4 Role of non-polar extract of *Aloe vera* gel on early pregnancy in letrozole-induced PCOS mouse model

4.1 Rationale of the study

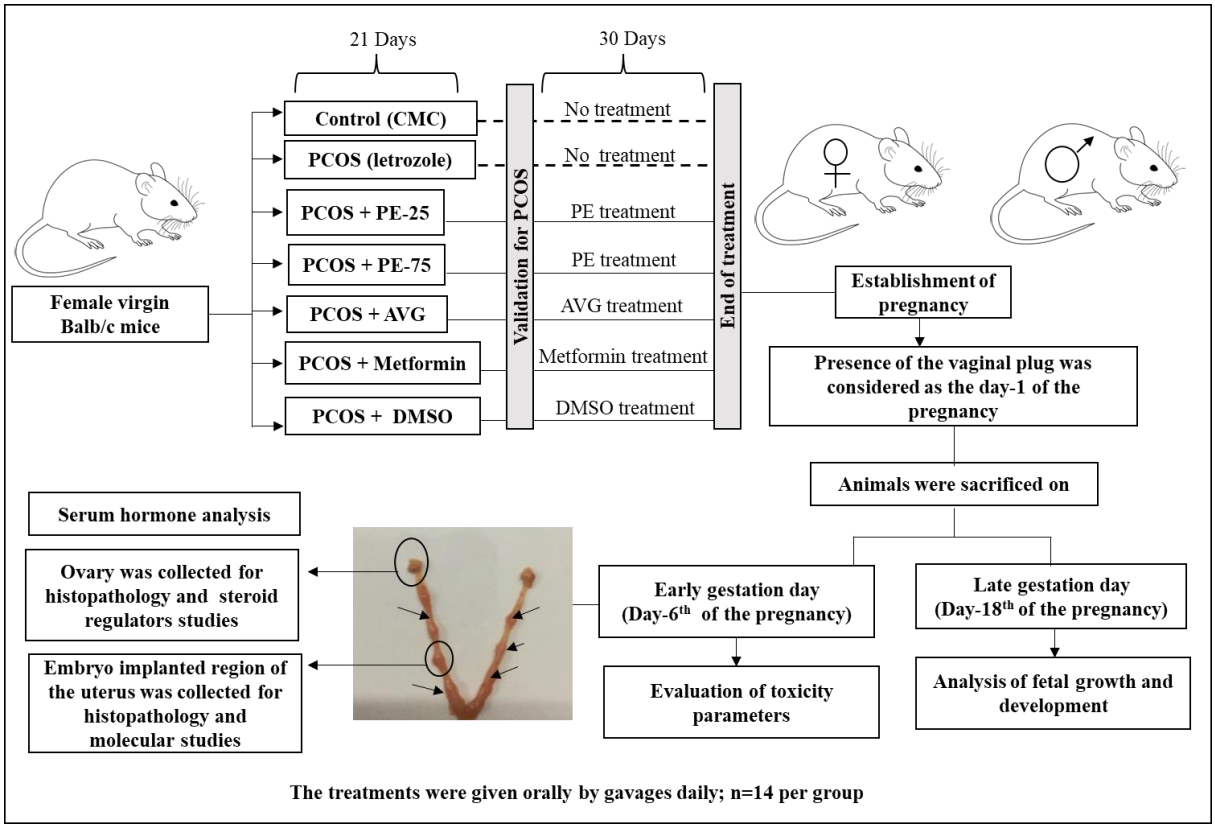
Data from the previous chapter have clearly demonstrated the molecular alterations underlying the early pregnancy loss of PCOS animals wherein the expression of ovarian regulators (*Lhr*, *Star*, *Cyp17a1*, *Cyp19a1*, *Ar*, *Pr*, *Esr1* & *2*) and early pregnancy markers (*Itga4*, *Itgb1*, *Hox10a*, *Mmp9*, *Timp3*, *Gp130*, *Stat3*, *Ar*, *Pr*, *Esr1* & *2*) were modulated in embryo implanted region of the uterus; translated as a poor fetal outcome. These markers try to establish an appropriate milieu that is crucial for the development and survival of the fetus. Thus, targeting them for therapeutic interventions could help in better pregnancy outcomes in PCOS phenotype. PCOS women who wish to conceive are pharmacologically approached by induction of ovulation (e.g, Clomiphene citrate) and insulin-sensitizing agents (e.g, Metformin) (Artini et al., 2018). Although, the use of these synthetic drugs is disputable due to chronic effects upon prolonged usage (Jorquera et al., 2020). Hence, the use of traditional medicine systems is currently being researched. In this context, *Aloe barbadensis* is used for female reproductive disorders (Sheba & S, 2021). However, scientific elucidation of *Aloe vera* gel for its role as a pre-conceptive agent remains undetermined.

In this context, the herb *Aloe vera* has been explored in our laboratory wherein, we demonstrated that *Aloe vera* gel (AVG) was efficient in controlling PCOS in terms of ovarian structure-function (Maharjan et al., 2010; Radha et al., 2014) and its complications (Desai et al., 2012d). Further, extraction of AVG using different solvent systems demonstrated that phytosterol containing a non-polar fraction of *Aloe vera* gel (25 µg/kg of body weight for 60 days) could modulate steroidogenic targets in the nonpregnant letrozole-induced PCOS rat model (Radha & Laxmipriya, 2016b). It is interesting to note that insufficient scientific data regarding the treatment of PCOS phenotype with indigenous plants when used as a pre-conceptive therapy. With this background, preliminary data observed that a non-polar fraction of *Aloe vera* gel (25 µg/kg of body weight for 30 days) could improve fetal growth and development on the day 18th of pregnancy in the letrozole-induced PCOS mouse model. This suggests that phytosterols present in the non-polar extract of AVG could direct the early gestation events to form proper fetus development. However, it is important to point out that

in silico or *in vitro* analysis could not answer the bioactivity of phytosterol during the pregnancy phase, as pregnancy is a holistic system whereas molecular crosstalk and overexpression of various proteins make it challenging to identify the role of phytocomponents of AVG in PCOS pregnancy. Therefore, extraction of the non-polar phytocomponents of AVG was carried out using petroleum ether by the Soxhlet method, and pivotal molecules of early pregnancy (Day-6th of pregnancy) were studied using *in vivo* mice model. Thus, the current study is directed toward investigating the probable molecular cascade to decipher the role of phytocompounds in the rectification of early pregnancy loss and may act as a pre-conceptive agent in the letrozole-induced PCOS mouse model. This modulation could result in the improvement of the embryonic-uterine network leading to better fetal outcomes with minimum side effects.

4.2 Material and methods

The letrozole-induced PCOS mouse model was developed and validated (Explained in chapter 3). After the validation of the model, the Petroleum ether extract of *Aloe vera* was prepared (Detailed methodology for the preparation of the plant extract is provided the chapter 2). Further, for evaluating the effect of the PE extract of AVG as a pre-conceptive agent on several parameters as previously explained in chapter 3, the animals were allocated into 7 groups as shown in figure 4.1. Moreover, the drug safety parameters were analysed after the treatment of the PE extract of AVG. Parallel to the *in vivo* experiment, characterization of the phytocompounds present in the PE extract of AVG was carried out using GC/MS analysis (The methodology described in chapter 2).



Control group: Received 1% carboxymethyl cellulose (CMC) daily for 21 days and served as untreated control.

PCOS group: 0.5 mg/kg/day letrozole daily for 21 days and served as untreated PCOS.

PCOS + PE-25 group: PCOS animals treated with petroleum ether extract of *Aloe vera* gel (25µg/kg/day) for 30 days.

PCOS + PE-75 group: PCOS animals treated with petroleum ether extract of *Aloe vera* gel (75µg/kg/day) for 30 days.

PCOS + AVG group: PCOS animals treated with *Aloe vera* gel (10 mg/day) for 30 days.

PCOS + Metformin group: PCOS animals were treated with Metformin (100 mg/kg/day) for 30 days and served as a positive control (Dey et al 2022).

PCOS + DMSO group: PCOS animals treated with DMSO (0.1%, 0.3 ml/day) for 30 days and served as vehicle/negative control.

Figure 4.1. Plan of work for evaluating the bioactivity of PE extract of AVG as a pre-conceptive agent in a letrozole-induced mouse model.

The dose selected for the study was based on previous lab studies wherein 25µg/kg/day was efficacious on female PCOS endocrinopathy (A. Dey et al., 2022). After the respective treatment regime, animals were sacrificed on the day-6^{th} & 18^{th} of the pregnancy, blood was collected and tissues were excised for molecular and toxicology studies.

4.3 Results

4.3.1 Phytochemical analysis of PE extract of AVG

Firstly, the lyophilized *Aloe vera* gel powder was extracted with a non-polar solvent (Petroleum ether - PE), and Phytochemical screening of the non-polar extract exhibited the presence of Phytosterols, Glycosides, and Terpenoids (Data not shown). The yield of the extract is 5.286%. The total phytosterols in the PE extract of AVG were found to be 771.8 ± 42.04 µg/g (Table 4.1).

Table 4.1. % Yield, nature, and quantitative estimation of the phytosterol in the Petroleum ether extract of *Aloe vera* gel.

	Petroleum ether extract of *Aloe vera* gel
Colour	Dark greenish yellow
Odour	Characteristic
Consistency	Sticky
% Yield dry weight (mg)	5.286%
Phytosterol concentration (µg/g)	771.8 ± 42.04 (n=3)

4.3.2 Gas Chromatography-Mass Spectroscopy (GC/MS) analysis

The non-polar phytocompounds from petroleum ether extract of *Aloe vera* were identified by GC-MS analysis based on a comparison of their retention time (min), peak area, peak height, and mass spectral patterns with the spectral database of authentic compounds stored in the National Institute of Standards and Technology (NIST) library. The report of GC/MS data reveals the name of the compound, the retention times, molecular formula, molecular weight, and peak area (%) of components in the PE extract of AVG (Table 4.2). The GC-MS chromatogram of petroleum ether extract of *Aloe vera* gel is shown in figure 4.2. Data from the GC-MS results revealed that the γ-Sitosterol, n-Hexadecanoic acid, Oleic acid, 9,12-Octadecanoic acid, Campesterol, and β sitosterol are present in the PE extract of AVG with an abundance of 45.25 %, 29.48 %, 5.40 %, 4.44 %, 3.60 %, and 2.53 % respectively.

Table 4.2. Phytochemical constituents identified in the petroleum ether extract of *Aloe vera* gel using gas chromatography-mass spectrometry. CAS: chemical abstract service.

Sr. no	RT (min)	Name of the compound	CAS	Molecular formula	Molecular weight	Peak Area%
1	5.780	Dodecanoic acid	143-07-7	$C_{12}H_{24}O_2$	200.32	0.14
2	5.854	Nonanedioic acid, dimethyl ester	1732-10-1	$C_{11}H_{20}O_4$	216.27	0.16
3	7.915	Tetradecanoic acid	44-63-8	$C_{14}H_{28}O_2$	228.37	0.96
4	8.927	Pentadecanoic acid	1002-84-2	$C_{15}H_{30}O_2$	242.4	0.31
5	9.375	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	82304-66-3	$C_{17}H_{24}O_3$	276.4	0.13
6	9.751	cis-Vaccenic acid	506-17-2	$C_{18}H_{34}O_2$	282.5	0.34
7	10.113	n-Hexadecenoic acid	57-10-3	$C_{16}H_{32}O_2$	256	29.48
8	10.659	Eicosanoic acid	506-30-9	$C_{20}H_{40}O_2$	312.5	0.47
9	10.897	Heptadecanoic acid	506-12-7	$C_{17}H_{34}O_2$	270.5	0.34
10	11.576	9,12-Octadecadienoic acid	60-33-3	$C_{18}H_{32}O_2$	280.4	4.44
11	11.648	Oleic Acid	112-80-1	$C_{18}H_{34}O_2$	282.5	5.40
12	11.834	Octadecanoic acid	57-11-4	$C_{18}H_{36}O_2$	284.5	0.91
13	13.095	Stearic acid, 2-hydroxy-1-methylpropyl ester	14251-39-9	$C_{22}H_{44}O_3$	356.6	0.23
14	14.463	9,12-Octadecadienoic acid (Z,Z)-	60-33-3	$C_{18}H_{32}O_2$	280.4	0.26
15	16.537	Erucylamide	112-84-5	$C_{22}H_{43}NO$	337.6	0.16
16	19.260	Stigmasterol acetate	4651-48-3	$C_{31}H_{50}O_2$	454.7	0.57
17	19.470	β-Sitosterol	83-46-5	$C_{29}H_{50}O$	414.7	0.23
18	19.725	Cholestane-3,5-diol, 5-acetate, (3β.,5α)-	41721-93-1	$C_{29}H_{50}O_3$	446.7	0.55

19	20.236	Cholest-8-en-3-ol, (3β)-	7199-91-9	$C_{27}H_{46}O$	386.7	0.15
20	20.892	Campesterol	474-62-4	$C_{28}H_{48}O$	400.7	3.60
21	21.520	γ-Ergostenol	516-78-9	$C_{28}H_{48}O$	400	0.53
22	21.748	Stigmasta-7,25-dien-3-ol, (3β.,5α)-	6785-58-6	$C_{29}H_{48}O$	412.7	0.40
23	22.179	γ-Sitosterol	83-47-6	$C_{29}H_{50}O$	414	45.25
24	22.839	β-Sitosterol	83-46-5	$C_{29}H_{50}O$	414.7	2.53
25	26.516	1,4-Epoxynaphthalene-1(2H)-methanol, 4,5,7-tris (1,1-dimethyl ethyl)-3,4-dihydro-	56771-86-9	$C_{23}H_{36}O_2$	344.5	2.45

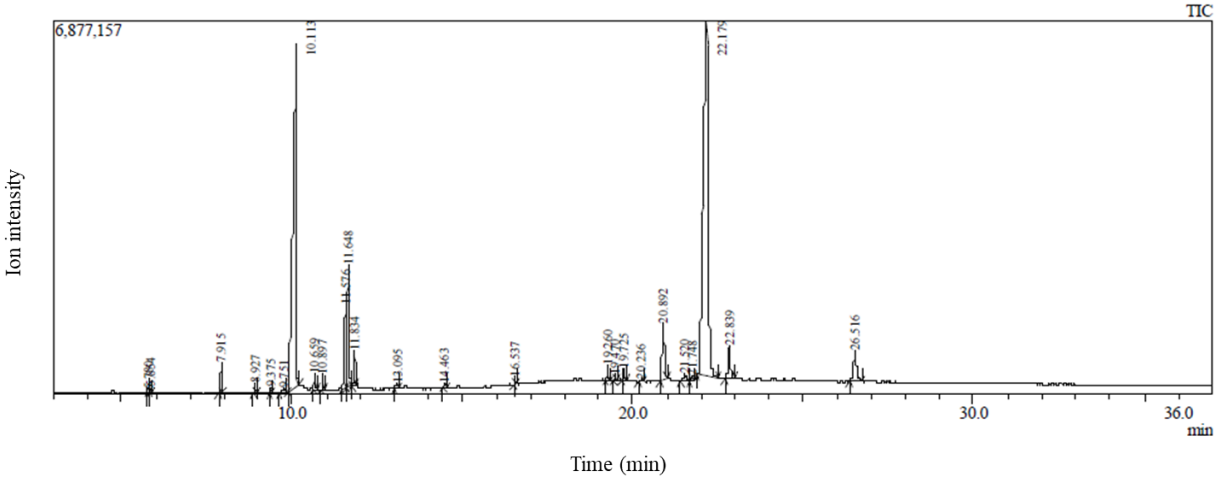


Figure 4.2. The chromatograph of petroleum ether extract (PE) of *Aloe vera* gel. The x-axis and y-axis represent the retention time and ion intensity respectively.

Moreover, the late gestation studies in the previous chapter have clearly indicated abnormal pregnancy outcomes in the letrozole-induced PCOS mouse model. Therefore, the efficacy of the PE extract of AVG was evaluated during the late gestation period of the letrozole-induced PCOS mouse model. The plan of work is provided in figure 4.1.

4.3.3 Effect of PE extract of AVG on fetal growth and development during the late gestation period in PCOS mice

The body weight of animals throughout the gestation period was monitored and the number, weight, and morphology of fetuses have been examined. During the late gestation period, the body weight of letrozole-treated animals was found to be gradually decreased while the treatment of the Metformin and PE extract of AVG (PE-25 and PE-75) in the PCOS animals exhibited a significant increase in the body weight (Figure 4.3). Moreover, the PCOS animals exhibited retarded fetal growth, and development with the decreased (**P<0.01) weight and a number of growing fetuses compared to the control animals. Treatment of PCOS animals with metformin, AVG, and PE extract of AVG treatment (PE-25 & 75) showed healthy growing fetus with increased ($^{\$\$\$}$P<0.001) in their numbers compared to the PCOS animals (Figure 4.4 and 4.5).

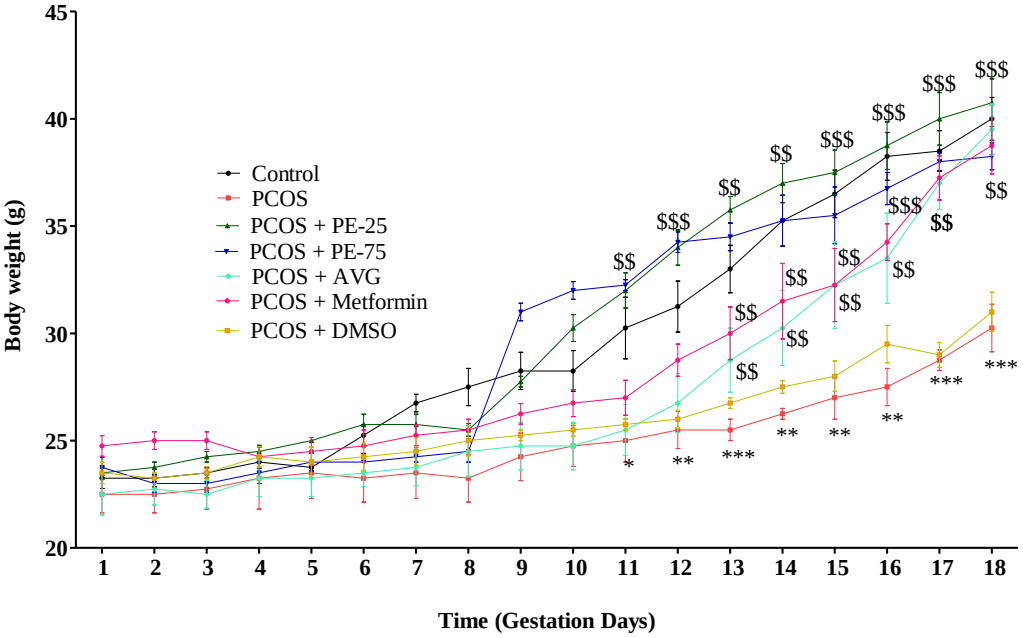


Figure 4.3. Effect of PE extract of AVG on Body weight throughout gestation period in letrozole-induced PCOS mice model. All the values are represented as mean ± SEM; N = 6 per group; **P < 0.01; ***P < 0.001 as compared to the control group, $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001 as compared to PCOS group.

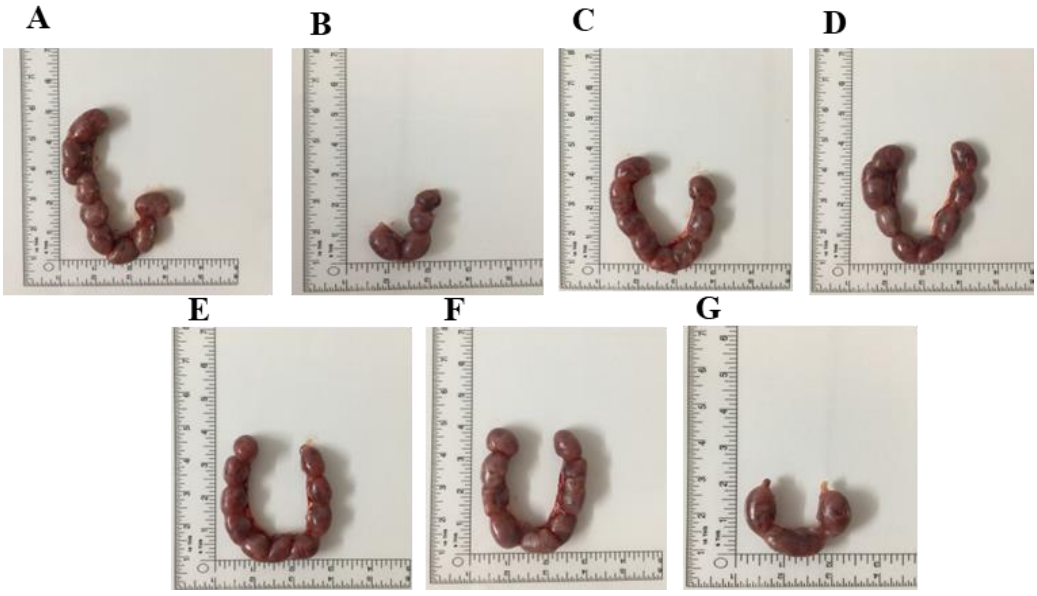


Figure 4.4. Effect of PE extract of AVG on fetal growth and development (Day 18th of pregnancy) in the letrozole-induced PCOS mice model. **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO.

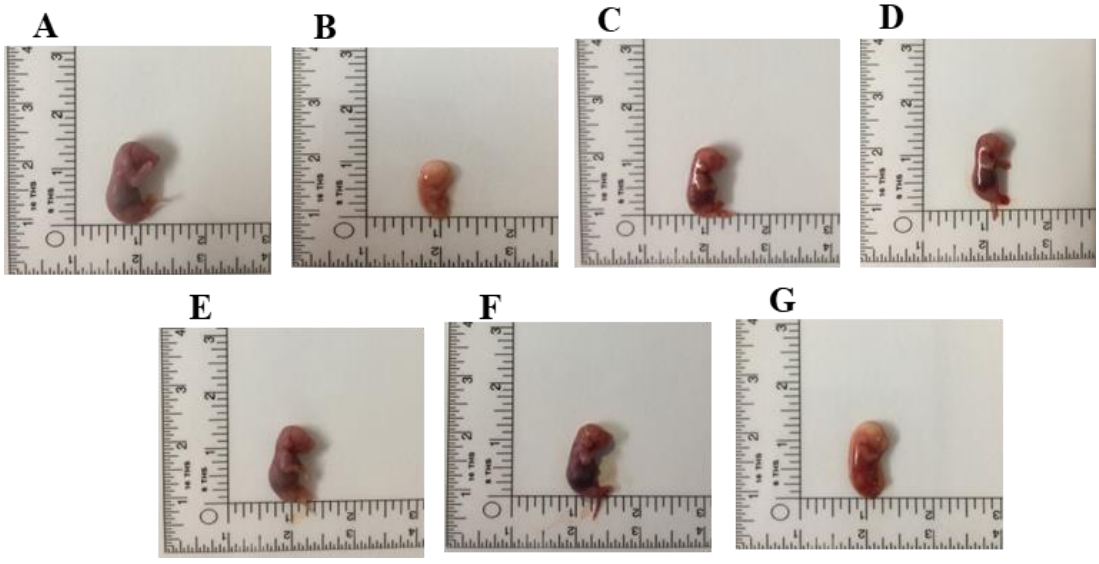


Figure 4.5. Effect of PE extract of AVG on pups growth (Day 18th of pregnancy) in the letrozole-induced PCOS mice model. **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO.

These results demonstrated that a PE extract of AVG for 30 days could improve fetal growth and development in the letrozole-induced PCOS mouse model. The results are comparable to the metformin treatment (Standard drug used for the treatment of PCOS). This suggests that phytosterol present in the non-polar extract of AVG has the potential to rectify the uterine milieu for better fetal outcomes which could be due to consequence of corrected microenvironment from the implantation events. Thus, the molecular cascade involved in the establishment of early pregnancy was studied in the letrozole-induced PCOS mouse model.

To study the early gestation events, female mice from all the groups were kept for mating, and the presence of a vaginal plug was considered as the day 1^{st} of pregnancy. Further, the animals were sacrificed on the day-6^{th} of pregnancy (The plan of work is given in figure 4.1). Initially, the body weight, plasma hormone profile, blood glucose levels, and ovarian histology were studied. Further, to study the effect of PE of AVG on early pregnancy complications, reproductive performance, number, and histology of embryo implanted region of the uterus were analysed. The results of early gestation studies are explained below.

4.3.4 Effect of PE extract of AVG on early pregnancy loss (Day-6) of PCOS mice

Before the establishment of early pregnancy in all the groups, the effect of PE extract of AVG and Metformin treatment on the estrus cyclicity has been monitored. The control group of the animals exhibited a normal cycle while treatment with letrozole exhibited a disturbed estrus cycle. Notably, the treatment of the PCOS animals with PE extract of AVG completely reversed the disturbed estrus cycle to normalcy. Similar, results were observed in the AVG and Metformin-treated groups (Table 4.3). The disturbed estrus cycle in PCOS animals could be due to the altered steroid hormone profile (Testosterone, progesterone, and estradiol) in the PCOS group on day 6 of pregnancy. As shown in figure 4.6 the plasma testosterone in the letrozole-treated group was significantly elevated than that in the untreated group (**$P<0.01$), which can be correlated with hyperandrogenaemia in PCOS. Interestingly, a decline in progesterone levels was observed in PCOS mice (***$P<0.001$). However, Metformin and PE extract of AVG treatment (PE-25 and PE-75) in PCOS animals significantly reduced testosterone ($^{\$}P<0.05$) levels. Progesterone levels significantly improved upon metformin ($^{\$\$\$}P<0.001$) and PE extract of AVG treatment (PE-75 $^{\$\$}P<0.01$, PE-25 $^{\$\$\$}P<0.001$) in the PCOS group whereas estradiol levels remain unaltered amongst all the groups. Since it is well known that continuance of the steroid milieu is essential for the ovarian structure and function, the histology profile of the ovary using a hematoxylin-eosin stain was analyzed.

The control group showed normal ovarian morphology (Mature follicles and corpora lutea) whereas the PCOS animals demonstrate reduced mature follicles, multiple peripheral cysts, and fewer corpus luteum as compared to control animals. Treatment of PCOS animals with metformin, whole AVG, and PE extract of AVG (PE-25 & 75) showed healthy growing follicles, and corpora lutea with a few cystic follicles (Figure 4.7). In addition, PCOS is associated with hyperinsulinemia, hyperglycemia, and an increase in body weight. Thus, the effect of the PE extract of AVG was monitored on these parameters. The enhanced insulin (**P<0.01) and blood glucose levels (**P<0.01) were observed in the letrozole-treated animals. However, the metformin and plant extract treatment (AVG & PE extract of AVG) in the PCOS group significantly reduced the insulin (PE-75 $^{\$\$}$P<0.01, PE-25, metformin, AVG $^{\$\$\$}$P<0.001) and glucose levels (PE-25 $^{\$\$}$P<0.01, AVG and Metformin $^{\$}$P<0.05). Further, the body weight of animals during the early gestation period (Days 1 to 6) did not show a significant difference among all the groups (Figure 4.8).

Moreover, to study the effect of plant extract treatment on early pregnancy loss, reproductive performance, number, and histology of the embryo implanted region of the uterus were analyzed on the day 6th of pregnancy. It was seen that the number of pregnant females was reduced in letrozole induced PCOS mouse model. However, upon treatment with metformin and PE extract of AVG in PCOS animals, an increase in the number of pregnant females was observed. Also, a fewer number of embryo implants were observed in PCOS animals compared to control animals (**P<0.01). Metformin ($^{\$\$\$}$P<0.001) and PE extract of AVG treatment (PE-25 $^{\$}$P<0.05 PE-75 $^{\$\$}$P<0.01) in PCOS animals significantly increased in the number of embryo implantation sites (Table 4.4). Figure 4.9. demonstrates the pictorial representation of implanted embryos in the uterus of all the groups. Histology of embryo implanted region of the uterus demonstrates that the embryo has attached to antimesometrial uterine lumen epithelium (marked by black arrows) and is surrounded by developing decidual cells in the control group. However, the PCOS group exhibited an accumulation of erythrocytes (marked by black arrows) caused by a gain in vascular permeability. Metformin, AVG, and PE extract of AVG treatment (PE-25 and PE-75) in PCOS animals showed attachment of the embryo to the uterine wall (Figure 4.10).

Table 4.3. Effect of PE extract of AVG on estrus cyclicity of letrozole-induced PCOS mice.

	Control	**PCOS**	**PCOS + PE-25**	**PCOS + PE-75**	**PCOS + AVG**	**PCOS + Metformin**	**PCOS + DMSO**
Normal Cycle	100%	-	75%	62%	90%	77%	-
Extended Proestrus	-	-	17%	23%	-	15%	20%
Extended Estrus	-	-	-	-	-	-	-
Extended Metaestrus	-	35%	-	-	10%	8%	20%
Extended Diestrus	-	65%	8%	15%	-	-	60%

n = 14 per group

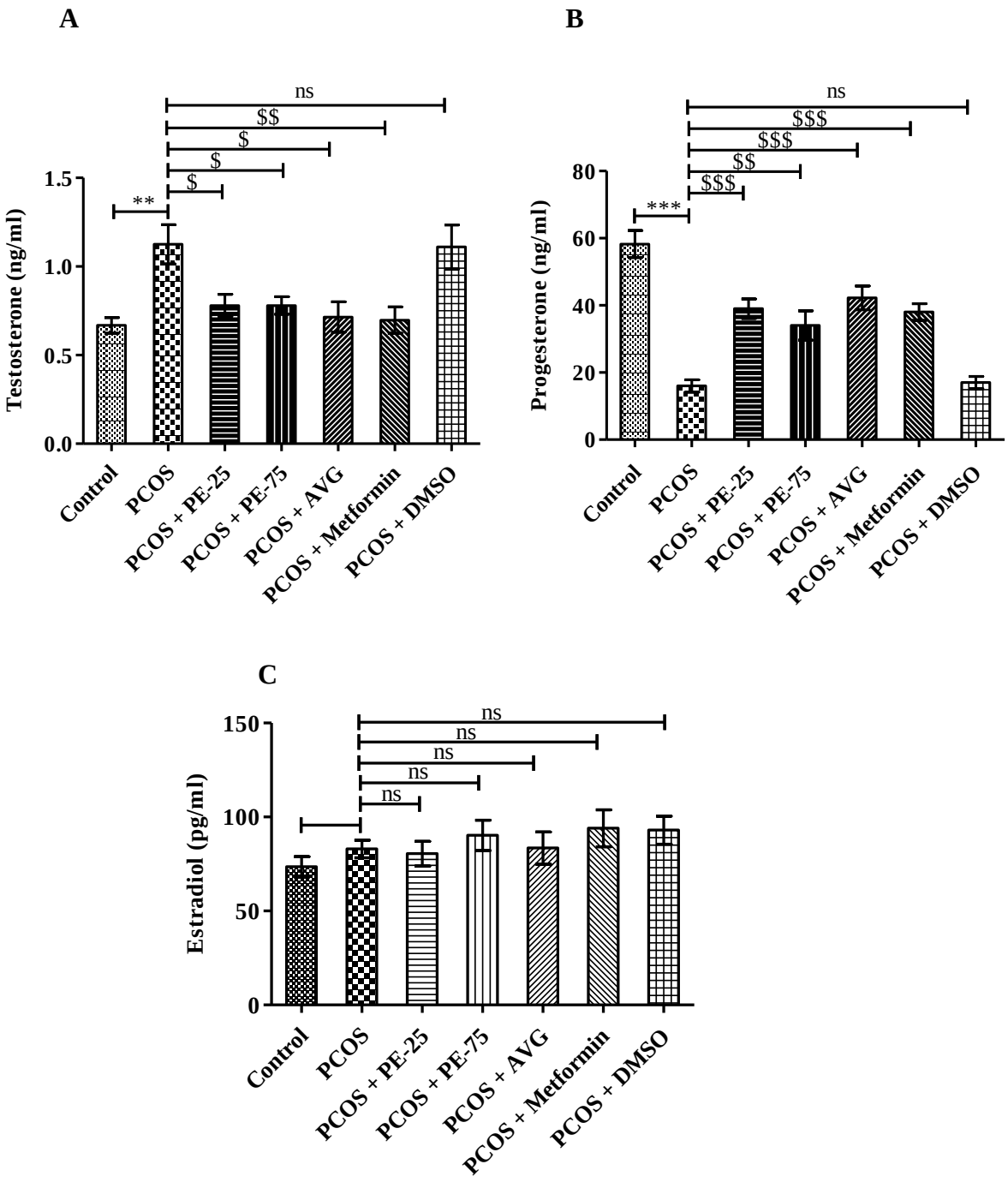


Figure 4.6. Effect of PE extract of AVG on steroid hormone profile on day 6th of pregnancy in the letrozole-induced PCOS mice model. **A.** Testosterone **B.** Progesterone and **C.** Estradiol. The values are represented as Mean ± SEM. N=6 per group. **P<0.01, ***P<0.001 as compared to control group; $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, ns-non-significant as compared to PCOS group.

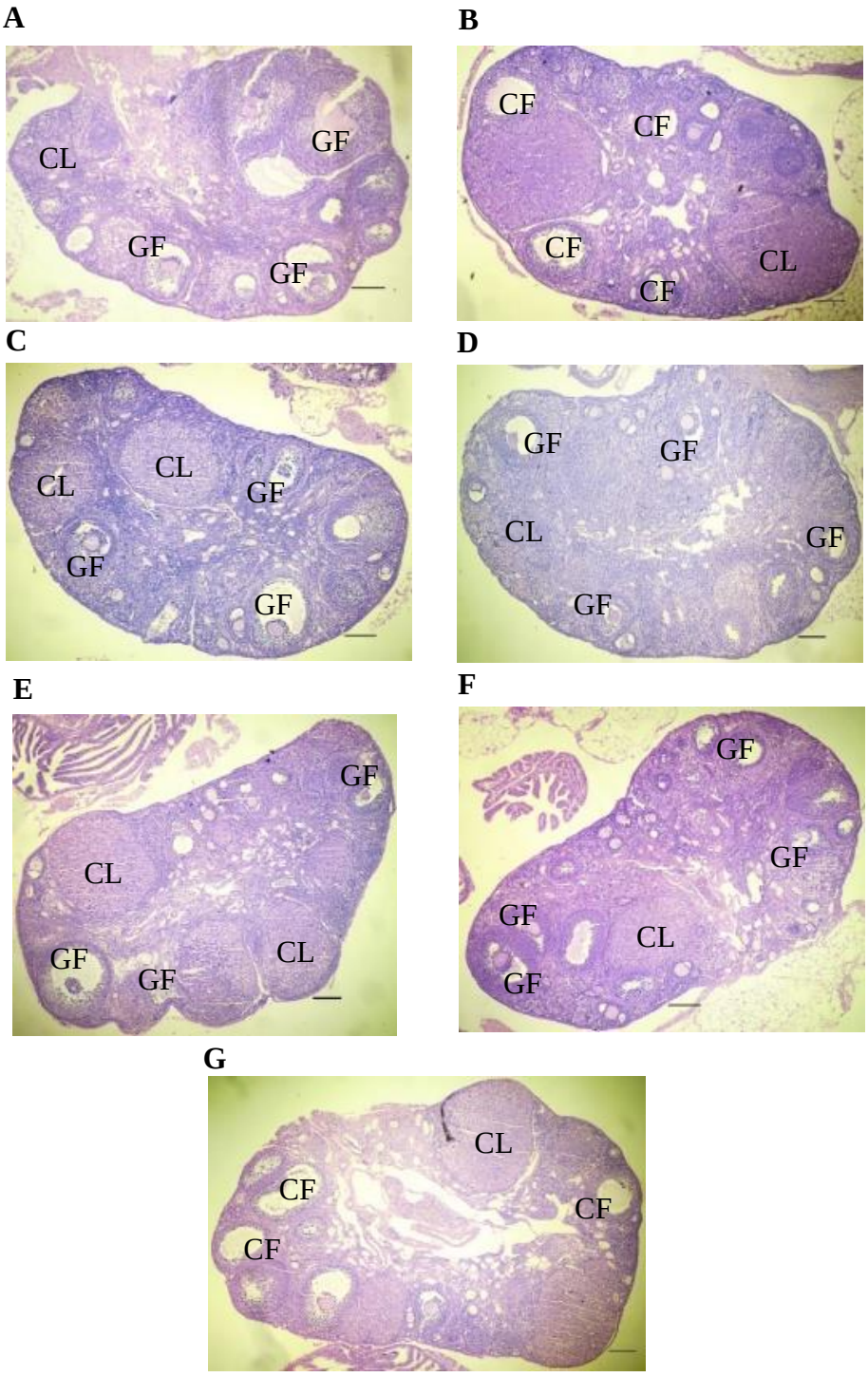


Figure 4.7. Effect of PE extract of AVG on ovarian histology (Day-6th of pregnancy) in the letrozole-induced PCOS mice model **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO. CL: corpus luteum; CF: cystic follicle; GF- Graafian follicle. Magnification 4X.

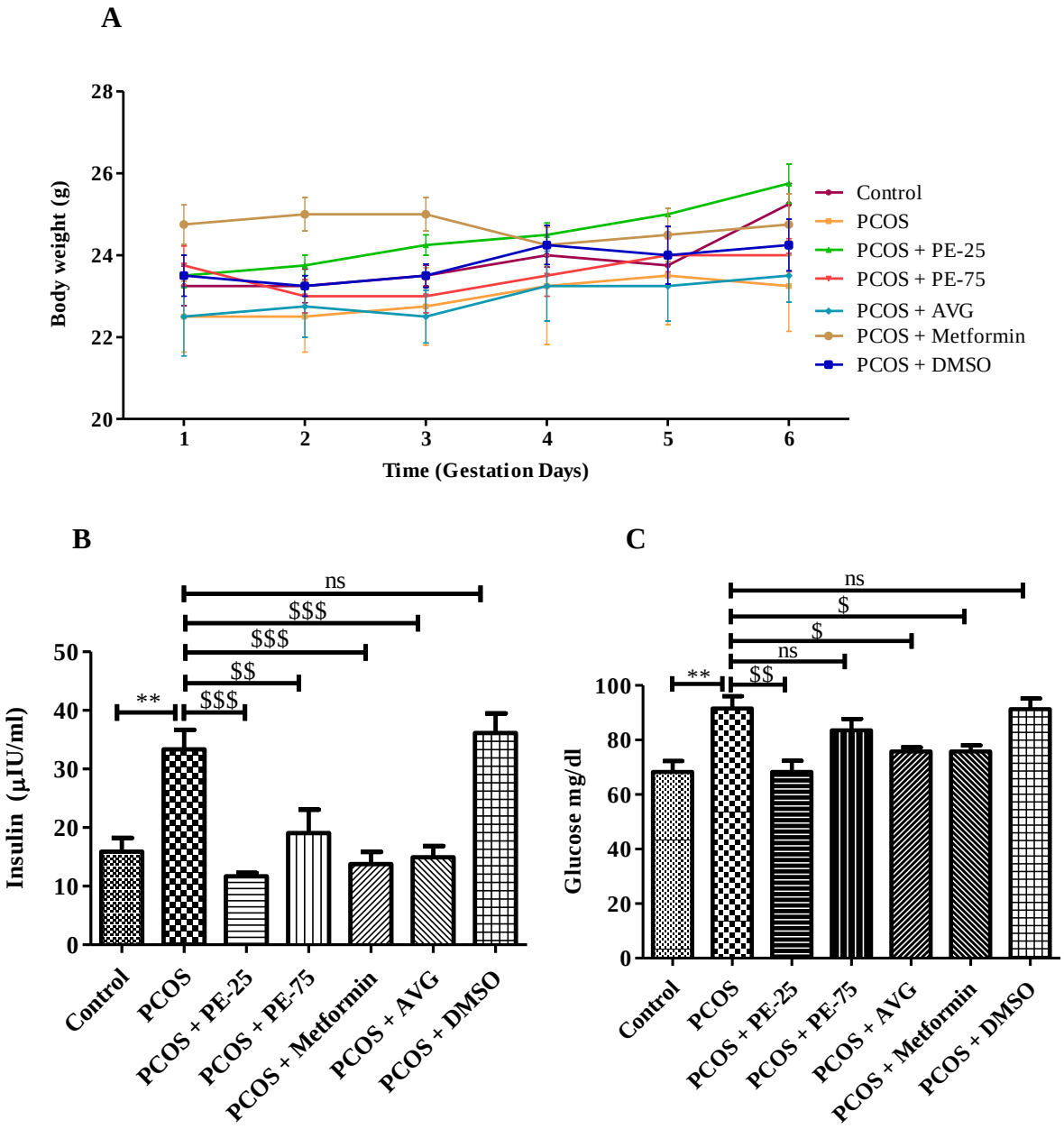


Figure 4.8. Effect of PE extract of AVG on the **A.** Changes in body weight during the early gestation period **B.** Insulin levels and **C.** Random blood glucose on the day 6th of pregnancy in the letrozole-induced PCOS mice model. All the values are represented as mean ± SEM; N = 6 per group; **P<0.01, as compared to the control group. $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, ns = not significant compared to the PCOS group.

Table 4.4. Effect of PE extract of AVG on reproductive performances for female fertility in the letrozole-induced PCOS mice model.

	Control	**PCOS**	**PCOS + PE-25**	**PCOS + PE-75**	**PCOS + AVG**	**PCOS + Metformin**	**PCOS + DMSO**
Females (n)	14	14	14	14	14	14	14
Mated females (n)	13	12	14	13	13	14	12
Pregnant Females (n)	12	8	13	12	11	12	9
Not pregnant females (n)	1	4	1	1	2	2	3
Time required for conception (in Days)	3.769 ± 0.2809	3.308 ± 0.3279 ns	2.462 ± 0.3323 ns	2.231 ± 0.3028 ns	2.231 ± 0.2571 ns	2.692 ± 0.3649 ns	2.308 ± 0.2627 ns
Mating Index* (%)	92.8571 ~93	85.7142 ~86	100	92.8571 ~93	92.8571 ~93	100	85.7142 ~86
Fertility Index (%)**	85.71429 ~86	57.14286 ~57	92.85714 ~93	85.71429 ~86	78.57143 ~79	85.71429 ~86	64.28571 ~65
Weight of Ovary (mg) (on day 6 of pregnancy)	8.833 ± 0.5239	7.867 ± 0.3383 ns	9.167 ± 0.3180 ns	8.133 ± 0.2728 ns	7.600 ± 0.6083 ns	7.867 ± 0.4372 ns	7.500 ± 0.6928 ns
Total number of embryo implants	9.000 ± 0.3162	5.800 ± 1.020 **	8.400 ± 0.4000 $	8.800 ± 0.4899 $$	9.200 ± 0.3742 $$	10.20 ± 0.3742 $$$	5.200 ± 0.8602 ns
Weight of embryo implanted site (mg)	9.433 ± 0.9387	8.302 ± 1.576 ns	9.333 ± 0.7126 ns	7.900 ± 0.6807 ns	8.100 ± 0.6083 ns	8.967 ± 0.8413 ns	8.526 ± 0.7311 ns

The values are represented as Mean ± SEM. **P<0.01, ns = not significant as compared to the control group; $P<0.05, $$P<0.01, $$$P<0.001, ns-non-significant as compared to PCOS

group. *Mating index = Mated females/ Total females kept for mating × 100. **Fertility index = Pregnant females/ Total females kept for mating females × 100.

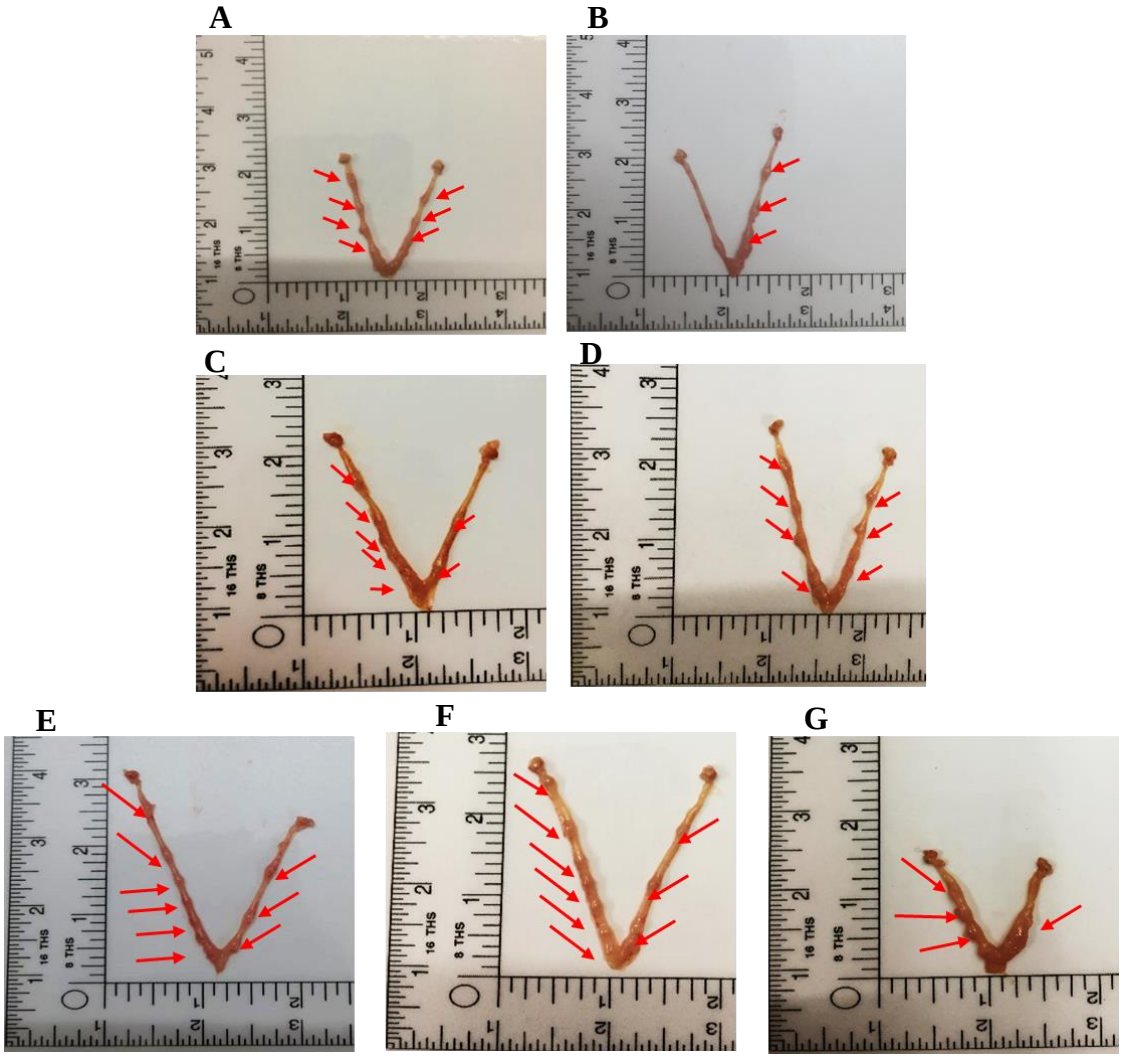


Figure 4.9. Effect of PE extract of AVG on a number of embryo implanted sites on day 6 of pregnancy in the letrozole-induced PCOS mice model. **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO. Arrows indicate the embryo implanted site of the uterus.

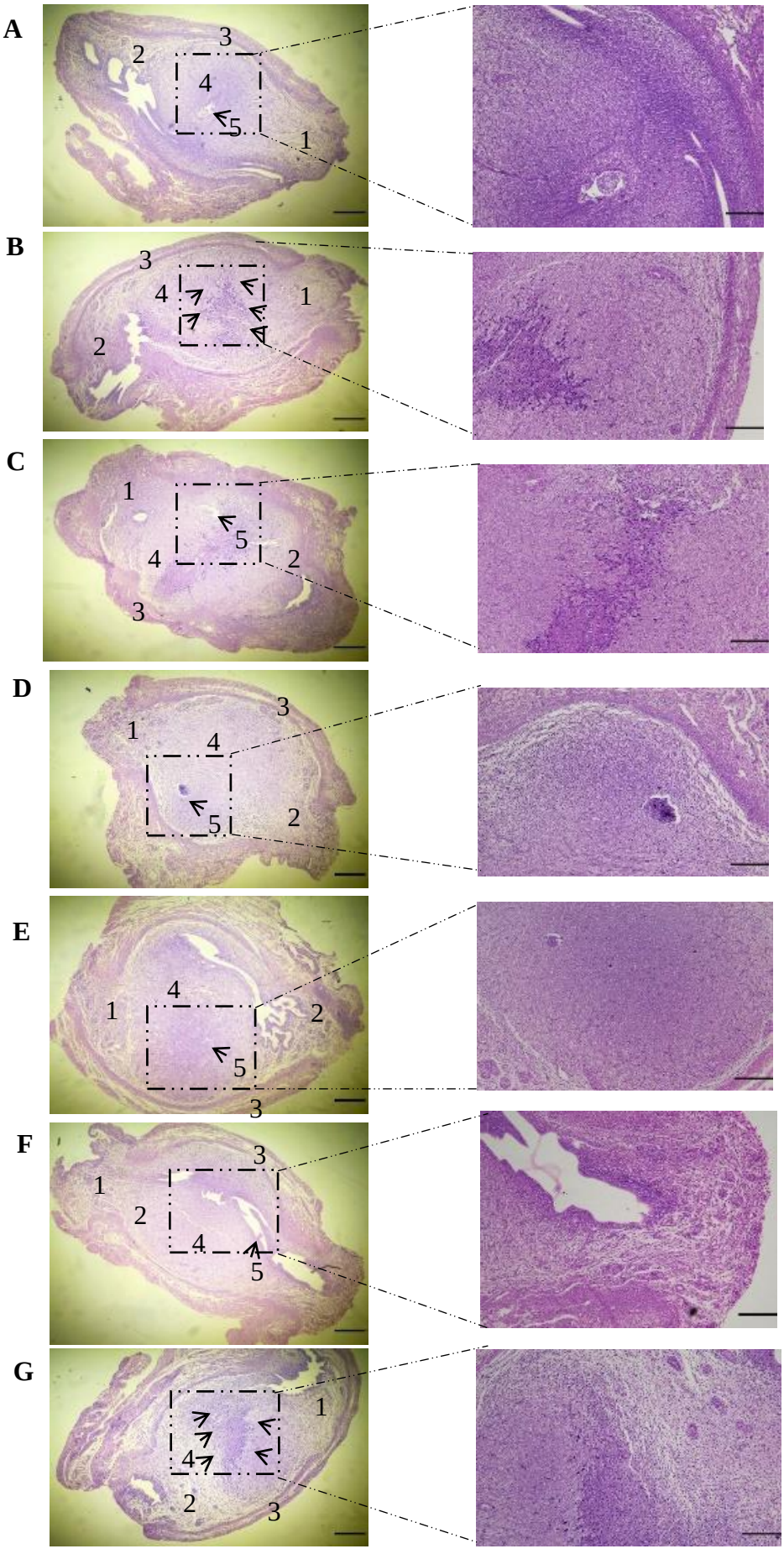

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Figure 4.10. Effect of PE extract of AVG on histology of embryo implanted region of the uterus on day 6 of pregnancy in the letrozole induced PCOS mice model. **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO. In the control group, the black arrows indicate embryo. In the PCOS & DMSO group, black arrows indicate the accumulation of erythrocytes. 1. Mesometrium. 2. Mesometrial endometrium. 3. Myometrium. 4. Anti-mesometrial decidua. 5. Embryo. Magnification 4X.

The above results signify the effect of PE extract of AVG on female reproductive dysfunction and its role in recovery from early pregnancy loss. The results are comparable to the positive control (Metformin) treatment used in the current experiments. Also, improved ovarian structure and steroid hormone production were observed on the day-6^{th} of pregnancy in PCOS animals. Therefore, in the next part of the study, the effect of plant extract on the key regulators of ovarian steroid production has been investigated.

4.3.5 Effect of PE extract of AVG on the ovarian gonadotropin receptors on day 6 of pregnancy in PCOS mice

Luteinizing hormone and Follicle-stimulating hormone (Gonadotropins) act on the ovaries via their receptors, LHR, Luteinizing Hormone Receptors, and FSHR, follicle-stimulating hormone receptors, respectively. Hence, in the ovary, gonadotropin receptor transcript levels were studied using quantitative real-time PCR. Transcriptional upregulation of *Lhr* (**P<0.01) was observed in the PCOS group compared to the control group with no difference in *Fshr*. Metformin and PE extract of AVG treatment in PCOS animals did not show a significant change in gene expression of *Lhr* and *Fshr* (Figure 4.11).

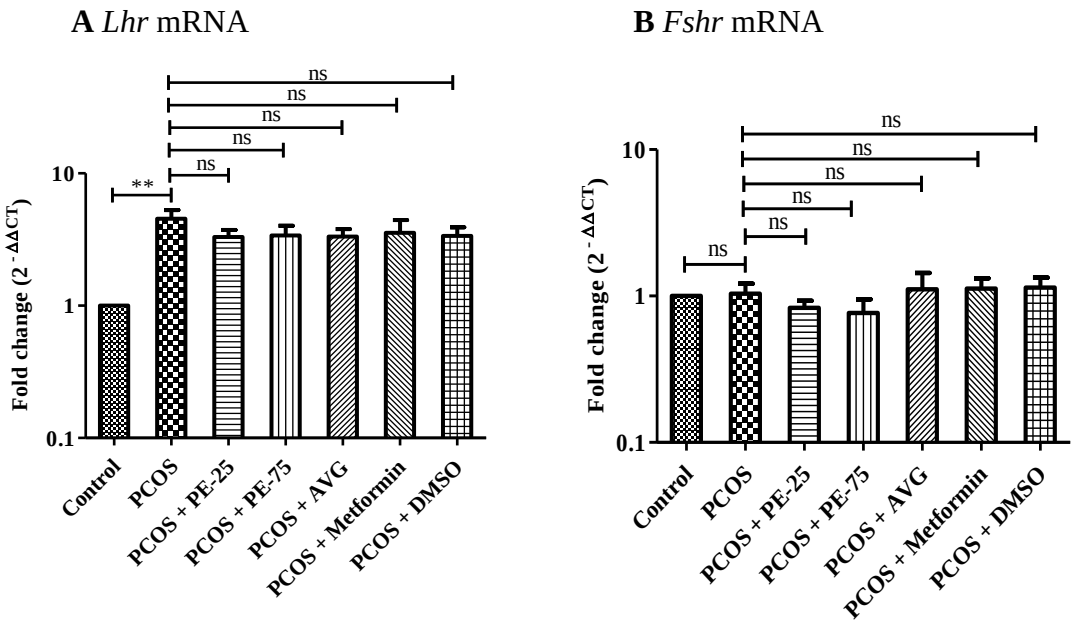


Figure 4.11. Effect of PE extract of AVG on the ovarian gonadotropin receptors. **A.** Luteinizing hormone receptors **B.** Follicle-stimulating hormone receptor. Values are mean fold changes in gene expression. Error bars represent SEM; N=6 per group. **P<0.01 as compared to the control group, ns-non-significant as compared to the PCOS group.

4.3.6 Effect of PE extract of AVG on the key markers of ovarian steroidogenesis on day 6 of pregnancy in PCOS mice

The binding of the LH and FSH to their receptors results in the stimulation of signalling pathway leading to enhanced steroid synthesis in the ovaries. The key gene encoding proteins of steroidogenesis, Steroidogenic Acute Regulatory (*Star*) protein, Cytochrome P450 17α-hydroxylase/C17, 20-lyase (*Cyp17a1*), and cytochrome P450 aromatase (*Cyp19a1*) were evaluated in the ovaries. When analysed for gene expression, *Star* (**P<0.01) and *Cyp17a1* (* P<0.05) were found markedly high in PCOS animals when compared with control animals. On contrary, *Cyp19a1* (**P<0.01) was downregulated in the PCOS ovary compared to the control. Metformin treatment in PCOS animals exhibited a decreased *Cyp17a1* (*P<0.05) expression with no changes in the *Star* and *Cyp19a1*. The PE extract of AVG treatment in PCOS animals demonstrates upregulation of the *Cyp19a1* (PE-25 $^{\$\$\$}$P<0.001, PE-75 $^{\$}$P<0.05, AVG $^{\$\$}$P<0.01) expression in the ovary with no difference in the *Star* and *Cyp17a1* (Figure 4.12).

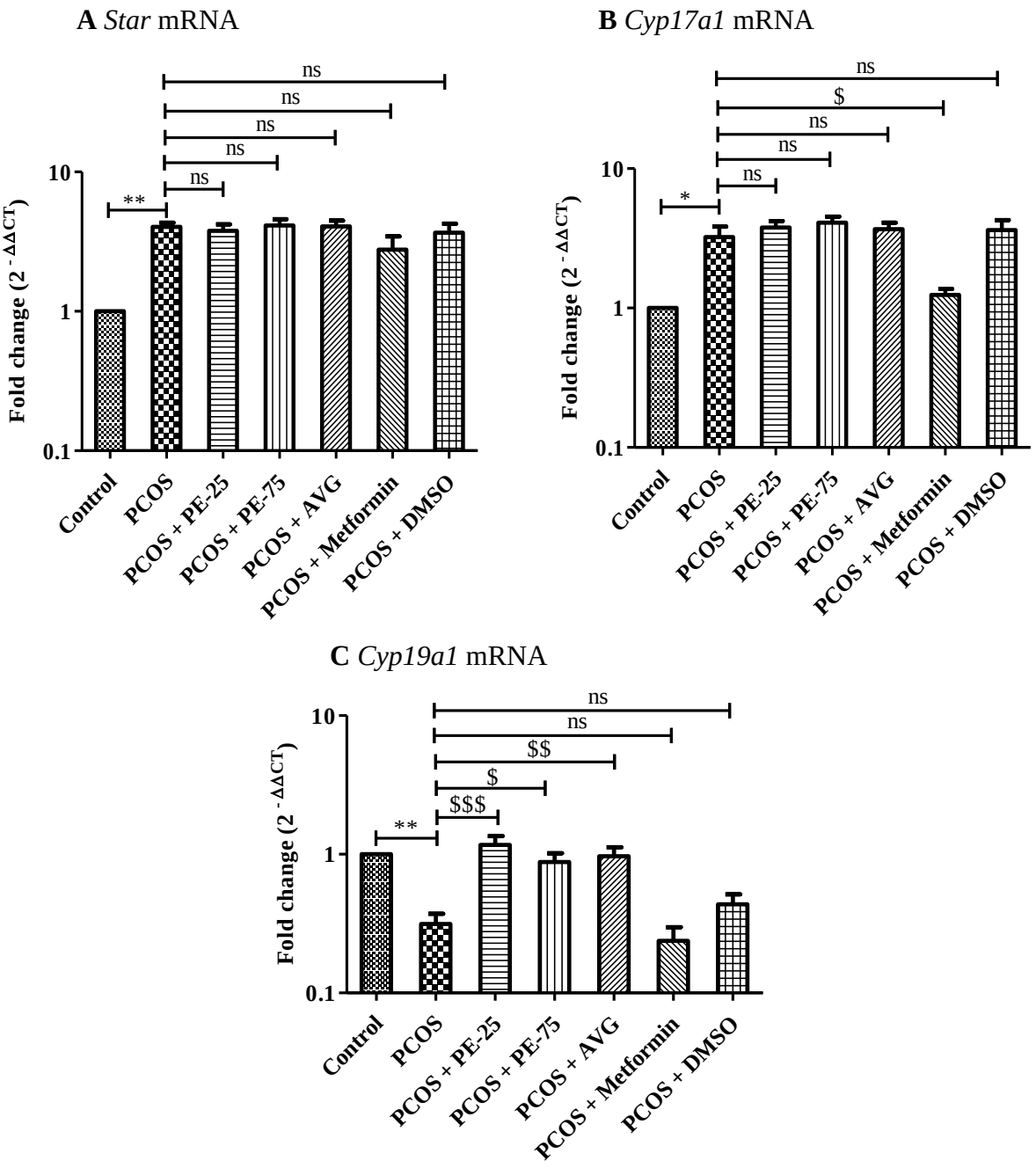
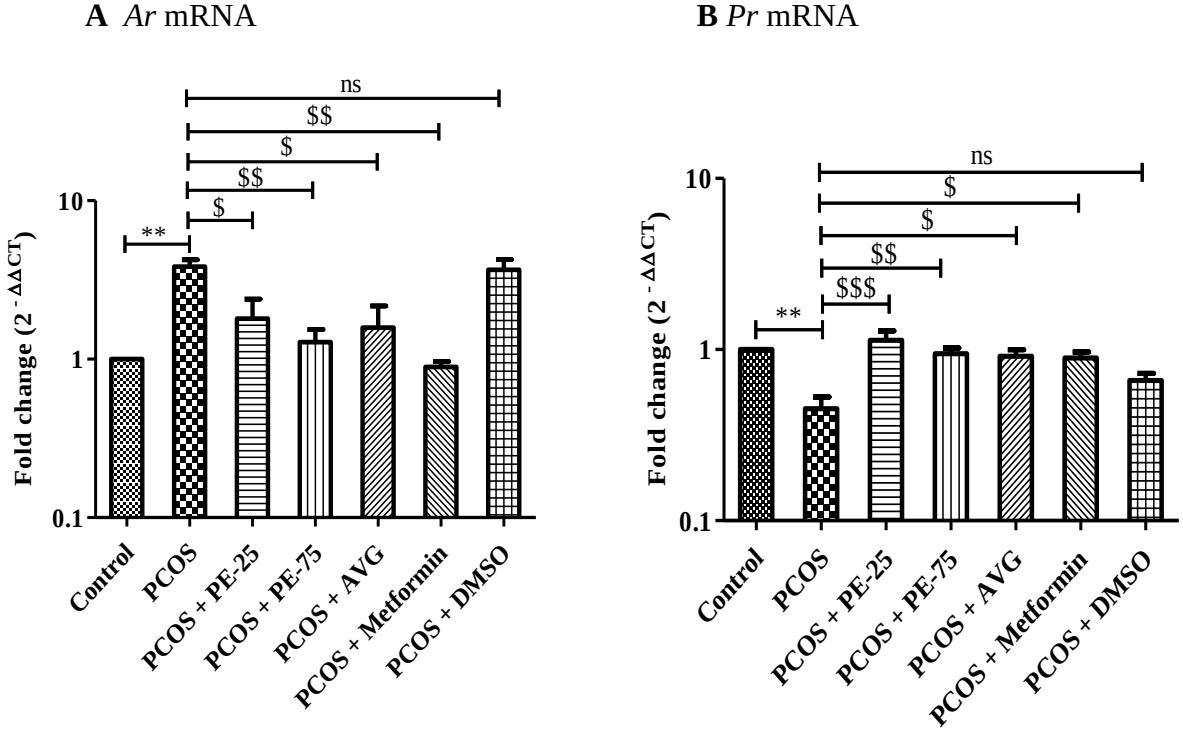


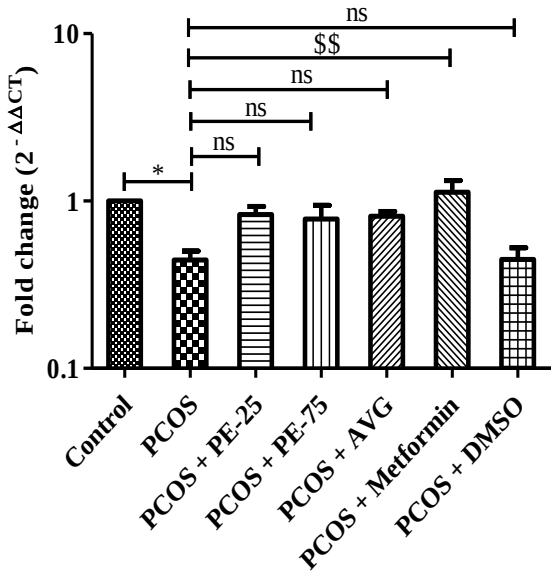
Figure 4.12. Effect of PE extract of AVG on the key mediators of ovarian steroidogenesis. **A.** Steroidogenic Acute Regulatory Protein **B.** Cytochrome P450 17α-hydroxylase/C17, 20-lyase **C.** Cytochrome P450 aromatase. Values are mean fold changes in gene expression. Error bars represent SEM; N=6 per group. *P<0.05, **P<0.01 as compared to the control group, $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 and ns-non-significant as compared to the PCOS group.

4.3.7 Effect of PE extract of AVG on the ovarian steroid receptors on day 6 of pregnancy in PCOS mice

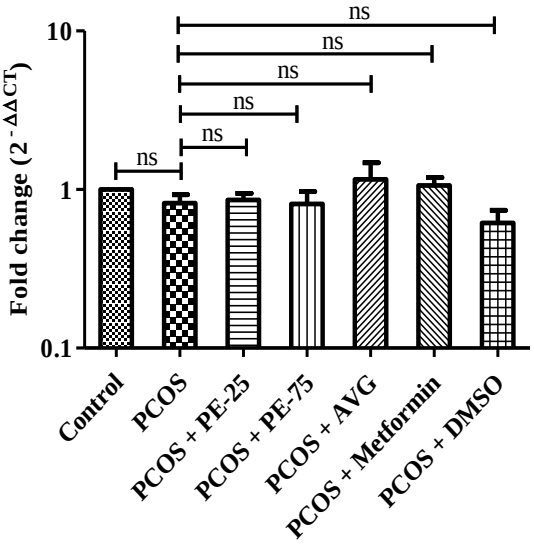
Steroids like progesterone, androgen, and estrogen mediate their autocrine effect through their receptors, progesterone receptor (*Pgr*), androgen receptor (*Ar*), and estrogen receptors α & β (*Esr-1* & *2*) respectively, and regulate ovarian function. The mRNA (**P<0.01) and protein (*P<0.05) expression pattern of *Ar* was significantly increased. On contrary, transcriptional and protein downregulation of *Pgr* (**P<0.01) was observed in the PCOS animals as compared to the control. In addition, gene expression of *Esr1* (*P<0.05) was decreased with no difference in the mRNA, and protein expression of *Esr2* in the PCOS ovary compared to the control. Oral administration of Metformin and PE extract of AVG in PCOS animals exhibited downregulation of *Ar* (Gene expression; PE-25, AVG $^{\$}$P<0.05, metformin $^{\$\$}$P<0.01, protein expression; PE-75 and metformin $^{\$\$}$P<0.01) and upregulation of *Pr* expression in the ovary (Gene expression; PE-25 $^{\$\$\$}$P<0.001, PE-75 $^{\$\$}$P<0.01, AVG and metformin $^{\$}$P<0.05, protein expression; Metformin $^{\$}$P<0.05). However, gene expression of *Esr1* was found to be increased ($^{\$\$}$P<0.01) upon the metformin treatment in PCOS animals, and the expression (mRNA and protein) of *Esr2* was not changed amongst all groups (Figure 4.13).



C *Esr1* mRNA



D *Esr2* mRNA



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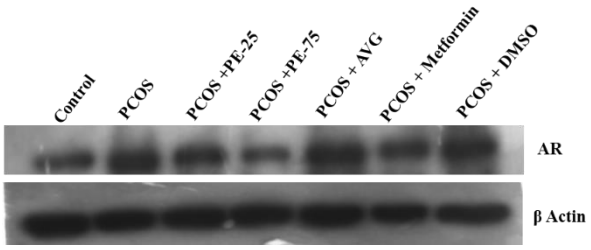

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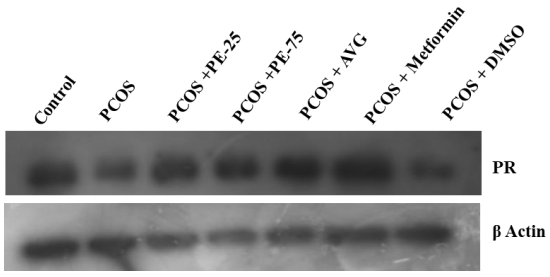
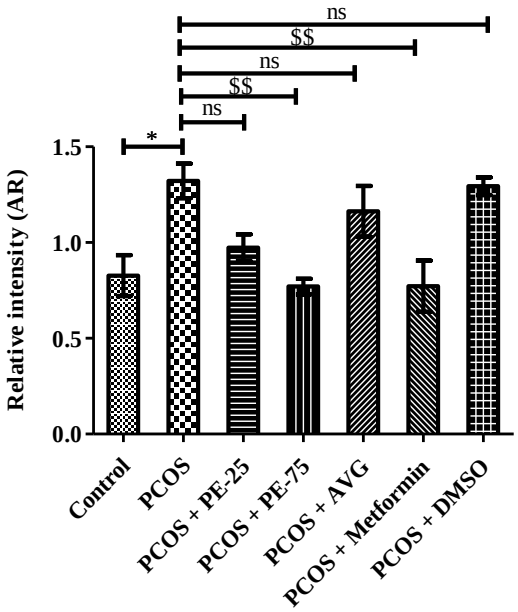
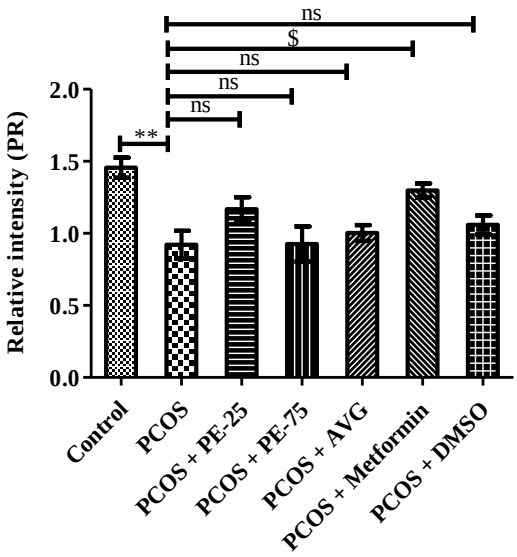






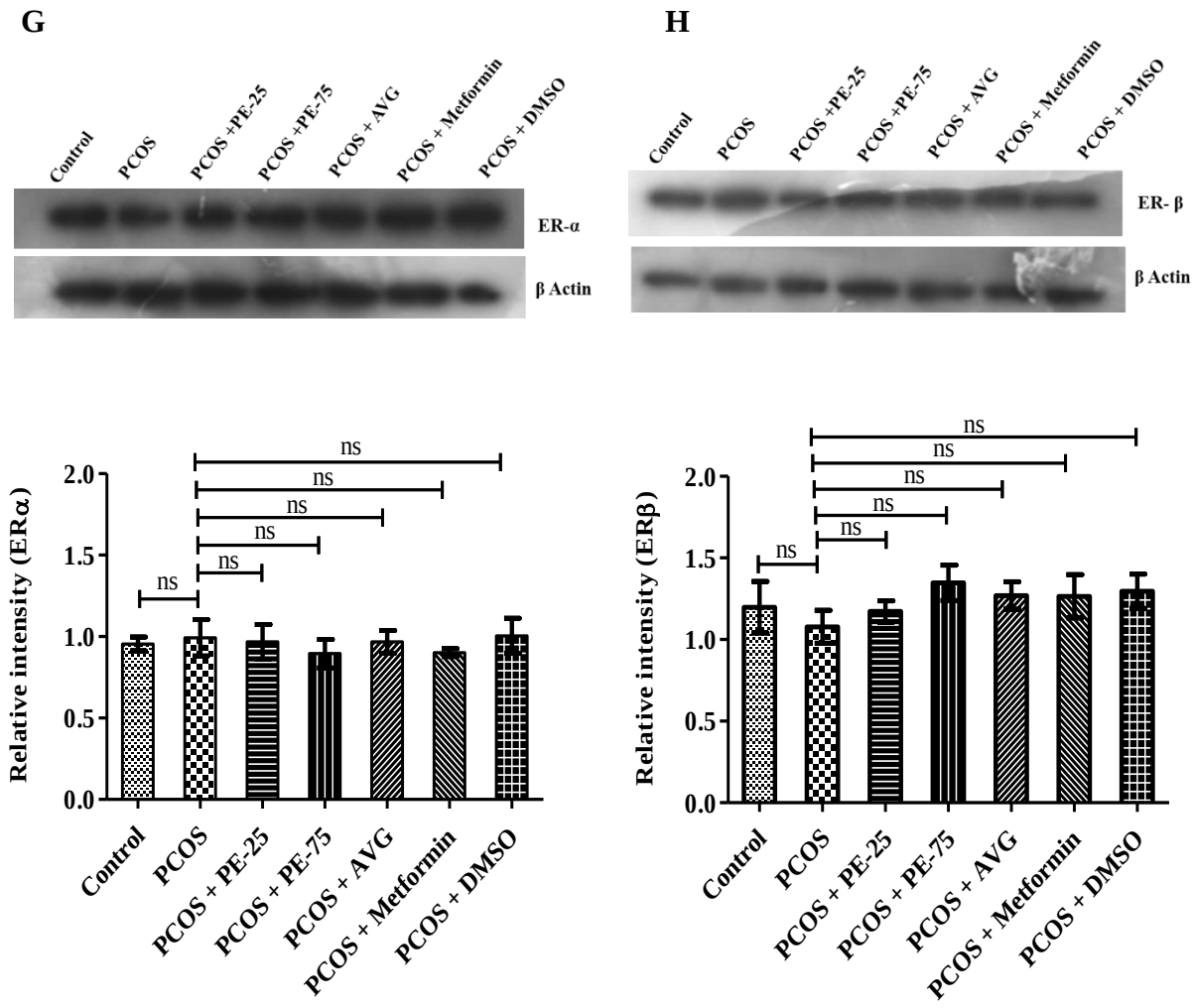


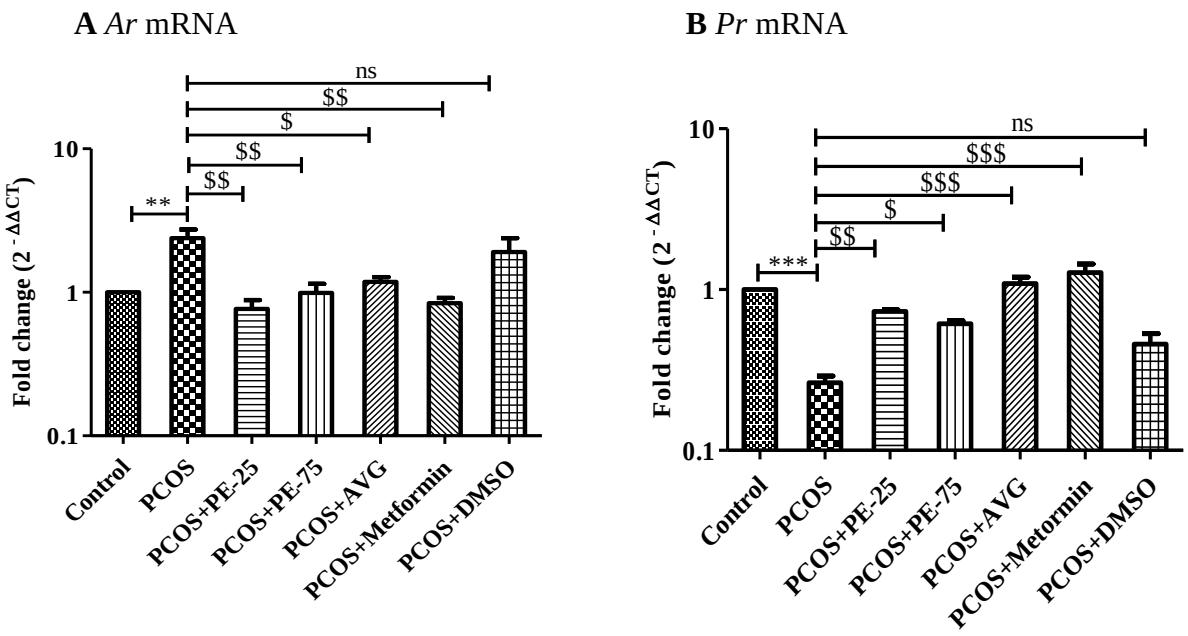
Figure 4.13. Effect of PE extract of AVG on the ovarian steroid hormone receptors. The upper graph represents values of mean fold changes in gene expression **A.** Androgen receptor **B.** Progesterone receptor **C.** Estrogen receptor-α **D.** Estrogen receptor-β and the lower graph represents the relative intensities of bands obtained on western blots in the letrozole-induced PCOS mice model. **E.** Androgen receptor **F.** Progesterone receptor **G.** Estrogen receptor-α **H.** Estrogen receptor-β. Error bars represent SEM; N=6 per group. * P<0.05, **P<0.01, ns-non significant as compared to the control group; $P<0.05, $$P<0.01, $$$P<0.001 and ns-non-significant as compared to the PCOS group.

The observed hyperactivation of steroidogenesis in PCOS ovaries reverted to normal after the oral administration of plant extract (AVG & PE extract of AVG). These observed results could be correlated to the improved hormone profile of PCOS animals. Further, steroid hormones exert their effect through their receptors and regulate the uterine milieu by activating downstream pathways in the maintenance of early pregnancy. Thus, the effect of the PE extract of AVG on the complex signalling of early pregnancy was investigated in the

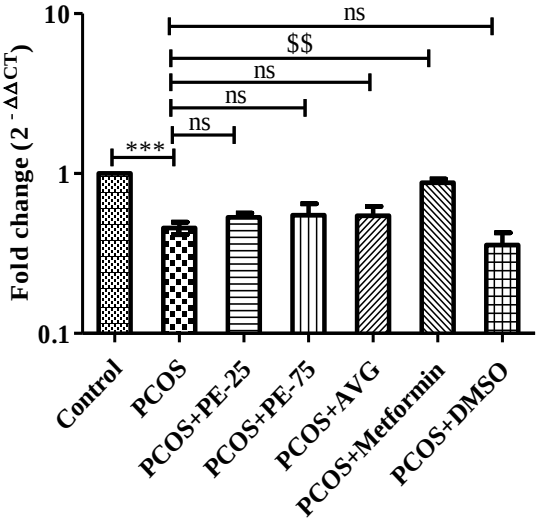
letrozole-induced PCOS mouse model. The plan of work is given in figure 4.1 and the results are explained below.

4.3.8 Effect of PE extract of AVG on the steroid receptor in the embryo implanted region of the uterus in PCOS mice

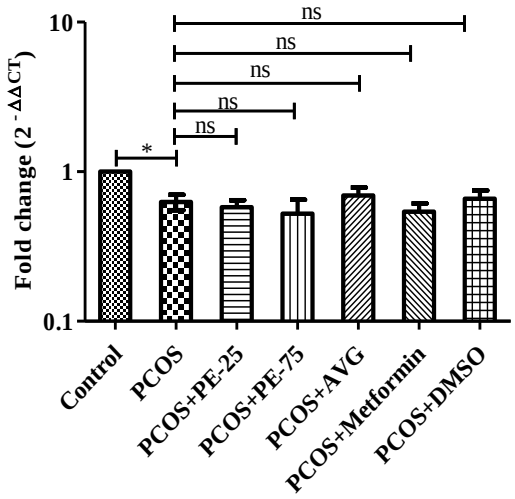
To accomplish a sequential event of pregnancy, the endometrium requires to undergo steroid-dependent changes. Steroids like testosterone, progesterone, and estrogen mediate their effect through their receptors, androgen receptor (*Ar*), progesterone receptor (*Pgr*), and estrogen receptor (*Esr1/2*), respectively. Transcriptional (**P<0.01) and protein (*P<0.05) upregulation of *Ar* was observed in PCOS animals. Metformin and PE extract of AVG treatment in PCOS animals showed downregulation of *Ar* ($^{\$\$}$P<0.01) with no difference in the protein expression amongst all the groups. On the contrary, protein expression and mRNA levels (***P<0.01) of the *Pgr* (**P<0.01), *Esr1* (***P<0.01), and *Esr2* (*P<0.05) were found markedly decreased in the embryo implanted region of the uterus in PCOS animals when compared with control tissues. PE of AVG treatment in PCOS animals showed upregulation of *Pgr* (Gene expression; PE-25 $^{\$\$}$P<0.01, PE-75 $^{\$}$P<0.05, metformin $^{\$\$\$}$P<0.001, protein expression; PE-25, PE-75, AVG $^{\$}$P<0.05 and Metformin $^{\$\$}$P<0.01). However, no differences were observed in mRNA and protein expression of *Esr1* & *2* whereas the metformin-treated group demonstrated upregulation of *Esr1* transcript levels ($^{\$\$}$P<0.01) (Figure 4.14).



C *Esr1* mRNA



D *Esr2* mRNA



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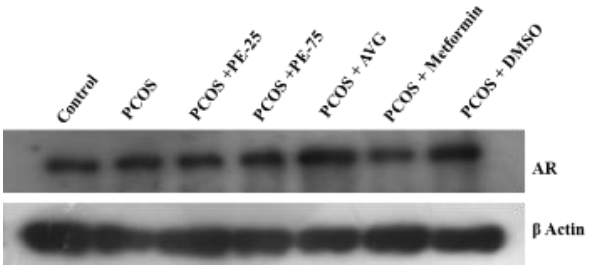

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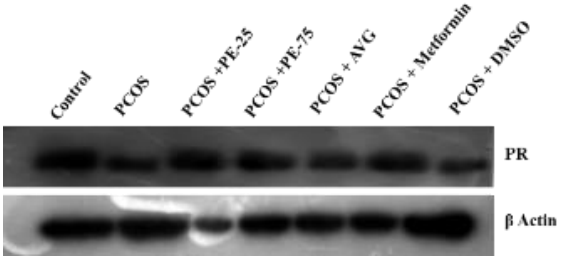
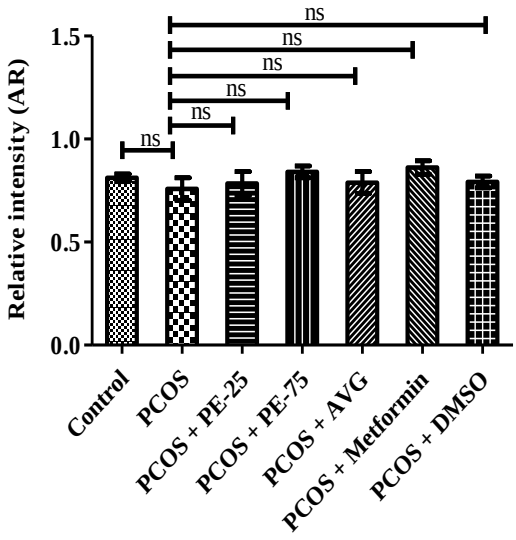
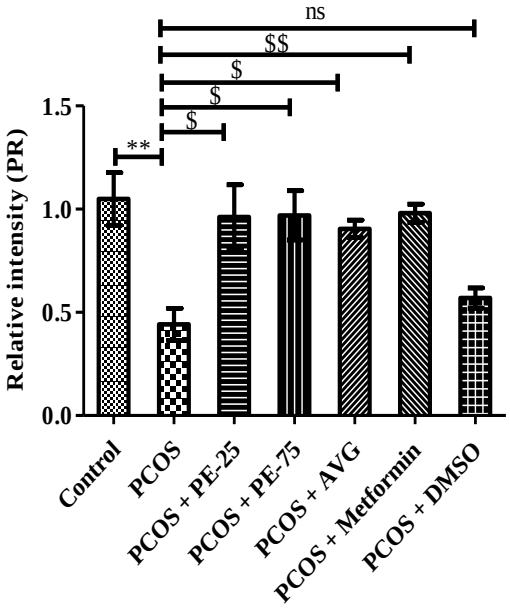






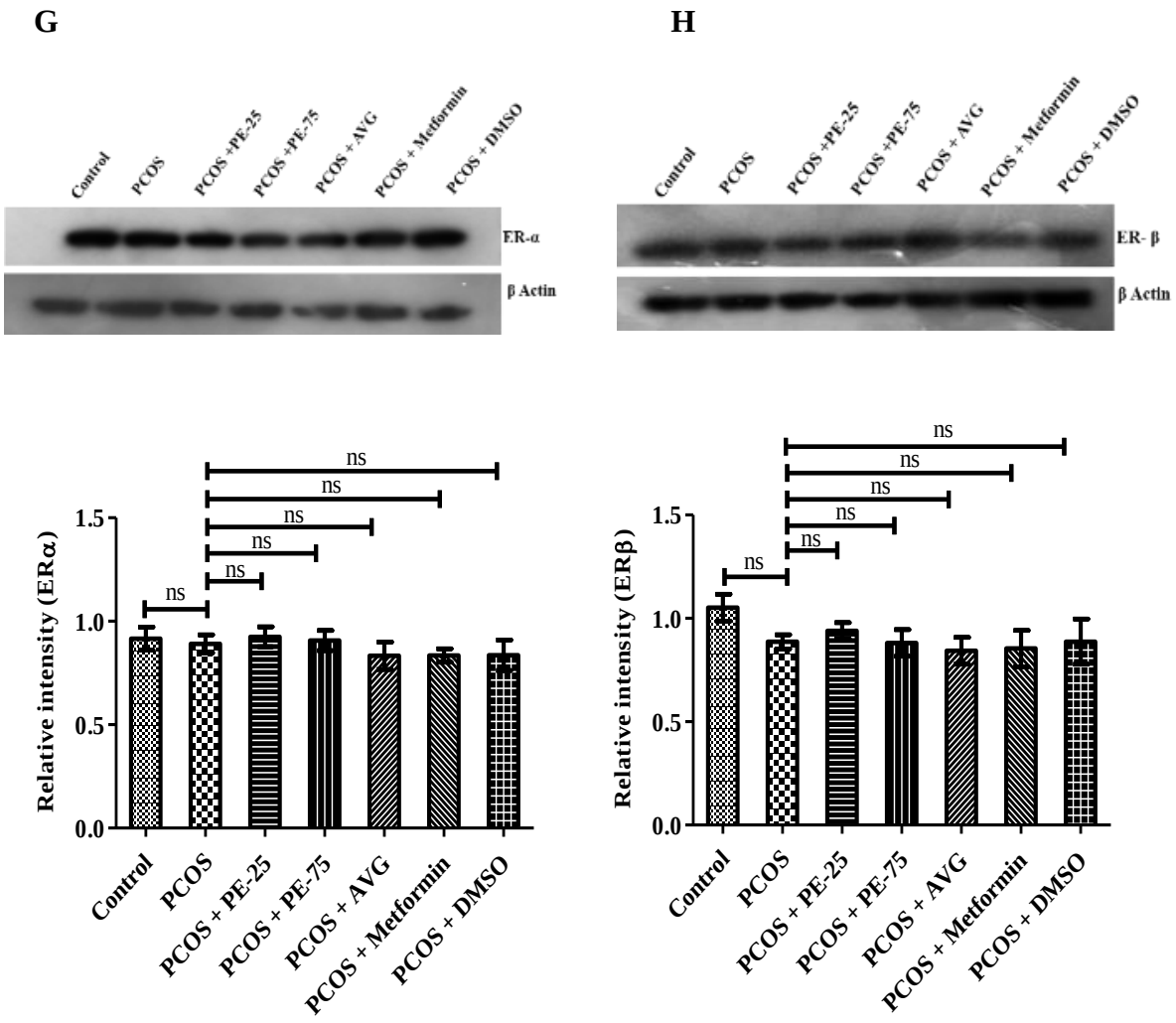


Figure 4.14. Effect of PE extract of AVG on steroid hormone receptors in the embryo implanted region of the uterus. The upper graph represents values of mean fold changes in gene expression **A.** Androgen receptor **B.** Progesterone receptor **C.** Estrogen receptor-α **D.** Estrogen receptor-β and the lower graph represents the relative intensities of bands obtained on western blots in the letrozole-induced PCOS mice model. **E.** Androgen receptor **F.** Progesterone receptor **G.** Estrogen receptor-α **H.** Estrogen receptor-β. Error bars represent SEM; N=6 per group. * P<0.05, **P<0.01, ns-non significant as compared to the control group; $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 and ns-non-significant as compared to the PCOS group.

4.3.9 Effect of PE extract of AVG on the feto-maternal interaction in the embryo-implanted region of the uterus in PCOS mice

Subsequently, integrin, α4β1 (*Itga4, Itgb1*) are known to involve in embryo-endometrium interaction in the establishment of a pregnancy. When analysed for gene expression, *Itga4* (*P<0.05) and *Itgb1* (**P<0.01) declined in the PCOS group when compared to the control

group. Metformin and whole AVG treatment in PCOS animals showed upregulation of *Itga4* (AVG $^{\$\$\$}$P<0.001), *Itgb1* (AVG and Metformin $^{\$\$}$P<0.01) (Figure 4.15).

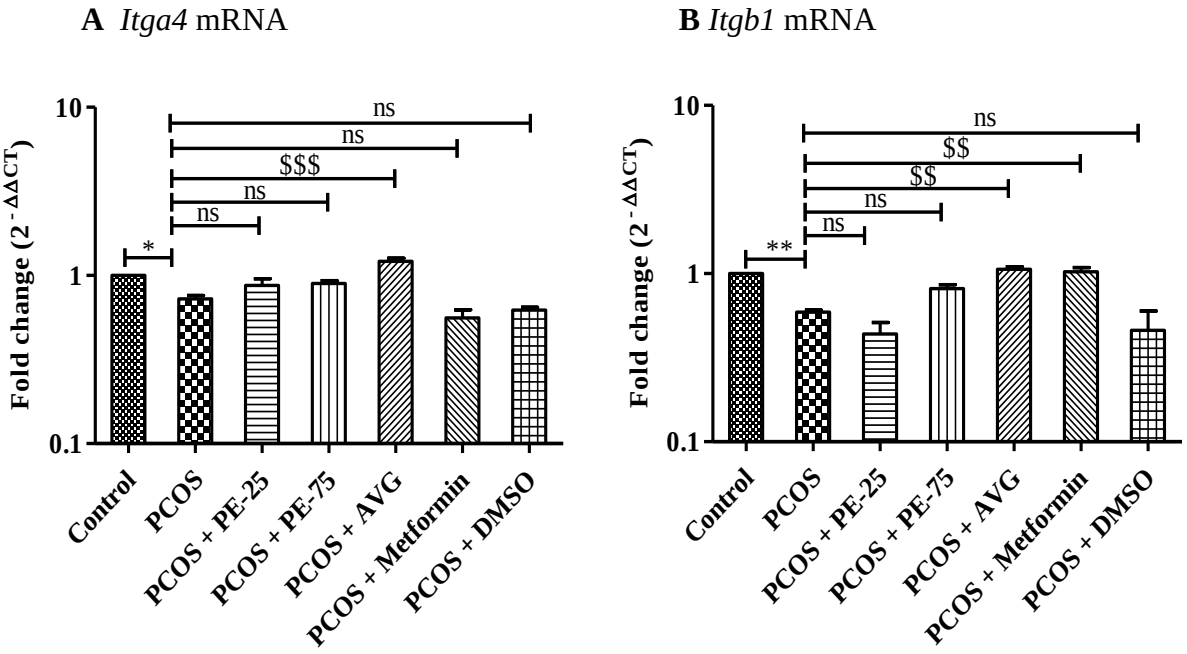


Figure 4.15. Effect of PE extract of AVG on integrin gene expression in the embryo implanted region of the uterus. Values are mean fold changes in gene expression, **A.** Integrin- α4 **B.** Integrin- β1 in the letrozole-induced PCOS mice model. Error bars represent SEM; N=6 per group. *P<0.05, **P<0.01 as compared to the control group; $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, ns-non-significant as compared to PCOS group.

4.3.10 Effect of PE extract of AVG on decidualization of embryo implanted region of the uterus in PCOS mice

Transcription factors, such as homeobox genes (*Hox10a)* are known to be involved in the decidualization of uterine stroma to complete the early stage of pregnancy. Transcript levels of *Hox10a* (**P<0.01) declined in the implanted region of the uterus in the PCOS group compared to the control group. Metformin and PE extract of AVG treatment in PCOS animals demonstrate upregulation (PE-25, metformin $^{\$\$}$P<0.01) of transcript levels (Figure 4.16).

A *Hox10a* mRNA

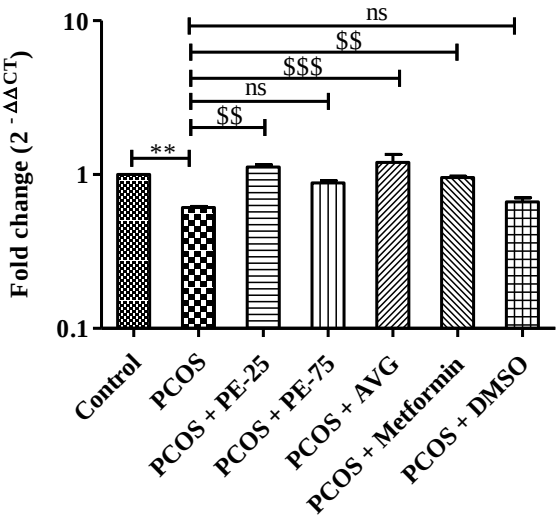


Figure 4.16. Effect of PE extract of AVG on transcription factor gene in the embryo implanted region of the uterus. Values are mean fold change in gene expression, **A.** Homeobox-10A in the letrozole-induced PCOS mice model. Error bars represent SEM; N=4 per group. **P<0.01, ns-non-significant as compared to control group; $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, ns-non-significant as compared to PCOS group.

4.3.11 Effect of PE extract of AVG on the MMP and TIMPs in the embryo implanted region of the uterus in PCOS mice

Invasiveness of the embryo to receptive endometrium required substantial degradation and remodelling of the ECM (extracellular matrix). Matrix metalloproteinases (*Mmp-2,9*) are responsible for the breakdown of ECM during the implantation process. The transcript level of *Mmp-9* was decreased (*P<0.05) in the implanted region of the uterus in PCOS animals compared to the control group. Treatment of Metformin and PE extract of AVG (PE-25, 75 $^{\$\$}$P<0.01, metformin $^{\$\$\$}$P<0.001) in PCOS animals exhibited an increase in gene expression whereas transcript level of *Mmp*-2 did not show a significant change in all groups. Further, the expression of MMPs is firmly regulated by their endogenous inhibitors, TIMPs (Tissue inhibitors of MMPs). Gene expression of *Timp-1* and *Timp-3* (***P<0.001) were reduced in the implanted region of the uterus in the PCOS group compared to the control group. PE extract of AVG treatment did not have any significant difference in gene expression of *Timp-*

1 and *Timp-3*. However, Metformin treatment in PCOS animals showed an upregulation of *Timp-1* ($^{\$\$\$}$P<0.001) & 3 ($^{\$\$}$P<0.01) (Figure 4.17).

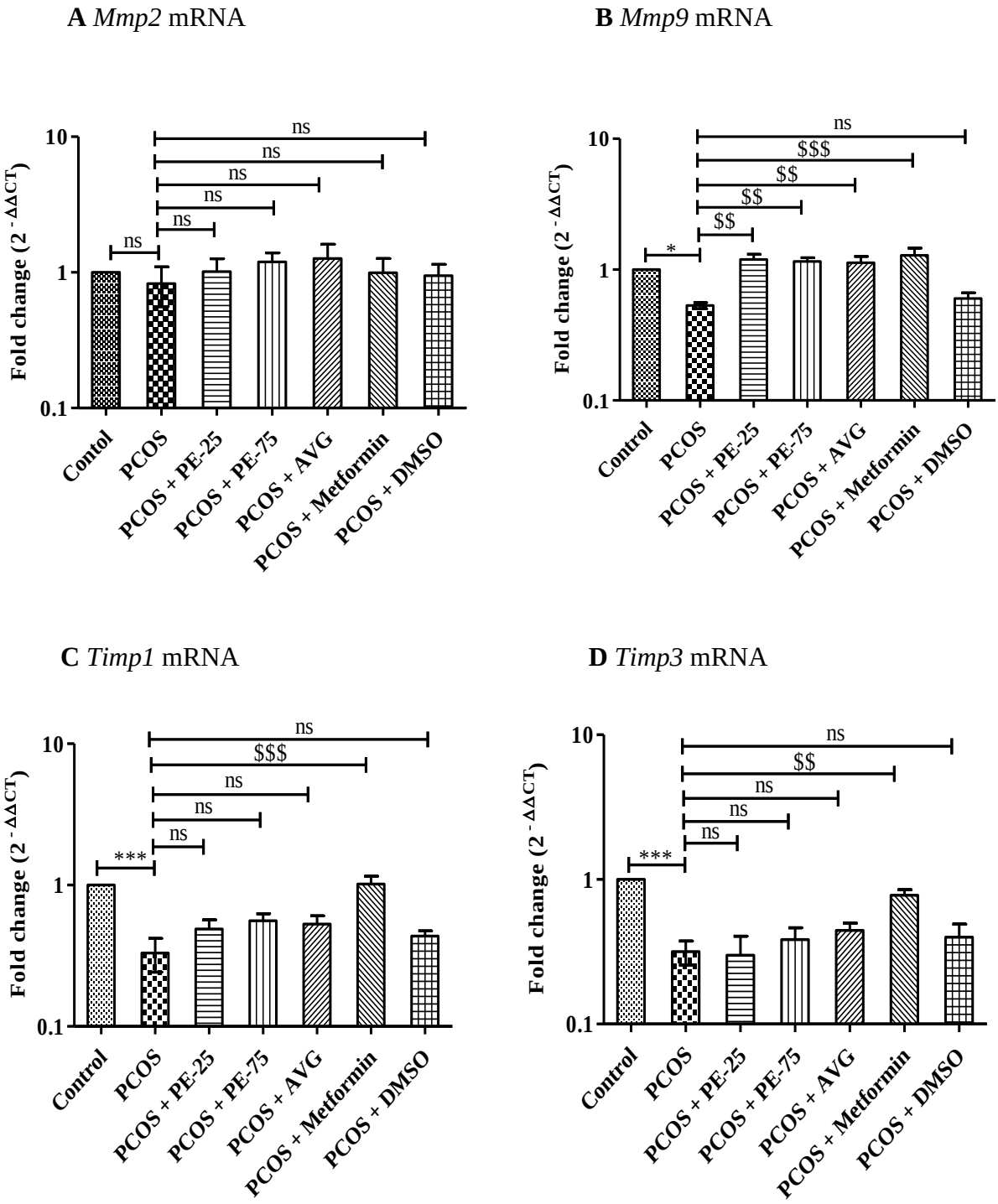


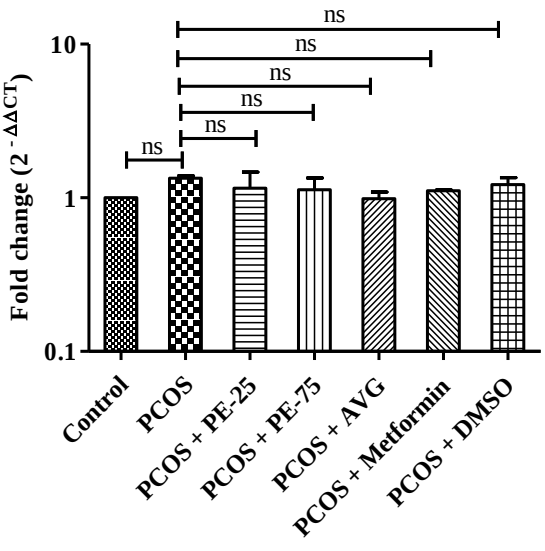
Figure 4.17. Effect of PE extract of AVG on matrix metalloproteinases and their inhibitors in the embryo implanted region of the uterus. Values are mean fold changes in gene expression

of **a.** MMP-2 and **b.** MMP-9 **c.** TIMP1 **d.** TIMP3 in the letrozole induced PCOS mice model. Error bars represent SEM; N=4 per group. * P<0.05, ***P<0.001, ns-non-significant as compared to control group; $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, ns-non-significant as compared to PCOS group.

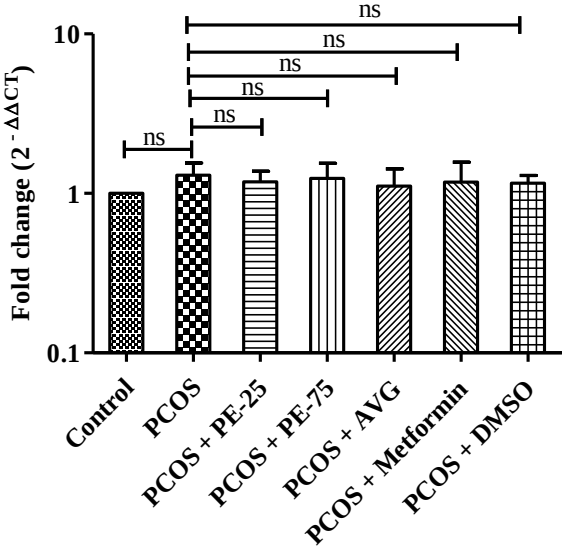
4.3.12 Effect of PE extract of AVG on LIF-STAT3 signalling pathway in the embryo implanted region of the uterus in PCOS mice

A tightly regulated rhythm between uterine maturation and embryonic development is crucial for a successful pregnancy. This function is mediated through the binding of cytokines on their receptors, mainly by the LIF-STAT pathway. When analyzed for key mediators of the pathway, no difference was observed in the transcript level of leukemia inhibitory factor (*Lif*) and leukemia inhibitory factor levels (*Lifr*) in all the groups. Glycoprotein 130 (*Gp130*) (***P<0.001) and Signal transducer and activator of transcription 3 (*Stat3*) (*P<0.05) were declined in the embryo implanted region of the uterus of PCOS animal as compared to the control group. However, oral administration of metformin and PE extract of AVG to PCOS animals showed significant upregulation of transcript levels of *gp130* (PE-25 & Metformin $^{\$\$\$}$P<0.001, PE 75 $^{\$}$P<0.05) and *Stat3* (PE 25, PE 75 $^{\$}$P<0.05 and Metformin $^{\$\$}$P<0.01) (Figure 4.18).

A *Lif* mRNA



B *Lifr* mRNA



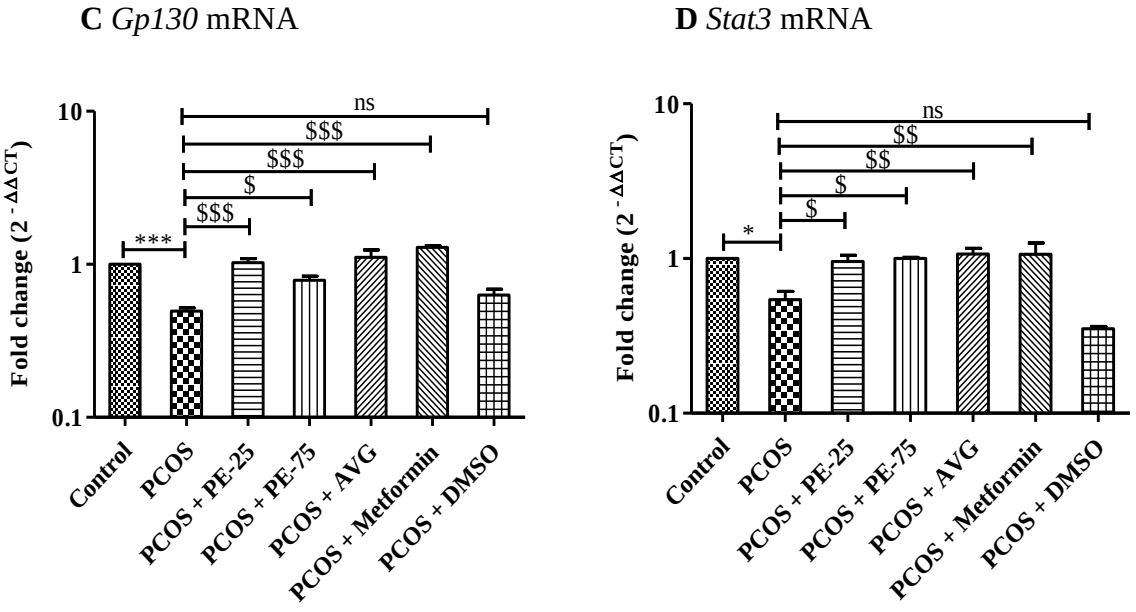


Figure 4.18. Effect of PE extract of AVG on key mediators of LIF-STAT3 pathway in the embryo implanted region of the uterus. Values are mean fold changes in gene expression, **A.** Leukemia inhibitory factor (LIF) **B.** Leukemia inhibitory factor receptor (LIFR) **C.** Glycoprotein 130 (GP130) **D.** Signal transducer and activator of transcription 3 (STAT3) in the letrozole-induced PCOS mice model. Error bars represent SEM; N=4 per group. * P<0.05, ***P<0.001 as compared to control group; $P<0.05, $$P<0.01, $$$P<0.001, ns-non-significant as compared to PCOS group.

The current study clearly demonstrated that the PE extract of *Aloe vera* gel has a potential efficacy on the reproductive performance of letrozole-induced PCOS females. Further, it was observed that the 25µg/kg/day of PE extract of AVG for 30 days is a sufficient dose to improve the molecular changes in the early pregnancy loss of the PCOS animals. Interestingly, the observed remedial potentiality of the PE extract of AVG was comparable to the metformin treatment which is a standard drug for the treatment of PCOS. Further, to translate any drug into clinical studies, the safety and assessment of toxicity become crucial. Thus, after the treatment with PE extract of AVG, estimation of the toxicity parameters was done in the letrozole-induced PCOS animals.

4.3.13 Effect of PE extract of AVG on toxicity parameters

No deleterious response or mortality was noticed in animals during the course of the treatments amongst all the groups. The body weight of animals during early gestation days did not show any significant change (Figure 4.8A). Also, the quantity of food and water consumption was found to be uniform during the whole treatment period among all the groups (Figure 4.19). During the phenotypic examination, the morphological features (fur, skin, eyes & nose) remained the same. Salivation, convulsions, diarrhea, or odd behaviors were not observed in the treatment group of animals (Table 4.5). Next, the effect of the PE extract of AVG on female reproductive performance during the early (Table 4.4 and Figure 4.9) and late phases (Figure 4.3, 4.4, and 4.5) of pregnancy were analyzed. Further, histopathological analysis of the ovary (Figure 4.7) and embryo implanted region of the uterus was done (Figure 4.10). There were no deleterious effects were observed in the reproductive performance after the treatment. Also, different biochemical parameters were examined on day 6^{th} of the pregnancy wherein random blood glucose (RBG) of the animals increased after the letrozole administration compared to the untreated animals, which is one of the characteristics of PCOS pathology. Upon oral administration of the PE extract of AVG, the RBG was reduced significantly in the PCOS animals (Figure 4.8C). Further, no significant differences were observed in the lipid profile (Total Cholesterol, Triglycerides, HDL cholesterol, LDL cholesterol) of the treated group as compared to the control group (Figure 4.20). Key markers of liver/kidney damage and their histopathological analysis were examined after the treatment with PE extract of AVG. The current experiment details demonstrate no significant difference in the liver and renal function tests (Table 4.6 and Figure 4.21). In the liver histoarchitecture, a systematic hepatic lobular array with a well-organized central vein (CV) was shown among all the groups, and hepatocytes were polyhedral in shape with tiny uniform nuclei (Figure 4.22). Kidney histopathology demonstrated well-organized and dense glomeruli, along with numerous tubules (distal tubules & proximal tubules) in all the groups (Figure 4.23).

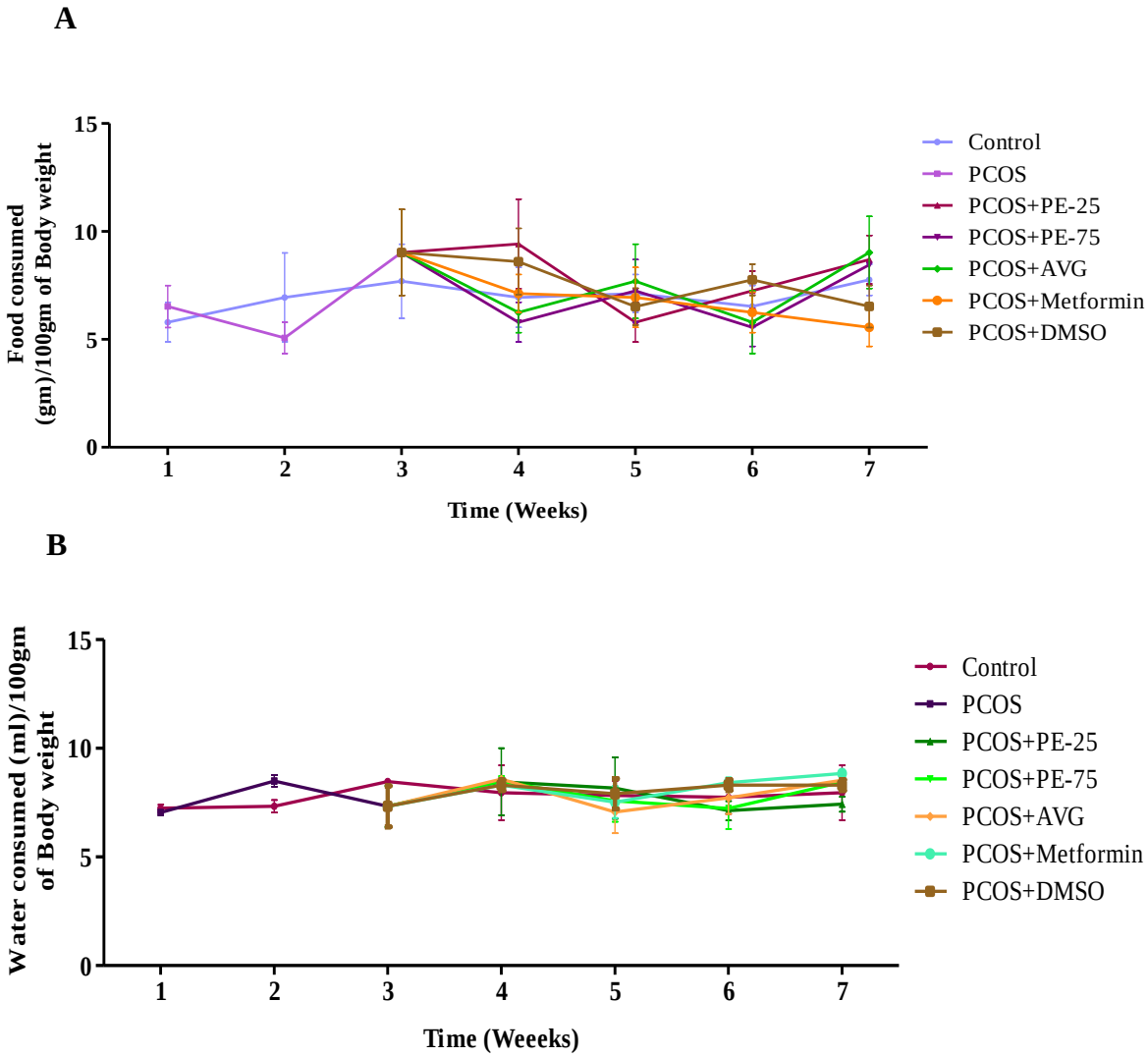


Figure 4.19. Effect of PE extract of AVG on **A**. Quantity of food consumed **B**. Quantity of water consumed during the entire experiment period.

Table 4.5. Effect of PE extract of AVG on the phenotypic characteristics of animals during the course of treatment.

	Control	**PCOS**	**PCOS + PE-25**	**PCOS + PE-75**	**PCOS + AVG**	**PCOS + Metformin**	**PCOS + DMSO**
Behavior	N	N	N	N	N	N	N
Convulsions	NO	NO	NO	NO	NO	NO	NO
Biting	NO	NO	NO	NO	NO	NO	NO
Locomotor activity	N	N	N	N	N	N	N
Tremors	NO	NO	NO	NO	NO	NO	NO
Eye Prominence	NO	NO	NO	NO	NO	NO	NO
Hair Coat	N	N	N	N	N	N	N
Lacrimation	NO	NO	NO	NO	NO	NO	NO
Salivation	N	N	N	N	N	N	N
Fecal excretion	N	N	N	N	N	N	N
Urine output	N	N	N	N	N	N	N

N= Normal; NO = Not Observed

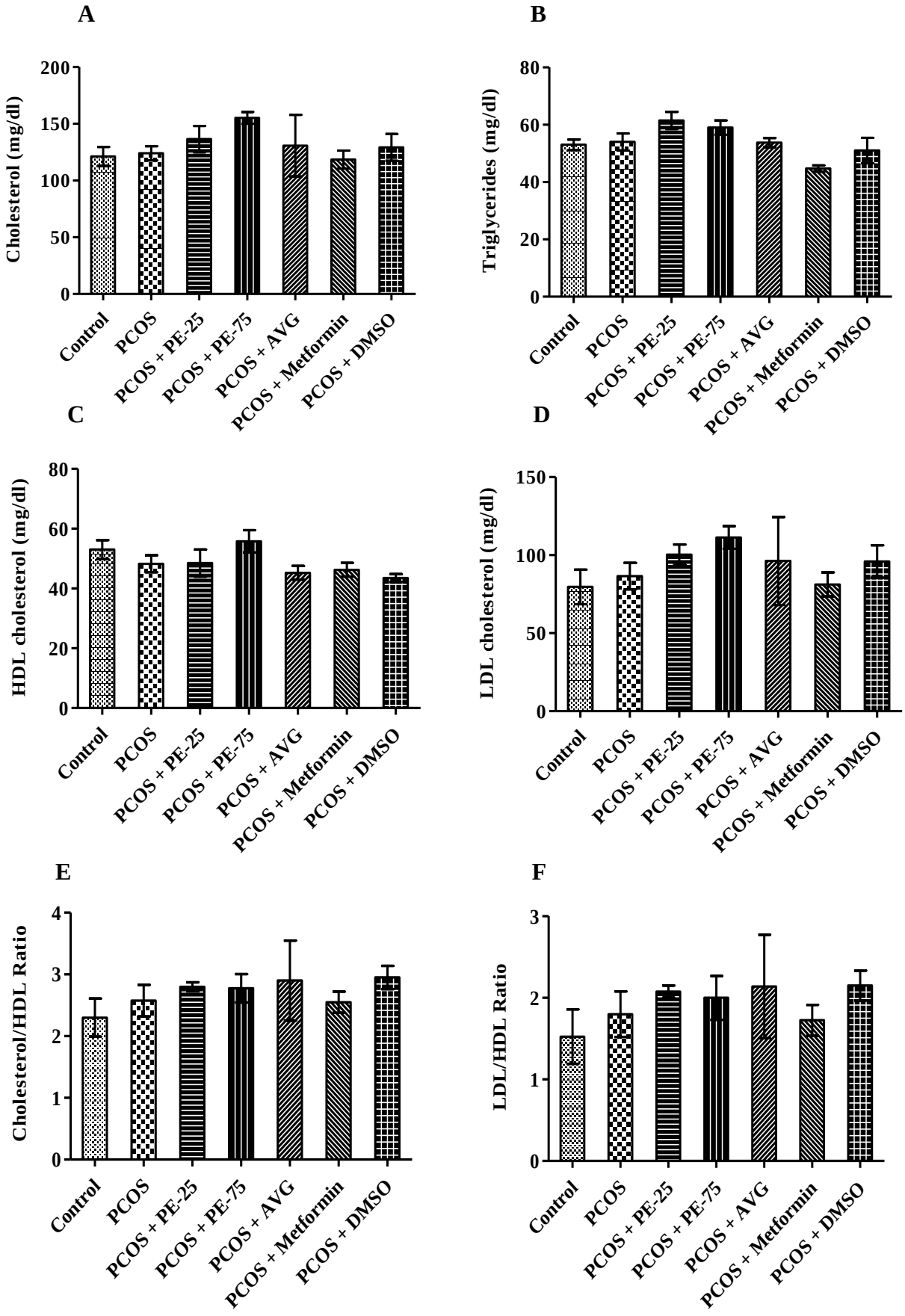

A
Cholesterol (mg/dl)
200
150
100
50
0
Control
PCOS
PCOS + PE-25
PCOS + PE-75
PCOS + AVG
PCOS + Metformin
PCOS + DMSO
B
Triglycerides (mg/dl)
80
60
40
20
0
C
HDL cholesterol (mg/dl)
D
LDL cholesterol (mg/dl)
150
100
50
E
Cholesterol/HDL Ratio
4
3
2
1
F
LDL/HDL Ratio

Figure 4.20. Effect of PE extract of AVG on lipid profile on the day 6th of pregnancy in letrozole-induced PCOS mice model. **A.** Total Cholesterol **B.** Triglycerides **C.** HDL cholesterol **D.** LDL cholesterol **E.** Cholesterol/HDL Ratio **F.** LDL/HDL ratio. All values are represented as Mean ± SEM; N=4 per group; non-significant results amongst all the groups.

Table 4.6. Effect of PE extract of AVG on liver function parameters on the day 6th of pregnancy in letrozole induced mice model.

	Control	**PCOS**	**PCOS + PE-25**	**PCOS + PE-75**	**PCOS + AVG**	**PCOS + Metformin**	**PCOS + DMSO**
ALT (IU/L)	26.50 ± 3.464	29.00 ± 2.273	26.00 ± 2.533	24.50 ± 1.548	23.75 ± 1.548	26.50 ± 1.323	25.00 ± 2.415
AST (IU/L)	95.50 ± 24.16	104.8 ± 20.02	77.50 ± 12.33	85.50 ± 9.613	86.75 ± 19.55	92.25 ± 21.95	77.75 ± 10.57
Alkaline Phosphatase (IU/L)	9.250 ± 1.493	10.00 ± 1.581	11.00 ± 2.160	10.25 ± 1.031	11.50 ± 1.190	10.50 ± 1.041	13.00 ± 1.958
Total protein (g/dl)	4.200 ± 0.3028	4.275 ± 0.3614	3.675 ± 0.2496	4.050 ± 0.3926	3.750 ± 0.2723	3.400 ± 0.4183	3.925 ± 0.3902
Albumin (g/dl)	1.650 ± 0.1323	1.700 ± 0.1414	1.425 ± 0.08539	1.600 ± 0.1683	1.500 ± 0.1472	1.300 ± 0.09129	1.500 ± 0.2121
Globulin (g/dl)	2.550 ± 0.1893	2.575 ± 0.2250	2.250 ± 0.1658	2.450 ± 0.2255	2.250 ± 0.1258	2.100 ± 0.4708	2.425 ± 0.1931
Albumin/ Globulin ratio	0.6500 ± 0.02887	0.6750 ± 0.02500	0.6500 ± 0.02887	0.6500 ± 0.02887	0.6500 ± 0.02887	0.7000 ± 0.1354	0.6250 ± 0.06292

All values are represented as Mean ± SEM; N=4 per group; non-significant results amongst all the groups.

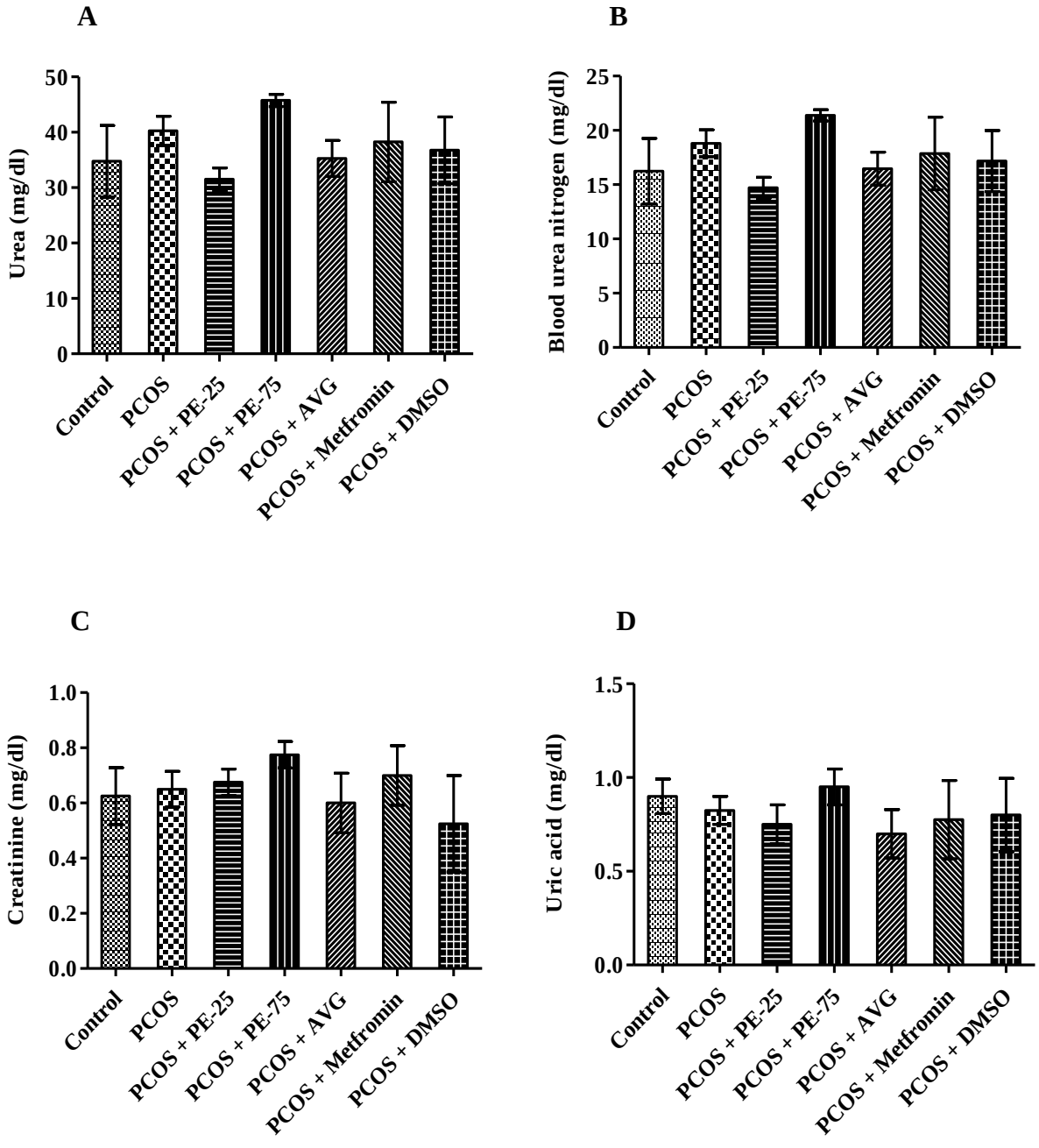


Figure 4.21. Effect of PE extract of AVG on renal function parameters on the day 6th of pregnancy in a letrozole-induced mouse model. **A.** Urea **B.** Blood urea nitrogen **C.** Creatinine **D.** Uric acid. All values are represented as Mean ± SEM; N=4 per group; non-significant results amongst all the groups.

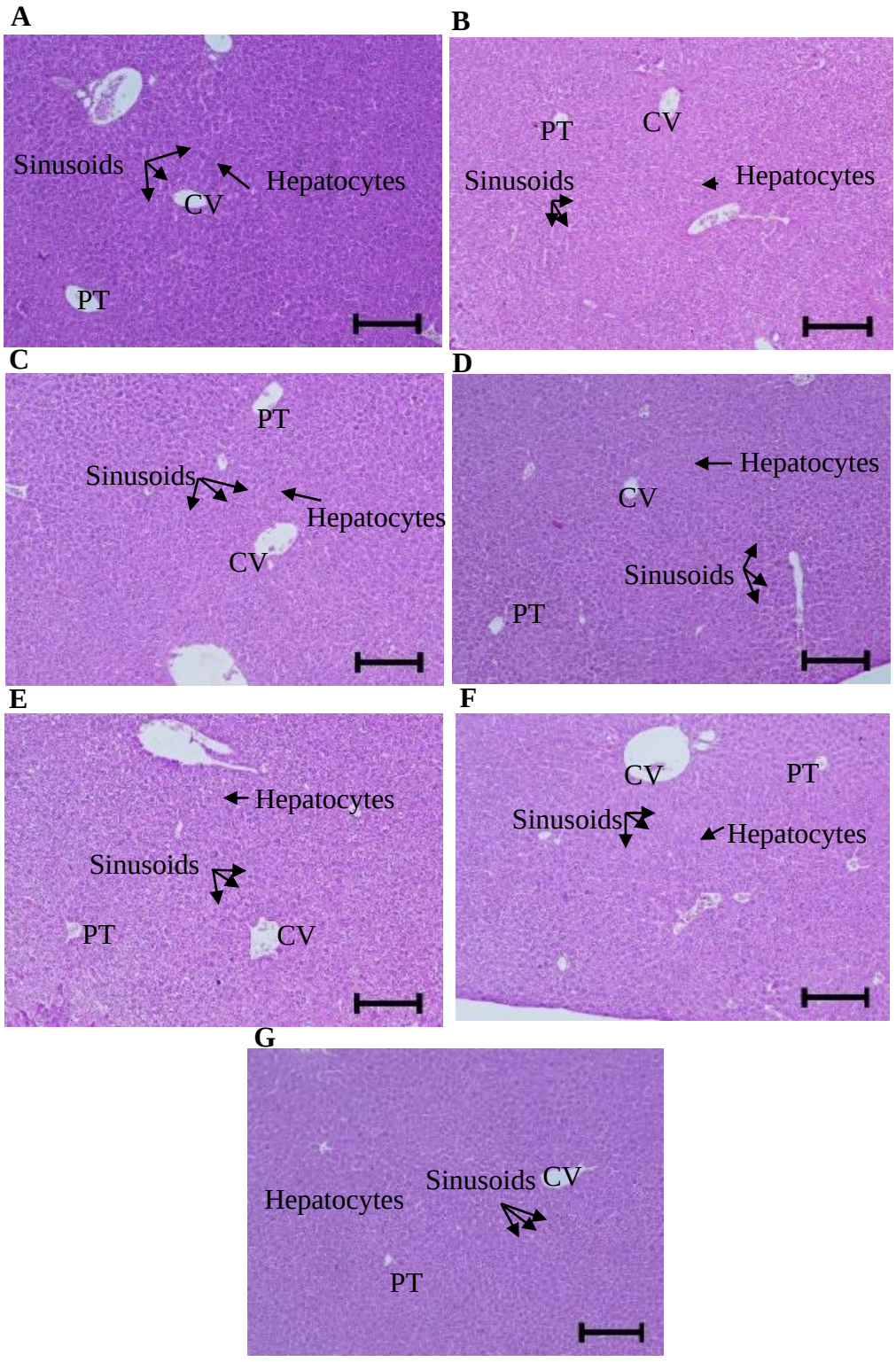


Figure 4.22. Effect of PE extract of AVG on histology of liver on the day 6 of pregnancy in the letrozole-induced PCOS mice model. **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO. CV, Central vein, PT, Portal triad. Magnification 10X

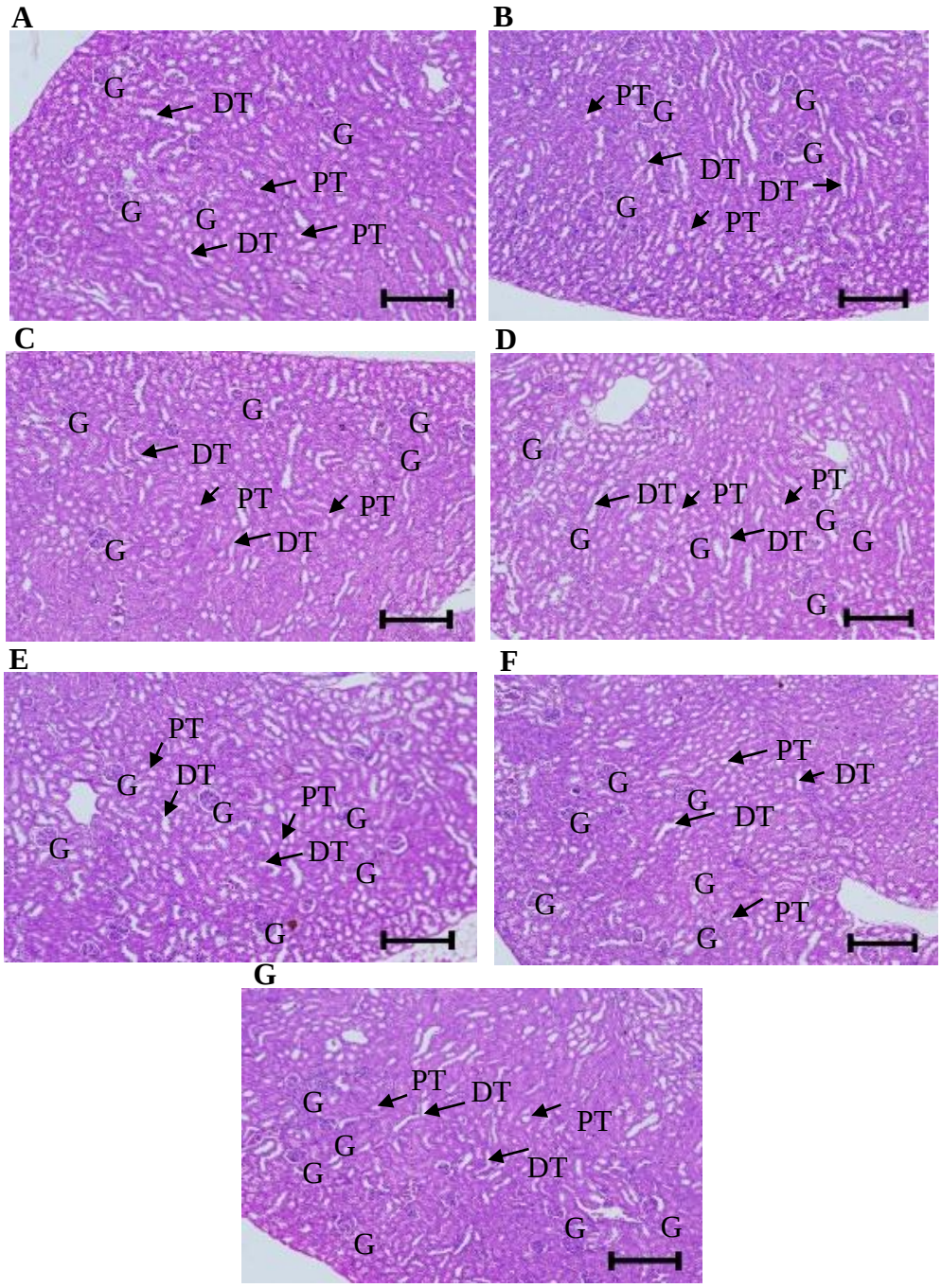


Figure 4.23. Effect of PE extract of AVG on histology of kidney on the day 6 of pregnancy in the letrozole-induced PCOS mouse model. **A.** Control **B.** PCOS **C.** PCOS + PE-25 **D.** PCOS + PE-75 **E.** PCOS + AVG **F.** PCOS + Metformin **G.** PCOS + DMSO. G, Glomeruli, DT, Distal tubules, PT, Proximal tubules. Magnification 10X.

All the above parameters indicate that the standardized dosage of the PE extract of AVG (25 and 75 µg/ kg body weight) doesn't exhibit any signs of toxicity for 30 days. However, the effect of the dose for longer period of time needs to be investigated.

4.4 Discussion

In PCOS pathology, pregnancy has been implicated with several complications that have translated as poor fetal growth and loss (Zhang et al., 2020). Along the same lines, the current study also exhibited retarded fetal growth and a lesser number of pups were born to PCOS animals. In this context, early gestational events are found to be the important window that directs proper fetal growth by the numerous molecular pathways. Thereby, this study emphasizes the molecular players that are important for the maintenance of embryonic-uterine cross-talk for proper embryonic development. Targeting them for therapeutic interventions could help us in the management of early pregnancy complications linked to PCOS pathology. In addition to this, the present study was an attempt to elucidate the therapeutic potential of phytocompounds present in petroleum ether (PE) extract of *Aloe barbadensis (Aloe vera* gel-AVG) when given as a pre-conceptive agent in the improvement of embryo-uterine transmission for the establishment of pregnancy in letrozole induced PCOS mouse model.

To understand the efficacy of petroleum ether (PE) extract of AVG on early pregnancy complications, firstly, letrozole induced PCOS mouse model was developed, and previously it was reported that 0.5 mg/kg/day of letrozole daily for 21 days was able to establish PCOS features in rodents (Chaudhari et al., 2018; Dey et al., 2022). Letrozole-induced female Balb/c mice showed elevated testosterone levels during early pregnancy. It could be correlated with the capability of aromatase inhibitor (letrozole), resulting in a low conversion of androgens to estrogens, as a consequence, an excessive accretion of androgens was observed in the ovary (Kafali et al., 2004). In addition, low levels of progesterone were observed, which can be associated with the disruption of corpus luteum formation in PCOS animals, similar observations were noted wherein PCOS patients showed abnormal ovulation (Huang et al., 2016). Interestingly, circulating estrogen did not change in PCOS when compared to the control group, which is in accordance with a study reported in the letrozole-induced mouse model (Kauffman et al., 2015). The altered hormone profile in PCOS animals might influence ovarian structure. When examined for ovarian histology, the number of peripheral cysts was observed, which is one of the characteristic features of PCOS (Fox et al., 1991; Adams et al.,1985). Reports have shown that altered hormone profiles in PCOS have a lower probability of childbirth, decreased pregnancy rates, and higher miscarriage rate. (Elenis et al., 2021; Su et al., 2017; Gaggiotti-Marre et al., 2019). Indistinguishable, results

were noticed in our study, the number of pregnant females and number of implanted embryos were significantly reduced in the PCOS animals.

Interestingly, treatment of PCOS animals with AVG, and PE extract of AVG (25 and 75 μg/kg/day) before conception demonstrated decreased circulating free testosterone. The efficacy of PE extract of AVG towards upregulation of *Cyp19a1*, leading to more conversation of androgens to estrogen, resulting in a low level of testosterone. The observed potency can be ascribed to the existence of certain phytocompounds in the PE extract of AVG. Our GC-MS data shows that the extracts contain n-Hexadecenoic acid, γ-Sitosterol, Oleic acid, campesterol, etc. This suggests that these compounds could be the active component that renders the above effects. Recent studies have demonstrated that campesterol, stigmasterol, β-sitosterol, and hexadecenoic acid present in *Caesalpinia bondue* seed extract might be helpful in regulating hyperandrogenism (Kandasamy & Balasundaram, 2021). As the increased testosterone level is the major predisposing factor that comes up with the numerous other clinical symptoms of PCOS; modulating testosterone levels after PE extract of AVG treatment could have favorable effects on reproductive abnormalities of PCOS pathology. Further, it is interesting to note that amelioration in progesterone content after the oral administration of PE extract of AVG (PE-25 & PE-75) in PCOS animals, could be correlated with the newly formed corpus luteum in the ovary. A similar line of results is seen in the non-pregnant letrozole-induced PCOS mice model (Dey et al., 2022). It has been reported that the normalization of progesterone content in PCOS rats treated with *Vitex Agnus* extract containing a complex mixture of total phenolic and flavonoid compounds (Saul, 2017). Thus, the PE extract of AVG treatment which is rich in phytochemicals like campesterol, γ-sitosterol, and hexadecenoic acid has potential efficacy in the improvement of progesterone levels in PCOS mice. The corrected hormone profile might be responsible for the healthy follicular development in the PCOS ovary, as the reduced number of peripheral cysts and increased number of corpus luteum was observed by plant extract treatment. A similar report has demonstrated improved histopathological changes in letrozole-induced PCOS mice after the treatment with campesterol, β sitosterol, and stigmasterol containing *Capparis spinosa* extract (Mously et al., 2020). Interestingly, our previous lab study exhibited that campesterol from partially purified non-polar phytocompounds of *Aloe vera* gel significantly reversed the hormone profile along with the ovarian structure in the PCOS mice. The results were comparable to those obtained with metformin treatment. Also, the quantification by HPLC revealed that the concentration of campesterol was found to be 15.4

µg/gm in the PE extract of AVG (Dey et al., 2022). Hence, it can be noted that campesterol present in the PE extract of AVG at both the concentration of the dosage (25 & 75µg/kg/day) was found to be similarly strong as AVG and metformin by modulating all the preliminary symptoms of PCOS pathology. However, 25 µg/kg/day of PE extract of AVG for 30 days was the minimum effective dose for improving the comorbidities associated with PCOS. As the consequence, enhanced pregnancy rates, number of pregnant females, and embryo implantation sites were observed on the day 6^{th} of pregnancy in PCOS animals. This could be due to the anti-androgenic, and progestogenic properties of the PE extract of AVG. Current pieces of evidence exhibited that the different plant extracts improve hyperandrogenaemia (Hossein et al., 2015), hyperinsulinemia, increase fertility index, gestation rate (Mvondo et al., 2020), progesterone content (Maleki et al., 2021), and ovarian morphology (Zangeneh et al., 2010; Yang et al., 2018) in PCOS animals. Also, one of the studies concluded that the phytochemicals (Campesterol, stigmasterol, β sitosterol, and fatty acid) containing *Moringa oleifera* hydroalcoholic extract has a potential effect on reproductive performance (receptivity, conception rate, and litter size) in female rabbits (Speiy et al., 2021). Thus, the potential of PE extract of AVG owing to the presence of campesterol, Oleic acid, γ-sitosterol, and hexadecenoic acid. In contrast, oral administration of 500 mg/kg/day of crude bark methanol extract (CBE) of *Dysoxylum alliarium* to female rats showed deteriorated epithelial layer, as well as vacuole formation in the underlying decidual zone in the endometrium during day 6 and 7 of pregnancy, subsequently abrogated embryonic development and reduced number of embryo implantation sites was observed. It has been speculated that phytoestrogens present in the extract are responsible for this effect (Das et al., 2013). One of the works of the literature shows that β sitosterol has estrogen-like properties and regulates P450scc activity, therefore compromising the conversion of cholesterol to pregnenolone (Gerber et al., 2015). Moreover, altered hormone production, changes in sexual development, decreased spawning rate, and egg production was observed in fish exposed to phytosterol (ThienponfL, 2008). Goldfish exposure to phytosterols (75µg/L13) showed modulation in gonadal steroidogenesis and decreasing steroid levels (Maclatchy et al., 1997). It has been observed that small doses of (0.5-50 mg/kg/day) phytosterol caused changes in the weight of reproductive organs (Malini & Vanithakumari, 1991; Baker et al., 1999; Reed, 2016). Also, a study from our lab denoted that phytosterol-containing non-polar extract of AVG could effectively manage reproductive and metabolic complications in letrozole-induced PCOS animals (Radha & Laxmipriya, 2016c).

In view of the above reports, it can be clearly seen that phytochemicals present in both dosages (25 and 75 μg/kg/day) of PE extract of AVG have the potential as an endocrine modulator. Further, our preliminary experiments showed a drastic improvement in the fetal outcome of PCOS animals when treated with PE extract of AVG. The betterment could be due to an improved uterine milieu. Although, molecular targets or modes of action of phytochemicals present in the AVG are not defined when used as a pre-conceptive agent. Thereby, the present study attempts to identify the effect of PE extract of AVG on molecular pathways involved in the establishment of pregnancy using the letrozole-induced PCOS mice model.

The data from the previous chapter have shown the altered ovarian steroidogenesis and autocrine regulation of steroid hormones associated with disturbed intra-ovarian function in the PCOS ovaries on day 6 of pregnancy. The results from the current chapter demonstrated that the oral administration of AVG, metformin, and PE extract of AVG in PCOS animals modulates the gene expression of steroidogenesis marker *Cyp19a1*, steroid hormone receptors, *Ar*, and *Pr*. In the previous lab study, similar results were noticed in the non-pregnant PCOS mice model wherein PCOS animals treated with campesterol containing isolate of PE extract of AVG and pure campesterol (0.01 μg/kg/day) showed the modulation in the transcript of *Cyp19a1*, *Ar*, and *Pr* in the ovary (Dey et al., 2022). Thus, it can be noted that the phytocomponents present in AVG have the potential to improve ovarian function at the molecular level. Moreover, the experiments from the current study exhibited that the protein expression of the ovarian PR has not changed after the plant extract treatment. However, the overexpressed AR protein was downregulated at the dose of 75 μg/kg/day (PE extract of AVG), treatment and the results are comparable to the whole AVG-treated animals. Therefore, it is interesting to point out that at the transcription levels, phytocomponents of AVG could act as the androgenic as well as progestogenic regulators whereas at the protein level the bio-actives of AVG act as the androgenic modulators in the ovary during early pregnancy in the PCOS pathology.

Further, in the uterus, gonadal hormones exert their effect through their receptors, progesterone receptor (PR), estrogen receptor (ERα/β), and androgen receptor (AR) respectively, and synchronize cell differentiation, proliferation and secretory protein production in the uterus during the implantation phase of the pregnancy (Pawar et al., 2014b). When examined for the expression of steroid hormone receptors in the implanted region of the uterus, on the day 6th of pregnancy, PCOS animals exhibited declined gene and protein

expression of PR. Progesterone signalling is known to have an inhibitory effect on the E/ER signalling pathway in stromal cells of the endometrium for the establishment of pregnancy (Wetendorf & DeMayo, 2014). Thus, in our experimental model, declined P/PR signalling did not show an inhibitory effect on E/ER, resulting in no difference in ER, and its target gene leukemia inhibitory factor (*Lif*) was observed. However, decline PR expression was improved by PE extract of AVG treatment in PCOS animals, even though ER and *Lif* did not show a significant difference, suggesting that both the doses (25 and 75 µg/kg/day) are safe and do not exhibit endocrine disruption potential. On the contrary, a higher concentration of phytosterols was studied for their efficacy wherein the high dose (5mg/kg/day), of dietary phytosterols mixture containing mainly β-sitosterol, has chronic effects on the reproduction of the mouse in which elevated plasma testosterone levels and reduced uterine weights in the pups of F (2) and F (4) generations have been observed. In addition, phytosterol exposure leads to an increase in the concentrations of plasma estradiol in the female pups of the F (3) generation and testicular testosterone content in the male pups of the F (2) generations (Ryökkynen et al., 2005). This indicates that the effects of phytosterols on the reproductive system may vary with doses. Furthermore, it is known that progesterone signalling inhibited androgen receptor (AR) expression, whereas estrogen dramatically elevated AR abundance in the stroma of ovariectomized mouse uteri during early pregnancy (Li et al., 2014). In this direction, the current study revealed that altered P/PR signalling in PCOS animals did not have a prohibited influence on AR expression, as the overexpression of AR (gene and protein) was observed in the embryo-implanted region of the uterus. Normal expression of AR was observed in PCOS mice upon the treatment with metformin, AVG, and PE extract of AVG. Hence, it could be noted that enhanced progesterone signalling by phytosterol-containing plant extract at both the concentration of dose 25 & 75 µg/kg/day have a modulatory effect on the androgen receptor. Therefore, considering all of the above evidence and results of the current *in vivo* study signifies that an increase in P/PR signals could be because of the progestin-like activity of one of the bio-actives, present in the PE extract of AVG. To substantiate our results, the previous study from our lab has demonstrated that n-Hexadecanoic acid and campesterol containing partially purified non-polar extract of *Aloe vera* gel significantly modulates ovarian steroid receptor gene expression in letrozole-induced PCOS mice (Dey et al., 2022). However, the role of individual bioactive in the rectification of the early pregnancy cross-talk at the molecular level needs to be validated.

Moreover, ovarian steroid hormones have been known to regulate the cell adhesion molecules during the attachment phase of the embryo (Merviel et al., 2001). *In vivo* and *In vitro* studies reported that progesterone is likely responsible for the regulation of integrins on the endometrium surface (Mokhtar et al., 2018; Chen et al., 2016). Apart from steroidogenic control, LIF and LIFR are responsible to stimulate the expression of integrins (αvβ3, β5) on the plasma membrane surface of endometrial cells, which is required in the adhesion of the blastocyst implantation (Chung et al., 2016). Results from the current study exhibited that low progesterone levels could be one of the contributory reasons causing the reduced integrin expression because no difference was observed in the gene expression of LIF & its receptor in the embryo implanted region of the uterus in PCOS animals. This suggests that the declined expression of integrins was not mediated via LIF & LIFR. In addition, the enhanced progesterone levels by treatment of PE extract of AVG in PCOS animals at both the concentration of dosage did not show any significant difference in the integrin expression suggesting that the non-polar phytocomponents of AVG could not take action on the integrins directly or indirectly. However, the study of protein levels of the integrins will further confirm this evidence. In contrast, the whole AVG and metformin-treated animals were showing upregulation in the integrin expression which could result in enhanced embryo-uterine attachment during early pregnancy in the letrozole-induced PCOS animals. Also, the studies are not available to confirm the direct or indirect role of phytocompounds on the LIF signalling.

Further, uterine decidualization, a key event during early pregnancy is known to regulate by homeobox transcription factors, HOX1OA, and HOX11A (Du & Taylor, 2016) and it is evident from the *in vitro* study that P/PR signalling upregulates the *Hox10a* while upregulation can be inhibited by progesterone signalling blocker in human endometrial stromal cells (Taylor et al., 1998). As the HOX10a is the downstream target of the progesterone in the uterus (Lim et al., 1999), declined gene expression of *Hox10a* in our study could correspond to the changes in progesterone signals of the letrozole-induced animals. Treatment of PCOS animals with metformin and PE extract of AVG exhibit upregulated *Hox10a* expression in the implanted region of the uterus. This result also demonstrates the progestogenic potential of phytosterols of AVG at the concentration of 25 and 75µg/kg/day, consequently engaging in the decidualization process during early pregnancy in PCOS animals.

During the blastocyst invasion and decidualization, the homeostasis of the MMPs, (Matrix metalloproteinases) and its tissue inhibitors (TIMPs) is believed to be crucial for embryo implantation and pregnancy (Curry & Osteen, 2001). In our experiment, the transcript level of *Mmp9*, *Timp1,* and *Timp3* were significantly reduced in the embryo-implanted region of the uterus in the PCOS animals. However, upon PE extract of AVG treatment in PCOS animals, only *Mmp9* showed upregulation, with no changes in *Timp1,3*. Further, estrogenic regulation of MMP 9 (Zhang et al., 2007), TIMP 1, and 3 (Nothnick et al., 2004) in ovariectomized female mice have been reported. In contrast, our experimental model system did not show estrogenic regulation of MMPs as identical estrogenic signals were observed amongst all the groups. Hence, it was observed that steroid fluctuation caused by phytosterol-containing PE extract of AVG in PCOS animals could not responsible for the stabilization of MMP and TIMPs. Still, there are not sufficient reports available to support these results.

Apart from the above molecules, the proper growth and development of the implanting embryo is a fundamental event in early pregnancy, and the LIF-STAT pathway is known to regulate this process (Kimber, 2005). When examined for key regulatory genes of this pathway, letrozole-treated animals did not show any significant changes in mRNA levels of *Lif* & *Lifr* in the embryo-implanted region of the uterus. Instead, the expression of *gp130* and *Stat3* significantly declined in the PCOS animals. Emerging evidence suggests that ovarian steroids are reported to play a critical role in regulating LIF, LIFR, and gp130 expression in the uterus throughout the implantation window period (Salleh & Giribabu, 2014). This is supported by the observation wherein exogenous administration of estrogen can induce LIF and LIFR expression in the endometrium of ovariectomized mice (Chen et al., 2000; Ni et al., 2002). Also, a high level of GP130 mRNA levels was detected in the uterus of ovariectomized mice treated with both estrogen and progesterone (Ni et al., 2002). In addition, uterine conditional ablation of STAT3 leads to hampered PR-mediated pathways and reduced PR protein expression *in utero*, suggesting that STAT3 has a pivotal role in PR-dependent pathways during early pregnancy in mice (Lee et al., 2013). In this study, disrupted LIF-STAT signalling was observed in the PCOS animals. However, the declined transcript levels of *Gp130* and *Stat3* were found to be increased upon PE extract of AVG treatment. Also, indirect regulation of the PR-mediated pathway by *Stat3* was observed upon treatment with the plant extract at both the concentration of dose (25 & 75µg/kg/day) as results in a healthy implanting embryo was observed in the uterus. A similar effect was observed in AVG and metformin-treated PCOS animals. The observed effect of each

molecule could be immediate or secondary cross-talk between steroids and bioactive present in the plant extract. Hence, further experiments need to be designed to confirm whether the improved LIF signalling by PE extract of AVG could be because of enhanced progesterone signals or whether increased gene expression of STAT3 has activated the PR-mediated pathways in the implanted region of the uterus. Moreover, herbs and their bio-actives in molecular signalling has been not documented except for few Chinese reports (Xu et al., 2021; Zhanet et al., 2022).

Based on all of the above molecular deficits in the PCOS pregnant uterine, it was speculated that the changes observed might be originated from the modification of the histological architecture structure of the implanted region of the uterus on the day 6^{th} of pregnancy. Moreover, healthy growing implanted embryos were found in the untreated animals, whereas in the letrozole-treated animals, the appearance of vascular permeability was observed in the implanted region of the uterus. This unacceptable uterine structure was retrograde after the treatment with phytosterols containing PE extract of AVG. As a consequence, switching of the uterine structure could give a fine-tuning in the molecular cascade, which gives rise to a better fertility index and increase embryo implants in PCOS pathology, indicating the role of phytosterols in the correction of uterine milieu adapted for better fetal outcomes. It was reported that the methanolic extract of *Talinum Paniculatum* root and leaf has estrogenic properties that were observed in ovariectomized rats, estrogenic activity was evaluated by determining uterine histoarchitecture and uterine weight. The phytosterols such as stigmast-22 en-3-ol, β-sitosterol, stigmasterol, and campesterol have been claimed to possess estrogenic activity due to their affinity to the estrogen receptor (Sukwan et al., 2013)**.** Also, the efficiency of dietary phytoestrogens in ovariectomized rat uteri showed an increase in uterine weight and height of luminal epithelium, uterine edema, hyperplasia of luminal epithelium, and increased estrogen receptor β expression (Helmy, 2014). Nevertheless, these findings are not in agreement with our results because phytocomponents present in the PE extract of AVG at the dosage of 25 & 75 µg/kg/day did not show a proliferative effect on the implanted region of the uterus, and weight gain in the embryo implanted region, suggesting phyto-progestin like effect rather than phytoestrogen.

Altogether, the present study demonstrated a minimum effective dose (25 µg/kg/day) of PE extract of AVG exhibited an anti-androgenic effect on the ovary and progestogenic effects on the uterus. On the basis of the results discussed above and from the previous lab study, the n-

Hexadecanoic and Campesterol of *Aloe vera* have been shown to refine the PCOS condition. Out of these two compounds, campesterol is thought to be one of the dynamic components of *Aloe vera* that could act as a pre-conceptive agent in PCOS pathology. The possible mechanism of a phytosterol-containing extract derived from *Aloe vera* gel may be due to its regulatory role in the transcription and translation of steroid receptors and key early pregnancy marker proteins, mainly by acting as a progestogenic agent. It assisted in the restoration of the majority of progesterone-responsive genes during the early phase of pregnancy in letrozole-induced PCOS animals. Additionally, the ovarian and uterine structure was improved by normalizing the hormonal milieu in letrozole induce PCOS animals. Remarkably, PE extract of AVG is similarly potent as AVG and metformin towards control of pregnancy complications associated with PCOS, indicating that oral administration of PE extract of AVG in the dose of 25 µg/kg/day before conception for 30 days is adequate for treatment of PCOS pregnancy complications without causing any adverse effect to animals.

The above data of this chapter clearly depict that the PE extract of AVG has proven efficacy as a pre-conceptive agent. However, the study warrants the further characterisation of non-polar phytocompounds of AVG that could possibly play a role as a ligand in modifying the targets of pregnancy directly or indirectly. Thus, understanding the possible interaction of the phytocomponents with key molecules of early pregnancy is vital. Although, the screening of molecular interaction between each phytocompounds and target molecules becomes tedious and impractical. It can be noted that a preliminary study of bioactive isolation and its characterization in *in-vitro* and *in-vivo* studies showed that campesterol and n-Hexadecanoic acid are possible ligands of *Aloe vera* that have proven to have the best efficacy as fertility agents in the non-pregnant state of PCOS (Dey et al., 2022). Hence, our next chapter has focused on the *in-silico* validation of these compounds (Campesterol and n-Hexadecanoic acid) as a pre-conceptive agent in PCOS pathology.

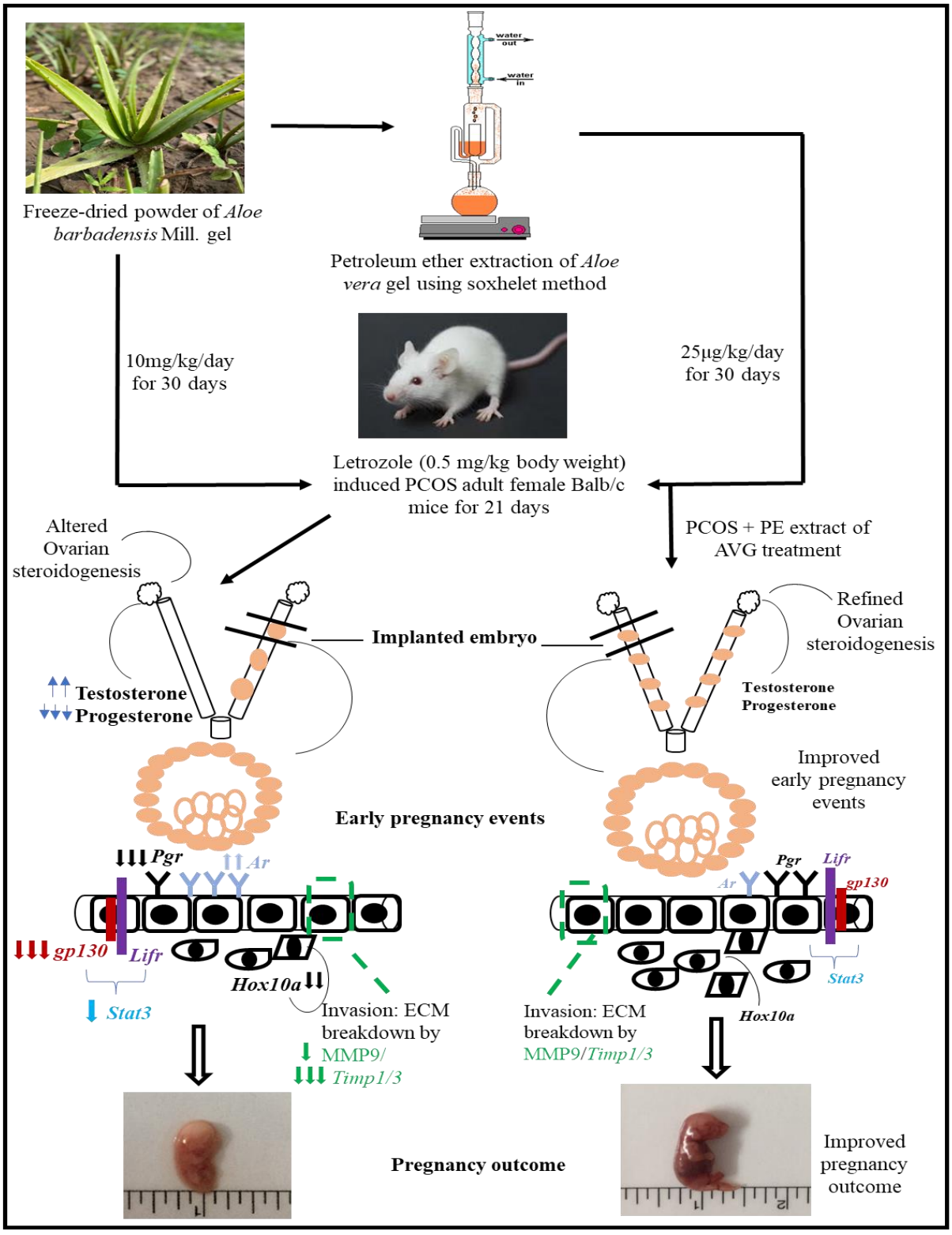


Figure 4.24. Diagrammatic summary of the current study. *Ar* androgen receptor, *Pgr* progesterone receptor, *Hox10a* homeobox transcription factor 10a, MMP Matrix metalloproteinase, *Timp* tissue inhibitor of metalloproteinase, *Lifr* leukemia inhibitory factor receptor, *Gp130* glycoprotein 130, *Stat3* signal transducer and activator of transcription 3.

4.5 Conclusion

The phytocomponents containing PE extract of *Aloe vera* gel exhibited progestin-like properties during early pregnancy abnormalities in the letrozole-induced PCOS mouse model. The key molecular players of early pregnancy events were investigated using an "*in vivo*" study. It can be concluded that the phytocomponents containing PE extract of AVG (PE-25) may exert agonistic effects on progesterone signalling and its regulators in early gestation. The results were comparable to those obtained with *Aloe vera* gel and metformin treatment, recommending that bio-actives present in the PE extract of AVG is a possible bio-functional compound(s) of *Aloe vera* gel that can be used as a pre-conceptive agent, will provide health-giving interventions, rendering therapies to prevent serious complications for the PCOS mothers. However, detailed molecular interpretation of the bio-functional molecules requires purification of non-polar phytocomponents from *Aloe vera* gel and its investigation towards a better uterine microenvironment in multi-etiological pathology with the least severe effects. Also, ***this is the first study to demonstrate the molecular mechanism of bio-actives of Aloe vera gel towards the management of early pregnancy complications when used as a pre-conceptive agent in PCOS phenotype.***