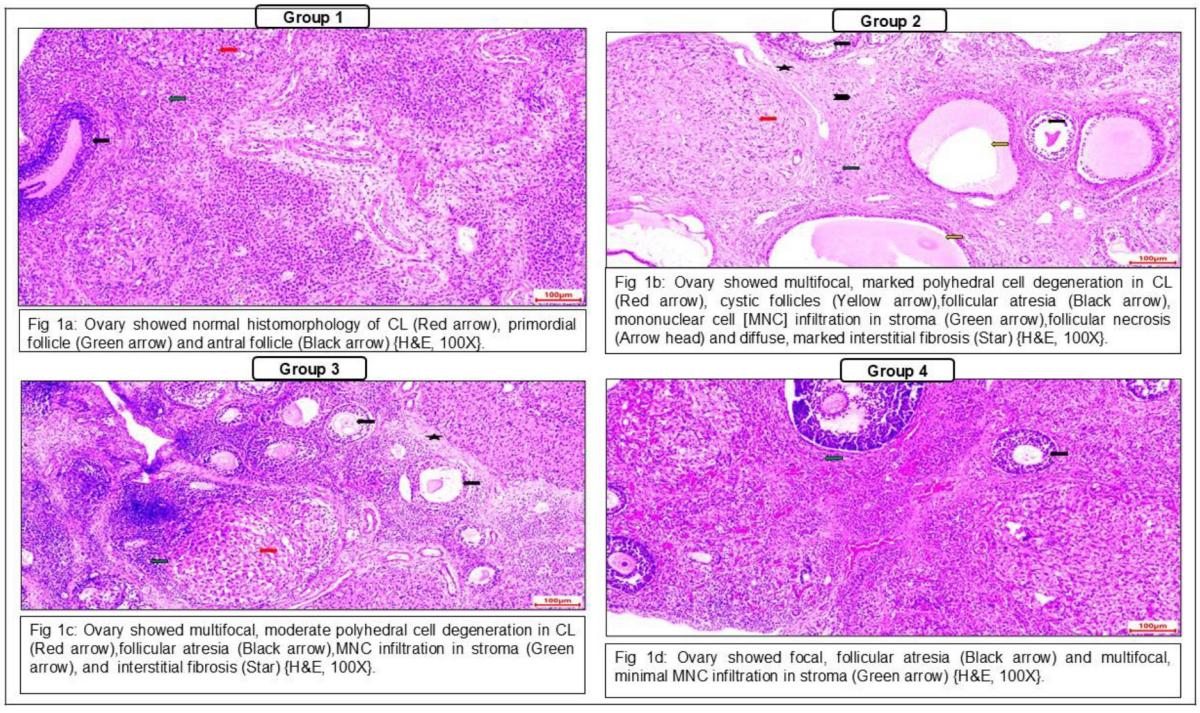


Figure 1(a-d). Showing ovarian sections of Groups (1-4), stained with H and E {100x}.

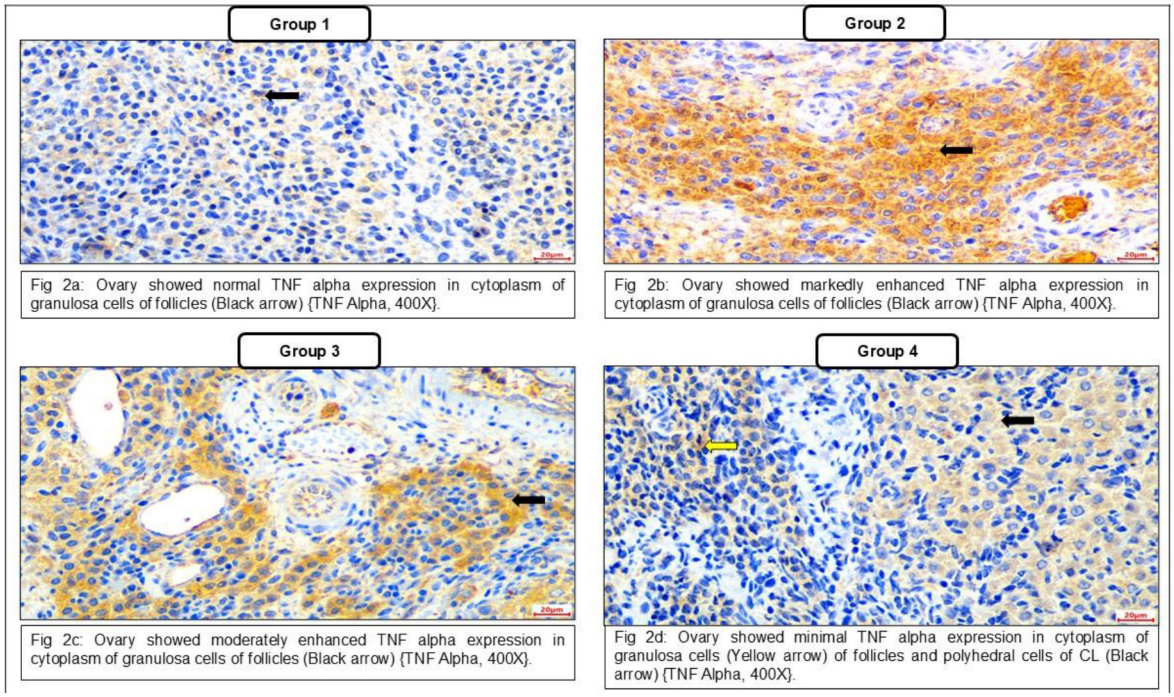


Figure 2(a-d). Showing TNF-alpha immunoexpression in ovarian tissues across the experimental Groups (1-4), TNF-α {400x}.

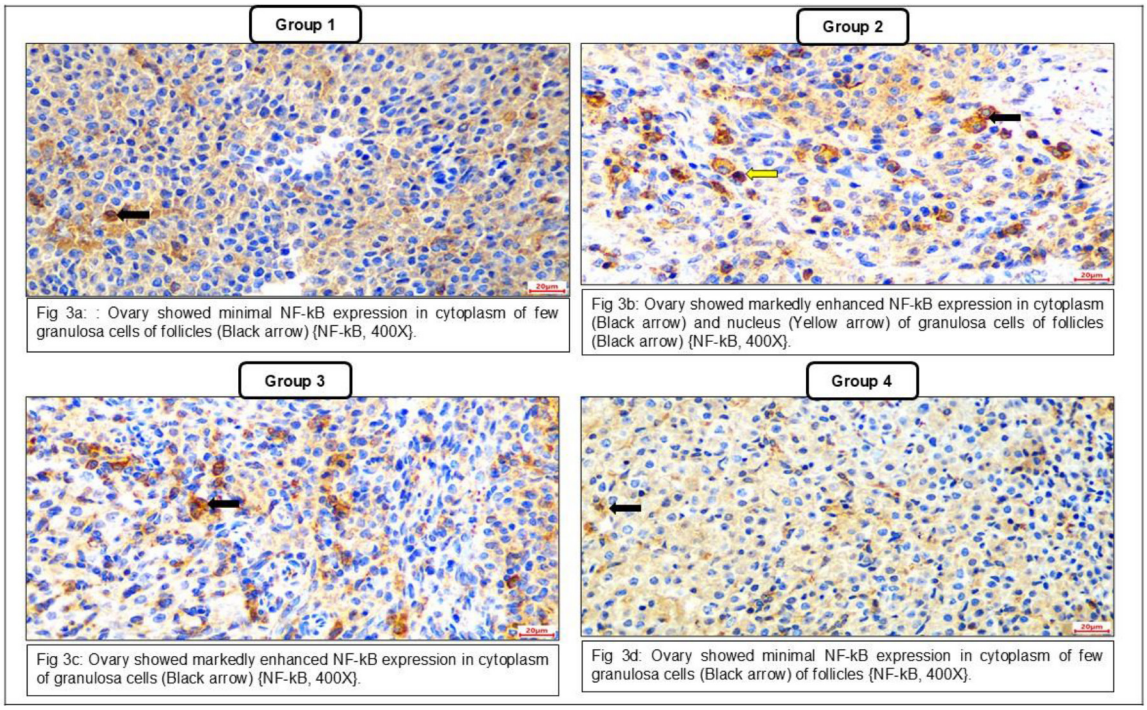


Figure 3(a-d). Showing NFkB immunoexpression in ovarian tissues across the experimental Groups (1-4), NFkB {400x}.

cells of CL and granulosa cells of ovarian tissue (Figure 3a). Markedly enhanced NF-kB expression in the cytoplasm and nucleus of granulosa cells was observed in Group 2, indicating the inflammatory activity of letrozole on ovarian tissue (Figure 3b). NF-kB will be activated through various signalling pathways during

an inflammatory response and then translocated to the nucleus, where it activates gene transcription and plays a critical role in regulating genes involved in inflammation. In Group 3, moderately enhanced NF-kB expression in the cytoplasm of granulosa cells(Figure 3c), in Group 4, minimal NF-kB expression in cytoplasm of few granulosa cells of follicles (Figure 3d), suggesting that the plant extract at high dose (500 mg/k) exhibits a notable anti-inflammatory effect by effectively reducing NFkB expression associated with letrozole-induced inflammation.

4. Discussion

Polycystic Ovarian Syndrome (PCOS) is a common endocrine disorder in women during their reproductive age[22,23]. Various factors, like hormonal, genetic, environmental, and metabolic, can be implicated in the aetiology of PCOS; however, the cause is still elusive. PCOS is most associated with oligo- or anovulation[24], hyperandrogenemia, obesity, and the presence of multiple ovarian follicles. As per the Rotterdam criteria, which were introduced in 2003, to diagnose at least two of the following three criteria must be present: (1) oligo- or anovulation, (2) multiple cystic follicles, and (3) hyperandrogenism[25]. Several studies reported that oxidative stress (OS) is one of the major causes in PCOS manifestation. OS will lead to the development of many diseases like neurodegenerative[26], cardiovascular diseases, diabetes, and cancers[6]. The World Health Organisation (WHO) reported that more than 116 million women globally were impacted by PCOS in the year 2012. A nationwide survey conducted in India in 2020 revealed that approximately 16% of female subjects aged between 20 and 29 years had PCOS[27]. Global prevalence of PCOS emphasises the importance of more awareness, early detection, and affordable treatment measures to enhance reproductive health among women. Use of some drugs, such as metformin and clomiphene citrate, results in some side effects, like nausea and abdominal pain. As a result, there is increasing interest in alternative treatments such as herbal medicines that are effective for PCOS. Such herbal medicines improve lipid peroxidation, insulin resistance, and inflammation, which are etiologies for PCOS[28]. Recent trends indicate a global shift towards traditional herbal medicine, valued for its therapeutic benefits and minimal toxicity. Traditional medicine remains the main source of primary health care for nearly 80% of the world population[29]. *Phyllanthus niruri*, commonly known as *Chanca piedra* in Spanish, *Pitirishi* in India. This herb is commonly used for renal stones, jaundice, diabetes, hepatitis, and bronchitis[8]. Medicinal effects are largely due to bioactive compounds like alkaloids, flavonoids, phenylpropanoids, and polyphenols[30]. The current study demonstrates that administering *P. niruri* at a high dose (500 mg/kg) effectively mitigates PCOS symptoms by normalising the oestrous cycle, lowering levels of insulin, testosterone, and AMH and elevating superoxide dismutase (SOD) activity. PCOS was successfully induced by administering letrozole for 21 days. This was confirmed by irregular oestrous cyclicity, mainly prolonged diestrus phase[31,32] and reduction in body weight. Letrozole is an aromatase inhibitor, which decreases the conversion of androgens into estrogen, resulting in hyperandrogenemia[33,34]. During the induction phase, Groups 2 to 4 (induced with PCOS) had irregular oestrous cycles, and those in the control group (Group 1) had normal cycling. After the treatment phase, a normal oestrous cycle was found in the animals given the high dose (Group 4). Group 3 animals, however, had little regularisation of their cycles.

In this study, increased levels of testosterone were observed in the PCOS-induced and low-dose treatment group, compared with the control, indicating hyperandrogenism. Disruption of the hypothalamo-pituitary-ovarian (HPO) axis in PCOS leads to greater secretion of luteinizing hormone (LH) from the anterior pituitary compared to follicle-stimulating hormone (FSH)[35]. Elevated LH levels stimulate theca cells to produce excess androgens, contributing to hyperandrogenism. Meanwhile, reduced FSH levels diminish estrogen production by granulosa cells, resulting in impaired follicular development and anovulation. These findings are consistent with previous studies[22,36,37]. In the high dose treatment group, testosterone levels are normal when compared with the control (Group1).

Anti-Mullerian Hormone (AMH), produced by granulosa cells of pre-antral and antral follicles, serves as an important biomarker for the diagnosis of PCOS[38,39]. Elevated AMH levels are known to

inhibit the development of a dominant follicle, leading to anovulation, a hallmark feature of PCOS. In the present study, Groups 2 and 3 exhibited significantly higher AMH levels compared to the control Group (Group 1), indicating disrupted follicular development. Interestingly, Group 4 showed a significant reduction in AMH levels, suggesting a potential therapeutic effect in restoring ovarian function.

In PCOS, decreased serum SOD levels have been reported[21,32]. In this study, Groups 2 and 3 showed a marked reduction in SOD levels compared to the control group. These findings correlate with earlier studies[11,40]. Interestingly, an elevation in SOD levels was noted in Group 4, contrary to the anticipated pattern.

Polycystic ovary syndrome (PCOS) is a hormonal disorder characterised by insulin resistance (IR) and hyperinsulinemia[41,42]. Body fat distribution is one of the key factors contributing to the development of IR in PCOS[43]. In this study, a significant increase in fasting insulin levels was observed in animals from Groups 2 and 3, indicating elevated IR. In contrast, animals in Group 4 showed a significant decrease in insulin levels after treatment with a high dose, suggesting a potential improvement in insulin sensitivity.

The Ovarian section of PCOS induced rats exhibited multiple cystic follicles[44], atresia, necrosis, degeneration of corpus luteum and stromal fibrosis. However, after treatment, significant recovery from ovarian damage, reduction in follicular cysts were observed in animals treated with high dose (Group 4). NF-kB is a primary transcription factor that plays a role in regulating immune and inflammatory responses[45]. NF-kB will be activated through various signalling pathways during an inflammatory response and then translocated to the nucleus, where it activates gene transcription and plays a significant role in the regulation of genes involved in inflammation. TNF alpha is a proinflammatory cytokine produced mainly by immune cells like macrophages, and it will be elevated during an inflammatory response in the body. In the current study, a significant increase in expression of TNF alpha and NF-kB in cytoplasm and granulosa cell nuclei was observed in Group 2, which could be due to the inflammatory activity of letrozole on ovarian tissue. Minimal expression was seen in Group 4, indicating the prominent efficacy of plant extract (high dose) on minimising inflammation induced by letrozole.

5. Conclusion

This study is the first to illustrate the therapeutic efficacy of *Phyllanthus niruri* in a Letrozole-induced PCOS in Wistar rats. Administration of *P. niruri* resulted in significant improvement in antioxidant activity and hormonal balance, including the reduction of elevated insulin, testosterone and AMH levels. Histopathological analysis revealed marked restoration of normal ovarian architecture, characterised by a reduction in cystic follicles and the reappearance of healthy growing follicles and corpora lutea. Furthermore, increased SOD activity, which supports a protective role against oxidative stress in ovarian tissues. These findings suggest that *Phyllanthus niruri* exerts multi-dimensional therapeutic effects by regulating endocrine function, improving ovarian histology, and mitigating oxidative stress, thus providing a potential herbal alternative for the management of PCOS. However, further clinical investigations and molecular research are needed to comprehensively understand its mechanisms of action and human therapeutic potential.

6. Author Contribution

N. Sree Devi: Conceptualisation, writing and editing the original paper. S. Saravana Kumar: Supervision, N. Hima Bindu, A. Gnanavel, S. Karthick: proof reading All authors have read and agreed to the published version of the manuscript.

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