Effect of *Benincasa hispida* T. Fruit Extract on Letrozole Induced Polycystic Ovary Syndrome in Female Rats

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Abstract

Polycystic ovary syndrome (PCOS) is a complex endocrine and metabolic disorder characterized by hyperandrogenism, anovulation, and insulin resistance. The present study evaluated the therapeutic efficacy of *Benincasa hispida* fruit extract (BHFE) in letrozole-induced PCOS in female rats. Fresh fruits were shade-dried and extracted with 60% ethanol using cold maceration. Preliminary phytochemical screening confirmed the presence of alkaloids, flavonoids, phenols, and steroids, while acute oral toxicity (OECD guideline 423) demonstrated safety up to 2000 mg/kg. Thirty female rats were divided into five groups: normal control, PCOS control (Letrozole 1 mg/kg *p.o.* for 21 days), standard (metformin 20 mg/kg), and test groups treated with BHFE (200 and 400 mg/kg *p.o.*) for 28 days post-induction. Parameters assessed included body weight, estrous cyclicity, oral glucose tolerance test (OGTT), serum lipid profile, insulin, sex hormones, reproductive organ weights, and ovarian histopathology. BHFE treatment significantly normalized body weight, improved estrous cyclicity, and restored hormonal balance by reducing luteinizing hormone and testosterone levels while increasing follicle-stimulating hormone and progesterone. It enhanced glucose tolerance, decreased serum insulin, and improved lipid profile by lowering total cholesterol, triglycerides, LDL, and VLDL while elevating HDL. Histopathological evaluation revealed restoration of normal ovarian architecture with reduced cystic follicles and reappearance of corpus luteum. The beneficial effects of BHFE are attributed to its antioxidant, anti-inflammatory, and hormone-modulatory properties. In conclusion, *Benincasa hispida* fruit extract exhibits potent protective and restorative effects in letrozole-induced PCOS, supporting its potential as a natural multi-targeted therapeutic agent for PCOS management.

Keywords: *Benincasa hispida*; Hormonal balance; Letrozole-induced PCOS; Metformin; Metabolic regulation

Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial endocrine disorder affecting women of reproductive age, characterized by hyperandrogenism, anovulation, and polycystic ovarian morphology [1]. It is often accompanied by metabolic disturbances such as insulin resistance, obesity, dyslipidemia, and an increased risk of type 2 diabetes and cardiovascular diseases. The etiology of PCOS is complex, involving genetic, hormonal, and environmental factors. Child and adolescent overweight and obesity were associated with significantly increased risk of later polycystic ovary syndrome symptoms [2].

Globally, prevalence estimates of PCOS are highly variable, ranging from 2.2% to as high as 26%. WHO estimates that it affected 116 million women are affected Worldwide in 2012(3.4% of women). In India the prevalence is gradually increasing. It is due to the lifestyle that people have adopted.The prevalence of PCOS among them was 22.5% by Rotterdam and 10.7% by Androgen Excess Society Criteria [3].

The pathophysiology of PCOS involves primary defects in the hypothalamic–pituitary axis, insulin secretion and ovarian function. The various pathogenetic mechanisms of PCOS include abnormal gonadotropin-releasing hormone (GnRH) regulation leading to increased luteinizing hormone (LH) and decreased FSH; decreased response of ovarian follicles to FSH; increased anti-Mullerian hormone, follicular arrest and increased secretion of testosterone, estradiol and dehydroepiandrosterone (DHEA). Obesity, especially abdominal fat deposition, is the major predisposing factor for the metabolic phenotype in PCOS [4].The insulin play an important role in the molecular mechanisms implicated in the androgenic hypersecretion and also the inhibition of hepatic synthesis of SHBG synthesis in liver cell. It prevents the normal follicular development in granular cell by a decrease in the level of follicle stimulating hormones which leads to follicular arrest [5]. Approximately, 25% to 30% of women with PCOS will show impaired glucose tolerance by the age of 30 and 8% of affected women will develop type 2 diabetes[6].

Current pharmacological interventions,depends on the patient’s desired outcomes whether the treatment of menstrual irregularity to achieve pregnancy or contraception, the anti-androgenic therapy for hyperandrogenic symptoms like hirsutism, the insulin sensitizer for insulin resistance associated with PCOS, and the ovulation inducer for infertility to induce ovulation[7]. All these therapeutic approaches are short-term symptomatic approaches; hence

there is a lack of long-term systemic approaches in the management of PCOS. Consequently, there is a growing interest in exploring alternative and complementary therapies, particularly those derived from natural products.

The usage and acceptability of complementary medicine by women has increased from 26% to 91% during the past ten years. Ayurvedic system of medicine has been emerging as one of the most commonly practiced alternative medicine for various health problems, including PCOS [8, 9]. Recent day global health debates getting significant attention towards traditional herbal medicines, Ayurveda is one of the most accepted traditional systems of Indian medicine.

Benincasa hispida (Thunb.), commonly known as ash gourd or winter melon is a traditional medicinal plant widely used in Ayurvedic and folk medicine. It belongs to the Cucurbitaceae family and is known for its diverse pharmacological properties including antioxidant, anti-inflammatory, antidiabetic, and diuretic effects. Traditionally used to treat neurological diseases, kidney disease, menstrual disorders, fever, cough accompanied by thick mucus and to fight intestinal worms [10-12]. The fruit is rich in bioactive compounds such as flavonoids, alkaloids, sterols, and phenolic acids, which contribute to its therapeutic potential [13]. Recent studies have indicated that *B. hispida* may exert beneficial effects on metabolic and hormonal imbalances, making it a promising candidate for the management of PCOS.

In light of the above, the present study aims to investigate the therapeutic effect of *Benincasa hispida* fruit extract on letrozole-induced PCOS in female rats.The study also planned to elucidate the potential mechanisms through which *B. hispida* exerts its effects, with a particular focus on its hypolipidemic, anti-diabetic, and hormone-modulatory properties.

Materials and Methods

Drugs and Chemicals

All the chemicals used in this study were of analytical grade. The following chemicals were used for the experimental study. Letrozole was purchased from the manufacturer of Wynclark Pharmaceuticals Private Limited, India.

Collection and Extraction of *Benincasahispida* fruit

The fresh fruits of *B. hispida*were collected from local market in and around of Coimbatore, all the collected fruits were screened. The whole fresh fruit of *B. hispida*were cut into small pieces and shade dried at room temperature.The dried materials were then coarsely powdered by a mechanical grinding and sieved (mesh no. 40). The powdered materials were extracted with 60% v/v ethanol by cold maceration technique for 48 hrs. After extraction, it was

filtered and dried at 60°C in rotaryevaporator to produce a semisolid mass.The driedextract was stored at 4°C in an air tight container until further use.

Phytochemical analysis

Qualitative analysis

The *Benincasahispida* fruitethanolicextract (BHFE)of was subjected to preliminary phytochemical screening for the presence or absence of phytoconstituents alkaloids, carbohydrates, flavonoids, steroids, proteins, tannins, phenols, terpens&steroiolsby standard methods [14].

Quantitative analysis

Estimation of total alkaloid content

To determine the total alkaloid content in the *Benincasahispida* fruitethanolic extract (BHFE) was dissolved with 1ml of 2N Hcl and filtered. The filtered solution was transferred into the separating funnel, and then 5 ml of phosphate buffer and 5 ml bromocresol green solution (BCG) were added. Then this mixer was diluted with chloroform. The absorbance of the test and standard solutions were determined at 470 nm. Atropine 20-100 µg/ml was used as standard. The total alkaloid content was expressed as mg of Atropine (AE)/g of extract [15].

Estimation of total flavonoid content

Total flavonoid content in the BHFE was estimated by $AlCl_3$ method. Quercetin was used as standard. The quercetin 1 mg/ml methanolic solution was prepared and different aliquots 20-100µg/ml from this solution was prepared with methanol. 3 ml of BHFE solution (1mg/ml solution in methanol) or standard solution was added into the test tube containing 1 ml of 2% $AlCl_3$methanolic solution and allowed to stand for 1 hour at room temperature. The absorbance of the solution was measured at 420 nm. The total flavonoid content was expressed as mg of Quercetin (QE)/g of the extract [16].

Estimation of total phenolic content

The total phenolic content in the BHFE was determined by the Folin-Ciocalteu method with slight modification. The 0.1 ml of extract (0.1 mg/ml in distilled water) was treated with 0.5 ml of Folin-Ciocalteau reagent and 1.5 ml of 7% sodium carbonate. The combined solution was shaken well and made up to 10 ml with distilled water. Then it was incubated in dark at room temperature for 2 hrs. Then the absorbance of the test and standard was taken at 725 nm in spectrometer against a reagent blank. Gallic acid (20-100 µg/ml) was used as standard. The total phenolic content was expressed as mg of gallic acid (GAE)/gm of extract [17].

Estimation of total steroidal content

The steroidal content in BHFE was estimated by the Liebermann-Burchard method with modifications. The extract 1 mg/ml was prepared by dissolving with chloroform, to that the freshly prepared Libermann-Burchard reagent (50 ml acetic anhydride and 5 ml of concentrated sulphuric acid) was added. Cholesterol (20-100 µg/ml) was used as standard. The absorbance of the test and standard was measured at 650 nm against a reagent blank. The total steroidal content in the BHFE was expressed as mg of cholesterol/g of ethanolic fruit extract of *B.hispida*[18].

Acute oral toxicity studies

Acute oral toxicity of *Benincasahispida* fruitethanolic extract (BHFE)was evaluated by the acute toxic class method as per OECD (Organization for Economic Co-operation and Development) test guideline 423 [19]. The female non-pregnant rats were acclimatized and housed in the experimental condition one week before the experiment. Before experimentation, the animals were overnight fasted and provided water *ad libitum.* The feed was withheld up to 4 hours after dosing. Three female rats were treated with a single oral dose of 2000 mg/kg was administered. After administration, each animal was individually observed for the first 30 minutes, followed by special attention for the first 4 hours and periodically for 24 hours, thereafter daily for 14 days. The evaluation parameters include changes in skin and fur, mucous membrane, eye, respiration, circulatory, central, peripheral nervous system, and somatomotor activity and behavioral changes. The occurrence of salivation, diarrhea, lethargy, tremors, convulsions, sleep, and coma should be monitored closely.

***In-vivo* PCOS study in Letrozole induced PCOS rat model**

Selection of experimental animals

The thirty colonyinbred virgin female Albino wistar rats, weighing between 180 and 230 g. The animals were housed under standard environmental conditions of 12/12 light/dark rhythm, maintained under controlled (23 ±2°C) room temperature, in polypropylene cages. The animals had at least two sequential estrous cycles that continued for fourdays were selected for the study. Estrous cycles were monitored by early morning vaginalsmear sampling.All the rats were acclimatized under laboratory conditions seven days prior to initiation of experiment. The Experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Sri Abirami College of Pharmacy, Coimbatore; Care and use of laboratory animals were confirmed to CCSEA guidelines (IAEC Reference No.: SACOP/Re/M. Pharm/04/2025 Dated: 21.06.2025).

Experimental design

Thirty female Wistar rats were randomly divided into five groups of six per each. Group I served as normal control received (0.5 ml of 0.5% CMC), Group II served as PCOS control received Letrozole (1 mg/kg b.w. *p. o.*) for 21 days, and Group III to V served as treatment groups received Metformin 20 mg/kg, *B. hispida* fruit extract 200 mg/kg & 400 mg/k *p.o.* respectively for 28 days.

Induction of PCOS in rats

The polycystic ovarian syndrome in rat model was induced by administering Letrozole 1 mg/kg orally once daily for 21 days [20]. The induction of PCOS was primarily confirmed by examining the vaginal smear and measuring the menstrual irregularity on 21stday after letrozole treatment. After induction of PCOS the drug treatments were provided further for 28 days.

Physical parameters

Bodyweight changes

The body weight of each rat was measured on day 0, after that weekly till continuation of the treatment using a standard animal weighing balance, and the final changes in body weight were recorded.

Feed and water intake analysis

The feed and water intake per treatment were measured daily using a standard weighing balance. The weekly mean intake of individual animals per day was calculated and recorded.

Vaginal smear observation

The estrous cycle regularity in rats was evaluated by the vaginal smear technique. The vaginal fluid was collected from day 14 to the end of the study treatment, on a daily morning between 8.00 AM to 9.00 AM. The vaginal fluid was collected by inserting the tip of a pipette filled with 10 µl of normal saline into the rat vagina at a depth of 5-10 mm and flushed in and out three times, if the solution became cloudy in the first flush, there was no need of further flushing. After collecting the vaginal fluid, a drop was placed on the glass slide and the smear was prepared. The prepared smear was dried and stained with 0.5% methylene blue aqueous solution. The stained slides were observed in the light microscope (40 x magnifications) to determine the stages of the estrous cycle based on the cell morphology [21].

Oral Glucose Tolerance Test (OGTT)

The Oral Glucose Tolerance Test (OGTT) was performed on day 21 after completion of letrozole administration and day 50 of the study following the post-drug treatments. Before the experiment animals fasted for 12 hours, and the glycemia was measured by tail vein blood sampling using AccuCheck Active glucometer (Roche Diagnostics Ltd, India). On the day of the experiment blood glucose level was assessed before (baseline) and after the single oral glucose (2 gm/kg b.w.) challenge at 30, 60, and 120 minutes [22].

Serum Biochemical Analysis

The serum biochemical parameters like serum lipid profile, sex hormone-binding globulin (SHBG), and serum insulin were estimated from the separated serum on day 50 of the study. The serum lipid profile like low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL) cholesterol, and triglyceride (TG) were analyzed by using the semi-auto analyzer kits techniques following the manufacturer's instruction (ARKRAY Healthcare Pvt. Ltd., Surat, India). The kits code are LDL cholesterol (71LS400-56), HDL cholesterol (71LS300-56), and for triglyceride (72LS100-60). The serum sex hormone-binding globulin (SHBG) and serum insulin levels were assayed by using an enzyme-linked immunosorbent assay (ELISA) kit (#EH421RB & #KAQ1251 respectively) upon the manufacturer's directions (Thermo Fisher Scientific, India).

Serum Sex Hormonal Assay

Theserum sexhormones testosterone, estradiol, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and progesterone was assayed on Cobas e411 immunoassay analyzers (Roche Diagnostics International Ltd., USA) using the particular ELISA kits technique according to the manufacturer's direction (Roche diagnostics, India). The product codes are testosterone (052022301090), estradiol (06656021500), LH (03561097190), FSH (117758635000), and progesterone (07092539500). The free androgen index (FAI) was calculated by the formula [23]

$$FAI = \frac{\text{Total testosterone}}{\text{SHBG}} \text{ X } 100$$

Reproductive Organ Weights

At end of the drug treatment period on day 50 of the study, the ovaries and uterus were removed from the sacrificed animals of each group. The adhering fat was removed and organ

weight was weighed. Bilateral ovaries of a rat were weighed and the mean value was calculated as ovary weight [24].

Histopathological Examination

Theright ovary of each rat was fixed in 10% neutral formal saline, dehydrated with the increasing concentration of ethanol, and after that immersed in xylene and embedded in paraffin wax. The blocks were longitudinally sectioned at 5 µm thickness from the center. The section was mounted on a slide and stained by the hematoxylin and eosin (HE) staining procedure.Some sections were stained by Masson's trichrome and examined under a light microscope with 40 X magnification for histopathological changes [25].

Statistical Analysis

All the data were expressed as mean ± standard error mean (mean ± SEM) to determine statistical significance. One-way analysis of variance (ANOVA) was used to compare rats over time or within groups, followed by post hoc Dunnett's multiple comparison test using Graphpad Prism V.8.0 statistical package. The level of P values <0.05 were judged as statistically significant.

Results

Extraction Value and Qualitative Phytochemical Analysis

The extraction value of the *Benincasahispida* fruitethanolic extract (BHFE) was calculated as 18.6% w/w yield percentage. The result of primary phytochemical screening of BHFE showed the presence of alkaloids, carbohydrates, flavonoids, steroids, proteins, tannins, phenols, terpens&steroiols.

Quantitative Phytochemical Estimation

The major phytoconstituents alkaloids, flavonoids, phenolic and steroidal total content in the *B. hispida* fruit extract is mentioned in table 1.

Table 1: Quantitative Phytochemical Estimationof *B. hispida* fruit extract

S. No.	Phytoconstituents	Quantity (mg/g)
1.	Total Alkaloid content	36.97 ± 1.48
2.	Total Flavonoid content	40.21 ± 2.47
3.	Total Phenolic content	23.00 ± 3.09
4.	Total Steroidal content	52.37 ± 4.53

N=3, values are expressed as mean ± SEM.

Acute oral toxicity study

The acute oral toxicity of *B. hispida*atthe limited dose of 2000 mg/kg revealed that normal weight gain in the treated animals. There were also no toxic symptoms, mortality, observational, behavioral and somatomotor changes observed after single dosing of BHFE. These results indicate that the BHFE was found to safer for acute use at the tested dose level of 2000 mg/kg. Hence the lethal dose of 50% (LD50) of *B. hispida* fruitextractis greater than 2000 mg/kg.

Effect of *B. hispida* fruit extract on Body Weight Changes in the Letrozole Induced PCOS Rats

Results in Table 2 showed the changes in body weight gain. The weight gain was significantly ($P<0.05$) higher after 21 days of letrozole treatment. On day 49 of the study after 28 days of drug treatment, PCOS control showed a significantly ($P<0.001$) weight gain as compared to the normal control. The treatment of Metformin and BHFE produce significant ($P<0.05$) less body weight gain than the PCOS control rats

Table 2: Effect of *B. hispida* fruit extract on Changes in Bodyweight Gain in the Letrozole Induced PCOS Rats

Treatment	Changes in bodyweight gain	
	Day 21	Day 49
Group-I (Vehicle Control)	19.25 ± 6.50	47.75 ± 5.75
Group-II (PCOS Control)	38.75 ± 7.21 [a]	88.75 ± 6.20[c]
Group-III (Metformin-20 mg/kg)	30.19 ± 4.20 [a]	58.75 ± 5.44[d]
Group-IV (BHFE-200 mg/kg)	34.50 ± 5.78 [a]	59.21 ± 2.00 [d]
Group-V (BHFE-400 mg/kg)	35.70 ± 2.17 [a]	51.79 ± 6.34 [d]

Values are expressed as mean ± SEM, N=6, Statistical significance represented as[a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ Vs Group I; [d] $P<0.05$;[e]$P<0.01$; [f]$P<0.001$ Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Effect of *B. hispida* fruit extract on the Feed Intake in the Letrozole-induced PCOS Rats

There was no significant difference in the feed intake and water intake observed in the treatment groups when compared to the normal control and PCOS control. The feed intake results were represented in Table 3. The water intake results were represented in Table 4.

Table 3: Effect of *B. hispida* fruit extract on the Feed Intake in the Letrozole-induced PCOS Rats

Treatment	Feed intake (g/day/rat)						
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 50
Group-I (Vehicle Control)	12.36 ± 0.70	12.42 ± 0.65	13.94 ± 0.79	13.77 ± 0.55	12.33 ± 0.17	12.14 ± 0.65	13.52 ± 0.43
Group-II (PCOS Control)	12.21 ± 0.47	12.60 ± 3.65	11.61 ± 0.71	13.36 ± 0.58	13.44 ± 0.58	12.87 ± 1.24	13.44 ±0.32
Group-III (Metformin-20 mg/kg)	12.40 ± 0.63	11.84 ± 1.27	12.14 ± 0.65	13.77 ± 0.55	12.34 ± 0.84	13.34 ± 0.60	12.89 ± 0.20
Group-IV (BHFE-200 mg/kg)	12.39 ± 0.62	12.61 ± 0.45	13.75 ± 1.77	13.21 ± 0.76	13.14 ± 0.45	12.50 ± 0.61	12.98 ± 0.75
Group-V (BHFE-400 mg/kg)	12.40 ± 0.50	11.43 ± 0.51	12.14 ± 0.45	13.21 ± 0.54	12.90 ± 0.72	12.50 ±0.55	12.90 ± 0.73

Values are expressed as mean ± SEM, N=6, Statistical significance represented as $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$ Vs Group I; $^{d}P<0.05$;$^{e}P<0.01$; $^{f}P<0.001$ Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Table 4: Effect of *B. hispida* fruit extract on the Water Intake in the Letrozole-induced PCOS Rats

Treatment	Water Intake (ml/day/rat)						
	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 50
Group-I (Vehicle Control)	13.86 ± 0.39	13.93 ± 0.51	14.29 ± 1.72	14.79 ± 1.98	14.64 ± 1.83	15.29 ± 2.17	14.07 ± 0.72
Group-II (PCOS Control)	14.86 ± 1.30	13.57 ± 1.23	12.57 ± 1.66	13.57 ± 0.37	12.79 ± 0.92	12.50 ± 1.99	14.86 ± 1.37
Group-III (Metformin-20	13.91 ± 0.56	14.79 ± 0.82	15.64 ± 2.90	12.43 ± 0.30	13.07 ± 1.30	13.79 ± 0.83	13.42 ± 2.07

mg/kg)							
Group-IV (BHFE-200 mg/kg)	13.64 ± 2.50	12.14 ± 0.59	13.39 ± 1.87	12.93 ± 0.48	13.28 ± 0.61	17.09 ± 2.90	15.79 ± 1.86
Group-V (BHFE-400 mg/kg)	14.60 ± 0.78	13.43 ± 0.69	12.36 ± 0.64	10.50 ± 1.54	13.64 ± 0.84	15.50 ± 1.64	14.71 ± 1.41

Values are expressed as mean ± SEM, N=6, Statistical significance represented as $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$ Vs Group I; $^{d}P<0.05$;$^{e}P<0.01$; $^{f}P<0.001$ Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests.

Effect of *B. hispida* fruit extract on the Estrus cycle in the Letrozole-induced PCOS Rats

The results in Table 5 represent the number of estrus cycles during the total 5 weeks of the study period. The number of estrus cycles was significantly ($P<0.001$) less in the PCOS control as compared to the normal control. It indicates the development of PCOS in the letrozole-treated rats. The metformin treatment and BHFE treatments showed significant improvement in the number of estrus cycles as compared to the PCOS control. In comparison between the treatments the *B.hispida* fruit extract treatment showed dose dependent improvement in the menstrual irregularity.

Table 5: Effect of *B. hispida* fruit extract on the Number of Estrus Cycles in the Letrozole-induced PCOS Rats

Treatment	Number of Complete Estrus Cycle in 5 Weeks
Group-I (Vehicle Control)	6.48 ± 0.43
Group-II (PCOS Control)	1.20 ± 0.21^{c}
Group-III (Metformin-20 mg/kg)	3.27 ± 0.31^{cf}
Group-IV (BHFE-200 mg/kg)	2.77 ± 0.21^{ce}
Group-V (BHFE-400 mg/kg)	4.07 ± 0.33^{cf}

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05; [b]P<0.01; [c]P<0.001 Vs Group I;[d] P<0.05;[e]P<0.01; [f]P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Oral Glucose Tolerance Test (OGTT)

The 21 days after letrozole treatment, the fasting blood glucose level was significantly higher in the letrozole-treated rats as compared to the normal control. After the oral glucose challenge, the blood glucose level in the letrozole-treated rats wassignificantly (P<0.001) higher when compared to the normal control. It indicates the development of insulin resistance in the letrozole-induced PCOS condition (Table 6).

The 28 days of post drug treatments(on day 50 of the study) the fasting blood glucose level was significantly (P<0.001) higher in the PCOS control when compared to the normal control. After the oral glucose challenge, the blood glucose level was significantly (P<0.001) higher in the PCOS control as compared to the normal control. The treatment of metformin and BHFE (200 mg/kg and 400 mg/kg) showed a significant (P<0.001) reduction in the blood glucose level after the oral glucose challenge when compared to the PCOS control. The results are represented in Table 7.

Table 6: Oral Glucose Tolerance Test (OGTT) in the Letrozole-induced PCOS Rats on Day 21

Treatment	Blood Glucose Level (mg/dl)			
	Baseline	After Oral glucose Challenge		
		30 mins	60 mins	120 mins
Group-I (Vehicle Control)	80.72 ± 6.84	156.00 ± 7.00	123.67 ± 5.04	85.38 ± 4.03
Group-II (PCOS Control)	102.01 ± 4.10[b]	189.20 ± 3.60[b]	157.33 ± 7.13[a]	116.33 ± 7.50[c]
Group-III (Metformin-20 mg/kg)	103.40 ± 6.66[b]	187.50 ± 4.98[a]	158.73 ± 8.45[a]	115.30 ± 9.80[c]
Group-IV (BHFE-200 mg/kg)	103.00 ± 2.65[b]	189.11 ± 7.80[b]	156.42 ± 5.52[a]	112.25 ± 6.62[c]
Group-V (BHFE-400 mg/kg)	104.67 ± 3.71[b]	186.60 ± 2.96[b]	158.29 ±9.53[a]	115.21 ± 5.13[c]

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05; [b]P<0.01; [c]P<0.001 Vs Group I. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Table 7: Effect of *B. hispida* fruit extract on the OGTT in the Letrozole-induced PCOS Rats on Day 50

Treatment	Blood glucose level (mg/dl)			
	Baseline	After oral glucose challenge		
		30 mins	60mins	120 mins
Group-I (Vehicle Control)	82.67 ± 5.33	159.53 ± 7.62	107.33 ± 4.23	93.40 ± 4.27
Group-II (PCOS Control)	159.70 ± 6.08[c]	218.33 ± 12.47[c]	186.22 ± 5.69[c]	159.56 ± 5.52[c]
Group-III (Metformin-20 mg/kg)	108.23 ± 5.21[bf]	182.73 ± 4.06[d]	113.45 ± 8.00[f]	97.72 ± 8.08[f]
Group-IV (BHFE-200 mg/kg)	102.76 ± 2.96[bf]	163.23 ± 8.37[f]	117.78 ± 11.46[f]	99.87 ± 9.24[f]
Group-V (BHFE-400 mg/kg)	94.23 ± 6.27[f]	158.45 ± 4.91[f]	112.34 ± 7.83[f]	98.30 ± 6.74[f]

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05;[b]P<0.01; [c]P<0.001 Vs Group I.; [d] P<0.05;[e]P<0.01; [f]P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Effect of *B. hispida* fruit extract on Serum Lipid Profile in the Letrozole-induced PCOS Rats

There was significant (P<0.001) elevation of VLDL, LDL cholesterol, and triglycerides serum levels and a significant (P<0.001) reduction of HDL level in the PCOS control when compared to the normal rats. These altered lipid profiles were significantly (P<0.001) improved by the drug treatments as compared to the PCOS control. The treatment of BHFEshowed dose-dependent improvement in the lipid profile (Table 8).

Table 8: Effect of *B. hispida* fruit extract on Serum Lipid Profile in the Letrozole-induced PCOS Rats

Treatment	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)
Group-I (Vehicle Control)	62.58 ± 7.73	19.44 ± 3.81	45.65 ± 4.87	80.43 ± 4.23
Group-II (PCOS Control)	168.82 ± 4.69^{c}	62.30 ± 5.75^{c}	20.65 ± 5.12^{c}	147.32 ± 6.88^{c}
Group-III (Metformin-20 mg/kg)	72.70 ± 12.09^{f}	25.33 ± 4.02^{f}	38.24 ± 6.23^{e}	91.32 ± 11.50^{e}
Group-IV (BHFE-200 mg/kg)	63.45± 7.70^{f}	27.72 ± 3.74^{f}	32.40 ± 3.66^{e}	89.07 ± 5.90^{f}
Group-V (BHFE-400 mg/kg)	60.24 ± 5.89^{f}	20.18 ± 2.25^{f}	40.73 ± 5.32^{f}	81.22± 8.74^{f}

Values are expressed as mean ± SEM, N=6, Statistical significance represented as ^{a}P<0.05;^{b}P<0.01; ^{c}P<0.001 Vs Group I.; ^{d} P<0.05;^{e}P<0.01; ^{f}P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Effect of *B. hispida* fruit extract on Serum Insulin and SHBG Level in the Letrozole-induced PCOS Rats

The serum fasting insulin level was significantly (P<0.001) higher and sex hormone-binding globulin (SHBG) levels were significantly (P<0.001) lower in the PCOS control when compared to the normal control rats. The treatment of metformin and *B. hispida* fruit extract (200 mg/kg and 400 mg/kg) showed a significant (P<0.001) reversal effect on the serum insulin and SHBG levels as compared to the PCOS control (Table 9).

Table 9: Effect of *B. hispida* fruit extract on Serum Insulin and SHBG Level in the Letrozole-induced PCOS Rats

Treatment	Fasting Insulin (mIU/l)	SHBG (nmol/l)
Group-I (Vehicle Control)	21.17 ± 3.29	68.64 ± 4.58
Group-II (PCOS Control)	42.56 ± 3.52^{c}	38.14 ± 3.53^{c}
Group-III (Metformin-20 mg/kg)	20.65 ± 2.33^{f}	63.75 ± 1.43^{f}
Group-IV	25.32 ± 2.32^{f}	58.36 ± 2.38^{f}

(BHFE-200 mg/kg)		
Group-V (BHFE-400 mg/kg)	22.45 ± 5.50[f]	59.42 ± 5.15[f]

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05;[b]P<0.01; [c]P<0.001 Vs Group I.; [d] P<0.05;[e]P<0.01; [f]P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Effect of *B. hispida* fruit extract on Serum Sex Hormones Level and Free Androgen Index (FAI) in the Letrozole-induced PCOS Rats

The PCOS control group showed a significant (P<0.001) decrease in the serum estradiol, progesterone, and FSH level, and also a significant (P<0.001) increase in total testosterone and LH levels than the normal control. These altered serum sex hormones level of testosterone, estradiol, LH, and FSH was high significantly (P<0.001) reversed by the metformin and BHFE 200mg/kg and 400 mg/kg treatment, and moderate significant (P<0.01) reversal effect on progesterone when compared to the PCOS control. Also the free androgen index (FAI) was significantly (P<0.001) increased in PCOS control when compared to the normal control. It confirms hyperandrogenism in PCOS conditions. All the treatments significantly (P<0.001) reduces the free androgenic index than the PCOS control. (Table 10-11).

Table 10: Effect of *B. hispida* fruit extract on Serum Sex Hormones Level in the Letrozole-induced PCOS Rats

Treatment	Estradiol (pg/ml)	Progesterone (ng/ml)	LH (ng/ml)	FSH (ng/ml)
Group-I (Vehicle Control)	38.21 ± 6.32	30.21 ± 3.61	6.80 ± 3.04	30.12 ± 2.49
Group-II (PCOS Control)	10.45 ± 2.91[c]	11.76 ± 1.92[c]	18.62 ± 1.78[c]	13.46 ± 3.60[c]
Group-III (Metformin-20 mg/kg)	30.64 ± 3.06[f]	29.45 ± 2.81[e]	7.24 ± 3.43[f]	22.86 ± 5.07[e]
Group-IV (BHFE-200 mg/kg)	29.08 ± 5.52[f]	28.64 ± 3.83[e]	10.20 ± 3.94[af]	24.25 ± 3.09[f]
Group-V	31.23 ± 1.18[f]	29.87 ± 2.35[e]	7.30 ± 5.66[f]	26.53 ± 5.62[f]

(BHFE-400 mg/kg)				

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05;[b]P<0.01; [c]P<0.001 Vs Group I.; [d] P<0.05;[e]P<0.01; [f]P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Table 11: Effect of *B. hispida* fruit extract on Total testosterone and Free Androgen Index (FAI) in the Letrozole-induced PCOS Rats

Treatment	Total Testosterone (ng/dl)	FAI
Group-I (Vehicle Control)	26.43 ± 2.00	38.51 ± 3.08
Group-II (PCOS Control)	91.34 ± 4.56[c]	239.47 ± 3.82[c]
Group-III (Metformin-20 mg/kg)	30.27 ± 6.34[f]	47.48 ± 4.17[f]
Group-IV (BHFE-200 mg/kg)	35.11 ± 7.33[f]	60.16 ± 5.49[f]
Group-V (BHFE-400 mg/kg)	29.37 ± 3.50[f]	49.43 ± 6.83[f]

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05;[b]P<0.01; [c]P<0.001 Vs Group I.; [d] P<0.05;[e]P<0.01; [f]P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Effect of *B. hispida* fruit extract on Reproductive Organs Weight in the Letrozole-induced PCOS Rats

A significant (P<0.001) increase in ovarian weight was observed in PCOS control as compared to the normal control. The treatment of metformin and BHFE (200 mg/kg and 400mg/kg) significantly (P<0.001) reduces the ovary weight in PCOS rats than the PCOS rats. There was no significant change in uterus weight was observed in the study groups (Table 12).

Table 12: Effect of *B. hispida* fruit extract on Reproductive Organs Weight in the Letrozole-induced PCOS Rats

Treatment	Relative Organ Weight mg/100g b. w.	
	Ovary	Uterus
Group-I (Vehicle Control)	92.34 ± 9.73	158.35 ± 11.04
Group-II (PCOS Control)	146.58 ± 5.43[c]	113.28 ± 8.95[b]
Group-III (Metformin-20 mg/kg)	116.42 ± 6.12[cf]	132.65 ± 8.00
Group-IV (BHFE-200 mg/kg)	115.23 ± 3.41[cf]	131.42 ± 10.31
Group-V (BHFE-400 mg/kg)	98.30 ± 3.92[f]	137.42 ± 9.32

Values are expressed as mean ± SEM, N=6, Statistical significance represented as [a]P<0.05;[b]P<0.01; [c]P<0.001 Vs Group I.; [d] P<0.05;[e]P<0.01; [f]P<0.001 Vs Group II. Data were analyzed by one-way ANOVA followed by post hoc Dunnett's multiple comparison tests

Effect of *B. hispida* fruit extract on Ovarian Histology in the Letrozole-induced PCOS Rats

The normal control rats showed the normal histological structure of the ovary like the presence of various stages of follicles including primary, growing antral follicles, mature follicles, and corpus luteum (Figure 1A). In comparison to normal control, letrozole-induced PCOS rats showed altered histoarchitecture of the ovary such as the absence of corpus luteum and increased cystic follicles and undeveloped follicles(Figure 1B). The metformin treatment illustratedthe signs of improvement as a decreased number of follicular cysts, numerous different stages of follicles, few atretic follicles, and the existence of corpus luteum (Figure 1C). The treatment of BHFE 200 mg/kg and 400 mg/kg showed better improvement in the histoarchitecture of the ovaries of PCOS rats i.e. increased number of developing follicles and presence of the corpus luteum (Figure 1 (D & E).

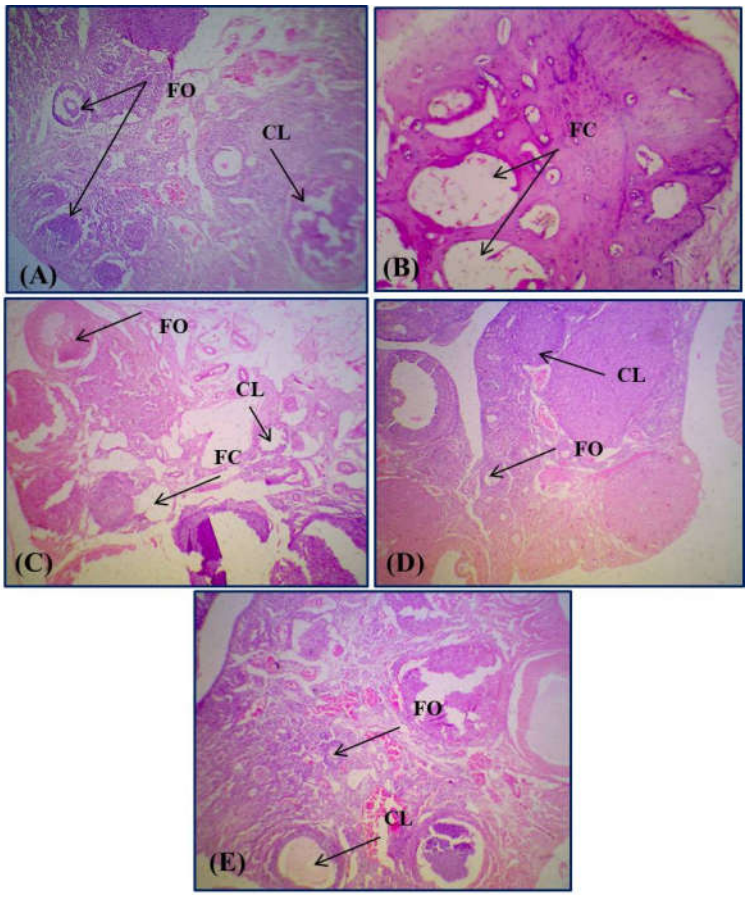

Figure 1: Histopathological Photo Microscopic Images of Ovary Sections (40x). (A) Normal control, (B) PCOS control, (C) Metformin 20 mg/kg, (D) BHFE-200 mg/kg, (E)BHFE-200 mg/kg

Discussion

Polycystic ovary syndrome (PCOS) is a complex endocrine-metabolic disorder characterized by ovarian dysfunction, hyperandrogenism, and metabolic derangements such as insulin resistance and dyslipidemia [26, 27]. Conventional treatments, including metformin, clomiphene, and letrozole, offer symptomatic relief but are often limited by side effects and lack of holistic efficacy [28]. In this context, herbal medicines have gained attention for their multi-targeted therapeutic actions. *Benincasa hispida* (ash gourd), a member of the Cucurbitaceae family, is traditionally recognized in Ayurveda and Traditional Chinese Medicine for its cooling, diuretic, and restorative properties [29]. Recent pharmacological studies have highlighted its antioxidant, anti-inflammatory, antidiabetic, and antihyperlipidemic activities, making it a promising candidate for PCOS management. Hence, this study is attempted to evaluate *B. hispida* fruit as an effective alternative medicine for PCOS.

The primary phytochemical analysis of this study showed the presence of alkaloids, carbohydrates, flavonoids, steroids, proteins, tannins, phenols, terpens & steroiols. The quantitative estimation of B.hispida fruit ethanolic extract showed the rich content of total steroids 52.37 mg/g, followed by total flavonoids 40.21 mg/g, total alkaloid content 36.97 mg/g, and total phenolic content 23.0 mg/g.

The safety of the *B.hispida* fruit extract was evaluated by acute oral toxicity study as per OECD guideline 423. The BSFE did not show any toxic symptoms or mortality at the limit dose of 2000 mg/kg *p.o.,* which showed that the BSFE is safe to use up to 2000 mg/kg.

In this study, PCOS in rats was developed by the administration of letrozole 1 mg/kg p. o. for 21 consecutive days. Letrozole-induced PCOS model is one of the most commonly used animal models, which mimics most of the clinical features of PCOS women. The clinical manifestation of PCOS includes hyperandrogenism, anovulation, and follicular cysts with metabolic complications like insulin insensitivity, hyperinsulinemia, dyslipidemia, and cardiovascular diseases [30]. The results of this study are also in line with the previous study reports.

Obesity and an increase in body weight augment the clinical symptoms of menstrual irregularity and infertility [31]. Similarly, the results of this study also represented that the letrozole-treated PCOS rats showed significant weight gain, which was caused by the deposition of abdominal fat. The increased concentration of testosterone in the peripheral blood vessels can be the reason for abdominal fat deposition [32]. The treatment of *B. hispida* fruit extract dose dependently reduce the body weight gain in PCOS rats, it might be due to its anti-androgenic property by reducing the serum testosterone concentration reduces the abdominal fat deposition.

The menstrual irregularity and chronic anovulation is the primary clinical feature of PCOS, 80% of PCOS women are associated with these symptoms [32]. The normal duration of the estrus cycle in rats is 4-5 days of four phases in the sequential order of proestrus, estrus, metestrus, and diestrus [21]. The induction of PCOS was confirmed by measuring the estrus cycle through daily vaginal smear examination. Letrozole-induced PCOS rats showed an irregular estrus cycle indicated by the arrested estrus cycle in the diestrus stage itself, which was represented by the presence of predominant leucocytes in the vaginal smear. It confirms the development of PCOS in letrozole-treated rats. Also, the number of estrus cycles in the PCOS control was significantly less when compared to the normal control estrus cycle. The estrus irregularity in the letrozole-induced PCOS rats is due to the alteration in the sex hormones and gonadotrophin concentrations, which controls the ovarian function and

follicular maturations [33]. The increased concentration of circulatory androgen and intraovarian androgens affects the follicular growth and maturation leading to the anovulatory irregular estrus cycle in the letrozole-induced PCOS rats [32]. The treatments of metformin and BHFE significantly regularize the estrus irregularity induced by the letrozole. In comparison between the treatments, the number of estrus cycles was higher in the BHFE 200 mg/kg and 400 mg/kg treated groups when compared to metformin treatment. It indicates that the B. hispida fruit extract has the potential to regularize the menstrual irregularity in PCOS women, which might be due to its ability to reduce circulatory androgen and ovarian androgen levels.

Insulin resistance (IR) is one of the key pathogenic factors of PCOS [34]; 60-70% of PCOS women are associated with insulin resistance. Insulin resistance with compensatory hyperinsulinemia plays a significant role in ovarian function; IR stimulates ovarian theca cells to produce androgen leading to the arrest of follicular maturation and anovulation in PCOS [35]. The result of this study indicates the development of insulin resistance in letrozole PCOS rats by showing increased fasting glucose and insulin levels with impaired glucose clearance in oral glucose tolerance tests following an oral glucose challenge. These results are in line with previous study reports. All the treatments significantly improve the insulin resistance in letrozole-induced PCOS rats by reducing fasting blood glucose and serum insulin levels, and promoting glucose clearance rate in the OGGT model. In comparison between the treatment groups, metformin and BHFE 400mg/kg treatment show superior results on insulin resistance in PCOS rats. Hence the results of this study indicate the treatment of BHFE at 400 mg/kg showed a superior effect on insulin resistance by improving hyperinsulinemia-mediated insulin resistance by increasing glucose uptake and promoting glucose clearance in PCOS rats.

Polycystic ovarian syndrome is commonly associated with dyslipidemia like increased serum low-density lipoprotein, very low-density lipoprotein cholesterol, and triglyceride levels, and also decreased high-density lipoprotein cholesterol levels, it has been estimated that 70 % of PCOS women have at least any one of this lipid abnormalities [36]. The hyperinsulinemia and hyperandrogenism in PCOS conditions stimulate lipolysis in adipocytes resulting in increased free fatty acid leading to dyslipidemia. Dyslipidemia is the most prevalent metabolic abnormality in PCOS patients [37, 38]. The results of this study also confirmed that letrozole-induced PCOS rats showed dyslipidemia like increased serum concentration of LDL, VLDL cholesterol, and triglycerides level and decreased HDL cholesterol levels. It might be due to the letrozole-induced hyperinsulinemia and hyperandrogenism-produced

dyslipidemia. All the treatments significantly reverse the lipid abnormality in the letrozole-induced PCOS rats. In comparison between the treatments, BHFE treatment showed dose dependent hypolipidemic effect. BHEF 400 mg/kg treatment possesses a superior hypolipidemic effect in all lipoprotein abnormalities. It might be due to its potent hypoglycemic and anti-androgenic properties.

Sex hormone abnormality is the major diagnostic feature of PCOS like elevated level of serum total testosterone and luteinizing hormone (LH) and decreased estradiol, follicle-stimulating hormone (FSH), and progesterone levels. Hyperandrogenism is the hallmark of PCOS [39]. Administration of letrozole blocks the ovarian conversion of androgen to estrogen leading to hyperandrogenism in the ovary, by decreasing the sex hormone binding globulin (SHBG) increase the peripheral circulatory androgen level in the letrozole-induced PCOS rats. Similar findings were observed in this study. The free androgenic index is the marker of hyperandrogenism in PCOS patients [23]. The results of this study indicate the development of hyperandrogenism in PCOS rats by showing the increased concentration of serum total testosterone and free androgenic index. The hyperandrogenism in PCOS rats feedbacks to the pituitary gland results in abnormal fluctuation increase in LH and decrease in FSH secretion. Also, a significant reduction in estradiol and progesterone was observed in the letrozole-induced PCOS rats. All the treatments significantly normalize the hormonal abnormality induced by the administration of PCOS. The treatment of BHFE showed dose dependent effect on normalizing the sex hormonal abnormality in PCOS rats. This impact could be attributed to its ability to help PCOS patients normalize their sex hormone imbalance.

The ovary weight was significantly increased in the POCS control rats; it might be due to the letrozole-induced hyperandrogenism, which may lead to the development of ovarian cysts with hyperplasia of theca cells and thickened ovarian capsules [40]. All the treatments significantly reduced the letrozole-induced ovarian weight. The treatment of BHFE on reduction in ovary weight might be due to its anti-androgenic property. It indicates that the treatment of BHFE by reducing ovarian hyperandrogenism alters ovarian morphology, and reduces ovarian cysts in letrozole-induced PCOS rats.

The ovarian histopathological of this study confirmed the alteration of ovarian function in PCOS rats. Whereas the ovaries of PCOS rats showed an increased number of follicular cysts and very few/or absence of corpus luteum, and the presence of fewer antral follicles with hyperplasia of theca cells and thickened ovarian capsules. Additionally, the cystic follicle sizes were larger than other follicles, which are correlated to the rise in intraovarian androgen

levels. The treatment of metformin showed different phases of follicles with the presence of corpus luteum indicating the recovery of ovary morphology in PCOS rats, but the presence of atretic follicles indicates an anovulatory cycle. The treatment of BHFE showed normal histology of the ovary with different stages of developing follicles, and an increase in the number of corpus luteum. The presence of corpus luteum indicates the occurrence of ovulation and the different stages of follicles indicate the regular estrus cycle. The results of this study confirm that the treatment of *B. hispida* fruit extract by reducing follicular cysts promotes follicular maturation and ovulation through improving insulin sensitivity and reversal of androgen-mediated alteration of ovarian morphology of PCOS rats.

This study concludes that *Benincasa hispida* fruit extract exhibits significant protective and restorative effects in letrozole-induced PCOS. By addressing both reproductive and metabolic abnormalities, BHFE offers a comprehensive therapeutic approach superior to symptom-specific pharmacological agents. Its favorable safety profile further enhances its potential for long-term use. However, while the preclinical findings are promising, further mechanistic studies and well-designed clinical trials are warranted to validate its efficacy and establish standardized therapeutic applications in women with PCOS.

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