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Original article

Protective effects of vanillic acid on letrozole-induced polycystic ovarian syndrome: A comprehensive study in female wistar rats

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ABSTRACT

Background: Polycystic ovarian syndrome (PCOS) is one of the known causes of anovulatory fertility in the world. Previous research has linked oxidative stress could contribute to PCOS, and vanillic acid has shown antioxidant potential. Hence, the present study evaluated the effect of vanillic acid on letrozole-induced polycystic ovarian syndrome in female rats.

Materials and methods: PCOS was induced in Wistar female rats with letrozole (1 mg/kg, orally) in carboxymethoxycellulose (1 % w/v), administered for 21 days. After induction, the standard group received clomiphene citrate (1 mg/kg, orally) while other treatment groups were administered with vanillic acid at doses 25, 50, and 100 mg/kg, orally for 15 days, and without treatment was considered a negative control group. Different parameters studied were body weight, ovary weight, blood glucose, lipid profile, hormonal levels [luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone], markers for oxidative stress (superoxide dismutase, reduced glutathione, catalase, and malonaldehyde), and histopathology of the ovary. Statistical analysis was done for the results and $p < 0.05$ was considered to indicate the significance.

Results: Vanillic acid-treated animals showed a concentration-dependent activity on the tested parameters. The highest tested dose (100 mg/kg) produced a more prominent effect in significantly ($P < 0.001$) decreasing the body weight, and ovary weight and improving the hormonal imbalance. Also, vanillic acid significantly ($P < 0.01$) reduced elevated blood sugar and lipid levels. Additionally, vanillic acid reduced oxidative stress significantly ($P < 0.001$) in the ovaries of female rats. Histopathological reports showed a reduction in cystic follicles and appearance of normal healthy follicles at different stages of development after the administration of vanillic acid. Furthermore, these effects were observed to be comparable with those recorded for standard drug, clomiphene.

Conclusion: The current study data suggests that vanillic acid has protected the letrozole-induced polycystic ovarian syndrome. In the event of several side effects associated with conventional treatments used for PCOS, the findings of this study suggest the promising role of vanillic acid. More research in this direction might identify the true potency of vanillic acid in the treatment of PCOS.

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1. Introduction

Polycystic ovarian syndrome (PCOS) is a frequently occurring heterogeneous metabolic and endocrine illness in reproductive-aged women accountable for infertility. This disease is characterized by androgen hyper-production (hyperandrogenism), ovulatory defects, and polycystic ovaries (Bayona et al., 2022).. The menstrual cycle is interrupted due to the blockage of ovulation, which causes amenorrhea. PCOS is recognized when numerous cysts are found in the ovarian follicles. Absence of the menstrual cycle inhibits fertilization and conception resulting in difficulties in the pregnancy. And about 90 % of women with PCOS will have difficulty in conception (Chiaffarino et al., 2022).

PCOS is associated with an amplified risk for obesity, diabetes mellitus type-2, infertility, endometrial cancer, bleeding, dyslipidemia, hypertension, and cardiovascular disease (Choudhury and Rajeswari, 2022). Oxidative stress (OS) is one of the chief issues that are reported to be intricate in the pathophysiology of PCOS. Several studies have shown a diminution in mitochondrial O_2 ingestion and glutathione (GSH) levels in PCOS patients which might lead to mitochondrial dysfunction (Dubey et al., 2021). In PCOS, the mononuclear cells are increased more in this inflammatory state in response to hyperglycemia and C-reactive protein. Hyperglycemia due to reactive oxygen species (ROS) from mononuclear cells activates the release of tumor necrosis factor (TNF-α) and increased nuclear factor-kappa B cells (NF-kB) (Xu et al., 2022). It resulted in increased insulin resistance which is mediated via TNF-α concentration. Subsequently, OS produces an inflammatory environment that increases insulin resistance, and directly stimulates hyperandrogenism (Schweighofer et al., 2023). In addition, gonadotropin hormone pathway as well as the altered steroidogenesis is related to the pathogenesis of PCOS (Khodaeifar et al., 2019). During these alterations, studies have indicated that PCOS patients has abnormal high level of LH that can be related to some of the early signs such as hirsutism, acne, alopecia and arrested growth of follicles leading to anovulation (Ainehchi et al., 2020).

Some of the herbal therapeutic plants have been used for the management of numerous ailments of reproductive system. Due to the adverse effects caused by allopathic medicines, there is a need for alternative safe medicine and plant products with minimal side effects are being tested for the management of diverse types of diseases including PCOS (Schweighofer et al., 2023). In this direction, identification and isolation of plant-based medications specifically which acts as phytoestrogens, antioxidant, and anti-androgenic in action has assumed importance (Arentz et al., 2014). Some of the important naturally obtained products reported to be effective in PCOS are quercetin and essential oil of Mentha spicata (Vaez et al., 2023; Sadeghi Ataabadi et al., 2017). The studies suggested that these agents reduced several pathological defects such as inflammation, hormonal imbalance, adipocity by acting at molecular and gene levels, presenting a promising role in the management of PCOS (Mihanfar et al., 2021).

Some evidence has indicated that an imbalance in antioxidant and reactive oxygen status is closely related to female infertility (Dubey et al., 2021). Phenolic compounds are a considerable part of plant nourishments. Due to their antioxidant property, they show a major part in the preclusion of many illnesses (Lyu et al., 2020). Vanillic acid is a phenolic derivative from eatable plants and fruits and is the oxidized form of vanillin. Several studies have reported vanillin as a nutraceutical because of its antitumor, anticlastogenic, and antimutagenic properties (Zhu et al., 2023). In addition, vanillic acid is known to possess antioxidant, free radical scavenging, anti-inflammatory (Jeong et al., 2018), and phytoestrogenic activities (Xiao et al., 2014).

Vanillic acid has been also described to decrease the expression of interleukin 1α, interleukin-6, and TNF-α in isoproterenol cardiotoxic rats. In high-fat diet-fed (HFD) rats, vanillic acid significantly reduced cyclooxygenase-2 (COX-2), monocyte chemoattractant protein-1 (MCP-1), insulin resistance, phosphoinositide-3-kinase (PI3K), and glucose transporter-2 (GLUT 2) and, exhibited the capability to forage hydroxyl superoxide anion as well as lipid radicals (Ashokkumar and Vinothiya, 2023). It has also shown a protective effect against hyperglycemia, hyperinsulinemia, hyperlipidemia, and in HFD-fed rats (Chang et al., 2015). Vanillic acid was reported by others to attenuate the expressions of dihydrotestosterone and 5α reductase in benign prostatic hyperplasia (Abdel Fattah et al., 2023). Nevertheless, the protective effect of vanillic acid on PCOS is not well documented in the literature. Hence, in this research, the protective role of vanillic acid against letrozole-induced PCOS was evaluated by analyzing the metabolic changes (body weight, blood glucose, and lipid profile), hormonal as well as antioxidant status, and histopathological alterations in ovaries of female rats.

2. Materials AND METHODS

2.1. Criteria for entering and closing the scientific study

The research was conducted as per the standard guidelines reported in the literature (Smith et al., 2018). Before designing the study, a literature review was done to identify the research problem. A hypothesis was developed to solve the problem and accordingly, the aim and objectives were proposed. To achieve this, the experimental design was prepared and subjected to approval from the Institutional Animal Ethics Committee. After approval, standard guidelines were followed for procuring the chemical, reagents, and animals. Good laboratory practices were adopted while handling them, including animal transportation, housing, handling, and experimentation that were conducted in approved research laboratories. The data from the study was collected and scientifically represented either as tables/figures to conclude. The euthanized animals were disposed of as per the guidelines and the left-over chemicals/reagents were returned to the central storage facility of the institution before closing the research project.

2.2. Medications and chemicals

Vanillic acid was procured from PC Chemicals, Mumbai, India. Letrozole was procured from Cipla Verna Indl. Estate, Goa, India. Clomiphene citrate (O-fert®, 50 mg) tablets were bought from Innovative Pharmaceuticals, Mumbai, India. Additional chemicals used in this study were of analytical quality and procured from the central warehouse of the institution.

2.3. Experimental animals

Thirty six female Wistar rats of age 2 – 3 months (150–200 g) were utilized in this investigation and were obtained from the animal house of Yashoda Technical Campus, College of Pharmacy, Wadhe, Satara, India. They were accommodated under regular laboratory settings at a temperature of 25 ± 2 °C, relative humidity 55 ± 5 %, and 12.00:12.00 hr dark: light cycle with regular laboratory diet (Animal Nutraceuticals, Satara) and water *ad libitum*. As per the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India the present experiment was carried out. The research procedure was sanctioned by the Institutional Animal Ethics Committee (Approval No. YSPM/YTC/PHARMA/35/2019).

2.4. Vaginal smear observation

Animals with a 4–5-day regular estrus cycle were used for the study. Each morning amongst 8:00 and 9:00 am, vaginal secretions were collected by using the cotton swab smear technique and assessed using crystal violet stain to check the estrus cycle, and only animals which exhibited regular estrus cycle were selected for study. The smears were carefully observed under a microscope to identify the stage of the estrous cycle. Some of the important characteristics that signify that the female rat is in the estrus phase are the abundant presence of nucleated

keratinized epithelial cells. These cells represented blue cytoplasm with jagged/angular edges and lacked nucleus. In addition, few neutrophils (round cells with small multi-lobulated nuclei), small nucleated epithelial cells (large and round nucleated cells with blue cytoplasm), and large nucleated epithelial cells (round to polygonal with irregular/jagged/angular borders with blue cytoplasm) can also be seen in the estrus phage (Mott and Goeders, 2023).

2.5. Induction of PCOS in female rats

Except control group, all the investigational rats were orally administered with letrozole (1 mg/kg) dissolved in 1 % CMC (Carboxy methyl cellulose) solution for 21 days to induce PCOS condition. The changes in body weight and blood glucose levels were reported to be the early markers for the induction of PCOS in experimental animals (Liyanage et al., 2021).

2.6. Treatment protocol

The study was conducted on 36 animals equally separated into six groups as follows:

Group 1: Control group- which received only 1 % Carboxymethyl cellulose solution.

Group 2: Negative Control - served as the PCOS group which received letrozole (1 mg/kg).

Group 3: Standard - letrozole + Clomiphene Citrate (1 mg/kg) (Morgante et al., 2018).

Group 4: Test group I- letrozole + Vanillic acid (25 mg/kg).

Group 5: Test group II-letrozole + Vanillic acid (50 mg/kg) (Kumari et al., 2021).

Group 6: Test group III-Letrozole + Vanillic acid (100 mg/kg).

Clomiphene citrate is one of the common medications used in the treatment of PCOS and so was utilized in the present study as a standard agent (Morgante et al., 2018). Clomiphene citrate in CMC (1 %) was treated orally in animals for 15 days. The test groups (groups- 4, 5, and 6) were treated with vanillic acid in 1 % CMC per oral route for 15 days at predetermined dosages. These groups (groups 3, 4, 5, and 6) received letrozole for 21 days followed by their respective treatments for 15 days, as described before.

On the 22nd day, the negative control group and on the 36th day, animals from other groups were euthanized with high CO_2 anesthesia. The experimental rats were euthanized under the supervision of a veterinarian who is also a member of the Institutional Animal Ethics Committee. After confirming the death of animals, ovaries were dissected and used to determine their weight and histopathology. Blood samples were gathered by cardiac puncture. The serum was parted by centrifugation which was further used for the various parameters like serum hormonal levels, and lipid profile. Ovaries were removed, cleaned, and weighed. Further subjected to oxidative stress parameters.

2.7. Metabolic markers

2.7.1. Ovary weight and body weight

The body weight of all animals was documented on the first day of every week till 5 weeks. At the culmination of the study, on the 36th day, ovaries were removed, and their weights were noted (Zhou et al., 2021).

2.7.2. Measurement of blood glucose level

Blood was taken on the 22nd, 28th, and 36th days for the estimation of glucose. Blood glucose level was measured using a Roche Diabetes Care Inc. glucometer (Tanaka et al., 2021).

2.7.3. Serum hormone analysis

At the end of the study, hormonal analysis was performed. Serum follicle-stimulating hormone (FSH), testosterone, and luteinizing hormone (LH) were studied using ELISA assay kits (Ghosh et al., 2022), procured from HiMedia Laboratories Pvt Ltd., India.

2.7.4. Assessment of lipid profile

The lipid profile [high-density lipoprotein (HDL), Low-density lipoprotein (LDL), triglycerides (TG) and total cholesterol (TC), were estimated by using enzymatic kits (Tanaka et al., 2021) procured from Aspen Laboratories Pvt, Ltd., India.

2.8. Oxidative stress markers

The oxidative stress in ovaries was assessed by measuring catalase, superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) levels. Ovaries were homogenized in 0.1 M phosphate buffer (pH 7.4). As such homogenate is used for protein estimation. The protein content, as well as malondialdehyde (MDA), catalase, SOD, and GSH, were estimated in the homogenate according to the procedure described in the literature (Li et al., 2021).

2.9. Histopathological examination

Ten percent formalin was used to fix the removed ovaries. They underwent tissue processing in accordance with histology protocol, which included washing with tap water, dehydrating with increasing alcohol grades, and clearing the tissue with xylene. Next, the paraffin embedding technique was applied. The blocks were put on slides after being sectioned with a microtome. Approximately 5 μm of the tissue was sectioned. Hematoxylin-eosin (HE) staining, dehydration, clearing, and mounting on DPX mountant (a mixture of distyrene, plasticizer and xylene) beneath glass coverslips were performed on these sections. The light microscope, which was attached to a camera (ESAW Labware Pvt. Ltd, Ambala, India) to take pictures, was used for observation (Li et al., 2019). These slides were observed under a magnification of 100× to record the major histopathological changes. The various developmental phases of ovary such as primary (marked as yellow arrow), secondary (white arrow), tertiary (green arrow), corpus luteum (pink arrow), and corpora haemorrhgaica (red arrow) were studied to determine the influence of various drug administration (Hoseinpour, et al., 2019).

2.10. Statistics

The results obtained from the study are represented as mean ± standard error (SEM) either as tabular columns or figures. The data was subjected to statistical analysis by using GraphPad Prism software version 5.0. The comparison between groups was done between control Vs other treated groups as well as between negative group (letrozole) Vs treatment groups and standard group (clomiphene) Vs treatment groups (vanillic acid). The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test to determine the significant difference between groups. A p-value less than 0.05 was considered to indicate the statistical significant variation.

3. Result

3.1. Morphology of ovary

The gross morphological characters observed in the normal and letrozole-treated ovaries are represented in Fig. 1. Administration of letrozole (negative group) to the rats has increased the fluid-filled cysts when compared to normal groups that did not receive any treatment. The size of the ovary also appeared to be increased after the letrozole administration. On the other hand, administration of different medications such as clomiphene and vanillic acid (at various doses tested) reduced these changes induced by letrozole. The size as well as the fluid-filled content in the ovaries was found to be decreased upon treatment with both vanillic acid and clomiphene.

3.2. Effect of vanillic acid on body weight and ovary weight

The negative group showed a noteworthy rise in body weight ($p < 0.001$) in contrast with the control group. Treatment of vanillic acid (100 mg) significantly ($p < 0.001$) inhibited the increase in body weight as matched with the negative group. Further, clomiphene-treated animals also displayed significant ($P < 0.001$) decline in the body weight.

The negative group exhibited a substantial upsurge in ovarian weight when matched with the control group. Vanillic acid treatment significantly reduced ovary weight at dosages of 25 mg/kg ($p < 0.01$), 50 mg/kg ($p < 0.001$), and 100 mg/kg ($p < 0.001$) in comparison with the negative group. The administration of clomiphene was also found to reduce ($P < 0.001$) the ovary weight in contrast with negative control. Besides, the weight of ovaries was significantly decreased by vanillic acid at a dose of 25 mg/kg ($p < 0.05$), 50 mg/kg ($p < 0.01$), and 100 mg/kg ($p < 0.001$) when compared with standard group (Table-1).

Values represent mean ± SEM; n = 6; Analysis of data was performed using one-way ANOVA followed by Tukey's multiple comparison tests; p value less than 0.05 was considered as statistically significant. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; [#]Values compared with normal control. [†]Values compared with negative control. [††]Values compared with standard.

3.3. Effect of vanillic acid on blood glucose level

The negative group revealed a significant rise in blood glucose levels compared with the control group. Furthermore, dosing of PCOS-induced rats with clomiphene markedly ($p < 0.001$) attenuated the blood glucose level. Vanillic acid administration also displayed a significant decrease in blood glucose levels at a dose of 25 mg/kg ($p < 0.05$), 50 mg/kg ($p < 0.001$), and 100 mg/kg ($p < 0.001$) as equated with the negative control group. Further, a significant ($P < 0.05$) decrease in blood glucose was observed when vanillic acid-treated animals were matched with the standard group (Fig. 2).

3.4. Effect of vanillic acid on serum hormonal parameters

The serum levels of LH and testosterone were remarkably elevated in the negative group ($p < 0.001$) while FSH was reduced significantly ($p < 0.001$) in contrast to the control group. Clomiphene Administration as standard drug was observed to reverse these changes in hormonal levels significantly ($P < 0.001$) upon comparison with the negative control animals. Vanillic acid at a dose of 25 mg/kg ($p < 0.01$), 50 mg/kg (0.01), and 100 mg/kg ($p < 0.001$) significantly decreased LH levels as matched with negative group animals. A noteworthy rise in FSH level was also observed in the vanillic acid-treated group at a dose of 25 mg/kg ($p < 0.05$), 50 mg/kg ($p < 0.001$), and 100 mg/kg ($p < 0.001$) when likened with the negative group. Testosterone levels were diminished considerably ($p < 0.001$) when different doses of vanillic acid were administered compared to the negative control rats. Further, when vanillic acid-treated groups were compared with standard, a significant ($P < 0.05$) difference was observed (Fig. 3).

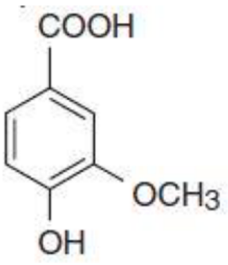

Fig. 1. Morphological characteristics of isolated ovary Note: A – Normal ovary; B – Fluid filled ovary with cysts.

Fig. 2. Effect of vanillic acid on blood glucose level. All data is expressed as Mean ± SEM; n = 6; Analysis of data was performed using one-way ANOVA followed by Tukey's multiple comparison test; p value less than 0.05 was considered as statistically significant. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; [#]Values compared with normal control. [†]Values compared with negative control. [††]Values compared with standard.

3.5. Effect of vanillic acid on lipid parameters

Significant changes occurred in serum lipid levels due to letrozole administration in the negative when matched with the control group. Cholesterol, triglycerides, and LDL levels were elevated as $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively whereas HDL was significantly reduced ($p < 0.001$) in the negative animal group. Fig. 6 indicates the result of vanillic acid on lipid profile. The standard group and treatment with vanillic acid at all tested dosages considerably decreased ($p < 0.001$) cholesterol, TG, and LDL levels while significantly elevated ($p < 0.001$) HDL levels as equated with the negative group (Fig. 4).

3.6. Effect of vanillic acid on antioxidant activity

The Malonaldehyde (MDA level was markedly augmented ($p <$

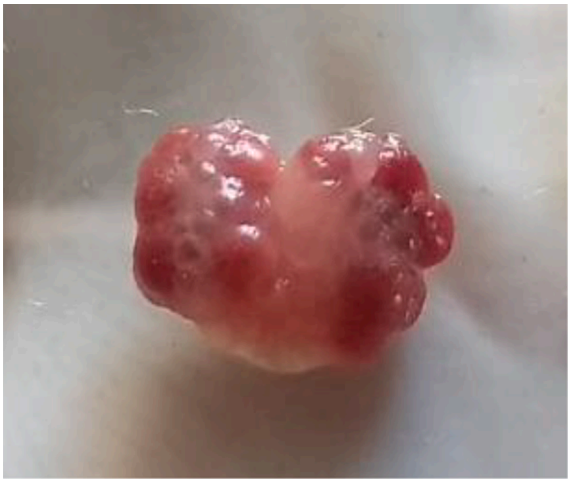

Fig. 3. Effect of vanillic acid on serum hormonal parameters Note: A – LH levels; B – FSH levels; C – Testosterone levels. Values represent mean ± SEM; n = 6; Analysis of data was performed using one-way ANOVA followed by Tukey's multiple comparison test; p value less than 0.05 was considered as statistically significant. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; [#]Values compared with normal control. [†]Values compared with negative control. [††]Values compared with standard.

0.001) in PCOS-induced animals when matched with control group animals. Vanillic acid-treated animals exhibited a significant decrease in MDA levels at doses 25 mg/kg ($p < 0.05$), 50 mg/kg ($p < 0.001$), and 100 mg/kg ($p < 0.001$) when equated to the negative group. The GSH, catalase, and SOD levels were significantly decreased in the negative group ($p < 0.001$). Vanillic acid showed its antioxidant effect by significantly increasing the activity of GSH, catalase, and SOD ($p < 0.001$) when matched with PCOS-induced animals. High doses (100 mg/kg) of vanillic acid exhibited significant elevation in the activity of GSH, catalase, and SOD ($p < 0.001$) when matched with the standard group of animals treated with clomiphene citrate (Figure-5).

Histopathological observation

4. Discussion

Present study indicated that experimental polycystic ovarian syndrome (PCOS) induced by the administration of letrozole significantly altered the body weight, ovary weight, blood glucose, lipid profile, antioxidant status, and histomorphological changes of ovaries in rats. Vanillic acid treated for 15 days was found to exhibit a dose-dependent defensive effect against the experimental PCOS induction by letrozole. The efficacy of vanillic acid appears to match with the clomiphene citrate (standard drug), which is routinely used in the management of PCOS (Figs. 1 to 6 and Table 1).

Letrozole (LTZ) is a non-steroidal aromatase inhibitor that induces many features of human PCOS. LTZ produces PCOS-like conditions by delaying the conversion of testosterone and androgen to estradiol and estrone respectively (Liyanage et al., 2021, Wu et al., 2022). Reduction in estrogen levels diminishes the negative feedback on LH production resulting in elevated levels of LH stimulating theca cells to secret testosterone (Esparza et al., 2020). In addition, LTZ-induced PCOS shows metabolic features like enhanced body weight and insulin resistance (Zhang et al., 2020; Rafiee et al., 2022). Observations suggested that such ovaries have thicker theca cell layers, many cysts with thin granulosa cell layers, and increased follicle atresia. Furthermore, administration of LTZ was reported to cause hyperglycemia and hyperlipidemia (Zuo et al., 2023).

The negative group was administered with letrozole, the testosterone levels were found to be significantly elevated. These observations are in accordance with the previous research conducted on LTZ. Due to the inhibition of the aromatase enzyme, it resulted in increased production of androgen and induction of PCOS (Franik et al., 2018; Alaee et al., 2022). Further, the treatment with vanillic acid in this study could normalize the hormonal level. Besides, in the negative group, the FSH level was significantly reduced, and the LH level was increased significantly matched to the control grouped animals. Treatment with intermediate and high doses of vanillic acid displayed a noteworthy diminution in LH and testosterone levels associated with an induction of FSH levels as compared with the negative group.

According to literature, abnormal ratio of gonadotropin is one of the initial steps in the pathogenesis of PCOS. During this phase, excess LH stimulates the production of androgen, which can cause cutaneous symptoms of hyperandrogenemia (hirsutism, acne) and anovulation (Khodaeifar et al., 2019). Besides, excess LH secretion compared to FSH level is also reported to contribute to hyperinsulinemia, adiposity and ovarian defects (Gargus et al., 2022). Further, LH also stimulates steroidogenesis by producing androgens from theca interna cells that gets converted to estradiol in granulosa cells by aromatase enzyme under FSH activation (Ainehchi et al., 2020). Down regulation of aromatase enzyme is partially responsible for altered steroidogenesis observed in PCOS (Unfer et al., 2020). The altered steroidogenesis in PCOS is associated with increased expression of P450scc in thecal stromal cells, increased CYP17A1 gene expression, diminished granulosa cell layer, heightened thecal cell layers with multiple large cysts, enhanced androsenedione as well as T levels and increased production of estradiol (Ainehchi et al., 2020; Unfer et al., 2020).

The AGE-RAGE (receptor for advanced glycation end products) interactions have also been proposed for the altered steroidogenesis in PCOS. It is hypothesized that interference in the AGE-RAGE signally cascade in granulosa cells causes attenuation of LH activity (Garg and Merhi, 2016). It was found that AGE produces inappropriate activation of ERK1/2 pathway in granulosa cells that will lead to altered steroidogenesis in these cells (Shah et al., 2023). Furthermore, the altered steroidogenesis is reported to elevate androgen production and abnormal follicular development (Ainehchi et al., 2020). Besides, augmented insulin resistance and induction of inflammatory responses are also linked to the altered steroidogenesis (Khodaeifar et al., 2019).

In our study, we found a substantial upsurge in body weight in PCOS-induced rats in contrast with the control group. As reported, this could be due to the deposition of abdominal fat (Shrivastava and Conigliaro, 2023). Ovarian weight was more than the control group rats after the induction of experimental PCOS. The observations suggested that treatment of vanillic acid prevented further increases in ovarian weight and body weight, suggesting the efficacy of vanillic acid in PCOS (Zuo

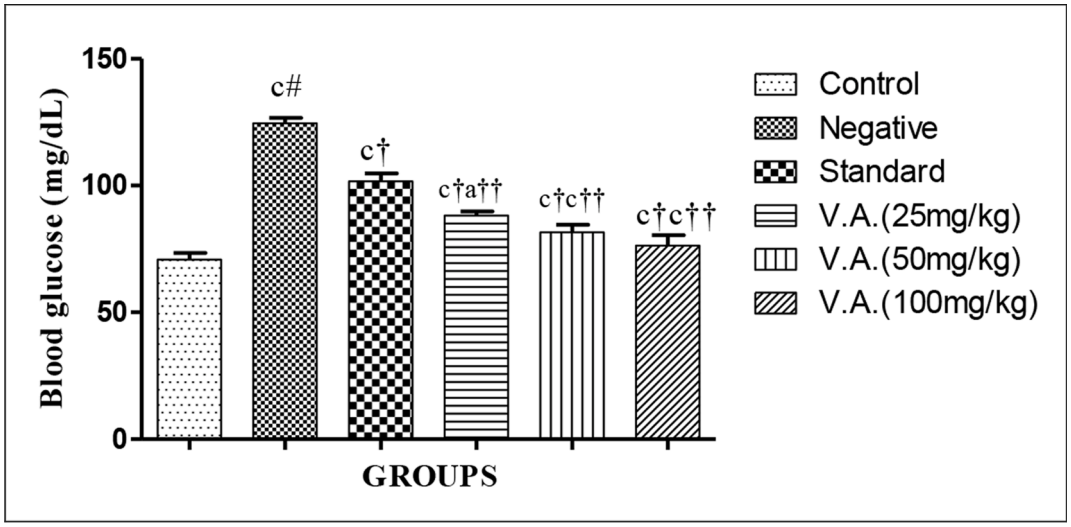

Fig. 4. Effect of vanillic acid on lipid profile Note: A – Cholesterol levels; B – LDL levels; C – Triglyceride levels; D – HDL levels; Values represent mean ± SEM; n = 6; Analysis of data was performed using one-way ANOVA followed by Tukey's multiple comparison test; p value less than 0.05 was considered as statistically significant. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; [#]Values compared with normal control. [†]Values compared with negative control. [††]Values compared with standard.

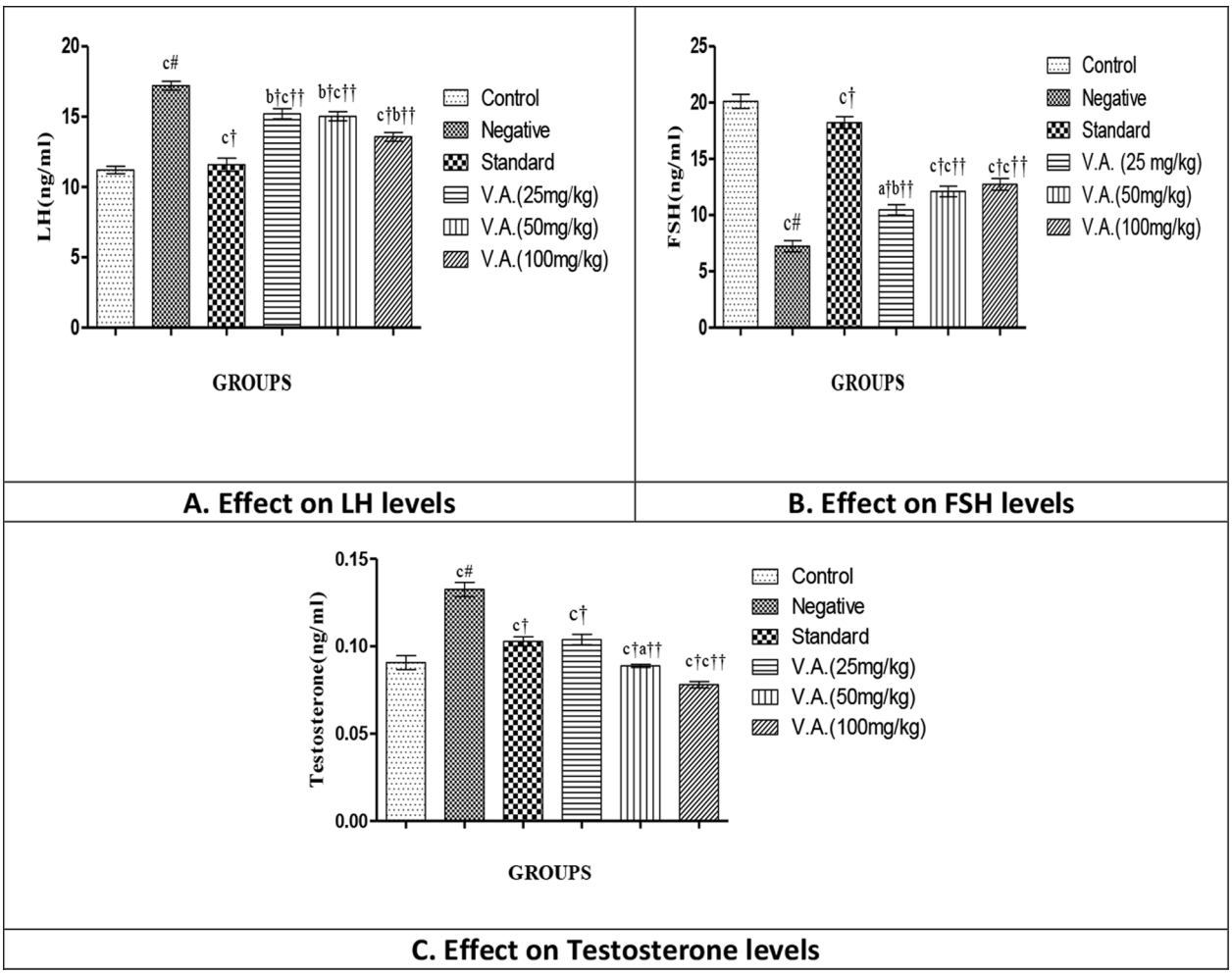

Fig. 5. Effect of vanillic acid on oxidative stress parameters Note: A – MDA levels; B – GSH levels; C – H_2O_2 levels; D – SOD levels. Values represent mean ± SEM; n = 6; Analysis of data was performed using one-way ANOVA followed by Tukey's multiple comparison test; a p-value less than 0.05 was considered as statistically significant. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$; [#]Values compared with normal control. [†]Values compared with negative control. [††]Values compared with standard.

et al., 2023).

PCOS is also strongly linked with diabetes mellitus type-2 and hyperglycemia which further leads to the development of insulin resistance (Choudhury and Rajeswari, 2022; Zhang et al., 2020). Studies have indicated that patients with PCOS have significantly higher glucose-insulin index and plasma insulin levels (Kruszewska et al., 2022). Hyperinsulinemia is reported to increase androgen production and elevate the circulating testosterone levels in the blood. This will cause premature luteinization of granulosa cells leading to disruption of granulosa cells and defective development of follicles in PCOS (Tong et al., 2022). Our study has observed a marked rise in blood glucose levels in negative group animals, and oral treatment of vanillic acid markedly inhibited the rise in blood sugar values. These findings support the earlier study, where compounds possessing a beneficial effect on PCOS also reduce hyperglycemia (Ashkar et al., 2020).

Dyslipidemia is present in 70 % of patients with PCOS. It is presented as high triglycerides, cholesterol levels, low HDL, and raised levels of LDL-C that may result in an abnormal lipid profile (Liu et al., 2022; Zhang et al., 2020; Zuo et al., 2023). In the current study, the same effect was seen in the negative group. The negative group exhibited an increase in cholesterol, TG's, LDL, and a decrease in HDL levels. Interestingly, vanillic acid treatment displayed its action by lowering cholesterol, TGs, and LDL and elevating HDL levels. Such a decrease in elevated lipid levels was reported to benefit patients with PCOS (Celik and Acbay, 2012).

The overproduction of ROS leads to oxidative stress, which may contribute to various diseased states affecting female reproduction (Dubey et al., 2021). Abnormal oxidative status was related to prolonged diseases such as cancer, diabetes, polycystic ovarian syndrome, and cardiovascular diseases. Oxidative stress (OS) is a major inducing factor in PCOS pathogenesis (Liu et al., 2022). Lipid peroxidation and abnormal ROS production causes oxidative stress and damages the cells. Malondialdehyde is the most extensively used as a marker for lipid peroxidation assessment which reflects OS due to which increased ROS leads to raised MDA levels (Xu et al., 2022). Earlier studies suggested that P13K/AKT/MTORE signaling pathways play essential role in PCOS. This cascade of events regulates the interaction of androgens, insulin, and growth factors. Oxidative stress is reported to cause destruction of these processes and contributes in dysregulation of androgen, gonadotropins, insulin levels and also increased atresia of follicles in PCOS (Soltani et al., 2023).

According to previous research, OS causes cell damage, loss of follicular function and contributes in premature ovarian failure (Majdi Seghinsara et al., 2019). Studies have indicated that OS can interfere in the proliferation of granulosa cells and affects its development as well as maturation of oocytes (Delkhosh et al., 2019). Besides, estrogen essential for proliferation of granulosa cells will be affected by excess OS, leading to development abnormalities in ovaries (Soltani et al., 2023). It has already been reported that OS is involved in increasing ovarian steroidogenesis enzyme activities which could result in stimulating

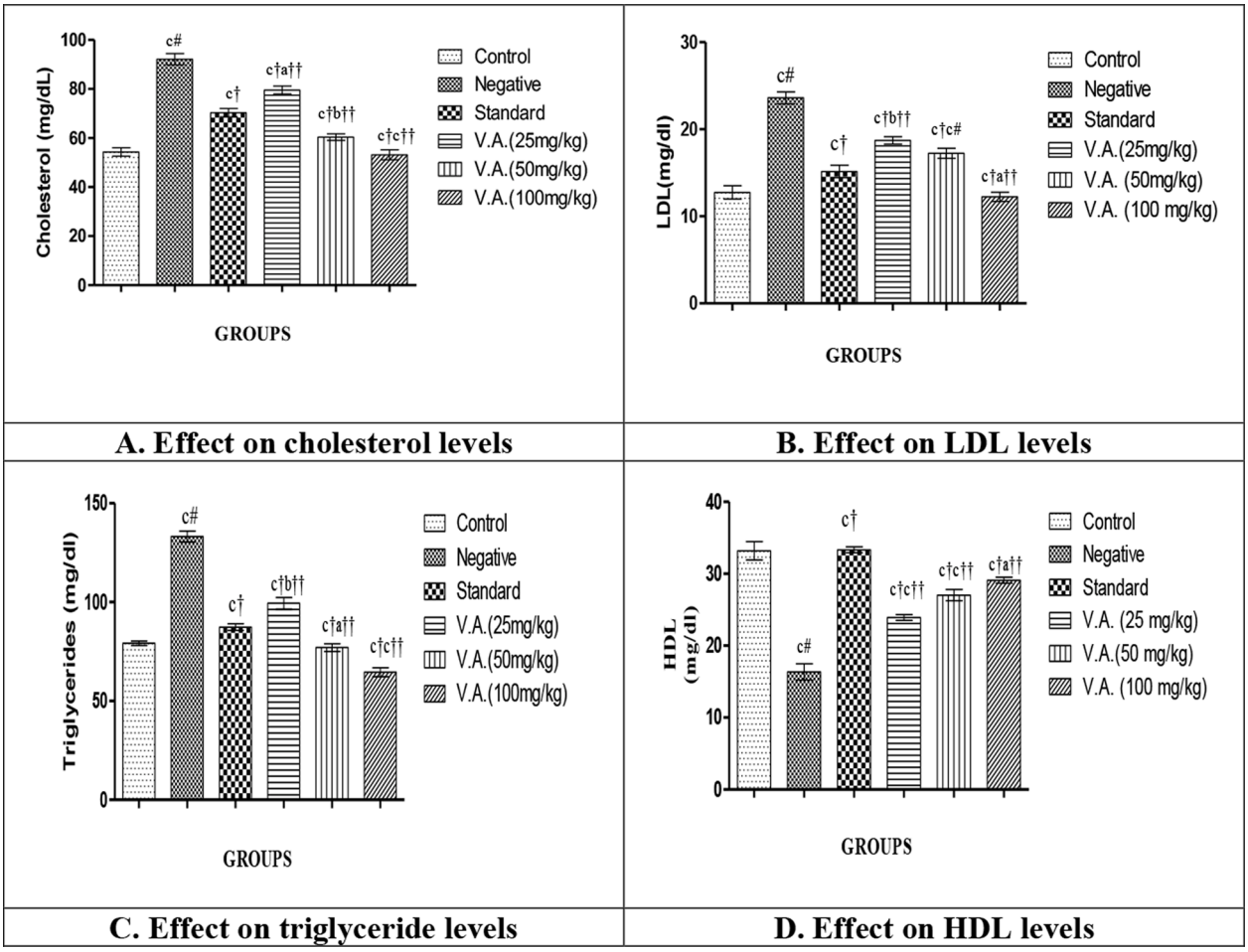

Fig. 6. Histopathological observation of ovaries.

Table 1
Effect of vanillic acid on body weight and ovary weight.

Group no.	Treatments	Body weight (gm)	Ovary weight (mg)
1	Control	189.2 ± 3.12	32.38 ± 1.48
2	Negative	250.3 ± 1.58$^{c\#}$	63.79 ± 0.90$^{c\#}$
3	Standard	209.8 ± 2.88^{c†}	48.37 ± 1.31^{c†}
4	Vanillic acid (25 mg/kg)	225.2 ± 2.07$^{c\dagger b\dagger\dagger}$	55.29 ± 1.14$^{b\dagger a\dagger\dagger}$
5	Vanillic acid (50 mg/kg)	194 ± 2.30$^{c\dagger b\dagger\dagger}$	40.16 ± 1.78$^{c\dagger b\dagger\dagger}$
6	Vanillic acid (100 mg/kg)	181.5 ± 2.47$^{c\dagger c\dagger\dagger}$	36.75 ± 1.30$^{c\dagger c\dagger\dagger}$

androgen production (Liu et al., 2022). Antioxidants play a crucial role in repairing the cell damage caused by reactive oxygen species (Moshfegh et al., 2022).

Additionally, women having PCOS showed abnormal OS that causes genetical instability and increases chances of cancer (Liu et al., 2022). In the current study, it was detected that the negative group exhibited significantly elevated MDA levels whereas catalase, superoxide dismutage (SOD), and GSH levels depleted significantly. On the other hand, vanillic acid was able to significantly lower the malondialdehyde level. Furthermore, administration with vanillic acid induced the activities of GSH, catalase, and SOD levels significantly. The observed results of vanillic acid have similarities in efficacy to clomiphene citrate (Peker et al., 2021), suggesting that vanillic acid has the potential to be an effective therapeutic intervention in the management of PCOS complications.

Earlier studies suggested that chronic oxidative stress results in inflammatory process, tissue remodeling and apoptosis (Ye et al., 2023). LET n the earlier studies have increased the markers of inflammation and apoptosis, suggesting its mechanism in PCOS (Areloegbe et al., 2022). Besides, the treatments that have promising effects in PCOS are found to reduce these markers such as NF-kB, TNF-α, and interleukin-6 (Ji et al., 2023). Insulin resistance is also reported to play a vital role in the dyslipidemic complications associated with PCOS (Kruszewska et al., 2022). Estimating the levels of insulin and detecting the action of vanillic acid on various inflammatory and apoptosis markers could be some of the future directions of research to determine the role and efficacy of vanillic acid in PCOS.

5. Conclusion

Overall results concluded that vanillic acid showed beneficial effects like clomiphene citrate. It also normalized body weight, hormonal imbalance, lipid profile, hyperglycemia, antioxidant status, and ovarian histomorphological alterations in PCOS rats induced by letrozole. Vanillic acid is abundantly present in fruits and vegetables, which is scientifically considered to be a healthy diet. More studies in this direction might determine the true potential of vanillic acid and might help in detecting a safe and efficacious beneficial intervention in the treatment of polycystic ovarian syndrome in females.

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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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