Beneficial effect of polyherbal formulation in letrozole induced Polycystic ovarian syndrome (PCOS)

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A B S T R A C T

Aim of the study: PCOS is an endocrine condition that results in enlarged ovaries with tiny cysts on the margins. The present study aims to investigate the beneficial effect of polyherbal formulation in Letrozole induced PCOS in female Albino Wistar rats.

Materials and methods: Acute toxicity study of the polyherbal formulation by administering a single dose (2000 mg/kg) was done in female Albino Wistar rats (20 weeks, 250 g) following OECD guideline 423. For PCOS induced study female Albino Wistar rats were divided into six groups (6 animals/group). Group I (control) was given 0.5% carboxymethylcellulose (CMC) suspension daily as vehicle control. Letrozole (1 mg/kg) was orally administered for 21 days in Group II to VI for induction of PCOS. After induction of PCOS, animals were treated with standard drug (Group III- Clomiphene citrate- 1 mg/kg) and polyherbal tablets (Group IV − 500 mg/kg, Group V− 750 mg/kg, and Group VI − 1000 mg/kg) up to 50 days. Vaginal smears were taken daily to check the estrous cycle. Body weight was measured weekly. Blood samples were withdrawn on 0, 21 and 50 days for the determination of fasting blood glucose, lipid profile, LH, FSH, and hormonal levels.

Results: Administration of letrozole caused the abnormality in serum sex hormone profile, lipid profile, glucose, and the estrous cycle. The treatment with polyherbal formulation significantly decreased (P < 0.0001) the level of testosterone and improved estradiol and progesterone levels (P < 0.0001). There was a decrease in elevated glucose levels from 71.51 ± 0.15 mg/dl in disease induced group to 57.33 ± 1.90 mg/dl in treatment groups. The triglycerides level was normalized to 33.41 ± 1.81 mg/dl and HDL level was increased to 40.63 ± 1.35 mg/dl in treatment groups. The polyherbal formulation by exerting its beneficial effect also caused the disappearance of the cysts in the ovaries.

Conclusion: The polyherbal formulation was found to be effective in PCOS. The effect may be attributed to the individual herbs reported having a significant effect on the pathophysiology of letrozole induced PCOS.

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1. Introduction

Polycystic ovarian syndrome (PCOS) is a hormonal disorder with several symptoms related to an imbalance of hormones, androgen excess, ovarian dysfunction, and polycystic ovarian morphology which affects women and girls in their reproductive period. The typical clinical features of PCOS include hirsutism, irregular menstruation, chronic anovulation, and infertility. Chronic hyperandrogenism is associated with impaired hypothalamic-pituitary feedback, luteinizing hormone (LH) hypersecretion, premature granulosa cell luteinization, aberrant oocyte maturation, and premature arrest of activated primary follicles. Women with PCOS also show the signs of insulin resistance and hyperinsulinemia and are at higher risk for early onset of type 2 diabetes and metabolic syndrome. Unrecognized or untreated PCOS is a risk factor for cardiovascular disease.

The diagnosis and treatment of PCOS are controversial with...
List of abbreviations

PCOS: Polycystic ovarian syndrome
OEC: Organization for Economic Cooperation and Development
CMC: Carboxymethyl cellulose
COCP: Combined oral contraceptive pills
FSH: Follicle stimulating hormone
LH: Luteinizing hormone
IAEC: Institutional Animal Ethical Committee
CPCSEA: Committee for Purpose of Control and Supervision of Experiments on Animals
HDL: High density lipoprotein
LDL: Low density lipoprotein
ANOVA: Analysis of variance
NC: Normal control
DC: Disease control
SC: Standard control
TG1-500: Treatment group – 1 (500 mg/kg)
TG2-750: Treatment group – 2 (750 mg/kg)
TG3-1000: Treatment group – 1 (1000 mg/kg)

2. Materials and methods

2.1. Preparation and evaluation of polyherbal tablet

The alcoholic extract of TFG (31 mg), CL (62 mg), and BA (31 mg) whereas hydroalcoholic (50:50) extract of SA (62 mg) and BV (62 mg) and CM (250 mg) purified in cow’s urine was used for the preparation of polyherbal tablet (500 mg). Pre-formulation study of the powder blend and post-compression evaluation of tablet was done by various parameters like weight variation, friability, hardness, thickness, diameter, disintegration time, in-vitro dissolution, and accelerated stability study.

2.2. Acute toxicity study

Acute toxicity study of the polyherbal formulation was performed following OECD guidelines 423.

2.2.1. Experimental animals

The animals for the acute toxicity study were approved by the Institutional Animal Ethics Committee (IAEC) of Parul Institute of Pharmacy & Research, approval no. 984/2019-09. Female Albino Wistar rats (6 females) of age 20 weeks and weight 200–250 g were used for the study.

2.2.2. Experimental design

The rats were acclimatized to their surroundings 5 days before the experiment by separating them from the rest of the animals and allotting different cages to them. The rats were identified by color marking and provided with rat feed and water ad libitum. Before...
dosing, the rats were weighed. The polyherbal tablet (500 mg) was crushed and suspended in CMC (0.5%) solution. The single-dose for the administration of polyherbal formulation (1.5 ml/animal) (2000 mg/kg) was administered. Following administration of a single dose of a polyherbal tablet, animals were observed for the clinical symptoms for 30 min, at the hourly intervals for the next 24 h and thereafter for total 14 days. The animals were observed for signs of convulsions, tremors, circling, depression, excitement, and mortality.

After 14th day, the animals were euthanized, and blood and all the vital organs like the heart, liver, kidney, ovaries, lungs, and brain were isolated, cleared from fat and stored in formalin solution. All the organs were fixed in paraffin wax and a histopathological study was carried out for the organs.38

2.3. Letrozole induced PCOS study

2.3.1. Experimental animals

Adult female Albino Wistar rats (200–250 g) were employed for the study. Animals were allowed to acclimatize for two weeks. Throughout the study all animals were caged in polyprene cages and maintained in a controlled environment of (22 ± 3 °C) temperature, (55 ± 5%) humidity and a 12 h light/dark cycle. Animals were fed with a standard diet and water ad libitum. The study was approved by the Institutional Animal Ethical Committee (IAEC) for the use of animals and care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with protocol no. 984/2019-09.

2.3.2. Drugs and reagents

Letrozole was purchased from Triveni Interchem Pvt. Ltd, Vapi, Gujarat, India. Clomiphene citrate was obtained as a gift sample from Shimoga chemicals, Maharashtra. All the chemicals were of analytical grade.

2.3.3. PCOS induction

All the experimental animals except the control group were orally administered with letrozole at a dose of 1 mg/kg dissolved in 0.5% CMC daily for a period of 21 days. Control group received vehicle only (0.5% CMC). Vaginal smears were collected daily and evaluated microscopically using crystal violet stain to confirm the induction of PCOS. The disease was confirmed by the irregularity of estrous cycle.

2.3.4. Study design

The study consisted of 36 female Albino Wistar rats equally divided into six groups designated as Group I (served as a control group), Group II (served as PCOS induced group), Group III (served as a standard group) and Group IV, V and VI (served as treatment groups).

Following letrozole administration, the standard group was administered with clomiphene citrate (active ingredient) at a dose of 1 mg/kg in 0.5% CMC. Treatment groups IV, V and VI were administered with polyherbal formulation with the dose of 500 mg/kg (dose 0.58 ± 0.02%). In vitro dissolution study showed that there was 0.58 ± 0.02% dissolution of letrozole. The tablet was evaluated using post-compression parameters. The optimized polyherbal tablet exhibited a hardness of 1.5 ± 0.06 kg/m², disintegration time of 30 ± 1 min, and friability of 0.58 ± 0.02%. In vitro dissolution study showed that there was 90% drug release at the end of 2 h. The accelerated stability study revealed that the content of markers in the tablet does not deviate from more than 10% of the initial content indicating the stability of the tablet.

3. Results

3.1. Preparation and evaluation of polyherbal tablet

The polyherbal tablet was evaluated using post-compression parameters. The optimized polyherbal tablet exhibited a hardness of 1.5 ± 0.06 kg/m², disintegration time of 30 ± 1 min, and friability of 0.58 ± 0.02%. In vitro dissolution study showed that there was 90% drug release at the end of 2 h. The accelerated stability study revealed that the content of markers in the tablet does not deviate from more than 10% of the initial content indicating the stability of the tablet.

3.2. Acute toxicity study

In an acute toxicity study after oral administration of a single dose of polyherbal formulation (2000 mg/kg) the rats were observed for 30 min at the hourly intervals for the next 24 h and thereafter for 14 days twice daily (morning and evening). The body weight was recorded daily for 14 days. No mortality and clinical signs of ataxia, convulsions, lacrimation, nasal/oral discharge and polyuria were observed. No remarkable changes or differences were observed in body weight. All the animals had normal behavior throughout the study. The blood, biochemical and hormone parameters were evaluated in animals (Table 1). The histopathology...
study of the vital organs like the heart, liver, kidney, brain, lungs and ovaries did not show any significant pathological changes (Fig. 1).

3.3. Letrozole induced PCOS study

3.3.1. Body weight

During the administration of letrozole for 21 days there was a significant increase in the body weight of all groups as compared to the normal control group. After the treatment with the standard drug and polyherbal formulation, remarkable decrease in weight was observed. Treatment groups with polyherbal formulation at a dose of 500, 750 and 1000 mg/kg showed a significant decrease (P < 0.0001) in body weight as compared to the disease control group on Day 50 (Fig. 2).

3.3.2. Vaginal smear test

A vaginal smear test showed the proestrous, estrous, metestruas, and diestrous phases of the reproductive cycle of the rats. After 21 days of administration of letrozole, the reproductive cycle becomes irregular. During that time the control group had a regular estrous cycle. The irregularity in the estrous cycle indicates the induction of the PCOS in rats. From 21 days to 50 days, the disease control group showed an irregular estrous cycle and exhibited a constant diestrus phase. The treatment group with polyherbal formulation and the standard control group showed improvement in the irregularity of the estrous cycle and a decrease in the length of the diestrus phase as compared to the disease group (Fig. 3).

3.3.3. Biochemical Parameters

Glucose: On Day 0 there was no significant difference in blood glucose between all the groups. On day 21 after administration of letrozole, there was a significant increase (P < 0.0001) in blood glucose observed in all the groups as compared to the normal control group. After treatment with polyherbal formulation, significant decrease (P < 0.0001) in blood glucose level was observed as compared to the disease control group. The decrease in the blood glucose level indicates a positive effect on hyperglycemia which is the major pathophysiological condition in PCOS (Table 2).

3.3.4. Lipid profile

The lipid profile was evaluated by measuring total cholesterol, triglycerides and HDL on days 0, 21 and 50. There was no significant difference found in the levels of total cholesterol while triglycerides levels were elevated and HDL was decreased in the animals after administration of letrozole on day 21 as compared to the normal control group. Post-treatment with polyherbal formulation and standard drug from 21 to 50 days normalized the elevated levels of triglycerides and HDL (Table 2).

3.3.5. Serum hormonal assay

Serum testosterone was significantly increased whereas progesterone and estradiol were decreased markedly in the animals after administration of the letrozole for 21 days as compared to the normal control group. Treatment with standard drug and polyherbal formulation cause a significant decrease in testosterone and the level of estradiol and progesterone was improved as compared to the disease control group (Table 3).

3.3.6. Ovary weight

There were a significant increase in ovary weight in letrozole induced PCOS rats (82.67 ± 2.05) as compared to the normal group (41 ± 0.82). After treatment with polyherbal formulation and standard drug, this condition was normalized with SC (54 ± 0.82), TG1-500 (55 ± 0.82), TG2-750 (51 ± 0.82) and TG3-1000 (51 ± 0.82) (Fig. 4).

As evaluated by two-way ANOVA.

3.3.7. Ovarian morphology

Letrozole administered group showed many small and multiple ovarian follicles and cysts with a smaller number of corpus luteum. No histological abnormalities were observed in the control group. The treatment group showed normal follicular development as compared to the disease control group, which showed a decrease in the number of cyst formation (Fig. 6).

4. Discussion

In the present study, letrozole was used for the induction of PCOS. Letrozole is a non-steroidal aromatase inhibitor. Oral administration of letrozole (1 mg/kg once daily for 21–28 days) in prepubertal or post-pubertal female rats can induce PCOS-like features. Letrozole increases free testosterone, FSH, and LH while decreasing progesterone and estrogen hormones in rats. There is also an increase in insulin resistance and weight gain observed in rats induced with letrozole. Due to the increased levels of androgens, there is follicular atresia and abnormal follicular development in the ovary.

In PCOS, hormone activity becomes irregular as ovulation is not occurring expectedly. Therefore, the body gives mixed signals and the menstrual cycle gets disturbed. It can change from irregular, infrequent periods (oligomenorrhea) or heavy to absent periods (amenorrhea). In our study, letrozole produces estrous irregularity due to an imbalance of hormones, circulating hyperandrogenism and excess intraovarian androgen. Administration of letrozole for 21 days caused the diestrus phase to continue for a longer period time in the disease control group and other groups. The treatment group and standard group after administration of polyherbal formulation and clomiphene citrate respectively showed improvement by regularizing estrous cycle and decreasing the length of the diestrus phase as compared to the disease control group. Moreover, the polyherbal formulation contains SA which is used to manage menorrhagia as it possesses estrogenicity activity. Further methanolic extract of SA reduces the thickening of the endometrium proliferation in the uterus by lowering the levels of lipopolysaccharides induced COX-2 enzymes in the rat uterus. It possesses antiproliferative and anti-keratinizing effects in the uterus through its anti-estrogenic and anti-inflammatory properties.

Insulin resistance accompanied by compensatory hyperinsulinemia is an important biochemical feature of PCOS which puts women at increased risk for diabetes. The results from our
study showed that the glucose level was elevated in the rats after administration of letrozole for 21 days. We found that the treatment with polyherbal formulation significantly decreased \((P < 0.0001)\) the glucose level in rats as compared to the disease control group. The activity of the polyherbal formulation may be attributed to the presence of TFG in the polyherbal formulation which is an insulin sensitizer and known to prevent diabetes.\(^{22,44}\)

Moreover, TFG seed extract showed significant results in 94% of patients regulating the menstrual cycle. It also significantly reduces ovary volume and size of cyst.\(^{47}\)

The ovarian and adrenal glands of women with PCOS are usually the sites of the production of elevated androgens. It is also proposed that women with PCOS have a hyperproduction of CYP17 enzymes, which are found to be responsible for forming androgens in the ovaries and adrenals. Ovaries make several androgens of which testosterone is the most prominent, others include androstenedione and dehydroepiandrosterone (DHEAS). The most typical feature of a polycystic ovary is the stroma and theca cells make excessive testosterone.\(^{48}\)

CM which is one of the major ingredients of the polyherbal formulation helps to regulate the normal hormonal levels as well as decreases morphological abnormalities in the ovarian follicles (Fig. 5).\(^{22,30,43}\) The mechanism of action of CM can be explained by antihyperglycemic, insulinogenic activity due to the steroidal lipids guggulsterone-E and guggulsterone-Z which possess antioxidant properties due to the presence of hydroxyl group at \(\alpha\)-position of double bonds similar to antioxidant vitamins.\(^{29}\)

PCOS is found to be associated with various patterns of dyslipidemia including higher levels of triglycerides, total cholesterol and low-density lipoprotein (LDL), and low levels of high-density lipoproteins (HDL). In our study, there was not any significant difference found in the total cholesterol level in disease control and treatment groups. The triglyceride levels were significantly increased \((P < 0.05)\) in disease induced group as compared to the normal control group after administration of letrozole for 21 days. Post-treatment with polyherbal formulation at all the dose levels significantly decreased \((P < 0.0001)\) triglycerides levels in treatment group as compared to the disease control group. Administration of letrozole significantly decreased \((P < 0.0001)\) triglycerides levels in treatment group as compared to the disease control group. Administration of letrozole significantly decreased \((P < 0.0001)\) the level of HDL in disease induced group as compared to the normal control group. The treatment with polyherbal formulation significantly increased \((P < 0.001)\) the levels of HDL in the treatment group at 750 mg/kg and 1000 mg/kg dose levels.

Polycystic ovaries are six-fold larger than normal ovaries in size. There is a large number of immature follicles which causes the change in the shape of the ovary. The ovary becomes whitish and there are multiple cystic follicles covered by a dense fibrous capsule. There is luteinization of the theca cells and thickening of tunica albuginea (connective tissue covering the ovaries). In our study, the treatment group showed a decrease in the ovary weight as compared to the disease control group. BV present in the polyherbal formulation corrects the pathophysiology of PCOS by diminishing cysts and preventing them from becoming larger in the ovaries.\(^{14,43}\)

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PCOS is treated by BA with berberine as an active component by addressing clinical, metabolic, and reproductive concerns. Curcumin an active ingredient of CL rectifies the abnormalities in the serum lipid, glucose and glycosylated hemoglobin levels. It also possesses anti-inflammatory activity on the granulosa layer of the corpus luteum. From the results, it can be identified that the

Fig. 3. Estrous cycle phase in rats. (A: Estrous phase shows a large number of anucleated keratinized epithelial cells, few small and large nucleated epithelial cells; B: Diestrous phase shows a greater number of neutrophils; C: Metestrus phase shows a large number of neutrophils and anucleated keratinized epithelial cells; D: Proestrus phase shows small nucleated epithelial cells.)

|                | NC        | DC    | SC    | TG1-500 | TG2-750 | TG3-1000 |
|----------------|-----------|-------|-------|---------|---------|----------|
| Glucose (mg/dl) |           |       |       |         |         |          |
| 0 day           | 61.19 ± 0.42 | 60.39 ± 0.23 | 61.06 ± 0.69 | 61.73 ± 1.16 | 62.06 ± 2.54 | 60.73 ± 0.56 |
| 21 days         | 61.43 ± 0.26 | 70.60 ± 7.54 | 71.43 ± 0.26 | 71.69 ± 6.90 | 71.43 ± 0.26 | 71.43 ± 0.26 |
| 50 days         | 61.54 ± 0.35 | 73.93 ± 2.82 | 54.78 ± 9.91 | 58.65 ± 3.73 | 58.21 ± 4.34 | 55.15 ± 8.67 |
| Total Cholesterol |          |       |       |         |         |          |
| 0 day           | 37.82 ± 2.43 | 38.84 ± 1.79 | 37.48 ± 1.29 | 38.42 ± 0.62 | 38.75 ± 1.09 | 39.16 ± 0.82 |
| 21 days         | 37.58 ± 1.35 | 38.75 ± 1.86 | 40.11 ± 2.20 | 40.80 ± 1.75 | 39.75 ± 0.51 | 40.47 ± 1.19 |
| 50 days         | 38.75 ± 1.86 | 39.44 ± 1.73 | 38.51 ± 1.62 | 37.56 ± 1.26 | 38.75 ± 0.48 | 38.18 ± 1.32 |
| Triglycerides   |           |       |       |         |         |          |
| 0 day           | 26.37 ± 0.45 | 24.56 ± 0.86 | 27.39 ± 0.90 | 25.39 ± 0.74 | 27.09 ± 1.27 | 26.61 ± 0.23 |
| 21 days         | 25.95 ± 0.30 | 38.02 ± 0.40 | 36.21 ± 0.28 | 35.69 ± 1.02 | 35.41 ± 0.87 | 35.48 ± 0.98 |
| 50 days         | 26.26 ± 0.49 | 38.54 ± 0.61 | 33.60 ± 0.67 | 32.68 ± 0.15 | 32.08 ± 1.32 | 32.31 ± 1.31 |
| HDL             |           |       |       |         |         |          |
| 0 day           | 54.49 ± 0.93 | 54.71 ± 0.12 | 55.11 ± 1.12 | 53.04 ± 0.83 | 55.83 ± 0.83 | 53.89 ± 0.45 |
| 21 days         | 54.78 ± 1.24 | 32.57 ± 0.49 | 32.15 ± 0.71 | 31.13 ± 0.67 | 30.92 ± 0.47 | 31.24 ± 0.80 |
| 50 days         | 54.44 ± 0.94 | 30.91 ± 0.48 | 45.05 ± 0.57 | 39.50 ± 1.05 | 40.26 ± 0.40 | 42.13 ± 0.64 |

Values are expressed as Mean ± SEM (n = 6).

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns-non significant.

As evaluated by two-way ANOVA followed by Bonferroni comparison. (NC: Normal control, DC: Disease control, SC: Standard control, TG1-500: Polyherbal formulation 500 mg/kg, TG2-750: Polyherbal formulation 750 mg/kg, TG3-1000: Polyherbal formulation 1000 mg/kg).

a NC vs DC.
b DC vs SC.
c DC vs TG1-500.
d DC vs TG2-750 and.
e DC vs TG3-1000.
5. Conclusion

Polyherbal formulation demonstrated a beneficial effect similar to Clomiphene citrate in treating PCOS conditions and inducing ovulation. It restored the hormone and lipid profile, glucose levels as well as ovarian morphology in letrozole induced PCOS animals. This activity may be attributed to the multiple pharmacological activities like estrogenic, antihyperlipidemic, hypoglycemic and antioxidant activity of various phytoconstituents present in the polyherbal formulation which could be useful in the effective management of PCOS and thereby preventing ovarian cell dysfunction and improving fertility. Together broad spectrum of the biological effects of polyherbal formulation makes it a promising alternative for treating clinical and pathological abnormalities in PCOS conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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Fig. 6. Effect of polyherbal formulation on ovarian morphology. (NC: Normal control shows the cortex with primary follicles with aggregation of granulosa cells showing intact oocyte and corpus luteum showing intact cells; SC: Standard control, TG1-500: Polyherbal formulation 500 mg/kg, TG2-750: Polyherbal formulation 750 mg/kg, TG3-1000: Polyherbal formulation 1000 mg/kg shows normal development of primary follicles and aggregation of the granulosa cells and corpus luteum was found which was distorted in the disease control group. It also showed the development of oocytes in the corpus luteum). (GC-Granulosa cells; CL-Corpus luteum; Oo-Oocyte).
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