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Evaluation of Protective Role of a Hesperidin on Letrozole induced Polycystic Ovarian Syndrome (PCOS) in Female Rats

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Abstract : Objective: To evaluate protective effect of Hesperidin (bioflavonoid, found in citrus fruits, such as lemon and orange) on Letrozole induced PCOS in female adult wistar rats. **Methods:** For inducing PCOS, letrozole (1 mg/kg) was administered p.o. for 21 consecutive days, followed by 15-days Hesperidin treatment at the doses 50 mg/kg, 100 mg/kg, and 200 mg/kg, p.o. using 0.5 percent w/v CMC as a vehicle. **Results:** Letrozole caused abnormalities in the ovarian weight, body weight, serum sex steroid profile such as FSH, LH, Testosterone levels and glucose levels. Most of the parameters were restored to normal levels, along with reduction of cysts in the ovaries due to Hesperidin.**Conclusion:** In female wistar rats, Hesperidin had a positive impact on PCOS caused by Letrozole. It had an effect similar to Clomiphene citrate, the most commonly used treatment for induction of ovulation in PCOS.
Keywords : Letrozole, PCOS, Hesperidin, Cysts, Clomiphene citrate.

1. Introduction:

Polycystic ovary (or ovarian) syndrome (PCOS) was first described by Leventhal and Stein in 1935[1]. Polycystic ovary syndrome (PCOS), a set of symptoms affecting women of childbearing age, is assumed to be epidemic in scope. Cysts form in the antral follicles of the ovaries as a result of an imbalance in the proportion of female sex hormones. PCOS is described as when multiple cysts develop in the ovarian follicles as a result of hormonal imbalance. In women, anovulation and the absence of a menstrual cycle inhibit fertilization and reproduction, making pregnancy difficult [2]. PCOS affects 6–10% of all women around the world[3].

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Women with PCOS has several risk factors which are associated with development of uterine cancer including fatness, hyperinsulinemia, diabetes mellitus and abnormal uterine bleeding[4]. The frequency of depression and anxiety is higher in women with PCOS than in general population. Mood disorders are capable of impairing quality of life, which are well-known in young adult women, concerned with fertility, and in women of all ages with respect to obesity, and clinical manifestations of excessive androgen[5].

In rats, various experimental models for PCOS have been developed, including the administration of Estradiol Valerate, DHEA, and an excess of prepubertal androgen[6].Despite the fact that these models cause PCOS, none of them are entirely persuasive and accurately represent the symptoms of human PCOS. Letrozole, a non-steroidal aromatase inhibitor, causes PCOS model that is similar to human PCOS in several respects. It prevents the conversion of testosterone and androstenedione to estradiol and estrone respectively and generates PCOS related syndrome by inducing hormonal imbalance, circulating hyperandrogenism and intra ovarian androgen excess resulting to formation of polycystic ovary[7].Because of the induced elevation of androgen levels in ovaries, follicular atresia and irregular follicular growth are seen[8].Letrozole induction has been linked to hyperglycemia, which can lead to insulin resistance and hyperlipidemia, which also lead to metabolic syndrome[9].Today, a variety of medications are used to treat PCOS and stimulate ovulation. However, extreme side effects such as arthritis, joint or muscle pain, and psychological symptoms have been identified as a result of these therapies. As a result, natural-source medicines with minimal to no side effects are becoming increasingly popular[10].

Hesperidin (5, 7, 30-trihydroxy-40-methoxy-flavanone7-rhamnoglucoside) is a bioflavonoid found in citrus fruits like orange and lemon, as well as plant-derived beverages like tea and olive oil, which have traditionally been used in herbal medicine[11]. Hesperidin has wide range of pharmacological properties, such as antioxidant[12], anti-inflammatory, antihyperlipidemic[13], properties.

Hesperidin modulates the different hallmarks of cancer notably cell death, inflammation and oxidative stress mechanism.Hesperidin is also one of the most essential bioflavonoid present in the Citrus genus (Rutaceae)[14].In DMI rats, hesperidin has a considerable reduction in total blood lipid profiles and plasma insulin concentrations, as well as anti-hyperglycemic and hypolipidemic activity[15]. Due to the stated activities, we hypothesised that Hesperidin could be useful in the treatment of Letrozole-induced PCOS in this investigation.

2. Materials and Methods:

2.1. Experimental animals

Virgin, cyclic, adult female Wistar Albino rats (150–200 g) were employed for the study. These animals were procured from registered breeder and acquainted in the quarantine area for one week.After acquaintation, animals were transferred to the standard laboratory conditions and allowed to acclimatise for two weeks in animal house of YSPM's Yashoda Technical Campus, Pharmacy, Wadhe, Satara. During the experimental study allanimals were caged in standardpolypropylene cages with maintained controlled environmentof 22 ±3℃ temperature, 50 ± 15% humidity and a 12 h light/dark cycle. They were fed with standard pellet diet and water provided*ad libitum*. The study was duly approved by Institutions AnimalEthics Committee(IAEC) for the use of animals and care of the animalswas carried out as per the guidelines of committee for the purposeof control and supervision of experiments on animals (CPCSEA).

2.2. Drugs and Reagents

Hesperidin was acquired from OZONE® INTERNATIONAL (INDIA). Letrozole was obtained from Sun Pharmaceutical Ind Limited.Clomiphene Citrate (Fertomid-50) tablets were procuredfrom Cipla, India. All other chemicals used were of analytical grade. Theserum follicle stimulating hormone (FSH), luteinizing hormone (LH) and Testosterone were measured via Enzyme Linked Immunosorbent Assay(ELISA) with the help of commercial kits (ELISA kit).Blood glucose level was measured using Accu-Check Active glucometer.

2.3. PCOS induction

All the experimental animals except control group were orally administered with Letrozole at a dose of 1 mg/kg dissolved in 0.5% Carboxy Methyl Cellulose (CMC) once daily for 21 days[7]. Control group received

vehicle only (0.5% CMC). Vaginal Smears were collected daily and evaluated microscopically using 0.1% Crystal violet stain (prepared by diluting 0.1 g of crystal violet in 100 ml of double distilled water followed by filtration through whatman filter paper) and examined under X10 objective lens of laboratory microscope to confirm the induction of PCOS.

2.4. Study design

Thirty-six female Albino Wistar rats were divided into six groups: group 1 (control group), group 2 (PCOS induced group), group 3 (standard group), groups 4, 5 and 6 (treatment groups). Following Letrozole administration, standard group was administered with Clomiphene Citrate at a dose of 1 mg/kgp.o.in 0.5% CMC and treatment groups 4, 5 and 6 were administered Hesperidinat the dose of 50 mg/kg, 100 mg/kg and 200 mg/kg p.o.respectively in 0.5% CMC for 15 days i.e., from day 22nd to 36th day. At the end of the treatment animals were fasted overnight and anaesthetized with diethyl ether. Blood was collected by puncturing retro-orbital sinusthen by centrifugation method serum was separated and used for estimation of hormones.

2.5. Biochemical estimations

2.5.1.Hormonal assay

The serum luteinizing hormone (LH), follicle stimulating hormone (FSH), and Testosterone were measured via Enzyme Linked Immunosorbent Assay(ELISA) with the help of commercial kits (ELISA kit).

2.6. Ovarian histomorphology

The excised ovaries were fixed in 10% Formalin. According to histological procedure, they were subjected to tissue processing by washing with water which was followed by dehydration through an ascending ethanol series, then cleared through xylene. Then paraffin embedding method was used. The blocks were sectioned at 5 µm thickness using microtome and were mounted on slides coated with poly-lysine. These blocks were stained with hematoxylin-eosin(HE), dehydrated, cleared and mounted on DPX mountantunder glass cover slips. The light microscope (100X) was used for observation of slides which was connected to a camera to capture images[16].

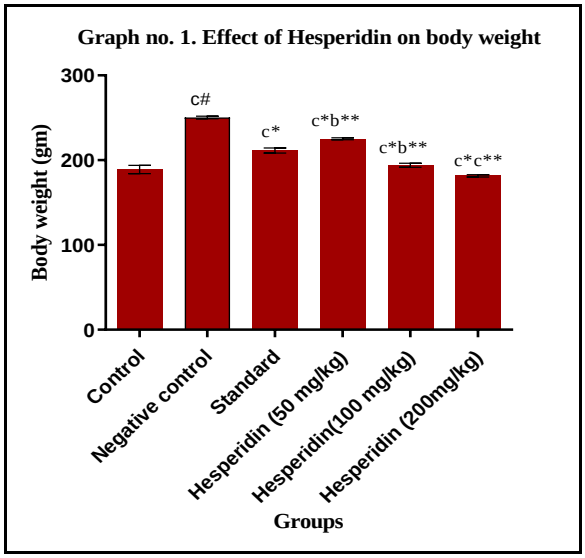
2.7. Statistical analysis

The statistical analysis was carried out with Graph pad prism 5.0 software. The data was statistically analyzed using one-way ANOVA method followed by Tukey's multiple comparison tests and p values ($p<0.05$, $p<0.01$, $p<0.001$)were considered to be statistically significant.

3. Results

3.1. Body weights

When body weight compared with control group, negative controlgroup showed significant elevation ($p<0.001$) in body weight. When standard and all treatment groups of hesperidin (50mg/ kg, 100mg/kg, 200mg/kg) compared to the negative group, the body weight decreased considerably ($p<0.001$).Low and Intermediate dose of hesperidin significantly ($p<0.01$) reduced the body weight as compared to standard group. High dose of hesperidin showed significantly ($p<0.001$) reduction in body weight as compared to standard group. (Graph no. 1)

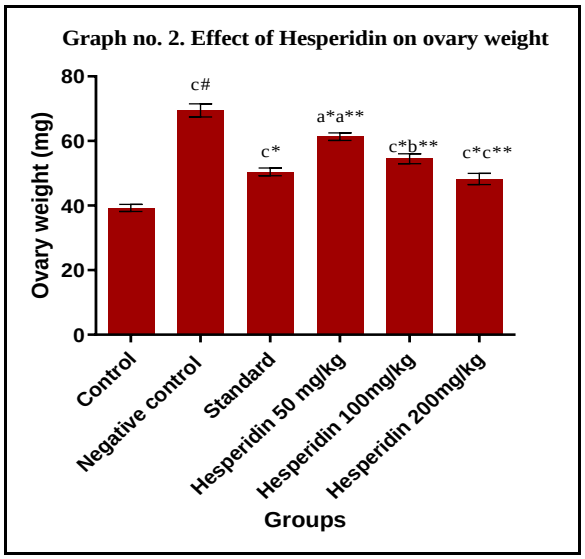


#= Data compared with control group
***= Data compared with negative control group**
****=Data compared with standard group**
a=p<0.05, b=p<0.01, c=p<0.001

3.2. Organ weights

3.2.1. Ovary weights

In terms of ovarian weights, there was significant increase in ovarian weight in negative group as compared with control group. After treatment with standard drug there was significant ($p<0.001$) decrease in ovarian weight as compared to negative group. When negative control groupcompared with intermediate and high dose of hesperidin, then it shows significantly ($p<0.001$)reduced ovarian weight. When low dose of hesperidin compared with standard and negative control group, then it indicated less significant decrease in ovary weight; whereas intermediate and high dose showed significantly ($p<0.01$ and $p<0.001$) decrease in weight of ovary respectively.(Graph no. 2)



#= Data compared with control group
***= Data compared with negative control group**
****=Data compared with standard group**
a=p<0.05, b=p<0.01, c=p<0.001

Table no. 1. Evaluation of Hesperidin on body weight and ovary weight of Letrozole induced PCOS in female rats

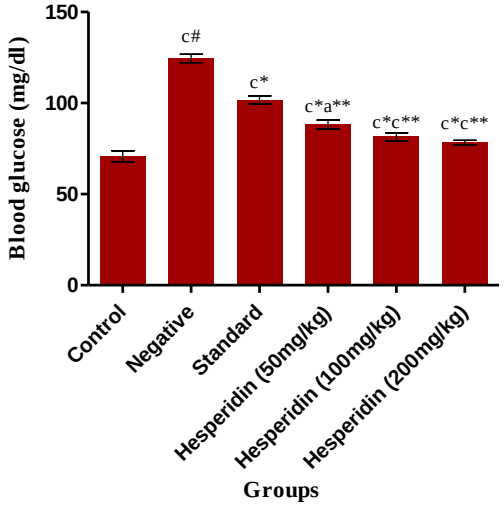
Group no.	Groups	Body weight(gm)	Ovary weight (mg)
1	Control	189.0±4.92	39.26±1.10
2	Negative	250.5±1.54$^{c\#}$	69.47±2.00$^{c\#}$
3	Standard	211.3±3.04^{c*}	50.41±1.20^{c*}
4	Hesperidin(50 mg/kg)	225.2±1.11^{c*b**}	61.33±1.15^{a*a**}
5	Hesperidin(100 mg/kg)	194±2.40^{c*b**}	52.49±1.53^{c*b**}
6	Hesperidin(200 mg/kg)	181.5±1.34^{c*c**}	48.22±1.74^{c*c**}

Control: CMC; Negative control: Letrozole; Standard: Clomiphene citrate; Low dose: Hesperidin 50mg/kg; Intermediate dose: Hesperidin 100mg/kg; High dose: Hesperidin 200 mg/kg

3.3. Fasting Blood Glucose(FBG) levels

Negative group exhibited significantly ($p<0.001$) elevated blood glucose level as compared to control group. When standard and all doses of hesperidin compared with negative group it showed significant ($p<0.001$) reduction of blood glucose level. Intermediate and high dose of hesperidin exhibited significantly($p<0.001$) decreasing level of blood sugar as compared with standard group. Low dose of hesperidin compared with standard then it indicated less significant decrease in ovary weight. (Graph no. 3)

Graph no. 3. Effect of Hesperidin on blood glucose level



#= Data compared with control group
***= Data compared with negative control group**
****=Data compared with standard group**
a=p<0.05, b=p<0.01, c=p<0.001

Table no. 2. Evaluation of Hesperidin on Fasting blood glucose(FBG) of Letrozole induced PCOS in female rats

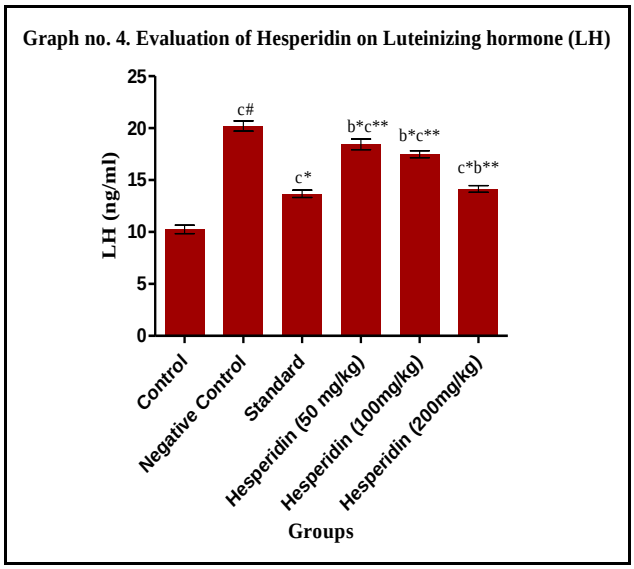
Group no.	Groups	Blood glucose (mg/dl)
1	Control	70.83±2.97
2	Negative	124.5±2.26$^{c\#}$
3	Standard	101.7±2.19^{c*}
4	Hesperidin(50 mg/kg)	88.17±2.46^{c*a**}
5	Hesperidin(100 mg/kg)	81.50±2.20^{c*c**}
6	Hesperidin(200 mg/kg)	76.33±1.27^{c*c**}

Control: CMC; Negative control: Letrozole; Standard: Clomiphene citrate; Low dose: Hesperidin 50mg/kg; Intermediate dose: Hesperidin 100mg/kg; High dose: Hesperidin 200 mg/kg.

3.4. Serum sex steroid profile

3.4.1.Evaluation of Hesperidin on Luteinizing hormone(LH) in Letrozole induced PCOS female rats

The serum levels of LH were remarkably increased in negative group ($p<0.001$) as compared with control group. All doses of Hesperidin showed significant reduction in LH level when compared with negative control group. Hesperidin (50mg/kg and 100mg/kg) showed significantly ($p<0.01$) decreased LH level as compared with standard. High dose of hesperidin shows decreased LH level significantly($p<0.01$) when compared with standard group. (Graph no.4)

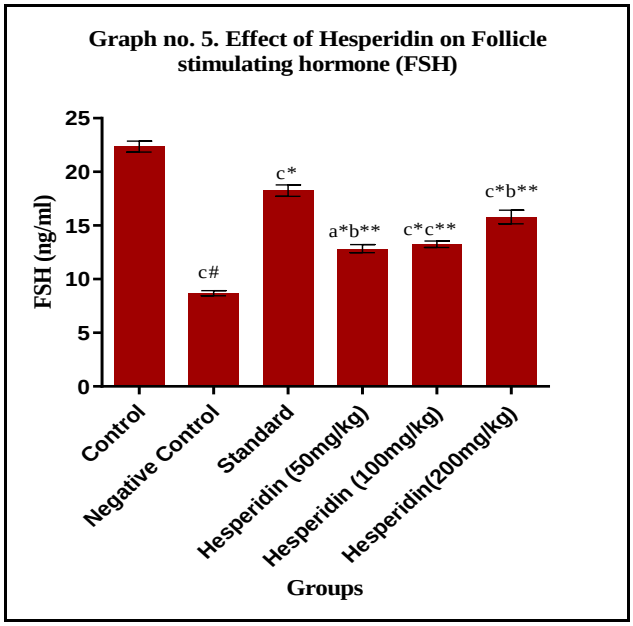


#= Data compared with control group
***= Data compared with negative control group**
****=Data compared with standard group**
a=p<0.05, b=p<0.01, c=p<0.001

3.4.2. Evaluation of Hesperidin on Follicle stimulating hormone(FSH) in Letrozole induced PCOS female rats

The serum levels of FSH were significantly reduced in negative group of animals when compared with control group of animals.When both intermediate and high dose of hesperidin (100mg/kg and 200mg/kg) was compared with negative group it showed significant ($p<0.001$) elevation in FSH levels. Intermediate and high dose of hesperidin exhibited significant ($p<0.001$) increased FSH levelsin comparison with standard group.

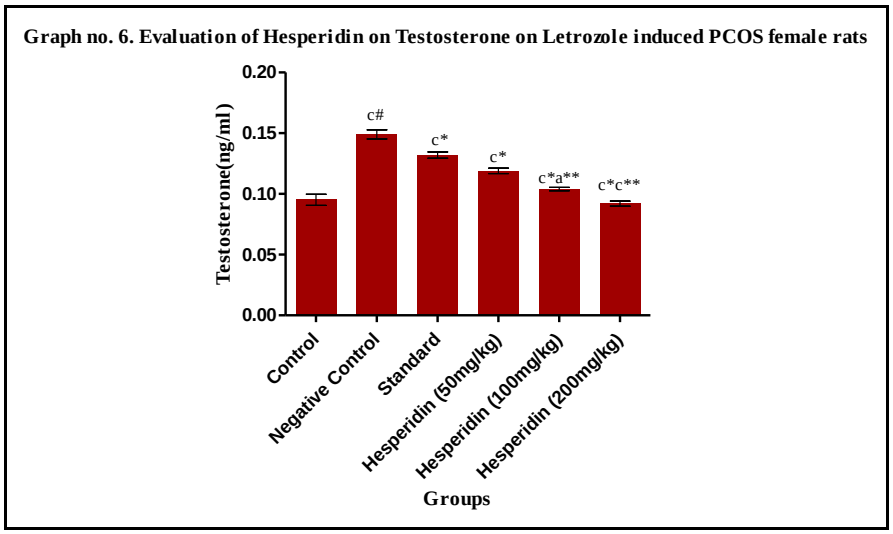
Low dose of hesperidin compared with standard and negative control then it indicated less significant decrease in FSH level. (Graph no.5)



#= Data compared with control group
***= Data compared with negative control group**
****=Data compared with standard group**
a=p<0.05, b=p<0.01, c=p<0.001

3.4.3. Evaluation of Hesperidin on Testosterone in Letrozole induced PCOS female rats

Testosterone significantly ($p<0.001$) raised in Letrozole induced group as compared with control group whereas standard and low, intermediate and high dose group of hesperidin significantly ($p<0.001$) reduced the levels of testosterone. Intermediate dose and high dose group of hesperidin significantly ($p<0.05$ and $p<0.001$ respectively) lowered the level of testosterone when compared with standard.(Graph no. 6)



#= Data compared with control group
***= Data compared with negative control group**
****=Data compared with standard group**
a=p<0.05, b=p<0.01, c=p<0.001

Table no. 3. Evaluation of Hesperidin on Luteinizing hormone(LH), Follicle stimulating hormone(FSH) and Testosterone on Letrozole induced PCOS female rats

Group no.	Groups	Luteinizing hormone(LH) (ng/ml)	Follicle Stimulating Hormone (FSH) (ng/ml)	Testosterone (ng/ml)
1	Control	10.25±0.40	22.36±0.51	0.095±0.004
2	Negative	20.21±0.48$^{c\#}$	8.69±0.23$^{c\#}$	0.149±0.003$^{c\#}$
3	Standard	13.68±0.35^{c*}	18.25±0.36^{c*}	0.132±0.002^{c*}
4	Hesperidin(50 mg/kg)	18.44±0.51^{b*c**}	12.85±0.37^{a*b**}	0.119±0.002^{c*}
5	Hesperidin(100 mg/kg)	17.39±0.34^{b*c**}	13.25±0.29^{c*c**}	0.104±0.001^{c*a**}
6	Hesperidin(200 mg/kg)	14.14±0.32^{c*b**}	15.80±0.63^{c*b**}	0.092±0.002^{c*c**}

Control: CMC; Negative control: Letrozole; Standard: Clomiphene citrate; Low dose: Hesperidin 50mg/kg; Intermediate dose: Hesperidin 100mg/kg; High dose: Hesperidin 200 mg/kg.

3.5. Histopathological changes

3.5.1. Histopathological observation of ovaries in Control group

Section of ovaries from control group animals showed healthy follicles with oocyte at different stages of development. The primary, secondary and tertiary follicles indicated by arrows. (Figure 1)The photograph shows normal developing stages of follicles in ovary.

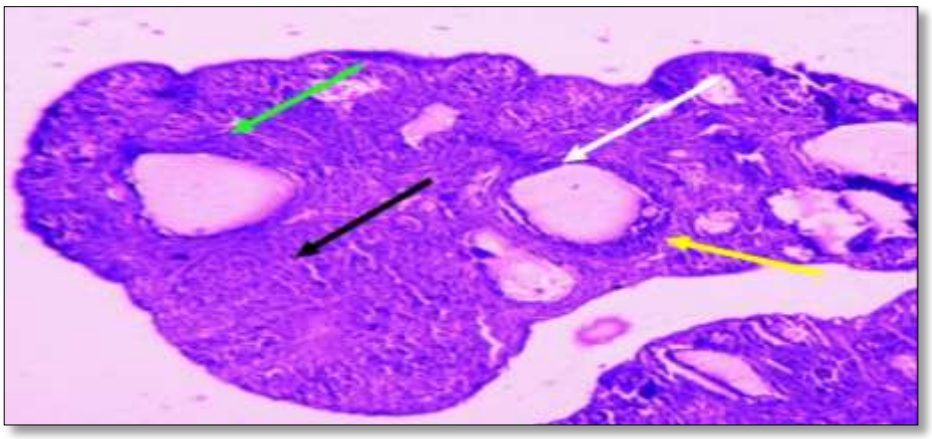


Fig no. 1 Histopathological observation of ovaries in Control group

Normal: Primary (white arrow), secondary (yellow arrow), tertiary (green arrow), corpus luteum (black arrow) (H&E stain100X)

3.5.2. Histopathological observation of ovaries in Negative Control group

Letrozole treated rats exhibited numerous cysts, with a very thin or no granulosa layer (Figure 2).Corpora lutea were completely absent indicating anovulation. Few follicles were observed at their early stages of development. In addition, they were accompanied withatretic follicles containing fluid filled antrum.

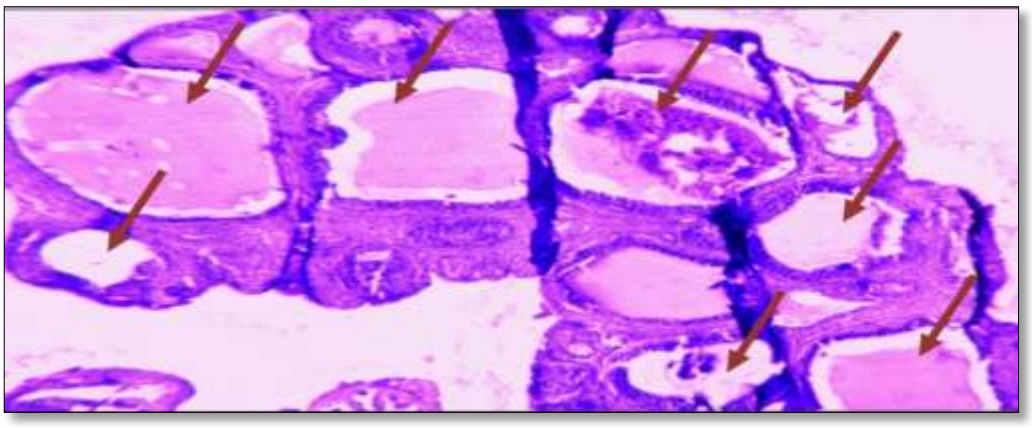


Fig no. 2. Histopathological observation of ovaries in Letrozole induced (Negative control) group

Negative: Cystic follicles (Brown arrow)

3.5.3.Histopathological observation of ovaries in Standard group

Clomiphene citrate treatment led to disappearance of cysts and appearance of healthy follicles and corporalutea. Decrease in cyst as compared to negative control group and shows developing follicles. (Figure 3)

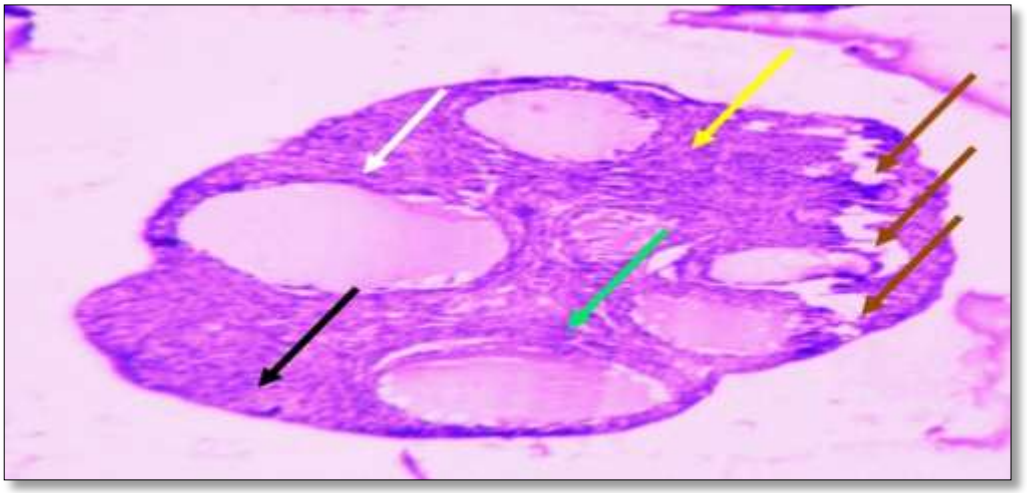


Fig no. 3. Histopathological observation of ovaries in Standard group

Standard: Primary (white arrow), secondary (yellow arrow), tertiary (green arrow), corpus luteum (black arrow), cystic follicles (brown arrow)

3.5.4. Histopathological observation of ovaries in Low dose (50 mg/kg) of hesperidin

Sections from low dose of hesperidin (50 mg/kg) group exhibited follicles larger in size and few corpora lutea. It also shows developing stages of follicles. (Figure 4)

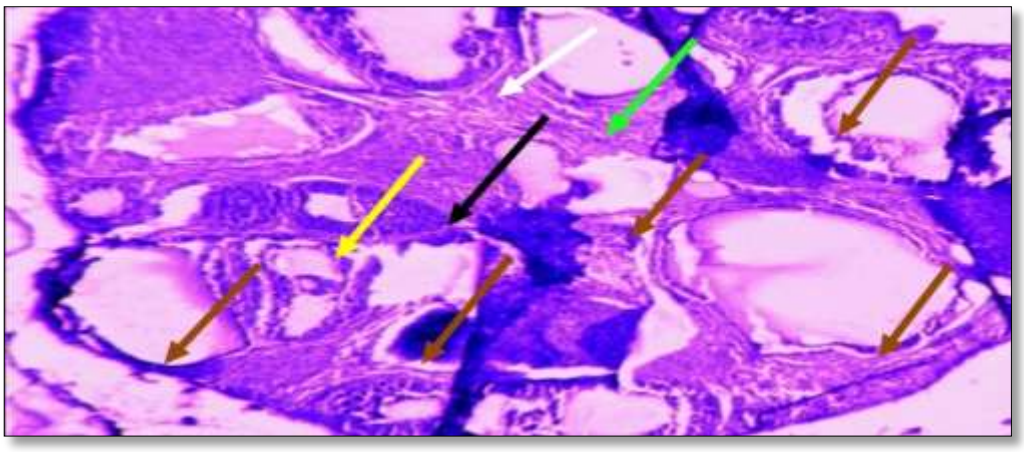


Fig no. 4. Histopathological observation of ovaries in Low dose (50 mg/kg) of hesperidin

Low Dose: Primary (white arrow), secondary (yellow arrow), tertiary (green arrow), corpus luteum (black arrow), cystic follicles (brown arrow)

3.5.5. Histopathological observation of ovaries in Intermediate dose (100 mg/kg) of hesperidin

Sections from intermediate dose of hesperidin (100 mg/kg) group exhibited few cysts and few corpora lutea. It also shows well differentiated developing stages of follicles. (Figure 5)

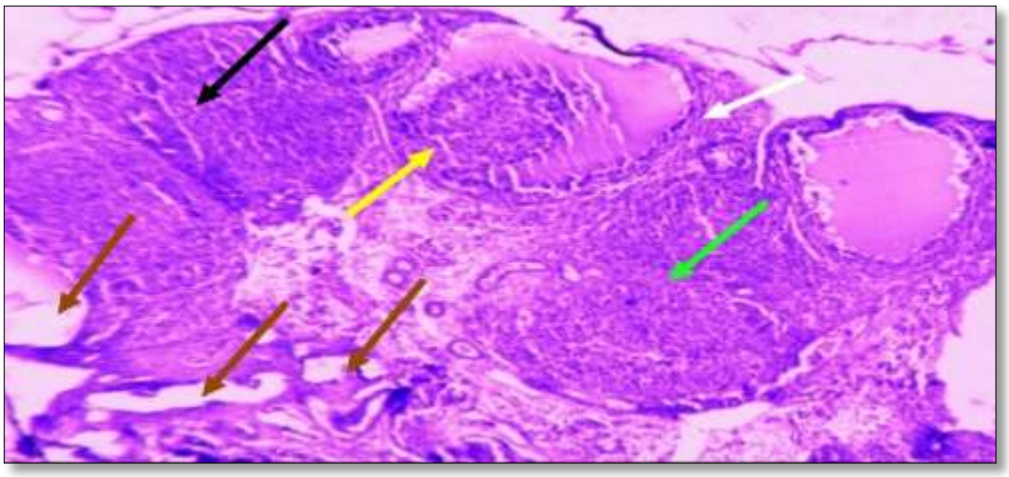


Fig no. 5. Histopathological observation of ovaries in Intermediate dose (100 mg/kg) of hesperidin

Intermediate Dose: Primary (white arrow), secondary (yellow arrow), tertiary (green arrow), corpus luteum (black arrow), cystic follicles (brown arrow)

3.5.6. Histopathological observation of ovaries in High dose (200 mg/kg) of hesperidin

Cysts were very less in number and normal sized healthy follicles at differentdevelopmental stages with oocytes were found in section fromhigh dose (200 mg/kg) group (Figure 6). Also with the high dosemany corpora lutea and antral follicles with clearly differentiated oocyte, granulosa cell layer were observed.

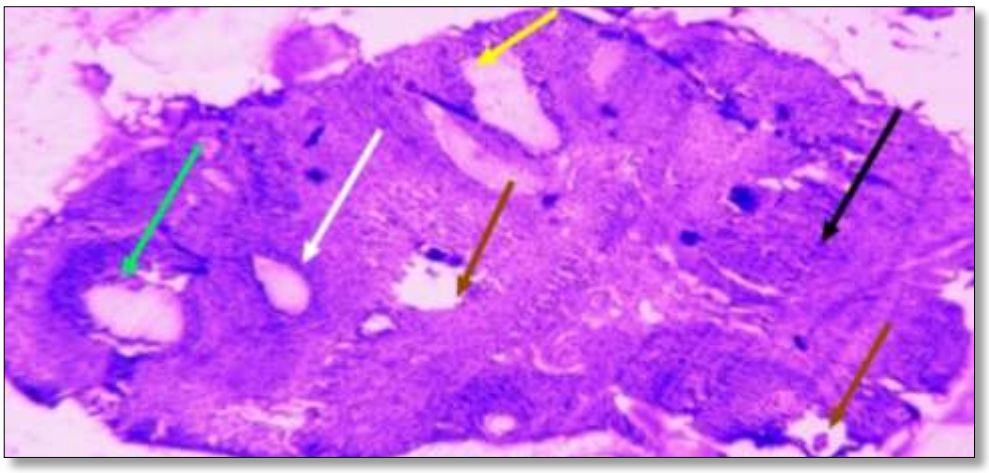


Fig no. 6. Histopathological observation of ovaries in High dose (200 mg/kg) of hesperidin

High Dose: Primary (white arrow), secondary (yellow arrow), tertiary (green arrow), corpus luteum (black arrow), cystic follicles (brown arrow)

4. Discussion

In this research, using the inhibitor of non-steroidal aromatase i.e. Letrozole, PCOS condition was developed in animals which presented biochemical and histological changes. The clinical and biochemical characteristics caused by PCOS in rats were investigated in this research.

The working of Letrozole induced PCOS model was confirmed by regular examination of vaginal smears and persistent vaginal cornification[17]. PCOS-induced rats enhanced body weight owing to abdominal fat deposition. But the body weight was considerably reduced by the therapy with hesperidin (50mg/kg, 100mg/kg, 200mg/kg). The weight of ovariesin negative group was greater than rats incontrol group, according to previous studies. The hesperidin therapy(50mg/kg,100mg/kg, and 200mg/kg)prohibited further rise in ovarian weight. The weight of ovaries in the negative group was greater than that of control rats according toobservations. Hesperidin therapy(50mg/kg,100mg/kg, and 200mg/kg) stopped further ovarian weight gain.

Letrozole induction of PCOS led in testosterone and LH levels being elevated while FSH level decreased. This imbalance in hormonal level led to an inconsistent cycle of estrous. The same circumstance have been noted in this research. Letrozole induced rats showed considerably higher concentrations of testosterone and LH when compared to control. Standard drug clomiphene citrate and hesperidin (100mg/kg, 200mg/kg) treated animals showed substantially reduced testosterone and LH levels. Hesperidin treatment showed FSH level elevation[18-20].

PCOS is also strongly linked with type-2 diabetes mellitus and insulin-resistant hyperglycemia[22]. In our analysis, there was a marked rise in blood glucose level in the negative group relative to the control group. Oral administration of hesperidin considerably inhibited increased blood sugar levels, indicating the impact of hesperidin on insulin resistance and diabetic conditions. Hesperidin showed beneficial effect against hyperglycemia as well as hyperlipidemia.

In ovaries, enhanced oxidant concentrations may change the steroidogenesis resulting in enhanced rates of androgen production and polycystic ovaries.[20-21] Many studies reported that oxidative stress is one of the various pathologicalfactors in women with PCOS[23].The histopathological report of Letrozole induced rats revealed the existence of polycysts in the ovary. If negative group compared to control group then negative group showed that more than two cysts were formed in the ovary. After therapy with hesperidin the cystic follicles reduced and also shows healthy follicles at different stages of development. This implies a marked recovery of ovarian tissue by hesperidin.

5. Conclusion

In normalizing the various parameters of PCOS condition in rats, the impact of hesperidin therapy with medium (100mg/kg) and high (200mg/kg) dose was observed to be similar with standard treatment.

In letrozole induced polycystic ovarian animals, hesperidin recovered the serum hormonal levels such as FSH,LH, and Testosterone, glycemic condition, along with body weight and ovarian weight. Thus, hesperidin might be helpful in managing PCOS condition due to restoration of irregular follicular phase and abnormal changes in the ovaries of rats.

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