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FORMONONETIN AS PROMISING THERAPEUTIC INTERVENTION FOR RESTORING OVARIAN, UTERINE, AND HEPATO-RENAL FUNCTIONS IN LETROZOLE-INDUCED,POLYCYSTIC OVARIAN SYNDROME *SPRAGUE DAWLEY* RATS

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Abstract:
Polycystic ovarian syndrome is an emerging health problem for females belonging to reproductive age across the globe. Conventional methods of treatment only provide symptomatic relief with many side effects. So to seek alternative treatment options, current study was designed to observe and compare the restorative and protective roles of formononetin (40 mg/Kg.b.w./day) on ovary, uterus, liver and kidneys of PCOS induced (via letrozole @ 1.0 mg/Kg b.w./day) female *Sprague dawley* rats (150-200 gm) by keeping cyproterone acetate (1.0 mg/Kg.b.w./day) as positive control. Statistically analyzed results ($p<0.0001$) showed that after treatment of PCOS induced rats with formononetin, there was remarkable restoration of FSH, estradiol and progesterone (3.14±0.98 ml U/mL, 5.8±1.2 pg/dL and 45± 0.01 pg/mL respectively) as compared to positive control (2.5±0.01 mlU/mL, 3.05±0.3 pg/dL and 48±5.2 pg/mL respectively) while there was decrease in the levels of bilirubin and uric acid (0.4±0.01 mg/dL and 2.3±0.7 mg/dL) as compared to positive control (0.9±0.01 mg/dL and 4.7±0.15 mg/dL) respectively. Serum ALT, AST, ALP, urea and creatinine were (67±0.01 IU/L, 255±0.3 IU/L, 315±0.0I IU/L, 41.3±1.5 mg/dL, 1.31±0.25 mg/dL) in experimental group as compared to control (78.3±1.8 IU/L, 134.3±4.5 IU/L, 618±0.0I IU/L, 55±0.01 mg/dL,0.8±0.1 mg/d) respectively. Histological examination experimental group showed restoration of normal ovarian stroma from typical pearl string cystic appearance, with normal hepato-renal tissues. So it is concluded that formononetin may helpful to cure or mange PCOS but more studies are require to establish hepto-renal safety.

Key words: Polycystic ovarian syndrome, Letrozole, Cyproterone acetate, Formononetin, Ovarian, Uterine, Hepato-renal

INTRODUCTION

Polycystic Ovarian Syndrome (PCOS) is a complex metabolic and endocrine disorder affecting women globally, with an overall prevalence of approximately 10%[1]. Studies indicate a higher incidence in Asian countries, with a rising trend in prevalence. PCOS is characterized by its heterogeneous nature, unclear etiology, and a broad clinical spectrum. Various hypothesis propose

genetic, protein, epigenetic, and environmental factors as contributors to the origin of the syndrome[2].The primary disruptions in PCOS involve hyperandrogenemia and hyperinsulinemia, and both are interconnected. Intact mitogenic and steroidogenic activity in the ovaries and decreasing hepatic synthesis of serum hepatic binding globulin (SHBG) contribute to hyperandrogenemia. This, in turn, may lead to insulin resistance (IR) by modifying muscle tissue composition and perpetuating a cycle of IR-hyperinsulinemia-hyperandrogenemia[3]. Hyperandrogenemia is associated with increased follicle growth and premature growth arrest of antralfollicles, resulting in the classical ovarian phenotype with a string of pearl-like appearance on ultrasound[4, 5]. Treating PCOS is challenging due to its complexity and associated risks, including obesity, cardiovascular diseases, infertility, and diabetes mellitus. Many pharmacological treatments are available like cyproterone acetaeto treat PCOS related hyperandrogenemia, but they offer symptomatic relief and related with known and unknown with side effects[6]. Seeking alternative treatments for this multifaceted disease, phytoestrogens like formononetin can be a potential agent. It is anisoflavone and obtained from many plants like soy bean (Glycine max), chick peas (Cicer arietinum L.), red clover (Trifoliumpratense) and Chinese medicinal plant Astragalus membranaceus. It is also known for anti- inflammatory, anti-cancer and antioxidant properties .More over it can regulate metabolic and endocrine disorders by binding to estrogen receptors and inhibiting 5α-reductase, thereby decreasing hyperandrogenemia in PCOS patients. Utilizing plant based, non-chemical sources to reduce hyperandrogenemia could offer advantages for PCOS patients[7, 8.]

MATERIALS AND METHODS

Animal selection

After ethical approval (Approval No: USM/Animal Ethics approval/2009/[45] [140]) adult female Sprague Dawley rats (150-200gm) had been housed in standard stainless steel cages at room temperature and 60-70 % relative humidity. Animals were fed with a standard laboratory diet and gave free access to water. Sick and ailing rats were excluded from the study. Animals were divided into the following four groups with three replications each as follows;

1. Vehicle: Received water only
2. Negative control group: Induced with PCO via 1mg letrozole/ kg b.w. of rat for 21 days[9].
3. Positive control group: PCO Induction+ cyproterone acetate (1mg/kg b.w. of rat) for 21 days[10].
4. Experimental group: PCO Inducted+ formononetin (40.0 mg/Kg b.w. of rats), administered as intraperitonially[11].

Collection and analysis of vaginal smears

PCOS induced rats have persistent diestrous phase. PCOS induction was confirmed by vaginal smears prepared under standard conditions[12].

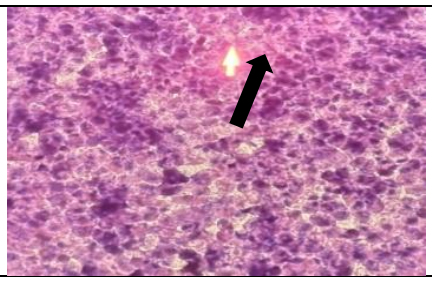
Collection and analysis of blood and tissues

After end of experiment rat were dissected by open chest method to collect blood in vacutainer tube systems and placed vertical upright position in racks in transportation boxes and stored in the dark, at 4 °C [13]. Hormones were measured through enzyme-linked immunosorbent assay ELISA kit method. Biochemical and enzymatic analysis was done through Spectrophotometer by standard method[3]. Ovaries, uterus, pancreas, liver and kidneys were preserved in 10% neutral formalin (MERCK) and dehydrated in descending grades of ethanol brought (MERCK), cleared in xyleneand embedded in paraffin wax purchased from BIOFAR chemicals. Sections of 4-5µm thickness had been cut and stained with hematoxylin (BIOFAR) and eosin and examined at 40 µm under microscope (model number XSZ-107BN, made in USA).

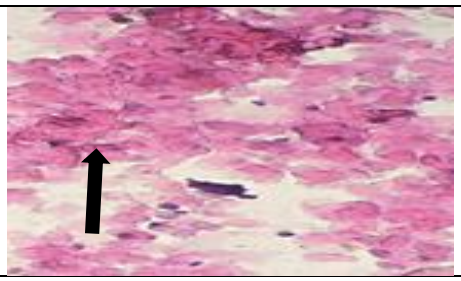
RESULTS

ANALYSIS OF VAGINAL SMEARS

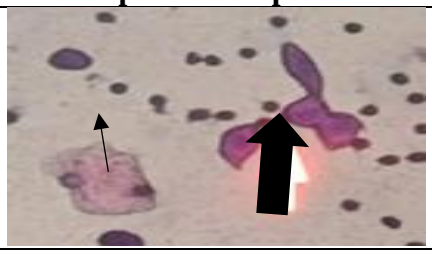
Stained vaginal smears of vehicle showed regular proestrous, estrous, diestrous and metestrous phases. In proestrous phase [Figure 1 (I)] well-formed round nucleated epithelial cells in clusters are seen. Estrous phase [Figure 1 (ii)] is characterized by cornified squamous cells found in clusters .The diestrous phase [Figure 1 (iii)] is characterized by prominent leukocytes with few epithelial and cornified cells. The metestrous [Figure 1 (iv)] is shown with large number of leucocytes and a small number of large, non-granular and anucleated cornified epithelial cells. The PCOS induced rats showed persistent diestrous phase with prominent leukocytes. [Figure 1(v)].



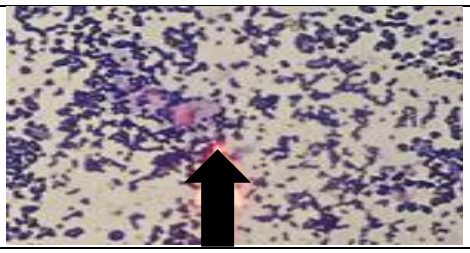
i) Proestrous phase: Arrow showing nucleated epithelial cells in clusters in proestrous phase



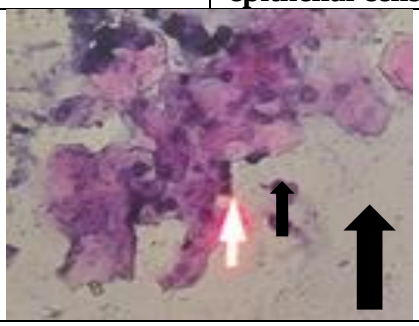
ii) Estrous phase:Arrow showing cornified squamous cells in clusters



iii)Diestrous phase: Arrow is showing prominent leukocytes in diestrous phase



iv) Metaestrous phase:Arrow highlighting relatively large number of leukocytes with cornified epithelial cells in metaestrous phase

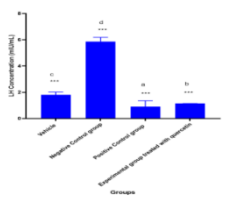


v)permanent Diestrous phase in PCOS induced rat: Arrow depicting prominent leukocytes

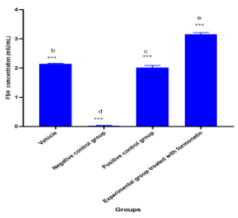
Figure 1 Phases obtained at vaginal smears i) Proestrous ii) Estrous iii) Diestrous iv) Metestrous phases of vaginal smears v) permanent Diestrous phase in PCOS induced rat Hormonal Profile of Animals

Statistically analyzed results showed that negative control group has significantly increased LH level (5.87±0.31mLU/mL) as compared to vehicle (1.8±0.21mLU/mL). The elevated LH level decreased slightly in experimental group at $p<0.0001$ (4.46±0.001 mlU/mL) but maximum reduction has been done in animals treated with cyproterone acetate (1.8±0.5 mlU/mL) {Figure 2(i)}. It has been proven statistically that in negative control group, FSH level decreased (0.03±0.01mLU/mL) as compared to vehicle (3.14±0.98mLU/mL) and experimental group

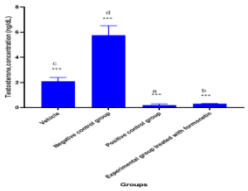
(3.19±0.01 mlU/mL), while rats in positive control group has slightly elevated FSH level (2.5±0.01 mlU/ml) at p<0.0001{Figure 2(ii)}. Statistically analyzed results showed significant elevation in testosterone levels in negative control group (6.15±1.2 ng/dL) as compared to vehicle (2.0±0.29 ng/dL). While both experimental and positive control groups has decreased testosterone levels, with maximum reduction by rats in positive control group (0.22±0.01 ng/dL and 0.33±0.01 ng/dL respectively) at p<0.0001 {Figure 2(iii)}. Negative control group has significant reduction in progesterone level (4.1±0.6 pg/dL) as compared to vehicle (7.5±0.5pg/dL) and this restoration was more evident in rats treated with formononetib (5.8±1.2 pg/dL) as compared to positive control (3.05±0.3 pg/dL) at p<0.0001 {(Figure 2(iv)}. Similarly level of estradiol significantly decreased in negative control group (28.3±20.12 pg/mL) as compared to vehicle (96.6 ±16.07 pg/mL) at p<0.0001 while estradiol level has been restored in experimental (45± 0.01 pg/mL) and positive control (48±5.2 pg/mL) groups {(Figure 2(v)}.



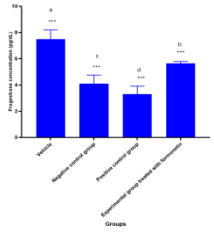
i) Concentration of LH (mLU/mL)



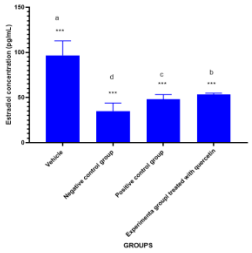
ii) Concentration of FSH (mLU/mL)



iii) Concentration oftestosteron (ng/dL)



iv) Concentration of progestrone (ng/dL)



v) Estradiol concentration (pg/mL)

Figure 2 Hormonal profile of animals.
(i) LH concentration **(ii)** FSH concentration **(iii)** Testosterone concentration **(iv)** Progestrone concentration **(v)** Estradiol concentration Comparison of animal groups from most significant results to less significant. ***= P<0.001, **= P<0.01, *= P<0.05

Enzymatic and Biochemical Profile of animals

Statistically analyzed data showed that ALT levels has been significantly increased in positive control and experimental groups (67±0.01 IU/L and 78.3±1.8 IU/L) respectively as compared to vehicle (28.6 ±7.09 IU/L) {Figure 3(i)}. Serum AST level had been decreased in negative control group (40.3±4.0IU/L), as compared to vehicle (44.3 ±3.7IU/L), while it has been increased in experimental group (255±0.3 IU/L) and positive control group (134.3±4.5 IU/L) {Figure 3(i)}. In positive control and experimental groups, there was maximum increase in the level of ALP (618±11.3 IU/L and 315±0.0I IU/L respectively) {Figure 3(i)}. Analysis showed that concentration of bilirubin had slight elevation in negative control group (0.8±0.1 mg/dl) as compared to vehicle (0.2 ±0.05 IU/L) and this level became normal when rats were treated with formononetin (0.4±0.01 mg/dL) and cyproterone acetate (0.9±0.01 mg/dL) {Figure 3(ii)}. Statistical analysis showed that blood urea significantly increased in negative control group (51.3 ±1.5 mg/dL) as compared to vehicle (25.1±1 mg/dL) and positive control group (41.3±1.5 mg/dL), while experimental group also showed eleveted level of blood urea (55.0± 0.01 mg/dL) {Figure 3 (iii)}. It was analyzed that creatinin was significantly raised in negative control group (0.72 ±0.1 mg/dL) as compared to

vehicle (0.31±0.05 mg/dL). In positive control group, its level was (0.8±0.1 mg/dL) which was elevated as compared to experimental group (1.31±0.25 mg/dL) {Figure 3 (iii)}. Statistically analysis of uric acid concentration showed that it was significantly decreased in negative control and experimental groups (2.7±1.93 mg/dL and 2.3±0.7 mg/dL respectively) as compared to vehicle (3.5 ±0.15 mg/dL) and positive control groups (4.7±0.15 mg/dL) {(Figure 3(iii)}.

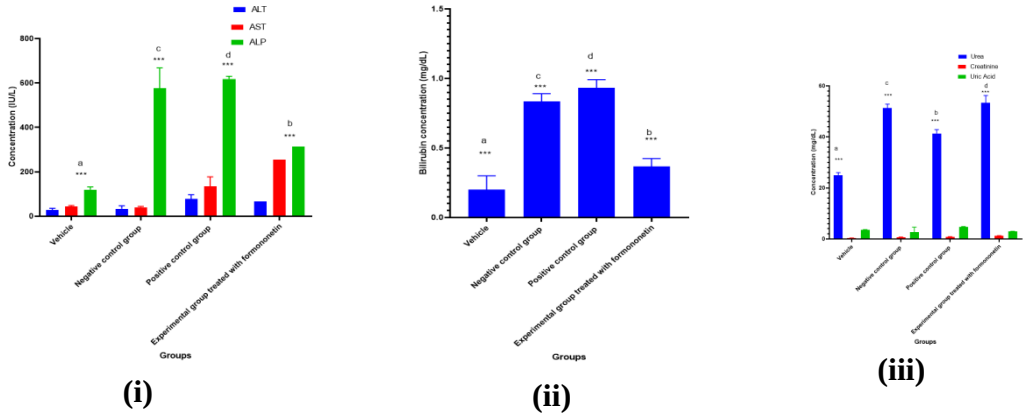
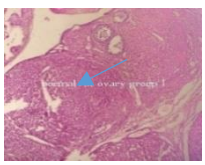


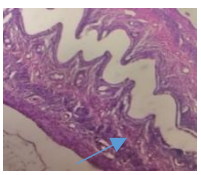
Figure 3 (i) Enzymatic profile (ii) Concentration of serum Bilirubin (iii) Urea, Creatinine and Uric acid concentration (a – d) = Comparison of animal groups from most significant results to less significant. ***= P<0.001, **= P<0.01, *= P<0.05

Histological Profile of animals

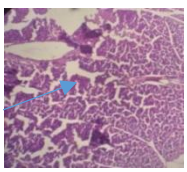
Histological analysis revealed multiple changes in ovarian, uterine, hepto-renal and pancreatic tissues which were ranging from normal to toxic levels in different groups of animals and is evident from [figure 4(I- XX)].In negative control group, histological features of ovary showed multiple sub capsular cystic follicles, like pearl of strings, while endometrium and myometrium of uterus were un-remarkable. Pancreas was found normal but kidney was showed with mild tubular interstitial nephritis and intra tubular casts. The liver histology showed that liver had mild peripheral inflammation [figure 4(VI- X)]as compared to vehicle which had normal ovarian parenchyma with corpus leuteum, unremarkable uterus kidney had normal glomeruli, tubules and interstitium and liver was observed with normal hepatic lobule, with no steatosis, hepatocystitis and ballooning or inflammation[figure 4(I- V)].In positive control group 3 with cyproterone acete histology of ovary is seen with relatively few cystic follicles. Uterus in secretory phase, liver, pancreas and renal histology is unremarkable[Figure 4(XI-XV)].In experimental group 4 with f formononetin ovary is seen with 3 to 4 residual cystic follicles are seen with corpus leuteum, liver, pancreas and renal histology is unremarkable[Figure 4 (XVI-XX)].



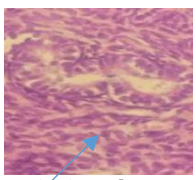
(I) Ovarian parenchyma shows corpus leuteum



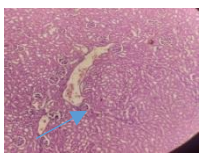
(II) Uterus endometrium and myometrium are un remarkable



(III) Pancreas. Showing islets of Langerhans



(IV) Kidney unremarkable with normal glomeruli tubules and interstitium



(V) Liver normal hepatic lobule, no steatosis, hepatocystitis,ballooning or inflammation

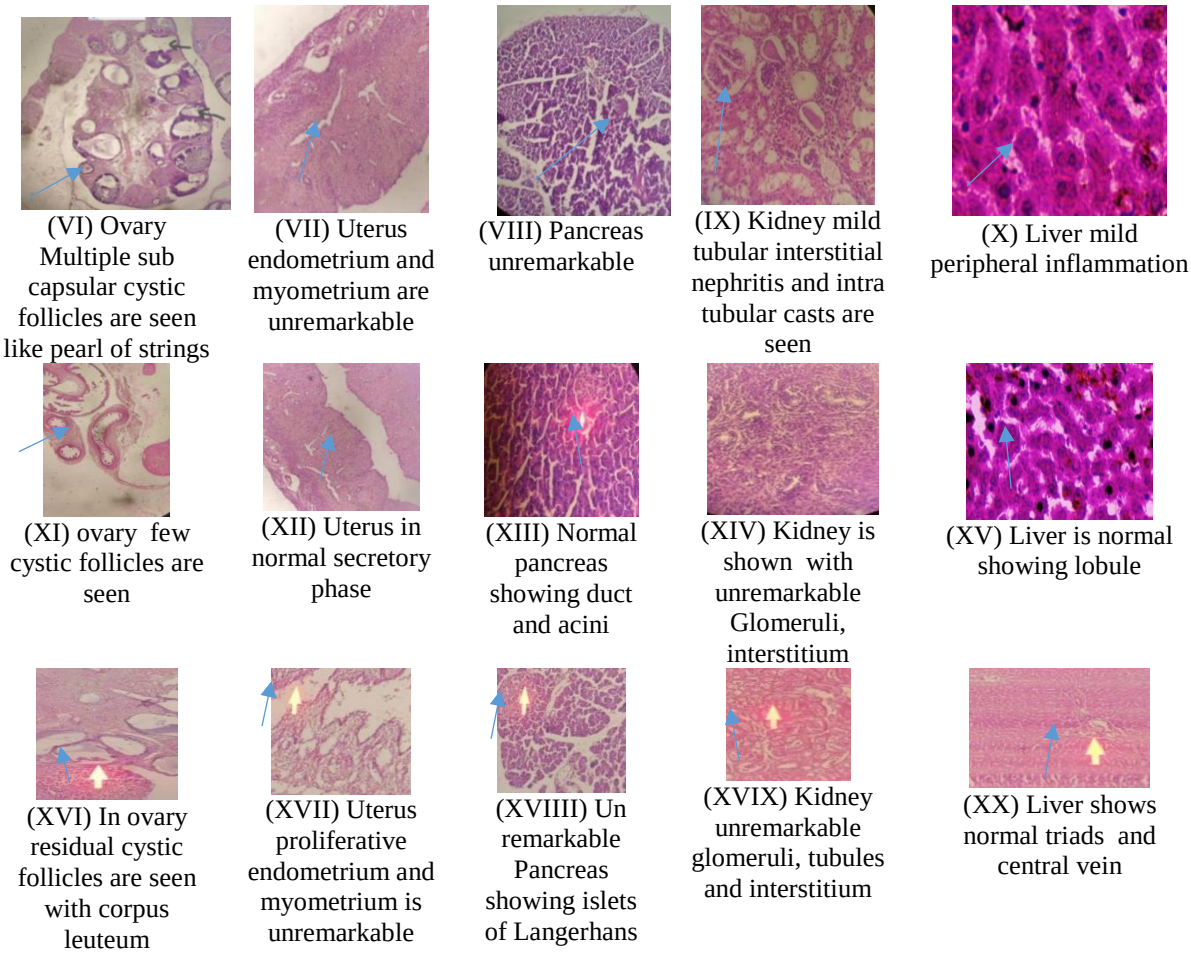


Figure 4 Histopathological features of animals at (40 µm) Vehicle (I-V), Negative control group (VI-X), Positive control group (XI-XV), Experimental group (XVI-XX)

DISCUSSION

PCOS related hyperandrogenemia is one of the leading cause of an ovulatory infertility worldwide[14,15]. It is commonly treated with systemic pharmacological agents. One of those agent is use of cyproterone acetate that work by directly inhibiting androgen synthesis, antagonizing androgen receptor effects, or inhibiting 5α-reductase activity. Additionally, it indirectly reduce hyperandrogenemia by suppressing excessive LH production[16]. Despite its beneficial role, it often lead to side effects like irregular menstrual bleeding and loss of libido[17]. Current study, focused on the in vivo characterization of formononetin, and its comparison with cyproterone acetate on PCOS induced rat model. Formononetin is a methylated is of lavones with estrogenic activity[18]. It is most commonly present in various legumes plants such as green peas, beans and liquorice roots. It is metabolized in the body to daidzein and genistein, respectively[19].Many research articles proved is of lavones as thepotential agent for reducing hyperandrogenemia by inhibiting testosterone 5α-reductase inhibitors along with antioxidant and anti-inflammatoryproperties[20].For this study, letrozole was used to induce PCOS in female Sprague Dawley rats. Letrozole is a drug belonging to third generation of non-steroidal aromatase inhibitors, so it prevents conversion of testosterone in estradiol that leads to hyperandrogenemia and hyperinsulinemia in animals and mimics the PCOS picture by increasing the LH and testosterone while decreasing FSH, estradiol and progesterone levels[21]. In our study PCOS induced animals exhibited all these characters as explained by[22]. Many articles suggest formononetin as a potential agent that may cure PCOS. But to our knowledge, no data is available to explain the sole role of formononetin on hormonal levels of PCOS patients. However many studies documented the beneficial role of plants that contain formononetin with other phytoestrogens on PCOS. All these plants were like current study reported about restoration

of FSH, LH and testosterone, estrogen, progesterone with phytoestrogens [23].The effects of hydro-alcoholic extract of raspberry fruit on ovarian follicles and serum parameters in poly cystic ovary syndrome-induced rat[24]. In PCOS the change of ovarian morphology is important diagnostic land mark and cause of many fertility related problems. Histopathological parameters of ovary, uterus, pancreas, kidneys and liver were found improved in fomononetin treated experimental animal group. To our knowledge, no research work was done before to explore effects of formononetin on PCOS. AST and bilirub in improvement in experimental group treated with formononetin was in accordance with[25]. (Jin et al .2017) who claimed hepato protectiverole of formononetin by improvement in AST and bilirubin levels may be through anti- inflammatory pathway. Renal marker improvement in formonentin treated group was in accordance with[26]. Renal markers were found to be improved in another study in which they found attenuated role of formononetin in the histopathological changes in kidneys and improvement in the levels of blood urea nitrogen and creatinine[27].

CONCLUSION

It is concluded that the formononetin had shown more promising results to cure PCOS along with reduction in hepato-renal toxicity by regulating hormonal and ovarian architecture in PCOS models. However more hepatic and renal protective role is needed to further evaluate and validate the study.

Patent
Not applicable

Funding:
Not applicable

Institutional Review Board Statement:
Current research work has been approved by ethical approval committee of institute of molecular biology and biotechnology, The university of Lahore under approval No(Approval No: USM/ Animal Ethics approval/2009/[45] [140])

Informed Consent Statement:
Not applicable

Data Availability Statement:
Not applicable

Conflict of Interest:
All authors declare no conflict of interest.

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Sample Availability:
Not applicable

Abbreviations:
PCOS Polycystic ovarian syndrome

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