

Effects of *Ficus deltoidea* Ethanolic leaves extract on female reproductive organs among Letrozole-induced polycystic ovarian syndrome rats

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Abstract: Insulin resistance and hyperandrogenism in polycystic ovarian syndrome (PCOS) have been implicated in the hypothalamic-pituitary-ovary (HPO) axis dysfunction, impacting on anovulation as well as menstrual irregularity. In the present study, *Ficus deltoidea* was evaluated to elucidate its effects on reproductive organs including ovary and uterus among letrozole-induced female Sprague Dawley rats (1.0 mg/kg bw, for 21 consecutive days). Experimental animals were divided into six groups (n=6). Five groups were induced into PCOS, while the sixth group received the vehicle and tagged as normal control (NC). The five PCOS induced groups were treated for 42 days with; (i) saline (negative control, PC), (ii) 10 mg/kg bw clomiphene citrate (positive control, PCC), and (iii) three test groups administered with different concentrations of the extract; 25 mg/kg bw (PFD25), 125 mg/kg bw (PFD125) and 250 mg/kg bw (PFD250). At the end of treatment period, ovary and uterus were collected; weighed and relative ovarian and uterine weights were calculated. Both tissues were processed using standard histological procedures. Treatment with 250 mg/kg bw *F. deltoidea* leaves extract significantly reduced the relative ovarian wet weight of PCOS females compared with PC rats (44.83±4.33 vs 63.44±4.03 mg/100 g bw respectively, p<0.05) and significantly increased relative uterine wet weight compared with those values of both PC and PCC rats. A significantly higher mean diameter of uterine horn was also observed (492.43±14.84 µm, p<0.05 (PFD250)). Qualitative assessment on histology of ovaries and uteri in PC group exhibited high incidence of cystic follicles, absence of corpora lutea structures with significantly thin uterus (111.87±20.95 µm, p<0.05). Treatment with the extracts showed less number of cystic follicles with presence of a number of corpora lutea and various stages of developing follicles implying ovulation had been restored among PCOS animal model in dose-dependent manner. In summary, *F. deltoidea* leaves ethanolic extract exerts protective effects against ovarian and uterine of letrozole-induced PCOS female rats, which sparks promising recovery for ameliorating PCOS especially on ovarian and uterine tissues.

Key words: *Deltoidea*; Polycystic ovarian syndrome; Cystic follicles; Corpus luteum; Anovulation; Infertility

1. Introduction

Approximately 50% of infertility cases are attributable to female factors, of which anovulation is the leading cause. Most cases of anovulation are due to the polycystic ovarian syndrome (PCOS), while the exact underlying mechanism of the syndrome remains unknown (Azziz et al., 2004). Current treatment modalities widely prescribed for PCOS women mainly depend on the severity of symptoms appeared on the individual patient. Anovulatory PCOS women who try to conceive are treated with ovulation inductors such as clomiphene citrate. Other patients with deteriorating insulin sensitivity are prescribed with insulin sensitizers such as metformin. However, these conventional synthetic drugs can cause many unwanted serious side effects such as risk of ovarian hyperstimulation, multiple pregnancies as well as psychological effects (Luciano et al., 2013). Thus, till now there is no satisfactory effective therapy to cure PCOS, which simultaneously can alleviate PCOS associated infertility problem. This has led to the search for

alternative medicinal plants which are therapeutically effective with minimal side effects as encouraged by World Health Organization.

Insulin resistance and hyperandrogenism in PCOS have been implicated in the hypothalamus-pituitary-ovary (HPO) axis dysfunction, impacting on anovulation as well as menstrual irregularity (Kort and Lobo, 2014). Thus, the main step in restoring ovarian function and a normal menstrual cycle in PCOS patients is by breaking the pattern of hyperinsulinemia, together with diet and lifestyle modifications. Indeed, insulin sensitizing agent had been reported to improve both insulin resistance and ovulatory dysfunction (Wang et al., 2007).

Progressive scientific research works have been conducted on various parts of *F. deltoidea*, a notorious medicinal herb native to Malaysia. It was proven that *F. deltoidea* is an excellent natural insulin sensitizing agent with many other properties as well. However, best to our knowledge, the effects of *F. deltoidea* on PCOS animal model remains elusive, in which there is paucity of reports regarding effects of *F. deltoidea* on reproductive aspect in letrozole-induced PCOS females. Thus, this study aims to ascertain the potential of *F. deltoidea*

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ethanolic leaves extract in improving reproductive abnormalities in PCOS animal model induced by letrozole.

2. Materials and methods

2.1. Chemical reagents

Powdered letrozole was purchased from Tokyo Chemicals Industry (Japan) while clomiphene citrate was obtained from MP Biomedicals (France).

2.2. Preparation of plant extract

Fresh leaves of *F. deltoidea* were collected from Forest Research Institute of Malaysia (FRIM), Kuala Lumpur. The sample was deposited at the Herbarium Unit, University Kebangsaan Malaysia and identification of the plant species was confirmed with voucher number UKMB40315.

Extraction began with washing the leaves thoroughly and oven-dried at 37±5°C, and later were ground to fine powder. The powder was soaked in 80% ethanol for three days at room temperature inside covered conical flasks. The mixture was then filtered. Evaporation of the ethanolic filtrate was conducted using rotary evaporator at 40°C. The yield appeared as dark brown semi-solid paste, stored at 4°C until use.

2.3. Experimental animals

The total number of rats used in this study was 36 female Sprague Dawley, age six weeks old, with 180 to 220 g body weight. The animals were procured from Chennur Supplier, Seri Kembangan and acclimatized at room temperature (25±2°C) with controlled light from 0800 to 2000 hours in the Animal Holding Facility, Faculty of Health Sciences, UiTM Puncak Alam and Selangor. The animals were given free access to food and water ad libitum. All procedures were carried out in accordance to the guide for care and use of laboratory animals as approved by the institutional Committee on Animal Research and Ethics (UiTM CARE), with reference no: 59/2014.

2.4. Induction of PCOS rats model

The animals were randomly divided into six groups A,B,C,D,E and F with six rats each (n=6). Five groups (B, C ,D, E and F) were administered with letrozole at the concentration of 1.0 mg/kg bw, dissolved in 2.0 ml/kg bw, of 0.5% carboxymethylcellulose (CMC). The induction lasted for 21 consecutive days through oral route, once daily. Group A was tagged as the normal control (NC) and was given 2.0 ml/kg bw the vehicle (saline), throughout 42 days of treatment period.

2.5. Confirmation on PCOS development in the animal model

2.5.1. Testosterone assay

Blood samples were withdrawn from retro-orbital for serum testosterone analysis using enzyme immunoassays (EIA) testosterone kit (Cayman Chemicals, USA). Prior to blood sampling, rats were first anesthetized using a combination of ketamine and xylazine intraperitoneally (0.1 ml/100 g bw).

2.5.2. Anestrus female

Vaginal lavage was performed by flushing a small volume of saline in Pasteur pipette into the opening of vagina canal of the rats. The fluid was withdrawn back into the same plastic Pasteur pipette by releasing its bulb for few times to ensure sufficient cells were collected. The fluid then was placed onto a glass slide, air dried and stained with crystal violet. Stages of estrous cycle were determined by identifying proportions of three types of cells under a microscope (McLean et al., 2012)

2.6. Experimental design

Rats exhibited higher titer of testosterone level than the normal rats with pronounced anestrus were recruited and subdivided into PCOS negative control (PC) group (Group B), positive control (PCC) treated with standard drug treatment (Group C) and extract treated groups (Groups D, E, and F).

Three doses of *F. deltoidea* leaves extract selected were 25, 125 and 250 mg/kg bw had been administered into three test groups and tagged as PFD25, PFD125 and PFD250 respectively.

The positive control group comprised of PCOS rats treated with 10 mg/kg bw clomiphene citrate (tagged as PCC) (Chaube et al., 2006). Meanwhile, the PCOS negative control and normal control groups were given saline. The treatments were administered for 42 days, once daily via oral gavage. The extract and clomiphene were suspended in saline (vehicle). The experimental design is summarized in Table 1.

Table 1: Experimental groups with their respective treatments

Group	Treatment	Remark
NC	Normal + saline	Normal control
PC	PCOS + saline	Negative control
PCC	PCOS rats+10mg/kg clomiphene	Positive control
PFD 25	PCOS + 25 mg/kg bw plant extract	Test groups
PFD125	PCOS + 125 mg/kg bw plant extract	
PFD250	PCOS + 250 mg/kg bw plant extract	

2.7. Euthanasia and tissue sampling

Twenty four hours after the last dose of each treatment, rats were checked for their reproductive cycle and sacrificed at the stage of estrus. Those

exhibited other stages than estrus (proestrus, metestrus and diestrus) were kept until the next estrus for euthanasia by single overdose injection of ketamine and xylazine (8.0:0.8 ml). After the death of the animal was confirmed, ovary and uterus were removed as a single unit and placed on filter paper. Fat bursa surrounding the organs was removed and any uterine luminal fluid found was blotted dry.

2.8. Wet weight of ovaries and uteri

Ovary and uterus were individually measured using analytical balance. Relative ovarian and uterine wet weights were calculated per 100 g bw.

2.9. Histology of ovary and uterus

Organs were rinsed with normal saline and fixed in 10% buffered formalin. Tissues were then processed in an automated tissue processor that involve dehydration of the samples by graded series of alcohol and xylene before tissues were embedded in paraffin wax, forming tissue blocks that were kept at 4°C before sectioning was performed. The tissues were sectioned at 4 µm using a microtome. This yielded tissues in paraffin ribbons, which were to float and lay straight in warm distilled water (37°C) inside a floating bath. Fishing technique was used to place the sections onto a pre-labelled glass slide and dried on a hot plate (50-55°C) until all paraffin melted. Later, the slides were kept in an oven (37°C) for 2 days to ensure tissues would not detached from slide during haematoxylin and eosin staining process. Haematoxylin stains basophilic nucleus with purple-blue. The pink eosin highlighted the eosinophilic cytoplasm. The slides later were mounted with cover slips using DPX mountant, dried at room temperature for two days prior to observation under a light microscope.

Slides were microscopically examined for any changes in organ structures. Stages of follicular development and regression were thoroughly examined from ovary slides, and mean diameter of uterus was measured using image analyzer, Soft Imaging System Analysis.

2.10. Statistical analysis

The collected data were analysed using the Statistical Package for Social Science (SPSS) version 20. Significance differences between means of the test and control groups were established by using Oneway Analysis of Variance (ANOVA) followed by post-hoc Duncan test for multiple group comparison. A value of p<0.05 was used to denote statistical significance. All results were expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. F. deltoidea leaves extract reduces ovarian weight

Table 2 shows the effects of F. deltoidea ethanolic leaves extract and clomiphene citrate on mean relative ovarian and uterine wet weights. It was found that letrozole-induced PCOS rats had significantly (p<0.05) higher mean relative ovarian weight (63.44±4.03 mg/100 g bw). Treatment with 25, 125 and 250 mg/kg bw of F. deltoidea ethanolic leaves extract, had significantly (p<0.05) reduced the mean relative ovarian wet weights in which the values were 50.27±4.59, 49.95±5.56, and 44.83±4.33 mg/100 g bw, respectively, showing gradual reduction in dose-dependent manner, suggesting F. deltoidea leaves has potential property in preventing further increase in ovarian weight, as the data was comparable to the mean values of those normal and reference drug treated rats which were 46.9 ± 4.10 and 63.449 ±4.03 mg/100 g bw, respectively.

3.2. F. deltoidea leaves extract reduces uterine weight

Mean relative uterine weight in PC group was significantly (p<0.05) lower than any other experimental groups with mean of 97.79±10.56 mg/100 g bw. Clomiphene citrate treatment had significantly (p<0.05) increased the mean relative uterine weight (113.28±8.36 mg/100 g bw) to a slightly higher value than that observed in PC group. Meanwhile, the extract treated groups showed significantly (p<0.05) higher values than both PC and PCC groups, in dose dependent manner. However, the effect of different doses of extract on uterine mean uterine weight was not significantly different with 147.62±25.9, 167.59±8.61, and 182.53±15.91 mg/100 g bw, for 25, 125 and 250 mg/kg bw of extract respectively.

Table 2: Effect of respective treatments on mean relative wet weights of ovary and uterus

Groups	Mean relative wet weight ± SEM (mg/100 g bw)	
	Ovary	Uterus
NC	46.94±4.10 ^a	262.41±15.23 ^d
PC	63.44±4.03 ^b	97.79±10.56 ^a
PCC	48.41±3.34 ^a	113.28±8.36 ^b
PFD25	50.27±4.59 ^a	147.62±25.93 ^c
PFD125	49.95±5.56 ^a	167.59±8.61 ^c
PFD250	44.83±4.33 ^a	182.53±15.91 ^c

Different superscripts within a column indicated significant difference at p<0.05

Table 3: Effect of respective treatments on mean diameter of uterine horn

Groups	Mean diameter of uterine horn ±SEM (µm)
NC	595.67±33.36 ^d
PC	111.87±20.95 ^a
PCC	383.57±14.49 ^b
PFD25	378.40±6.31 ^b
PFD125	473.33±43.68 ^c
PFD250	492.43±14.84 ^c

Different superscripts within a column denoted significance difference at p<0.05

However, those values were significantly lower than the mean relative uterine weight of the NC group (262.41 ± 15.23 mg/100 g bw).

3.3. Uterus histology: *F. deltoidea* leaves extract increases diameter of uterine horns

The diameter of uterine horn was measured and the data was presented in Table 3. The mean uterine diameter from PC rats was significantly ($p < 0.05$) lower (111.87 ± 20.95 μ m) than that in NC group (595.67 ± 33.36 μ m) with approximately 82% reduction of the normal uterine diameter.

Six weeks of treatments with different concentrations of *F. deltoidea* leaves extract on PCOS rats, the mean diameters of uterine horns were found to be significantly ($p < 0.05$) higher than that of PC rats, and the increment was mostly pronounced in rats treated with 250 mg/kg bw of the extract with value 492.43 ± 14.84 μ m. PCOS groups treated with 25 mg/kg bw and 125 mg/kg bw of the extract had slightly lower mean diameter of uterine horn (378.40 ± 6.31 μ m and 473.33 ± 43.68 μ m respectively) than that observed in PFD250 rats (492.43 ± 14.84 μ m). PCC rats treated with 10 mg/kg bw clomiphene citrate, however, showed less effect, in which the mean diameter of uterine horns was 388.57 ± 14.49 μ m, a value which was lower than any of the *F. deltoidea* leaves extract-treated groups, significantly lower than the NC group but higher than that of the PC group. Fig. 1 shows the photomacrographs of both organs that shows how uterine diameter was measured, while Fig. 2 shows photomicrographs representatives of uterine histology. No significance change on uterine structure was observed except for the difference in

uterine diameter among experimental animals due to the respective treatments.

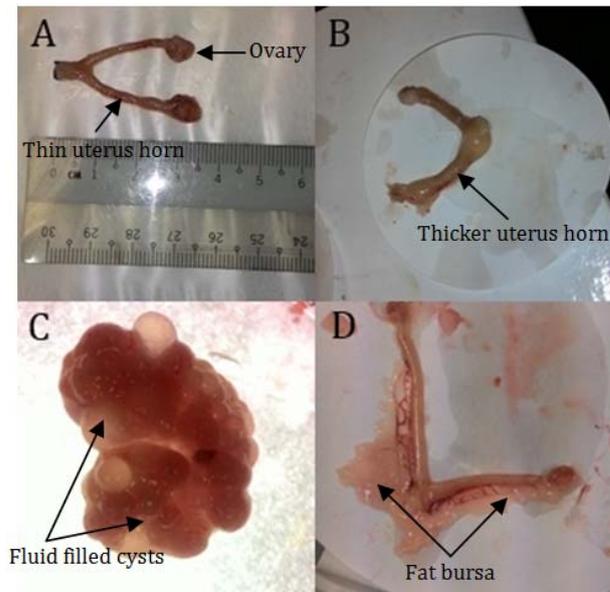


Fig. 1: Macroscopic observation on uterus and ovary

Photomacrographs of uterus and ovary retrieved at the end of experiment. Fig. 1A, thin uterus of rat from letrozole induced (PC) group; Fig. 1B, thick uterus retrieved from NC rat, after uterine luminal fluid was removed; Fig. 1C, fluid-filled-cysts were observed from ovary of PC rat under stereoscopic microscope, (8x), showing many fluid filled cysts, while Fig. 1D, uterus retrieved from rat treated with 250 mg/kg bw *F. deltoidea* leaves extract, surrounded by fat bursa.

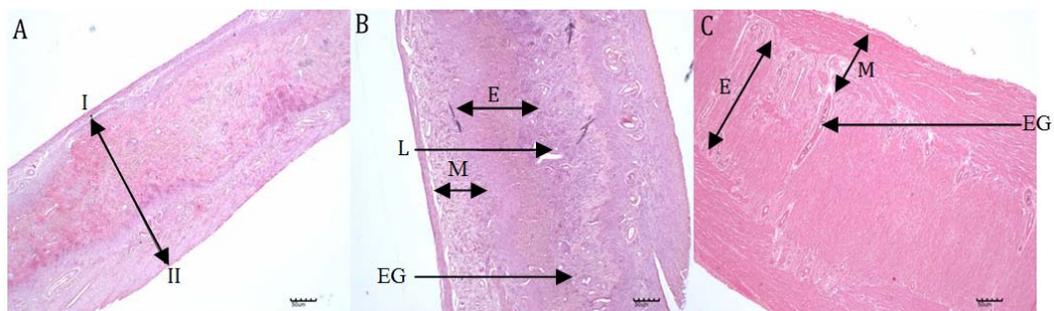


Fig. 2: Photomicrographs of uterus at 40x

Fig. 2A, uterus from PCOS induced rat; Fig. 2B, uterus from PFD250 rat and Fig. 2C, uterus from NC rat. Diameter of uterine horn is measured from point I to II on the captured image as in Fig. 2A. Abbreviation as shown on the images: Lumen (L), endometrium (E), myometrium (M), and endometrial gland (EG). The scale bar represented 50 μ m.

3.4. Ovarian histology: *F. deltoidea* leaves extract improves ovarian structure in PCOS rats

Multiple atretic follicles showed in Figs. 3A and 3B were the evidence of PCOS ovary with several

large cysts and scant granulosa cells were observed in the cortex region of ovary from letrozole-induced PCOS rats. These stages of atretic follicles were classified as severe category. In addition to marked atresia, luteal tissue was absent (Fig. 3A). In contrast, ovaries from NC rats exhibited follicles in various stages of development including primary, secondary and graafian follicles as well as newly formed corpora lutea (Figs. 3C and 3D). Ovaries from clomiphene citrate treated rats (PCC) on the other hand showed moderate ovarian recovery, since many cystic follicles were still present even though regression of corpora lutea were evidenced (Fig. 3E). Meanwhile, treatment with *F. deltoidea* leaves

extract resulted in marked recovery of ovary. Inside it there were healthy growing follicles of various stages of development with less cystic follicles. The recovery was mostly pronounced in ovaries of rats treated with 250 mg/kg bw *F. deltoidea* leaves extract, which showed the least number of cystic follicles and thicker granulosa and theca layers with

normal stages of follicular development (Fig. 3H). On the other hand, photomicrographs from ovaries of rats treated with 25 mg/kg bw (Fig. 3F) and 125 mg/kg bw of *F. deltoidea* leaves extract (Fig. 3G) revealed minimal changes in the ovarian follicular cyst number with fewer corpora lutea structures.

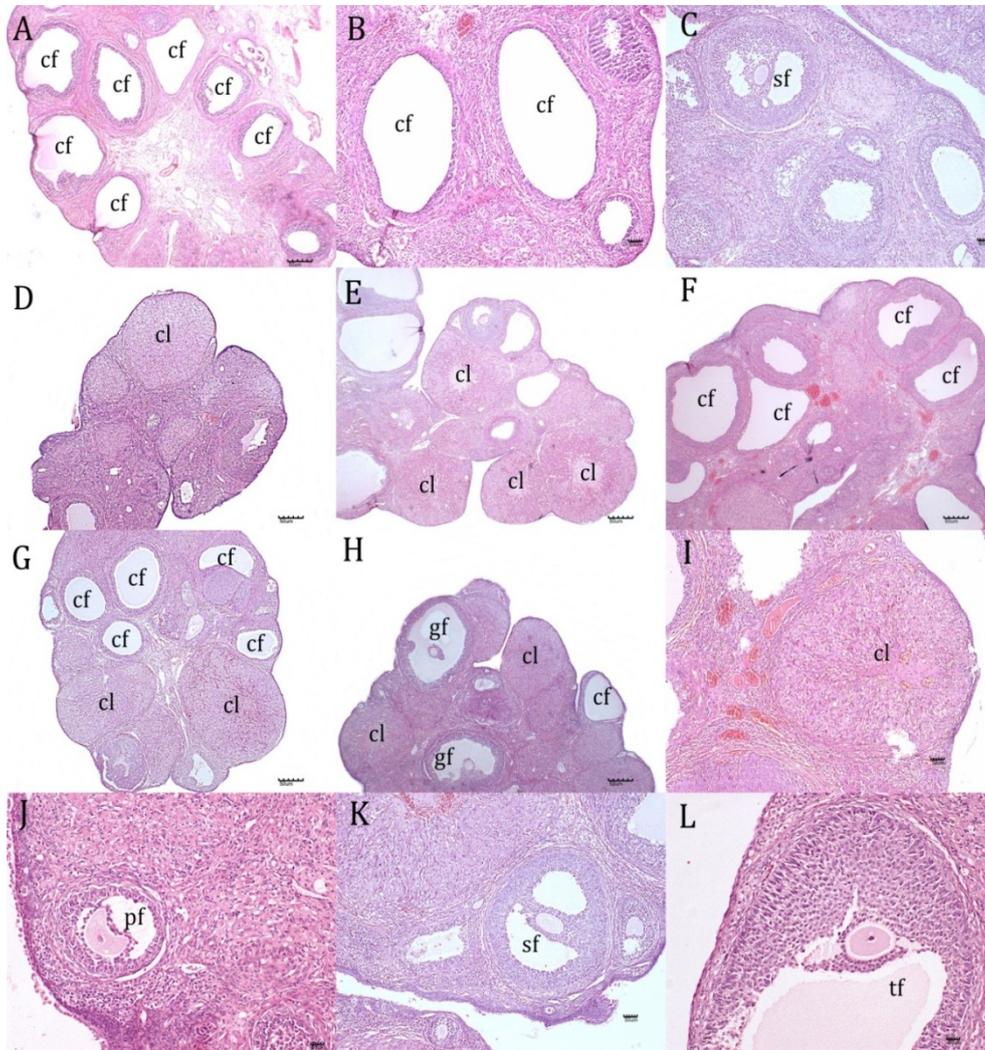


Fig. 3: Photomicrographs of ovaries from all experimental groups (n=3)

Fig. 3A is cross section of ovary from PC group which shows large number of cystic follicles (cf) with thin granulosa layers (40x); Fig. 3B, enlarged section of cystic follicle structure from Fig. 3A (100x). Fig. 3C is section of ovary from NC rat exhibiting normal secondary follicle (sf) with defined granulosa surrounding an oocyte and small antrum (40x); Fig. 3D, is section of normal ovary showing regression of empty follicles after ovulation, the corpus luteum (cl), (40x); Fig. 3E, is section of ovary from PCC group, showing a number of fresh luteal tissue (40x); Fig. 3F is section of ovary from PFD25 rat, showing the least recovery as numerous clear cystic follicles; Fig. 3G is section of ovary from PFD125 rat with corpora lutea structures and few cystic follicles still present; Fig. 3H is section of ovary from PFD250 rat showing significance diminished number of cystic follicles, with a number of regressed luteal tissue (corpora lutea), and graafian follicles (gf), with intact

oocytes surrounded by cumulus mass (40x); Fig. 3I - 3L are sections of ovaries from PFD250 rats showing developing follicles at various stages (100x); newly formed corpus luteum (Fig. 3I), primary follicle (pf) with proliferating layer of granulosa (Fig. 3J), secondary follicle with more layers of granulosa (Fig. 3K) and preovulatory tertiary follicle (tf), with big antrum and oocyte surrounded by cumulus cells (Fig. 3L). The scale bar represented 50 μ m.

4. Discussion

Letrozole-induced PCOS model impressively recapitulates endocrine, ovarian and metabolic aspects of the syndrome (Kauffman et al., 2015) and the model have been accepted universally. Letrozole is a non-steroidal aromatase inhibitor, which leads to suppression in estradiol production due to deficiency in activity of aromatase that would trigger

reasonable intraovarian disturbances in steroidogenesis expected to results in increased androgen production and PCOS development (McNeilly and Colin Duncan, 2013).

Ovarian weight in letrozole-induced PCOS rats was higher than normal rats. This finding is in line with other reports (Padmanabhan and Veiga-Lopez, 2013; McNeilly & Colin Duncan, 2013; Mannerås et al., 2007). Treatment of letrozole-induced PCOS rats with *F. deltoidea* ethanolic leaves extract had reduced the mean ovarian weight in dose dependent manner implies *F. deltoidea* leaves could prevent further increase in the ovarian weight in PCOS animal model, which might be related to the reduction in the number of cystic follicles developed within ovarian tissue.

On the other hand, significant reduction of uterine weight which was lower than the normal rats was observed in letrozole-induced rats. This problem could be due to the presence of inadequate level of circulating estrogen in the animal model as letrozole suppressed the activity of aromatase in biosynthesis of estrogen from androgens. Estrogen plays a pivotal role in stimulating growth of uterine thickness during estrous cycle in preparing uterus for implantation after a successful fertilization. Treatment with *F. deltoidea* ethanolic leaves extract however, has led to significant increase in uterine weight in a dose dependent manner suggesting the active compounds in *F. deltoidea* leaves possess phytoestrogenic property. Indeed, a healthy and normal sized uterus (particularly endometrium) is crucial for implantation and growth of fetus (Gleicher et al., 2011; Weissman et al., 1999).

Folliculogenesis is an ordered process, in which primordial follicles are recruited into cohorts of growing follicles from which one follicle is selected to develop mature oocyte under the influence of complex hormonal and intraovarian paracrine signals creating changing intrafollicular hormonal milieu. The events include appropriate cumulus cell-oocyte signaling that governs oocyte competence to complete meiosis, becomes capable of fertilization and initial embryonic development to achieve live births (Dumesic et al., 2008). However, many of these mechanisms are perturbed in PCOS.

Meanwhile, the ovary of letrozole-induced PCOS rats was evidenced with many cysts and thin granulosa cells, in which according to Baravalle et al. (2006), the cyst formed from antral follicles by apoptosis of the oocytes and granulosa cells with epithelization of basal layer of granulosa that escapes apoptosis. No corpus luteum was observed. These histological findings are indicative of biologically active FSH, LH, and lack of interplay between granulosa cells and theca cells, which would otherwise lead to ovulation (Kafali et al., 2004). Some reports showed the evidence of cysts could be due to endocrine imbalances in hypothalamus-pituitary-ovary (HPO) axis (Baravalle et al., 2006). Absence or diminishing numbers of corpora lutea with prolonged anestrus indicated anovulation (Jadhav et al., 2013), were both found in letrozole-

induced (PC) rats. Treatment with *F. deltoidea* leaves extract showed marked recovery in ovarian tissue as least number of cystic follicles was recorded in all treated groups with increasing number of corpora lutea and various stages of follicles development appeared in the ovary.

5. Conclusion

Treatment of PCOS-induced rats with *F. deltoidea* ethanolic leaves extract; especially with 250 mg/kg bw exhibited significant recovery in both ovarian and uterine tissues structure and function. In summary, *F. deltoidea* leaves might be beneficial in the prevention and maintenance of PCOS. Findings in the current study may provide primary information for designing further investigation on the PCOS ameliorating the potential of *F. deltoidea* as a natural option to combat the syndrome.

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