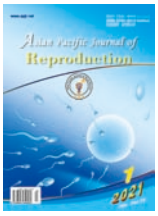

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Ethanolic extract of *Azadirachta indica* ameliorates ovarian defects through phosphoinositide−3 kinase inhibition in a rat model of polycystic ovary syndrome

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ABSTRACT

Objective: To assess the therapeutic potential of ethanolic extract of *Azadirachta (A.) indica* in rats with polycystic ovary syndrome (PCOS).

Methods: Thirty-five prepubertal female Sprague Dawley rats were randomly divided into five groups with 7 animals in each group. Group 1 received 0.5% carboxy methyl cellulose orally. Groups 2 to 5 received testosterone propionate (0.2 mg/kg, *s.c.*) dissolved in olive oil daily for 42 days to induce PCOS. In addition, group 3 was administered with *A. indica* extract (100 mg/kg, 0.5% carboxy methyl cellulose orally) from the 7th to 12th week, group 4 received quercetin (100 mg/kg, 0.5% carboxy methyl cellulose orally) and group 5 received wartmannin (100 mg/kg, 0.5% carboxy methyl cellulose orally). At the end of treatment, blood was collected for biochemical evaluation. Total follicular count and uterus corpus luteum count followed by *PI3K* gene expression in the ovary and uterus were evaluated.

Results: The ethanolic extracts of *A. indica* significantly reduced body weight, ovary weight and uterus weight of rats. Extracts of *A. indica* also significantly increased the levels of serum glucose, total cholesterol, triglyceride, low-density lipoprotein, very low-density lipoprotein, insulin, testosterone, and luteinizing hormone. Treatment also reduced lipid peroxidation and increased antioxidant parameters in the liver homogenates of PCOS-induced rats. Histological examination of the ovary and uterus confirmed PCOS occurrence and remission state in the PCOS-induced and treated groups, respectively. Moreover, *A. indica* and quercetin significantly downregulated *PI3K* gene expression. Histopathological results of the ovary and uterus also proved the protective role of *A. indica*.

Conclusions: *A. indica* leaf extract has beneficial effects in the treatment of PCOS by downregulation of *PI3K* gene expression.

KEYWORDS: *Azadirachta indica*; PI3 kinase; Quercetin; Steroidogenesis; Testosterone propionate; Wartmannin

1. Introduction

Polycystic ovary syndrome (PCOS) is a metabolic and clinically heterogeneous reproductive endocrine disease with a high prevalence in women of childbearing age characterized by polycystic ovaries, hyperandrogenism, anovulation, acne, increase in body weight, irregular menstruation, and oligo/anovulation. It is one of the main causes of infertility in females. The major clinical manifestations include irregular menstruation, amenorrhea, acne, and hursutisum[1]. The most common treatments for PCOS include hormonal contraceptives, progestin, letrozole, clomiphene or gonadotropin, and metformin. All these are short term symptomatic therapies and associated with mild to severe side effects. Taking together all these factors, there is a need for a safe, cost-effective, and long-term management strategy.

Azadirachta (*A.*) *indica*, known as neem, is a member of the Meliaceae family and has been widely used in Chinese, Ayurvedic, and Unani system of medicines in the treatment and prevention of various diseases[2]. *A. indica* contains various constituents including quercetin, β-sitosterol, nimbin, nimbidin, nimbolide, and limonoids and plays an important role in management of various disease like arthritis, diabetes, ulcers, cancer, bacterial and fungal infections through modulation of various pathways[3−5]. Quercetin is polyphenolic flavonoids abundantly present in fresh leaves of *A. indica* and is reported to have a beneficial role in treating inflammation, hypertension, mood disorders, obesity, antioxidant and gastrointestinal protective action[6]. While studying natural molecules from plant origin, we previously found that quercetin exhibited a beneficial role in the animal model of PCOS through inhibition of phosphoinositide-3 kinase (*PI3K*)[7]. It has been reported that *PI3K* in the ovary controls the androgen synthesis.

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Inhibition of *PI3K* can be a promising target in the treatment of PCOS. Hence, this study is to evaluate the therapeutic potential of *A. indica* rich in quercetin in rat model of testosterone propionate-induced PCOS. Gene expression is also determined to investigate the underlying mechanism.

2. Material and methods

2.1. Materials

Quercetin powder was procured from Otto Chemie Pvt. Ltd, India. Testosterone propionate was procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Diagnostic kits for the estimation of serum glucose, cholesterol, triglyceride, low-density lipoprotein (LDL) cholesterol, very-low-density lipoprotein (VLDL) cholesterol, and high-density lipoprotein (HDL) cholesterol, were acquired from Lab-care Diagnostics Pvt. Ltd. India. Enzyme-linked immunoabsorbent assay (ELISA) kits for the estimation of insulin, testosterone, and luteinizing hormone (LH) were obtained from Krishgen Biosystems, India. Chemicals for gene expression studies like FastRNA® Pro Green kit were obtained from MP Biomedicals. Revert Aid First Strand cDNA Synthesis Kit and polymerase chain reaction (PCR) master mix were procured from Thermo Scientific, India. GeneRuler™ 100 bp DNA Ladder from Fermentas life sciences and primers from Eurofins Genomics were procured. Other chemicals used were of analytical grade.

2.2. Sample collection and extraction

The fresh leaves of *A. indica* were obtained from the herbal garden of the Institute of Pharmacy Nirma University in the month of March 2015 by picking. Authentication was done by Department of Pharmacognosy, Institute of Pharmacy, Nirma University, Ahmedabad, and voucher specimen was deposited (NIP/PCOL/2016). Leaves (1 kg) were washed with tap water followed by distilled water to remove dust particles. Leaves were then dried in hot air oven at 60 ℃ for 24 h. Dry leaves were then grinded to make it in a powder form using grinder. The powder was passed through sieve number 60. The sieved powder (250 g) was extracted with ethanol (500 mL, 36 h) in soxhlet apparatus. Obtained extract was subjected to rotary evaporator, subsequently concentrated under reduced pressure (invaccum at 40 ℃) evaporated to dryness and stored at 4 ℃ in air tight bottle.

2.3. Phytochemical analysis of A. indica extract and identification of the active component

Morphological and physicochemical analysis were carried out to identify and confirm the collected leaves. The extract was analyzed by the Mayer's test, cyanidin test, Borntrager test, Libermann Burchard test, foam test and ferric chloride tests for determination of the presence of alkaloids, flavonoids, glycosides, phytosterols, saponins and tannins, respectively. Quantitative estimation of quercetin in the ethanolic extract of *A. indica* was carried out by using thin layer chrometography method.

2.4. Animals

Thirty-five healthy prepubertal (three weeks old) Sprague Dawley female rats weighing 50-75 g were acquired from Zydus Research Centre, Ahmedabad, India and housed in a pathogen-free environment at the animal house of the Institute of Pharmacy, Nirma University. Animals were housed under well-controlled temperature (22±5) ℃, humidity (55±5)%, and 12 h/12 h light-dark cycle with a well-ventilated animal house under a natural photoperiodic condition in polypropylene cages with free access to food and water *ad libitum*.

2.5. Treatment protocol

These 35 female rats were randomly divided into five groups (n=7 in each group). The PCOS was induced by subcutaneous injection of testosterone propionate (0.2 mg/kg) dissolved in olive oil daily for 6 weeks[8]. Group 1 was the normal control group, receiving 0.5% carboxy methyl cellulose orally. Group 2 was PCOS-induced group administered with testosterone for 6 weeks without treatment. Group 3 was PCOS-induced group and administered with *A. indica* extract (100 mg/kg, 0.5% carboxy methyl cellulose orally), Group 4 was PCOS-induced group and administered with quercetin (100 mg/kg 0.5% carboxy methyl cellulose orally), Group 5 was PCOS-induced group and administered with standard drug- wartmannin (100 mg/kg, 0.5% carboxy methyl cellulose orally. After 6 weeks of induction, microscopic observation of vaginal smears was carried out to confirm PCOS status of animals. Doses for *A. Indica* 100 mg/kg was selected based on preliminary study done taking 400 mg/kg as the highest dose, 200 mg/kg as middle dose and 100 mg/kg as the lowest dose[9]. Treatment started on the 7th week. At the end of the 12th week (on day 42 of treatment), the rats were sacrificed for morphological, biological, and histopathological evaluation.

2.6. Morphological and histopathological parameters

Body weight changes in all groups were noted daily till the end of the experiment. After 12 weeks, animals were sacrificed by using high dose of barbiturate and ovary and uterus were removed and weighed. Histopathological evaluation of ovary and uterus was carried out. The ovary and uterus were stored in 10% formalin for fixation. After fixation, the organs were trimmed and embedded in paraffin block after dehydration. Sections were trimmed by using microtome and mounted on the glass slides for staining using hematoxylin and eosin. They were observed with a light microscope

at magnification of 40× (Olympus CX23, Gurgaon, India) for total follicular count (follicle, and cystic follicle) and presence of corpus luteum. The adenomyosis as well as thickness of endometrium and myometrium in myometrium was observed in uterine sections.

2.7. Serum biochemical and hormonal parameters

Glucose levels and total lipid profile like total cholesterol, triglyceride, HDL, LDL, and VLDL cholesterol in serum were evaluated by using lab-care diagnostic kits. Serum insulin levels were estimated by using Insulin ELISA kit. Serum LH and testosterone levels were measured by using ELISA kit.

2.8. Oxidative stress parameters

Liver tissues were finely sliced and homogenized in chilled Tris buffer. The homogenate were centrifuged and clear supernant was used for estimation of various antioxidant parameters like reduced glutathione (GSH), superoxide dismutase (SOD), catalase, and nitric oxide (NO) level. GSH was estimated as per prescribed protocol by Patel *et al*[9]. SOD was estimated as per method described by Weydert and Cullen[10]. Catalase and NO level were estimated by method by Hadwan[11] and Masic *et al*[12] respectively. MDA formation was determined by the method of Noeman *et al*[13]. Result of antioxidant activity in liver was expressed in terms of total protein content which was estimated as protocol by Demirkan *et al*[14].

2.9. Gene expression

The primers for PCR study were designed through Oligo Analyzer 3.1 software and nucleotide tool from NCBI database. RNA was isolated from the ovarian tissue by FastRNA® Pro Green kit. The RNAs of 1.9-2.0 ratio were taken into consideration for cDNA synthesis by Revert Aid First Strand cDNA Synthesis Kit. The forward primer: 5′-CTGCTGTAGGCCGAGTAAG-3′ and reverse primer: 5′-GTGAGACCCCAAGTCCATCG-3′ were used. Further housekeeping gene used was GAPDH. The synthesized cDNA was amplified with initial denaturation, denaturation, annealing, extension, and final extension temperatures 94 ℃, 95 ℃, 49 ℃, 72 ℃, 72 ℃, respectively. Obtained PCR products were analyzed with gel electrophoresis followed by gel doc analysis.

2.10. Statistical analysis

Results were represented as mean±standard deviation (mean±SD). Statistical analysis was performed by using Graph Pad Prism 5 Statistical software. Statistical differences between the means of various groups were evaluated by using one-way analysis of variance followed by Tukey's test and data were considered to be statistically significant at P value <0.05.

2.11. Ethics statement

This study was approved by the Institutional Animal Ethics Committee of the Institute of Pharmacy, Nirma University, Ahmadabad (protocol No. IP/PCOL/MPH/17/007). The study also followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forests and Climate Change, Government of India.

3. Results

3.1. Pharmacognostic and phytochemical analysis of leaf of *A. indica*

The morphological characteristics like colour, odour, and taste confirmed the morphological and physical characteristics of collected leaves. In phytochemical analysis ethanolic extract of *A. indica* showed the presence of glycoside and flavonoids having highest concentration, while alkaloids and tannins having moderate concentration, saponins and phytosterols having low concentration. The presence of quercetin was confirmed by using thin layer chrometography with a reproducible Rf value of 0.60-0.63.

3.2. Effect of treatments on morphological parameters

Testosterone propionate significantly increased body, ovary, and uterus weight in the PCOS control group as compared with the normal control group. Treatment with *A. indica*, quercetin and wartmannin significantly reduced body weight as compared with the PCOS control group. In addition, treatment with *A. indica* and quercetin significantly decreased ovary and uterus weight as compared with the PCOS control group, while wartmannin did not significantly reduce both ovary and uterus weight (Figure 1).

3.3. Effect of treatments on histopathological change

In the normal control group, there were no morphological changes in ovaries and uterus, while in the PCOS control animals an increase in follicular count with large follicular cysts and absence of corpus luteum were observed in ovaries. *A. indica* treatment significantly reduced the number of follicles and there were fewer granulose cells, which were responsible for the cyst formation. Similar result was observed in the quercetin and wartmannin treatment groups (Figure 2). Similarly, the PCOS control group showed a development of adenomyosis in endometrium as well as myometrium as compared with normal control group. Treatment of *A. indica*, quercetin, and wartmannin showed a significant reduction in adenomyosis and thickness of endometrium and myometrium (Figure 3).

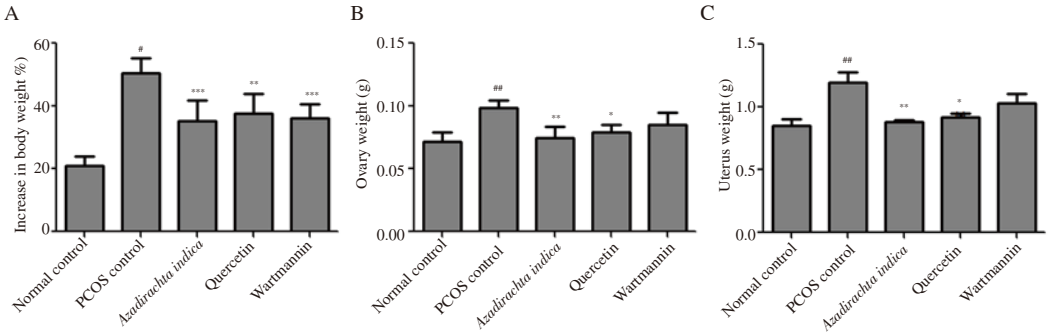

Figure 1. Effect of treatments on body weight (A), ovary weight (B), and uterus weight. Values are expressed as mean±SD; n=7 in each group. # and ##: Significantly different from the normal control group, P<0.05, P<0.01, respectively. *, **, and ***: Significantly different from the PCOS control group, P<0.05, P<0.01, P<0.001, respectively.

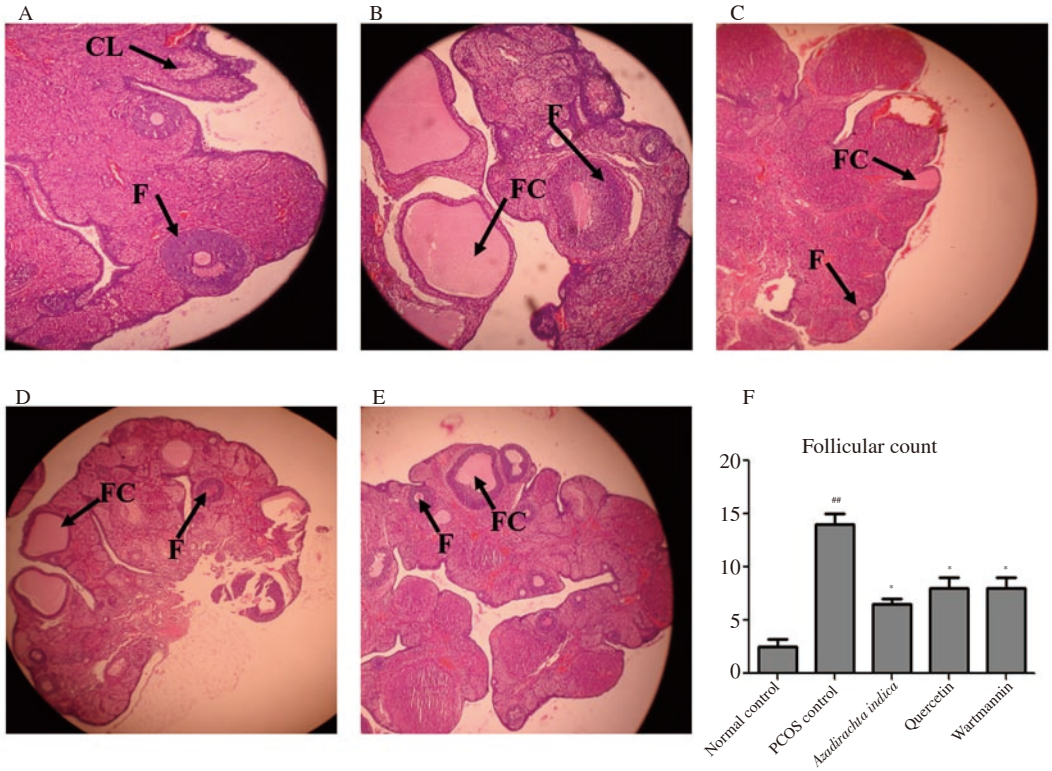

Figure 2. Effect of treatments on histopathology of rat ovaries. Histological sections are stained with hematoxylin and eosin (magnification: 40×). A: The normal control group shows mature follicle with corpus luteum. B: The PCOS control group shows many follicular cysts with absence of corpus luteum. Treatment with *Azadirachta indica* (C), quercetin (D) and wartmannin (E) reduce the number of follicular cysts. CL: Corpus luteum; F: Follicles; FC: Cystic follicles. ##: Significantly different from the normal control group, P<0.01; *: Significantly different from the PCOS control group, P<0.05.

3.4. Effect of treatments on serum glucose and lipid profile

Serum glucose, cholesterol, triglyceride, LDL and VLDL levels were significantly increased in the PCOS control group as compared with the normal control group, while serum HDL level was decreased. Treatment with *A. indica* and wartmannin significantly reduced serum glucose level, while a slight reduction of serum glucose level was observed in the quercetin treated group. *A. indica* significantly decreased serum cholesterol, triglyceride, LDL, and VLDL levels, but there was no significant reduction in cholesterol, triglyceride, LDL or VLDL levels in the quercetin and wartmannin treatment groups. And no significant improvement in serum HDL levels was observed in any treatment group (Table 1).

3.5. Effect of treatments on hormone levels

The PCOS control group showed a significant increase in serum insulin, LH, and serum testosterone levels as compared with the normal control group (P<0.001). Treatment with *A. indica* and quercetin significantly reduced serum insulin, LH and serum testosterone levels, while wartmannin did not produce any significant reduction in above hormonal levels as compared with the PCOS control group and other treatment groups (Figure 4).

3.6. Effect of treatments on oxidative stress parameters and total protein

The total protein levels were normal in all groups. There was a significant reduction in GSH, catalase, SOD, and reduced NO levels in the PCOS control group. Treatment with *A. indica* and quercetin significantly increased GSH, catalase and SOD levels, and reduced NO levels, while no significant difference was observed between the wartmannin treatment group and the PCOS control group (Table 2).

3.7. Effect of treatment on PI3 kinase inhibition

PCOS control animals showed a significant increase in *PI3K* mRNA expression in the ovarian theca cells as shown in Figure 5A. Treatment with *A. indica*, quercetin, and wartmannin significantly decreased *PI3K* mRNA expression as shown in Figure 5B.

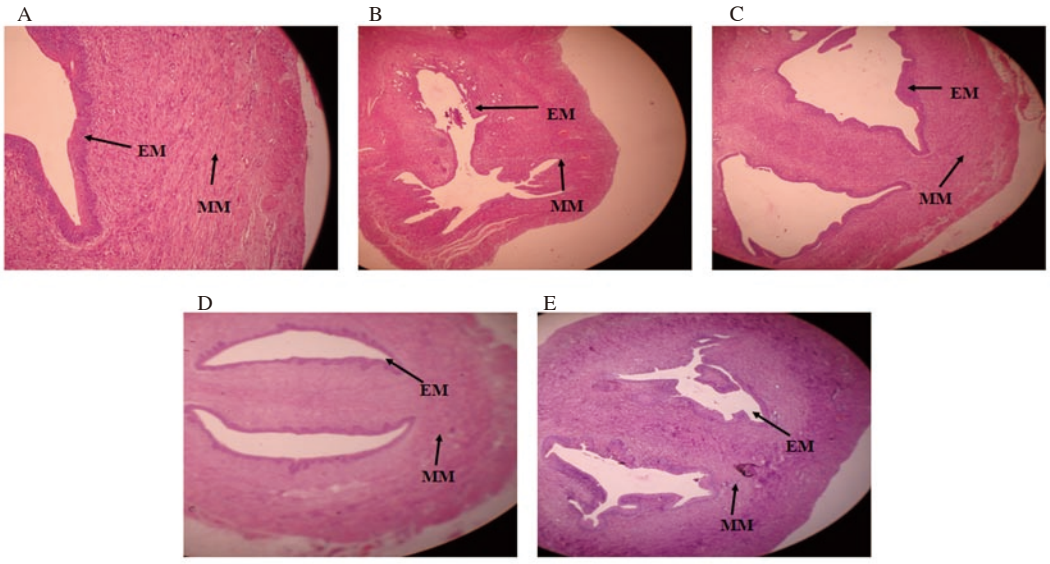

Figure 3. Effect of treatments on histopathology of the uterus. Histological sections are stained with hematoxylin and eosin (magnification: 40×). A: The normal control animals show normal architecture; B: The PCOS control group shows adenomyosis of the endometrium and myometrium with increased thickness; C: The *Azadirachta indica* treatment group shows mild adenomyosis with intact endometrium; D: The quercetin treatment group shows moderate adenomyosis with reduced thickness of the endometrium and myometrium; E: The wartmannin treatment group shows reduced adenomyosis with reduced thickness of the endometrium. MM: Myometrium; EM: Endometrium.

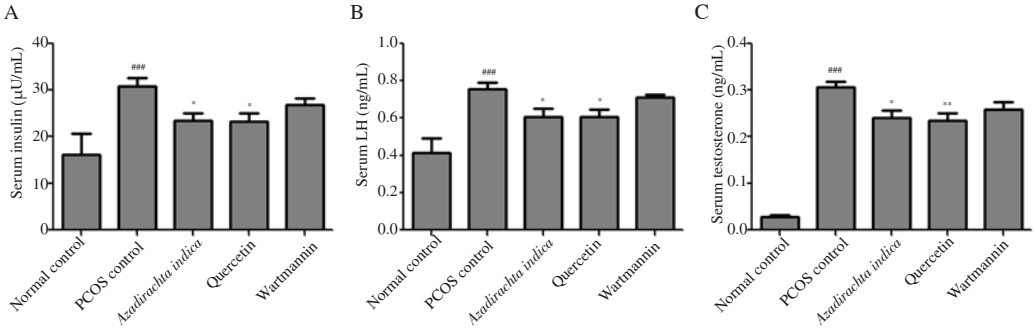

Figure 4. Effect of treatments on hormone levels after 12th week. A: Serum insulin levels; B: Serum luteinizing hormone (LH) levels; C: Serum testosterone level. Values are expressed as mean±SD; n=7 in each group. $^{###}$: Significantly different from the normal control group, P<0.001. * and **: Significantly different from the PCOS control group, P<0.05, P<0.01, respectively.

Table 1. Effect of treatments on biochemical parameters.

Parameters	Normal control	PCOS control	*Azadirachta indica*	Quercetin	Wartmannin
Serum glucose (mg/dL)	82.28±13.10	133.70±27.10$^{\#}$	95.67±18.3*	103.40±19.40	91.14±23.40***
Serum cholesterol (mg/dL)	179.70±21.80	227.40±16.40$^{\#}$	192.60±12.20**	215.50±8.10	198.40±12.03
Serum triglyceride (mg/dL)	92.35±10.70	143.70±27.30$^{\#}$	80.22±35.7*	112.80±37.90	112.90±25.40
Serum HDL (mg/dL)	67.95±21.00	58.97±9.71	81.63±12.7	60.10±14.00	74.48±18.10
Serum LDL (mg/dL)	90.00±15.70	129.30±27.10$^{\#}$	92.81±14.90*	130.70±9.51	116.90±14.40
Serum VLDL (mg/dL)	18.48±2.13	28.73±5.46$^{\#}$	16.04±7.13*	22.56±7.57	22.57±5.09

Values are expressed as mean±SD; n=7 in each group. $^{\#}$: Significantly different from the normal control group, P<0.05. *, **, ***: Significantly different from the PCOS control group, P<0.05, P<0.01, P<0.001, respectively. HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very-low-density lipoprotein.

Table 2. Changes in oxidative stress parameters.

Parameters	Normal control	PCOS control	*Azadirachta indica*	Quercetin	Wartmannin
Total protein (mg/mL)	1.45±0.21	1.54±0.11	1.49±0.14	1.55±0.09	1.61±0.05
GSH (μg/mg protein)	4.31±0.41	2.41±0.14$^{\#}$	4.66±0.52**	4.11±0.11*	3.93±0.19
SOD level (U/mg protein)	0.060±0.005	0.011±0.005$^{\#}$	0.051±0.002**	0.037±0.009*	0.048±0.007
Catalase (μmol/mg protein)	21.25±0.84	54.05±2.21$^{\#}$	40.90±1.06**	45.24±2.81*	46.09±1.50
NO (μmol/mg protein)	3.85±0.05	1.92±0.38$^{\#}$	3.66±0.11*	2.74±0.05	2.65±0.52

Values are expressed as mean±SD; n=7 in each group. $^{\#}$: Significantly different from the normal control group, P<0.05. *, **: Significantly different from the PCOS control group, P<0.05, P<0.01, respectively. GSH: glutathione; SOD: superoxide dismutase; NO: nitric oxide.

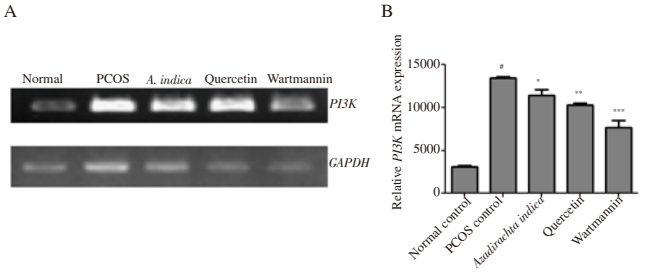

Figure 5. Effect of treatment on phosphoinositide-3 kinase (*PI3K*) expression. A: Representative images; B: *PI3K* mRNA expression. Values are expressed as mean±SD; n=7 in each group. $^{\#}$: Significantly different from the normal control group, P<0.05. *,**,***: Significantly different from the PCOS control group, P<0.05, P<0.01, P<0.001, respectively.

4. Discussion

PCOS is the most common endocrine disease and is more likely to develop obesity, insulin resistance and have a greater risk of hypertension, ovarian cancer, depression, and miscarriage[15,16]. Several reports show the efficacy of various drugs used for PCOS[17]. In the present study, prepubertal androgen model is used because it produces polycystic ovaries, blocks ovulation and attenuates progesterone production. Moreover, high insulin levels in the testosterone-treated rats can lead to insulin resistance[18]. This model mimics some of the important characteristics of human PCOS.

A. indica has major chemical constituents like azadirachtin, nimbinin, nimbidin, lomonoids, margosa, isomeldenin, quercetin, nimbadiol, nimocinol, *etc*. Quercetin is one of the major bioactive flavonoids found in the leaves of *A. indica* and quantitative analysis indicates that the aqueous leaf extract contains 6 to 8 mg% (w/w) quercetin[19,20]. Our previous studies showed that quercetin has a beneficial effect in treating PCOS by inhibiting *PI3K* which attributes to a decrease in the expression of *CYP17A1* gene, a key player in steroidogenesis. This result led us find out whether *A. indica* leaf extract rich in quercetin can produce protection against PCOS.

Weight gain is a common feature of PCOS. In the present study, animal weight was increased PCOS rats and treatments with quercetin and *A. indica* leaf extract significantly reduce the weight gain induced by testosterone propionate. It has been reported that testosterone propionate increases body weight due to hormonal imbalance and insulin resistance in PCOS[21]. Quercetin is reported to reduce insulin resistance[22]. Therefore, quercetin reduces body weight in testosterone-induced animals *via* reducing insulin resistance.

Prenatal exposure to high androgen doses induces abnormal follicular growth, resulting in cyst formation and consequently the increase in ovary and uterus weight[23]. Earlier reports showed that rats treated with androgens show a significant increase in ovary and uterus weights[24]. In our study, testosterone propionate administration was found to exhibit increases in ovary and uterus weight, and treatment with *A. indica*, quercetin and wartmannin produced a decrease in ovary and uterus weight, which indicates beneficial effects in PCOS.

Histopathological alterations in the ovaries of prenatal androgenized female rats suggested that androgen affects the ovary development and functions[25]. Further, altered expression of caspase and matrix metalloproteinase, which are markers of apoptosis and necrosis, was reported to increase in atretic follicles in the animal model of PCOS[21]. Active constituents isolated from *A. indica* were reported to reduce caspase-dependent apoptosis by targeting *PI3K*/Akt pathway[26]. Thus, in the present study, histopathological result shows that PCOS rats exhibited an increase in follicular and corpus luteal count in the ovary. Treatment with *A. indica* extract significantly improved histological changes in the ovarian cortex, which may be through *PI3K*/Akt signaling pathway. It suggests that *A. indica* extract could improve the follicular development in PCOS rats.

The leaf of *A. indica* has been reported to have a significant reducing effect on the blood glucose level in adrenaline-induced hyperglycaemia model[27]. Data of the present study also show a decrease in glucose level by treatment with *A. indica* leaf extract but quercetin did not produce changes in glucose levels, which indicates that other constituents present in *A. indica* leaf extract may be responsible for glucose-lowering effect.

Dyslipidaemia is very common in PCOS and characterized by elevated levels of cholesterol triglyceride, LDL and lowered levels of HDL[28]. In the present study, treatment with *A. indica* lowers the cholesterol and triglyceride level and also shows a decrease in LDL and VLDL, while quercetin did not produce reduction in lipid levels, which indicates that the impact of quercetin supplementation on plasma lipid levels is less as compared with the whole leaf extract.

Ovulation is strongly dependent on the stimulation of LH and its effect on the androgen production in the ovarian theca cells

by activation of the *PI3K*/Akt pathway[29,30]. Administration of testosterone propionate at gestational age shows a increase in serum testosterone and LH levels which were found consistent with reproductive features of clinical PCOS. Numerous reports have described that *PI3K* is involved in LH-induced Akt phosphorylation in theca cells which results in an increase in ovarian steroidogenesis[6]. Thus, the inhibition of *PI3K* leads to a decrease in the secretion of LH and testosterone hormone and this kinase might be potential target for the treatment of PCOS. The data presented here showed that the treatments with *A. indica*, quercetin, and wartmannin lower the testosterone and LH levels. Thus, protection produced by *A. indica* leaf extract may be due to inhibition of *PI3K*.

PCOS is characterized by hyperinsulinemia and insulin resistance. It has also been shown that *PI3K* inhibitors inhibited impairment of insulin secretion[31]. The present study evaluated the therapeutic effect of *A. indica*, quercetin, and wartmannin in PCOS induced rats. The present results showed that *A. indica* and quercetin have equivalent efficacy in reducing hyperinsulinemia in PCOS induced rats. The present findings provided evidence that the efficacy of *A. indica* and quercetin against PCOS is also through improvement in insulin resistance.

Various reports prove that testosterone stimulates the androgen to produce ovarian steroidogenic enzymes by increasing enzymatic oxidative level; this will cause inflamed ovary and uterus. According to a previous report, ethanol extract of *A. indica* leaves containing phenolic compounds strongly influences the activity of antioxidant enzymes and thus lead to protective effect[32]. In this study, PCOS induced rats show a high level of lipid peroxidation and decreased levels of antioxidant enzymes and the *A. indica* treatment group significantly reduced oxidative stress-related parameters. Thus, it seems that the alleviating effect of *A. indica* leaf extract against oxidative stress is ascribed to its phenolic component like quercetin.

To explore the potential mechanism of the therapeutic effect of *A. indica* on PCOS, gene expression of *PI3K* mRNA was carried out. Research studies from recent years have proven the potential relationship of *PI3K* signaling pathway and polycystic ovary syndrome[33,34]. *PI3K* is the direct mediator of insulin-induced thecal steroidogenesis[35]. In the present study, the treatment with *A. indica*, quercetin, and wartmannin showed an inhibitory effect of *PI3K* mRNA expression. In a word, *A. indica* treatment demonstrated the great efficacy in ameliorating PCOS through regulating *PI3K* signalling pathway followed by metabolic and endocrine effect.

Present study have evaluated beneficial effect of *A. indica* and quercetin in animal model of androgen excess through up-regulation of *PI3K* signaling pathway. However, the clinical manifestations of PCOS are complex which limits translational studies using animals.

In conclusion, the ethanolic extract of *A. indica* and quercetin has the beneficial effect in PCOS, which is evident from the biochemical parameters and hormonal parameters, improvement in morphological and histopathological changes of ovaries. The mechanism of action of *A. indica* and quercetin is also confirmed by gene expression studies.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Authors′ contributions

Shraddha Patel contributed in terms of performing the experiment and submitting the data for further analysis. Snehal Patel designed the research work and analyzed the data with inferences. The remaining authors Harsh Maru, Vishal Chavda and Jigar Shah contributed in terms of technical support and manuscript preparation.

References

[1] Desai NA, Patel SS. Increased insulin-like growth factor-1 in relation to cardiovascular function in polycystic ovary syndrome: Friend or foe? *Gynecol Endocrinol* 2015; **31**: 801-807.

[2] Alzohairy MA. Therapeutics role of *A. indica* (nem) and their active constituents in diseases prevention and treatment. *Evid Based Complement Alternat Med* 2016; **2016**: 7382506-7382517.

[3] Bandyopadhyay U, Biswas K, Sengupta A, Moitra P, Dutta P, Sarkar D, et al. Clinical studies on the effect of Neem (*A. indica*) bark extract on gastric secretion and gastroduodenal ulcer. *Life Sci* 2004; **75**: 2867-2878.

[4] Ebong PE, Atangwho IJ, Eyong EU, Egbung GE. The antidiabetic efficacy of combined extracts from two continental plants: *A. indica* (A. Juss) (neem) and *Vernonia amygdalina* (Del.) (African bitter leaf). *Am J Biochem Biotechnol* 2008; **4**: 239-244.

[5] Paul R, Prasad M, Sah NK. Anticancer biology of *A. indica* L. (neem): A mini review. *Cancer Biol Ther* 2011; **12**: 467-476.

[6] Kumar D, Rahal A, Malik JK. Neem extract. In: Gupta Ramesh K. *Nutraceuticals*. Boston: Acedemic Press; 2016, p. 585-597.

[7] Shah KN, Patel SS. Phosphatidylinositide 3-kinase inhibition: A new potential target for the treatment of polycystic ovarian syndrome. *Pharm Biol* 2016; **54**: 975-983.

[8] Shah K, Patel S. Phosphatidylinositide-3 kinase: A newer molecular target in metabolic and hormonal pathway of polycystic ovary syndrome. *Exp Clin Endocrinol Diabetes* 2014; **122**: 261-267.

[9] Satyanarayana K, Sravanthi K, Shaker IA, Ponnulakshmi R. Molecular approach to identify antidiabetic potential of *Azadirachta indica*. *J Ayurveda Integr Med* 2015; **6**(3): 165-174.

[10]Patel SS, Goyal RK, Shah RS, Tirgar PR, Jadav PD. Experimental study on effect of hydroalcoholic extract of *Emblica officinalis* fruits on glucose homeostasis and metabolic parameters. *Ayu* 2013; **34**(4): 440-444.

[11]Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc* 2010; **5**(1): 51-66.

[12]Hadwan MH. Simple spectrophotometric assay for measuring catalase activity in biological tissues. *BMC Biochem* 2018; **19**: 7-15.

[13]Masic A, Santos ATL, Etter B, Udert KM, Villez K. Estimation of nitrite in source-separated nitrified urine with UV spectrophotometry. *Water Res* 2015; **85**: 244-254.

[14]Noeman SA, Hamooda HE, Baalash AA. Biochemical study of oxidative stress markers in the liver, kidney and heart of high fat diet induced obesity in rats. *Diabetol Metab Syndr* 2011; **3**(1): 17-25.

[15]Demirkan G, Salomon AR, Gruppuso PA. Phosphoproteomic analysis of liver homogenates. *Methods Mol Biol* 2012; **909**: 151-163.

[16]El Hayek S, Bitar L, Hamdar LH, Mirza FG, Daoud G. Polyystic ovarian syndrome: An updated overview. *Front Physiol* 2016; **5**(7): 124-139.

[17]Teede H, Deeks A, Moran L. Polycystic ovary syndrome: A complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. *BMC Med* 2010; **8**: 41-51.

[18]Ryu Y, Kim SW, Kim YY, Ku SY. Animal models for human polycystic ovary syndrome (PCOS) focused on the use of *indirect hormonal perturbations: A review of the literature. Int J Mol Sci* 2019; **20**(11): 2720-2747.

[19]Ghowsi M, Yousofvand N, Moradi S. Effects of *Salvia officinalis* L. (common sage) leaves tea on insulin resistance, lipid profile, and oxidative stress in rats with polycystic ovary: An experimental study. *Avicenna J Phytomed* 2020; **10**(3): 263-272.

[20]Subapriya R, Nagini S. Medicinal properties of neem leaves: A review. *Anticancer Agents Med Chem* 2005; **5**: 149-156.

[21]Sithisarn P, Gritsanapan W. Variability of antioxidative quercetin content in siamese neem tree leaves in Thailand by TLC-densitometry. *Acta Hortic* 2008; **78**: 161-168.

[22]Honnma H, Endo T, Henmi H, Nagasawa K, Baba T, Yamazaki K, et al. Altered expression of Fas/Fas ligand/caspase 8 and membrane type 1-matrix metalloproteinase in atretic follicles within dehydroepiandrosterone-induced polycystic ovaries in rats. *Apoptosis* 2006; **11**: 1525-1533.

[23]Arias N, Macarulla MT, Aguirre L, Martínez-Castaño MG, Portillo MP. Quercetin can reduce insulin resistance without decreasing adipose tissue and skeletal muscle fat accumulation. *Genes Nutr* 2014; **9**: 361-370.

[24]Sotomayor-Zárate R, Tiszavari M, Cruz G, Lara HE. Neonatal exposure to single doses of estradiol or testosterone programs ovarian follicular development-modified hypothalamic neurotransmitters and causes polycystic ovary during adulthood in the rat. *Fertil Steril* 2011; **96**: 1490-1496.

[25]Wu XY, Li ZL, Wu CY, Liu YM, Lin H, Wang SH, et al. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. *Endocr J* 2010; **57**: 201-209.

[26]Wang F, Yu B, Yang W, Liu J, Lu J, Xia X. Polycystic ovary syndrome resembling histopathological alterations in ovaries from prenatal androgenized female rats. *J Ovarian Res* 2012; **5**: 15-22.

[27]Kumar D, Haldar S, Gorain M, Kumar S, Mulani FA, Yadav AS, Miele L, Thulasiram HV, Kundu GC. Epoxyazadiradione suppresses breast tumor growth through mitochondrial depolarization and caspase-dependent apoptosis by targeting PI3K/Akt pathway. *BMC Cancer* 2018; **18**: 52-69.

[28]Satyanarayana K, Sravanthi K, Shaker IA, Ponnulakshmi R. Molecular approach to identify antidiabetic potential of *A. indica*. *J Ayurveda Integr Med* 2015; **6**: 165-174.

[29]Liu Q, Xie YJ, Qu LH, Zhang MX, Mo ZC. Dyslipidemia involvement in the development of polycystic ovary syndrome. *Taiwan J Obstet Gynecol* 2019; **58**: 447-453.

[30]Fukuda S, Orisaka M, Tajima K, Hattori K, Kotsuji F. Luteinizing hormone-induced Akt phosphorylation and androgen production are modulated by MAP Kinase in bovine theca cells. *J Ovarian Res* 2009; **2**: 17-25.

[31]Chakrabarti J. Serum leptin level in women with polycystic ovary syndrome: Correlation with adiposity, insulin, and circulating testosterone. *Ann Med Health Sci Res* 2013; **3**(2): 191-196.

[32]Marshall JC, Dunaif A. Should all women with PCOS be treated for insulin resistance? *Fertil Steril* 2012; **97**: 18-22.

[33]Gupta NK, Srivastva N, Bubber P, Puri S. The antioxidant potential of *A. indica* ameliorates cardioprotection following diabetic mellitus-induced microangiopathy. *Pharmacogn Mag* 2016; **12**: S371-S378.

[34]Li T, Mo H, Chen W, Li L, Xiao Y, Zhang J, et al. Role of the PI3K-Akt signaling pathway in the pathogenesis of polycystic ovary syndrome. *Reprod Sci* 2017; **24**: 646-655.

[35]Wu C, Jiang F, Wei K, Jiang Z. Exercise activates the PI3K-AKT signal pathway by decreasing the expression of 5 -reductase type 1 in PCOS rats. *Sci Rep* 2018; **8**: 7982-7991.

[36]Pezet S, Marchand F, D'Mello R, Grist J, Clark AK, Malcangio M, et al. Phosphatidylinositol 3-kinase is a key mediator of central sensitization in painful inflammatory conditions. *J Neurosci* 2008; **28**: 4261-4270.