Therapeutic Effect of *Tinospora cordifolia (Willd)* Extracts on Letrozole-Induced Polycystic Ovarian Syndrome and its Complications in Murine Model

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ABSTRACT

BACKGROUND: Tinosopora cordifolia (*Willd)* (TC) is commonly used in Ayurvedic medicine since long time for number of ailments and its preparations are also considered by food safety and standards authority of India as nutritional supplement. However the scientific evidence on its possible safety and efficacy in polycystic ovarian syndrome and associated complications was not studied in detail.

OBJECTIVES: The purpose of this investigation is to examine whether or not *TC* can have therapeutic effects on letrozole induced PCOS and related complications such as body weight, dyslipidaemia, glucose tolerance, hormonal regulation, insulin resistance and sensitivity, severity of PCOS and histopathological changes in ovary using mice animal model.

DESIGN: Present study is a preclinical study involving laboratory animals.

METHODS AND ANALYSIS: After verifying the absence of PCOS, the animals began receiving Letrozole, which lasted for 21 days. Fasting blood glucose (FBG), the oral glucose tolerance test (OGTT), triglycerides, cholesterol, and weight were recorded. The levels of hormones like oestrogen, progesterone, insulin, testosterone, luteinising hormone (LH) and follicle stimulating hormone (FSH), histopathology was carried out.

ETHICS: The Institutional Animal Ethics Committee at DITU gave its clearance to the animal experimentation on July 10, 2021 (DITU/IAEC/21-22/07-06).

DISCUSSION: The majority of cornified epithelial cells were seen in groups treated with *TC* extract during the estrous phase of the cycle. Mice exposed to *TC* retained normal body weight. FBG, 1- and 2-hour OGTT, triglyceride and cholesterol levels were all significantly improved by extracts. Estradiol, progesterone, testosterone, insulin, LH and FSH concentration were all corrected in *TC*-treated animals. The HOMA-IR, HOMA-Beta and QUICKI values were also corrected with *TC* extracts. The morphological and microscopic features of the ovary were also greatly enhanced. Based on these findings, we conclude that treating PCOS mice with *TC* extracts significantly ameliorates the disease and severity down to nil-to-moderate levels by reducing hyperinsulinemia, hyperandrogenism, dyslipidaemia, enhancing insulin sensitivity, correcting oestrogen, progesterone, LH and FSH levels via enhanced ovarian function. Further molecular and cellular level of study is recommended for further elaboration of mechanism of action.

PLAIN LANGUAGE SUMMARIES

- *Tinospora cordifolia* satva, oil and hydroalcoholic extract were studied in letrozole-induced PCOS in mice model
- Anti PCOS efficacy of 3 preparations studied with respect to their mechanism of action in detail
- For the first time proposing method of calculating severity of PCOS in animal model
- *Tinospora cordifolia* oil preparation completely reversed PCOS effect of letrozole and made them normal
- Histopathological and morphological studies support the biochemical claims

KEYWORDS: Metabolic syndrome, insulin resistance, PCOS severity score, dyslipidaemia, ovary, insulin sensitivity

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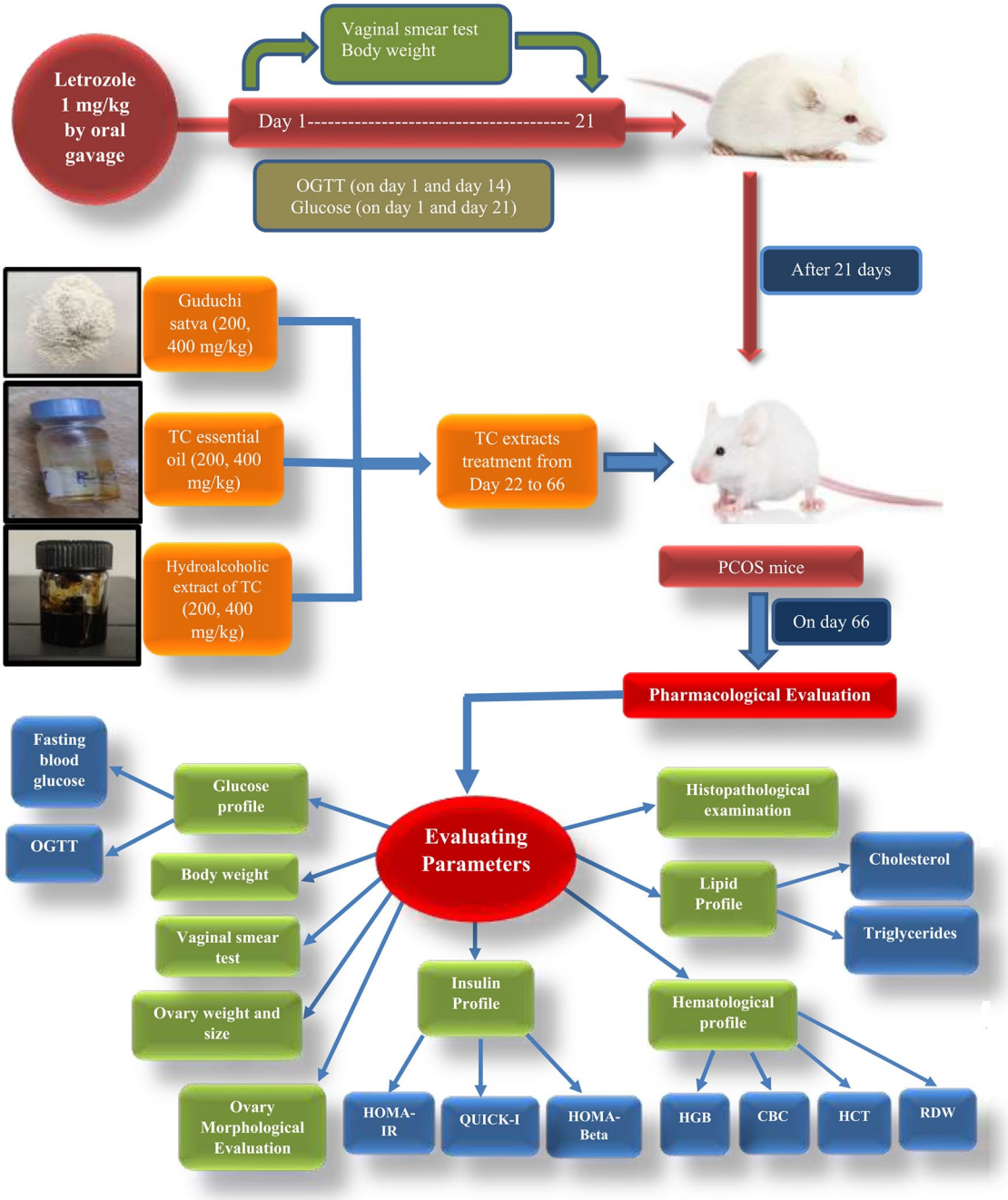
GRAPHICAL ABSTRACT



Introduction

Polycystic ovarian syndrome (PCOS), affects about 7.5% of the women of childbearing age. Hyperandrogenism associated with PCOS is shown by acne, irregular periods, oligo-ovulation/anovulation and unwanted hair growth on the face and body. When a woman has this disorder, it often leads to infertility due to hormonal dysregulation.[1] Women with PCOS often have hyperinsulinemia due to abnormal steroid and gonadotropin production with an increased luteinising hormone (LH) level due to higher rate of production and release.[2] Unlike increase in FSH concentration, increases in LH concentration facilitate the androgenesis in ovary. It is also believed that the lack of aromatase activity or other intra-ovarian anomalies in steroidogenesis may contribute to ovarian failure in PCOS.[3] Hormonal imbalance, systemic hyperandrogenism and ovarian androgenism have all been linked to a deficiency in aromatase enzyme activity responsible for determining the rate of biosynthesis of oestrogens from androgens.[4] In the ovary, aromatase converts C19 androgens produced by the granulosa cells into estradiol. PCOS is linked to follicular atresia and aberrant

follicular development, as demonstrated by letrozole-induced PCOS pre-clinical models. It was also reported that in the micro ovarian environment oestrogen receptor B expression decreased compared to normal animals.[5]

Clinical hyperandrogenism, is one of the key diagnostic criterion for PCOS[6] proven to increase the risk for diabetes, obesity, cancer, infertility and coronary heart disease. Reducing insulin resistance lowers ovarian androgen production and improves menstrual cyclicity.[1] While there are a number of drugs such as clomiphene citrate, metformin, orlistat and few other drugs shown promise in relieving only PCOS-related symptoms and cause unwanted side effects, such as psychological disturbances,[7] lactic acidosis[8] and muscle pain[9] researchers are still striving for a cure.

Many underutilised herbal therapies have been demonstrated to be useful in preventing and treating PCOS, despite the fact that the mechanism of action of herbal medications is not well-studied.[10,11] Recently concluded study reported that combination or symptomatic therapy with herbal extracts shown better results with respect to fertility.[12] Similarly, other literature review shows that the hydroalcoholic extracts of *Apium graveolens* and *Cinnamon zeylanicum* have strong antioxidant properties thereby protecting the ovary from damage.[13] Among the earliest and most popular plants used in Ayurvedic medicine is a shrub called *Tinospora cordifolia (Willd.) Miers ex Hook.f. & Thomson* (*TC*), plant name has been checked with http://www.worldfloraonline.org accessed on 14/01/2023, which is part of the family Menispermaceae.[14] In Ayurvedic and ethnic medicine *TC* has several medical uses due to its lack of side effects and a wide range of beneficial effects, including those on periods, allergies, arthritis, fever, leprosy, diabetes, stress and malaria. The stem has a number of medical purposes, including those of a diuretic, a thirst quencher, a source of iron and vitamins, a jaundice cure, and a bitter stomachic. When combined with other drugs, *TC* serves as an antidote for snakebite and scorpion stings.[15] The use of *TC* has been shown to hasten the recovery of diabetic foot ulcers and reduce the negative effects of radiation and chemotherapy.[14]

Chronic mild inflammatory process in the body is reported to have the role in pathogenesis of ovarian cyst and insulin resistance. The natural anti-inflammatory property of TC may aid in lowering insulin resistance, increasing metabolic rate and activating all tissues in the body.[16] Alkaloids (Tinosporine, mangoflorine, berberine [BER], choline, jatrorrhizine, palmatine [PAL], tembeterine), Steroids (β-Sitosterol, 20a-Hydroxy ecdysone), Terpenoids (Tinosporide, tinosporaside, ecdysterone, cordifolioside A, B and C, cordifoliside D and E), glycosides, sesquiterpenoids, essential oils, polysaccchrides, Quercetin, luteolin and kaempferol and a combination of fatty acids are the plant's primary chemical constituents (Figure 1).[17,18] Syringin, Berberine and Rumphioside-I were the alkaloids studied, and Insilico analyses showed that they significantly inhibited insulin receptor substrate (IRS1 and IRS2) receptors via the antagonistic ligand. Isoquinoline alkaloids are of tremendous interest due to their many therapeutic applications. The plant *TC* is said to be particularly high in isoquinoline alkaloids such as berberine, magnoflorine, palmatine and jatrorrhizine[19] and in another study berberine, isoquinoline alkaloids reversed ovarian apoptosis and morphological damage. Ber's effects on ovarian granulosa cell proliferation and death were decreased by blocking the PI3K/AKT pathway. For instance,[20] the prominent presence of these active chemical constituents in TC propose the hypothesis that TC may also have effect on these secondary signalling mechanism involved in pathogenesis of PCOS.

TC, known as Guduchi in Ayurveda and Siddha's 3000 BCE Materia Medica, is a hepatoprotective, immune-modulating and lifespan-extending drug.[21] After adequate preclinical with an illustration of mechanism of action, it is likely that *TC* extracts will be useful in the treatment of PCOS.[22] The pathogenesis of PCOS is commonly linked to mild inflammation, hyperglycaemia, hyperlipidaemia, infertility and oxidative stress. *TC* is proven to be anti-inflammatory,[23] anti-hyperglycaemia,[24] anti-hyperlipidaemic,[25] improvement in fertility[26] and helps in overcoming oxidative stress.[27] Present study is comprehensive and carried out with an objective to understand potential use of TC in clinical practice as nutritional supplement in PCOS treatment and/or management. Therefore this study is designed with basic goals of (1) prepare a water extract (satva) of *TC* as recommended by food safety standard authority of India and ayurvedic system of medicine, hydroalcoholic extract and essential oil of TC,[28,29] (2) examine their effects on letrozole-induced PCOS and (3) identify the mechanism of action of its effects using metabolic, endocrine, morphological and histological analyses.

Materials and Methods

Collection and authentication of TC-

The specimen had been obtained from the herbal garden of the institute and near the campus and authenticated at HNBG University, Srinagar, Uttarakhand, India. (Ref: Plant Taxn/356/2822-A).

Reagents and chemicals

All of the solvents and chemicals of analytical grade used in this research study were purchased from Merck, Mumbai, India. Metformin (USB Pvt. Ltd., India) and letrozole (Sun Pharmaceuticals, India) were purchased from local market. Insulin testing kits were provided by Wuhan Fine Biotech Co., Ltd. (Batch: M0260H030) as complementary. Cholesterol and triglyceride testing kit were purchased from Transasia Biomedicals Ltd., Mumbai. The ELISA kits used to analyse levels of progesterone, testosterone and estradiol were purchased from Abbott Laboratories, USA.

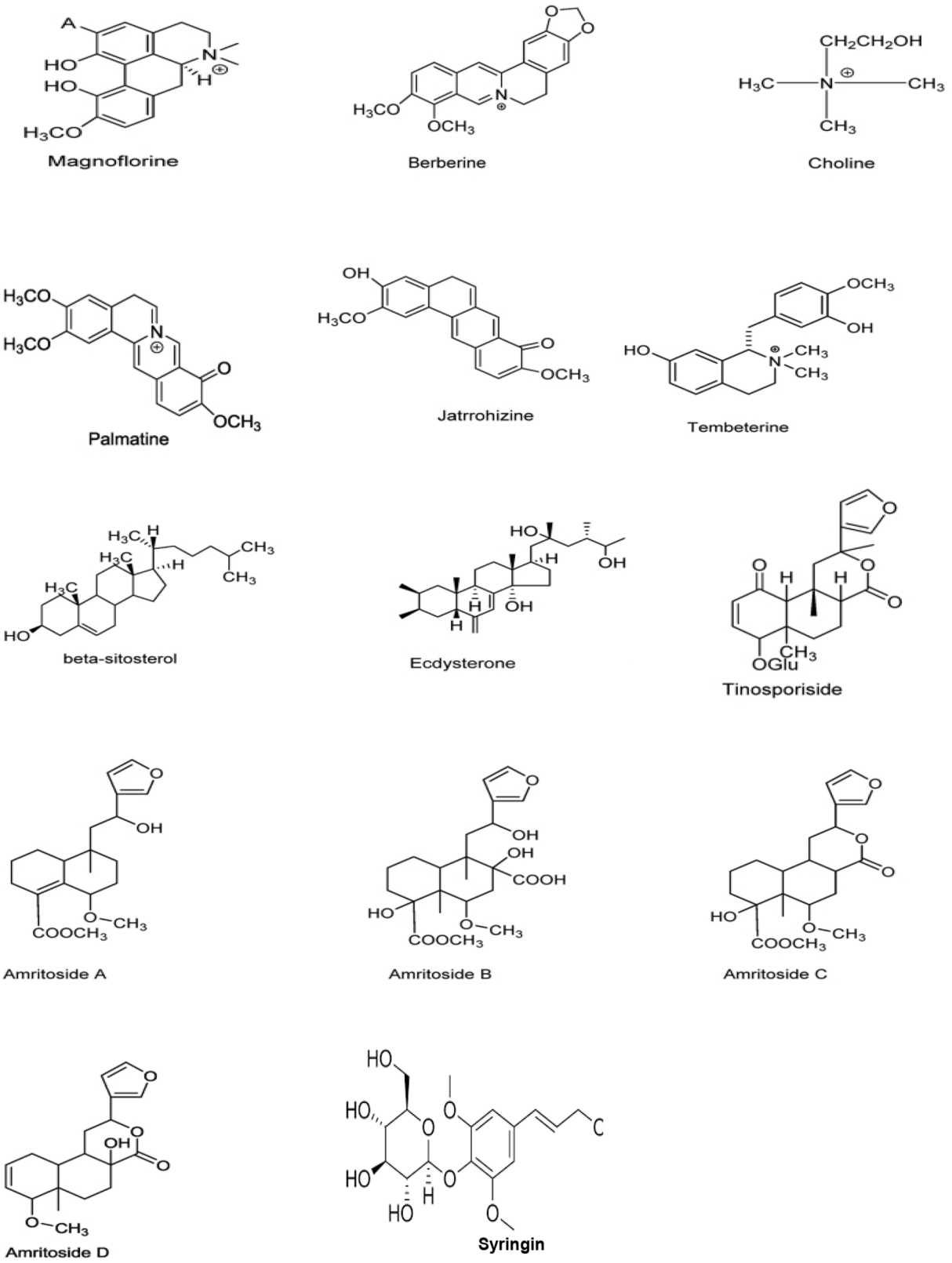


Figure 1. Chemical structure of *Tinospora cordifolia* constituents.

Instruments

The following instruments were used to conduct this research: Hemoglucometer (Dr. Morepen Glucose Monitor-Gluco One BG03, India), Rotary Microtome, Kwality fluorescent microscope with camera, and a cell analyser (Globolytics instruments Pvt. Ltd, India). Biochemical analyser, microplate reader for ELISA tests (Micro lab, India).

Preparation of hydroalcoholic extract

TC stems were collected and sterilised using 1% $KMnO_4$ solution. The stems were trimmed into smaller bits and air dried in the shade and then crushed up in a steel blender. For 7 days, with occasional shaking and warming, 1 kg of powdered *TC* stems were immersed in a hydroalcoholic solution (70:30) in a 5 L flat bottom flask and cold extracted. After 7 days, the mixture was filtered through a Buchner funnel to create the clear filtrate. In order to remove any remaining moisture from the filtrate, we used rotational vacuum evaporator in a water bath at 60˚C. After 15 days in a desiccator, the hydroalcoholic extract was completely dry and ready for usage.[30]

Formulation of guduchi satva

The fresh *TC* stems used to make guduchi satva were washed well with clean water, then chopped into pieces 1 to 2 inches in length, crushed into a coarse slimy mess and soaked in water for an entire day. It was carefully macerated and filtered through 4 layers of muslin the next day. After letting the filtrate (the remaining liquid after extraction) settle for as long as 5 hours, the supernatant liquid was delicately drained off, and the starchy sediment was scraped into a tray. Until pharmacological evaluation undertaken, this starchy substance was lyophilised at −55˚C to generate a white crystalline powder.[31]

Isolation of essential oil

The leaves of *TC* were harvested and then washed with running water. The essential oil was obtained by subjecting 100 g of newly harvested *TC* leaves to a Clevenger apparatus for hydrodistillation for a period of 4 hours. The hydrodistillation process resulted in an average yield of oil, which was reported.[32]

Animal handling

The National Institute of Biologicals in Noida, India, provided 6-week-old female Swiss albino mice weighing 30 to 40 g. Six mice were caged in sterilised plastic well-ventilated cages with 12 hours of darkness and 12 hours of light cycle. Mice were acclimated for a week before the PCOS study. Throughout the duration of the trial, the mice had unrestricted access to pelleted food and water. The Institutional Animal Ethics Committee at DITU approved the study on July 10, 2021 (DITU/IAEC/21-22/07-06).

Selection of dose/toxicity study

The toxicity of *TC* extract was evaluated in line with OECD 423 standards. Before the testing animals were fasted for 6 hours, however they continued to have access to water during the experiment. Oral needles were used to administer a single dose of the test sample (2000 mg/kg bw) in 1% CMC for hydroalcoholic extract and satva to mice. Whereas sesame oil was used for dilution and administration of TC essential oil to mice. Mice were continually observed for 3 to 4 hours after receiving medicine, and again 24 hours later. The animals were observed for any behavioural abnormalities, such as hair loss, erratic breathing, convulsions or diarrhoea, as well as indicators of lethargy, discomfort or death for 14 days. However, the chronic toxicity study was carried out for 21 days with a dose of 400 mg/kg/day p.o. and above signs were observed for any toxicity along with physical features such as hair loss, erratic breathing, convulsions or diarrhea.

Establishment of PCOS model

There were 54 animals, and they were all divided into 9 groups of 6 each. All of the animals' estrous cycles were checked for 3 estrous cycles with vaginal smear tests. Only animals with a normal estrous cycle were used in this experiment. Every group of animals was given 1 mg/kg of Letrozole p.o. every day for 21 days in 1% CMC, except for Group I, which was a normal control. To prove that PCOS was caused, vaginal smears were taken every day and observed under a microscope with methylene blue dye (1). Changes in the estrous cycle to diestrous phase led towards the disease development. Group I (NC) only got 1% CMC, Group II was a disease control and Group III (Metformin 250 mg/kg, orally once daily) was treated starting on day 22 for the rest of the study (days 22-66).[33,34]

Animal groups

Nine groups of 6 animals each in group I, II, III, IV, V, VI, VII, VIII and IX represents normal control, negative control (Letrozole treated only), positive control (250 mg/kg/day Metformin PO), *TC* satva treated 200 mg/kg/day, *TC* satva treated 400 mg/kg/day, *TC* oil treated 200 mg/kg/day, *TC* oil treated 400 mg/kg/day, *TC* HA extract treated 200 mg/kg/day and *TC* HA extract treated 400 mg/kg/day respectively.

Change in body weight

The animals were weighed periodically and their body weight was recorded throughout the procedure.[1]

Estrous cycle monitoring and vaginal smear cytology

A vaginal smear test was done every day to find out when the mice were in estrous phase. The estrous cycle in mice was examined by smearing the vaginal lining with a pipette. The 4 stages of the estrous cycle are called estrus, diestrus, metestrus and proestrus. Estrous phase was confirmed by the appearance of cornified-epithelial cells. Metestrus phase has epithelial cells, leukocytes and cornified cells with nuclei. Epithelial cells with nuclei predominate in proestrus phase, although leukocytes are few.[31] After flushing mice with 0.2 to 0.3 mL of saline, with a micropipette with the help of the same we collected

vaginal fluid to analyse the estrous cycle. Droplets of this solution were placed on slides under coverslips and observed under microscope with 10× and 45× lenses detecting methylene blue stained cells.[1] Presence of diestrus phase indicates that arresting of estrous cycle similar to amenorrhea in human.

Measurement of fasting blood glucose (FBG) and oral glucose tolerance test (OGTT)

The FBG level was measured using the tail incision procedure with a hemoglucometer on day 0, 15, 21, 44 and 66. For the OGTT measurement, the mice were given glucose by oral at a rate of 2 g/kg/body weight after fasting for a period of 6 hours. On days 1, 15 and 44, a hemoglucometer was used to measure the amount of glucose in the blood at 60 and 120 minutes during the 1 hour-OGTT and the 2 hour-OGTT, respectively.[1]

Blood collection and measurement of blood profiles

On day 66 of the study, retro-orbital and cardiac blood was collected and preserved in BD Vacutainers with potassium EDTA to prevent clotting till used.[1] Haemolysis, clotting, or clot-forming samples were discarded. The blood samples were analysed with GI-HA3000 for cell counts within 8 hours of blood collection, at 18 to 26°C for the following characteristics: absolute RBC count (10^{12} cells/L), haemoglobin concentration (g/L), haematocrit (%), platelets (10^9/L), mean corpuscular volume (fL), mean corpuscular HGB concentration (pg), mean corpuscular HGB distribution width (%), mean platelet volume (fL), procalcitonin concentration (%) and platelet large cell ratio.

Collection of plasma and detection of biochemical indexes

On last day of experiment, that is, 66th day, from each mice more than 2 mL of blood was withdrawn by cardiac puncture.

Following collection of plasma after centrifuge was kept at −80°C in a deep freezer. Fasting insulin levels were detected by using diagnostic kit supplied by Wuhan Fine Biotech Co., Ltd (Batch: M0260H030). Based on the results of FBG and insulin level we computed HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), HOMA-Beta (Homeostasis Model Assessment-Beta) and QUICKI (Quantitative insulin-sensitivity check index) levels using following formula.[33,35,36]

$$\text{HOMA-IR} = \text{Fasting Glucose}\ (\text{mg/dL}) \times \text{Fasting Insulin}\ (\mu\text{U/mL})/405)$$

$$\text{QUICKI} = 1/\left[\begin{array}{l}\log(\text{Fasting Insulin}\ \mu\text{U/mL}) \\ +\log(\text{Fasting Glucose mg/dL})\end{array}\right]$$

$$\text{HOMA-}\beta = (\text{Fasting Insulin} \times 20)/(\text{Fasting Blood Glucose} - 3.5).$$

Cholesterol and Triglycerides were analysed by using test kits purchased from Transasia Bio-medicals Ltd. Mumbai as per the manufacturer instructions.

Plasma estradiol, progesterone, and testosterone were assayed at local pathology laboratory by immunosorbent Sandwich ELISA colorimetric method in a 96 well plate micro-plate reader (Multiskan™ GO, Thermo Fischer scientific) using Abbott Laboratories, USA diagnostic kits.[37] There was limited serum sample ~50 µL available for LH and FSH estimation to meet minimum required volume of 150 µL. Therefore, serum sample was pooled from all 6 animals for LH and FSH into 1 each of respective group and estimated by immunological assay using ERBA Fertikit further computation of LH/FSH ratio.

Measurement of weight, size and morphological changes of the ovaries

After blood collection, animals were euthanised with overdose of ketamine and their ovarian weight was measured using digital balance and the size was measured using digital vernier calipers. The morphology were assessed by naked eye observation.[38]

Histological examination

The isolated ovaries from the mice were stored in formalin at a 10% concentration. For the purpose of analysing microscopic changes, 5 µM ovarian slices were used followed by staining with haematoxylin and eosin highlighted anomalies in the histopathological pictures.[1]

Severity of PCOS

Based on the previous studies and features of PCOS in animals we are proposing the following method of calculating the severity score of PCOS in mice and categorise it as nil, mild, moderate, severe and very severe.

$$\begin{aligned}\textbf{Severity Score} = &\left(\begin{array}{l}\text{Presence of oocyte} \\ +\ \text{Cystic follicle}\ (\text{no.})\end{array}\right) \\ &*(\text{Ovary diameter}\ (\text{mm})) \\ &+ (\text{Ovarian Weight}\ (\text{mg})/10)/10 \\ &*(\text{Insulin}\ (\text{IU/mL}) + \text{Testosterone}\ (\text{nmol/L}))\end{aligned}$$

*presence of oocyte = 0, Absence of oocyte = 1

Very severe: >10; Severe: 7 to 10; Moderate 4 to 7; Mild: 1 to 4; Nil: <1

Statistical analysis

For the statistical study, we utilised GraphPad Prism, version 9.0. Means and standard errors of the mean are shown for every data (SEM) as mean ± SEM. Size and weight of ovary, blood profile, oestrogen, progesterone, testosterone, HOMA-IR, HOMA-Beta, QUICKI, LH, FSH, LH/FSH, severity scores were analysed by unpaired *T* test whereas body weight, fasting blood glucose and OGTT were analysed by paired *T* test followed by one-way analysis of variance (ANOVA) and then Dennett's multiple comparisons test (DMCT) as a post-hoc analysis. *P* value of less than .05 was considered statistically significant. *, **, *** indicate statistical significance $P < .05$, $P < .01$, $P < .001$ respectively.

Result

Toxicity study

Swiss albino mice (n = 6) were given a single high dosage of 2000 mg/kg body weight of *TC* extract and monitored daily for 21 days to determine its toxicity. In chronic toxicity study animals were observed with maximum dose of preparations, that is, 400 mg/kg per day for 21 days. Throughout the course of the study, not a single mouse displayed any toxicity indications, proving that the extracts are completely safe for use on mice.

Estrous cycle determination

The normal control group (group I) had a normal estrous cycle (estrous, proestrus, metestrus, and diestrus phases) throughout the research. Figure 2 depicts a healthy mouse's estrous cycle. Group II animals with PCOS had delayed estrous cycles that lingered in diestrus/PCOS. As most vaginal smear cells were leukocytes, group III (Metformin) remained in diestrus. Groups IV, V, VI, VII, VIII and IX exhibited the estrous stage of the cycle, which comprises most cornified epithelial cells. In the estrus stage, predominantly cornified epithelial cells are present (2a). Pro-estrus stage characterised by nucleated cornified epithelial cells with a minor number of leukocytes (2b), Metestrus stage containing cornified epithelial cells, leukocytes and nucleated cornified epithelial cells (2c). In the diestrus stage mostly leukocytes are present (2d). Whereas in PCOS stage predominantly leukocytes were present (2e).

Ovarian size, weight and body weight

As depicted in Figure 3a the diameter of letrozole treated mice was highly significantly ($P < .001$) increased to 4.17 ± 0.22 mm compared to 2.56 ± 0.15 mm diameter of the control. This shows that the letrozole treatment induces hypertrophy of ovary. Highly significant ($P < .001$) reduction in diameter of the ovary was observed by *TC* preparation treatment. Similar results were seen with metformin treatment. The weight of the ovary was also computed in this study (Figure 3b). Letrozole treatment highly significantly (<.001) increased the weight of the ovary almost to double the normal control group. Treatment of *TC* for 66 days did not allow the weight of the ovary to increase and maintained almost normal weight. *TC* treatment reduced the letrozole induced ovarian hypertrophy and ovarian weight highly significantly ($P < .001$). On day 21, all treated mice gained weight significantly (Figure 3c). Letrozole substantially ($P < .05$) raised body weight on Day 66. *TC* therapy dose-dependently ($P < .01$) reduced mice's body weight ($P < .01$ and $P < .001$) compared to negative control group. After 66 days, the *TC* treated mice weighed almost as much as the normal control group.

Letrozole raised blood glucose levels ($P < .001$) on day 21 of experimental animals compared to day 1 and the normal control group. On day 21, *TC* significantly lowered blood glucose in mice compared to letrozole. On day 66, letrozole-treated animals with *TC* preparations had lower blood glucose levels than negative controls ($P < .001$). Satva therapy outperformed oil and HA extract with respect to efficacy. Fasting blood sugar (FBS) highly significantly ($P < .001$) increased compared to normal control group and from 115.5 ± 6.79 to 193.7 ± 12.64 mg/dL in letrozole treated group of mice on day 15 and further increased to 239.2 ± 10.86 mg/dL compared to normal control group. Treatment of mice with *TC* and metformin has no significant effect on day 15. However, long term treatment with metformin and *TC*, that is, 44 days highly significantly ($P < .001$) reduced the FBS levels to almost normal. The point to underline here that the fall in blood sugar levels in *TC* treated groups especially satva was better than metformin treatment (Figure 4a).

1-Hour oral glucose tolerance test

The results (Figure 4b) of a day-1, 1-hour oral glucose tolerance test (OGTT) were similar across all experimental animal groups. On day 15, letrozole-treated group showed significantly greater intolerance to oral glucose than in the control group ($P < .001$). In mice, *TC* oil treatment significantly ($P < .001$) attenuated intolerance on days 15 and 44, relative to the negative control group. Oral glucose intolerance was considerably reduced ($P < .05$) in those treated with satva and HA extract compared to those treated with letrozole alone. All *TC* preparations showed substantial and superior on 15-day therapy reductions in 1-hour oral glucose tolerance by day 44.

2-Hour oral glucose tolerance test

In this 2-hour oral glucose tolerance test we observed (Figure 4b) that oral glucose intolerance due to letrozole occurs only after 15 days. Again on day 15 the most promising results are seen with *TC* oil treatment compared to satva and HA extract. Letrozole highly significantly ($P < .001$) increased from first day 148.8 ± 9.094 mg/dL to 219.8 ± 14.05 mg/dL on day 15 and further to 264.7 ± 7.027 mg/dL on day 44. Hypoglycaemic effects are seen on day 44 by satva treatment. A dose dependent highly significant ($P < .001$) effect was observed with *TC* satva, oil and HA extract treatment on day 44.

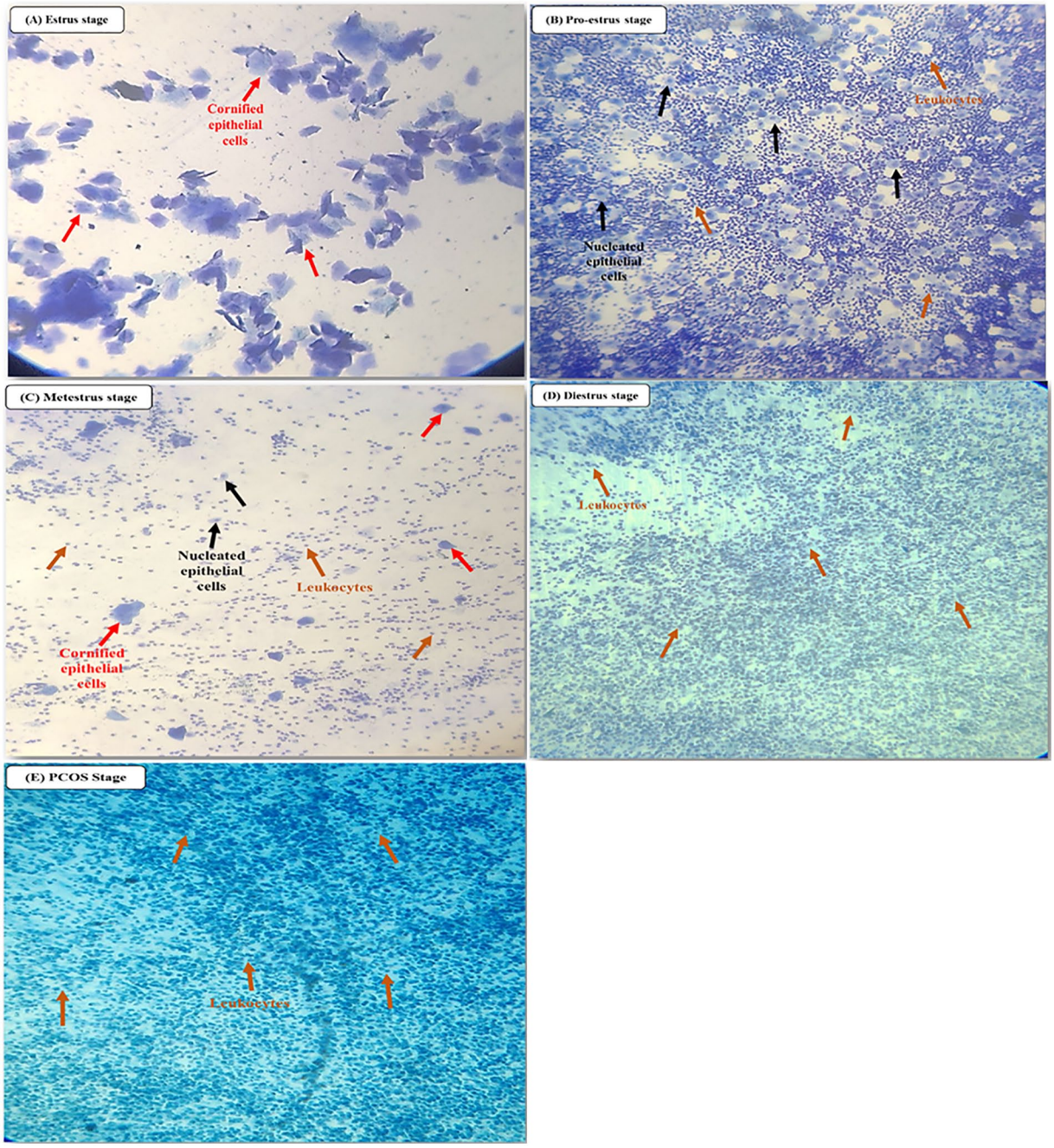


Figure 2. Stages of the menstrual cycle was estimated in the normal healthy and PCOS mice.
The different stages of the menstrual cycle was estimated in the normal healthy and PCOS mice. In the estrus stage, predominantly cornified epithelial cells (red arrow) are present (a). Pro-estrus stage characterised by nucleated cornified epithelial cells (red arrow) with a minor number of leukocytes (brown arrow) (b), Metestrus stage containing cornified epithelial cells (red arrow), leukocytes (brown arrow) and nucleated cornified epithelial cells (black arrow) (c). Diestrus stage: in the stage, mostly leukocytes (brown arrow) are present (d). PCOS stage: predominantly leukocytes (brown arrow) are present (e).

Blood profiles

Treatment of mice with letrozole increased RBC and platelet counts highly significantly ($P < .001$) along with MCV, HCT, MCH, RDW-SD, RDW-CV and PCT. Whereas the HGB, MCHC, PDW, MPV and P-LCR were significantly reduced. Treatment of mice with *TC* preparations significantly reversed effects of letrozole and brought the levels almost equal to the normal group of mice (Table 1).

Lipid profiles

The levels of cholesterol and triglycerides in the letrozole group were considerably higher than those in the normal group ($P < .01$). The blood cholesterol level in mice given metformin at a dose of 250 mg/kg was lowered very considerably ($P < .01$) and extremely significantly ($P < .001$). Serum cholesterol (Figure 5a) and triglyceride (Figure 5b) levels were statistically significant ($P < .01$) to highly statistically significantly ($P < .001$) depressed

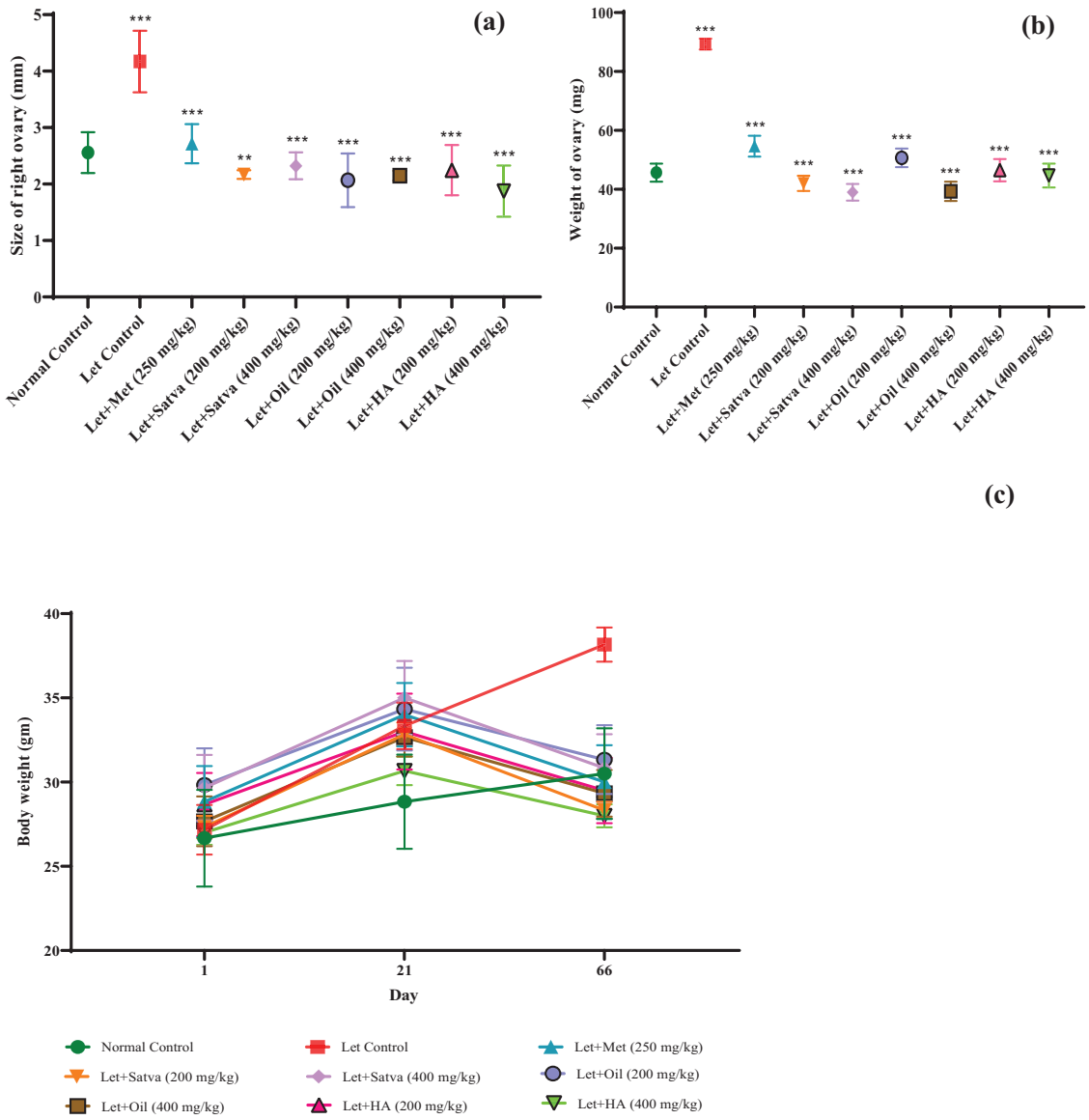


Figure 3. Impact of *TC* extract on letrozole induced PCOS mice size of the ovary, ovary weight and body weight.
Mice with PCOS produced by letrozole were given *Tinospora cordifolia* extracts to investigate change in their right ovary size (3), ovary weight (3) and body weight (3).

by all *TC* therapy groups. The most interesting fact is that the level of triglycerides and cholesterol were almost normalised.

Hormonal profile

All mice treated with letrozole were in diestrous phase therefore it was believed to have minimum change in the level of hormones. When comparing treated mice to controls, blood levels of estradiol (Figure 6a) and progesterone (Figure 6b) were found to be considerably ($P < 0.05$) lower after letrozole therapy, whereas testosterone (Figure 6c) levels were seen to be significantly ($P < .001$) more. Serum testosterone dropped in all *TC* treatment groups, but the highest doses of satva, oil and hydroalcoholic extracts decreased it to virtually normal levels. Letrozole's effect on estradiol was not reversed by metformin therapy, although the highest dose of satva, oil and HA extracts substantially ($P < .05$) raised estradiol levels compared to the letrozole-treated group. Serum progesterone levels rose significantly ($P < .001$) due to the combined effects of metformin and satva compared to the control group. Oil and HA extracts showed a negligible effect on progesterone levels. Treatment of mice with letrozole increased LH from normal 3.02 to 6.32, FSH from normal 2.01 to 4.85 whereas the LH/FSH ratio reduced from normal 1.5 to 1.3. The most effective action on elevated LH and FSH (Figure 6d) due to letrozole was observed by *TC* oil treatment dose dependently. The LH/FSH ratio of letrozole 1.3 was reduced to 0.71 and 0.9 by the oil at 200 and 400 mg/kg respectively. In all *TC* extract treatment groups the FSH was higher than LH. However, metformin treatment failed to reverse the effect of letrozole.

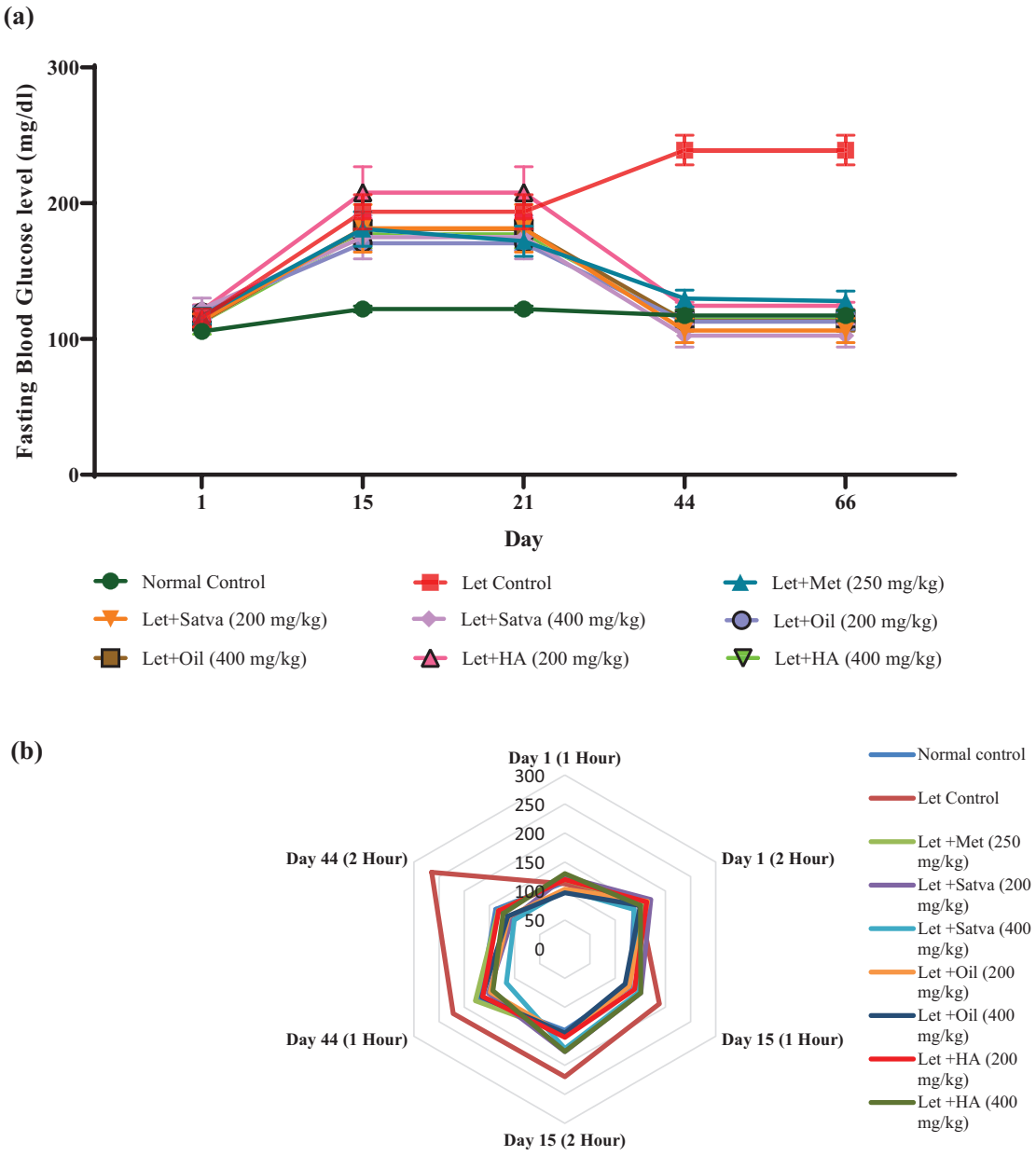


Figure 4. Impact of *TC* extract on Letrozole induced PCOS mice FBG and OGTT after 1 and 2 hours.
Tinospora cordifolia extract therapy altered FBS levels (a) in Letrozole-induced PCOS mice. Fasting blood glucose levels were monitored at different times during treatment with *Tinospora cordifolia* extracts in mice with PCOS produced by Letrozole. *Tinospora cordifolia* extracts were tested for oral glucose tolerance test (b) in mice with PCOS caused by Letrozole.

Insulin profile

Significant ($P < .05$) hyperinsulinemia (Figure 7a) was witnessed by treatment of mice with letrozole compared to normal control. Very significant ($P < .01$) decrease in serum insulin level was observed by treatment of *TC* in letrozole pretreated group of mice. Treatment with satva failed to stimulate beta cells in letrozole treated mice as indicated in HOMA-Beta index (Figure 7b). However, a dose dependent activity of beta cells was observed by treatment with oil and HA extracts. Similarly, the HOMA-IR index (Figure 7c) was highly significantly ($P < .001$) reduced by TC treatment in mice. Dose independent effect of *TC* on letrozone induced QUICKI (Figure 7d) was observed very significantly ($P < .01$) with 400 mg/kg dose of HA extract, oil, and satva compared to negative control. However, the lower doses of satva, oil and HA extracts significantly improved QUICKI.

Morphological observation of ovaries

Observation of the ovary through naked eyes shows that the ovaries in normal control group was clear and no black spots observed (Figure 8a). The letrozole treatment increased the volume and weight (Figure 8b) along with appearance of black spots and granular changes. Treatment with metformin reduced the black spots and granulation however not as clearer as TC

Table 1. Effect of *Tinospora cordifolia* extracts on Letrozole induced PCOS mice blood profile changes.

TREATMENT	NORMAL CONTROL	LET CONTROL	LET + MET (250 MG/KG)	LET + SATVA (200 MG/KG)	LET + SATVA (400 MG/KG)	LET + OIL (200 MG/KG)	LET + OIL (400 MG/KG)	LET + HA (200 MG/KG)	LET + HA (400 MG/KG)
RBC	1.87 ± 0.095	4.4 ± 0.27***C	1.92 ± 0.12***C	1.81 ± 0.075***C	2 ± 0.19***C	1.73 ± 0.15***C	2.02 ± 0.12***C	2.03 ± 0.07***C	1.97 ± 0.13***C
PLT	397.7 ± 36.34	821.3 ± 40.45***C	492.3 ± 16.44***C	471.8 ± 46.7***C	416.3 ± 26.22***C	454 ± 27.81***C	417.3 ± 35.36***C	437.3 ± 23.91***C	436.8 ± 32.38***C
HGB	13.88 ± 0.85	5.27 ± 1.08***C	12.33 ± 0.45***C	12.12 ± 0.58***C	12.52 ± 0.44***C	12.1 ± 0.36***C	12.73 ± 0.54***C	12.23 ± 0.61***C	12.23 ± 0.88***C
MCV	84.52 ± 3.088	93.53 ± 2.64*	82.08 ± 3.81*	81.85 ± 2.5**a	83.83 ± 3.17*	80.72 ± 4.02*a	81.47 ± 1.99**a	82.28 ± 2.58**	81.38 ± 2.20**a
HCT	9.59 ± 0.41	15.47 ± 0.79***C	10.04 ± 0.87***C	9.94 ± 0.68***C	10.81 ± 1.007**C	10.2 ± 0.95***C	10.61 ± 0.65***C	10.42 ± 0.53***C	10.86 ± 0.50***C
MCH	30.12 ± 0.81	35.18 ± 1.59**	30.6 ± 1.20*	30.03 ± 0.99*	32.13 ± 1.59	30.07 ± 1.76*	34.37 ± 3.26	27.78 ± 1.11**a	30.95 ± 1.14*
MCHC	31.33 ± 1.24	12 ± 2.19***	27.83 ± 3.56**	30.67 ± 6.35**	27 ± 1.75***	32 ± 8.31*	35 ± 4.84***a	31 ± 5.19**	33.17 ± 10.23*
RDW-SD	12.33 ± 0.24	18.45 ± 0.11***C	14.08 ± 1.46**	18.27 ± 0.33	17.88 ± 0.21*	19.3 ± 0.73	18.09 ± 0.25	18.05 ± 0.15*	17.58 ± 0.18**
RDW-CV	11.73 ± 0.084	11.88 ± 0.338	11.15 ± 0.52	11.23 ± 0.19	11.05 ± 0.29*	11.98 ± 0.20	11.87 ± 0.20	11.18 ± 0.098*	10.87 ± 0.08**a
PDW	11.93 ± 0.17	8.2 ± 1.23**C	14.95 ± 1.007***C	12.13 ± 0.29**C	12.13 ± 0.31**C	12.18 ± 0.30**C	11.9 ± 0.17**C	11.78 ± 0.51*b	12.6 ± 0.52**C
MPV	5.38 ± 0.28	3.9 ± 0.25**b	4.98 ± 0.31*a	4.75 ± 0.13**	4.78 ± 0.12**	5.22 ± 0.39**b	5.38 ± 0.23***b	5.13 ± 0.22**a	5.67 ± 0.33***c
PCT	0.32 ± 0.013	1.49 ± 0.16***c	0.50 ± 0.22**b	0.79 ± 0.22*a	0.67 ± 0.23**a	0.82 ± 0.18*	0.59 ± 0.21**b	0.55 ± 0.17**b	0.42 ± 0.069***c
P-LCR	5.2 ± 0.30	2.27 ± 0.35***	5.68 ± 2.17	4.38 ± 0.58**	5.8 ± 0.51***	4.92 ± 1.15*	7.32 ± 0.58***b	7.62 ± 1.02***b	7.48 ± 0.43***b

P value of less than .05 was considered statistically significant.
*, **, *** indicate statistical significance $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively.
The level of significance by One Way ANOVA are indicated by $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$.

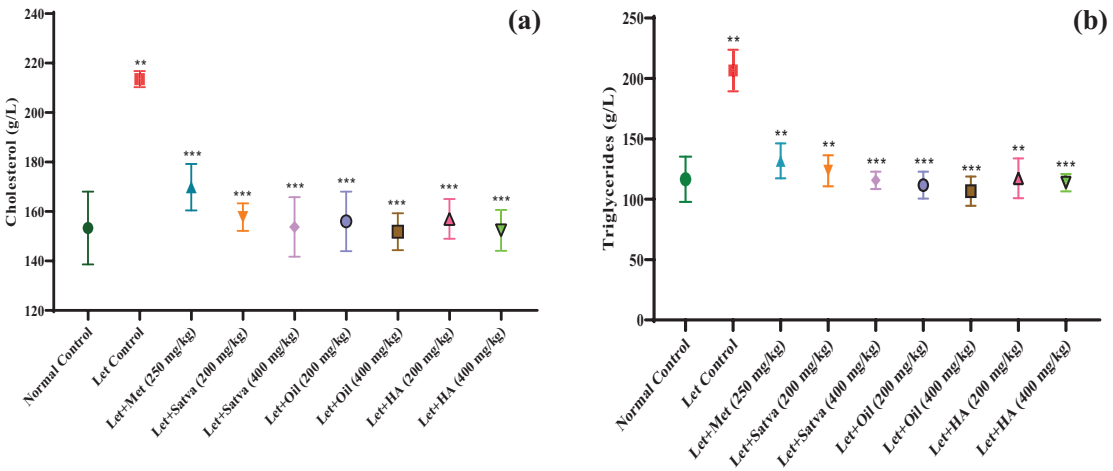


Figure 5. Impact of *TC* extracts on Letrozole induced PCOS mice Lipid profile.
Tinospora cordifolia extracts therapy altered cholesterol (a) and triglycerides levels (b) in Letrozole-induced PCOS mice.

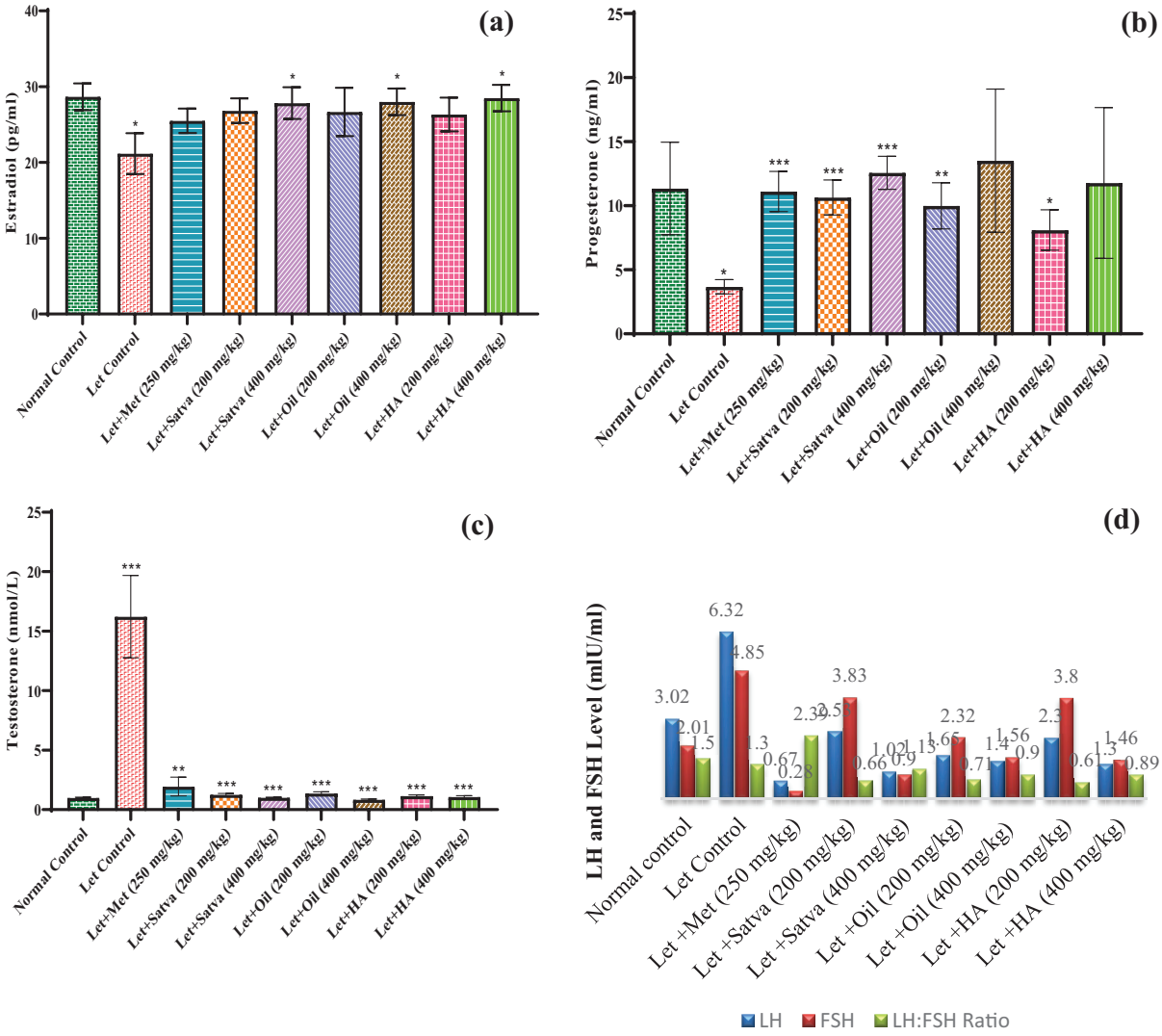


Figure 6. Impact of *TC* extracts on Letrozole induced PCOS mice Hormonal profiles.
Treatment with extracts of *Tinospora cordifolia* had an impact on Estradiol (a), Progesterone (b), Testosterone (c), LH and FSH levels (d) in Letrozole-induced PCOS mice.

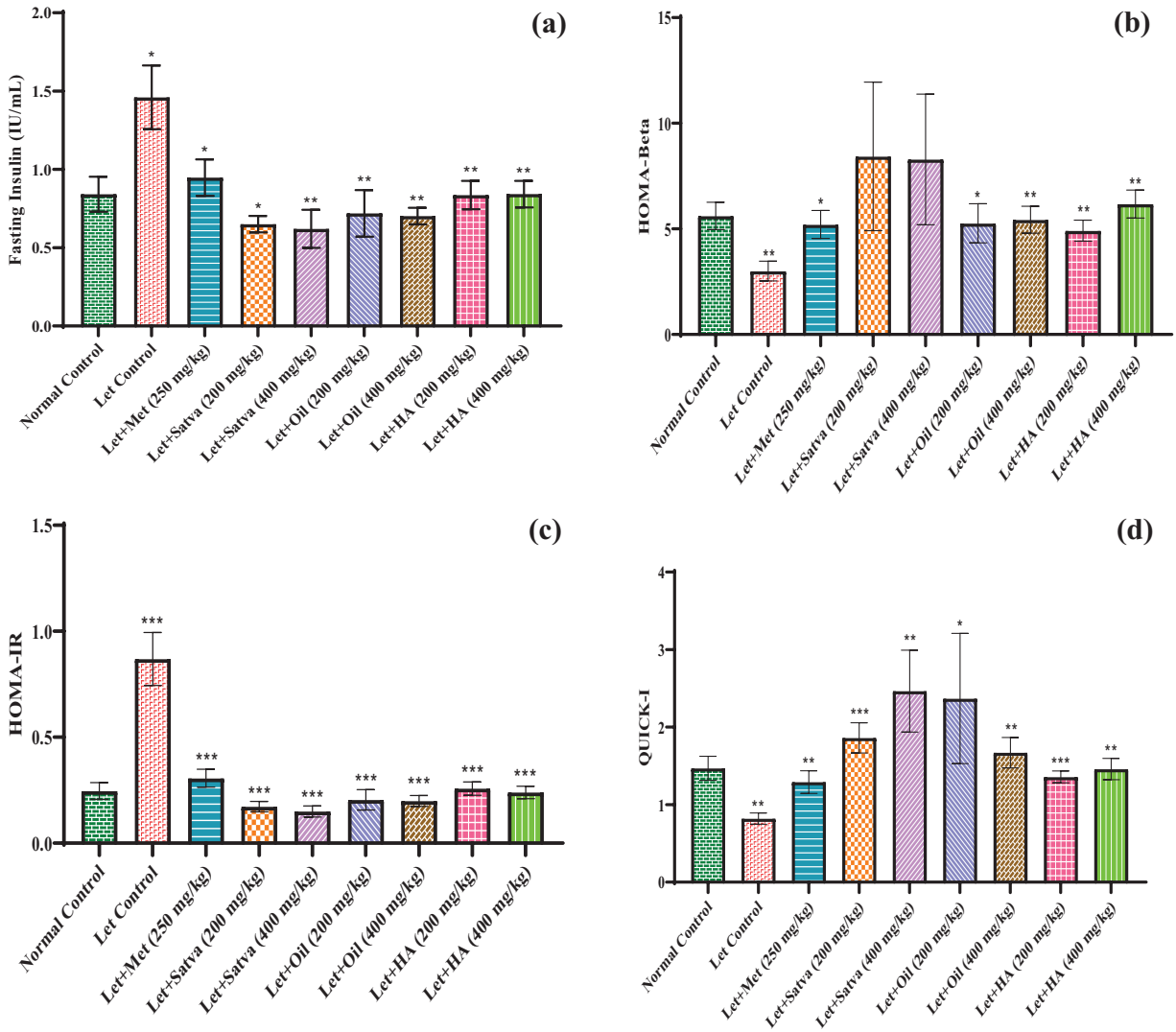


Figure 7. Impact of *TC* extracts on Letrozole induced PCOS mice serum insulin profile and insulin activities.
Treatment with extracts from *Tinospora cordifolia* had an impact on serum Insulin level in mice with PCOS induced by Letrozole. Serum insulin level (a), HOMA-Beta (b), HOMA-IR (c) and QUICKI (d).

treated groups (Figure 8c). Treatment of mice with TC oil in letrozole induced PCOS has absolutely fine and as clearer as normal (Figure 8f and g). Treatment with TC satva (Figure 8d and e) and HA extract (Figure 8h and i) also significantly reduced the alteration in appearance of ovary.

Histopathological examination of the ovaries

Oocytes were found inside a few of the follicles that were present in the normal control group's ovaries (Figure 9). Theca cells were polarised mesenchymal cells that have a spindle-like form and typically have 2 to 3 layers of thickness. These cells were discovered outside of the follicle-associated basal lamina. An immature interstitial compartment was found outside of the theca layer. The cells that made up this compartment were formed from steroidogenic theca cells that had been left over from atretic follicles. After *TC* therapy, PCOS-induced mice ovaries showed a larger number of cystic follicles, stomal nodules and stromal hyperplasia and fewer preantral follicles, antral follicles and corpus luteum compared to controls. Ovarian Histopathology study of after *TC* treatment in addition, there were no corpora lutea present, which is proof that ovulation had not taken place. Ovary slice from a *TC* satva-treated mouse demonstrates the presence of a medulla, graafian follicle and follicular cyst. Medulla, cortex, and follicular cysts are seen in the group that was treated with metformin. The components of the normal control group's ovary could be seen, each with a clearly defined structure, when the organ was cut transversely. There were un-ruptured primordial follicles, as well as primary follicle, secondary follicle and Graafian follicles. The blood arteries were normal, and the surface epithelium was bordered with low cuboidal to flattened cells. The stroma is made up of theca cells that range in shape from round to spindle. The mice that were given letrozole showed stromal nodules and follicular cysts coupled with an infrequent Graafian follicle. Additionally, these mice had multiple follicular cysts linked with hyperplasia of theca cells. The follicular cyst, medulla

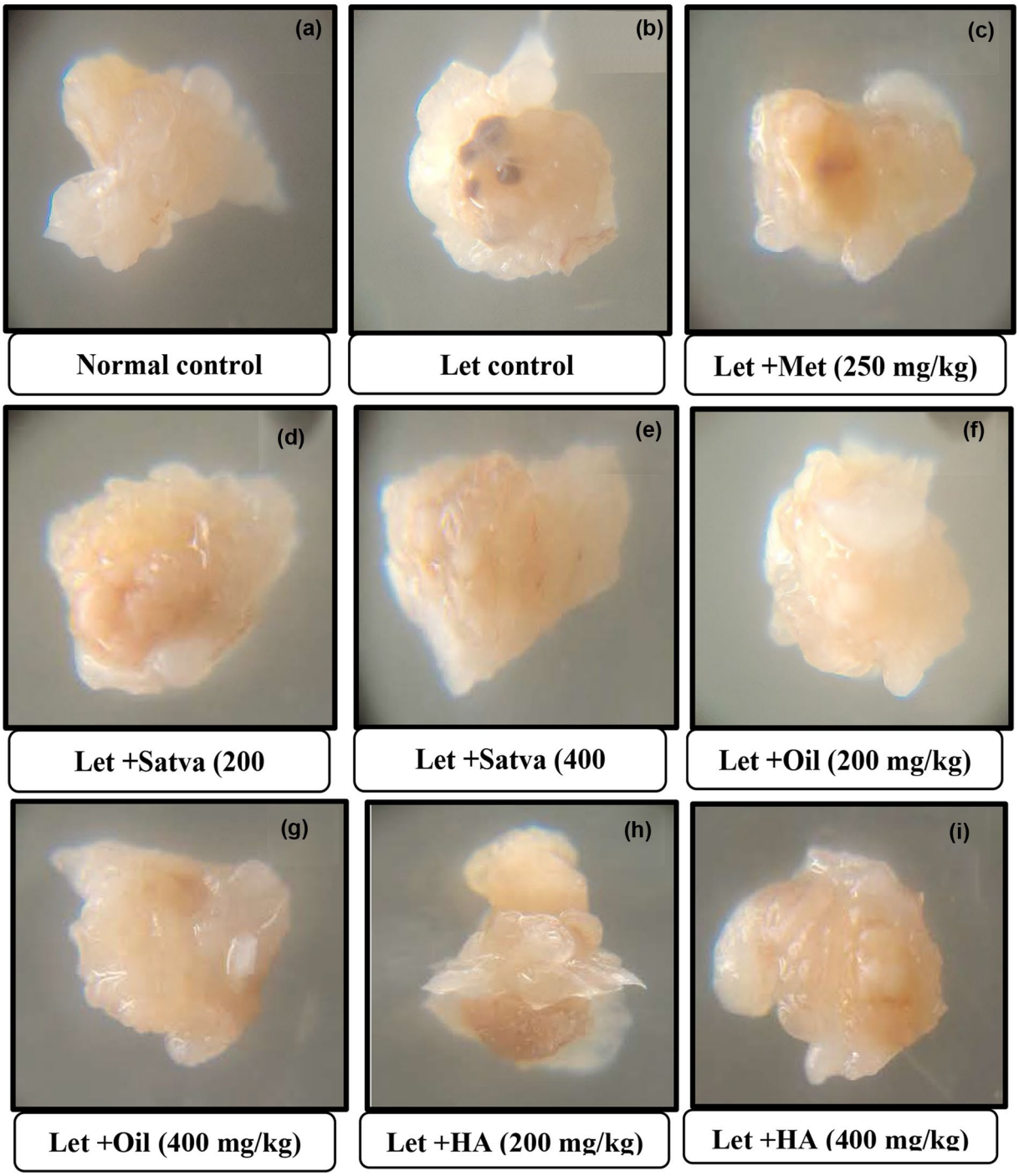


Figure 8. Impact of *TC* extracts on Letrozole induced PCOS mice Morphological Observation of Ovaries.
Analysing the ovarian morphological alterations brought on by *Tinospora cordifolia* extract therapy in Letrozole-induced PCOS mice. Comparison of normal ovary with that of PCOS and *TC*-treated ovaries. There were 9 groups: (a) the normal control, (b) the letrozole-only group, (c) the metformin-treated positive control, (d) the *TC* satva 200 mg/kg group, (e) the *TC* satva 400 mg/kg group, (f) the *TC* oil 200 mg/kg group, (g) the *TC* oil 400 mg/kg group, (h) the *TC* HA 200 mg/kg group and (i) the HA 400 mg/kg treated group.

and Graafian follicle were all visible in the ovary of mice that had been treated with *TC* oil. In conclusion, the ovary of a mouse that had been treated with *TC* hydroalcoholic extract exhibited a medulla, cortex and follicular cyst. Because of this, the findings suggest that there was a significant improvement in the histopathological signs of the mice that were given *TC* satva and oil as treatment.

Severity score measurement

In this study we proposed to evaluate the severity score for PCOS in mice model. The severity in letrozole treated mice was computed to be very severe compared to other groups of the study. Highly significant ($P < .001$) reduction in severity was observed by *TC* extract treatment. Metformin treatment reduced the severity to severe whereas treatment of mice with

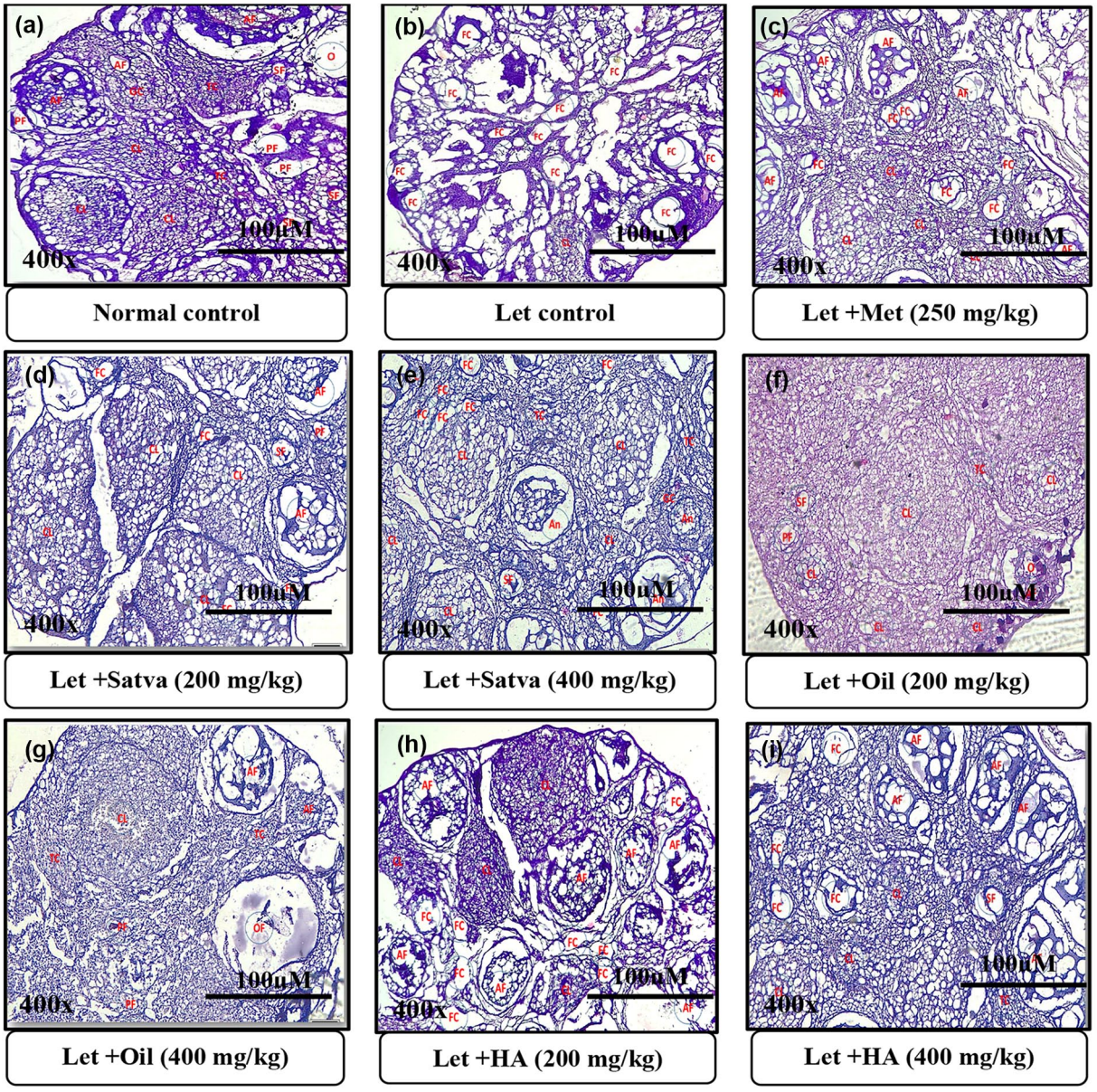


Figure 9. Impact of *TC* extracts on Letrozole induced PCOS mice Histopathological Examination of the Ovaries.
Analysis of Tinospora cordifolia extract-induced ovarian histological alterations in Letrozole-induced PCOS mice. Histological examination of normal ovarian tissue compared to those of PCOS and *TC*-treated ovaries. At 400× magnification, histological alterations in mouse ovarian tissues 100 µM were analysed using haematoxylin and eosin (H & E) staining. There were 9 groups: (a) the normal control, (b) the letrozole-only group, (c) the metformin-treated positive control, (d) the *TC* satva 200 mg/kg group, (e) the *TC* satva 400 mg/kg group, (f) the *TC* oil 200 mg/kg group, (g) the *TC* oil 400 mg/kg group, (h) the *TC* HA 200 mg/kg group and (i) the HA 400 mg/kg treated group.
PAF, Preantral Follicle; AF/An, Antral Follicle; FC, Cystic Follicle; CL, Corpus Luteum; BL, GC, granulosa cell; O, oocyte; PF, Primary follicle; SF, Secondary follicle.

TC satva and HA extracts reduced the severity to moderate. *TC* oil at a dose of 400 mg/kg successfully reversed letrozole induced PCOS to normal whereas the *TC* oil dose of 200 mg/kg reduced severity to mild (Figure 10).

Discussion

The letrozole-induced PCOS mouse model is most reproducible due to its clinical resemblance to features such as ovarian enlargement, hyperandrogenism, insulin resistance, missing or irregular cycle, haemorrhagic cysts, and other metabolic characteristics. In our studies, the effects of long-term letrozole therapy were similar to earlier studies given that PCOS in female mice was hyperandrogenic, which caused reproductive, metabolic and endocrine issues.[1] PCOS lowers progesterone and oestrogen during follicle formation owing to corpus luteum regression. LH levels rise because they are released more frequently and at higher rates than FSH, which promotes androgen synthesis in the ovaries.[5] Most commercially available drugs treat PCOS only symptoms, therefore researchers are always looking for options to treat the route cause.[8,9] Herbal medications have the potential to prevent and manage PCOS, but few studies have examined their mechanism of action.[10,11] *Glycyrrhiza glabra* and *Cinnamomum cassia* reduces testosterone levels, oligo-/amenorrhoea and PCOS respectively.[5] Objective

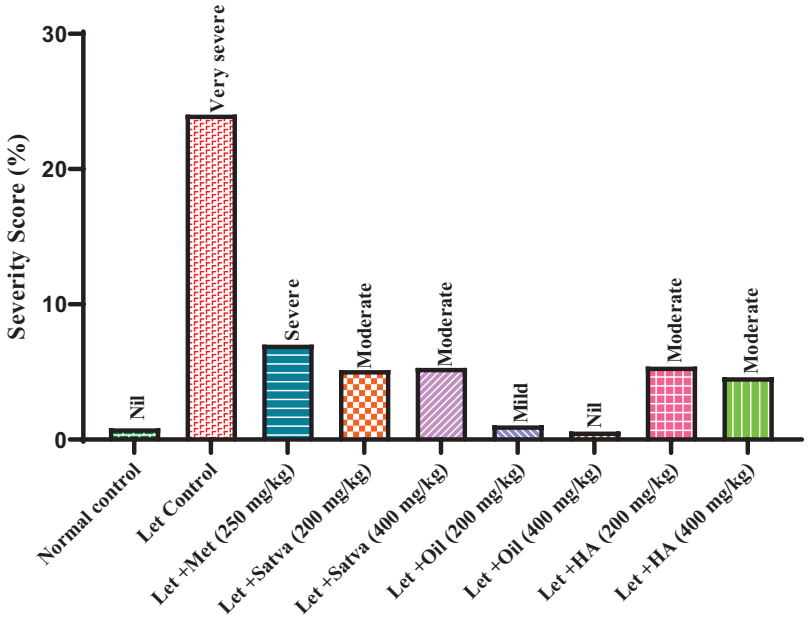


Figure 10. Impact of *TC* extracts on Letrozole induced PCOS mice severity score.

of present study was to prepare extracts of TC in different forms by following different methods and comprehensively evaluate their effect on PCOS related insulin resistance, insulin sensitivity, lipid profile, hormonal profile and ovary and propose possible mechanism of action.

In this study, we used a letrozole-induced PCOS mouse model. During the study, the PCOS group animals' estrous cycles were significantly delayed, with the diestrus phase lasting significantly longer than the other 3 estrous phases due to daily letrozole treatment.[5] The evaluation of *TC*-treated groups revealed that the majority of cornified epithelial cells are present during the estrous phase of the cycle restoring normal circulation concentrations of the testosterone, oestrogen, progesterone and gonadotrophins may be related to *TC*'s restorative impact on the mouse oestrous cycle. Ovarian function, such as follicular maturation and hormonal imbalance, were normalised by a regular oestrous cycle, which is regulated by these hormones.[39,40]

On the 21st day of the study, all of the mouse groups had a statistically significant increase in body weight. On day 66, letrozole caused a statistically significant ($P < .05$) increase in body weight compared to the normal control group of mice, which may have been the result of fat accumulation in the abdomen region.[28,41] Insulin is essential for promoting cellular absorption of amino acids, regulating lipolysis and promoting skeletal muscle development. This confirms what previous studies found, which was a considerable rise in the female mice's body weight through increased protein synthesis and protein breakdown is slowed with the help of IGF I and II. But *TC*-treated mice demonstrated dose-dependent very substantial ($P < .01$) and significant ($P < .001$) reductions in body weight gain compared to the negative control group. After 66 days of therapy, the mice's body weight had approximately wrapped up to that of the normal mice. This result agrees with what has been seen before because it controls cellular homeostasis and energy metabolism.[28]

Mice treated with letrozole showed substantial increase in RBC, platelet, MCV, HCT, MCH, RDW-SD, RDW-CV and PCT counts, all of which were statistically significant ($P < .001$) due to hyperandrogenism notwithstanding this, the HGB, MCHC, PDW, MPV and P-LCR were all reduced substantially. Mice given *TC* preparations have seen a dramatic reversal of letrozole's effects, with blood oestrogen levels returning to near-normal levels. These findings are consistent with a prior study showing that *TC* mitigates the harmful effects of lead via its antioxidant capabilities.[42,43]

This study's findings that Letrozole-induced PCOS mice exhibit hyperglycaemia are in line with those of earlier research.[44] The highest percentage of reduction in the increased blood glucose level was seen after treatment with *TC* satva. Glucose 6-phosphatase and fructose 1, 6-diphosphatase inhibition, liver glycogen content restoration, oxidative stress inhibition, gluconeogenesis inhibition and glycogenolysis inhibition are all mechanisms by which *TC* is shown to control blood sugar levels.[45] Reported insulin-mimicking and insulin-releasing actions have been found in *TC*'s isoquinoline alkaloids, including palmatine, jatrorrhizine and magnoflorine.[46]

There were significant differences between the 1- and 2-hour OGTT results of the letrozole-treated group and the control group on day 15. After administration of *TC* oil, satva or hydroalcoholic extracts to mice, glucose intolerance was significantly reduced on days 15 and 44 compared to the control group. Positive effects on glycaemic levels were seen with the test preparations, which may be explained by an increase in the

concentration of the anti-diabetic phytoconstituents. The anti-diabetic activities could be due to decreasing hepatic phosphorylase activity and increasing glucose absorption by peripheral tissues and organs, including the liver.[47]

Letrozole-treated mice exhibited hyperinsulinemia, a marker of insulin resistance, in contrast to their normal control group. Similar to the hyperinsulinemia/hyperandrogenism combination seen in PCOS, insulin resistance can significantly raise levels of circulating proinflammatory cytokines.[48] In mice pretreated with letrozole, administration of *TC* resulted in a significant decline in blood insulin levels, demonstrating a reversal of insulin resistance. It is evident that letrozole caused a rise in the HOMA-IR index, which was then reversed by *TC* therapy, lent more credence to these findings. The mechanism whereby the active components of *TC* increase muscle glucose absorption is supported by the Insulin Sensitivity Index QUICKI. Treatment with *TC* oil and HA extracts resulted in a dose-dependent increase in beta cell activity HOMA-Beta leading to increased oral glucose tolerance. These findings are consistent with earlier findings that *TC* reduced fructose-induced increased blood sugar, insulin and triglycerides.[49] Recently published study on berberine alkaloid which is also an important chemical constituent discovered in the *TC* plant stem has been shown to significantly enhance glucose tolerance.[50] Treatment with berberine significantly reduces fasting plasma glucose, improves insulin sensitivity and decreases LDL-C in type 2 diabetic rats, demonstrating its potent anti-hyperglycaemic and hypolipidemic effects. The expression of GLUT4 in tissues was dramatically increased after being exposed to berberine. Decreased expression of muscle and adipose GLUT4 in diabetic persons is one of the most important reasons of IR, since it is well known that absorption of glucose into cells depends on the help of membrane GLUT4.[51] In IR animal models, berberine was found to improve insulin sensitivity through activating AMP-activated protein kinase.[52]

Elevated TGs and low HDL cholesterol are hallmarks of dyslipidaemia, which is frequently seen in PCOS also reflected in currently used animal model. There is a wide range of potential triggers for dyslipidaemia in PCOS patients. A major contributor is insulin resistance, which increases lipolysis and modifies hepatic lipase and lipoprotein lipase expression. Total cholesterol, triglycerides and low-density lipoprotein (LDL) levels were all increased, but high-density lipoprotein (HDL) levels were lowered, after letrozole medication for PCOS.[53] In comparison to the letrozole control group, serum cholesterol and triglyceride levels were significantly ($P < .001$) lower in the *TC* treatment groups. The most intriguing finding is that the animals' triglyceride and cholesterol levels were reduced to within normal range similar to recently published research.[54] Earlier research has shown that the lipid-lowering properties of *TC*'s active ingredients, including its alkaloids, glycosides, flavonoids, saponins and tannins, may be responsible for the regulated gestational weight gain seen in mice.[17]

Serum testosterone and LH levels were measured and found to be considerably elevated in negative control group compared to normal mice, showing hyperandrogenism, proving the efficacy of our PCOS model. According to previous studies, the nonsteroidal aromatase inhibitor letrozole blocks the conversion of testosterone to estradiol. Because of this, oestrogen production reduces.[28,48] similar results were observed in the mice who had PCOS caused. The blood levels of testosterone and LH were found to be significantly decreased while oestrogen and progesterone levels rose significantly after repeated ingestion of satva, oil and hydroalcoholic extracts. This corresponds with what has been seen up until now. Letrozole was found to significantly increase the LH: FSH ratio, a defining feature of PCOS.[28] The ratio of LH to FSH drops significantly after treatment with satva, oil or hydroalcoholic extracts. The hormone levels of LH and FSH were restored to normal with the use of *TC* extracts.

Ovaries are primarily responsible for systemic hyperandrostenedione and testosterone through steroidogenesis, due to an increased number of theca cells and increased rate of mRNA transcription, which is a hallmark of polycystic ovaries. The hyperstimulation of thecal androgen production is likely influenced by both elevated LH concentrations and increased insulin concentrations resulting from insulin resistance. There may be additional variables within the ovary that enhance LH's stimulating effects on androgen production in the theca cells.[55,56] In this study, we analysed the morphology of the right ovary and the histopathology of the left ovary from each mouse. Hypertrophy, as well as the formation of black pa*t*ches and granular alterations, were seen with the naked eye after letrozole treatment.[57] Mice given *TC* oil that had PCOS produced by letrozole appeared to recover to normal health. Similar improvements were seen after *TC* satva and HA extract were treated to the ovary. After treating PCOS mice with *TC*, follicle numbers were restored and the ovaries were protected from oxidative stress, as was previously described.[58] It is proven that antioxidants have significant role to play in restoring the normal functioning of ovaries as presented in previous minocycline study which repaired oxidative damage through regulation of apoptotic-related gene regulation by decreasing the superoxide dismutase and glutathione peroxidase.[59] Similarly, treatment of mice with Co-enzyme Q10 decreased reactive oxygen species and atretic follicles in the ovary.[60] These results are supported by another study in which herbal extract of Olea europaea proven to have strong antioxidant properties to prevent ovary from oxidative damage.[61] Ovary weight was nearly twice that of the negative control group.[58] Following 66 days of treatment with TC, the ovarian weight of PCOS mice remained nearly normal. These are in line with the study in which supplementation with TC root extract significantly reduced the risk of morphological abnormalities in the ovaries due to radiation exposure.[62]

PCOS reduces ovarian physiological activity, since cystic follicles are more common than developing follicles and the corpus lutea.[63] *TC* treated groups had less cystic follicles than PCOS. PCOS groups had significantly fewer Graafian follicles and corpus lutea than controls and these effects were reversed by *TC* treatment, suggesting better ovarian function.[64] The *TC* group developed corpus luteum faster than the PCOS group, showing that *TC* treatment reduces cystic issues. The TC treatment groups had more primordial follicles and oocytes than the PCOS group. The literature indicates that PCOS raises androgens, causing anovulation and follicular atresia as androgens reach the granulosa layer of preantral follicles, link to cell receptors and kill cells.[63,64] It was also illustrated that androgens promote pyknotic granulosa cells, degrade oocytes and deteriorating follicles.

To aid clinical practitioners of traditional, herbal, and complementary medicine in making a decision on the use of TC preparations in the therapy of PCOS, we included all clinical indicators linked with PCOS in this preclinical study. However, while designing the study power analysis was not done to calculate the sample size. It is also important to consider the fact that potential for producing formulations containing the active elements of TC and its use in normal practice would be improved through the identification, isolation and evaluation of the active constituent(s).

Conclusion

Based on the results of the study we conclude that treatment of PCOS mice with *TC* satva preparation was the most promising with respect to reduction in fasting blood glucose, HOMA-IR, improved insulin secretion, oral glucose tolerance, QUICKI, lipid profile and decreasing hyperandrogenism, improving oestrogen, progesterone, LH and FSH levels and improved the ovaries weight and size when compared to hydroalcoholic extract and essential oil. Whereas the hydroalcoholic extract of TC improved HOMA-Beta level and TC essential oil improved the oral glucose tolerance. Based on the literature we proposed that the active constituents of TC such as Syringin, Berberine and Rumphioside-I alkaloid and others could be responsible for these activities. Therefore, *TC* could serve as a beneficial drug or nutritional supplement in all different forms for the treatment of PCOS and its complications.

Abbreviations

A4 Androstenedione
ANOVA Analysis of variance
CON Control
DHEA Dehydroepiandrosterone
DHT Dihydrotestosterone
E2 Estradiol
FSH Follicle-stimulating hormone
HA Hydroalcoholic
LET Letrozole
LH Luteinising hormone
P4 Progesterone
PCOS Polycystic ovary syndrome
SEM Standard error of the mean
SHBG Sex hormone–binding globulin
T Testosterone
TC *Tinospora cordifolia*

Declarations

Ethics approval and consent to participate

The Institutional Animal Ethics Committee at DITU gave its clearance to the animal experimentation on July 10, 2021 (DITU/IAEC/21-22/07-06). Consent to participate is not applicable.

Consent for publication

Not applicable.

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Author contributions

RR has involved in data curation; formal analysis; funding acquisition; investigation; methodology; resources; software; validation; visualisation; writing – original draft; writing – review and editing. AKS has involved in conceptualisation; formal analysis; methodology; supervision; writing – review and editing. HRC has involved in conceptualisation; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualisation; writing – original draft; writing – review and editing.

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Availability of data and materials

All data generated in the study are provided in the manuscript. No extra data is available.

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