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Therapeutic potentials of *Iris ensata* in management of polycystic ovarian syndrome (PCOS) utilizing letrozole-induced rat model: biochemical and hormonal variations

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Abstract

A multifactorial endocrinopathy affecting women in their reproductive years, polycystic ovarian syndrome (PCOS) causes insulin resistance (IR), hyperandrogenism, cardiovascular issues, obesity, and menstruation difficulties. The present study was designed to investigate the therapeutic potentials of the methanolic extract of *Iris ensata* and standard drug metformin (MET) on letrozole (1 mg/kg) induced against estrus cyclicity, biochemical and hormonal aspects of polycystic ovary syndrome in Wistar rats. Twenty-four Wistar rats were divided into four groups including a plain control group, a negative control group (PCOS), a positive control group and a treatment group. The positive control group received MET (150 mg/kg body weight) for 21 days. PCOS was induced with letrozole (1 mg/kg body weight) for 21 days. The treatment group was treated with a methanolic extract of *Iris ensata* for three weeks after the induction of PCOS for 21 days. Body weight and estrous cycle phase were measured every day. Rats were sacrificed after 21 days of treatment. Blood samples were collected on 14, 21 and 42 days for the measurement of biochemical profile i.e. glucose, triglycerides, total cholesterol and hormone levels i.e. testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol. There was letrozole-induced PCOS characterized by irregular estrus cyclicity, elevated triglycerides, total cholesterol, luteinizing hormone (LH), testosterone and estradiol concentrations compared with the control. These reproductive, biochemical, and hormonal alterations were alleviated by *Iris ensata* extracts. For instance, *Iris ensata* restored the estrus cyclicity with a remarkable effect after 21 days of treatment. Moreover, *Iris ensata* significantly decreased ($p<0.001$) LH and testosterone levels, but increased ($p<0.01$) estradiol (methanolic extract; 125 mg/kg) concentration. *Iris*

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ensata alleviated reproductive, biochemical and hormonal alterations in PCOS rats and could be useful in the management/treatment of reproductive and metabolic disorders related to PCOS.

Keywords: Polycystic, Genetic disorders, Obesity, Cardiovascular complications, Letrozole, Estrous cycle, Vaginal smear

Introduction

Polycystic ovarian syndrome (PCOS) is a highly prevalent disorder that causes several aspects of a woman's physical health and has long-term consequences that occur well beyond sexual maturity (Azziz et al. 2004; Yildiz et al. 2012; Purunen et al. 2011). It is an endocrine issue that impacts approximately 6-10% of pregnant females (Bozdag et al 2016). Irregular periods, subclinical confirmation of excessive androgens and multiple cysts on ultrasound are mostly common clinical features (Rotterdam Consensus 2004). Females with PCOS have an elevated risk for heart disease and co-morbidities along with dyslipidemia, insulin resistance (IR) and diabetes (Zhao et al 2016, Gandevani et al 2016). Conversely, there has been a growing fact that PCOS in women is much more likely to develop symptoms of anxiety and depression (Dokras et al 2012; Barry et al 2011) and may lead to infertility due to anovulation (Adams et al 1986; McGovern 2007). Ovarian disorders affect 75-85% of PCOS patients, though menstruation does happen periodically in these women (Aziz et al. 2009). The condition of anovulation is not always associated with irregular menstrual cycles in PCOS women, for instance, found that 16% of 316 with normal menstrual cycles have anovulation along with elevated male sex hormone levels i.e. 27 to 34-day intervals (Noroozzadeh et al. 2017). As per the WHO, conventional and natural drugs are used by around 70% of the world's population (Zhong et al. 2003). About 80% of India's rural communities focus on herbal and natural medicines for everyone medical care (Agnihotri et al. 2013). In both males and females, the hypothalamic-pituitary-adrenal system has a vital contribution for developing and controlling the reproductive organs. One such axis function was shown to be greater in patients with PCOS (Legro et al. 2003). To date, numerous interventions for polycystic ovarian disease have been formulated, including dietary changes, treatment plans and surgery (Azziz et al. 2016). Taking drugs including clomiphene citrate, metformin, letrozole and tamoxifen has been considered as the most excellent treatment (Marx 2003). Hot flushes, arthritis, muscular discomfort, irritability, depression and bloating are some of the moderate to extreme health risks of such medications (Blaschke et al. 1999). As a result,

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there may be a significant necessity to investigate a more appropriate therapeutic method for this disease. Despite the adverse effects of these medications, finding and formulating dietary supplements would be critical. In the previous centuries, medicinal plants have received particular consideration. Today, thanks to several surveys done under the heading of herbal medicine; most plants have proven to have a significant impact already been reported to be important in alleviating PCOS in humans (Gharagozloo et al. 2010).

The *Iris* plant, which refers to the Iridaceae family, is distributed worldwide, with almost 300 creatures called for their floral and therapeutic purposes. The *Iris* species are beneficial in combating respiratory asthma, cancer, inflammation, liver and uterine disorders (Park and Pezzutto 2002). Numerous compounds were isolated from different *Iris* species based on comprehensive phytochemical studies, i.e. flavonoids, stilbene glycosides, quinones, isoflavonoids and triterpenoids (Purev et al. 2002). The flavonoids and isoflavonoids are biologically active secondary phytometabolites that are eaten at a dietary level by mammals (Rahman et al. 2002). *Iris ensata* extract is said to have anti-hyperglycemic and hypoglycemic properties in rodents (Ahmad et al. 2012). Due to its broad medicinal benefits, *Iris ensata* was preferred to see how it affects letrozole-induced PCOS in Wistar rat model.

Methodology

Maintenance of Animals

Female Wistar rats aged 6-8 weeks, weighing an average of 190 ± 10g, were procured from the IIIM animal house, Jammu. The animals were housed in standard polypropylene cages and maintained in air-conditioned animal houses (20-25^{0}C relative humidity 70-75%) in a 12-hour light-dark cycle. The animals were fed on a standard laboratory diet and water ad libitum. Following proper approval from the Institution Animal Ethics Committee (IAEC) with the ethical certificate number (FVSc/VCC-9/19/286/-87), all experimental protocols and procedures were carried out in compliance with institutional regulations and national criteria for animal experimentation.

Experiential Design

Letrozole Induced PCOS in Rat Model

The 24 adult female Wistar rats were used for the experimental studies. All animals were randomly divided into 4 groups.

Group 1: The animals of this group served as control and received normal saline water (0.9% NaCl Solution) as the vehicle, a balanced pellet diet and water ad libitum for 42 days.

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Group II: The animals in this group received letrozole (6mg/kg body weight) orally for 21 days for the induction of PCOS and then left untreated served as PCOS control (positive control) for 42 days.

Group III: The animals in this group received letrozole (1mg/kg body weight) orally for 21 days for the induction of PCOS and then treated with *Iris ensata* (IE) (125 mg/kg) for 21 days.

Group IV: The animals in this group received letrozole (1mg/kg body weight) orally for 21 days for the induction of PCOS and then treated with MET (150 mg/kg) only for 21 days.

Determination of estrous cycle (vaginal smear)

Before beginning treatment, all of the rats included in this study had to have regular estrous cycles, which were verified by looking at vaginal smears under a light microscope for two consecutive cycles (about eight to ten days). Estrous cyclicity was monitored by daily observations of vaginal smears of all females for 28 days. The procedure was conducted and interpreted as per the standard method (Marcondes et al., 2002).

Biochemical profiling:

Blood samples for biochemical parameters measurement were obtained after deep anesthesia from the abdominal aorta during the estrus phase on day 14, 21 and 42. Blood samples were centrifuged at 6000 rpm for 5 min for subsequent measurement of biochemical parameters. The levels of glucose, triglycerides and total cholesterol were measured using an auto-analyzer.

Hormonal Estimations:

The blood sample was collected through cardiac puncture and then incubated at 37˚C for 1 hr. The sample was then centrifuged at 1000×g for 20 minutes. Serum was separated and then used for the hormonal analysis at different intervals i.e. after 21 and 42 days of treatment in both controls as well as experimental groups and analyzed in the laboratory of Veterinary Biochemistry using Elisa Reader.

Results

Observation of cytology of vaginal smears

Vaginal smears were prepared to track the oestrus cycle in letrozole-induced PCOS rats and investigate the estrous cycle phase. Proestrus, estrus, metestrus, and diestrus (Fig. 1A–E) displayed a consistent 4-to 5-day estrous cycle for Group I, lasting up to 42 days. Using light microscopy, the proestrus period (Fig. 1A) was observed to contain oval, nucleated epithelial

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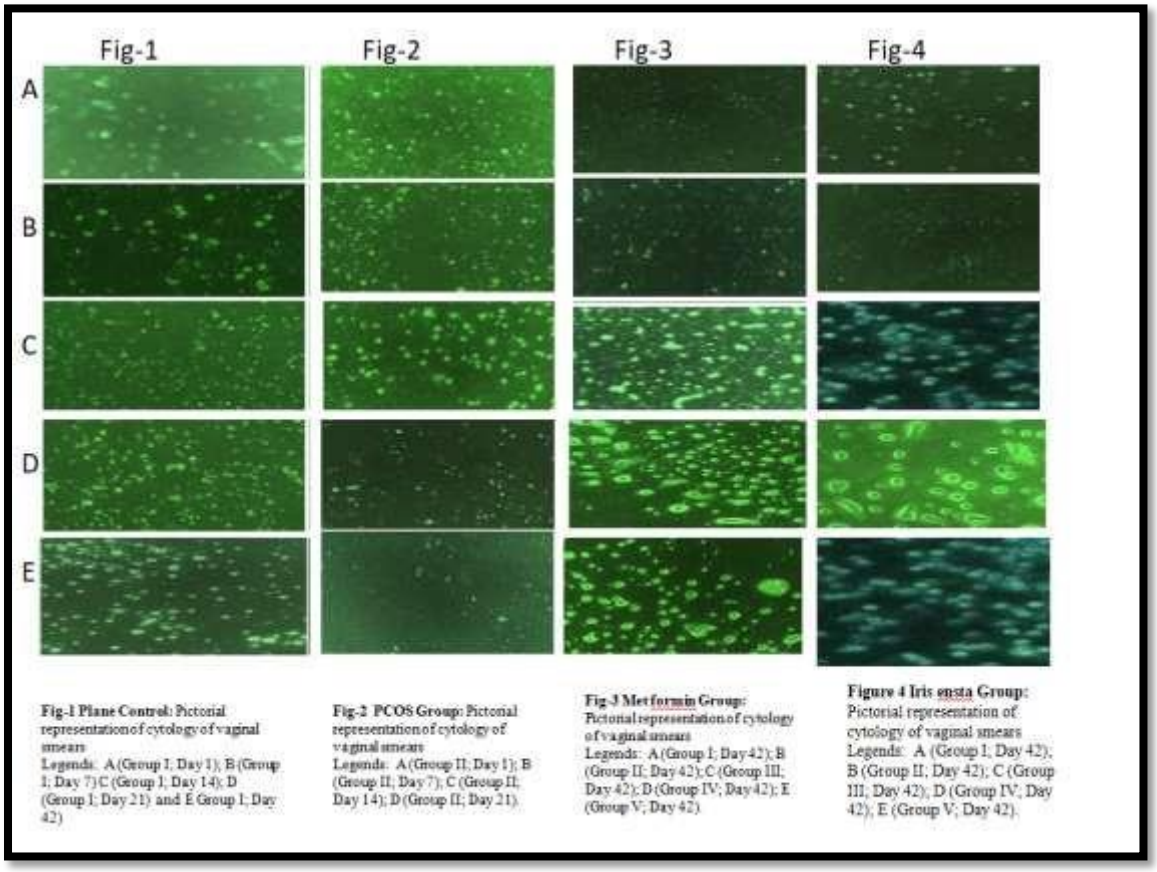
cells and a small number of keratinocytes; the estrus period (Fig. 1B) displayed irregularly shaped epithelial cells and a small number of nuclear epithelial cells; the diestrus period (Fig. 1C) displayed a large number of leukocytes and a few nuclear epithelial cells; the estrus period (Fig. 1D) displayed irregularly shaped keratinized epithelial cells and a small number of nuclear epithelial cells was observed during the diestrus period (Fig. 1ERats in group II received a letrozole solution and showed signs of extended diestrus. From the 12th to the 42nd day, a stained smear under light microscopy showed a significant number of leukocytes and a few nuclear epithelial cells, indicating that the PCOS rats in group II had continuously stayed in diestrus (Fig. 2A-E). Estrous cycles considerably enhanced in groups III and IV (metformin and *Iris ensta*), and during estrus on day 42, abnormally formed keratinized epithelial cells were seen (Figs. 3A-E; Fig. 4A-E).



Biochemical and Hormonal profiling

To understand the progression of PCOS through various parameters biochemical, hormonal, and genomic determinants and, henceforth, their effective targeting in the amelioration of PCOS, we evaluated the ameliorative effect of IE (*Iris ensata*) on biochemical, hormonal as

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well as genomics determinant in letrozole induced PCOS animal model. An aromatase inhibitor (letrozole) was used for the induction of polycystic ovary syndrome (PCOS). Polycystic ovary condition was developed in female Wistar rats by the oral administration of letrozole (1mg/kg/0.2ml/daily) for 21 days. Letrozole-induced PCOS female Wistar rats showed marked changes in body weight and estrous cycle. In addition to this, administration of letrozole also caused significant changes in the biochemical parameters including fasting glucose, cholesterol, triglycerides and circulating hormone levels i.e. testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol. *Iris ensata* (IE) and metformin (MET) treatments showed ameliorative effects against letrozole-induced symptoms. To find the significant differences, the numerical data were analyzed by using one-way ANOVA.

Effect of IE extract and MET on blood glucose in LET-induced PCOS in female Wistar rats after 30 days

In this study, we observed that glucose levels were significantly higher ($p<0.001$) in LET-treated rats as compared to the control group. However, in LET+IE and LET+MET-treated rats, a significant reduction ($p<0.001$) in blood glucose levels was observed as compared to the LET-treated group (Fig. 5).

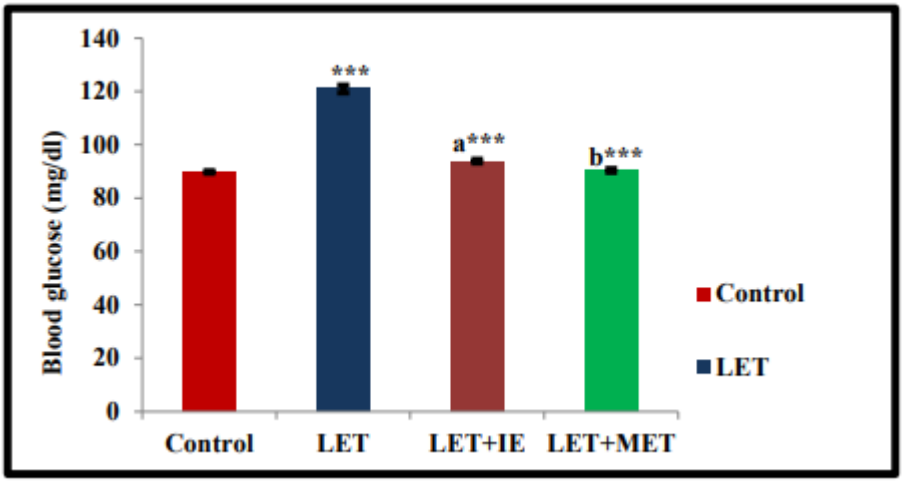

Fig 5: Blood glucose of control LET (letrozole), LET+IE (Letrozole + *Iris ensata*) and LET+MET (Letrozole + Metformin), treated female Wistar rats after 30 days

Effect of IE extract and MET on serum cholesterol levels in LET-induced PCOS in female Wistar rats after 30 days

The letrozole-induced PCOS rats showed significantly higher ($p<0.001$) cholesterol levels than the control group. However, these PCOS rats when treated with IE and MET for 30 days showed a significant drop ($p<0.001$) in serum cholesterol levels in comparison to the LET-

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treated group. Furthermore, the PCOS female Wistar rats when treated with LET+ IE had a much decrease in the serum cholesterol levels observed as compared to the LET+MET treated group (Fig.6).

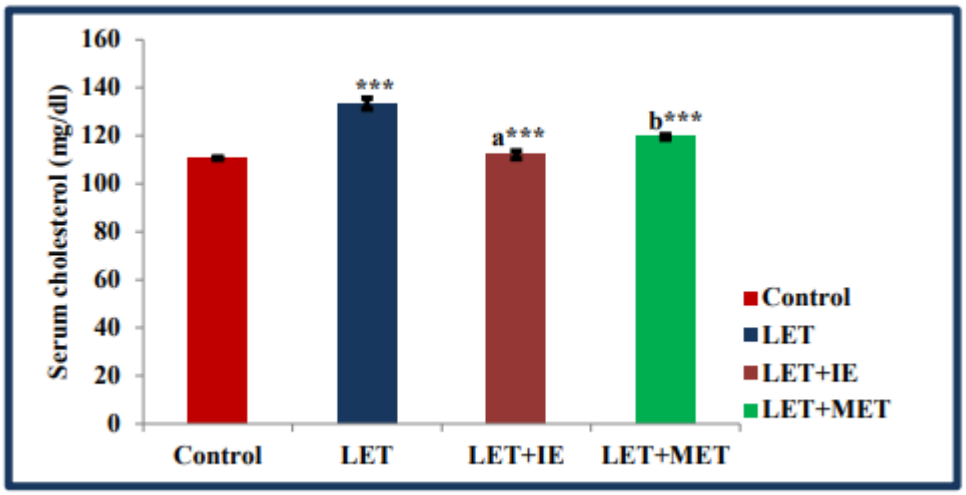

Fig 6: Serum cholesterol levels of control, LET, LET+IE and LET+MET treated female Wistar rats after 30 days

Effect of IE extract and MET on serum triglycerides (TG) levels in LET-induced PCOS in female Wistar rats after 30 days

In this study, we found that LET treatment led to a significant increase in serum TG of rats in comparison to the control. However, in the groups that were treated with LET+IE and LET+MET, a significant drop in the levels of TG was observed as compared to only LET treated group (Fig. 7).

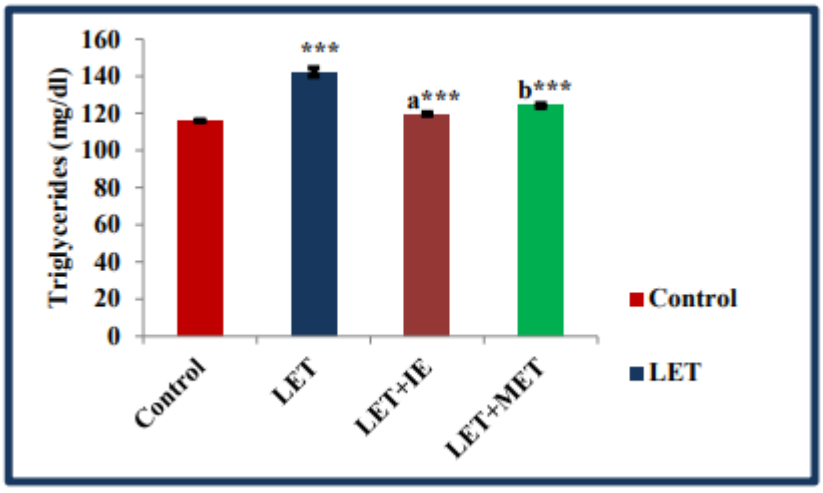

Fig 7: Serum triglyceride levels of control, LET, LET+IE and LET+MET treated female Wistar rats after 30 days

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Effect of IE extract and MET on serum testosterone (TET) levels in LET-induced PCOS in female Wistar rats after 30 days

The results confirmed that the LET treatment in Wistar rats led to an increase in TET levels as compared to the rats serving as control. However, in the groups that were treated with LET+IE and LET+MET, a significant drop in the levels of TET was observed as compared to the group that was treated only with LET (Fig. 8).

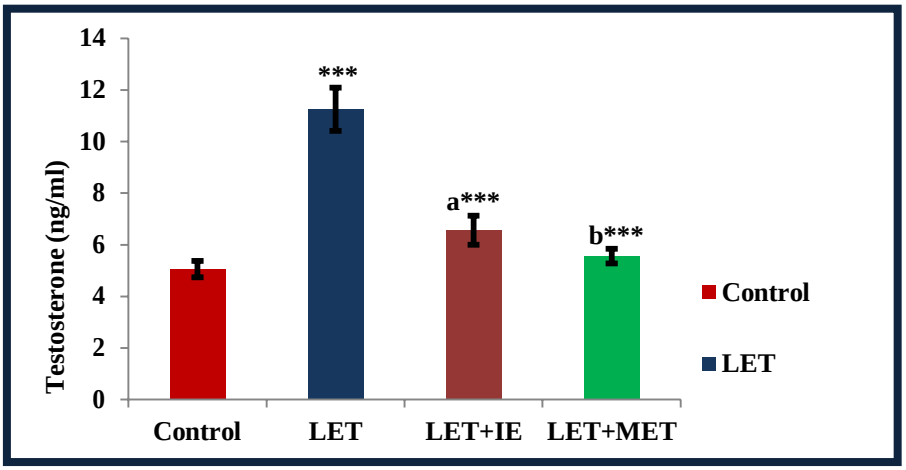

Fig 8: Serum testosterone levels of control, LET, LET+IE and LET+MET treated female Wistar rats after 30 days

Effect of IE extract and MET on serum Luteinizing hormone (LH) levels in LET-induced PCOS in female Wistar rats after 30 days

The results showed that LET treatment in Wistar rats led to an increase in serum LH levels as compared to the rats serving as control. However, the groups that were treated with LET+IE and LET +MET, showed a significant drop in the levels of LH as compared to the group which was treated only with LET (Fig. 9).

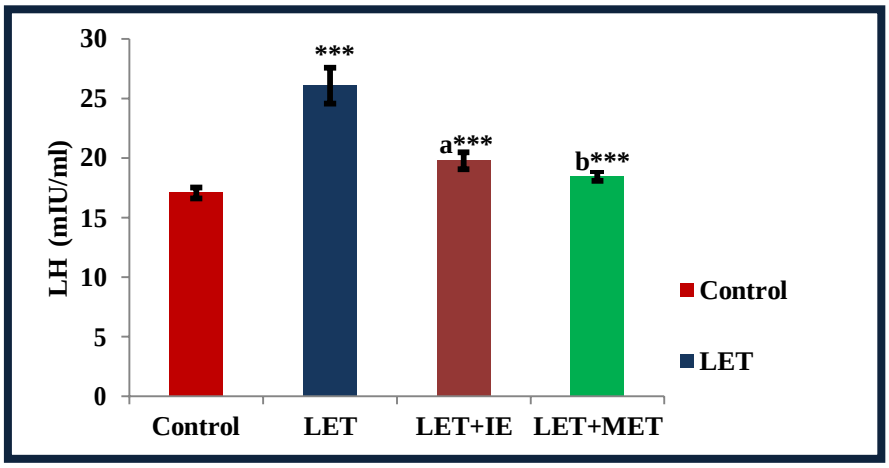

Fig 9: Serum testosterone levels of control, LET, LET+IE and LET+MET treated female Wistar rats after 30 days

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Effect of IE extract and MET on serum estradiol levels in LET-induced PCOS in female Wistar rats after 30 days

The results confirmed that LET treatment in Wistar rats led to adecline in serum estradiol levels as compared to the rats serving as control. However, the groups which were treated with LET+IE and LET +MET, showed a significant increase in the levels of estradiolas compared to the group that was treated only with LET (Fig. 10).

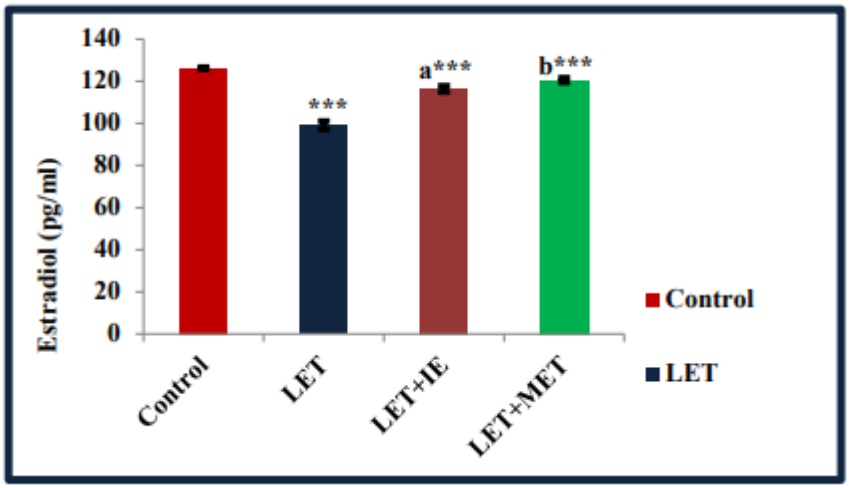

Fig 10: Serum estradiol levels of control, LET, LET+IE and LET+MET treated female Wistar rats after 30 days

Effect of IE extract and MET on serum follicle stimulating hormone (FSH) levels in LET-induced PCOS in female Wistar rats after 30 days

The results confirmed that LET treatment in Wistar rats led to adecline in serum FSH levels as compared to the rats serving as control. However, the groups which were treated with LET+IE and LET +MET, showed a significant increase in the levels of FSH as compared to the group that was treated only with LET (Fig. 11).

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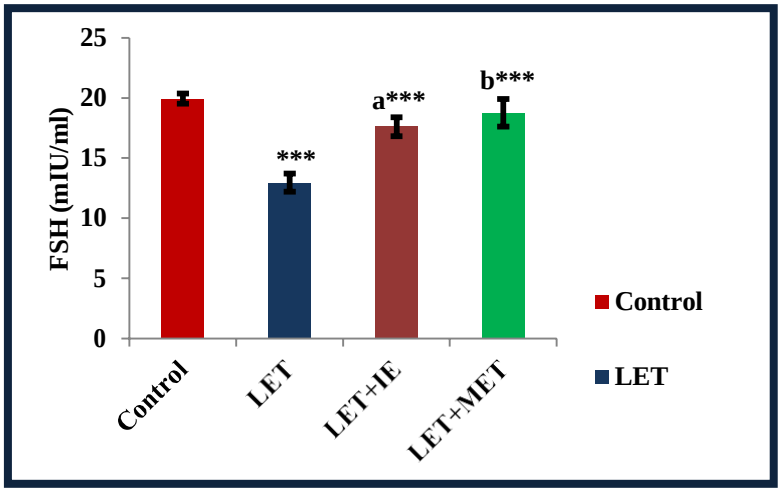

Fig. 11: Serum FSH levels of control, LET, LET+IE and LET+MET treated female Wistar rats after 30 days

Discussion

The present study unveiled the complex underlying mechanisms of polycystic ovarian syndrome (PCOS), a complicated disorder influenced by several biochemical, hormonal and genetic factors. The effectiveness of *Iris ensata* extract (IE) in relieving symptoms in PCOS rats establishes a promising basis for further investigation into their mechanisms of action and possible therapeutic use in the treatment of PCOS in humans. Significantly, the study emphasizes the need to simultaneously focus on several pathways to effectively treat the multifactorial aspects of PCOS. An in-depth analysis of the different stages of the estrous cycle using vaginal smears in rats with letrozole-induced PCOS provides a thorough understanding of the PCOS syndrome and the effects of prospective treatments. This study aimed to examine the therapeutic effects of *Iris ensata* extract (IE) and metformin (MET) on Polycystic ovary syndrome (PCOS) in female Wistar rats. The PCOS was developed in the animals by administering letrozole which acts as an aromatase inhibitor and can generate settings similar to polycystic ovary syndrome (PCOS), mimicking the symptoms observed in humans. These symptoms include disturbances in the estrous cycle, changes in body weight and variations in biochemical and hormonal profiles. Different animal groups were used to establish the potential use of *Iris ensata* extract. Group I, which served as the control, presented a consistent estrous cycle lasting 4-5 days, including the stages of proestrus, estrus, metestrus and diestrus. Light microscopy revealed detailed cellular features during each phase, highlighting the unique cycle alterations in vaginal cytology that are consistent with a healthy estrous cycle. In contrast, Group II rats that received letrozole treatment had an altered reproductive cycle, mostly in the diestrus phase from the 12^{th} to the 42^{nd} day. The

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extended diestrus phase, marked by a high number of leukocytes and a few nuclear epithelial cells, indicates a disruption in ovulation, which is a defining feature of PCOS. The letrozole-induced model accurately reproduces the anovulatory state and hormonal imbalance seen in clinical PCOS, highlighting the usefulness of this model for understanding the development of the disease. The administration of metformin (Group III) and *Iris ensata* (Group IV) as therapeutic interventions for letrozole-induced PCOS rats led to notable enhancements in the estrous cycle by the 42nd day. Both therapies seemed to reinstate the estrus phase, as shown by irregularly shaped keratinized epithelial cells, suggesting a return to ovulation and a possible improvement in PCOS symptoms. The administration of letrozole caused significant changes in fasting glucose, cholesterol, triglycerides, and hormone levels, including testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol. The observed abnormalities closely resemble the metabolic and endocrine disturbances seen in clinical patients with PCOS. Both the IE and MET therapies showed substantial therapeutic benefits on the symptoms generated by letrozole, affecting the levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol in a significant manner.

Ultimately, the study provides valuable insights into the therapeutic possibilities of IE and MET in PCOS, thereby facilitating the development of integrated treatment strategies for effectively treating this intricate disorder. Further research is necessary to understand the genetic basis of PCOS and how it interacts with biochemical and hormonal imbalances, which will ultimately help design specific and effective therapies.

Conclusion

The use of *Iris ensata* in the PCOS rat model improved hormonal balance, ovarian morphology, and metabolic parameters, similar to metformin, thus suggesting potential therapeutic benefits for PCOS and could be utilized as a promising natural alternative or complementary treatment.

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