4

Identification of the targets of the isolated phytocomponents of Non-polar fraction of *Aloe vera* Gel Extract using *"in-silico"* approach and its validation

4.1. Rationale

During the last few decades, drug discovery and development has evolved into a multidisciplinary field, with many factors playing and continuing to play a role in the successful transformation of a bioactive compound into a potential drug entity (Strovel et al., 2016). Due to the multiple components and its association with multiple targets, conventional experimental assays are laborious and time consuming. Thereby, computational pharmacology, as a multidisciplinary strategy for the emerging development of efficacious drugs. In this line, herbal research has exploited the technology of " *in silico" validation* due to the varied presence of several bioactive that could possibly interact with several known or unknown targets in complex diseases like PCOS.

Based on existing protein data banks, *"in-silico"* computational docking modelling predicts the most likely possible natural hits to connect with protein/receptor binding site and pharmacophore residues. Docking is an *"in-silico"* screening approach wherein, it is combined with experimental techniques to improve hit to lead optimisation by reducing the time and cost variables involved in drug development. The importance of molecular docking is growing as more proteins' structures are determined using XRD and NMR methods and contributed to the protein data bank. In addition to this, *"in-silico"* drug-likeness prediction, in combination with other ADME (absorption, distribution, metabolism, and excretion) parameters, opens up a slew of possibilities for speeding up the discovery of novel targets and, eventually, compounds with anticipated biological activity.

ADME, which constitutes the pharmacokinetic profile of a drug molecule, is very essential in evaluating its pharmacodynamic activities. Due to poor ADME characteristics, over 40% of pharmacological compounds fail in clinical trials (Darvas et al. 2002). Late-stage failures are a

major contributor to the constantly rising expense of new medication development. The use of computer-based approaches to incorporate pharmacokinetic concerns at earlier phases of drug development programmes (Hodgson 2001) is becoming much more popular (Lipinski et al. 1997; Gleeson et al. 2011; Kang, 2013). Drug-likeness is an important *"in-silico"* criterion for evaluating drug candidates throughout the early stages of drug development. This parameter may be defined as a way to link a compound's physicochemical properties to its biopharmaceutical properties in the human body, particularly its impact on oral bioavailability. The physicochemical properties that are necessary to increase the likelihood of oral bioavailability have been formalized into the 'rule of five' by Lipinski et al., (2001). According to the rule, an orally active drug has no more than one violation of the following: (i) Hydrogen bond donors $\leq$5; (ii) Hydrogen bond acceptors $\leq$ 10; (iii) Molecular mass $\leq$ 500 daltons; (iv)Octanol-water partition coefficient log P $\leq$ 5.6. The compounds which have more than one violation of these rules are not considered as orally active drug candidates.

Computational models produce valuable predictions that requires thorough validation with further experimental data utilising approaches of *"in-vitro"* and *"in-vivo"* models, depending on the needs of the research. In the present study, aim was to identify bioactives from the non-polar extract of Aloe vera gel with favourable pharmacokinetics properties and with functional capacity to interacting and modulating the process of steroidogenesis and metabolism. To achieve this goal, an *"in-silico"* analysis was performed through a pipeline comprised of Absorption, Distribution, Metabolism, and Excretion (ADME) properties prediction. Also, molecular docking analysis was done to understand the binding affinities and interactions of the major phytocompounds from partially purified non-polar phytocompounds isolated from *Aloe vera* gel towards the steroidogenic and metabolic regulators. The results obtained from *"in-silico"* studies were further validated using an *"in-vitro"* cell-based bioassay system.

4.2. Materials and methods

Data from the previous chapter has demonstrated that the bioactive partially purified non-polar phyto-components (PPNPP) of *Aloe vera* gel- LP1, LP2, LP3, LP4 and LP5 contains n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate with an abundance of 97.07%, 96.04%, 94.03%, 92.45% and 87.49% respectively. Therefore, n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate were considered as the ligands for the *"in-silico"* studies. The detailed protocol followed for *"in-silico"* studies has been mentioned in Chapter 2. Briefly, the above-

mentioned ligands were docked with the key steroidogenic and metabolic targets that includes Follicle Stimulating Hormone Receptor (FSHR), Steroidogenic Acute regulatory protein (StAR) ,3-beta hydroxysteroid dehydrogenase (3b-HSD), Aromatase, Estrogen Receptor alpha, Estrogen Receptor beta, Androgen receptor, Progesterone receptor and Phosphorylated Insulin Receptor tyrosine kinase. Docking studies have been carried out by using GLIDE (Grid-based Ligand Docking with Energetics) software v5.5 developed by Schrödinger. All the steps followed while performing the docking studies are mentioned in details in Chapter 2. The docking score, potential energy of binding, and hydrogen bonds produced with the surrounding amino acids were all utilised to predict their binding affinities and correct alignment inside the active sites of the afore-mentioned targets. Further, assessments of the pharmacokinetic properties of Absorption, Distribution, Metabolism and Excretion (ADME) of the PPNPPs were calculated by SwissADME tool, which predicts physically significant and physiochemical descriptors of potential drug compounds. Based upon the "drug likeness" properties and *"in-silico"* molecular docking studies, further validation of the targets was performed using an *"in-vitro"* bioassay. Detailed description of the methodology followed for the *"in-vitro"* bioassay has been mentioned in Chapter 2.

4.3. Results

4.3.1. Effect of PPNPPs isolated from *Aloe vera* gel on the steroidogenic and metabolic targets using *"in-silico"* approach

"In-silico" molecular docking and drug likeness studies of the above mentioned PPNPPs isolated from *Aloe vera* gel are discussed below.

4.3.1.1. Docking Studies of PPNPPs isolated from *Aloe vera* gel

Molecular docking was performed by GLIDE to evaluate the interaction mode of major components of partially purified non-polar phytocomponents (PPNPP) of *Aloe vera* gel against steroidogenic and metabolic targets and the magnitude of interaction between them was assessed. As explained elaborately in Chapter 2, the ligands (n-Hexadecanoic acid, Cholesta-3,5-diene, Campesterol acetate, b-Sitosterol and Stigmasterol acetate) were selected on the basis of their abundance in the LP1, LP2, LP3, LP4 and LP5. Selection of targets was done on the basis of previous lab studies (Maharjan and Nampoothiri, 2016a). The structures of key steroidogenic and metabolic targets were obtained from RCSB Protein Data Bank, whose

description has been provided in the material and methods section. The various steps involved in preparation of the ligand and target structure has already been discussed in Chapter 2.

The key parameter created as a result of molecular docking is binding energy. It provides information on the intensity and affinity of the ligand-receptor interaction. The weaker the contact, the higher the binding energy, and vice versa. As a result, we looked for the ligand with the lower binding energy, thus conferring good affinity towards the target, among the test compounds, during docking process. The phytocompounds that exhibited binding affinity towards more than one targets, but only the one's exhibiting least docking score and least binding energy was considered as the suitable ligand-target interaction. The docking scores of the tested compounds with the steroidogenic and metabolic regulators are presented in Table 4.1. In this study, the phytocompounds that demonstrated good measurable binding affinities for the target residues was considered. The binding affinities were indicative of the ligand's contribution to and flexibility for the target. The present study also showed the H-bond distances and their contacts types for each molecule (Figure 4.1). Amongst the various ligands, Campesterol acetate (major component of LP3) exhibited greater affinity towards key steroidogenic targets like Progesterone receptor, Steroidogenic Acute Regulatory protein, and Aromatase with a docking score of -9.988, -8.312 and -6.913 respectively. Estrogen Receptor beta and3-beta hydroxysteroid dehydrogenase were found to have good interactions with beta-Sitosterol (LP4) and Stigmasterol acetate (LP5) with the docking score of -7.166 and -6.512 respectively. n-Hexadecanoic acid (LP1) was found to dock with Androgen Receptor and phosphorylated Insulin Receptor tyrosine kinase with a docking score of -7.419 and -3.205 respectively. However, none of the PPNPPs isolated from *Aloe vera* gel exhibited any interaction with other important targets like Estrogen Receptor alpha and Follicle stimulating Hormone Receptor (Data not shown).

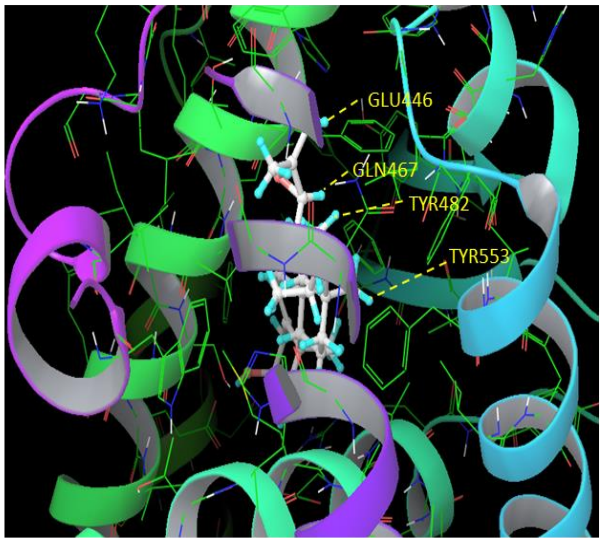
All the test compounds showed hydrogen bond interactions with different residues (Figure 4.1). The results revealed that Campesterol acetate (LP3) is showing hydrogen bond interaction with GLU 446, GLN 467, TYR 482 and TYR 553 residues of the active site of Steroidogenic acute regulatory protein. Further, Campesterol acetate exhibited hydrogen bond interactions with Aromatase and Progesterone Receptor at MET 374 and ASN 719, THR 894 residues respectively. The obtained results suggest that Campesterol acetate, which is a major phytochemical present in LP3 is a key modulator of steroidogenic targets like StAR, Aromatase and PR. On the other hand, n-Hexadecanoic acid was found to strongly interact with Androgen receptor by forming hydrogen bonds with ASN 705 and THR 877 residues, suggesting it to

play an important role in regulation of hyperandrogenism, which is a major co-morbidity associated with PCOS. In addition to this, n-Hexadecanoic acid was also found to interact with phosphorylated Insulin Receptor tyrosine kinase by establishing hydrogen bonds with MET1079, with greater negative binding energy, suggesting strong affinity between the ligand and the target, in spite of having low docking score. Docking score just is the scoring function used to predict the binding affinity of both ligand and target once it is docked. On the other hand, binding energy is the sum of all the intermolecular interactions that is present between the ligand and the target (Pantsar and Poso, 2018).

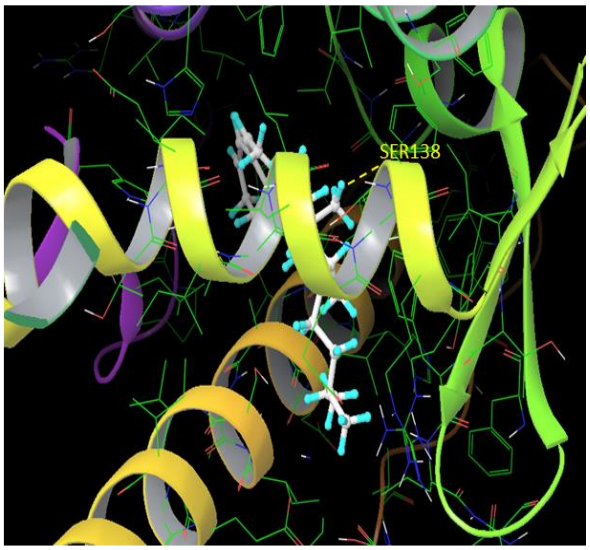
Table 4.1: Induced fit docking results of partially purified non-polar phytocomponents of *Aloe vera* gel docked with key steroidogenic and metabolic targets.

Sl. No	Protein name (Targets)	PDB Accession no.	Docked Ligands	Docking Score	Potential Energy of Binding (eV)	Residues involved in H-bonding interactions
1.	Steroidogenic Acute Regulatory protein	3H3Q	Campesterol acetate	-8.312	-62.099	GLU 446, GLN 467, TYR 482, TYR 553
2.	3-beta hydroxysteroid dehydrogenase	1HXH	Stigmasterol acetate	- 6.512	-23.729	SER138
2.	Aromatase	3S79	Campesterol acetate	-6.913	-28.963	MET 374
4.	Estrogen Receptor beta	2J7Y	b-Sitosterol	-7.166	-32.982	LEU 387, ARG 394
5.	Androgen receptor	2AM9	n-Hexadecanoic acid	-7.419	-43.819	ASN 705, THR 877
6.	Progesterone receptor	1A28	Campesterol acetate	-9.988	-58.006	ASN 719, THR 894
7.	Phosphorylated Insulin Receptor tyrosine kinase	1IR3	n-Hexadecenoic acid	-3.205	-44.117	MET1079

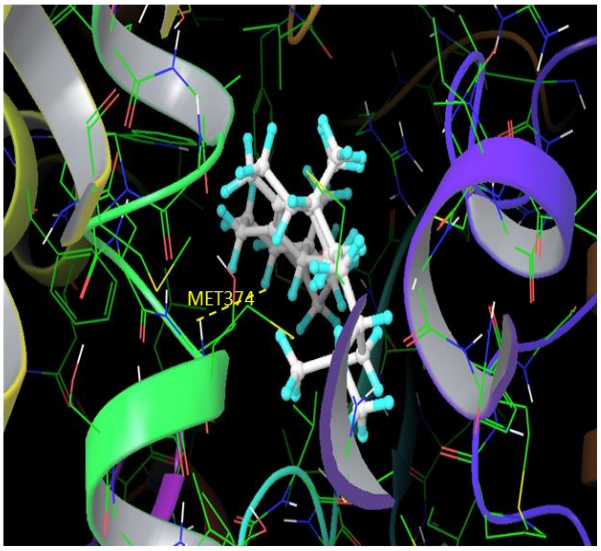
[A]


GLU446
GLN467
TYR482
TYR553

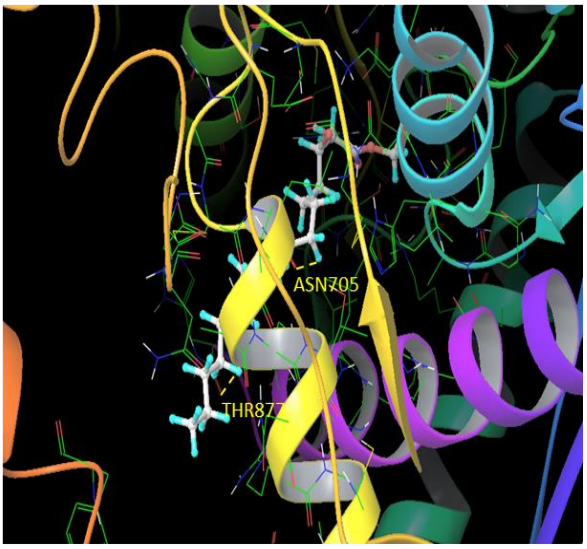
[B]


SER138

[C]


MET374

[D]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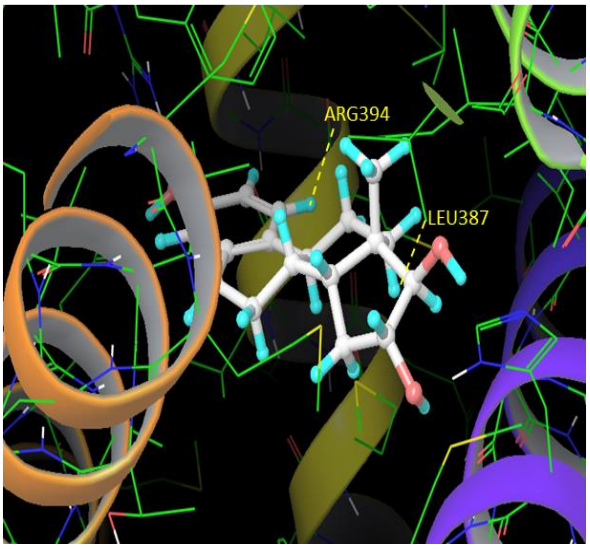
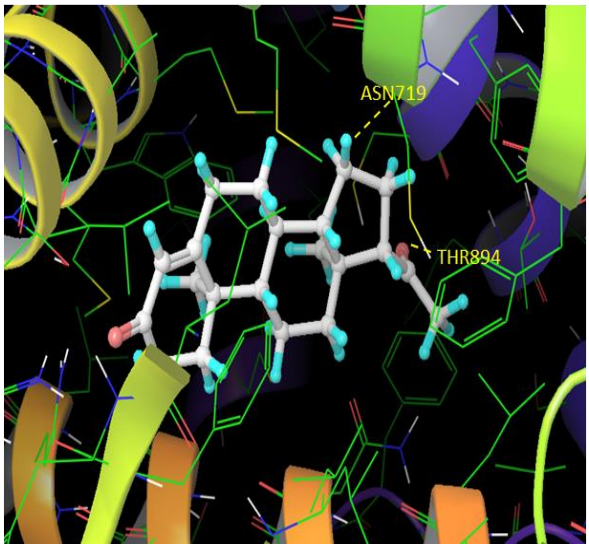
THR877

[E]


ARG394
LEU387

[F]


ASN719
THR894

[G]

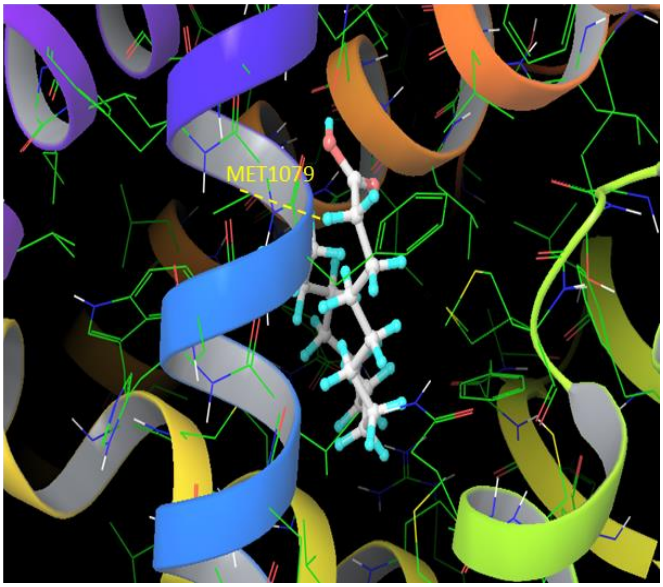


Figure 4.1: Representative image of the molecular docking interactions [A] Steroidogenic acute regulatory protein with Campesterol acetate, [B] 3-beta hydroxysteroid dehydrogenase with Stigmasterol acetate, [C] Aromatase with Campesterol acetate, [D] Androgen Receptor with n-Hexadecanoic acid, [E] Estrogen Receptor beta with β- Sitosterol, [F] Progesterone Receptor with Campesterol acetate and [G] Phosphorylated Insulin Receptor tyrosine kinase with n-Hexadecanoic acid. Residues and hydrogen bond contacts (yellow dotted lines), and the 2D template representing the types of contacts formed between the ligand and target.

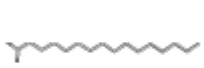
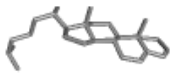
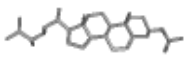
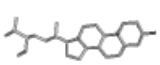
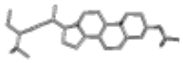
4.3.1.2. Absorption, Distribution, Metabolism and Excretion (ADME) studies

It is now recognized that selection of a 'robust' drug candidate requires a balance between efficacy, safety and drug metabolism and pharmacokinetic properties. ADME analysis was performed virtually to know the pharmacokinetic or drug-likeness property of the PPNPPs isolated from the *Aloe vera* gel. The Lipinski's screening is an essential filter that determines if a compound is suitable for drug designing. Data from Table 4.2 demonstrates that all the compounds followed the Lipinski's rule of five with not greater than one violation with respect to molecular weight (≤500 KDa), number of H-bond donors (≤5), number of H-bond acceptors (≤10) and an octanol-water partition coefficient (logPo/w ≤5). The partition coefficient between n-octanol and water (logPo/w) is the classical descriptor for lipophilicity. Aqueous solubility is an important parameter in determining the oral bioavailability, LP1 was found to be moderately soluble. However, LP2, LP3, LP4 and LP5 were poorly soluble in water. Except LP2, all other compounds have their log Kp values < -2.0 and hence may have good skin permeability. Predicting blood-brain barrier (BBB) permeability is essential for drug

development as any molecule cannot exhibit pharmacological activity without transiting this barrier. As per data obtained, none of the PPPNPPs isolated from *Aloe vera* gel mentioned above can readily cross the BBB except LP1. LP1 (n-Hexadecanoic acid) can penetrate central nervous system. SwissADME allows you to determine if a substance is a P-Gp (P-glycoprotein) substrate or an inhibitor of the most major cytochromes P450 (CYP) isoenzymes (Daina et al., 2017). P-Gp is the most important member among ATP-binding cassette transporters or ABC-transporters, and plays a key role to facilitate active efflux through biological membranes, for instance from the gastrointestinal wall to the lumen or from the brain (Montanari and Ecker, 2015). Apart from this, P-Gp has a number of functions, one of which is to protect the central nervous system (CNS) against xenobiotics (Szakács et al., 2008). Identification of phytochemicals interaction with CYP (cytochromes) is also a critical parameter of ADME analysis. This isoenzyme superfamily plays an important role in drug clearance via metabolic biotransformation (Testa and Kraemer, 2008). It has been proposed that CYP and P-Gp can work together to metabolise small compounds in a synergistic manner to increase tissue and organism safety (van Waterschoot and Schinkel, 2011). Inhibition of these isoenzymes is undoubtedly one of the most common causes of pharmacokinetics-related drug-drug interactions, which can result in toxic or other undesirable side effects owing to decreased clearance and build-up of the drug or its metabolites (Hollenberg, 2002; Huang et al., 2008; Kirchmair et al., 2015). Data from our analysis demonstrates that except LP5, all the phytochemicals have higher probability to be a substrate for P-Gp. In addition to this, all the PPNPPs isolated from *Aloe vera* gel were not the inhibitors for key CYP isoforms like CYP1A2 and CYP3A4, suggesting these phytocomponents may not be a candidate for drug-herb interaction that may influence pharmacokinetic parameters and may be considered as “safe" for human health.

Table 4.2. ADME of the partially purified non-polar phytocomponents of *Aloe vera* gel

ADME Toxicity	**LP1** n-Hexadecanoic acid	**LP2** Cholesta-3,5-diene	**LP3** Campesterol acetate	**LP4** beta-sitosterol	**LP5** Stigmasterol acetate

Structure					
Molecular weight (g/mol)	256.4	368.6	400.7	414.71	454.7
H-bond donors	0	1	1	1	0
H-bond acceptors	2	2	1	2	1
LogP$_{(o/w)}$	0.8	3.9	2.6	3.7	1.2
Water Solubility	Moderately soluble	Poorly soluble	Poorly soluble	Poorly soluble	Poorly soluble
Pharmacokinetics					
GI Absorption	High	Low	Low	Low	Low
BBB Permeant	Yes	No	No	No	No
P-Gp Substrate	Yes	Yes	Yes	Yes	No
CYP1A2 inhibitor	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No
Log K_p (skin permeation)	-2.77 cm/s	-1.60 cm/s	-2.35 cm/s	-2.20 cm/s	-2.59 cm/s
Drug likeliness					
Lipinski rule	1 Violation	1 Violation	1 Violation	1 Violation	1 Violation
Bioavailability Score	0.85	0.55	0.55	0.55	0.55
Medicinal Chemistry					
Leadlikeness	2 Violations	2 Violations	2 Violations	2 Violations	2 Violations

These results suggest that all the compounds reported in the current study possessed desired properties and could be developed into potential drug candidates. However, the data needs to be validated using an *"in-vitro"* or *"in-vivo"* method to understand their physiological implication. *"In-vitro"* methods can be carried out rapidly and are economical way to screen large number of phytocompounds, therefore, a bioassay was designed using a steroidogenic human granulosa-like tumor cell line, KGN.

4.3.2. Effect of PPNPPs isolated from *Aloe vera* gel on the steroidogenic and metabolic targets using *"in-vitro"* approach

Validation of the results obtained in the "in-silico" study was performed by incubating the phytochemicals with the KGN cell-line for 24 hours. Forskolin is a known stimulator of cAMP pathway, which in turn activates steroidogenesis (Haggard et al., 2018) and has been used as a positive control. On the other hand, Letrozole is a widely used selective inhibitor of steroidogenesis as it blocks the key enzymes (Bhatnagar, 2007) and thus has been used as a negative control in the present study.

4.3.2.1. Effect of PPNPPs isolated from *Aloe vera* gel on the KGN cell-line morphology and viability and calculation of IC_{50} value

The IC_{50} value is correlated with drug potency, i.e. the amount of drug necessary to produce the effect—the lower the IC_{50} value, the more potent is the drug. Data from Figure 4.2 demonstrates that there is a dose dependent decline in the cell-viability of KGN cell-line when incubated with the PPNPPs and the petroleum ether extract of *Aloe vera* gel. Table 4.2 depicts the IC_{50} values obtained for LP1, LP2, LP3, LP4, LP5 and PE were 25.3, 31.7, 8.9, 28.7, 4.0 and 70.45 ng/ml respectively. Hence, these were the doses used for the respective PPNPPs (LP1- LP5) in all the further cell- based assays. The treated and non-treated cells when observed under the microscope demonstrated healthy growing cells (Figure 4.3). However, treatment with Forskolin (positive control), LP1, LP3, LP4 and PE demonstrated oil droplets in the cell body, suggesting that the steroidogenesis is altered in these cells after treatment with the phytocompounds.

Table 4.2: IC_{50} values at 24 hours exposure of the PPNPPs of *Aloe vera* gel on the KGN cell-line

Isolates	**IC_{50} values (ng/ml)**
LP1	25.27 ± 0.04
LP2	31.697 ± 0.05
LP3	8.936 ± 0.002
LP4	28.698 ± 0.002
LP5	4.016 ± 0.01
PE extract	70.452 ± 0.01

N=6. The values are represented as Mean ± SEM.

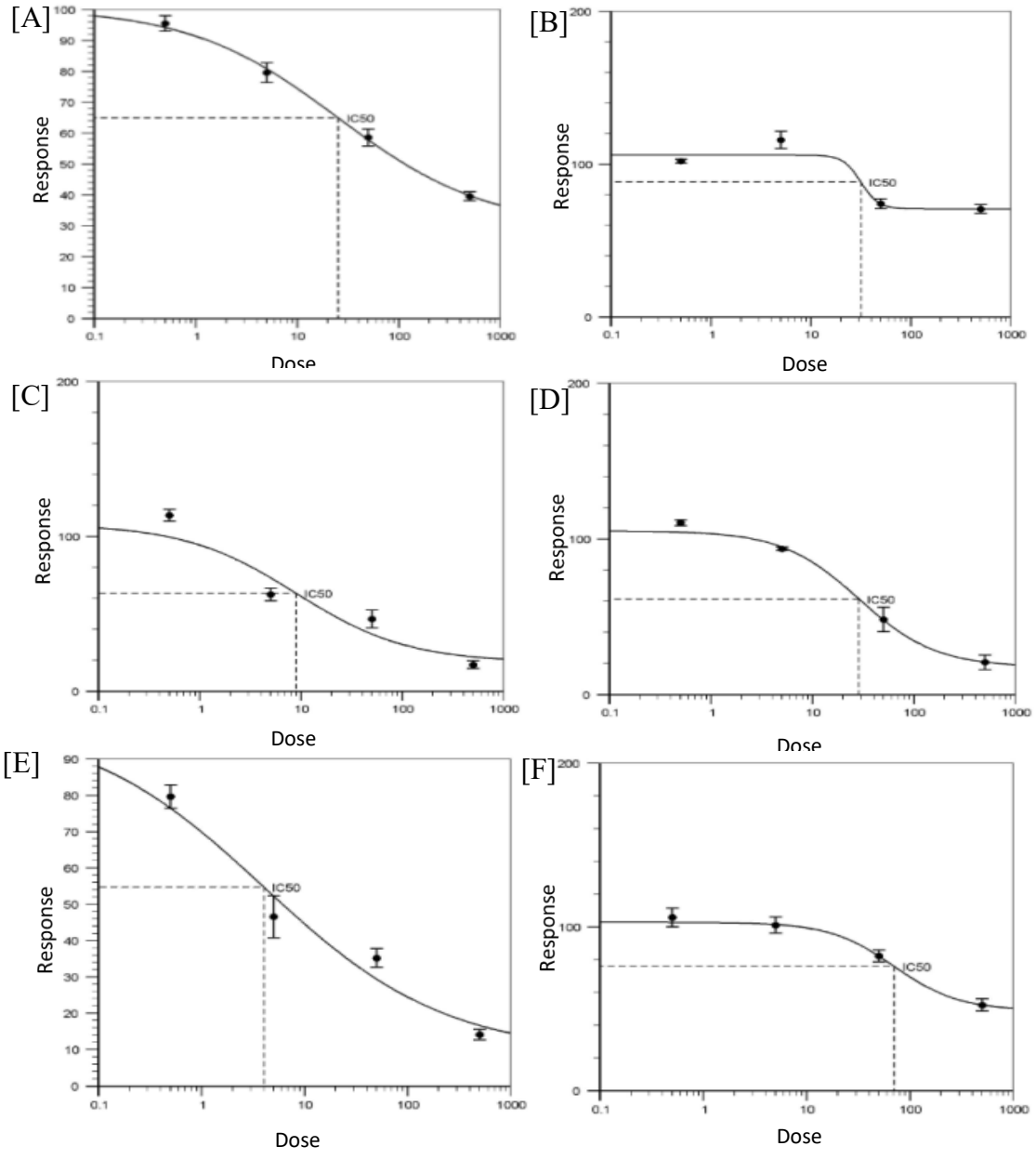


Figure 4.2. Dose dependent effect of the PPNPPs isolated from *Aloe vera* gel on the cell viability of KGN cell-line. N=6. The values are represented as Mean ± SEM. (A) LP1, (B) LP2, (C) LP3, (D) LP4, (E) LP5 and (F) Petroleum ether extract of *Aloe vera* gel.

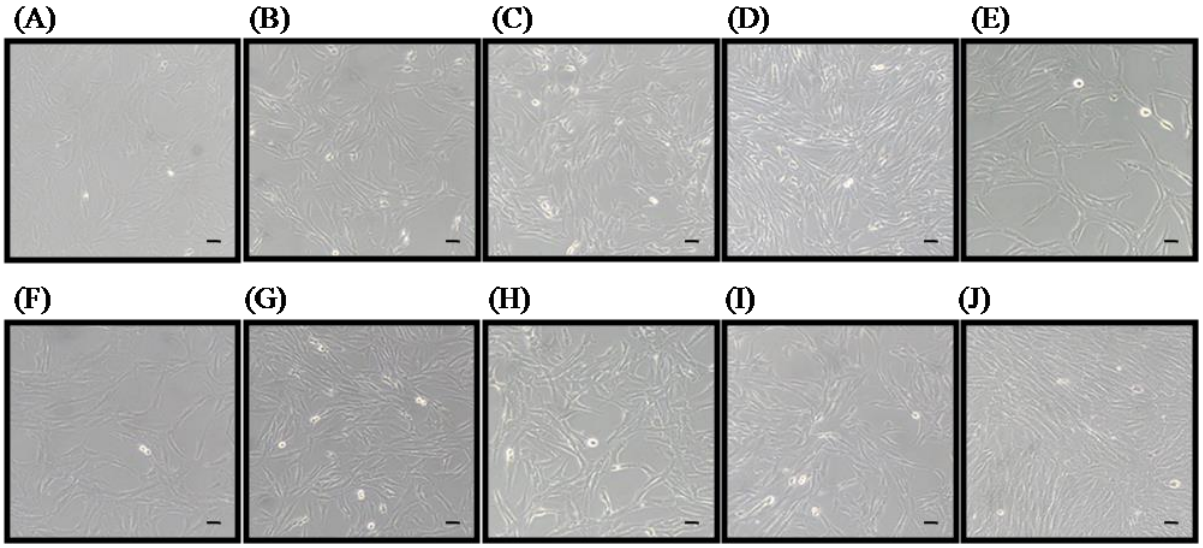


Figure 4.3. Morphology of KGN cell line upon subjecting them to PPNPPs isolated from *Aloe vera* gel at the IC_{50} concentration. N=8. Observed under 10X magnification. (A) Control, (B) DMSO, (C) Forskolin, (D) Letrozole, (E) LP1, (F) LP2, (G) LP3, (H) LP4, (I) LP5 and (J) Petroleum ether extract of *Aloe vera* gel

4.3.2.2. Effect of PPNPPs isolated from *Aloe vera* gel on the hormone secretion by KGN cell-line

The spent media were pooled for each treatment group within six trials, then assessed for the hormone (testosterone, estradiol and progesterone) secretion by the KGN cell-line. Figure 4.4 demonstrated that there is a significant increase in the testosterone levels in the spent media of the KGN cell-line upon treatment with Letrozole ($P<0.001$), LP2 ($P<0.05$), LP4($P<0.05$) and LP5 ($P<0.05$). However, the testosterone levels were reduced significantly in LP1 treated group ($P<0.05$). The estradiol levels were significantly increased in the spent media upon treatment with Forskolin ($P<0.001$), LP3 ($P<0.001$), LP4 ($P<0.001$) and Petroleum ether extract of *Aloe vera* gel ($P<0.01$) whereas, Letrozole treatment significantly reduced the Estradiol levels in the spent medium. Further, there was a significant increase in the progesterone levels when the cells were treated with Forskolin ($P<0.001$), LP1 ($P<0.05$), LP3 ($P<0.01$), LP4 ($P<0.05$) and Petroleum ether extract of *Aloe vera* gel ($P<0.01$).

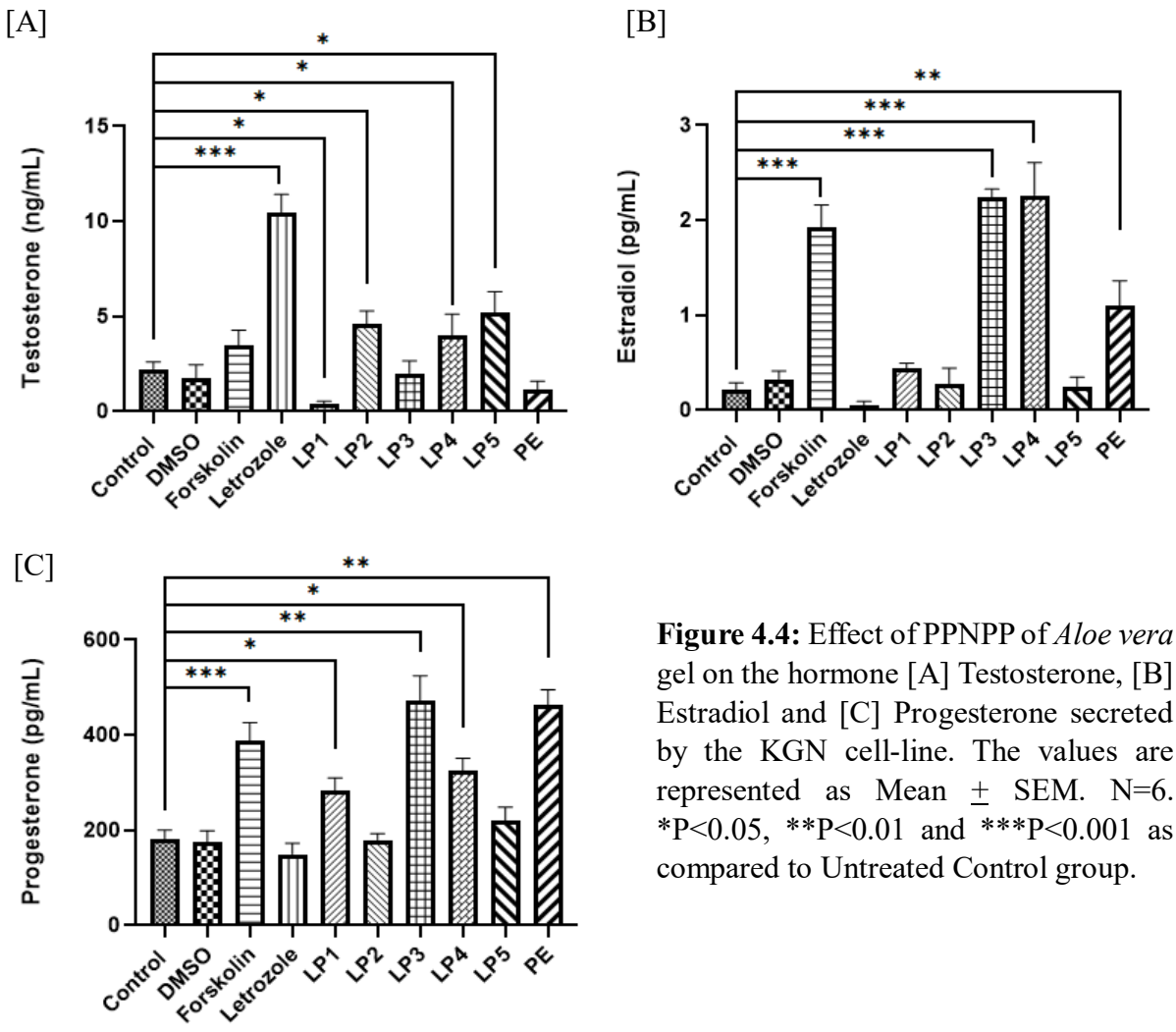


Figure 4.4: Effect of PPNPP of *Aloe vera* gel on the hormone [A] Testosterone, [B] Estradiol and [C] Progesterone secreted by the KGN cell-line. The values are represented as Mean ± SEM. N=6. *P<0.05, **P<0.01 and ***P<0.001 as compared to Untreated Control group.

4.3.2.3. Effect of PPNPPs isolated from *Aloe vera* gel on the gene expression of steroidogenic regulators

It is clear from above, the phytocomponents have a steroidogenic potential. Further, it was essential to understand these modulations seen was a result of molecular alterations. Thereby, the changes in transcript levels of steroidogenic regulators were studied. To estimate the transcript levels of steroidogenic regulators, we performed real-time PCR using specific primers for *Star*, *HSD3b1* and *Cyp19a1*. The mRNA was extracted from the KGN cell-line post treatment and the cDNA was prepared which was used as a template for the gene expression studies. There was a significant increase in the transcription of *Star* upon treatment with Letrozole (P<0.01). On the other hand, treatment with Forskolin (P<0.01), LP3 (P<0.05) and petroleum ether extract of *Aloe vera* gel (P<0.01), significantly reduced the mRNA levels of *Star*, suggesting that the cholesterol uptake, which is the rate limiting step of steroidogenesis, is being inhibited by LP3 as well as the petroleum ether extract of *Aloe vera* gel. The mRNA

levels of *Hsd3b1* significantly increased when KGN cell-line was treated with LP3 (P<0.05) and LP5 (P<0.05). The obtained results were comparable to that of PE treated group (P<0.001). The expression of *Cyp19a1*, encoding for the protein Aromatase (rate limiting enzyme for testosterone to estradiol conversion), was found to significantly decrease upon treatment with Letrozole (P<0.001). However, upon treatment with the phytocompounds, there was a significant increase in the expression of *Cyp19a1*, with LP3 and petroleum ether extract of *Aloe vera* gel, exhibiting maximum potential.

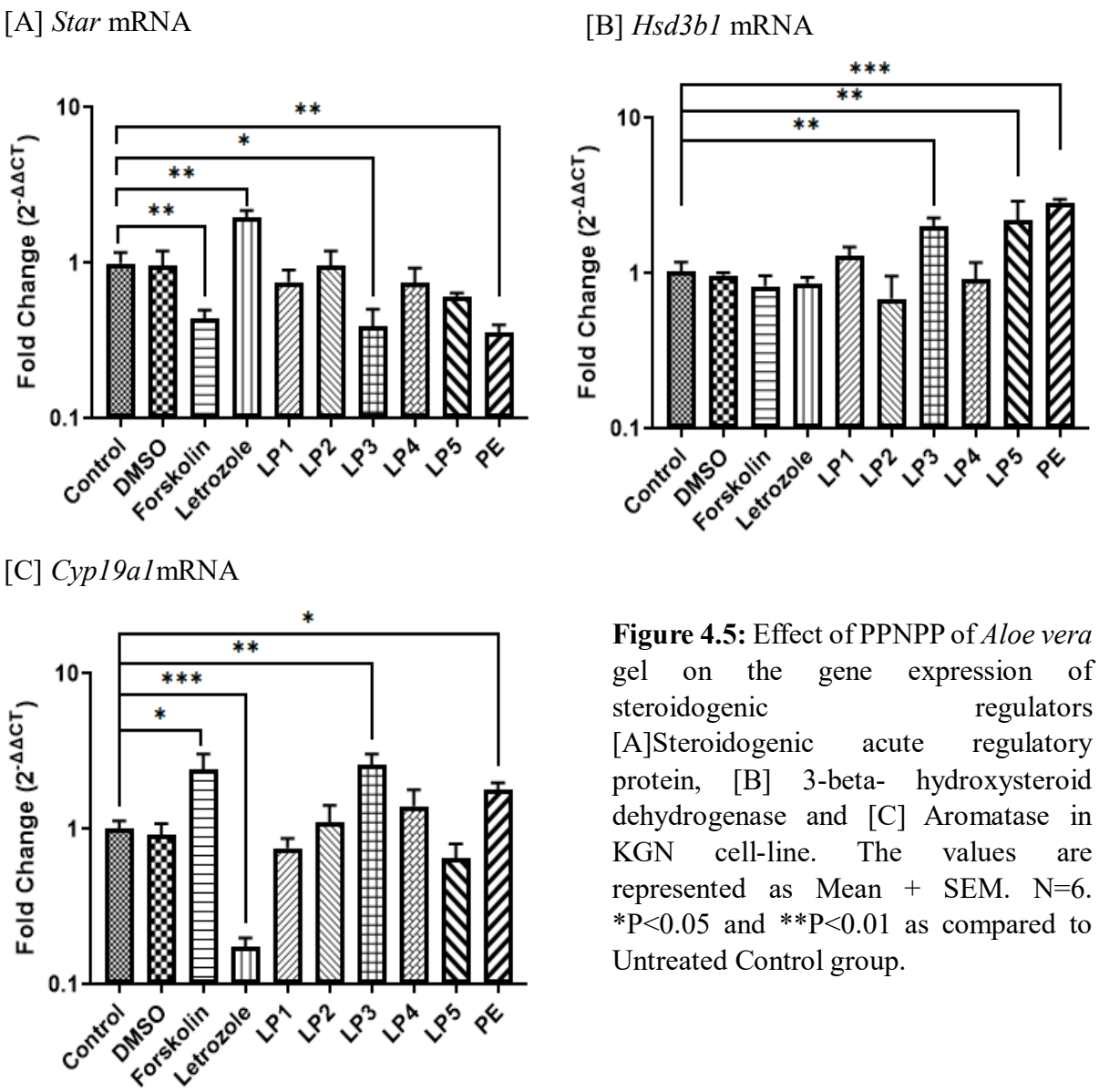
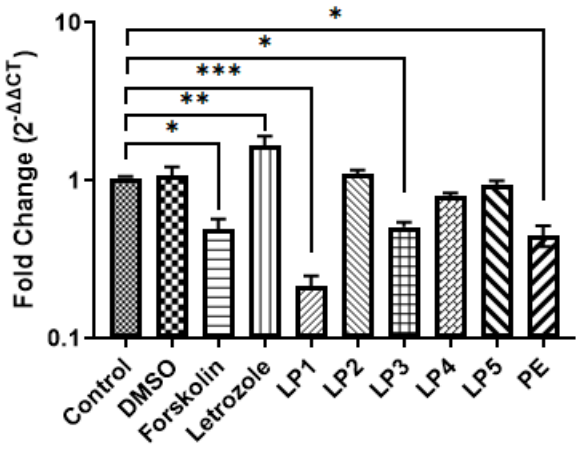


Figure 4.5: Effect of PPNPP of *Aloe vera* gel on the gene expression of steroidogenic regulators [A]Steroidogenic acute regulatory protein, [B] 3-beta- hydroxysteroid dehydrogenase and [C] Aromatase in KGN cell-line. The values are represented as Mean + SEM. N=6. *P<0.05 and **P<0.01 as compared to Untreated Control group.

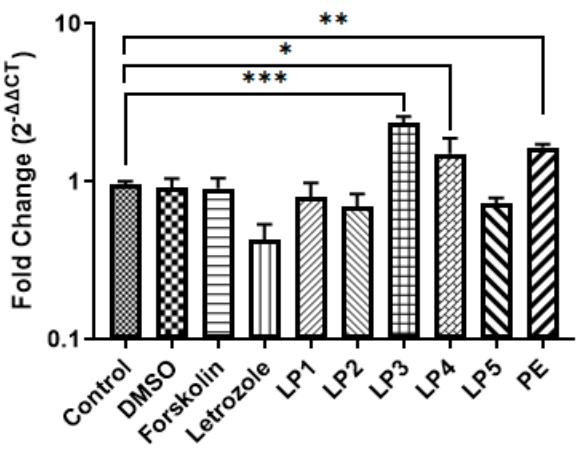
4.3.2.4. Effect of PPNPPs isolated from *Aloe vera* gel on the gene expression of steroid receptors

Estimation of the expression levels of mRNA encoding the steroid hormone receptors, including Androgen Receptor (*Ar*), Estrogen Receptor-beta (*Esr-2*) and Progesterone Receptor (*Pgr*) was performed by using real- time PCR with their specific primers. Data from Figure 4.6 shows that treatment with Forskolin ($P<0.05$), LP1($P<0.001$) and LP3 ($P<0.05$) caused a significant decrease in the gene expression of *Ar*, which was comparable to the decline observed in case of petroleum ether extract treated group ($P<0.05$). There was significant increase in the estrogen receptor beta expression in the LP3 ($P<0.001$) and LP4 ($P<0.05$) and PE ($P<0.01$) treated groups, suggesting a synergistic effect. Interestingly, there was a significant increase in the progesterone receptor expression in the KGN cell-line upon treatment with Forskolin ($P<0.05$), LP3 ($P<0.001$) and Petroleum ether extract of *Aloe vera* gel ($P<0.01$), whereas the expression of progesterone receptor reduced drastically upon treatment with Letrozole ($P<0.001$).

[A] *Ar* mRNA



[B] *Esr-2* mRNA



[C] *Pgr* mRNA

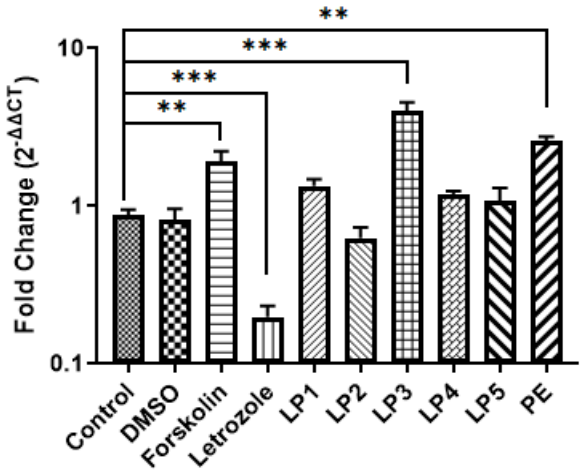


Figure 4.6: Effect of PPNPP of *Aloe vera* gel on the gene expression of steroid receptors [A] Androgen Receptor, [B] Estrogen Receptor- beta and [C] Progesterone Receptor in KGN cell-line. The values are represented as Mean + SEM. N=6. *P<0.05, **P<0.01 and ***P<0.001 as compared to Untreated Control group.

4.3.2.5. Effect of PPNPPs isolated from *Aloe vera* gel on the gene expression of key metabolic regulator-Insulin receptor

As discussed in Chapter1, insulin signalling plays an important role in regulating the steroidogenic as well as metabolic pathways. Hence, we accessed the transcriptional levels of Insulin Receptor (*Insr*) mRNA using real-time PCR. Data from Figure 4.7 demonstrates that treatment of KGN cell-line with Forskolin ($P<0.05$), LP1($P<0.01$) and Petroleum ether extract of *Aloe vera* gel ($P<0.05$) significantly downregulated the mRNA levels of *Insr*. The observed results suggest that LP1 and PE might be an effective regulator of insulin receptor transcription.

Insr mRNA

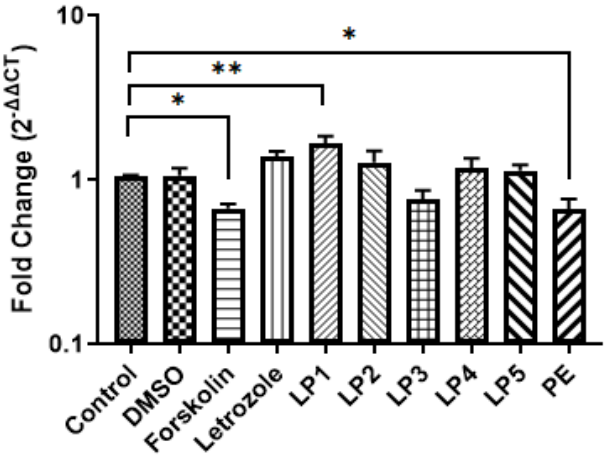


Figure 4.7: Effect of PPNPP of *Aloe vera* gel on the gene expression of Insulin Receptor in KGN cell-line. The values are represented as Mean ± SEM. N=6. *P<0.05 and **P<0.01 as compared to Untreated Control group.

The results demonstrate that LP3 is a potent stimulatory modulator for estradiol and progesterone biosynthesis whereas, LP1 has a good anti-androgenic property as well as the potential to modulate the insulin receptor expression.

4.4. Discussion

There are several studies which highlight the implication of phytochemicals on the endocrine system (Solís-S et al., 2017; Arpi and Laxmipriya, 2019; Lambert and Edwards, 2017). Modulation of catalytic activity of steroidogenic or steroid-catabolizing enzymes could be one of the effects of phytochemicals in maintaining the steroid mileu (Hilscherova et al., 2004). Feeding of plant derived sterols, reduced testosterone production by modulating GnRH and GnIH expression in the brain and testes of male Japanese quail (*Coturnix coturnix japonica*) (Qasimi et al., 2018). Another study suggests that flavonoids have inhibitory effect on cortisol production in human adrenocortical H295R cells by inhibiting the activities of P450scc, 3b-HSD type II, P450c17, P450c21 and P45011 (Ohno et al., 2002). Another *"in-vitro"* study demonstrates that treatment with quercetin does not affect granulosa cell growth but impairs

steroidogenesis and angiogenesis in swine granulosa cells (Santini et al., 2009). In yet another study, it was found that unsaturated fatty acids stimulated steroidogenesis in cultured rat adrenocortical cells (Sarel and Widmaier, 1995) whereas, treatment of bovine granulosa cells with saturated free fatty acids (palmitic acid and stearic acid) are known to induce apoptosis, causing potential reproductive abnormalities such as amenorrhea, which is often observed in obese women. However, on the contrary, oleic acid (monounsaturated fatty acid) supplementation has shown to protect exocrine pancreatic cells from palmitic acid-induced apoptosis thus reducing the rate of pancreatic diseases caused by obesity (Sharma et al., 2019). The bioactivities depend upon the type of fatty acids; saturated and trans-fatty acids were found to decrease insulin concentrations hence, leading to insulin resistance. On the other hand, polyunsaturated fatty acids (PUFA) increased plasma insulin concentration and decreased insulin resistance (Sears and Perry, 2015). It is to be noted that, insulin resistance lies in the core of all metabolic and endocrine disorders.

Present study demonstrates the anti-androgenic role of LP1 (containing 97.07% n-Hexadecanoic acid) by decreasing the testosterone levels in the spent media by probably decreasing the Androgen Receptor mRNA levels as seen from the *"in-silico"* and *"in-vitro"* studies. This bioactivity can be extremely beneficial for management of endocrine pathologies such as PCOS that has hyperandrogenism as one of the major associated comorbidities. In relation to this, there exists a strong association of fatty acids with the normal functioning of the endocrine system. Dietary fatty acids have significant impact on hormone, neuropeptide concentrations as well as their receptors (Bhathena, 2006). Studies in mice have shown that dietary intake of omega-3 and omega-6 fatty acids has positive association with the implantation rate by modifying the uterine phospholipid fatty acid composition and arachidonic acid levels (Akerele and Cheema, 2016). Studies demonstrate that omega-3 polyunsaturated fatty acids, present in abundance in fish oil was found to ameliorate high-fat diet induced reproductive dysfunction in male C57BL/6 mouse by modifying the rhythmic expression of testosterone synthesis related genes (Wang et al., 2018). These reports clearly suggest that fatty acids have efficacy to modulate steroidogenic targets, supporting our data. This is the first study that demonstrates that the fatty acid isolated from *Aloe vera* gel have steroid modulatory properties. The observed effects may be due to the various stable interactions with the Androgen receptor and other steroidogenic targets, as observed in the molecular docking experiments. Several studies from past 20 years have highlighted the crosstalk of hyperandrogenism and insulin resistance in the pathogenesis of PCOS (Diamanti-Kandarakis

et al., 2019). Even though, LP1 was also found to have less docking score when docked with phosphorylated Insulin Receptor tyrosine kinase, it exhibited greater negative binding energy, suggesting that it possesses good affinity towards the target (Wang and Zhu, 2016). Further validation by *"in-vitro"* studies, confirmed the potential of LP1 to regulate the gene expression of insulin receptor, thereby, suggesting its role as a metabolic regulator.

In addition to the fatty acids, phytosterols are also known to modulate steroidogenesis. Data from the current study has clearly shown that LP3 (containing 94.04% Campesterol acetate) and LP4 (containing 92.45 % beta-sitosterol isolated from *Aloe vera* gel can enhance the progesterone and estradiol levels in the spent medium. These bioactive molecules can regulate the transcription of key steroidogenic proteins and receptors such as Progesterone Receptor, Estrogen Receptor beta, StAR and Aromatase by binding to them with stable interactions. In relation to the results obtained, data from literature shows that b-Sitosterol exposure in gold fishes (*Carassius auratus)* leads to decreased circulating hormone, cholesterol concentrations and intra-mitochondrial cholesterol pools due to altered gonadal StAR transcript abundance (Sharpe et al., 2006). Similar cholesterol lowering and estrogenic responses have been observed in juvenile gold fishes when they were provided feed supplemented with *Aloe* (Palermo et al., 2013). Also, fatty acids and phytosterols present in Saw Palmetto extract demonstrate anti-androgenic effect in male Syrian Hamster by inhibiting 5α-reductase enzyme (Opoku-Acheampong et al., 2015).

Also, certain terpenoids are known to be steroidogenic modulators, wherein, Gibberellic acid treatment in male diabetic rats, exhibited enhanced activity of steroidogenic markers-3β-HSD, 17β-HSD, elevated tissue testosterone content, increased StAR expression and androgen binding protein levels (Premalatha et al., 2014). On the other hand, study by Taxvig et al., 2009, showed that treatment with phytoestrogen mixture leads to increased estradiol production and decreased testosterone production in H295R human adrenal corticocarcinoma cells, indicating an induced aromatase activity. Also, exposure of the MCF7 human breast adenocarcinoma cells and JEG-3 choriocarcinoma cells to mixture of isoflavonoids and coumestrol lead to cell growth and induced aromatase activity along with an increase in progesterone receptor protein expression as well as a decreased ER alpha expression. These studies show that phytochemicals can stimulate or inhibit biologic responses in vertebrates by mimicking or modulating the action or production of endogenous hormones. Also, daily intake of bioactive terpenoids can modulate the activities of ligand dependent transcription factors like peroxisome proliferator-activated receptors (PPARs), which are dietary lipid sensors that control energy homeostasis,

thereby, they pose therapeutic potential for management for endocrine disorders and metabolic syndromes. These studies demonstrate that phytochemicals indeed exhibit steroidogenic and metabolic modulatory properties and can be used for pathological conditions, which involve dysregulated steroidogenesis and impaired ovarian microenvironment such as PCOS. As a consequence of this, there is an increasing urge by the scientific community to identify bioactive molecules for management of PCOS. In this regard, several herbs (Abasian et al., 2018) have been screened for their efficacy, however, a very few bioactives have been investigated such as Curcumin (Reddy et al., 2016), Menthol (Mesbahzadeh et al., 2017), Tanshinones (Shen et al., 2013) etc. As previously mentioned, *Aloe vera* gel has been found to be effective in management of ovarian structure-function in letrozole induced PCOS rat model (Maharjan et al., 2010; Desai et al., 2012; Radha et al., 2014; Nampoothiri et al., 2015; Radha and Laxmipriya, 2016a; 2016b; Dey et al., 2017), this study was further interesting as it identified and elucidated the role of bioactive molecules of *Aloe vera* gel- LP1 and LP3 as safe and probable therapeutic option for management of steroidogenic and metabolic alterations, as observed in multi etiological pathology like PCOS.

4.5. Conclusion

In the present study, we studied an *"in-silico"* methodology combining molecular docking and ADME prediction, to identify the targets of the partially purified non-polar phytocompounds isolated from *Aloe vera* gel to modulate the key steroidogenic and metabolic regulators. *"In-silico"* prediction of adsorption, digestion, metabolism, excretion (ADME) profile based on physicochemical properties and Lipinski's rule-of-five showed that the compounds were non-toxic and had desirable drug-like properties. The study determined that LP1 and LP3, consisting of 97.07% and 94.03% of n-Hexadecanoic acid and Campesterol acetate respectively, exhibit steroidogenic modulatory activity by modulation of key targets like Steroidogenic acute regulatory protein, Aromatase, 3-beta hydroxysteroid dehydrogenase, Androgen Receptor, Progesterone Receptor and Estrogen Receptor beta. The results obtained by *"in-silico"* analysis were further validated by incubating the phytochemicals in an *"in-vitro"* culture of KGN cell-line (human ovarian granulosa-like tumor cell line). The identification of such bioactivity facilitates the design of therapeutic protocols and compositions which may be useful in symptoms associated with reproductive endocrinopathies such as PCOS.