6 Summary

Polycystic Ovary Syndrome is a heterogenous endocrinopathy in reproductive-aged women with discomposure to endocrine, reproductive and metabolic functions (Adam Balen, 2004). This multi-etiological pathology is associated with clinical pregnancy complications, an increased rate of spontaneous abortion/early pregnancy loss, and preterm delivery (Palomba et al., 2015; Azizia & Hardiman, 2011). However, molecular alterations in PCOS pregnancy that originates from the mother, embryo, or both are still not clear. The ethical restrictions and a lack of mechanistic studies have excluded studies on embryo-endometrium interlinkage in PCOS patients. Thereby, there exists a need for a model system wherein molecular events of early pregnancy could be studied with ease and dissection of pathways. The previous study from the lab has clearly shown that letrozole (0.5 mg/kg/day) when administrated orally for 21 days daily exhibits reproductive and metabolic alteration signs similar to the human PCOS condition in mice (Dey et al., 2022). Moreover, the preliminary data from the current study exhibited retarded fetal growth and development as observed on the day 18th of pregnancy in PCOS mice. Thus, it is possible that early gestation events are found to be important widows that direct proper fetus development. Hence, the present study emphasizes the probable regulatory mechanism for the organization of early pregnancy events in the letrozole-induced PCOS mouse model. Further, targeting them for therapeutic interventions could help us in the management of early pregnancy complications linked to PCOS pathology. In this direction, 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.

In order to examine the pregnancy complications associated with PCOS pathology, the letrozole-induced PCOS mouse model was developed, validated (Explained in chapter 3), and hormone profile, reproductive performance, and key molecular markers were evaluated in the embryo-implanted region of the uterus on the day 6th of pregnancy. The results showed an altered hormone profile (high testosterone, low progesterone with unchanged estrogen levels) in the PCOS animals on day 6 of pregnancy compared to control animals, which could alter the ovarian structure. Also, when examined for ovarian histology, the number of peripheral cysts was observed in PCOS ovaries. However, the treatment of PCOS animals with AVG,

and PE extract of AVG (25 and 75 μg/kg/day) before conception demonstrated decreased circulating free testosterone and increased progesterone content. 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. 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 favourable effects on reproductive abnormalities of PCOS pathology. Further, it is interesting to note that amelioration in progesterone content could be correlated with the newly formed corpus luteum in the ovary. 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.

The imbalanced steroid hormone production in PCOS animals could result from the hyperactivation of ovarian steroidogenesis. When analysed for the ovarian steroidogenic modulators, the overexpression of luteinizing hormone receptors (*Lhr*), Steroidogenic Acute Regulatory protein (*Star*), and cytochrome P450-17α-hydroxylase/C17, 20-lyase (*Cyp17a1*) with a decreased expression of cytochrome P450 aromatase (*Cyp19a1*) was observed. These results indicated that ovarian dysfunction on day 6 of pregnancy in PCOS is a cumulative result of excess androgen with a poor progestogenic response. Further, the autocrine role of steroid hormones in ovarian function has been reported (Drummond, 2006). Thus, the overexpression of androgen receptors and decreased progesterone receptors in the ovary could extensively affect ovarian follicle maturation, as demonstrated in our histopathological analysis. Also, the results of our study indicated that the number of pregnant females and the implanting embryos in the uterus were significantly reduced in the PCOS animals. Altogether, data indicate that an altered hormone production could modulate the growth and survival of a fetus in PCOS pathology. With an aim of a quest for herbal management, it is seen that the oral administration of AVG, metformin, and PE extract of AVG in PCOS animals could modulate the gene expression of steroidogenesis marker *Cyp19a1*, steroid hormone receptors, *Ar*, and *Pr* in the ovary. Thus, it can be noted that the phytocomponents present in AVG have the potential to improve ovarian function at the molecular level. Moreover, 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.

The observed potency and efficacy can be ascribed to the existence of certain phytocompounds in the PE extract of AVG. The GC-MS data shows that the extracts contain several bioactive including n-Hexadecenoic acid, γ-Sitosterol, Oleic acid, campesterol, etc. This suggests that these compounds could be the active component/s that render the above effects. Interestingly, our previous lab study exhibited that campesterol and n-Hexadecanoic acid were the major compounds present in partially purified non-polar phytocompounds of *Aloe vera* gel that significantly reversed preliminary symptoms of PCOS pathology in the non-pregnant state (Dey et al., 2022). Thus, it can be noted that these bioactive/s could be responsible to render the above-observed effects. Although, the molecular targets or mode of action of phytochemicals present in the AVG are not defined when used as a pre-conceptive agent.

Thereby, the present study attempted to understand molecular deficits of pregnancy loss in the PCOS phenotype. In the embryo-implanted region of the uterus, steroid hormones mediate their effect through their receptors for the establishment of pregnancy. When examined for the expression of steroid hormone receptors in the implanted region of the uterus on the day 6^{th} of pregnancy, PCOS animals exhibited overexpression of AR and declined PGR expression. Progesterone signalling is known to have an inhibitory effect on the E/ESR and androgen signalling pathway in stromal cells of the endometrium for the establishment of pregnancy (Wetendorf & DeMayo, 2014; Li et al., 2014). It can be noted that in our experimental model, the decline in P/PGR signalling did not show an inhibitory effect on estrogen and androgen, resulting in no difference in the estrogen-responsive gene (LIF) and AR expression respectively. Thus, the reduced P/PR signalling may further dysregulate the downstream targets required for the establishment of pregnancy. Although, the decline in PR expression was improved by PE extract of AVG treatment in PCOS animals, even though ER and its responsive gene (LIF) did not show a significant difference. Also, 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 AR and could activate the downstream targets required for the establishment of pregnancy.

Further, the numerous scientific evidence (Discussed in chapter 3) are denoting that progesterone is known to influence various signalling molecules in the uterus for the establishment of pregnancy, in this direction, the data from the current study demonstrated that the reduced progesterone content in the letrozole-induced PCOS animal could be one of the contributory factors causing the reduced integrin (Itgα4, β1- Required for the attachment of the embryo to uterus) and homeobox protein 10A expression (Hox10a- Decidualization marker) in the embryo-implanted region of the uterus compared to the control animals. As a consequence of this aberrant early embryonic-uterine attachment and decidualization may alter the pregnancy outcomes in the PCOS animals, as observed by the poor fetal outcome. Moreover, 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. 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. In addition, the HOX10a was found to improve in the implanted region of the uterus after the treatment with metformin and PE extract of AVG. 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 blastocyst invasion, homeostasis of the proteases (MMPs) and their inhibitors (TIMPs) are important. When analysed for their expression, the matrix metalloproteases (MMP-9) and their inhibitors (TIMP-1 and 3) were found to be decreased in the embryo-implanted region of the uterus of PCOS animals. The estrogenic regulation of the MMPs and TIMPs has been reported in the uterus (Zhang et al., 2007; Nothnick et al., 2004). However, in the present study, PCOS animals did not exhibit any change in estradiol content as compared to the control animals. Thus, it can be noted that the imbalance in the expression of the proteases and their inhibitors may be attributed to improper blastocyst invasiveness during early gestation in PCOS. Whereas, upon PE extract of AVG treatment in PCOS animals, proteases (MMP-9) showed upregulation, with no changes in inhibitors (TIMP1,3). Thus, the 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.

The proper growth and development of the implanting embryo is a crucial step during early pregnancy. The key mediator of the LIF-STAT signalling pathway directs this fundamental event in the uterus (Salleh & Giribabu, 2014). When analyzed for the key markers of the LIF signalling pathway, letrozole-treated animals did not show any significant changes in the mRNA levels of LIF and LIFR in the embryo-implanted region of the uterus compared to that of control animals. Alternatively, the expression of GP130 and STAT3 significantly declined in the PCOS animals. Emerging evidence suggests that estrogen can induce LIF and LIFR expression in the endometrium of ovariectomized mice (Chen et al., 2000; Ni et al., 2002). In addition, it is reported that STAT3 has a pivotal role in PR-dependent pathways during implantation in mice (Lee et al., 2013). In this study, disrupted LIF-STAT signalling was observed in the PCOS animals. The unaltered estrogen levels in the PCOS group did not influence LIF and LIFR expression in the implanted region of the uterus. However, it couldn't be confirmed whether the unbalanced LIF signalling could be because of altered progesterone signals or whether declined STAT3 does not activate the PR-mediated pathways in the embryo-implanted region of the uterus. Moreover, the declined transcript levels of *Gp130* and *Stat3* were found to be increased upon PE extract of AVG treatment. Thus, it can be noted that PR-mediated pathways can be indirectly regulated by STAT3 upon treatment with the plant extract at both the concentration of dose (25 & 75µg/kg/day) as results in a healthy implanting embryo observed in the uterus. A similar effect was observed in AVG and metformin-treated PCOS animals. 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.

With all of the 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 embryo implanted region of the uterus on the day 6^{th} of pregnancy. The healthy-growing implanted embryos were found in the untreated animals, whereas in the letrozole-treated animals, the appearance of vascular permeability was observed. These inherent changes in the structure area of the uterus could be implicated in endometrial dysfunction in pregnant PCOS mice. Moreover, the molecular rectification in the embryo

implanted region of the uterus after the treatment of PE extract of AVG, whole AVG, and metformin in PCOS animals, might be originated from the improved histological architecture structure of the implanted region of the uterus. 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 animals, indicating the role of phytosterols in the correction of uterine milieu adapted for better fetal outcomes.

Till now, the present study demonstrated that in the letrozole-induced PCOS animals, the majority of the molecular alterations were due to the aberrant PR expression and its signalling, which further dysregulates the expression of the key markers that are required for the uterine-embryonic cross-talk during the early gestation period (Diagrammatically summarized in chapter 3, figure 3.16). This could be the reason for the early pregnancy complications/early fetal loss observed in the PCOS animals. Moreover, the phytocompounds containing PE extract of AVG therapy to PCOS animals demonstrated a minimum effective dose (25 µg/kg/day) of PE extract of AVG exhibited a progestogenic effect on the uterus. 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 along with the key early pregnancy marker proteins, mainly by acting as a progestogenic agent (Diagrammatically summarized in the chapter 4, figure 4.24). It assisted in the restoration of the majority of progesterone-responsive genes during the early phase of pregnancy in letrozole-induced 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.

However, the study warrants the further characterization 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). Therefore, these compounds (Campesterol and n-Hexadecanoic acid) have been validated as a pre-conceptive agent in PCOS pathology using *in silico* molecular docking study. Further, the n-Hexadecanoic acid and campesterol were selected as ligand and prime proteins of early pregnancy (Steroid receptors, adhesion phase markers, decidualization markers, proteases/their inhibitors, and key mediators of the LIF-STAT pathway) were chosen as targets for molecular interaction study.

The n-Hexadecanoic acid and campesterol were showing molecular interaction with target proteins of early pregnancy and that could act as agonists and/or antagonists to prevent early pregnancy loss in PCOS pathology. Although, it is interesting to note that campesterol has the most significant binding with the target proteins compared to the n-Hexadecanoic acid. Moreover, it has been visualized that campesterol has well-established bonds in the binding pocket of the progesterone receptor with the highest docking score. The binding interactions are alkyl, Pi-Alkyl, and van der Waals with residues PRO A:696, ARG A:766, TRP A:769, LYS A:769, and HIS A:770. Also, the *in-silico* study has laid the foundation for identifying the best target for phytosterol that mimics, progesterone in early pregnancy molecular events. In addition, the *in vivo* experiment demonstrated that campesterol containing PE extract of AVG (PE-25) may exert agonistic effects on progesterone signalling and its regulators during early gestation. Thus, the correlation between the *in vivo* data and the binding affinities could impart valuable insight into the therapeutic procedure which may help in the pregnancy complications associated with infertility like PCOS.

Thus, the overall study depicts that a major factor in abnormal pregnancy or poor fetal outcome in PCOS pathology is the result of steroidogenic modulation which leads the way to influence downstream partners at the materno-fetal interface. This alteration could be managed by campesterol containing PE extract of AVG, thus correcting the embryo-uterine microenvironment of PCOS mothers. The observed result could be due to the effect of AVG on improving the downstream targets indirectly or by binding with the key markers of pregnancy, in consequence, better pregnancy/fetus outcomes were observed in the PCOS phenotype, also this is the first study to show the potency of non-polar phytosterols of AVG as a pre-conceptive nutraceutical in management of PCOS pregnancy.

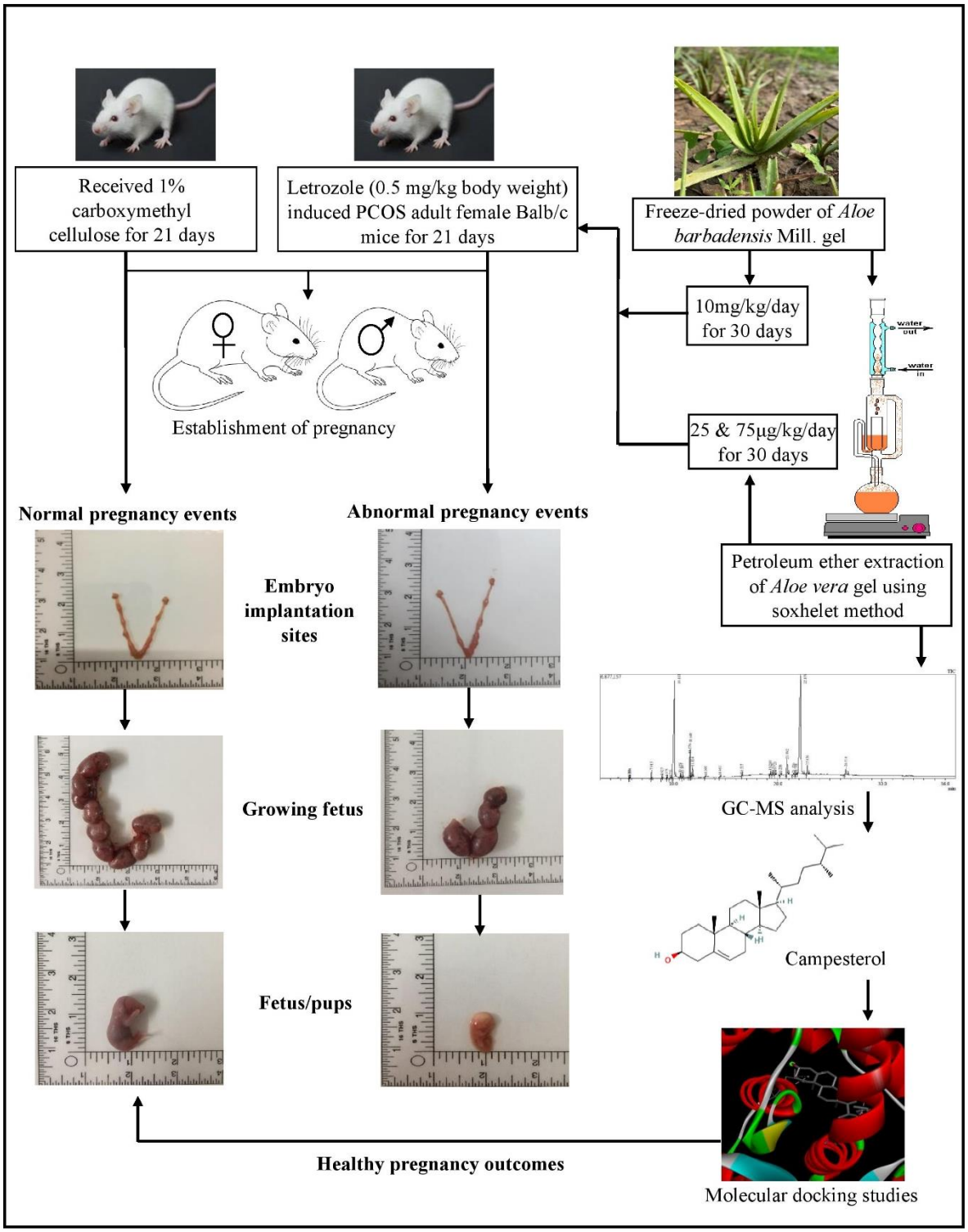

Figure 6.1 Diagrammatic summary of the overall study.

6.1 Overall study highlights:

- PCOS mice model was developed using letrozole (0.5 mg/kg/day for 21 days), following female mice were mated with the age-control males.
- Early (Day-6^{th}) and late gestation (Day-18^{th}) abnormalities were observed in the letrozole-treated mice compared to the untreated mice.
- Reduced fertility index and a number of embryo implanted sites were observed on the day-6^{th} of pregnancy along with altered hormone profiles and ovarian histology noticed in PCOS mice.
- Modulated histological structure of the embryo implanted region of the uterus was observed in PCOS mice.
- Hyperactivation of ovarian steroidogenesis in PCOS animals leads to alter steroid receptor expression in the embryo-implanted region of the uterus.
- Further, deranged adhesion molecules, decidualization markers, imbalanced proteases, and their inhibitors along with disturbed LIF-STAT signalling were examined in the implanted region of the uterus in PCOS mice.
- From all of the above molecular defects, it can be concluded that the effect of letrozole on the embryo-uterine tissue could be due to the aberrant progesterone signalling in PCOS mice, thus translating into a fetal loss or poor pregnancy outcomes.
- In the next part of the study, the therapeutic efficacy of Petroleum ether (PE) extract of *Aloe vera* (AVG) as a possible pre-conceptive agent was studied in PCOS pathology.
- Further, early (Day-6^{th}) and late gestation (Day-18^{th}) abnormalities have been restored upon Petroleum ether (PE) extract treatment of *Aloe vera* (AVG) thus, overcoming fertility in this endocrinopathy.
- Reduced fertility index and a number of embryo implanted sites were enhanced upon the treatment with PE extract of AVG.
- Upon the treatment with PE extract of AVG, hormone profile and ovarian histology were found to be normal on the day-6^{th} of pregnancy in PCOS mice.
- The structure of the embryo implanted region of the uterus in PCOS animals reverted to normal after the treatment with PE extract of AVG.

- Improved ovarian steroidogenesis after the treatment with PE extract of AVG in PCOS mice could further have a modulatory effect on steroid receptor expression in the embryo-implanted region of the uterus.
- An upgraded decidualization marker, Matrix metalloprotease (MMP-9), and LIF-STAT signalling pathway was found in the implanted region of the uterus in PCOS mice after the treatment of PE extract of AVG.
- The effect of the PE extract of AVG on the PCOS pregnant uterus suggests that the majority of molecular restoration could be due to the enhanced progesterone signalling in PCOS mice.
- The minimum effective dose of PE extract of AVG for improvement of pregnancy loss was found to be 25µg/kg/day for 30 days.
- The PE extract of AVG is equally potent as metformin and whole AVG towards control of pregnancy complications in PCOS mice.
- The oral administration of the PE extract of AVG at doses of 25 and 75µg/kg/day for 30 days did not show any adverse effect on animals; thus, proving it to be a safe herbal alternative.
- Further, characterization of the phytoconstituents of the PE extract of AVG was done by GC-MS analysis wherein n-Hexadecanoic acid and Campesterol were major components along with other phytosterols.
- On the basis of previous lab study, n-Hexadecanoic acid and Campesterol was selected for the molecular docking study
- The *in-silico* molecular docking study confirms the significant binding interaction of campesterol with the key pregnancy markers compared to n-Hexadecanoic acid, as campesterol formed well-established bonds in the binding pocket of the Progesterone receptors compared to selected targets.
- *In vivo* and *in silico* results signifies that the PE extract of AVG may exert agonistic effects on the progesterone signalling pathway for the establishment of pregnancy in PCOS.
- Hence, the PE extract of AVG could be considered as a pre-conceptive agent without having toxicity in the management of PCOS pathology.

All-inclusive, this investigation has given a newly added platform to dissect the molecular variation at the maternal-fetus interface underlying PCOS mothers. Also, bioprospecting *Aloe vera* as a remedial substitute for pregnancy complications in PCOS

gives an insight into its efficacious mechanism and is possibly inferred as a "*pre-conceptive agent*". This proves its pharmacological and ethnobotanical significance in the treatment of multiorgan endocrinopathy, namely PCOS. Consequently, adding to its overall potential and economic viability at national and international levels.

6.2 Future Directions:

- A study of the protein expression of certain key early pregnancy markers will give better insight into the regulatory mechanism of early pregnancy loss.
- As the molecular derangements obtained in the current study are progesterone-dependent, further exogenous chemical agonist administration-based studies may be attempted in the PCOS model and early pregnancy complications can be investigated.
- The long-term effect of the studied dose (PE extract of AVG - 25 and 75 µg/kg/day for 30 days) needs to be investigated for its toxic effects if any.
- Detailed molecular interpretation of the bio-functional molecules of *Aloe vera* requires purifications and isolations of non-polar phytocomponents and their study toward a better uterine microenvironment.
- It is important to point out that the investigation of the individual bioactive (Campesterol) as a pharmacodynamic modulator at varying doses and time has to be attempted before drawing its potential in translational research.
- Also, apart from pharmacodynamic properties, the pharmacokinetics (both single and multiple dose studies) of isolated bioactive should be evaluated.