5 Identification of the targets of non-polar phytocomponents of *Aloe vera* gel extract towards modulation of early pregnancy using the *"in-silico"* approach

5.1 Rationale of the study

In the previous chapter, an *in-vivo* study demonstrated the therapeutic potential of phytosterol-containing non-polar (Petroleum ether-PE) extract of *Aloe barbadensis* for the improvement of early pregnancy loss in the letrozole-induced PCOS mouse model. It was observed that 25µg/kg/day of PE extract of *Aloe vera* gel (AVG) for 30 days was the minimum effective dose for improving pregnancy rate and embryo implantation on the day-6th of pregnancy. This could be due to phytosterols present in the PE extract of AVG and their interaction with the molecular targets of early pregnancy. In this regard, a detailed interpretation of molecular interactions of phytosterol when used as a pre-conceptive agent is still not known. One of the ways to predict the possible interaction between a putative ligand and a possible receptor is through *in-silico* approaches.

Due to the availability of several identified protein data banks, the *"in-silico*" computational docking tool allows us to predict the most likely possible natural hits to connect with protein/receptor binding sites and pharmacophore residues. Therefore, it will give insights into the molecular signalling pathways and create molecular networks. Thus, *in-silico* molecular docking comes up with an advantageous application for drug design and analysis. This technique contributes to the prediction, visualization, and investigation of plant-derived drugs for human health (Chandran & Patwardhan, 2017). It has been well known that the medicinal properties of plants can be allocated to phytochemicals present in them which in turn can be responsible for their physiological action on the human body (Okwu, 2008; Saxena et al., 2013). In the screening of bioactive compounds of plants and their interconnection with multiple targets, traditional experiment systems are tough and tedious. In addition, pregnancy is a complex process with overlapping expression patterns making it challenging to investigate their stage-specific role in PCOS phenotype. Thereby, the traditional systems in combination with bioinformatics techniques have led to the envisioning and uncover the actions of phytocompounds by identification of their molecular targets in PCOS pregnancy.

With reference to the above line of study, chapter 4 has potentially identified the presence of several phytocompounds in the PE extract of AVG. It is clear from earlier lab data (Dey A, Ph.D. thesis, unpublished data), though several phytosterols are present in the petroleum extract of *Aloe vera* gel, the fractions containing n-HA and Campesterol were most effective in modulating the PCO milieu as compared to other phytosterol containing fractions when incubated with KGN cell type. Further, this fact was confirmed that these bio-actives n-Hexadecanoic acid (n-HA) and campesterol from partially purified non-polar phytocomponents of AVG exhibited ovarian and metabolic modulators in non-pregnant letrozole-induced PCOS mouse model (Dey et al., 2022). Hence, these two compounds 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 the molecular *in silico* study.

With this background, the main focus of this chapter was to identify the molecular interaction of the primary targets of early pregnancy with the selected ligands (Phytocompounds of the AVG). Further, this computational approach gives an insight into the mechanism underlying the pregnancy loss of PCOS animals.

5.2 Materials and methods

"*In-silico*" analysis of non-polar phytocomponents of *Aloe vera* gel with key early pregnancy markers

5.2.1 Molecular Docking Study

The data from the previous chapter demonstrated that the PE extract of AVG contains 25 different chemical structures. Amongst the identified phytochemicals, n-Hexadecanoic acid and campesterol were selected for the *in-silico* studies. These compounds were screened against a number of key important molecules (explained below) of early pregnancy in order to check their binding affinity in the active site of the protein. The docking studies which include protein preparation, ligand preparation, and grid generation were performed using MGL Tools1.5.7rc1 and Autodock vina software. The docking score and amino acid residues involved in the interaction were utilized to anticipate binding affinities towards the active binding pocket of the targets.

5.2.1.1 Ligand preparation and Grid generation

The identified ligand structures from the results of the GC-MS were drawn using ChemDraw software and prepared using Autodock from MGL Tools 1.5.7rc1 in which all the bonds of ligands were set rotatable. After ligand preparation, grid generation was done using AutoGrid. In Autogrid, a grid map was prepared using a grid box. The grid size used is 40 x 40 x 40 Xyz points with a grid spacing of 0.375 and grid center dimensions (x, y, z) 57.437, 22.957, and 0.357 respectively. The docking studies were performed using Autodock vina in which the ligand and protein both kept rigid and the pose which was having lowest binding affinity was extracted for further analysis and, was aligned with the target protein structure.

5.2.1.2 Protein Preparations

The three-dimensional structure of all the proteins (Key early pregnancy markers) include steroid receptors (Androgen receptor, Progesterone receptor, Estrogen receptor α, Estrogen receptor β), adhesion phase markers (*Integrin β1* & α4), decidualization marker (Homeobox 10A), proteases, and their inhibitors (Matrix metalloproteinases 2 & 9, Tissue inhibitor of Matrix metalloproteinases 1 & 3), key mediators of the LIF-STAT pathway (Leukemia inhibitory factor, Leukemia inhibitory factor receptor, Glycoprotein 130, and Signal transducer and activator of transcription 3) was selected as a target in the study. All the protein structures were retrieved from the RCSB-Protein data bank site. The details of their PDB IDs, resolution, and method by which structure was obtained are mentioned in Table 5.1. The protein structures were prepared using Discovery studio 2020 in which water molecules and other small molecules were removed. Then using the graphical user interface of autodock tools 1.5.7rc, the polar hydrogens, Kollman charges, fragmental volumes, and salvation parameters were assigned to the protein.

Table 5.1. Details of the targets obtained from the RCSB-Protein data bank.

Targets	PDB ID	Source	Resolution Å	The method by which structure was obtained
Androgen receptor (AR)	4OHA	Homo sapiens	1.42	X-ray diffraction
Progesterone receptor (PR)	1A28	Homo sapiens	1.80	X-ray diffraction
Estrogen receptor α (ERα)	6B0F	Homo sapiens	2.86	X-ray diffraction
Estrogen receptor β (ERβ)	3OLS	Homo sapiens	2.20	X-ray diffraction
Integrin *β1* (ITGB1)	4DX9	Homo sapiens	*2.99*	X-ray diffraction
Integrin α4 (ITGA4)	4HKC	Homo sapiens	2.2	X-ray diffraction
Homeobox 10A (HOX10a)	3K2A	Homo sapiens	1.95	X-ray diffraction
Matrix metalloproteinases 2 (MMP2)	1RTG	Homo sapiens	2.6	X-ray diffraction
Matrix metalloproteinases 9 (MMP9)	1GKC	Homo sapiens	2.30	X-ray diffraction
Tissue inhibitors of Matrix metalloproteinases 1 (TIMP1)	1UEA	Homo sapiens	2.80	X-ray diffraction
Tissue inhibitors of Matrix metalloproteinases 3 (TIMP3)	3CKI	Homo sapiens	2.30	X-ray diffraction

Leukemia inhibitory factor (LIF)	1EMR	Homo sapiens	3.50	X-ray diffraction
Leukemia inhibitory factor receptor (LIFR)	3E0G	Homo sapiens	3.1	X-ray diffraction
Glycoprotein 130 (GP130)	1BQU	Homo sapiens	2.0	X-ray diffraction
Signal transducer and activator of transcription 3 (STAT3)	6NUQ	Homo sapiens	3.15	X-ray diffraction

5.3 Results

5.3.1 Interaction of the identified non-polar phytocomponents of *Aloe vera* gel with the key intermediates of early pregnancy using the *"in-silico"* approach.

To achieve a successful pregnancy, the first step is embryo implantation, wherein two-way communication between a competent blastocyst and receptive uterus gives rise to attachment and invasion of the embryo to the uterine epithelium, following the decidualization of the uterine stroma (Lee et al., 2007). Each step of the initial pregnancy involves an interplay of the various signalling pathways. The detailed regulators involved in the mechanism are explained in chapter 1. In the current part of the study, the interaction between key regulators (targets) of early pregnancy and phytocompounds (ligands) has been examined. The phytocompounds were identified by GC-MS analysis, and the γ-Sitosterol (45.25%), n-Hexadecanoioc acid (29.48%), Oleic acid (5.40%), 9,12-Octadecanoic acid (4.44%), Campesterol (3.60%) were found to be the most abundant in PE extract of AVG (Chapter 4). Although, the *in vitro*, *in vivo*, and *in silico* analysis from the previous lab study confirms that the n-Hexadecanoic acid and Campesterol are the two bioactive of AVG responsible for the management of the PCOS (Dey et al., 2022). Hence, these two phytocomponents were chosen as a ligand for further studies. The binding interactions were analyzed using AutoDock Vina software. The target structures were retrieved from the RCSB Protein Data Bank. A description of the preparation of the ligand and target structure has been provided in

the material & methods section. The results for both compounds were discussed below. The following sections will discuss the detailed interaction of both molecules (n-Hexadeconic acid and campesterol) with the selected molecular targets.

5.3.2 Docking studies of n-Hexadecanoic acid from the non-polar fraction of *Aloe vera* gel

Firstly, **steroid receptors** were used as the target, wherein, in the binding pocket of the **Androgen Receptor**, n-HA acid shows a binding score of -3.7 with alkyl and Pi-alkyl interactions with amino acids PHE A: 804, ARG A:752, TRP A:751, and PRO A:801. Further, when the active site of the **Progesterone Receptor** was docked with n-HA, it demonstrated a -6.0 -docking score, alkyl, pi-alkyl, and van der Waals interactions with LEU A:763, PHE A:778, LEU A:721, VAL A:760, LEU A:718. Similarly, **Estrogen Receptor α** and n-HA revealed a docking score of -5.6 as well as the alkyl and Pi-Alkyl interaction with amino acid residue LEU A:525, LEU A:349, ALA A:350, LEU A:387, PHE A:404, LEU A:391, LEU A:428, LEU A:346, and MET A:388. In the binding pocket of **Estrogen Receptor β**, n-HA exhibits a docking score of -3.5 and alkyl interactions with MET:494, LEU A:331, VAL A:328, ILE A:310, LYS 314, and LEU A:324 residues. From the results, it can be noted that among all the steroid receptors, the Progesterone Receptor has maximum interaction with n-HA.

Furthermore, the interconnection between **receptivity markers** (Integrin β1 and α4) and n-HA were studied. In the binding cavity of **Integrin β1**, n-HA has been showing the carbon-hydrogen bond with ILE A:111, Pi-Sigma interaction with TYR A:172, alkyl and Pi-alkyl interaction with amino acid residue ILE A:65, PHE A:110, and TYR A:67 together with docking score of -5.0 whereas **Integrin α4** demonstrates docking score of -3.8 as well as conventional hydrogen bonding with ASP A:92 and alkyl and Pi-alkyl interaction with Val A:51, ALA A:23, ILE A:93, and TYR A:48 residues. From these two receptivity markers, integrin β1 showed better interaction with the n-HA.

In this direction, the **decidualization marker**, the **homeobox transcription factor 10A**, and n-HA display a docking score of -3.4, with the alkyl and Pi-alkyl interactions with ALA A:293, TRP A:294, ILE A:290, and LEU A:312 amino acid residues. It has shown that n-HA interacts with the decidualization marker.

In addition, when looking into the **proteases**, **matrix metalloproteases 2**, and n-HA showed a docking score of -3.7 including the carbon-hydrogen interaction with residue LYS A: 576, Pi-Sigma interaction with residue TRP A:610, alkyl and pi-alkyl interactions with residue TYR A:581, ALA A:609, and PHE A:588. In the active site of **matrix metalloproteinases 9**, n-HA denotes a docking score of -5.3 along with the pi-Sigma interactions with HIS A:401, PHE A:110 and Alkyl and Pi-Alkyl interactions with LEU A:187, HIS A:190, LEU A:188, TYR A:179, HIS A:411 amino acid residues. Along with the study of MMP, it is necessary to examine the interaction of the **inhibitors of proteases**, TIMP, (Tissue inhibitors of Matrix metalloproteinases 1 & 3), **TIMP 1**, with n-HA has shown a docking score of -5.6, in addition, conventional hydrogen bonding interaction with ARG A:233 and alkyl and Pi-alkyl interaction with TYR A:223, LEU A:197, VAL A:198, LEU A:218, HIS A:201, and LEU A:164 residue was observed whereas, in **TIMP 3** binding site, n-HA has demonstrated a docking score of -4.4 together with alkyl interactions with residues ILE A: 282, ILE A:279, ILE A:252, and ILE A:255. The outcome of the result revealed that n-HA exhibits a stronger interaction with the protease, MMP-9, and its inhibitor, TIMP-1.

Next, the key intermediate of the **LIF-STAT pathway**, **leukemia inhibitor factor**, and n-HA examined a docking score of -5.1 including conventional hydrogen bonding with LEU A:162 and alkyl and Pi-Alkyl interactions with PRO A:148, LEU A:166, TYR A:90, and VAL A:85 residues. In the binding site of the **leukemia inhibitory factor receptor**, n-HA demonstrated a docking score of -4.2 and carbon-hydrogen interaction with ASN A:339 and alkyl interaction with residues ARG A:333, ALA A:336, ALA A:315, PRO A:304, and VAL A:307. In the binding pocket of **Glycoprotein 130**, n-HA indicates a -3.2 -docking score, and the Alkyl interaction with ILE A:154, VAL A:168, conventional hydrogen bonding with GLN A:153, TYR A:143, and carbon-hydrogen bond with residue PRO A:155. In the binding cavity of the **Signal transducer and activator of transcription 3**, n-HA shows alkyl interactions with ILE A: 258, ARG A:325, CYS A:251, PRO A:336, PRO A:256, ALA A:250 amino acid residue as well as -3.7-docking score. The results indicated that the Leukemia inhibitor factor and its receptor demonstrated a greater interaction with n-HA.

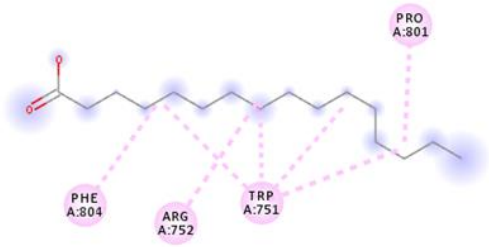
All-inclusive, the **Progesterone receptor** was found to be maximally docked with the n-HA (docking score of -6.0) when compared to all other targets. The representative image of the 2D and 3D molecular docking interactions of n-HA with the steroid receptors, adhesion phase markers, decidualization marker, proteases, and their inhibitors, key mediators of the LIF-STAT pathway is presented in figure 5.1.

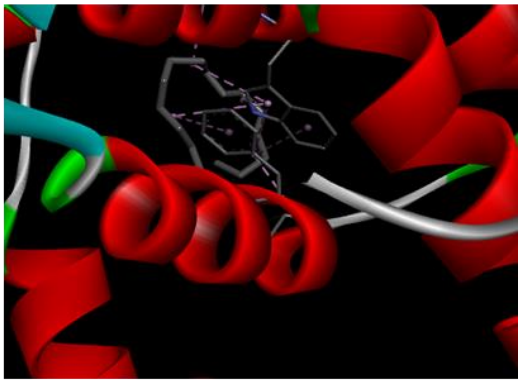
Table 5.2. Binding interactions of n-Hexadecanoic acid with the key molecules of early pregnancy.

Targets	PDB ID	Docking Score	Amino acid interactions
Androgen receptor (AR)	4OHA	-3.7	Alkyl and Pi-alkyl interactions with PHE A: 804, ARG A:752, TRP A:751, and PRO A:801
Progesterone receptor (PR)	6F88	-6.0	Alkyl, Pi-alkyl, and van der Waals interactions with LEU A:763, PHE A:778, LEU A:721, VAL A:760, LEU A:718
Estrogen receptor α (ERα)	6B0F	-5.6	Alkyl and Pi-Alkyl interaction with LEU A:525, LEU A:349, ALA A:350, LEU A:387, PHE A:404, LEU A:391, LEU A:428, LEU A:346, and MET A:388
Estrogen receptor β (ERβ)	3OLS	-3.5	Alkyl interactions with MET:494, LEU A:331, VAL A:328, ILE A:310, LYS 314, and LEU A:324
Integrin β1 (ITGB1)	*4DX9*	-5.0	Carbon-hydrogen bond with ILE A:111, Pi-Sigma interaction with TYR A:172, alkyl and Pi-alkyl interaction with ILE A:65, PHE A:110, and TYR A:67
Integrin α4 (ITGA4)	4HKC	-3.8	Conventional hydrogen bonding with ASP A:92 and alkyl and Pi-alkyl interaction with Val A:51, ALA A:23, ILE A:93, and TYR A:48
Homeobox 10A (Hox10a)	3K2A	-3.4	Alkyl and Pi-alkyl interactions with ALA A:293, TRP A:294, ILE A:290, and LEU A:312
Matrix metalloproteinases 2 (MMP2)	1RTG	-3.7	Carbon-hydrogen interaction with LYS A: 576, Pi-Sigma interaction with TRP A:610, alkyl and Pi-alkyl interactions with TYR A:581, ALA A:609, and PHE A:588

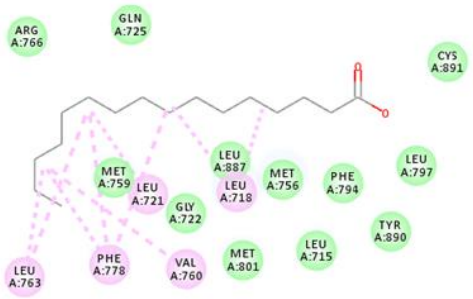
Matrix metalloproteinases 9 (MMP9)	1GKC	-5.3	Pi-Sigma interactions with HIS A:401, PHE A:110, alkyl and Pi-Alkyl interactions with LEU A:187, HIS A:190, LEU A:188, TYR A:179, HIS A:411
Tissue inhibitor of Matrix metalloproteinases 1 (TIMP1)	1UEA	-5.6	Conventional hydrogen bonding interaction with ARG A:233, alkyl and Pi-alkyl interaction with TYR A:223, LEU A:197, VAL A:198, LEU A:218, HIS A:201, LEU A:164
Tissue inhibitor of Matrix metalloproteinases 3 (TIMP3)	3CKI	-4.4	Alkyl interactions with ILE A: 282, ILE A:279, ILE A:252, and ILE A:255
Leukemia inhibitory factor (LIF)	1EMR	-5.1	Conventional hydrogen bonding with LEU A:162 and alkyl and Pi-Alkyl interactions with PRO A:148, LEU A:166, TYR A:90, and VAL A:85
Leukemia inhibitory factor receptor (LIFR)	3E0G	-4.2	Carbon-hydrogen interaction with ASN A:339 and alkyl interaction with ARG A:333, ALA A:336, ALA A:315, PRO A:304, and VAL A:307
Glycoprotein 130 (GP130)	1BQU	-3.2	Alkyl interaction with ILE A:154, VAL A:168, conventional hydrogen bonding with GLN A:153, TYR A:143, and carbon-hydrogen bond with PRO A:155
Signal transducer and activator of transcription 3 (STAT3)	6NUQ	-3.7	Alkyl interactions with ILE A: 258, ARG A:325, CYS A:251, PRO A:336, PRO A:256, ALA A:250

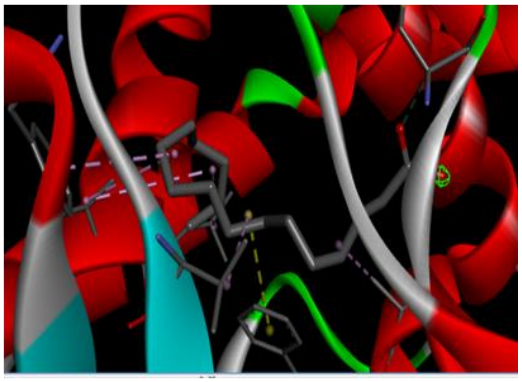
[A] Androgen receptor



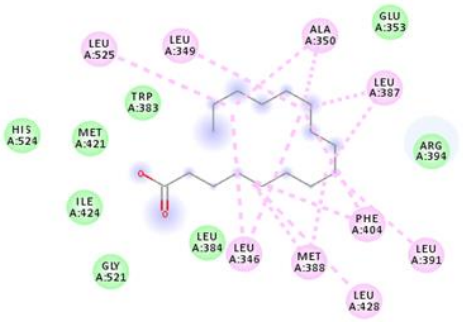


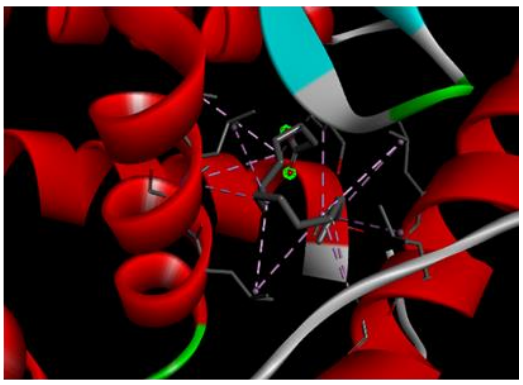
[B] Progesterone receptor



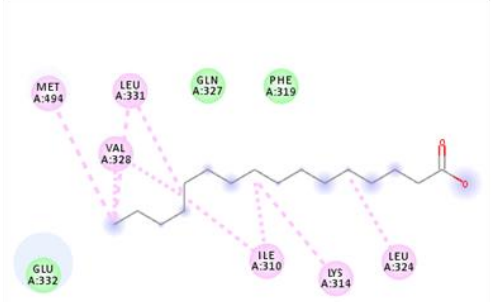


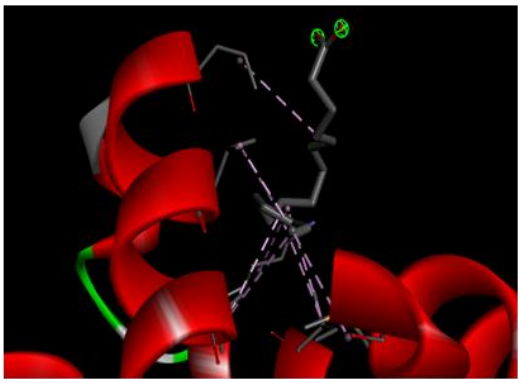
[C] Estrogen receptor α



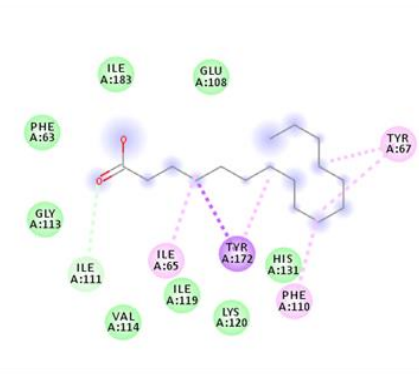


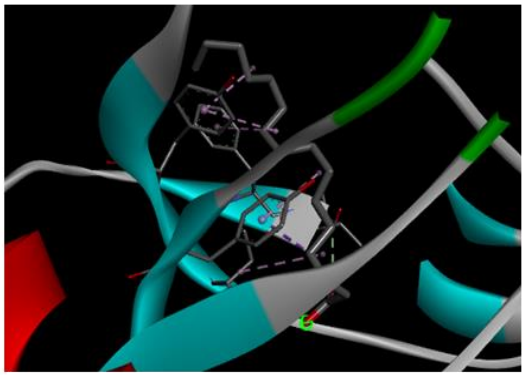
[D] Estrogen receptor β



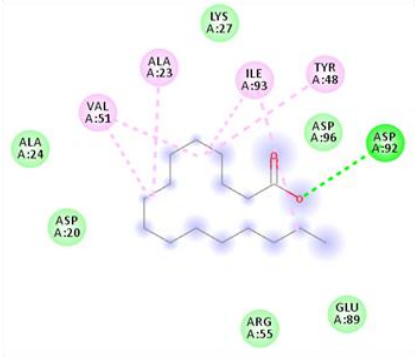


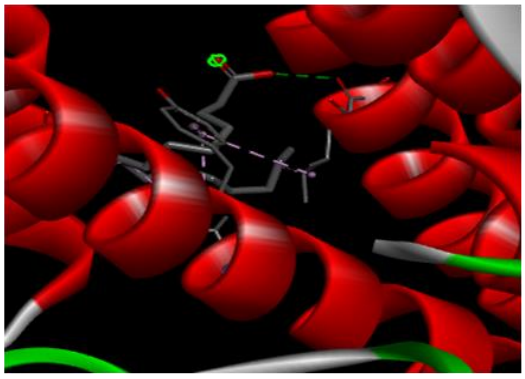
[E] Integrin β1



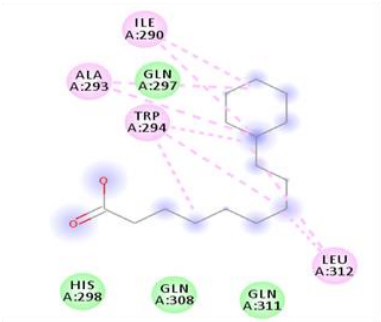


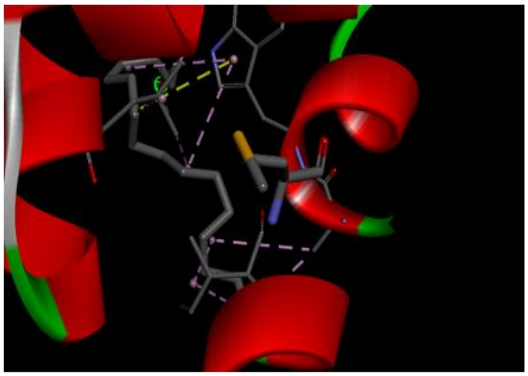
[F] Integrin α4



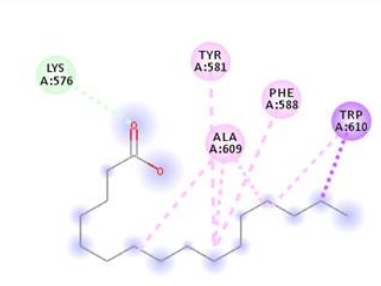


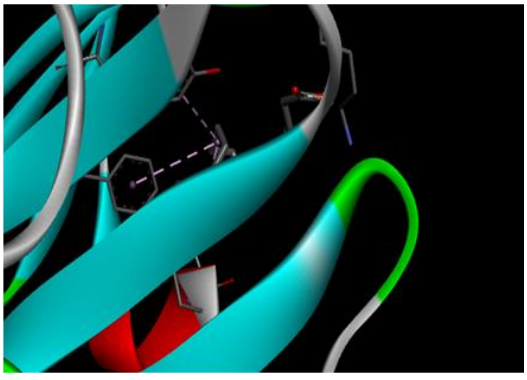
[G] Homeobox 10A



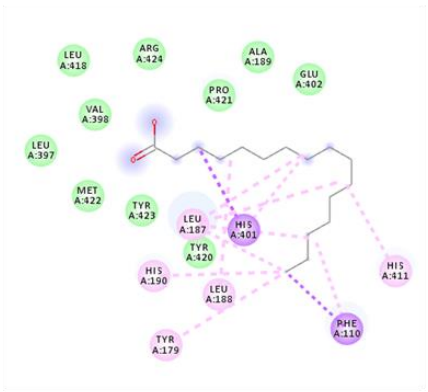


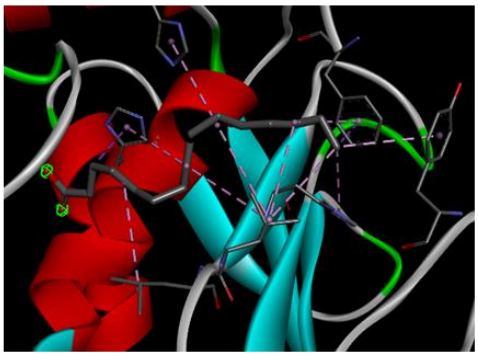
[H] Matrix metalloproteinases 2



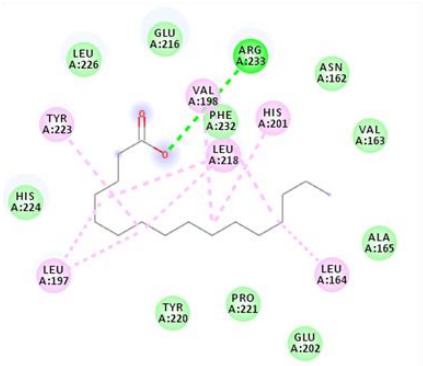


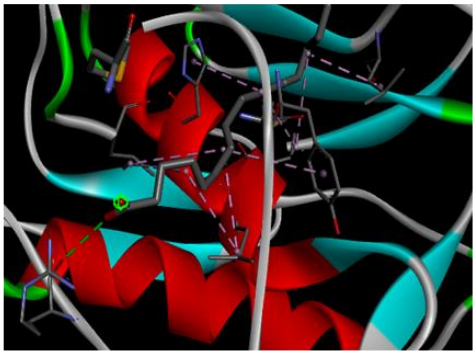
[I] Matrix metalloproteinases 9



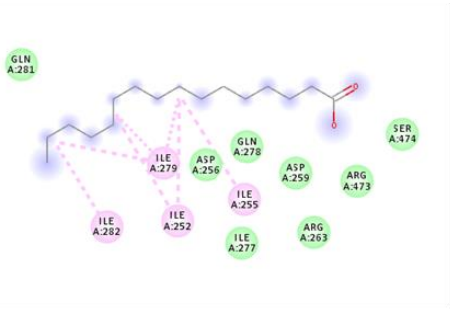


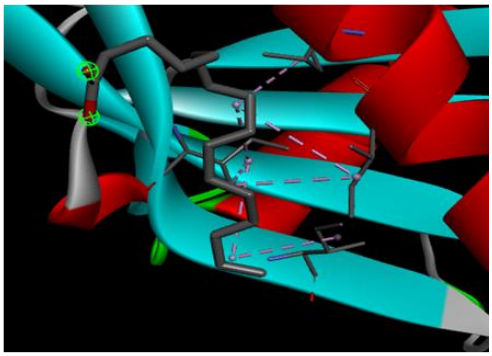
[J] Tissue inhibitor of matrix metalloproteinases 1



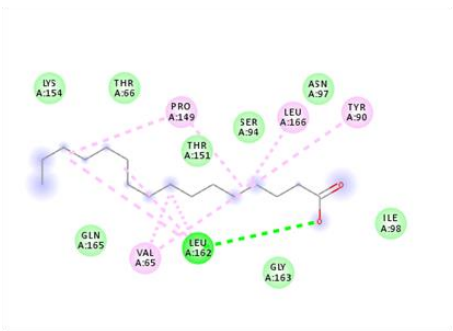


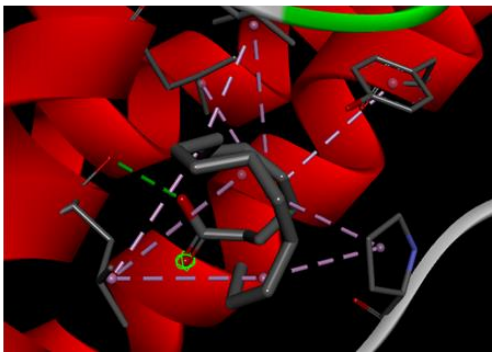
[K] Tissue inhibitor of matrix metalloproteinases 3



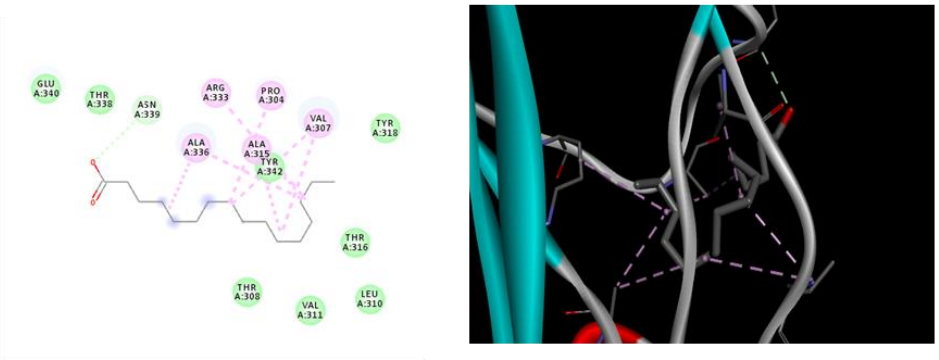


[L] Leukemia inhibitor factor

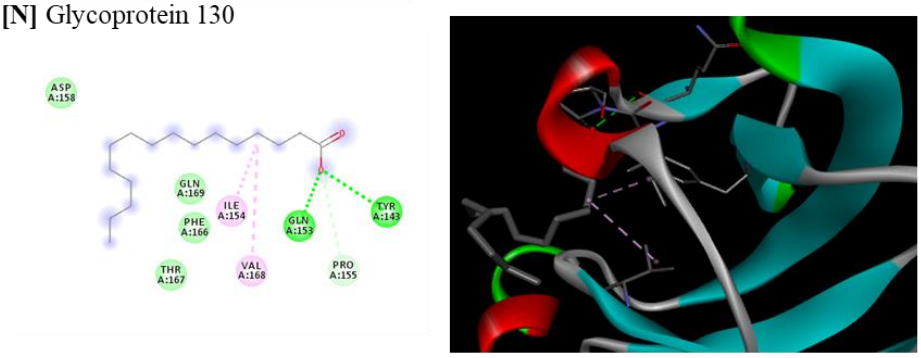




[M] Leukemia inhibitor factor receptor



[N] Glycoprotein 130



[O] Signal transducer and activator of transcription 3

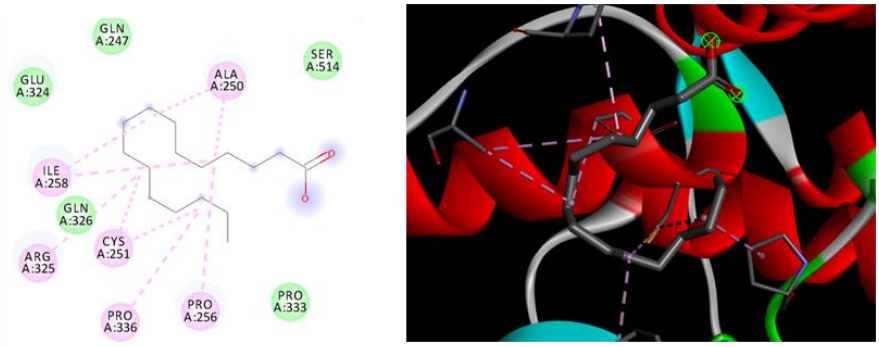


Figure 5.1. Representative image of the 2D and 3D molecular docking interactions of n-Hexadecanoic acid with key molecules of early pregnancy.

5.3.3 Docking studies of campesterol from the non-polar fraction of *Aloe vera* gel

Similarly, the selected targets (discuss above with the n-HA) have been examined for their interaction with the campesterol. Firstly, **steroid receptors** were examined, and it has been observed that in the binding pocket of the **Androgen Receptor**, campesterol showed a binding score of -8.0 and alkyl interactions with residues LEU A: 805, PRO A:801, PRO A:682, and ALA A:748. Further, in the active site of the **Progesterone Receptor**, campesterol demonstrated a -9.5-docking score, alkyl, Pi-Alkyl, and van der Waals interactions with residues PRO A:696, ARG A:766, TRP A:769, LYS A:769, HIS A:770.

Estrogen receptor α and campesterol revealed a docking score of -8.8 as well as the Alkyl interaction with ILE A:326, PRO A:324, and LEU A:320. Also, in the binding pocket of **Estrogen receptor β**, campesterol exhibited a docking score of -6.5 together with the conventional hydrogen bonding with ASP A:489 and alkyl interaction with VAL A:307, LEU A:490 residues. The results indicated that among all the steroid receptors, the Progesterone receptor demonstrated a greater interaction with campesterol.

Furthermore, the interconnection between **receptivity markers** (Integrin β1 and α4) and campesterol were studied. In the binding cavity of **integrin β1**, campesterol had shown the Pi-sigma interaction with TYR A:91, alkyl and Pi-alkyl interaction with LEU A: 88, ILE A:71, and LEU A:169 residue together with a docking score of -7.1 whereas **integrin α4** demonstrated a docking score of -6.9 as well as alkyl interaction with ILE A:217. From these two receptivity markers, integrin β1 showed better interaction with campesterol.

Further, the **decidualization marker**, the **homeobox transcription factor 10A**, and campesterol display a docking score of -6.8, with alkyl and Pi-alkyl interaction with TRP A:294, LEU A:312, ILE A:290, VAL A:286. It has shown that campesterol interacts with the decidualization marker.

In addition, when looking into the **proteases**, **matrix metalloproteases 2**, and campesterol showed a docking score of -7.0 including the alkyl interaction with PRO A:527 amino acid residue. In the active site of **matrix metalloproteinases 9**, campesterol denotes a docking score of -8.0 along with the alkyl and Pi-alkyl interaction with LEU A: 187, HIS A: 411, PHE A: 110, TYR A: 179 amino acid residues. Next, when analysed for the **inhibitors of proteases**, tissue inhibitors of matrix metalloproteinases 1 & 3 (TIMP 1 & 3), **TIMP 1**, and campesterol have shown a docking score of -8.2, in addition, Pi-sigma interaction with residue PHE A:83 and alkyl/Pi-alkyl interaction with residue TYR A:168, PHE A:86, ALA A:169, PHE A:210, TYR A:155, and HIS A:166 whereas, in **TIMP 3** binding site, campesterol have demonstrated docking score of -7.9 together with alkyl and Pi-alkyl interactions with residue PHE A:323and LEU A:380. The outcome of the result revealed that campesterol exhibits a stronger interaction with the protease, MMP-9 and its inhibitor, TIMP1.

Next, the key intermediate of the **LIF-STAT pathway**, the **leukemia inhibitor factor**, and campesterol examined a docking score of -6.5 including the alkyl and pi-alkyl interactions with residues LEU A:57, PHE A:42, and LYS A:171. In the binding site of the **leukemia**

inhibitory factor receptor, campesterol demonstrated a docking score of -6.9 and Pi-alkyl interaction with residues PHE A:97 and TRP A:195. In the binding pocket of **glycoprotein 130,** campesterol indicates a -5.5-docking score and the alkyl interaction with LYS A:189 amino acid residue. In the binding cavity of the **signal transducer and activator of transcription 3**, campesterol shows conventional hydrogen bonding with AMINO Acid ARG A:350 and alkyl interaction with residues PRO A:336, ILE A:258, CYS A:251 as well as a -7.3-docking score. The results indicated that the signal transducer and activator of transcription 3 demonstrated a greater interaction with campesterol.

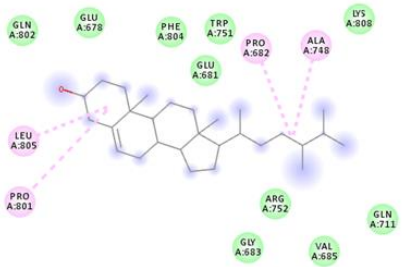
Altogether, the progesterone receptor was found to be maximally docked with the campesterol (docking score of -9.5) when compared to all other targets. The representative image of the 2D and 3D molecular docking interactions of campesterol with the steroid receptors, adhesion phase markers, decidualization marker, proteases, and their inhibitors, and key mediators of the LIF-STAT pathway is presented in Figure 5.2 and Table 5.3.

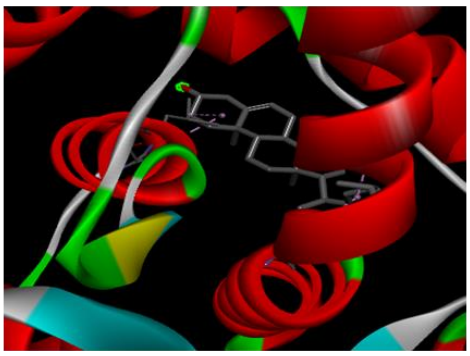
Table 5.3. Binding interactions of campesterol with the key molecules of early pregnancy.

Targets	**PDB ID**	**Docking Score**	**Amino acid interactions**
Androgen receptor (AR)	4OHA	-8.0	Alkyl interactions with LEU A: 805, PRO A:801, PRO A:682, and ALA A:748
Progesterone receptor (PR)	6F88	-9.5	Alkyl, Pi-Alkyl, and van der Waals interactions with PRO A:696, ARG A:766, TRP A:769, LYS A:769, HIS A:770
Estrogen receptor α (ERα)	6B0F	-8.8	Alkyl interaction with ILE A:326, PRO A:324, and LEU A:320
Estrogen receptor β (ERβ)	3OLS	-6.5	Conventional hydrogen bonding with ASP A:489 and alkyl interaction with VAL A:307, LEU A:490
Integrin β1 (ITGB1)	*4DX9*	-7.1	Pi-sigma interaction with TYR A:91, alkyl and Pi-alkyl interaction with LEU A: 88, ILE A:71, and LEU A:169
Integrin α4 (ITGA4)	4HKC	-6.9	Alkyl interaction with ILE A:217

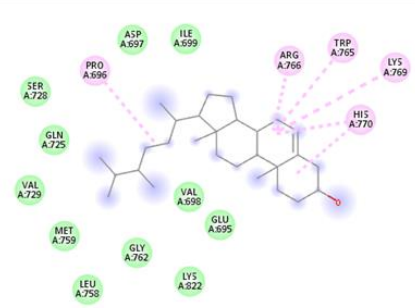
Homeobox 10A (HOX10A)	3K2A	-6.8	Alkyl and Pi-alkyl interaction with TRP A:294, LEU A:312, ILE A:290, and VAL A:286
Matrix metalloproteinases 2 (MMP2)	1RTG	-7.0	Alkyl interaction with PRO A:527
Matrix metalloproteinases 9 (MMP9)	1GKC	-8.0	Alkyl and Pi-alkyl interaction with LEU A: 187, HIS A: 411, PHE A: 110, TYR A: 179
Tissue inhibitor of Matrix metalloproteinases 1 (TIMP1)	1UEA	-8.2	Pi-sigma interaction with PHE A:83 and alkyl/Pi-alkyl interaction with TYR A:168, PHE A:86, ALA A:169, PHE A:210, TYR A:155, HIS A:166
Tissue inhibitor of Matrix metalloproteinases 3 (TIMP3)	3CKI	-7.9	Alkyl and Pi-alkyl interactions with PHE A:323 and LEU A:380
Leukemia inhibitory factor (LIF)	1EMR	-6.5	Alkyl and Pi-alkyl interactions with LEU A:57, PHE A:42, and LYS A:171
Leukemia inhibitory factor receptor (LIFR)	3E0G	-6.9	Pi-alkyl interaction with residues PHE A:97 and TRP A:195
Glycoprotein 130 (GP130)	1BQU	-5.5	Alkyl interaction with LYS A:189
Signal transducer and activator of transcription 3 (STAT3)	6NUQ	-7.3	Conventional hydrogen bonding with AMINO Acid ARG A:350 and alkyl interaction PRO A:336, ILE A:258, CYS A:251

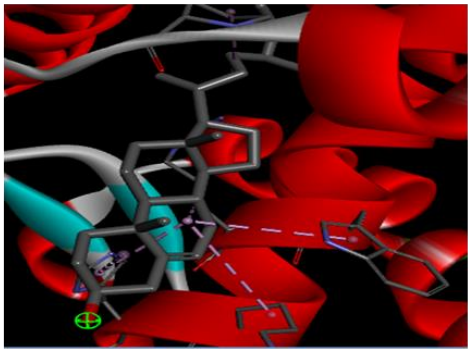
[A] Androgen receptor



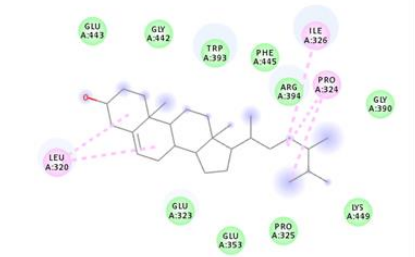


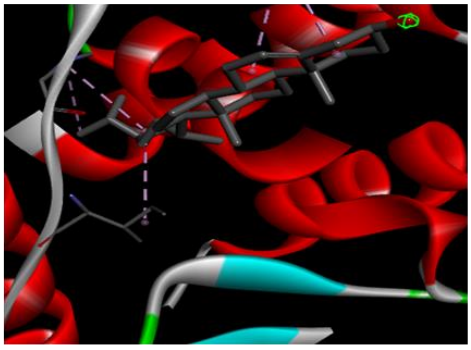
[B] Progesterone receptor





[C] Estrogen receptor α





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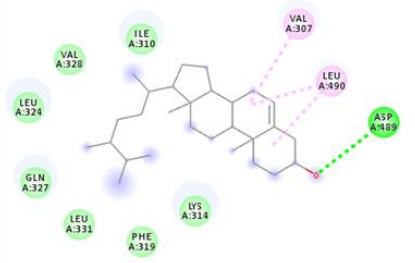
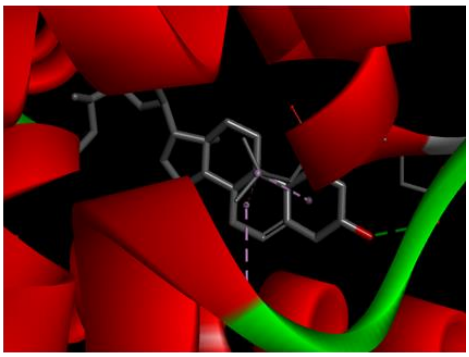
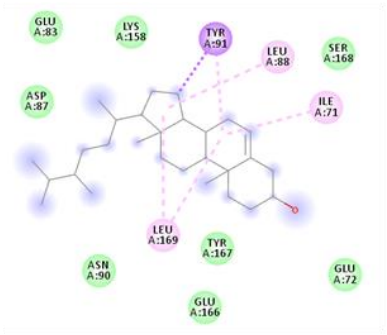
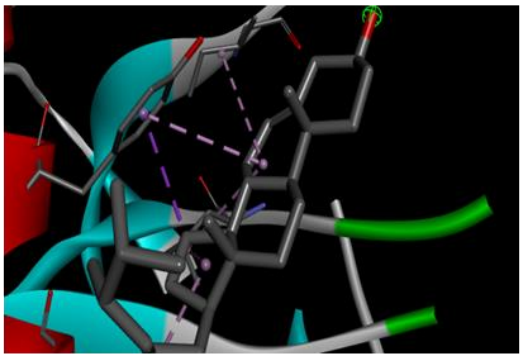


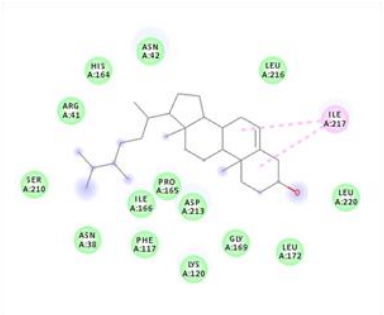


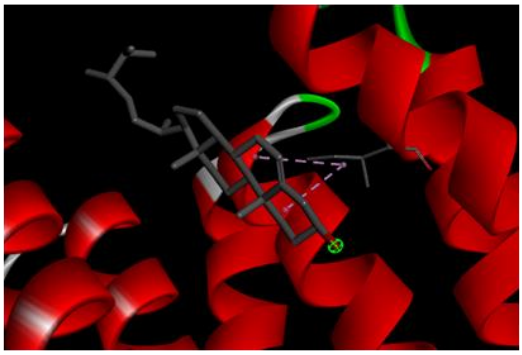
[E] Integrin β1



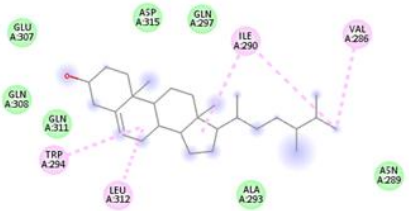


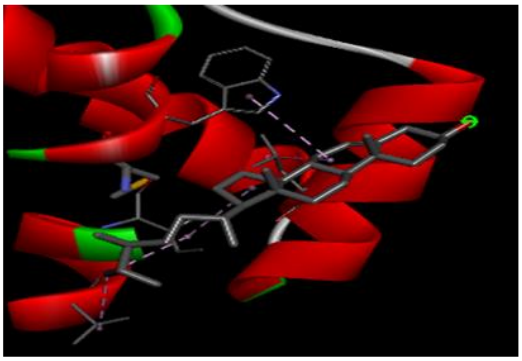
[F] Integrin α4



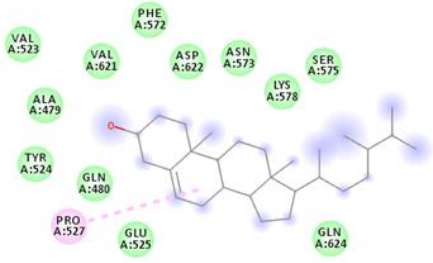


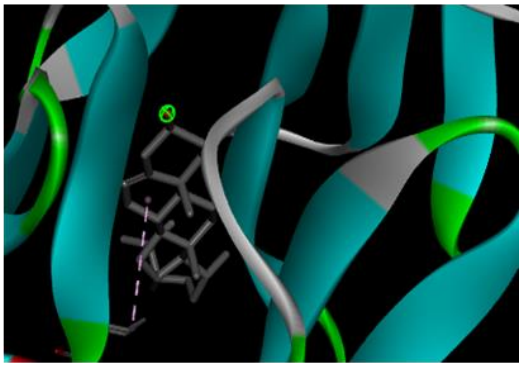
[G] Homeobox 10A



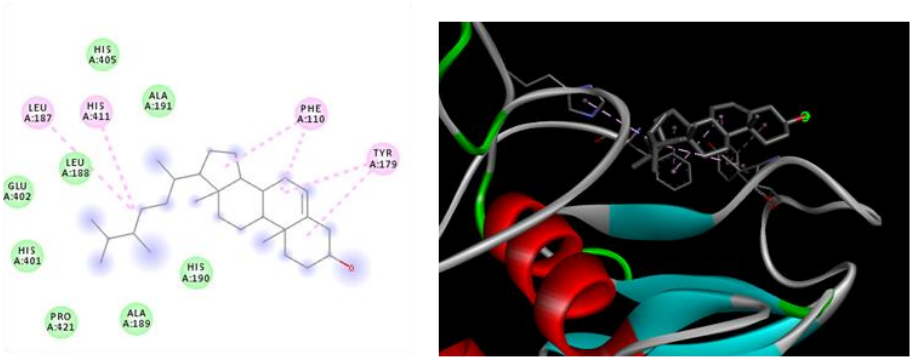


[H] Matrix metalloproteinases 2

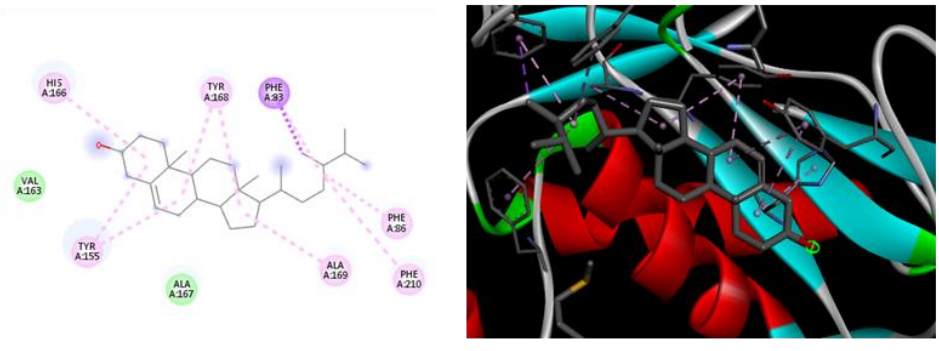




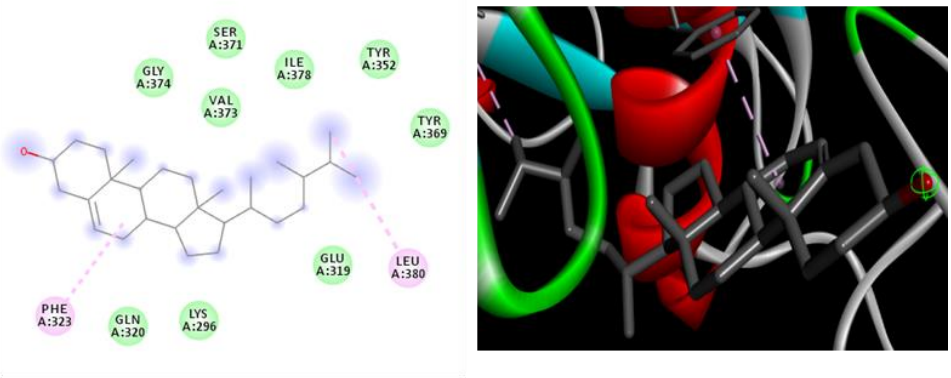
[I] Matrix metalloproteinases 9



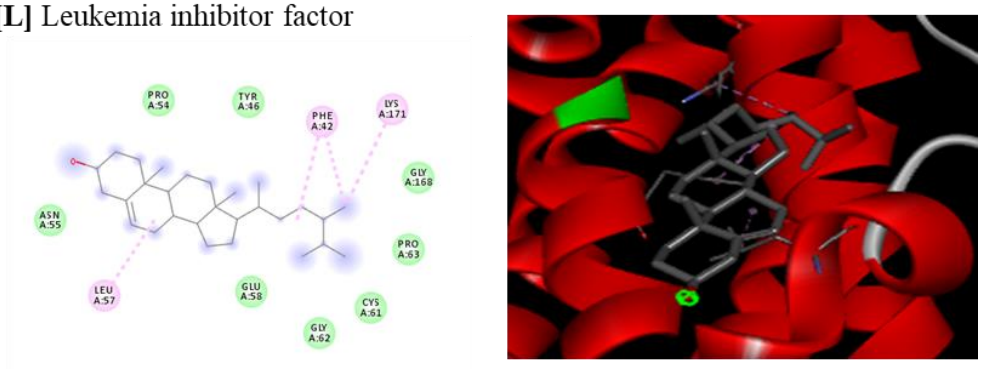
[J] Tissue inhibitor of matrix metalloproteinases 1



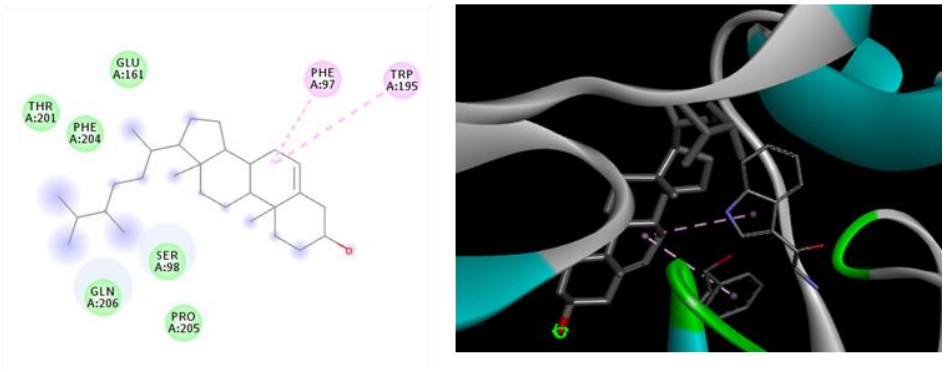
[K] Tissue inhibitor of matrix metalloproteinases 3



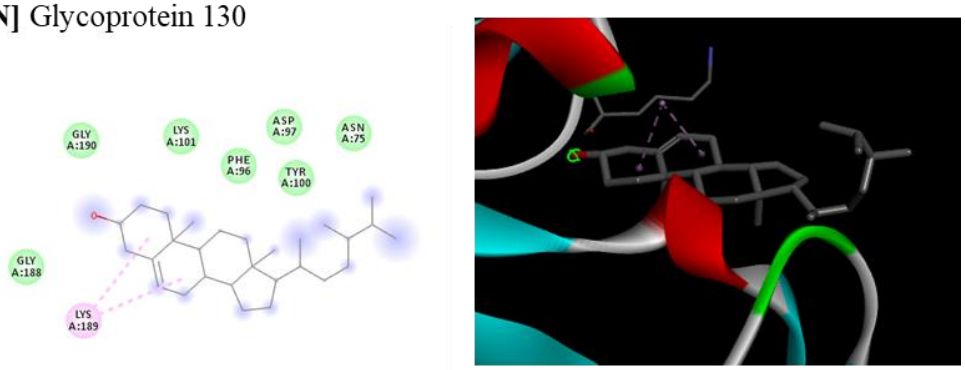
[L] Leukemia inhibitor factor



[M] Leukemia inhibitor factor receptor



[N] Glycoprotein 130



[O] Signal transducer and activator of transcription 3

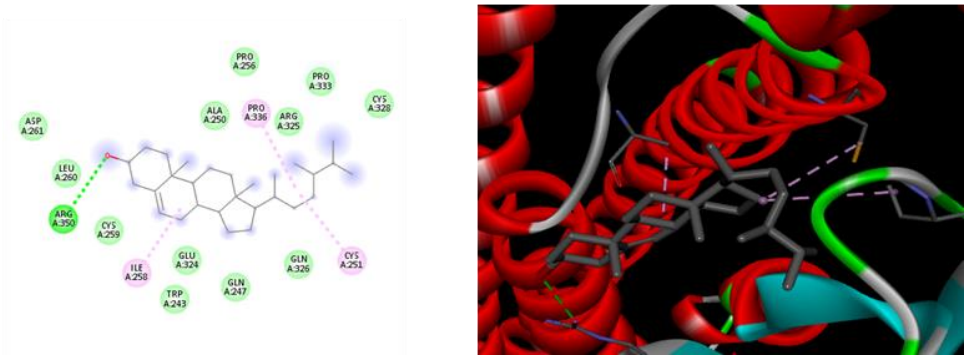


Figure 5.2. Representative image of the 2D and 3D molecular docking interactions of campesterol with key molecules of early pregnancy.

To sum up, it has been observed that amongst all the selected targets, progesterone receptor, integrin β1, homeobox 10A, matrix metalloproteinases 9, and tissue inhibitor of matrix metalloproteinases 1, signal transducer and activator of transcription 3 showed the maximum correlation with both the phytocompounds (n-HA and campesterol) in the terms of docking score. However, campesterol has been showing a higher binding affinity towards all the above-listed targets. From that, the progesterone receptor was showing maximum interaction.

The comparative docking results of non-polar phytocomponents (n-HA and campesterol) of *Aloe vera* gel docked with key targets of early pregnancy molecules are presented in Table 5.4.

Table 5.4. Comparative docking results of n-Hexadecanoic acid and Campesterol of *Aloe vera* gel with key molecules of early pregnancy.

Targets	**PDB ID**	**Docking Score with Campesterol**	**Docking Score with n-Hexadecanoic acid**
Androgen receptor (AR)	4OHA	-8.0	-3.7
Progesterone receptor (PR)	6F88	-9.5	-6.0
Estrogen receptor α (ERα)	6B0F	-8.8	-5.6
Estrogen receptor β (ERβ)	3OLS	-6.5	-3.5
Integrin β1 (ITGB1)	*4DX9*	-7.1	-5.0
Integrin α4 (ITGA4)	4HKC	-6.9	-3.8
Homeobox 10A (HOX10A)	3K2A	-6.8	-3.4
Matrix metalloproteinases 2 (MMP2)	1RTG	-7.0	-3.7
Matrix metalloproteinases 9 (MMP9)	1GKC	-8.0	-5.3
Tissue inhibitor of Matrix metalloproteinases 1 (TIMP1)	1UEA	-8.2	-5.6
Tissue inhibitor of Matrix metalloproteinases 3 (TIMP3)	3CKI	-7.9	-4.4

Leukemia inhibitory factor (LIF)	1EMR	-6.5	-5.1
Leukemia inhibitory factor receptor (LIFR)	3E0G	-6.9	-4.2
Glycoprotein 130 (GP130)	1BQU	-5.5	-3.2
Signal transducer and activator of transcription 3 (STAT3)	6NUQ	-7.3	-3.7

5.4 Discussion

Aloe barbadensis (*Aloe vera*-AVG) has been explored in our laboratory wherein reproductive and metabolic complications linked with PCOS have been studied using *in vivo* experiments (Maharjan et al., 2010; Radha et al., 2014; Desai et al., 2012). Furthermore, solvent-based extraction demonstrated that a non-polar extract of *Aloe vera* enhanced ovarian structure-function in PCOS animals (Radha & Laxmipriya, 2016b). Also, in the current study, *in vivo* experiments denoted that a non-polar (petroleum ether-PE) extract of *Aloe vera* could exert an agonistic effect on progesterone signalling during early gestation events and prevent serious complications in PCOS mothers (Chapter 4). This indicates that phytocompounds present in the petroleum ether extract of AVG can act on the uterus directly or indirectly to form a maternal-fetal interface for proper fetal development. Thereby, it would be interesting to understand the probable interaction of bio-actives present in the petroleum ether extract of AVG with the important targets of early pregnancy. The extract of *Aloe vera* has several compounds (Described in chapter 4), in this direction; our lab demonstrated, that the two bioactive, n-Hexadecanoic acid and campesterol from partially purified non-polar phytocomponents of *Aloe vera* have been shown to modulate reproductive and metabolic functions in the non-pregnant PCOS animals (Dey et al., 2022). Hence, the n-Hexadecanoic acid (n-HA) and campesterol have been chosen as ligands for the molecular docking study and were screened against a number of key molecules of early pregnancy in order to check their binding affinity at the active site of the protein.

During the initial phase of pregnancy, steroids and their receptor signalling cascade play a crucial role (Wang et al., 2013; Pawar et al., 2014; Kowalski et al., 2004). Our data shows that phytocomponents enriched fraction could modulate the steroid receptors in the embryo-uterine tissue of PCOS mice (Chapter 4). Moreover, it has been reported that the phytocomponents have heterogeneity of steroidal or non-steroidal activities in rodents, and *in vitro* experimental models indicating their physiological efficacy (Sunita & Pattanayak, 2011). In addition, phytocompounds are able to interact with steroid receptors and can function as agonists or antagonists depending on the presence or absence of natural ligands (Shanle & Xu, 2011). The results from the current chapter show that both campesterol and n-HA have the potency to interact with the androgen receptor (AR), progesterone receptor (PR), and estrogen receptor (ERα/β). Amongst all these, it is interesting to note that campesterol has the most significant binding for all steroidal receptors especially more effectively towards progesterone receptors. The campesterol bind to PR with alkyl, pi alkyl interactions between hydrophobic aromatic groups of the ligand along with several polar amino acids and thus having thermo stable interaction which can further effectively couple signal transduction downstream. In this line, no data have been reported with PR as a direct target for the campesterol. Although, *in silico* findings indicates that fatty acids namely octadecanoic acid (stearic acid) identified from the *Scoparia Dulcis* showed binding interaction with ERα/β, and these results are reflective of the phytoestrogenic property *in vivo* wherein the expression of proliferating cell nuclear antigen (PCNA) was increased in endometrium tissue (Wangsa et al., 2020). Also, the most potent compounds hexadecanoic acid, octadecanoic acid, demecolcine, and γ-sitosterol identified from *Cynoglossum zeylanicun* have a strong affinity toward estrogen receptors and showed tissue-specific estrogenic activity with endometrium proliferation in the ovariectomized mice (Gowala et al., 2020). These are several phytocomponents that have estrogenic activity as seen in the literature. Even though in our study, phytocompounds (n-HA and campesterol) are showing interaction with estrogen receptors, in the *in vivo* experiments, phytocompounds do not modulate the expression of estrogen receptors in the embryo-uterine tissue and the estrogen content at the systemic levels. Further, it can be noted that letrozole induced mouse model did not show a significant change in the estrogen concentration, this effect of letrozole is similar to the other reported data (Kauffman et al., 2015). But it could be noted that the strongest interaction of campesterol with the PR, has modulatory potential for progesterone and its downstream pathways (Chapter 4). Further, the activated progesterone signalling has an inhibitory effect on the AR in the embryo-uterine tissue of PCOS animals and campesterol

has shown an affinity with AR, this direct binding efficacy of the phytocompound suggests that the campesterol could also act as an antagonist to the AR. This observation is in line with the report suggesting that there are various steroid analogs that can bind and act as antagonists to AR (Gauthier et al., 2012). In this line, the previous study from our lab showed n-HA and campesterol from the *Aloe barbadensis* Mill. exhibited an antagonist effect on the androgen/AR in the letrozole-induced non-pregnant PCOS mice (Dey et al., 2022). However, in this study n-HA demonstrated only an anti-androgen effect. It is clear from this and earlier chapter data that phytosterol treatment alleviates progesterone content which could be due to the effect on ovarian StAR expression (Radha & Laxmipriya, 2016a). The increased progesterone could also upregulate PR and increase the efficacy of phytosterol in promotion of the fetal development. Thus, in the current study well-built molecular interaction of campesterol with PR and *in vivo* data gives evidence of progestin-like activity of campesterol during early pregnancy loss in PCOS phenotype.

Furthermore, the LIF-STAT pathway is known to regulate the growth and development of the implanting embryo during early pregnancy (Kimber, 2005). When performed the docking of the key regulators, Leukemia inhibitory factor, Leukemia inhibitory factor receptor, Signal transducer, and activator of transcription 3, and Glycoprotein 130 of the LIF-STAT pathway with the n-HA and campesterol, these phytocomponents were showing the docking with the LIF (IL-6 family cytokines). Hydrogen bonding and alkyl, pi interactions of LIF with n-HA and campesterol have been observed; mainly with several hydrophobic core amino acids having stronger hydrophobic interaction. However, the free energy indicates that campesterol is the better ligand as compared to n-HA. One of the studies showed that a Chinese drug kirenol binds with IL-6 with similar energy as campesterol (Wu et al., 2017), thus suggesting phytochemicals can bind to the interleukins. Next, the LIF receptor, LIFR also showed a better interaction with campesterol. Major interactions are pi and alkyl pi of phenylalanine and tryptophan, aromatic amino acids which are stable interactions (Gallivan & Dougherty, 1999), and the contribution of indole ring to the stability has been proposed. These kinds of interactions have not been reported with these phytocompounds. However, results from our *in vivo* experiments revealed that no significant changes were observed in the expression of LIF and LIFR in the embryonic-uterine tissue (Chapter 3). This could be because of the estrogenic regulation of these markers in the endometrium (Chen et al., 2000; Ni et al., 2002), and the PCOS animal model used in the current study has not shown a difference in the hormone estrogen levels. However, the direct interaction of campesterol with the LIF and

LIFR could further modulate their binding affinity towards the specific common co-receptor for the IL-6 family (Glycoprotein 130-gp130). In this context, the molecular docking study visualized that campesterol has a stronger binding capacity to the gp130. There are no reports documenting this type of ligands and targets relation, in spite of that, it has been reported that cholesterol can bind to lysosomal glycoprotein (Li & Pfeffer, 2016). In a similar manner, as campesterol mimics the cholesterol structurally, the interacting campesterol having 3β hydroxy moiety may form a stable connection with the gp130; however, this fact needs to be confirmed using molecular tools. Also, a report suggested that sterols could interact with the P-glycoprotein and reform its structure/function by taking off part of the drug-binding pocket or by interacting with the putative consensus cholesterol binding motif detected within the transmembrane domains (Clay et al., 2015). Moreover, the binding of the LIF to LIFR leads to the activation of STAT3, which further has an impact on the modulation of embryo-uterine functions during embryo implantation (Suman et al., 2013). This downstream target of LIF signalling exhibited a higher docking score with the campesterol. The major non-polar amino acids seem to play the role in the alkyl interaction of the coiled-coil domain. This domain is very important for STAT3 recruitment to the receptor leading to subsequent tyrosine phosphorylation and tyrosine phosphorylation-dependent activities (Zhang et al., 2000). The data obtained from *in vivo* study observed that in PCOS animals declined transcript levels of *Gp130* and *Stat3* were found to be increased upon PE extract of AVG treatment suggesting n-HA and campesterol have a direct effect on the LIF signalling activators. Moreover, uterine conditional ablation of STAT3 leads to hampered PR-mediated pathways and reduced PR protein expression *in utero*, suggesting that STAT3 has a pivotal role in PR-dependent pathways during implantation in mice (Lee et al., 2013). Thus, it is noted that the strongest binding affinity of campesterol with STAT3 could lead to activating PR-mediated pathways in the embryo-implanted region of the uterus.

Moreover, progesterone has been known to regulate the cell adhesion molecules during the embryo-uterine attachment phase of pregnancy (Merviel et al., 2001; Chen et al., 2016). The extracellular matrix (ECM) ligands play critical roles in cell-cell adhesion that occur during implantation (Singh & Aplin, 2009b). ECM and its receptors modify numerous key physiological activities in cells, counting embryogenesis and fetal development (Lessey, 1998). Integrins transmit signals bidirectionally across the cell membrane (Abram & Lowell, 2009; Lu et al., 2016) and are categorized on the basis of the coupling of α and β subunits (Tolomelli et al., 2017). The α4 subunit can combine with β1 subunits for further signal

transduction. It has been reported that out of 39 mutations inside the ligand binding of α4 (Residues 108-268) (Kamata et al., 1995), Tyrl87, Trp188, and Glyl90 are crucial for ligand interactions of α4β1. These data strongly suggest that these residues are important for ligand binding. Also, the site-directed mutagenesis technique demonstrated that the residues namely D130, S132, N224, D226, E229, D233, D267, and D295 had been shown to be important for ligand binding for β1 integrin (You et al., 2002). When the binding interaction of n-HA and campesterol with the integrin α4β1 was examined in the current study, the campesterol was found to be more docked with both integrin α4 and β1, especially with β1. The residues LEU169, TRY91, LEU88, and ILE71 are trying to establish an adhesion to the integrin. These amino acids being hydrophobic can potentially interact with the hydrophobic core of campesterol, thus stabilizing the ligand and receptor more effectively than HA. This fact needs to be more explored in the future for understanding the potential of this phytocompound. However, the expression of the integrin (α4β1) was not changed upon the treatment with the petroleum ether extract of *Aloe vera* in the PCOS animals. On the contrary, the whole *Aloe vera* gel extract-treated group of animals showed changes in the integrin expression in the implanted region of the uterus. Thus, it can be noted that the whole *Aloe vera* gel extract exists as a cocktail of another group of phytocompounds which may activate the signal transduction or recruit the other molecules that are required for signalling.

Apart from the above-mediated events, blastocyst attachment with the uterine epithelium is followed by the decidualization of the stromal cells. The transcription factors within the endometrium that can play a role in decidualization have been recognized wherein Homeobox protein10A (HOX10A-transcription factor) deficient mice exhibit compromised decidualization of the endometrium during implantations (Benson et al., 1996). The function of HOX proteins is based on their capacity to control gene expression as DNA-binding proteins (Pearson et al., 2005). Domain-swapping experiments have demonstrated that the homeobox binding domain plays a leading role in dictating the functional accuracy of the protein (Chan & Mann, 1993). Moreover, it has been reported that the homeobox binding domain of Zeb1(transcription factor) can interact with the phytocompound, kaempferol with a binding energy of -5.34 k/cal/mol and includes a conventional hydrogen bond involving Asn13 (Amin et al., 2017). Thus, it can be said that the phytocompounds can interact with the transcriptional factor, even so, there is no clear evidence for the n-HA and campesterol. The results of the current molecular docking study indicate that campesterol has been showing the best affinity with the HOX10a. The amino acid involves in the interactions are TRP294,

LEU312, ILE290, and VAL286 with a docking score of -6.8. Moreover, as HOX10a is the downstream target of the progesterone in the uterus (Lim et al., 1999), the declined gene expression of *Hox10a* in the PCOS animals could correspond to the changes in progesterone signals in the letrozole-induced animals. Treatment of PCOS animals with metformin and PE extract of AVG exhibit upregulating *Hox10a* expression in the implanted site of the uterus. It is interesting to note that phytocomponents of *Aloe vera* could directly interact with HOX10 or the enhanced progesterone signalling could lead to activation of the HOX-mediated signalling in the PCOS animals.

During the blastocyst invasion and decidualization, the homeostasis of the Matrix metalloproteinases (*Mmp9)* and its tissue inhibitors of MMPs (*Timps*) is considered to be crucial for embryo implantation and pregnancy (Curry & Osteen, 2001). It plays an integral role in tissue remodelling which is regulated by the hormones- progesterone and estrogen (Zhang et al., 2000). The n-HA and campesterol have been shown to interact with the Homopexin motif of the proteases and from both the proteases (MMP-2 & 9), the MMP-9 was found to be strongly attached to the campesterol. The collagenase-like 1 region and proline residue have a pyrrolidine side chain that could make contact with the campesterol ligand. It is proposed that proline-containing sequences do not bind tightly but it is important for the modulation of protein as interaction is hydrophobic (Kay et al., 2018). Further, n-HA binds to the same motif with different amino acids through pi sigma and pi alkyl interactions. However, campesterol has a better binding affinity, indicating that proline amino acid interaction could be stronger. Apart from this, a study by Wang et al., 2021 suggested that Chinese extract - *Guizhi Fuling Wan* containing campesterol, and sitosterol could alter targets involving angiogenesis, and tissue remodelling in endometriosis suggesting, these phytocomponents could act as a uterine modulator. In a similar line, a recent study showed that the sitosterol present in *Sedum Sarmentosum* can bind to MMP-2 and affect atherosclerosis (Liu et al., 2022), thus implying sterols potentially bind to MMP-2. In this regard, in our current study, the PE extract-treated group has shown the potent activation of MMP-9 over MMP-2 in the implanted region of the uterus (Chapter 4). Thus, the binding of campesterol in the collagenase I domain is more efficacious, though the role of binding in the Homopexin motif can't be neglected. Moreover, endogenous inhibitors of proteases, TIMPs bind MMPs in a 1:1 stoichiometric fashion, therefore a variation in MMP: TIMP ratio can lead to tissue degradation (Vincent et al., 2015). In the current study, MMPs and TIMPs have been altered in PCOS animals. However, the phytocomponents containing extract treatment

demonstrated the upregulation of the MMP9, and expression of the TIMP1 & 3 did not change. Thus, the interaction of the TIMPs with the phytocompounds could not be enabled to switch the expression transcriptionally, as the TIMP1 and 3 are extensively glycosylated, activity-based assays need to be done in the future.

To summarize, it has been observed that n-HA and campesterol were showing molecular interactions with the key molecular players of early pregnancy and that could act as agonists or antagonists to prevent early pregnancy loss in the PCOS phenotype. In addition, the molecular docking relationship between the conventional drug metformin, and the above-mentioned targets has been examined. The studies have indicated a large number of indirect or direct targets of metformin including mTOR (Dowling et al., 2007), AMP-activated kinase (Li et al., 2011), protein-threonine kinase, LKB1 (Shaw et al., 2005) and, mitochondrial complex-I (Owen et al., 2000; Bridges et al., 2014), hexokinase-II (Salani et al., 2013), and glycerophosphate dehydrogenase (Madiraju et al., 2014) respectively. All these studies have recognized the mode of action of metformin. In the present study, the metformin was found to be docked with the selected targets with a low affinity compared to the n-HA and campesterol (Results not shown). Although, the absence of structural data for the metformin-target complex, it would not be the definitive index to prove the direct potency. This draws attention to the phytocompounds (n-HA and campesterol), as a better candidate for endometrial defective PCOS patients.

5.5 Conclusion

The aim of the current study was to examine the ligand-protein molecular docking to anticipate the binding affinity of the n-HA and campesterol from the PE extract of *Aloe barbadensis* with the crucial proteins involved in the early pregnancy sequence. 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. 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. Also, this chapter has laid the foundation for identifying the best target for phytosterol that mimics, progesterone in early pregnancy molecular events. ***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.