CHAPTER 7

EFFECT OF PETROLEUM ETHER (P1) EXTRACT OF THE ALOE VERA GEL IN PCOS RAT MODEL

7.1 INTRODUCTION

Aloe vera has been used for many centuries for its curative and therapeutic properties. Although diverse ingredients from the inner gel have been identified, but therapeutic effects have not been correlated well with each individual component. *Aloe vera* contains 75 potentially active constituents: Polysaccharides, vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acids and amino acids (Shelton 1991; Atherton 1998).

Several reports have suggested various efficacies such as hypoglycaemic, antitumor, anti-inflammatory, antioxidant and laxative effects of *Aloe vera* gel (Rajasekaran et al. 2004; Tanaka et al. 2006). These beneficial properties of *Aloe* leaf have been attributed to the various phyto-compounds found in the inner leaf parenchymatous tissue (Boudreau and Beland 2006; Hamman 2008). The presence of sterols, phenols, alkaloids, polyphenols, flavonoids and lignin have attributed to various efficacies (Madhavi et al. 1995; Veerapur et al. 2009). Recently, several researchers have tried to check in the efficacy of phyto-components from AVG in the content of its hypoglycemic and anti-inflammatory potential (Rajasekaran et al. 2006; Saleem 2009).

One of the well studied phyto-component in *Aloe vera* gel is phytosterol, which has various biological properties (Jones et al. 1999; Ostlund Jr 2004). Phytosterols (plant sterols) are secondary plant metabolites which structural and biological counterparts of cholesterol. Plant sterols are responsible for permeability and fluidity of cell membranes. Further, they act as precursors of brassinosteroids, thus regulating storage and transport processes, transform into numerous other metabolites such as glycoalkaloids and saponins (Piironen et al. 2000). Similarly to cholesterol, most phytosterols have a steroid nucleus and a hydroxyl group in the 3β-position at carbon atom three (C3) (Moreau et al. 2002).

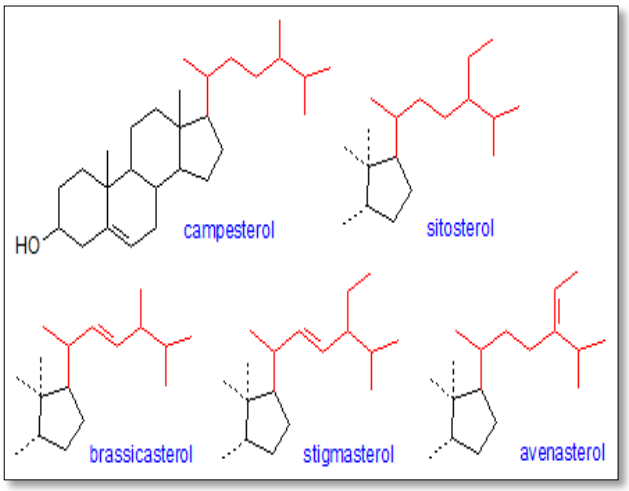


Figure 7.1 Structures of main plant sterols.

Structural differences with cholesterol are mainly found inside chain substitution at C24, an additional double bond between C22 and C23 and/or, in the case of plant stanols, saturation taken place at steroid nucleus. The addition of a methyl/ethyl group at the C-24 position of the side chain sterol (campesterol/β-sitosterol, respectively) whereas in the case of plant stanols, the saturation of the Δ5 double bond (campestanol and sitostanol) increases the hydrophobicity of the molecule, thereby reducing their absorption (Heinemann and Ankenbauer 1993). Phytosterols can be hydrogenated to obtain phyto-stanols. Both esters are chemically stable materials, having comparable chemical and physical properties to edible fats and oils. The substances are insoluble in water, but soluble in non-polar solvents, such as hexane, iso-octane and 2-propanol.

Isolated phytosterols have modulatory action that lead to prevention of visceral fat obesity and improved hyperglycemia, hyperlipidemia and insulin resistance in metabolic syndrome (Misawa et al. 2008). In this context, Tanaka et al. (2006) tried to isolate various phytosterols and purify the active compounds from fraction of *Aloe vera* gel that ameliorated hyperglycaemia in diabetic C57BL/ KS-Lepr db (db/db) mice, where the mice lack functional leptin receptor (Lee et al. 2001). In addition these, β-sitosterol was shown to have glucose lowering effect in type 2 diabetes patients (Sutherland et al. 2009). Recent study demonstrated that oral administration of anti-diabetic phytosterols isolated from *Aloe vera*, caused a reduction in pro-inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1) and serum adiponectin level, which are risk factors involved in cardiovascular disorders (Misawa et al. 2012).

An important co-morbidity of hyperglycemia is Dyslipidemia. Thereby, research has focused on β sitosterol for the development of steroidal drugs and functional food ingredients among all phytosterols as it exerts hypo-cholesterolemic effects by inhibiting the absorption of cholesterol in the intestines through competition with LDL-cholesterol (Gylling and Miettinen 1996) and also decreases in total, along with LDL-cholesterol concentrations of 5–15% as evident from several studies (Jones et al. 1999; Ostlund Jr 2004). *Aloe vera* gel contains various phytosterols wherein β-sitosterol, stigmasterol, lupeol and campesterol are in abundance (Hamman 2008). Also, earlier chapter demonstrated that 70% of P1 fraction (Petroleum ether extracted fraction) is enriched with β sitosterol and other

phytosterols. These phytosterols present in *Aloe vera* gel is known to have cholesterol lowering property wherein "i*n vitro"* study has demonstrated that phytosterols activates liver X receptor [a nuclear receptor- controls the expression of ATP-binding cassette sub-family G member 5 (ABCG5/8)], thereby increasing the expression of ATP Binding Cassette (ABC) transporters and the transport of cholesterol from enterocytes back into the intestinal lumen, which in turn leads to reduction in cholesterol absorption from intestinal wall (Plat and Mensink 2005). In clinical study, esterified sterol and stanol mixtures consumption resulted in reduction in plasma Triglycerides (TG) concentrations (Jones et al. 2000). This could be correlated with hypoglycaemic effect rendered by AVG. Also, molecular level studies have shown that decrease in the expression levels of hepatic genes encoding gluconeogenic enzymes [Glucose 6-phosphatase, Phospho-enolpyruvate carboxykinase (PEPCK)], lipogenic enzymes (ACC, FAS), and sterol regulatory element-binding proteins 1(SREBPs 1) whereas increased the expression of hepatic β-oxidation enzymes [apocarotenoid oxygenase (ACO), Carnitine palmitoyltransferase I (CPT1)] and Peroxisome Proliferator Activated Receptor alpha (PPARα) expressions could alter dyslipidemia. Also, feeding of 0.5% stigmasterol for 6 weeks to Wistar and Wistar-Kyoto (WKY) rats significantly suppresses HMG-CoA reductase activity and results in approximately 11% reduction in plasma cholesterol levels (Batta et al. 2006). In addition to this, β-sitosterol also has direct effect on signalling pathway wherein it stimulates phosphorylation of AMPK and its downstream target lipogenic enzyme-Acetyl-CoA carboxylase (ACC). The increased phosphorylation of AMPK and ACC was remarkably inhibited by the AMPK-specific inhibitor Compound C (Zhou et al. 2008).

Apart from anti-hyperglycaemic and anti-hyperlipidemic properties, *Aloe vera* gel is known to have wound healing property wherein some phytosterols glycosides have been identified that increase the production of angiogenic factors and/or the expression of their respective receptors suggesting phytosterols also act as novel factor in angiogenesis (Ovesna et al. 2004). It is known to regulate key molecules that is involved in inflammation, the immune response, anti-cancer defences, and apoptosis (Awad and Fink 2000; Bouic 2002). *"In vitro"* study suggested that phytosterols also affect cell cycle kinetics wherein β-sitosterol induced cell cycle arrest at the G2/M transition (Awad et al. (2001); Vivancos and Moreno 2005)

reported that β-sitosterol increased the activities of antioxidant enzymes, superoxide dismutase and glutathione peroxidase indicating that phytosterols can protect cells from damage by reactive oxygen species. It may possible that phytosterols from AVG be effective in management of metabolic syndrome by increasing overall antioxidant potential.

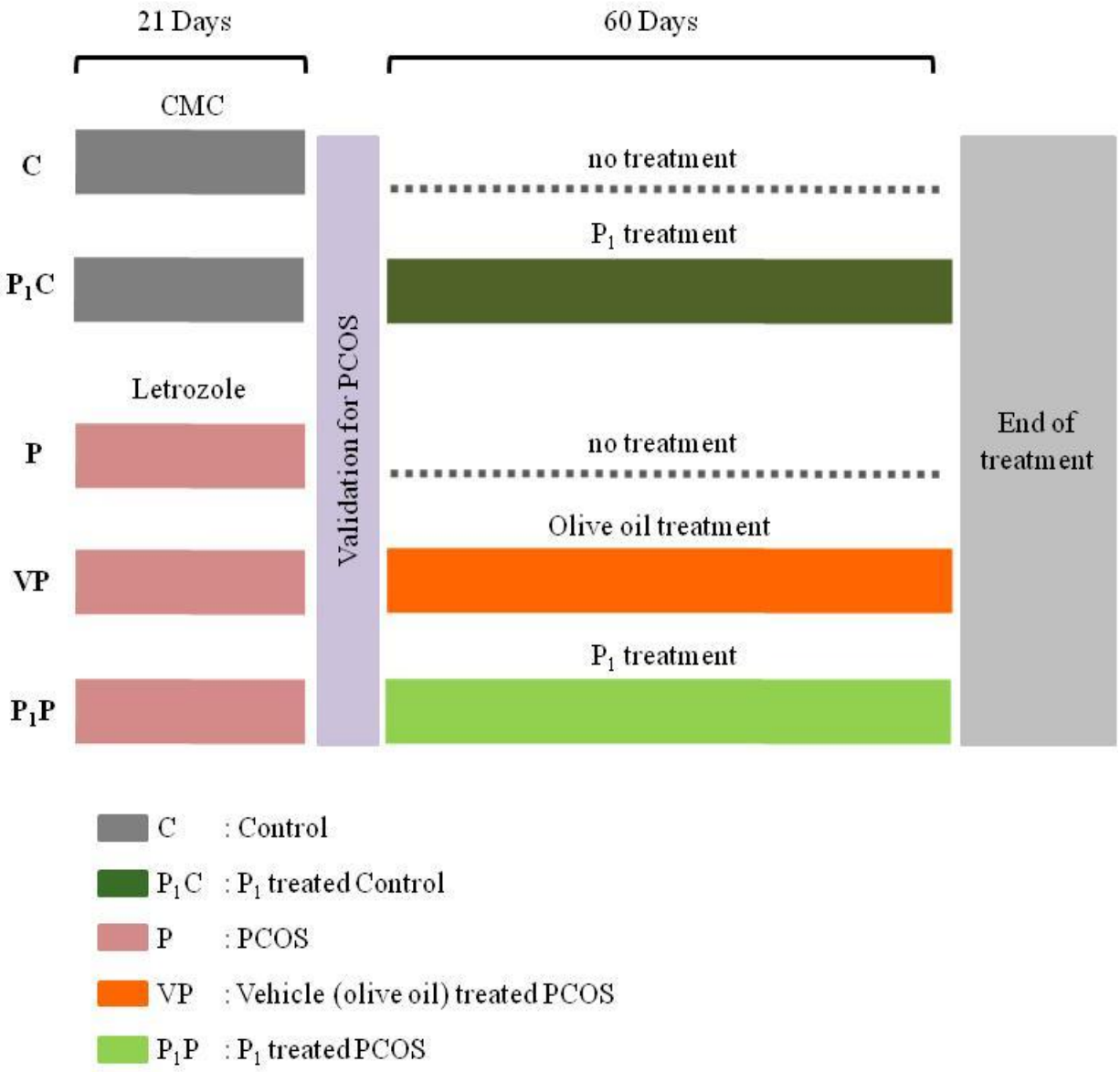
As PCOS a disease condition wherein hyperglycaemia and dyslipidemia are secondary manifestations in addition to ovarian dysfunction. Also, it is clear that AVG has enriched β sitosterol, stigmasterol, lupeol and fatty acids derivatives as shown in the current study. Thereby, these phyto-components independently or synergistically could act at various targets as above mentioned mechanisms. Steroidogeneis is functional attribute of ovary which is under the regulation of hypothalamus-pituitary axis. "*Ex vivo"* study from previous chapter showed wherein non-polar P1 fraction extracted by petroleum ether indicated that it could directly modulate ovarian steroidogenic enzyme activity. Thereby, current chapter attempts to understand *"in vivo"* role of non polar P1 fraction from AVG on steroidogenesis with special reference to ovary.

7.2 MATERIALS AND METHODS

7.2.1 PREPARATION OF P1 FRACTION FROM ALOE VERA GEL

Initially, epidermis had removed from the leaf and homogenate of the *Aloe vera* gel was prepared. After that equal volume of *Aloe* gel and petroleum ether was taken in volumetric flask (1:1) ratio, vigorously stirred and kept in shaker for 24 hrs at room temperature. Then, organic layer evaporated at room temperature. Quantification of total sterols content was assayed from dried extract of petroleum ether (P1 fraction) by Sabir et al. (2003). Earlier chapter suggested that 10 mg dry weight of *Aloe vera* gel demonstrated significant modulation in ovarian structure-function. It is evident that form HPLC analysis that 10 mg weight contained ~25 µg phytosterols. Thereby, ~25 µg of non polar fraction of AVG was used for "*in vivo"* study wherein it was resuspended in olive oil according to dry weight of *Aloe vera* gel (10 mg).

7.2.2 PLAN OF WORK



Adult virgin Charles foster female rats (2-3 months; 200 ± 15 g) were used for the study

C : 1% Carboxy methyl cellulose (CMC) for 21 days

P : 0.5 mg of letrozole per kg body weight for 21 days

P_1C : Control animals treated with 25 µg P_1fraction dissolved in 1 ml of olive oil

VP : PCOS animals treated with1 ml of olive oil

P_1C : PCOS animals treated with 25 µg P_1fraction dissolved in 1 ml of olive oil

All treatments were given daily by oral gavages

n = 6-8 for each group

7.2.3 ANIMAL TREATMENT

Animals were initially divided into 2 groups wherein one group received 1% CMC (carboxymethylcellulose) and served as vehicle control (C). The other groups of animals were treated orally with letrozole daily for 21 days (0.5 mg/kg body weight). Letrozole treated animals demonstrated insulin resistance, disturbed estrus cyclicity and were considered as PCOS group (P). One set of animals were treated with partially purified P1 fraction (~25 μg/ml) for 2 months after the induction of PCOS (P1P). Next group of PCOS animals received Olive oil (Vehicle for P1 fraction) (VP). In addition to this, untreated animals receiving P1 fraction were designated to be herbal control (P1C) during the course of experiments.

7.2.3 BIOCHEMICAL PARAMETERS

During course of experiment regime, several biochemical parameters have been checked at time period of 30 days of treatment as well as at the end of experiment (6o days). Parameters such as Serum glutamate pyruvate transaminase (SGPT) and serum creatinine levels were analyzed to evaluate toxicity of P1 fraction of *Aloe vera* gel whereas Oral Glucose Tolerance Test (OGTT) was performed to assess glucose tolerance in all the groups of animals. Apart from glucose tolerance test, lipidemic status was assessed in serum of all animals in all groups using kits.

Steroid hormones: Testosterone, Estradiol, Progesterone and Insulin levels were checked in the serum by ELISA.

At the end or experiment regime, animals of all groups were sacrificed in diestrus stage. Following this, ovaries, uterus, brain and liver tissues of the animals were excised out wherein activities of steroid biosynthetic enzymes- 3β Hydroxy steroid dehydrogenase (3β-HSD) and 17β Hydroxy steroid dehydrogenase (17β-HSD) were estimated in reproductive tissues-ovary, uterus and brain region-hypothalamus, pituitary. Also, steroid metabolizing enzymes of phase I metabolism -17β Hydroxy steroid oxidoreductase (17β-HSOR) and cytochrome P_{450} and enzymes for Phase II metabolism - UDP-glucuronosyltransferase (UDPGT) and glutathione S-transferase (GST) activities were studied in liver tissues in all groups of animals. The protocols for the assays have been mentioned earlier in material and methods section.

Total RNA was extracted from ovarian tissue by TRIZOL method and studied for gene expression of StAR, Aromatase, Androgen receptor (AR), Insulin receptor

(IR), Luteinizing hormone receptor (LHR), Follicle stimulating hormone receptor (FSHR) by Reverse transcriptase Polymerase Chain Reaction (RT-PCR) method and were normalized using internal control-GAPDH.

In addition to this, ovarian tissue was excised, kept in lysis buffer and stored at -80°C. Later, tissue was processed for western blot analysis to check the key protein expression of StAR, 3β-HSD, Aromatase and Androgen receptor (AR) and β actin as internal control.

To analyze changes occur in ovarian structure after treatment of P1 fraction, immediately after sacrifice of the animals, ovaries were removed and kept in 10 % formalin for 24 hrs and tissues were further processed for sectioning with 5µm thickness. After that tissues were fixed with Hematoxylin-eosin stain and sections were observed in light microscope. After observation, images were taken with 4 x magnifications.

Detailed methods were discussed above are explained in materials and methods.

7.3 RESULTS

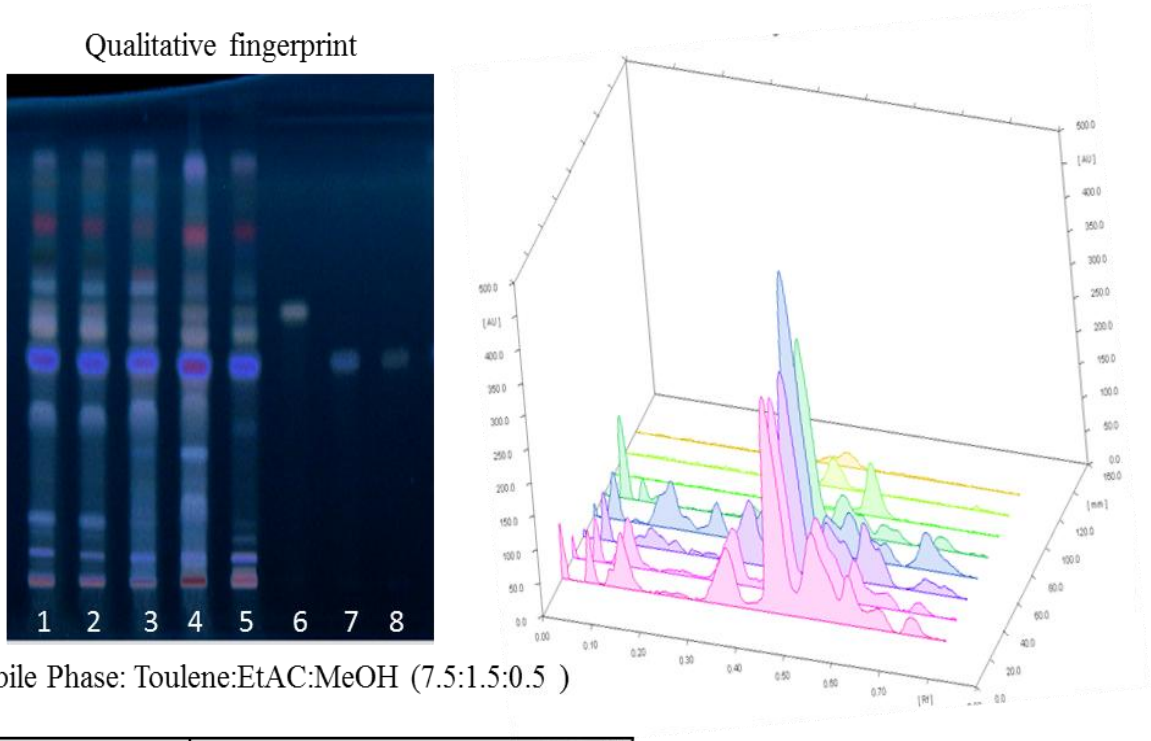
The phytochemical analysis of *Aloe vera* gel extracted with petroleum ether exhibited various phytosterols in non-polar fraction has been named as (P1) fraction. Also, "*ex-vivo*" study suggested that P1 fraction has direct effect on ovarian steroidogenic enzyme activity. Hence, in view of this, aim of present chapter was to evaluate the "*in vivo"* effect of P1 fraction of *Aloe vera* gel in letrozole induced PCOS rat model.

In this context, letrozole induced PCOS rats (P group) were treated with P1 fraction using olive oil as vehicle (~25µg/orally) for 60 days (P1P group). In addition to this, there were PCOS animals which received olive oil and served as vehicle group (VP) while control group (C group) received P1 fraction was termed as herbal control (P1C group).

Data from previous chapters have demonstrated that 10 mg dry weight of *Aloe vera* gel for 60 days was the minimum effective dose required for the management of clinical pathophysiological symptoms of PCOS. In addition to this, a time-dependent profiling of phytocomponents present in the P1 fraction was performed by HPTLC to check their stability upon storage. Results demonstrate that the P1 extract was stable

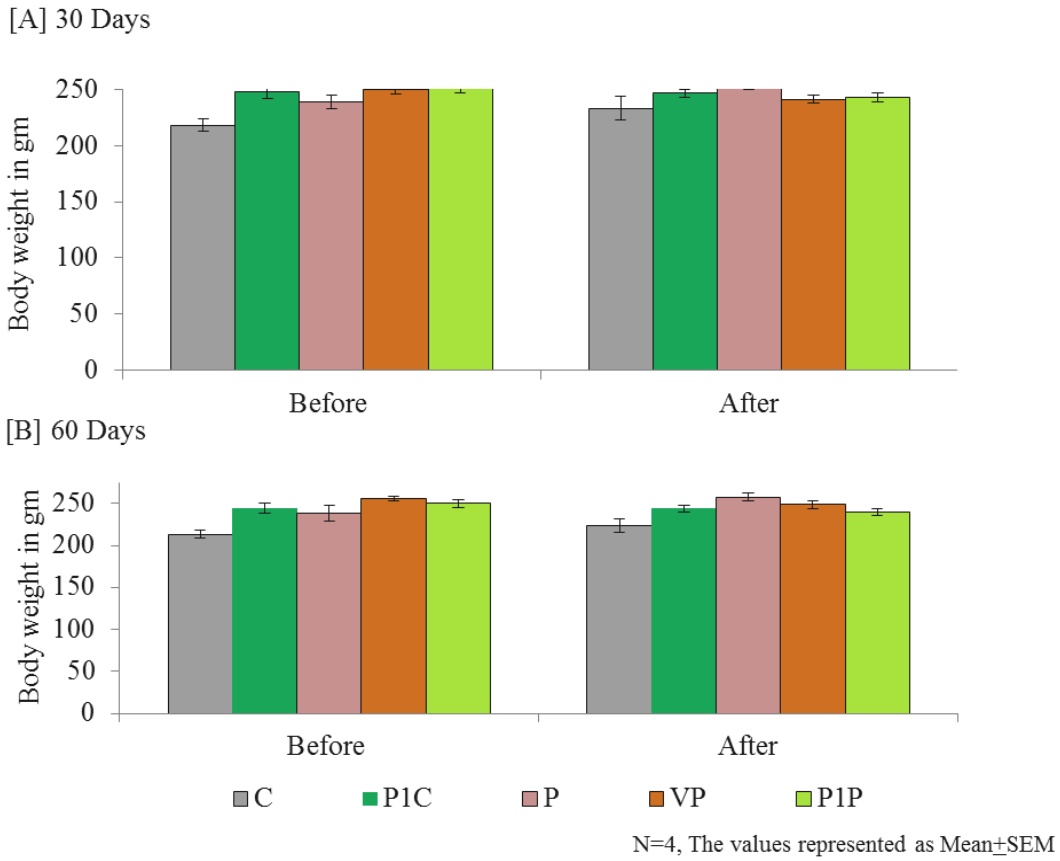
upto 60 days (Figure 7.3.1). Hence, the P1 treatment was continued for 60 days.

Figure 7.3.1 Stability of P1 fraction of *Aloe vera* gel



Track 1: 15 days	Track 5: Fresh *Aloe*
Track 2: 30 days	Track 6: Lupeol 50 ppm
Track 3: 45 days	Track 7: Betasitosterol 25 ppm
Track 4: 60 days	Track 8: Stigmasterol 10 ppm

Figure 7.3.2 Effect of P1 fraction of AVG on Body weight in letrozole induced PCOS rats

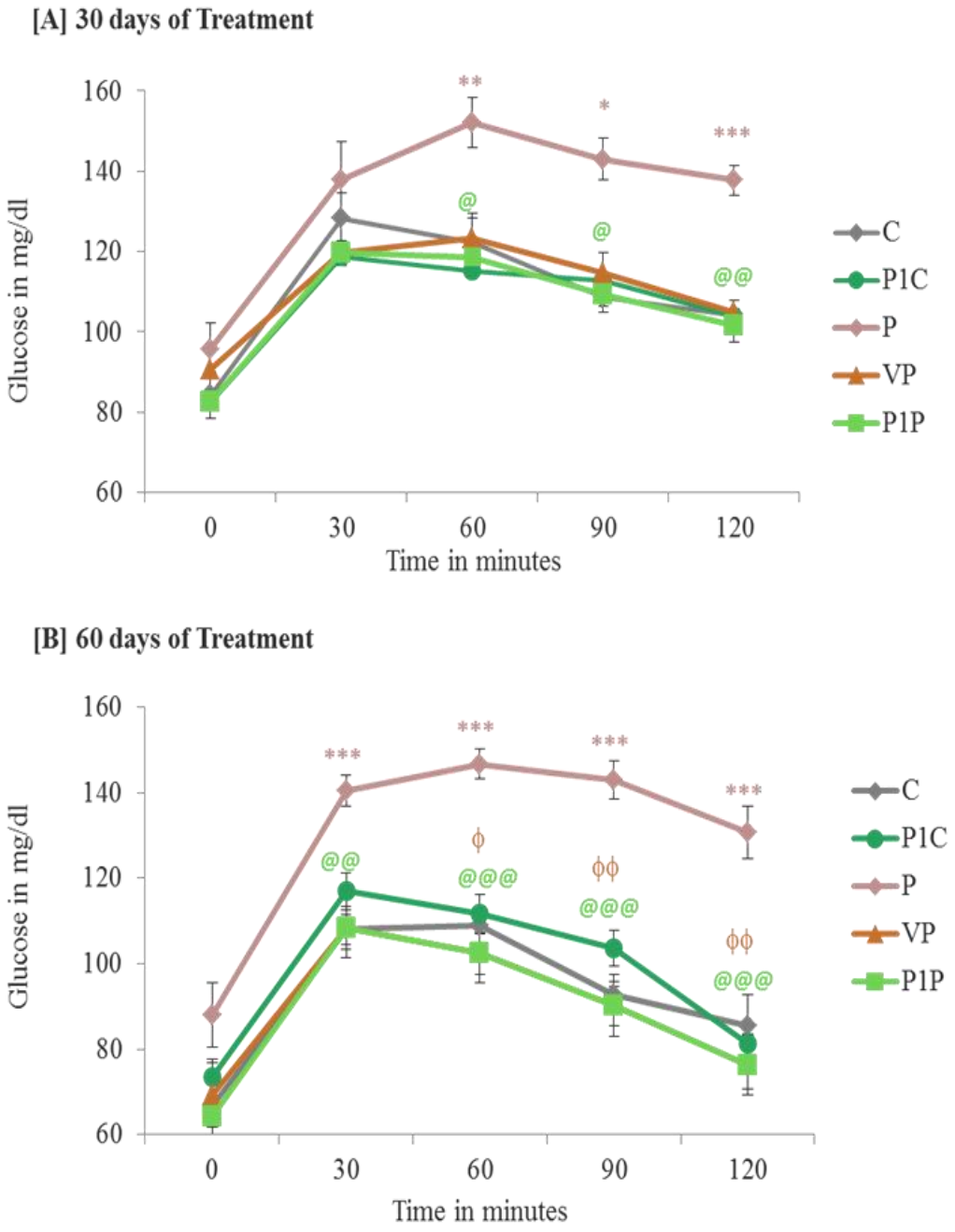


N=4, The values represented as Mean±SEM

There was no significant alteration in the body weight of animals after treatment with P1 fraction of *Aloe vera* gel (Figure 7.3.2).

Oral glucose tolerance test was performed to evaluate the efficacy of P1 fraction of *Aloe vera gel* (AVG) on the glucose tolerance in Letrozole induced PCOS rats. After 30 days, Letrozole induced PCOS rats (Group P) exhibited a change in glucose profile wherein glucose level was elevated in OGTT profile (**p<0.01, **p<0.01, ***p<0.001) at 60, 90 and 120 minutes of the test respectively as compared to control group. At 30 days of time interval, P1 fraction treated PCOS rats (P1P) demonstrated a significant change at 60, 90 and 120 minutes point of OGTT profile (@p<0.05, @@p<0.01, @@p<0.01) whereas PCOS rats treated with olive oil (VP) showed significant change at 90 and 120 minute (@p<0.05) in OGTT profile as compared to PCOS group. Herbal control group (P1C) depicted similar results to control rats (Figure 7.3.3 (A)). At end of experiment regime (60 days), OGTT profile was evaluated again in all groups of animals to check glucose tolerance. Letrozole induced PCOS rats (P).

Figure 7.3.3 Effect of P1 fraction of AVG on Oral Glucose Tolerance Test (OGTT) in letrozole induced PCOS rats



N=4, The values represented as Mean±SEM
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
*p<0.05, **p<0.01, ***p<0.001 as compared to Control group
@p<0.05. @@p<0.01, @@@p<0.001 as compared to PCOS group
ϕp<0.05,ϕϕp<0.01 as compared to PCOS group

Table 7.3.1 Effect of P1 fraction of AVG on insulin status in letrozole induced PCOS rats

GROUPS	Insulin (μIU/ml)	HOMA-IR
C	12.3±0.6	1.8±0.1
P1C	13.2±1.2	2.5±0.1
P	21±1.0**	4.5±0.3***
VP	9.1±1.2@@@	1.4±0.1@@@
P1P	13.5±0.76@	2.5±0.2@@

N=4, The values represented as Mean±SEM
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
**p<0.01, ***p<0.001 as compared to Control group
@@p<0.01, @@@p<0.001 as compared to PCOS group.

HOMA IR = Fasting insulin x Fasting glucose / 405
Normal insulin resistance : < 3
Moderate Insulin resistance : Between 3 – 5
Severe Insulin resistance : > 5

During the course of experiment, liver toxicity markers like Serum Glutamate Pyruvate Transaminase (SGPT) and creatinine was evaluated. P1 fraction treated groups (P1P) exhibited non-significant change in levels of the toxicity markers. In addition to this, letrozole treated PCOS group (P) as well as olive oil-vehicle treated animals (VP) demonstrated no change in the above parameters upon treatment indicating no toxic effects of phytosterols fraction on PCOS rats during study (Figure 7.3.4).

In PCOS, excessive androgen contributes to alteration in lipid metabolism. The body composition is influenced by testosterone, which decreases subcutaneous fat lipolysis along with increased abdominal fat deposition that leads to central obesity in PCOS females (Vilmann et al. 2012). Hence, lipid profile was evaluated in PCOS rats (P) wherein it exhibited elevation in triglycerides level (***p<0.001) as compared to control group whereas no change was observed in total cholesterol level. In PCOS rats, LDL level was also elevated (***p<0.001) while no significant changes were

observed in HDL and VLDL levels. Elevated TG levels implicated the presence of metabolic syndrome in PCOS.

This hypertriglyceridemia has been normalized with P1 fraction (P1P) treatment (@@@p<0.001). P1 fraction demonstrated more significant change as compared to Vehicle group (VP) group (#p<0.05) suggested that P1 fraction rich in various phytosterols has more lipid lowering efficacy. In PCOS rats, LDL levels were also reduced after P1 fraction treatment (@@@p<0.001) whereas no significant changes were observed in HDL and VLDL levels. Vehicle group (VP) also showed significant change in LDL level (@@@p<0.01) but P1 fraction again exerted more significant change as compared to vehicle (VP) group (###p<0.001) (Table 7.3.2).

Table 7.3.2 Effect of P1 fraction on Lipid profile in letrozole induced PCOS rats

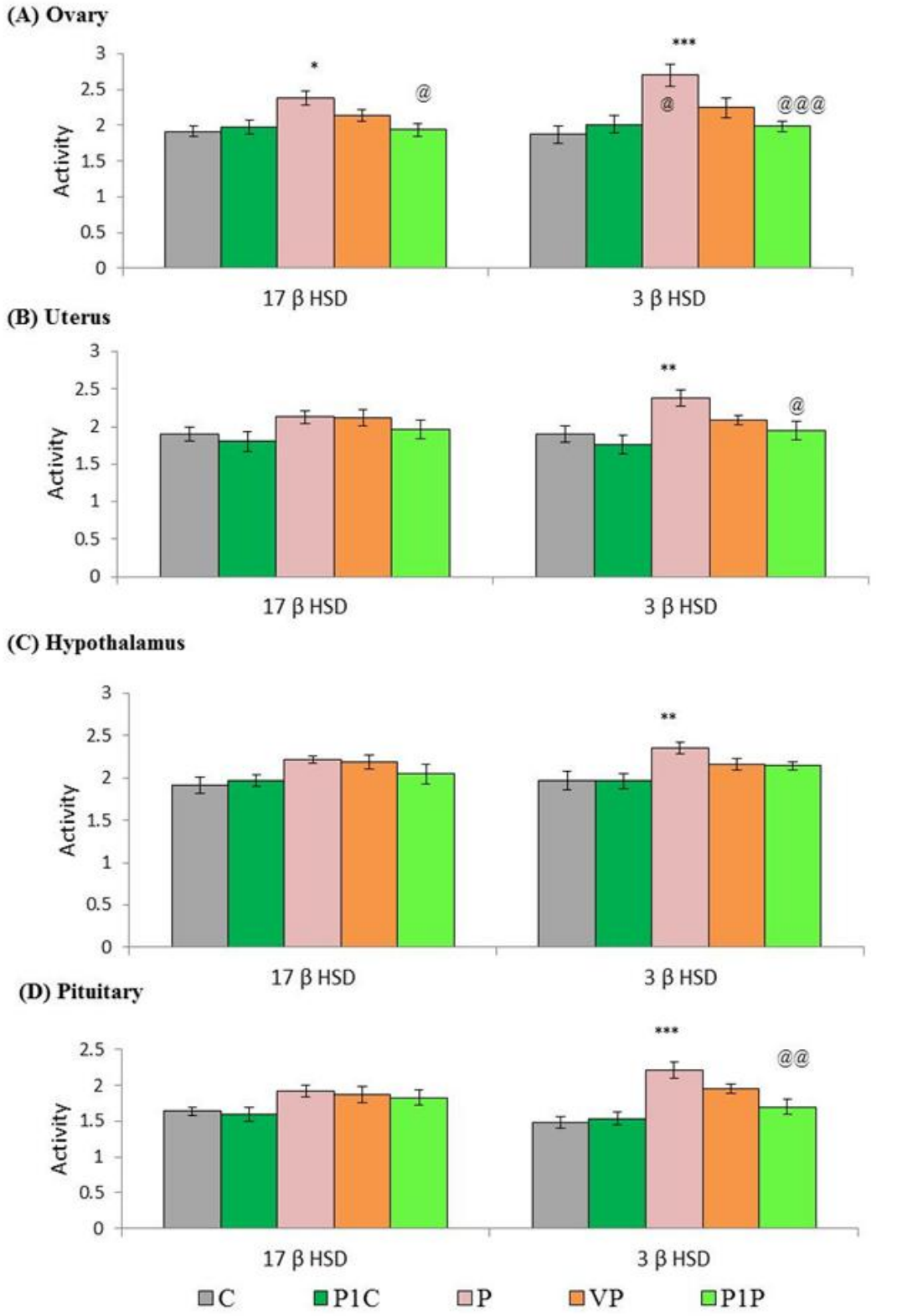
	Triglycerides	Total Cholesterol	LDL	HDL	VLDL
C	70.8±2.9	48.3±1.7	51.9±3.6	31.1±2.0	13.8±0.69
P1C	71.6+4.9	52.6±5.7	49.6±3.1	27.6±2.8	16.0±1.5
P	96.0±6.3***	55.8±3.4	108.0±10.2***	24.5±2.3	22.6±2.0
VP	78.7±2.3@	43.6±3.7	79.11±5.5@@@	34.1±1.4	13.7±1.3
P1P	64.2±2.4@@@#	45.3±4.3	45.8±2.2@@@###	28.7±1.4	13.2±0.4

N=4, The values represented as Mean±SEM
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
***p<0.001 as compared to Control group
@p<0.05, @@@p<0.001 as compared to PCOS group.
#p<0.05, ###p<0.001 as compared to Vehicle group.

Steroidogenesis plays a major role in regulation of the ovarian functioning and PCO condition is characterized by altered steroidogenesis leading to impairment in folliculogenesis (Diamanti-Kandarakis 2008). Thereby, key steroidogenic enzymes- 3β hydroxysteroid dehydrogenase (3β HSD) and 17β hydroxysteroid dehydrogenase (17β HSD) were studied in reproductive tissues: ovary, uterus and brain tissues: hypothalamus, pituitary in all groups of animals.

Letrozole induced PCOS rats (P) demonstrated an elevation in activity of both enzymes in ovary (*$p<0.05$, ***$p<0.001$). There was no change observed in 17β hydroxy steroid dehydrogenase (17β HSD) while 3β hydroxy steroid dehydrogenase (3β HSD) activity was elevated in uterus, hypothalamus and pituitary as compared to control group (**$p<0.01$, ***$p<0.001$). P1 fraction treated PCOS (P1P) group exhibited significant reduction in both enzymes activities (@$p<0.05$, @@@$p<0.001$) in ovary whereas vehicle (VP) group demonstrated a change in 3β HSD activity (@$p<0.05$) as compared to PCOS rats. Also, P1P group exerted change in uterine 3β HSD (@$p<0.05$) an enzyme involved in androgen production whereas no change was observed in vehicle (VP) group. In hypothalamus region, there was no significant change in P1 treated group as well as vehicle (VP) group as compared to PCOS rats. In pituitary region, PCOS rats demonstrated significant change in 3β HSD activity upon P1 fraction treatment (P1P) (@@$p<0.01$) whereas no significant change was observed in vehicle (VP) group (Figure 7.3.5).

Figure 7.3.4 Effect of P1 fraction of on Steroidogenic enzyme activity in letrozole induced PCOS rats



N=4, The values represented as Mean±SEM
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
*p<0.05, **p<0.01, ***p<0.001 as compared to Control group. @p<0.05, @@p<0.01 as compared to PCOS group.

In addition, serum steroid hormones levels were estimated in all groups of animals wherein letrozole induced PCOS (P) group demonstrated elevated level of serum testosterone level (*p<0.05) which reverted back to normal after P1 fraction (P1P) treatment (@p<0.05). PCOS rats also exhibited a decrease in level of progesterone (**p<0.01) and estradiol level (*p<0.05) which returned to normal values after P1 treatment (P1P) (@p<0.05). Vehicle (VP) group exhibited no significant changes in steroid hormones level (Table 7.3.3). Thereby, indicating no role of olive oil in steroid metabolism.

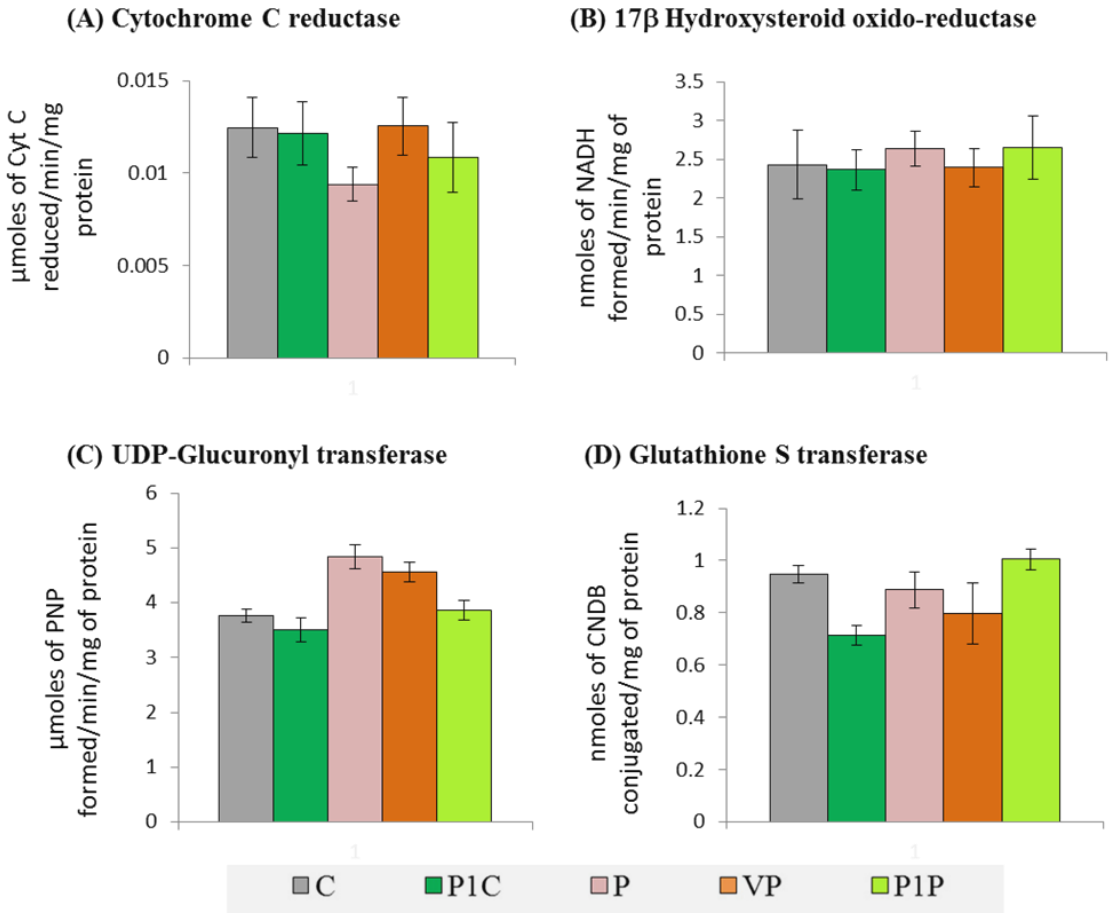
Table 7.3.3 Effect of P1 fraction of on Hormonal profile in letrozole induced PCOS rats

Groups	Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
C	0.42±0.04	72±2.5	14±1.5
P1C	0.48±0.26$^{@}$	70.6± 17.1	7.7± 1.6
P	1.13±0.15*	49±6.5*	5.5±1.2**
VP	0.96±0.24	56±6.5	7.9±1.2
P_1 P	0.48±0.21$^{@}$	75.3±10.7$^{@}$	11.5±3.5$^{@}$

N=3,Mean±SEM,
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS;
P_1P=P_1 treated PCOS
*P<0.05, **p<0.01 as compared to Control group; $^{@}$ p<0.05 as Compared to PCOS Group.

Balance of synthesis and catabolism depicts steroid levels. Apart from the steroid biosynthesis enzymes, the efficacy of P1 fraction of *Aloe vera* gel on steroid metabolizing enzymes were also estimated in all groups of animals after treatment. There was no significant changes observed phase I (Cytochrome c reductase, 17 beta Hydroxysteroid oxido reducatase) and phase II (UDP-glucoronosyl transferase, Glutathione S transferase) enzymes in all groups (Figure 7.3.5).

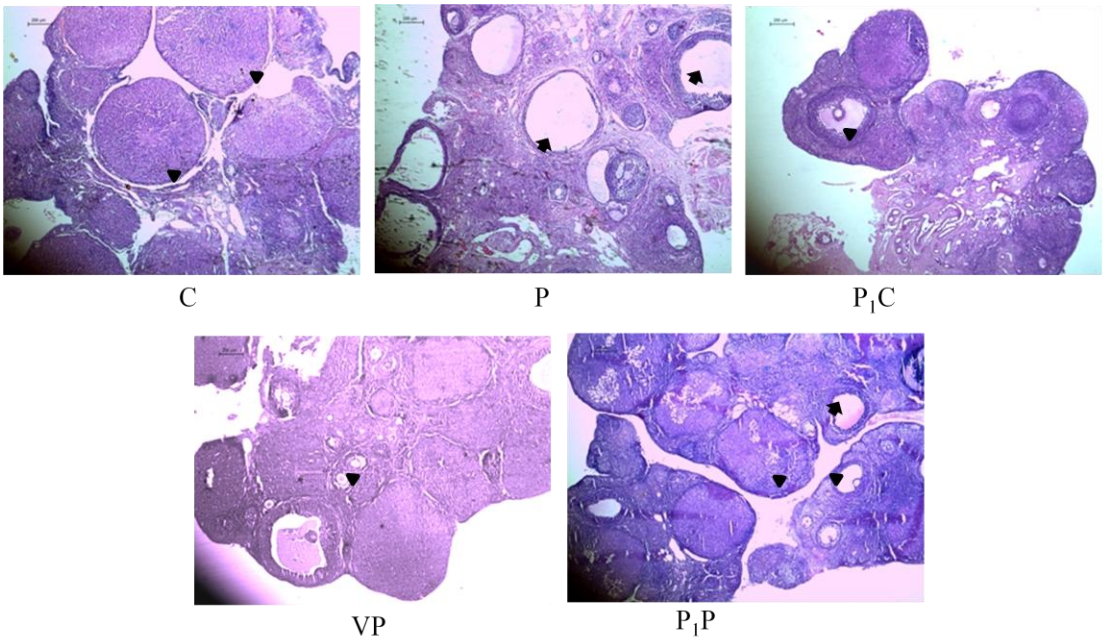
Figure 7.3.5 Effect of P1 fraction of AVG on Liver steroid metabolizing enzymes activities in letrozole induced PCOS rats



N=4, The values represented as Mean±SEM
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS
P_1P=P_1 treated PCOS

PCOS also characterized by presence of peripheral cysts, which is hallmark of PCO phenotype (Kovacs and Briggs 2015). Hence, histological changes were evaluated in current study wherein peripheral cysts with follicular fluid and arrested antral follicles were observed in letrozole induced PCOS rats (P) as compared to control. These PCOS rats after treatment of P1 fraction (P1P) demonstrated a reduction in peripheral cysts with increased growing follicles in ovary. Herbal control (P1C) also showed normal follicle growth similar to control rats. Vehicle (VP) group also exhibited reduced peripheral cysts in ovary as compared to PCOS rats (Figure 7.3.6).

Figure 7.3.6 Effect of P1 fraction of AVG on Ovarian histological changes in letrozole induced PCOS rats



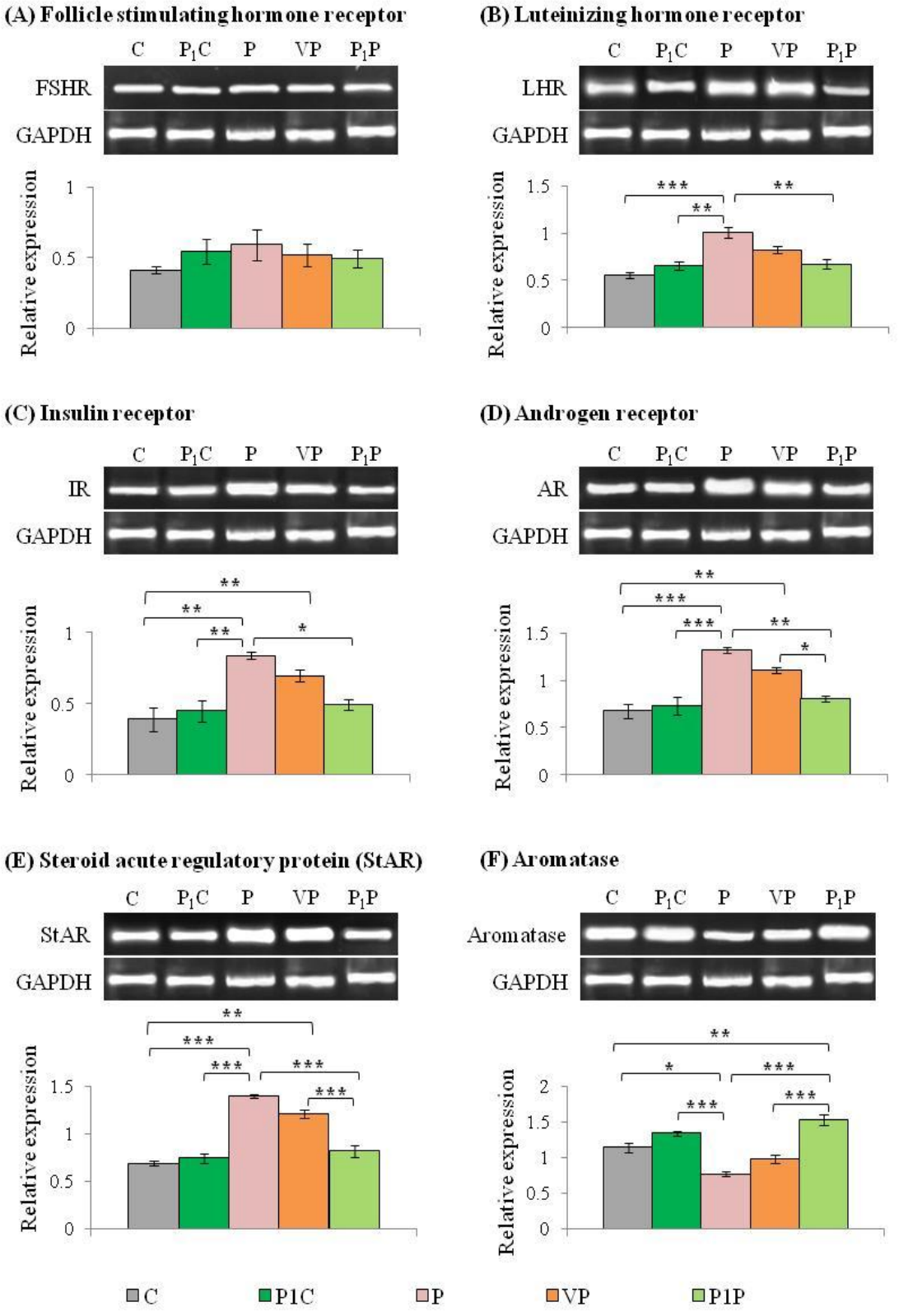
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
N=3, All sections taken in diestrus stage. Magnification- 4X
▲ : Growing follicles ◆ : Cyst

Ovarian steroid hormones perform several important actions related to reproductive organ function and their effects are mediated through interaction with specific proteins (Drummond and Findlay 1999; Salvetti et al. 2010). Several studies have shown that PCO affects the expression of steroidogenic proteins such as aromatase, 3β-HSD, LHR and StAR in the ovaries of letrozole treated rats(Zurvarra et al. 2009). Thereby, transcripts levels were evaluated in all groups of animals. Figure elucidated the effect on P1 fraction on transcript level of key protein in steroidogenesis wherein PCOS rat demonstrated a elevated gene expression of StAR (***p<0.001), insulin receptor (IR) (**p<0.01), Luteinizing hormone receptor (**p<0.01) and androgen receptor (AR) (***p<0.001) whereas decrease was observed in gene expression of in aromatase (*p<0.05) as compared to control group. However, there was no significant change was observed in gene expression of follicle stimulating hormone receptor (FSHR) in PCOS (P) group. After P1 treatment, there were significant changes observed in these key steroidogenic protein wherein substantial decrease in StAR (***p<0.001), insulin receptor (IR) (*p<0.05), LHR (**p<0.01) and AR (**p<0.01) transcripts level as compared to PCOS rats.5) whereas significant increase was observed in transcript level of aromatase (***p<0.001) in P1

fraction treatment (P1P). However, no significant changes were observed in all transcript level in vehicle (VP) group. Also, P1 fraction treated PCOS rats (P1P) demonstrated more significant change in gene expression of above key protein – androgen receptor (**p<0.01), StAR (***p<0.001), (***p<0.001) as compared to vehicle (VP) group suggested that P1 fraction has more efficacy in comparative study (Figure 7.3.7)

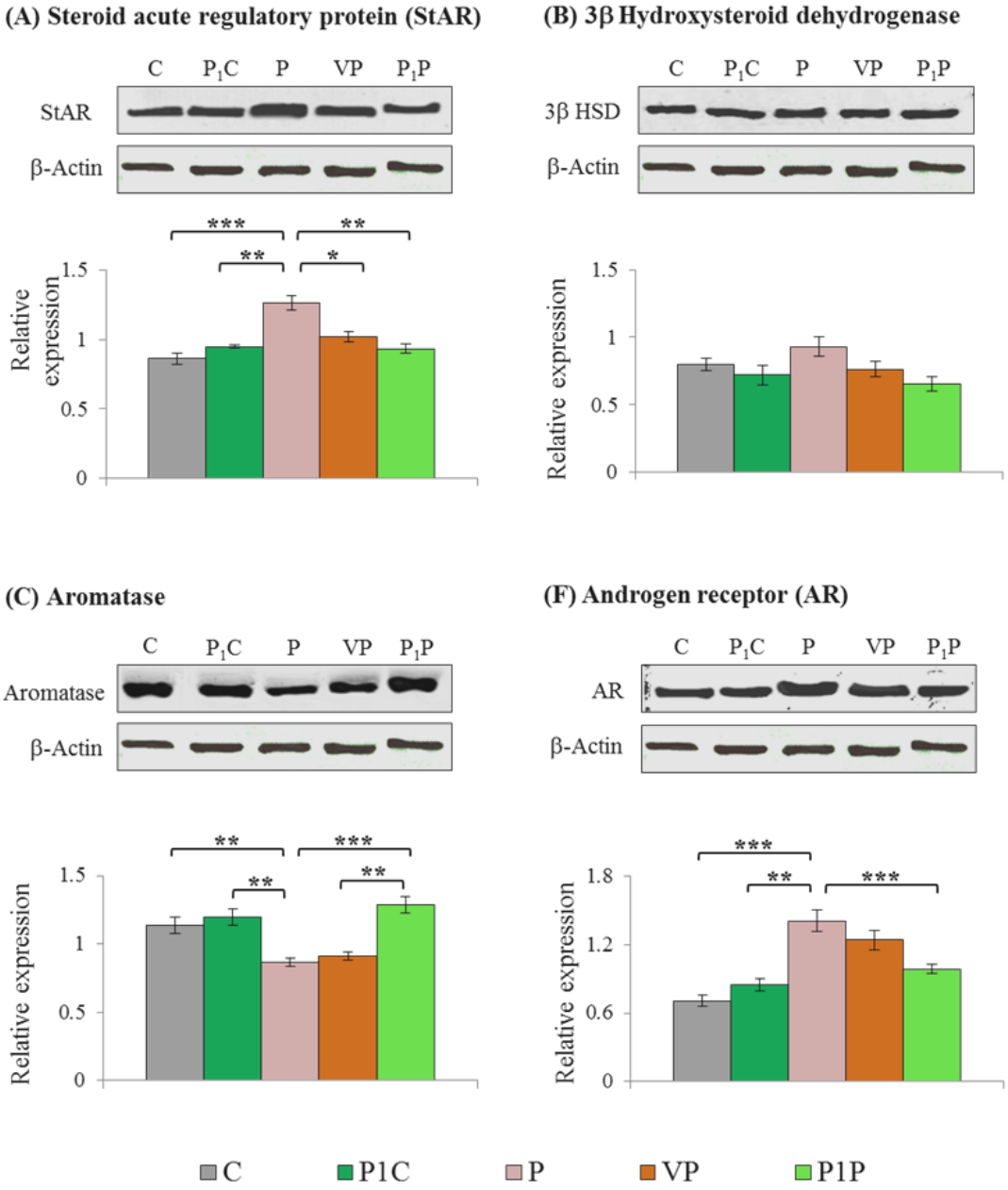
In ovarian steroidogenesis, StAR plays a major role in cholesterol transfer from outer membrane to inner membrane of mitochondria for steroid biosynthesis. Hence, relative protein expression of key regulatory proteins involved in steroidogenesis was evaluated wherein PCOS group demonstrated an elevation in relative protein expression of StAR (***p<0.001), androgen receptor (AR) (***p<0.001) while significant decrease in expression of aromatase (**p<0.01) as compared to control group (*p<0.05); while it reverted back as similar to control StAR (**p<0.01), androgen receptor (AR) (***p<0.001), aromatase (***p<0.001) when treated with P1 fraction (P1P). However, no significant change was observed in protein expression of 3β hydroxy steroid dehydrogenase (3β HSD) in all groups of animals. Vehicle (VP) group also showed significant change in StAR (*p<0.05) whereas no change was observed in AR and aromatase protein expression. In addition, P1P group exhibited more significant change in protein expression of aromatase as compared to vehicle (VP) group (**p<0.02), which elucidated efficacy of P1 fraction over vehicle treatment (Figure 7.3.8)

Figure 7.3.7 Effect of P1 fraction on Transcript of key protein involved in regulation of Steroidogenesis in letrozole induced PCOS rats



Relative expression = Expression of Target gene/Expression of GAPDH
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
n=3-4 per group; All values are presented as Mean ±SEM; *P<0.05; **P<0.01; ***P<0.001

Figure 7.3.8 Effect of P1 fraction on protein expression involved in regulation of Steroidogenesis in letrozole induced PCOS rats



Relative expression = Expression of Target gene/Expression of β-Actin
C=Control; P_1C= P_1 treated control; P=PCOS; VP=Vehicle (Olive oil) treated PCOS; P_1P=P_1 treated PCOS
n=3-4 per group; All values are presented as Mean ±SEM; *P<0.05; **P<0.01; ***P<0.001

7.4 DISCUSSION

Aloe species are now considered a very interesting source of bioactive compounds amongst which phytosterols play a major role (Tanaka et al. 2006; Misawa et al. 2012). Present study was an attempt to investigate the efficacy of partially purified P1 fraction (NPP) of *Aloe vera* gel with impact on modulation of ovarian structure-function in letrozole induced PCOS rat model. Our earlier published data suggested that treatment of *Aloe vera* gel restored glucose intolerance and dyslipidemia along with cyclicity. It has also modulatory effect on ovarian steroidogenesis (Maharjan et al. 2010; Desai et al. 2012).

In view of this result, current chapter investigates *"in vivo"* effect of partially purified non polar fraction of *Aloe vera* gel on letrozole induced PCOS rats. During experimental regime, stability of P1 fraction of *Aloe vera* gel as various phyto-components (volatile in nature) present in gel was assayed. HPTLC data suggested phytosterols are stable up to 60 days (Time period of *in vivo* treatment being 60 days). During course of experiments, levels of toxicity markers were estimated wherein no significant changes were observed in Serum Glutamate Pyruvate Transaminase (SGPT) and creatinine level. This may be because of low dose of phytosterols (~25 μg) that did not exert any toxic effects in PCOS rats.

Letrozole induced PCOS positive rats' demonstrated PCO phenotype including irregular cyclicity, increased body weight, glucose intolerance and altered steroid hormones profile. After validation of PCOS rat model, these PCOS positive rats were treated with partially purified non polar P1 fraction of *Aloe vera* gel (~25μg/orally) for 60 days. P1 fraction treated PCOS rats exhibited restored the glucose sensitivity as medicated by improved HOMA-IR implicating that phytosterols of P1 has good glucose lowering property (Tanaka et al. 2006). There are various reports that suggesting anti-hyperglycaemic property of phytosterols that is identified from *Aloe vera* gel which improved glucose intolerance (Tanaka et al. 2006; Misawa et al. 2008)) has also demonstrated that sterols isolated from *Aloe vera* reduced the expression of gluconeogenic enzymes (G6 Pase and PEPCK) and also stimulated glucose catabolism along with suppressed *de novo* glucose production. Also, phytosterols have direct effect on glucose metabolism wherein synergism of β-sitosterol and stigmasterol produced hypoglycaemic activity (Jamaluddin et al. 1994).

Lupeol is one of content phyto-component of the P1 fraction is known for anti-diabetic antioxidant properties (Gupta et al. 2012). *Aloe vera* gel also consists of phytosterol glycosides that also have reported anti-hyperglycemic property (Rajasekaran et al. 2004; Huseini et al. 2012). Thus, anti-hyperglycaemic property majorly due to phytosterols present in P1 fraction, which may enhance glucose absorption and improve glucose intolerance in PCO condition. As P1 fraction is enriched with 70 % β sitosterol content (data from chapter 3) and remaining were stigmasterol, lupeol along with some steroid glycosides as evident from current study (Waller 1978). These phyto-components independent/synergistically may cause an alteration in glucose homeostasis by above stated reports. Thereby, P1 is proven to be good hypoglycaemic agent.

It is known hyperglycemia leads to hyperinsulinemia. Also, it considered as one of the main complication that add to metabolic complexity in PCO phenotype. Present study showed that PCOS rats with elevated insulin level exhibited high HOMA-IR index suggesting hyperinsulinemia with insulin resistance. Also, insulin receptor up-regulation is noted in PCOS phenotype. This further strengthens that letrozole induced PCOS rats are insulin resistant. The values returned to normal level when treated with P1 fraction (phytosterols rich) suggesting that it has potential to sensitize the insulin receptor and reduce insulin level in PCO condition; thereby reverting insulin resistant state to sensitive status as indicated by improved HOMA-IR change. This could be evident from that reports which indicate the potentiality of phytosterols of AVG as insulin sensitizer (Misawa et al. 2012). Phytosterols isolated from different plants also reported to have anti hyperglycaemic effect and improved insulin sensitivity (Russell et al. 2002; Tanaka et al. 2006).

Apart from glucose metabolism, PCOS rats also exhibited elevated triglycerides and LDL level which are important contributors for lipid metabolism (Hussain and Alam 2014). In present study, dyslipidemia was again restored after treatment of P1 fraction indicating lipid lowering property of P1 fraction. Clinical study also suggested that consumption of phytosterol-β-sitosterol helps to decrease plasma low density lipoprotein (LDL) and triglyceride (TG) levels; although there was usually no change was observed in high-density lipoprotein (HDL) (Jones et al. 2000). In this context, phytosterols isolated from AVG is known to have direct effect

on lipogenic enzymes that involved in lipid biosynthesis wherein these isolated phytosterols administration induces down regulation of fatty acid synthesis and a tendency for up-regulation of fatty acid oxidation in the liver that lead to reduction in intra-abdominal fat and improvement of hyperlipidemia (Misawa et al. 2012). Lupeol, a antioxidant rich compound present in P1 fraction also has properties to normalize the lipid profile by decreasing LDL and total cholesterol level along with improved antioxidant status (Sudhahar et al. 2006). P1 fraction containing β-sitosterol, has a beneficial role in the reduction of the intestinal uptake of cholesterol and lowering the concentration of cholesterol in dietary mixed micelles via a dynamic competition mechanism (Mel'nikov et al. 2004). Our study has also similar kind of alteration which may due to synergistic action of phytosterols present in AVG. However, role of each phyto-components present in P1 need to be studied further.

Role of AVG has on ovarian steroidogenic enzyme activity has been evaluated in earlier chapter. In this context, P1 fraction treated PCOS rats demonstrated a reduction in both enzymes activities in reproductive tissues- ovary and uterus. This may be because of modulatory effect of phytosterols on key protein StAR -an important steroidogenic that is involved in transfer of cholesterol for the steroid production (Sharpe et al. 2007). Apart from reproductive region, P1 could significantly modulate enzyme activity in brain region-pituitary. This may be due to phytosterols rich content of P1 fraction has β sitosterols and stigmasterol (Gilman et al. 2003). This can also modulates brain function (Carter et al. 2007). This fact is further strengthened by a study that diet enriched in phytosterols has also been shown to prevent and/or delay the onset of Alzheimer's disease (Insulin resistant brain) in animals (Novak 1999). Ras et al. (2014) demonstrated that phytosterol esters have a role in countering hypercholesterolemia-related changes in the brain, by decreasing the cholesterol levels, increasing the phospholipid levels and increasing the level of antioxidant enzymes.

Our *"ex vivo"* experiments also elucidated that phytosterols rich P1 fraction has direct effect on ovarian steroidogenesis that can modulate steroidogenic enzyme activity significantly.

Altered steroidogenesis in PCOS caused an imbalance in steroid hormones

status which is one major hallmark of its aetiology. In present study, PCOS rats also demonstrated high testosterone with a decrease in progesterone and estradiol level. This altered hormonal profile was restored in P1 fraction treated rats which indicates modulatory action has taken place in ovarian steroidogenesis by inhibiting rate limiting key protein that involved in androgen production and modulate the androgen-estrogen flux in ovary. These changes lead to regulate the steroid hormones biosynthesis and normalized hormone levels. Reduced steroidogenic enzyme- 3 β HSD activities is also correlated with decreased testosterone production and improved progesterone production in P1 fraction treated PCOS rats. In this view, β-Sitosterol has been shown to have estrogen-like effects in fish (Stevenson et al. 2011; Sriraman et al. 2015). β-Sitosterol, which differs from cholesterol by the addition of an ethyl group on C24 (Figure 2), causes a decrease in plasma Testosterone (Van Der Kraak et al. 1998; Gilman et al. 2003). It binds to rainbow trout hepatic estrogen receptors (Tremblay and Van Der Kraak, 1998) and modulates the steroidogenic pathway at its first step, the conversion of cholesterol to pregnenolone (P5) by cytochrome P450 side-chain cleavage (P450scc) (MacLatchy and Vanderkraak 1995).

In this context, regulation of steroidogenesis governed by level of gonadotropin receptors and steroid receptors expressed on ovarian cells wherein expression of LHR and Androgen receptor is shown to be elevated on the stimulation by high insulin level in PCOS rats which further lead to high theca androgen modulation- Testosterone, DHEA, androstenedione. This may be due to high 3β-HSD enzyme activity, which is attributed by increased expression of transcript and protein. Also, heightened androgen receptor (AR) expression further increases the androgen production through 3β-HSD activity. Also, it has been demonstrated that insulin acts directly via its own insulin receptor at physiological concentrations in cultured polycystic ovary theca cells (Nestler 1997; Nelson-Degrave et al. 2005) and increases androgen production (Nelson et al. 1999), which is significantly greater in ovarian theca cells than from normal women. Thereby, increased expression level of insulin receptor (IR) in PCO rats could also contribute to rise in androgen content as seen in present study. Insulin alone can also increase androstenedione production and can synergize with LH to increase androgen biosynthesis in PCO condition (Nelson et al. 1999). This elevated IR expression was reduced when treated with P1 fraction of

AVG. This may be due to phytosterols present in P1 fraction that can act as insulin receptor sensitizer in diabetic rat (Tanaka et al. 2006). This decreased expression also correlated with improved HOMA IR.

In addition to this, LH also can upregulate StAR expression via cAMP signalling in ovary by binding with its receptor LHR present on the ovaries (Bauer et al. 2000). Elevated expression of LHR was noted in the current study. This could be due to increase serum LH concentrations, as reported by many scientists (Maliqueo et al. 2012; Kauffman et al. 2015). Transcript level of LHR was decreased while treated with P1 fraction of AVG. This may be because of the estrogenic effects of β-sitosterol on the pituitary wherein studies of phytoestrogens in mammals have demonstrated that genistein decreases GnRH-induced luteinizing hormone (LH) in rats (Hughes et al. 2004) and that coumesterol (phytosterol) decreases the amplitude of LH pulses in ewes (MacLatchy and Vanderkraak 1995). Also, β-Sitosterol reduces the rate of the side chain cleavage reaction, as compared to cholesterol activity (Masuo et al. 1980). Decreased pregnenolone production was concomitant with decrease in T production (MacLatchy and Vanderkraak 1995). This suggests that b-sitosterol could be directly affecting steroidogenic enzymes within the biosynthetic pathway.

With reference to above context, aromatase also plays a crucial role in steroid status wherein it converts theca androgens into estrogens, which have role in follicular development and regulation of feedback mechanism of reproductive cycle. In present study, transcript level as well as relative protein expression level was decreased in PCOS rats suggesting disturbed testosterone: estrogen flux due to lower conversion and thus leading to excess androgen production though ovarian steroidogenesis. Also, decreased aromatase expression is well correlated with low level of estradiol as seen in PCOS rats. However, altered expression level observed in PCO phenotype was reverted back to normal when treated with P1 frqaction of AVG. This may be attributed to the fact that StAR protein has been normalized by phytosterols, which reorients its flux where androgen is converted into estrogens efficiently by aromatase. Also, it is to be noted P1 components independently have potential to up-regulate the steroidogenic proteins as evident from P1 group.

Any functional modulation is mainly reflection of structural aberrations. Structurally, PCO rats demonstrated the presence of multiple fluid filled peripheral cysts in the ovary. Several studies have shown similar results (Kafali et al. 2004). P1 fraction treated PCOS rats demonstrated a reduction in peripheral cysts in ovary and increased growing follicles with presence of corpus luetum that mainly indicates successful ovulation and release of dominant mature graffian follicle. The restoration in the ovarian structure and function can be attributed to phytosterols that lead to modulation in the HPO axis. This modulation helped in maturation of follicles and release matured ova during ovulation. Reports have suggested that various medicinal ayurvedic herbs that helped to restore the ovulation and fertility (Adimoelja 2000; Sasikala et al. 2010). However, role of individual phytosterol at molecular level needs to be studied.

7.5 CONCLUSION

Current chapter focused on evaluation of "*in vivo"* efficacy of non polar P1 fraction of *Aloe vera* gel in letrozole induced PCOS rat model wherein P1 fraction (enriched with β sitosterol, stigmalsterol, lupeol as major phytosterols) treated PCOS rats demonstrated glucose sensitivity and regular cyclicity. It also restored lipid profile and disturbed hormonal profile that again helped to repair ovarian dysfunction in PCO phenotype. Apart from organ level, P1 fraction of *Aloe vera* gel also significantly affects transcripts level of StAR, LHR, androgen receptor (AR), aromatase and Insulin receptor (IR) as well as relative protein expression of StAR, 3β HSD and aromatase in PCOS rats, which may be one of possible reason for modulated steroidogenesis. This implies that P1 fraction rich in β sitosterol, stigmasterol, lupeol and other sterol derivatives that has potential to act at various targets for restoration of ovarian function in PCO phenotype. Overall, study implies that non polar P1 fraction containing phytosterols that could be active phyto-component involved in modulation of hyperglycaemic, hyperinsulinemic condition leading to functional ovulation. These phytocomponents might help us to design future drug that can improve ovarian structure-function. **Overall, studies prove that phytosterols containing P1 fraction of *Aloe vera gel* could be possible novel drug in management of PCOS.**

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