2 Materials and Methods

Chemicals/Reagents

Letrozole tablets (Concentation-2.5mg/tablet), marketed under the brand name letronat were procured from Natco Pharma Ltd. The blood glucose levels were measured by OneTouch Select Plus (Johnson & Johnson Ltd.). Ethanol was procured from HiMedia Laboratories Pvt. Ltd. All other reagents of analytical grade were purchased from Sisco Research Laboratories Pvt. Ltd., India. Hormones- testosterone, estradiol, progesterone, and insulin were assayed using ELISA kits (DBC Canada). RNAiso Plus was procured from Takara Inc. High-Capacity cDNA Reverse Transcription Kit was procured from Applied Biosystems. SYBR Green (Power SYBR Green PCR Master Mix Life Technologies, USA). ECL reagent (Immobilon Western Chemiluminescent HRP substrate, Millipore, USA). Primers used in the study were designed by the primer blast tool of NCBI and synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Details of the primer sequences are given in Table 2.1 The list of the antibodies used in the current study is provided in Table 2.2.

This section has been divided mainly into four parts:

2.1 *In-vivo* analysis of pregnancy complications associated with PCOS.

2.2 *In-vivo* analysis of the PE extract of AVG, when given as a pre-conceptive agent was studied in the PCOS mouse model.

2.2. Phytochemical analysis of Petroleum ether (PE) extract of *Aloe vera* gel (AVG).

2.4. *In-silico* analysis of the non-polar phytocomonents of *Aloe vera* gel with the early pregnancy marker.

2.1 *In-vivo* analysis of pregnancy complications associated with PCOS

2.1.1 Animal housing and maintenance

Adult virgin female Balb/c mice (Approximately, 3 months old) weighing 20-25g were chosen for the study which was housed in a standard controlled animal care facility, in cages (four mice/cage), and maintained in a temperature-controlled room (22-25°C, 40-50%

humidity) on a 12: 12-hour dark-light cycle. The animals were maintained under standard nutritional and environmental conditions throughout the experiment. Animals were allowed to acclimatize for one week before starting the experiments. All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature (22-25°C). Experimental protocols used in the study were approved by the Institutional Animal Ethical Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara. Also, experiments were performed in compliance with the ethical standards of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

2.1.2 Letrozole induced PCOS mouse model

PCOS mouse model was developed using Letrozole, a non-steroidal aromatase inhibitor (Kafali et al., 2004). The procedure was as follows:

Reagents:

I. 1% Carboxy methyl cellulose (CMC): 1 gm of CMC in 100 ml of distilled water was boiled in a water bath with continuous stirring until the CMC powder was completely dissolved. The solution was cooled at room temperature and stored at 15°C till further use. This was used as the vehicle.

II. Letrozole dose preparation: Letronat™ tablets containing 2.5 mg letrozole were purchased from Natco Pharma Ltd., Hyderabad, India. The tablets were crushed using a mortar pestle and the powder was weighed and mixed with 0.5 ml of 1% CMC and administered orally to animals at a final dose of 0.5 mg letrozole/kg body weight.

2.1.3 Procedure/Drug administration and experimental design

Thirty adult virgin (2-3 months) female Balb/c mice weighing 20-25g were chosen for the study. Animal maintenance and housing conditions are discussed in the above section. Experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara, Gujrat (Ethical Approval Number - MSU/BIOCHEMISTRY/IAEC/2019/4). Firstly, the animals were categorized into two groups:

Group I (Control group n=15) received 1% Carboxymethyl cellulose (CMC) orally daily for 21 days and served as untreated Control.

Group II (PCOS group n=15) received letrozole (0.5 mg/kg body weight) daily for 21 days and served as PCOS.

After 21 days of the treatment, the induction of PCOS was validated according to the Rotterdam criteria (2003) wherein the body weight of animals, Oral glucose tolerance test, estrus cycle profile, hormone analysis (Testosterone), and ovarian histology were analyzed.

2.1.3.1 Estrus cycle

Estrus cyclicity was monitored every morning during the whole period of treatment by examining vaginal lavage. Cells on vaginal walls were collected by washing the vagina with normal saline (0.9% NaCl) and smeared on glass slides. The slides were examined under the microscope for the relative abundance of different types of cells and the following stage has been identified:

Proestrus stage: The presence of round-shaped nucleated epithelial cells and some cornified epithelial cells indicated the proestrus stage.

Estrus stage: Estrus smear predominates with anucleated cornified cells.

Metestrus stage: This stage is comprised of an equal proportion of leukocytes and cornified squamous epithelial cells.

Diestrus: The stage comprised predominantly of leukocytes.

2.1.3.2 Oral Glucose Tolerance Test (OGTT)

An oral glucose tolerance test (OGTT) was performed according to the protocol provided by Buchanan et al., 1991. The mice were fasted overnight (16 h) before the morning of the OGTT. The mice were orally fed glucose (2 g/kg as a 20% glucose stock solution). Glucose levels were measured by tail vein blood sampling using a blood glucose meter (OneTouch Select Plus (Johnson & Johnson Ltd., India) at 0 (before glucose load), 30, 60, 90, and 120 min after glucose administration. OGTT profile was analyzed by plotting the graph of glucose concentration in mg/dl vs. time in minutes.

Unit: mg/dl.

Normal Values: Laboratory mouse overnight fasting blood glucose levels: 65-90 mg/dl and rat-85-100 mg/dl respectively (Sun et al., 2016; Wang et al., 2010).

2.1.3.3 Hormone Estimation

Serum from blood was used as a sample to estimate the testosterone, estradiol, progesterone, and insulin levels using ELISA kits (Diagnostics Biochem Canada (DBC)- for testosterone, estradiol, progesterone measurement and DiaMetra for insulin). All measurements were taken according to the manufacturer's instructions. Each sample was assayed in duplicate. The sensitivity of the kits was 0.022 ng/mL, 10 pg/mL, and 0.1 ng/mL for testosterone, estradiol, and progesterone kits respectively. The working range was 0.08 to 16.7 ng/mL, 20 to 3200 pg/mL, and 0.3 to 60 ng/mL of testosterone, estradiol, and progesterone respectively. The intra-assay coefficient of variation (CV) was 6.6 % to 9.6 %, 4.6 % to 9.3 %, and 10.2 % to 10.6 % for testosterone, estradiol, and progesterone kits respectively. The inter-assay coefficient of variation (CV) was 6.1 % to 7.3 %, 6.2 % to 10.1 %, and 10.2 % to 12.6 %, for testosterone, estradiol, and progesterone kits respectively. The recovery range was between 80.5 % to 110.1 %, 90.3 % to 116.2 %, and 78 % to 124 % for testosterone, estradiol, and progesterone kits respectively.

Sample preparation

Blood was collected and serum was separated by centrifuging tubes at 3000 g for 10-15 minutes. The supernatant was collected in fresh tubes and utilized immediately for hormone estimation or stored at -80°C until use. ELISA was performed according to the manufacturer's protocol.

2.1.3.4 Histological examination of the ovaries

Reagents:

10% neutral buffered formalin (NBF):

Formalin: 10 %

$NaH_2PO_4.H2O$: 0.4 %

Na_2HPO_4: 0.65 %

The ovaries from both groups were collected and fixed in NBF. For histological examination, 5 μm thick sections were cut on a microtome and stained with Hematoxylin-Eosin. Histo-anatomical changes in ovaries were screened for and micrographs were taken on a Leica DM2500 microscope through a Leica EZ camera under 4X magnification.

After the validation of the above PCOS parameters, the experiments of the current study were divided into two parts:

I. The pregnancy-related alterations/complications associated with PCOS were investigated in the letrozole-induced PCOS mouse model. The procedure for drug administration is provided in the section 2.1.3 and the methodology for the establishment of pregnancy is explained in the following section 2.2.3.

II. The therapeutic potential of PE extract of AVG when given as a pre-conceptive agent was studied in the letrozole-induced PCOS mouse model. The detailed experimental design is explained below.

2.2 The efficacy of the PE extract of AVG, when given as a pre-conceptive agent in the PCOS mouse model

2.2.1 Animals

Ninety-eight adult virgin (3-4 months) Balb/c female mice weighing 20-25g were chosen for the study. The maintenance of the experimental animals has been carried out as explained in the section 2.1.1. Experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara (Ethical Approval Number (MSU/BIOCHEMISTRY/IAEC/2019/1&4).

2.2.2 Drug administration and experimental design

Firstly, the animals were categorized into two major groups- Control (n=14) and PCOS (n=84). The control animals received 1% carboxymethyl cellulose (CMC) orally every day for 21 days. While the PCOS group was given an oral dose of letrozole (0.5mg/kg body weight) daily for 21 days. After validation of the PCOS phenotype, these animals were distributed into 7 groups of 14 animals/groups as follows:

The procedure for the preparation of the PE extract of *Aloe vera* gel is provided in the section 2.3.2.

Group, I (Control group) received 1% CMC orally daily for 21 days and served as untreated control.

Group II (PCOS group) received letrozole (0.5 mg/kg body weight) daily for 21 days and served as untreated PCOS (Kafali et al., 2004).

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

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

Group V (PCOS + AVG group) PCOS animals were treated with oral doses of *Aloe vera* gel (10 mg/ day) for 30 days (Maharjan et al., 2010b).

Group VI (PCOS + Metformin group) PCOS animals were treated with an oral dose of Metformin (100 mg/kg/day) for 30 days and served as a positive control (Zaafar et al., 2014; Dey et al.,2022).

Group VII (PCOS + DMSO group) PCOS animals were treated with an oral dose of DMSO (0.1%, 0.3ml/day) for 30 days and served as vehicle/negative control.

Dose selection criteria for PE extract of AVG (PE-25 and PE-75): It is known that the efficacy of phytocomponents is concentration-dependent (Dey & Nampoothiri, 2019). Hence, on the basis of previous lab studies, the two different concentrations (25 & 75µg/kg/day of dose have been selected for the current study (Radha & Laxmipriya, 2016a; Dey et al.,2022).

After the different treatment regimens, next, to study the pregnancy complications linked with PCOS and the efficacy of the PE extract of AVG as a pre-conceptive agent, pregnancy was established in all the groups.

2.2.3 Establishment of pregnancy

All Balb/c female mice from each of the groups were allowed to mate with age-match male mice of the same strain (Female: male-2:1). The following morning, females were checked for the presence of a vaginal plug. The presence of the vaginal plug was considered as day 1 of pregnancy and the following studies have been carried out:

2.2.3.1 Early gestation studies

For the early gestation study, the body weight of the animals was monitored (Days 1st to 6th of pregnancy). The animals were sacrificed on the morning of day 6 (Adhesion of embryo to uterus). Before sacrificing animals, blood samples were collected by cardiac puncture. The serum was separated and kept in a freezer at – 80 °C for determining serum hormone levels

(Testosterone, progesterone, estradiol, and insulin). Further, at the end of the experiment, animals were sacrificed, and the embryo-containing region of the uterus was excised, trimmed, and appropriate parts were separated. One part is stored in RNAiso Plus reagent at -20 °C for the gene expression studies, one part is stored at -80 °C for the protein expression studies and, the remaining implanted site and ovary from both the groups were dissected and stored in 10% buffered formalin, for histopathological investigations (Procedure followed for the histopathology is explained in section 2.1.3.4) on the day-6th of pregnancy.

2.2.3.2 Late gestation studies

For late gestation analysis, the body weight of the animals was monitored (Days 1st to 18th of pregnancy). The animals were sacrificed on the day 18th of pregnancy. Moreover, the morphological analysis of developing fetuses, number, and weight of fetuses was examined.

2.2.4 Analysis of fertility parameters

The effect of letrozole and PE extract of AVG was analysed on the reproductive performance of animals wherein the number of mated females, and pregnant/non-pregnant females were examined. Also, the time required for conception (in days), mating and fertility index was calculated for each animal.

$$\text{Mating index (\%)} = \frac{\text{Mated females}}{\text{Total females kept for mating}} \times 100$$

$$\text{Fertility index (\%)} = \frac{\text{Pregnant females}}{\text{Total females kept for mating}} \times 100$$

2.2.5 Gene expression analysis

The details of the methodology used for transcriptional studies have been provided below.

2.2.5.1 RNA Isolation

1. Diethyl pyrocarbonate (DEPC) water: 1 ml of DEPC was added to 1 litre of distilled water and kept overnight on a magnetic stirrer for a completely homogeneous solution. The solution was autoclaved and used for the preparation of all the regents in RNA isolation.
2. Tris Borate EDTA Buffer (TBE) (10X; 1L)

   Tris base 108 g

Boric acid 55 g

0.5 M EDTA 40 ml

Volume made to 1 litre with DEPC water

3. RNA loading dye (5X; 1 ml)

   500 mM EDTA 8 µl

   40% Formaldehyde 72 µl

   Glycerol 200 µl

   Formamide 30 µl

   2.5% Bromophenol blue 30 µl

   10X TBE 400 µl

   Volume made to 1 ml with DEPC water

4. Other reagents used

   TRIzol reagent (Phenol + GITC)

   Chloroform

   2-Propanol

   75% Ethanol prepared in DEPC water

   1% Agarose in TBE buffer

   25 mM Ethidium bromide

All glassware and disposables were sterile and all surfaces involved were cleaned prior to the experiment. The isolation was carried out using a clean pair of gloves.

2.2.5.2 RNA isolation protocol

For RNA isolation, tissues (ovary and embryo implanted region of the uterus 10-100 mg) were homogenized in 1 ml of TRIzol reagent. This was followed by the addition of 200µl Chloroform. Tubes were allowed to stand on ice for 15 minutes with occasional gentle shaking. The mixture was then centrifuged at 12,000 g for 20 minutes at 4°C. The organic and aqueous phases are separated with visible white interphase. The upper layer was carefully taken into a new microcentrifuge tube (strictly without contamination from the lower layers) containing 500 µl isopropanol. This was gently mixed and refrigerated for at least 1 hr or overnight (maximum) to allow precipitation. The tube was then centrifuged at 12,000 g for 15 min. at 4°C. The supernatant was discarded and the pellet, which contains RNA, was washed with 1 ml of 75% ethanol and centrifuged again. After a total of three such washes, the tube was opened and left on ice for the pellet to dry (over-drying was avoided since it can lead to

trouble with the dissolution of the RNA in water). After excess ethanol had dried off, the pellet obtained from tissues was dissolved in 30 µl of DEPC water.

2.2.5.3 Quantification of RNA

1 µl RNA solution obtained from the above isolation step was diluted in 1 ml DEPC water and read at 260 nm and 280 nm on a UV-Vis spectrophotometer. The following formula was used to analyse the quantity of RNA:

$$RNA\ (\mu g/\mu l) = 40 \times OD\ 260$$

A ratio of OD 260 to OD 280 was used to check for the quality of the RNA preparation. A ratio >1.8 was accepted as indicative of the good purity of the RNA solution. Further, 2 µg of the RNA was electrophoresed on a 1% Agarose gel containing, Ethidium bromide. Three distinct and sharp bands reflected the good integrity of the RNA.

2.2.6 cDNA synthesis

Isolated RNA was reverse transcribed using the Verso cDNA synthesis kit (Thermo Scientific™, USA). The cDNA reaction was carried out for both tissues (ovary and embryo implanted region of the uterus) in a total volume of 20 µl as follows:

Component	**Volume**
RNA (1µg/µl)	1 µl
Oligo dT primers	1 µl
dNTP mix	2 µl
DEPC water	6 µl
Samples were heated at 65°C for 10 minutes to remove any 2° structure of RNA	
Kept on ice for 2 minutes and the following components were added	
5X RT Buffer	4 µl
Reverse Transcriptase (RT)	1 µl
RT enhancer	1 µl
DEPC water	4 µl
Samples were heated at 42°C for 30 minutes The reaction was terminated by heating the samples at 95°C for 2 minutes	

The cDNA synthesized was stored at -20°C till further use.

2.2.7 Real-time PCR

For the current study, all quantitative gene expression analyses were carried out by real-time PCR using Power Up™ SYBR™ Green master-mix (Applied Biosystems, Thermo Fisher Scientific™) on a Quant-Studio 5.0 Real-Time PCR system (Life Technologies, USA). Primers were procured from IDT (CA, USA). List of the primer used in the current study is provided in the Table 2.1.

2.2.7.1 Real-time PCR protocol

For each reaction mix, a mix with the following composition was prepared:

Component	**Volume**
2X SYBR Green master-mix	5 µl
Forward Primer (5µM)	0.5 µl
Reverse Primer (5µM)	0.5 µl
cDNA Template	1 µl
Milli-Q water	4 µl

For every sample, each gene was assayed in triplicate. The reactions were run in 96-well plates (ABI) sealed with clear sealing films. The following program was used for amplification:

Temperature	**Time**	
50°C	2 min	
95°C	2 min	
95°C	15 sec	
60°C	1 min	40 cycles

This was followed by a melt-curve analysis with the following program:

Temperature	**Time**
95°C	15 sec
60°C	1 min
95°C	1 sec

2.2.7.2 Data analysis of Real-time PCR

Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of the internal control gene or reference gene (β-Actin) from those of the target gene. The mean of these normalized Cq values was plotted.

$$\Delta Cq = Cq\ of\ target\ gene - Cq\ of\ reference\ gene$$

Moreover, fold change in expression was calculated by the ΔΔCq method of Livak and Schmittgen (2001). For fold change of gene expression in sample 2 as compared to that in sample 1:

$$\Delta\Delta Cq = \Delta Cq\ of\ gene\ in\ sample\ 2 - \Delta Cq\ of\ gene\ in\ sample\ 1$$

ΔΔCq values were plotted on a graph with a vertical axis following a logarithmic scale.

2.2.8 Protein Estimation

Principle: The method of Lowry et al. (1951) was followed. Tyrosine & Tryptophan present in tissue homogenates react with Folin Ciocalteau reagent (FC reagent) to give blue coloured complex. The colour is formed due to the reaction of alkaline copper with protein and the reduction of phospho-molybdate by the protein. The intensity of colour depends on the amount of aromatic acids present, whose absorbance is measured at 660 nm.

Reagents:

I. Lowry's Reagent:
   Reagent A: 2 % Sodium Carbonate 0.1N NaOH
   Reagent B: 1 % Copper Sulfate
   Reagent C: 2 % Sodium Potassium Tartarate

To make 100 ml of Lowry's reagent (Reagent-D), add 98 ml of reagent A, 1 ml of reagent B & 1 ml of reagent C (freshly prepared).

II Folin Ciocalteau reagent (FC reagent) (Freshly prepared): Mix equal volumes of FC reagent and distilled water (1:1 ratio).

III BSA Standard concentration: 1mg/ml.

Working range: 5 to 50 µg

Procedure:

Reagent	Blank	Test
Reagent-D	1.0 ml	1.0 ml
Distilled Water	0.1 ml	0.09 ml
Sample	-	0.01 ml
Keep at Room Temperature for 10 minutes		
FC Reagent	0.1 ml	0.1 ml
Mix well & Keep at Room Temperature for 20 minutes (In the Dark)		
Read absorbance at 660 nm		

Calculation: Protein was quantified using a standard graph. **Unit:** mg/ml

2.2.9 Gelatin zymography

Reagents:

1. **Phosphate-buffered saline (10X)**

NaCl	1.37 M
KCL	27 mM
Na_2HPO_4	100 mM
KH_2PO_4	18 mM
Tris - HCl	1 M
pH set to 7.4	

Tissue homogenates (10% for embryo-uterine tissue) were prepared in PBS, followed by centrifugation at 10000g for 20 minutes at 4°C. Protein was estimated from the supernatant by Lowry's method (Methodology has been described above in the section).

1. **Sample Buffer/Loading Buffer (2X)**

Tris - HCl	1 M
SDS	20%
Glycerol	10%
Bromophenol blue	0.1%
pH set to 6.8	

2. **Running Buffer (1X)**

Tris base	25 mM
Glycine	250 mM

SDS	0.2%

pH set to 8.3

3. **Gel stock (30%)**

Acrylamide	29%
Bis-acrylamide	1%

The solution was kept in dark overnight at room temperature followed by filtration through Whatman paper no. 1 before use.

4. **Resolving gel buffer (7.5 %; 10 ml)**

30% Gel stock	2.5 ml
Distilled water	3.7 ml
1.5 M Tris-Cl (pH 8.8)	2.5 ml
1 % Gelatin	1 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.010 ml

APS and gelatin solution must be prepared fresh. APS and TEMED must be added just before pouring the resolving gel buffer into the PAGE assembly.

5. **Stacking gel buffer (5.5%; 5 ml)**

30% Gel stock	0.850 ml
Distilled water	3.4 ml
1 M Tris-Cl (pH 6.8)	0.625 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

APS solution must be prepared fresh. APS and TEMED must be added just before pouring the resolving gel buffer into the PAGE assembly.

6. **Calcium assay buffer (CAB incubation buffer)**

Tris base	50 mM
NaCl	0.2 M
$CaCl_2$	0.5 mM
NaN_3	0.02%
Brij-35	0.02%

pH set to 8.3

7. **Triton-X washing buffer**

Triton-X	2.5 %

The solution must be prepared fresh.

8. **Coomassie stain**

CBB-G250	0.1%
Methanol	40%
Acetic acid	10%

9. **Destaining solution**

Methanol	30%
Acetic acid	5%

2.2.9.1 Protocol for Gelatin Zymography

Gel casting: Resolving gel buffer was poured between PAGE plates in the casting assembly, immediately followed by butanol. The gel was left undisturbed and allowed to fully polymerise for half an hour. The butanol was removed and the gel was washed thoroughly with distilled water and stacking gel buffer was poured on top of it. The comb was inserted immediately.

Sample preparation: Sample buffer (Loading buffer) at final working concentration, protein sample as per the required loading amount, and water were added to prepare the final sample. The sample must be prepared freshly, and placed on ice till the sample was loaded onto the gel.

Washing of gel: Gel was washed with the 2.5 % Triton-X on the gel rocker for 2 hrs. Next, discard the Triton-X and washed the gel with CAB buffer for 30 min.

Incubation of gel: After washing of gel with CAB, the gel was kept in the water bath at 37^{o} C with newly added CAB buffer.

CBB staining/destaining: Gel was stained in CBB staining solution for 1 to 2 hrs. This was followed by destaining for 2 to 4 hours or until a clear band was observed.

2.2.10 SDS-PAGE and Western blot

Reagents:

1. **RIPA Buffer**

Tris base	50 mM

NaCl	150 mM
Triton X-100	1%
SDS	0.1%
Sodium deoxycholate	0.5%
pH set to 8.	

Protease inhibitor (Sigma, USA) was added freshly before use as per the manufacturer's instructions.

Tissue homogenates (10% for ovary and embryo-uterine tissue) were prepared in RIPA buffer containing protease inhibitors. The samples were kept in cold condition for 1 to 2 hours to ensure complete lysis, followed by centrifugation at 10000g for 20 minutes at 4°C. Protein was estimated from the supernatant by Lowry's method, which was assayed as per the method described in the section.

2.2.10.1 SDS-PAGE

1. **Sample Buffer/Loading Buffer (5X)**

Tris base	250 mM
SDS	10%
Glycerol	50%
Bromophenol blue	0.1%
pH set to 6.5.	

β-Mercaptoethanol was added freshly during sample preparation at a final concentration of 100 mM.

2. **SDS-PAGE running Buffer (1X)**

Tris base	25 mM
Glycine	250 mM
SDS	0.2%

3. **Gel stock (30%)**

Acrylamide	29%
Bis-acrylamide	1%

The solution was kept in dark overnight at room temperature followed by filtration through Whatman paper no. 1 before use.

4. **Resolving gel buffer (12%; 20 ml)**

30% Gel stock	8 ml

Distilled water	6.6 ml
1.5 M Tris-Cl (pH 8.8)	5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.012 ml

APS solution must be prepared fresh. APS and TEMED must be added just before pouring the resolving gel buffer into the PAGE assembly.

5. **Stacking gel buffer (4%; 7 ml)**

30% Gel stock	1.162 ml
Distilled water	4.8 ml
1 M Tris-Cl (pH 6.8)	0.882 ml
10% SDS	0.07 ml
10% APS	0.07 ml
TEMED	0.007 ml

APS solution must be prepared fresh. APS and TEMED must be added just before pouring the resolving gel buffer into the PAGE assembly.

6. **Coomassie stain**

CBB-R250	0.1%
Methanol	40%
Acetic acid	10%

7. **Destaining solution**

Methanol	30%
Acetic acid	5%

2.2.10.2 Protocol for SDS-PAGE

Gel casting: Resolving gel buffer was poured between PAGE plates in the casting assembly, immediately followed by butanol. The gel was left undisturbed and allowed to fully polymerise for half an hour. The butanol was removed and the gel was washed thoroughly with distilled water and stacking gel buffer was poured on top of it. The comb was inserted immediately.

Sample preparation: The sample was prepared as per the required loading amount in the sample buffer (Loading buffer), and water was added to prepare the final working

concentration. The solution was heated at 80°C for 10 min. and placed on ice till the sample was loaded onto the gel.

CBB staining: Gel was stained in CBB staining solution overnight. This was followed by destaining for 2 to 4 hours.

2.2.11 Western Blot

1. **Transfer Buffer**

   Tris base 25 mM

   Glycine 192 mM

   Methanol 20%

pH set to 8.3. Methanol is added fresh before use. Buffer made ice-cold for use.

2. **Tris buffer saline (10x; 1 litre)**

   Tris base 24 g

   NaCl 88 g

Adjust the pH to 7.4 and make up the volume up to 1 litre with distilled water.

3. **Washing buffer (TBS-T)**

   0.1% Triton X-100 in TBS (1x)

4. **Blocking buffer (TBS-MT)**

   5% skimmed milk powder in TBS-T

5. **Antibody dilution buffer**

   BSA and Sodium azide was added to TBS-T to a final concentration of 5% and 0.02% respectively.

2.2.11.1 Western blot protocol

After PAGE, the gel was equilibrated in a transfer buffer for 30 min. A transfer stack was then prepared with the gel and membrane sandwiched between two Whatman filters papers (no. 1)
and a sponge pad of the same dimensions on either side. The transfer was carried out in cold conditions in a transfer tank at 100 V for 90 min.

The membrane was stained in 0.5% Ponceau stain to check the quality of the transfer. The reversible stain was washed off with TBS and the membrane was processed for antibody probing.

Antibody probing: Membrane was blocked with a blocking buffer at room temperature for 1 hour. It was then incubated in the primary antibody at 4◦C overnight. The membrane was washed with washing buffer thrice for 10 minutes each time. This was followed by incubation with HRP-conjugated secondary antibody for 1 hour. The membrane was washed again in washing buffer thrice as in the previous step. Bands were developed by treating the membranes with ECL reagent (Immobilon Western Chemiluminescent HRP substrate, Millipore, USA) as per the manufacturer's instructions, followed by exposing it to the X-ray film for 30 seconds. The bands on the X-ray film were developed by immersing the X-ray film in developer solution
for 1 minute followed by dipping it into fixer solution as per the instructions (Fujifilm, Japan). The protein bands were quantified using ImageJ software.

2.2.12 Drug safety parameter analysis

During the different drug regimens (Provided in the Section 2.2.2), the animals have been examined for any sign of toxicity. The animals from the control and treated groups were weighed before the test, weekly throughout the study, during the gestation period, and on the day of sacrifice. The mortality of all the animals was checked once every day. Throughout the investigation, each animal's general physical observations were made daily. The fur, eyes, nose, abdomen, and external genitals were examined, as well as the presence of fluids and excretions, and autonomic nervous system activity (e.g., lacrimation, piloerection, breathing rhythm, and reaction to handling) were monitored once daily. Further, the food and water consumed by all the treated animals have been noted daily. Also, the reproductive performance of animals was examined.

The analysis of certain biochemical parameters is also very important when evaluating the toxic effect of plant extracts. At the end of the 51 days of treatment, the animals were kept for mating, and on the day-6^{th} of pregnancy, the biochemical parameters like blood glucose levels were measured by tail vein blood sampling using a blood glucose meter (OneTouch Select Plus (Johnson & Johnson Ltd., India). Immediately after euthanizing the animals, the blood samples were collected by cardiac puncture and the serum was isolated and analyzed for further analysis like lipid profile: Serum cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), and triglyceride. The liver and kidney are more predisposed to the toxic effects of xenobiotics, as it is the major site for their metabolism and excretion (George, 2011). Hence, the key indicators of liver and kidney functions, such as liver

function indicators: Serum ALP, serum glutamic-oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT), albumin, globulin, total protein, and liver histopathology have been studied. Also, the renal function indicators: Serum creatinine, urea, uric acid, and kidney histopathology were analyzed in all groups of animals. All the above-discussed parameters were determined using Auto-analyzer (Erba, EM200).

2.3 Phytochemical analysis of Petroleum ether extract of *Aloe vera* gel

2.3.1 Plant Material

Commercially available freeze-dried *Aloe vera* gel [*Aloe barbadensis*] was obtained from M/s. Aum Agrifresh Foods, Vadodara [Product Code- 066; Batch No-06510; Manufactured in Sept'17; ISO 22000, HALAL certified]. It is manufactured without the use of matrix, preservatives, and additives. The authentication certificate and the quality control parameters are provided in Figure 2.1.

DEPARTMENT OF BOTANY

Faculty of Science

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
VADODARA- 390 002, Gujarat, (INDIA)
Telephone: (0265) 2791891

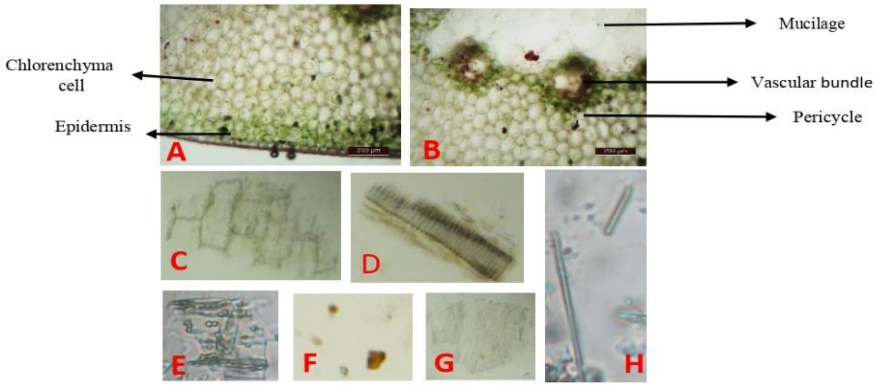
Padamnabhi S. Nagar
Associate professor

No. FS/BOT/PSN/ 15222 Date: 15-02-2022

CRTIFICATE OF AUTHENTICATION

This is to certify the plant powder provided by Ms. Shivani Dhadhal, Department of Biochemistry, The Maharaja Sayajirao University of Baroda, is that of *Aloe barbadensis* L. belonging to family of Liliaceae based on its pharmacognostic features.

Authentication was done on the basis of below characters.



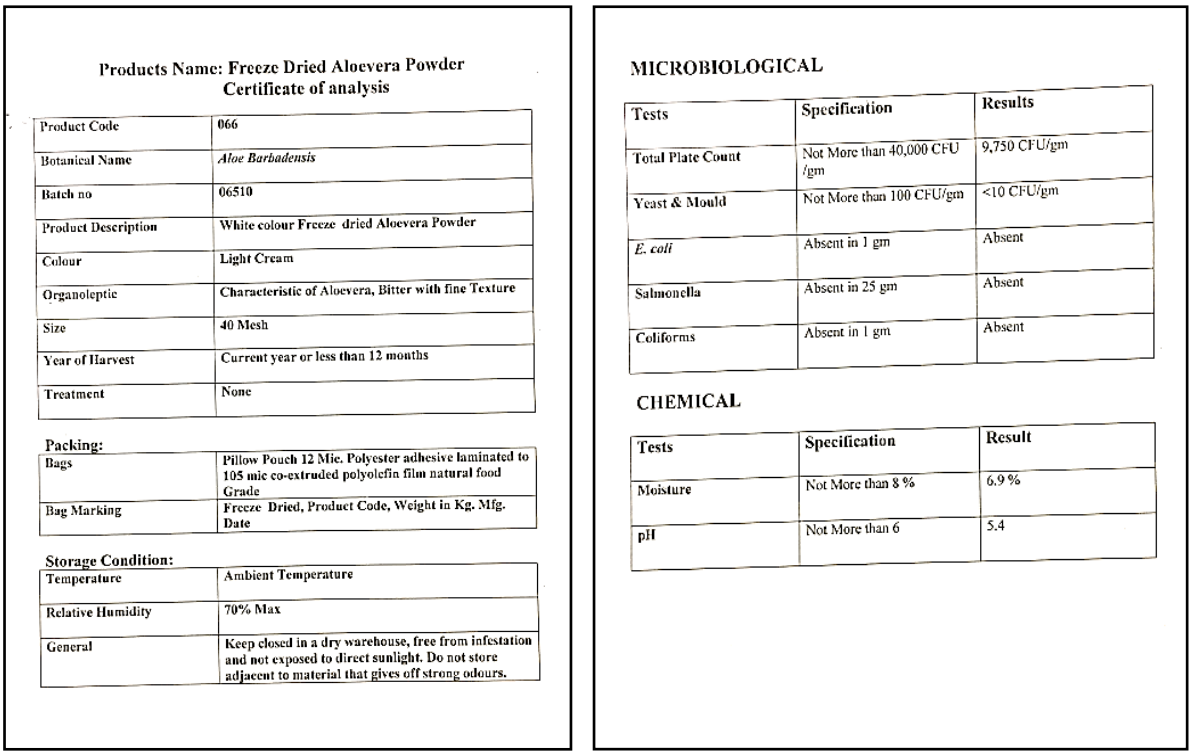
Description of Figure:

Microscopic: A and B are the T.S of the Aloe Leaf. Detailed T.S shows a Layer of Epidermis and covered with thick layer of cuticle. Blow this layer 10 to 15 rows of Chlorenchyma mesophyll cell. Isolated vascular bundles running parallel with epidermis at a short distance each bundle being capped with thin-walled pericyclic parenchymatous cell filed with aloetic juice. Inner cells of mesophyll being filled with mucilage.

Powder Study: C: Mucilage containing mesophyll cell; D: Fragment of xylem vessels; E: Chlorenchymatous Mesophyll cell; F: cell contain secondary metabolites; G: Mucilage; H: Acicular crystals of calcium oxalate.

(P.S. Nagar)

Associate Professor



Products Name: Freeze Dried Aloevera Powder
Certificate of analysis

Product Code	066
Botanical Name	*Aloe Barbadensis*
Batch no	06510
Product Description	White colour Freeze dried Aloevera Powder
Colour	Light Cream
Organoleptic	Characteristic of Aloevera, Bitter with fine Texture
Size	40 Mesh
Year of Harvest	Current year or less than 12 months
Treatment	None

Packing:

Bags	Pillow Pouch 12 Mic. Polyester adhesive laminated to 105 mic co-extruded polyolefin film natural food Grade
Bag Marking	Freeze Dried, Product Code, Weight in Kg. Mfg. Date

Storage Condition:

Temperature	Ambient Temperature
Relative Humidity	70% Max
General	Keep closed in a dry warehouse, free from infestation and not exposed to direct sunlight. Do not store adjacent to material that gives off strong odours.

MICROBIOLOGICAL

Tests	Specification	Results
Total Plate Count	Not More than 40,000 CFU /gm	9,750 CFU/gm
Yeast & Mould	Not More than 100 CFU/gm	<10 CFU/gm
E. coli	Absent in 1 gm	Absent
Salmonella	Absent in 25 gm	Absent
Coliforms	Absent in 1 gm	Absent

CHEMICAL

Tests	Specification	Result
Moisture	Not More than 8 %	6.9 %
pH	Not More than 6	5.4

Figure 2.1. Authentication certificate and quality control parameters of the plant material used in the present study.

2.3.2 Extraction of phytoconstituents by Soxhlet method

According to the previously described (Dey et al., 2022) method, the extraction of the non-polar phytochemicals from the *Aloe vera* gel was done using the Soxhlet extraction method. The plant material (100g) was further subjected to soxhlet extraction at a temperature of 40°C for 6-8 hours using petroleum ether (1 Litre) as the solvent. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40-50°C) in a rotavapor apparatus. The nature and yield of the extract were noted. The extract was stored in a refrigerator at 4°C till further use.

2.3.3 Quantitative analysis of Phytosterols (Kenny, 1952)

Reagents:

I. Liebermann–Burchard (LB Reagent): 0.5 ml of sulfuric acid was dissolved in 10 ml of acetic anhydride. Covered and kept in the ice bucket.
II. Standard stigmasterol solution: 5 mg of standard stigmasterol was dissolved in 5 ml of chloroform and mixed well.

Range of standard: 10-100 µg/ml.

Sample Preparation:

2 ml of extract was added to 2 ml of chloroform (1:1). The organic phase (1 ml) was separated and used for the estimation of total sterols.

Procedure:

Reagents	**Blank**	**Standard**	**Test**
Chloroform	1.0 ml	1.0 ml	1.0 ml
Std.	-	0.01 - 0.1ml	-
LB Reagent	1.0 ml	1.0 ml	1.0 ml
Incubate at room temperature for 15 minutes & absorbance taken at 625 nm.			

Calculations:

$$\text{Phytosterol content (µg/ml)} = \frac{\text{O.D. of Test}}{\text{Slope}} \times 2 \text{ (Dilution Factor)}$$

2.3.4 Gas Chromatography-Mass Spectrometry (GC/MS) analysis

GC/MS study includes the following steps:

1. Injection of extract into Gas Chromatograph column
2. Separation of its components as they flow through the analytical column
3. Analysis in the Mass Spectrometer (MS) based on mass/charge ratio.

2.3.4.1 Sample preparation

For the identification of bioactive phytochemicals from plant-derived matrices, details of the sample preparation are mentioned below.

Extraction conditions and sample preparation for plant-derived matrices for GC/MS analysis

1 ml of Dichloromethane was added to 1 mg of accurately weighed petroleum ether extract of *Aloe vera* gel and vortexed till completely soluble. 2 µl of prepared samples were injected into the programmed GC/MS instrument.

2.3.4.2 Instrumentation

The GC/MS analysis of the phytocompounds was done using a SHIMADZU QP2010 ULTRA system comprising a Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with Rxi-1ms fused silica column (30 × 0.25 μm ID × 0.25 μm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with an ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 0.9 ml/min, and an injection volume of 2 μl was employed (a split ratio of 10:1). The linear velocity of 36.0 cm/sec, pressure of 82.4 kPa and purge flow of 3.0 ml/min was used for the study. The injector temperature was maintained at 300°C, the ion-source temperature was 200 °C, and the oven temperature was programmed from 150 °C (isothermal for 2 min), with an increase of 10 °C/min to 300°C (isothermal for 20 min). The interface temperature was 260°C Mass spectra were taken at a scanning interval of 0.30 secs and fragments from 35 to 1000 m/z. The detector voltage was 0.8 kV and the scan speed was 3333. The solvent delay was 0 to 3 min, and the total GC/MS running time was 37 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total area. The mass detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

2.3.4.3 Identification of phytocomponents

Interpretation on mass-spectrum GC-MS was conducted using the database of the National Institute Standard and Technology (NIST) having more than 62,000 patterns. The interpretations of the mass spectrum of identified components were compared with the mass spectra of known components from the NIST library, through which the molecular weight, molecular formula, retention time, and peak area percentage were determined.

2.4 *In-silico* analysis of the non-polar phytocomonents of *Aloe vera* gel with the early pregnancy marker

The detailed methodology of the *in-silico* study is explained in chapter 5. The overall steps involved are explained below.

2.4.1 Molecular docking study

The previous lab study demonstrated that n-Hexadecanoic acid and Campesterol from partially purified non-polar phytocomponents of AVG exhibited ovarian and metabolic modulators in the non-pregnant letrozole-induced PCOS mouse model (Dey et al., 2022). Thus, two phytocompounds (n hexadecenoic acid and campesterol) of the PE extract were screened against a number of key molecules of early pregnancy in order to check their binding affinity in the active site of the protein. In the docking study, protein preparation, ligand preparation, and grid generation were performed using MGL Tools1.5.7rc1 and Autodock vina software.

2.4.1.1 Protein Preparation

The three-dimensional structure of all the target proteins listed in chapter 5 was obtained from the RCSB-Protein data bank site. The protein structures were prepared using Discovery studio 2020. Next, using the graphical user interface of autodock tools 1.5.7rc, polar hydrogens, Kollman charges, fragmental volumes, and salvation parameters were assigned to the protein.

2.4.1.2 Ligand preparation and Grid generation

The ligand structures isolated for docking studies 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 the docking studies, ligand and protein both kept rigid and the pose which was having lowest binding affinity was extracted.

2.4.2 Statistical analysis

The values are presented as mean ± standard error mean in all the experiments. Statistical analysis was done using student's t-test and one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests (GraphPad Prism 5 software, La Jolla, CA). p-values when less than 0.05 were considered to be statistically significant at the 95% confidence limit.

Table 2.1. List of primers used in the study.

Genes	Accession Number	Sequence (5' →3')
Actb	NM_031144	CCCGCGAGTACAACCTTCTTG
		GTCATCCATGGCGAACTGGTG
Ar	NM_013476.4	TATGGGGACATGCGTTTGGA
		AGGTCTTCTGGGGTGGAAAG
Esr1	XM_017313797.1	AATTCTGACAATCGACGCCAG
		GTGCTTCAACATTCTCCCTCCTC
Esr2	NM_207707.1	GACGAAGAGTGCTGTCCCAA
		GGGGTACATACTGGAGTTGAGG
Pgr	NM_008829.2	ATGGTCCTTGGAGGTCGTAA
		CAACACCGTCAAGGGTTCTC
Mmp2	NM_008610	GTGGTCCGCGTAAAGTATGG
		TAAACAAGGCTTCATGGGGG
Mmp9	NM_013599	AGCCGACTTTTGTGGTCTTC
		GCGGTACAAGTATGCCTCTG
Lifr	NM_013584	CGTGGCATTGGCTCCTG
		TTGTACCCCTCTCTTCAGACC
Itgav	XM_017592332.1	TGGCAGGGTCAGCTCATTTC
		TCACCATTGAAGTCTCCCACG
Itgb3	NM_153720.1	GCTTACCAGCAACCTTCGGA
		GTAGCCAAACATGGGCAAGC
Itga4	NM_010576.4	GAATCTCCTCCACCTACTCACAG
		CCAACGGCTACATCAACATATCC
Itgb1	NM_010578.2	ACCAAAGTCTGAGGAATAAAC
		TGGTTTCAGACTCCTTATTTG
Hox10a	XM_008762948.2	AGAAGGACTCCCTGGGCAAT

		TCTGGTGCTTCGTGTAAGGG
Hox11a	NM_001129878.1	CGGTGGCTCCGGTGG
		TTGACTTGACGGTCGGTGAG
Timp1	NM_001294280.2	CCCTTTGCATCTCTGGCATC
		GCATTTCCCACAGCCTTGAA
Timp3	NM_011595.2	GGCCTCAATTACCGCTACCA
		CTGATAGCCAGGGTACCCAAAA
Lif	NM_022196.2	GTCTTGGCCACAGGGATTGTG
		TGGTTCATGAGGTTGCCGTG
Stat3	NM_011486.5	GGGCCATCCTAAGCACAAAG
		GGTCTTGCCACTGATGTCCTT
Gp130	NM_010560.3	CAACACCAAAGTTCGCTCAA
		GCCGTCCGAGTACATTTGAT

Table 2.2. Details of antibodies used in the study.

Primary Antibody	**Procured from**	**Dilutions used**
β-Actin	Cell signaling technology # 4967	1:10000
Androgen receptor (AR)	Prof. Rakesh Tyagi, JNU, India	1:5000
Progesterone receptor (PR)	Abcam (ab63615)	1:2000
Estrogen receptor-α (ER-α)	Santacruz Biotech #543 (Dr. Anil Pillai, Medical college of Georgia)	1:5000
Estrogen receptor-β (ER-β)	Abcam #3577 (Dr. Anil Pillai, Medical college of Georgia)	1:5000