# 2
# Materials and methods

**Chemicals**

Pregnant Mare's Serum Gonadotropin (PMSG), Insulin (40 IU), Bovine Serum Albumin (BSA), 5-Androsten-3b-ol-17-one sulfate sodium salt, Transferrin, Forskolin, Cholesterol, 5-α-Cholestane, β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene, SigmaFAST™ protease inhibitor cocktail was procured from Sigma Aldrich India. Human Menopausal Gonadotrophin (HMG) [HUMOG®-150 I.U.] was procured from Bharat Serums and Vaccines Limited. Letrozole tablets-2.5mg, marketed under the brand name Letronat was procured from Natco Pharma Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), EGTA (ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid), Sucrose, Trypan Blue and Dimethyl sulfoxide (DMSO), Gas Chromatography and HPLC grade solvents were procured from HiMedia Laboratories Pvt. Silica gel 60G $F_{254}$ pre-coated thin layer chromatography plates were procured from Merck, India. Sterile cell culture treated plastic wares were procured from Corning Inc. DMEM: F12 (1:1), Fetal Bovine Serum- South American origin and Penstrep solution were procured from GIBCO®. KGN cell-line was obtained from RIKEN Bioresource Center (Tsukuba, Japan) and was a kind gift from Yoshihiro Nishi. Hank's Balanced Salt Solution (HBSS) and Phosphate Buffered Saline (PBS) were prepared manually in the laboratory using the standard recipe. The blood glucose levels were measured by OneTouch Select Plus (Johnson & Johnson Ltd.). Total Cholesterol (CC2-CLE.005), LDL-Cholesterol (CC3-LDL.022), HDL-Cholesterol (CC2-HDC.013) and Triglycerides (CC2-TGS.18M) kits were purchased from Reckon Diagnostics P. Ltd, India. Hormones- insulin, testosterone, estradiol and progesterone were assayed using direct ELISA kits (DBC Canada). TRIzol™ Reagent was procured from Invitrogen. Verso cDNA synthesis kit was procured from Thermo Scientific™. PowerUp™ SYBR™ Green master-mix was procured from Applied Biosystems, Thermo Fisher Scientific™. Primers for key steroidogenic and metabolic genes were designed by primer blast tool of NCBI and synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Details of the primer sequences are given in Table 2.3.

This section has been divided mainly into 5 parts:

2.1 Phytochemical analysis

2.2 Pharmacokinetics and tissue distribution study

2.3 *In-silico* analysis and its validation by steroidogenesis modulatory bioassay

2.4 *In-vitro* analysis in PCO-like ovarian cellular models

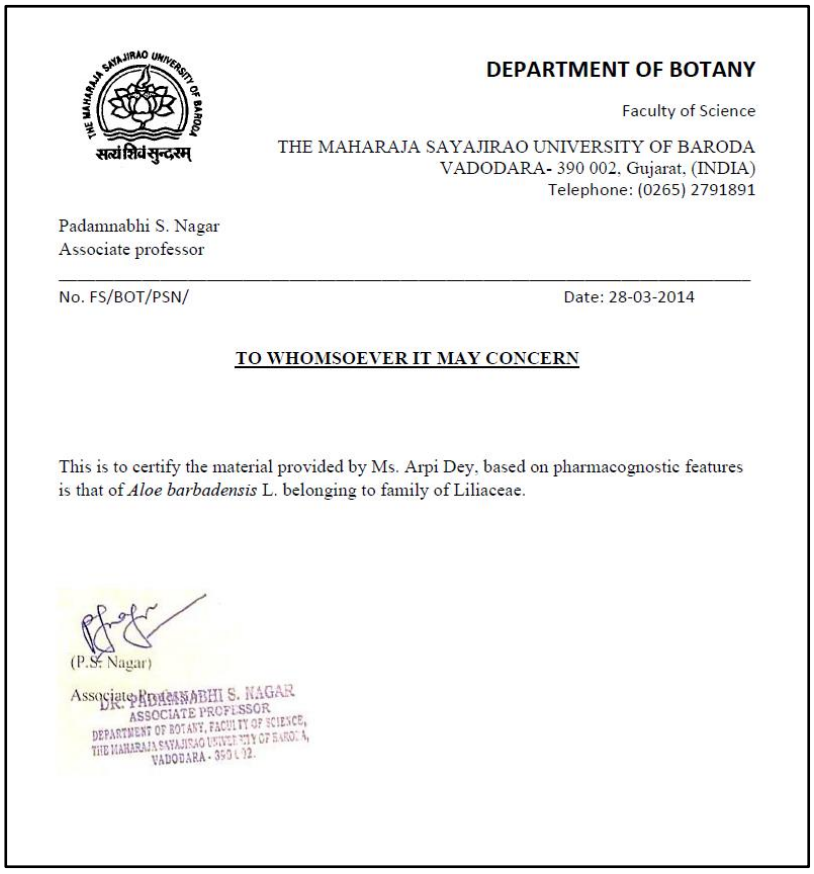
2.5 *In-vivo* analysis in PCOS rodent model

## 2.1 Phytochemical Analysis

All the materials and methods used in this section includes qualitative and quantitative analysis of the plant material.

### 2.1.1. 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. Certificate of authentication and quality control parameters are attached in Figure 2.1.



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Associate professor

No. FS/BOT/PSN/ Date: 28-03-2014

**TO WHOMSOEVER IT MAY CONCERN**

This is to certify the material provided by Ms. Arpi Dey, based on pharmacognostic features is that of *Aloe barbadensis* L. belonging to family of Liliaceae.

(P.S. Nagar)
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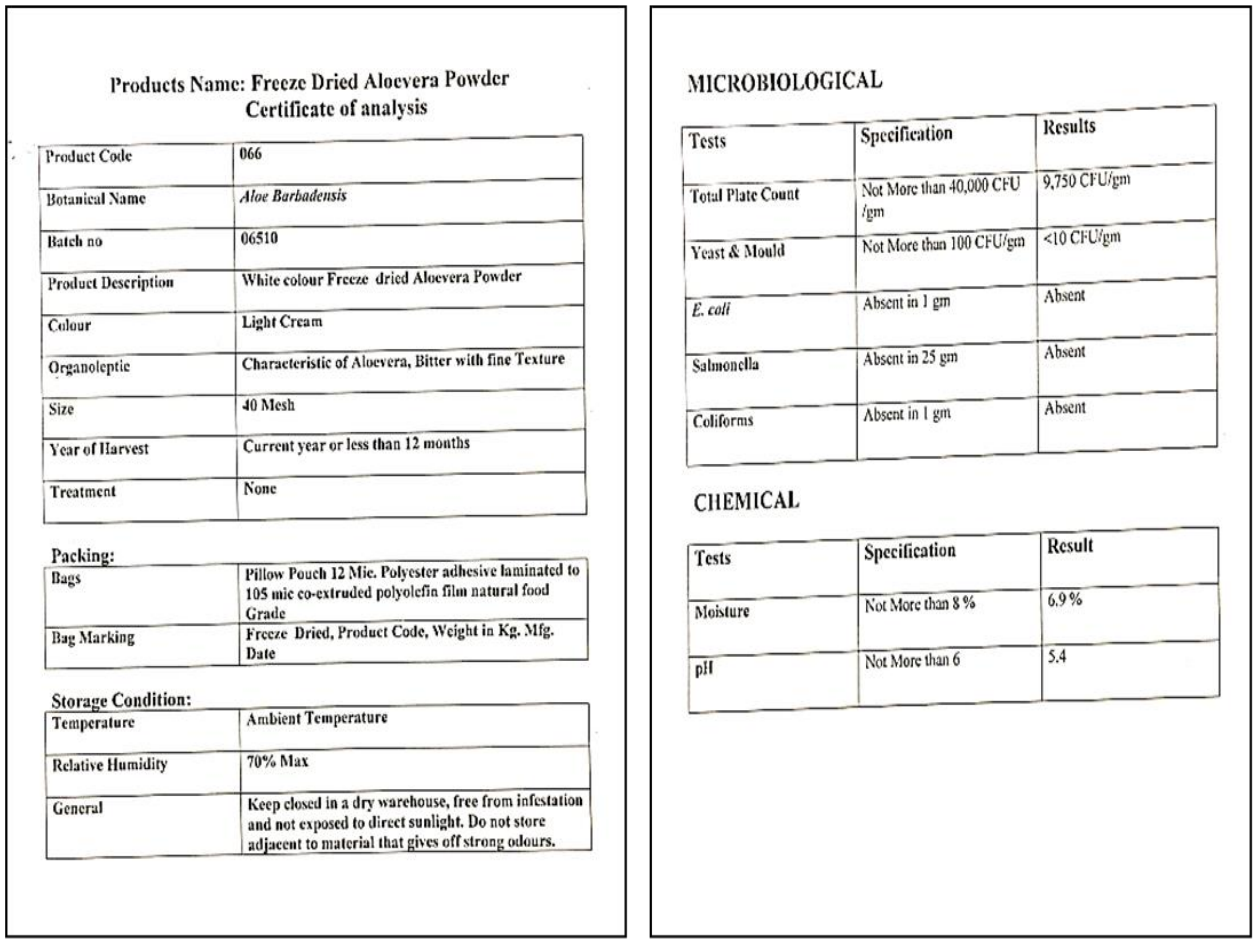

Products Name: Freeze Dried Aloevera Powder
Certificate of analysis

| | |
|---|---|
| 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

### 2.1.2. Extraction method

For extraction of the non-polar phytochemicals from the *Aloe vera* gel, the plant material was initially extracted with organic non-polar solvents like n-hexane, petroleum ether, chloroform and ethyl acetate by overnight shaking method. The mixture was left undisturbed for 4-6 hours and the organic phase was separated in a fresh beaker. The solvent was allowed to evaporate and the dry weights of the extracts were obtained. These extracts were further used for their preliminary phytochemical screening. Extraction in more polar solvents resulted in high extract yield but low fatty acids, phytosterol and terpenoid content as compared to more non-polar ones. Amongst all the non-polar solvents used, petroleum ether extract was found to be rich in non-polar phytochemicals. However, the yield obtained was very low, hence, we opted for a second mode of extraction i.e., Soxhelet, in order to increase the extraction efficiency of phytochemicals. In this regard, the plant material (500g) was further subjected to Soxhelet

extraction at a temperature of 40°C for 6-8 hours using petroleum ether (1 Liter) as the solvent (Figure 2.2). 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 was noted. The extract was stored in a refrigerator at 4°C till further use.

**Figure 2.2** Setup for Soxhelet extraction of non-polar phytocomponents of *Aloe vera* gel

### 2.1.3. Preliminary Phytochemical Analysis

A systematic and complete study of plant extracts includes a complete investigation of both primary and secondary metabolites derived from plant metabolism. The different qualitative chemical tests were performed for establishing profiles of given extracts for their nature of chemical composition. The extract obtained above was tested for the following qualitative chemical tests for the identification of various phytoconstituents and shown in Table 2.1

### 2.1.3.1. Qualitative Analysis

**Table 2.1:** Phytochemical Screening Tests

| **Major groups of phytochemical constituents** | **Test** | **Reagents and assay procedure** | **Inference** |
|---|---|---|---|
| Steroidal Glycosides | Baliget test | Solution A: 1% Picric acid in Ethanol<br>Solution B: 10% NaOH<br>Solution A and Solution B were mixed in 1:1 ratio and added drops by drop to 1 ml of sample. | Orange to deep red colour |
| | Killer-Killani Test | 1 ml of extract was mixed with 2 ml of Glacial acetic acid containing 1-2 drops of 2% $FeCl_3$.To this 2 ml of conc. $H_2SO_4$ was added along the walls of the test tube. | Formation of a brown ring at the interphase |
| Phytosterols | Libermann-bachmann | 1 ml chloroform was added to 1 ml of acetic anhydride and this mixture was cooled in ice and few drops of conc. $H_2SO_4$ were added. This reagent was added to 1 ml of extract. | Blue, Green, Red,<br>Orange colour change with time |
| | Salkouski Test | 1 ml chloroform and 1 ml conc. $H_2SO_4$ was added to 1 ml of the extract. | Formation of Red colour |
| Alkaloids | Mayor's test | Solution A: 1.358 g of Mercuric chloride was added in 60 ml distilled water.<br>Solution B: 5.0g KI was added in 10 ml of distilled water.<br>Both solutions were mixed and the final volume was made up to 100 ml | A white or creamy precipitate |

| | | | |
|---|---|---|---|
| | | with distilled water. To this reagent few drops of extract was added. | |
| | Wagner's test | 1.27g Iodine and 2g KI were added to 5 ml distilled water. The volume was made up to 100 ml with distilled water. This reagent was added by side of the test tube to 1 ml of extract**.** | Reddish brown precipitate |
| | Dragendroffes' s test | Solution A: 0.85 g bismuth nitrate in a solution of 10 ml acetic acid + 40 ml water<br>Solution B: 8 g KI in 20 ml water. Both solutions were mixed in equal proportion and this reagent was added to1ml of extract. | Prominent yellow precipitates |
| Phenolic Compounds | $FeCl_3$ | To the extract, few ml of distilled water and few drops of neutral 5% $FeCl_3$ solution were added. | Dark Green: Phenolic compounds<br>Blue colour: Tannins |
| | Gelatin test | To the extract, few ml of distilled water and 1% solution of gelatin containing 10 % NaCl were added. | Formation of white precipitate |
| Flavonoids | Lead acetate test | To 1 ml of extract, 1 ml of 10% lead acetate was added**.** | Formation of yellow precipitate |
| Terpenoids | | To the extract, 2ml of chloroform was added and shaken vigorously. The organic phase was separated and evaporated. 2ml of conc. $H_2SO_4$ was added and heated for 2 minutes. | Formation of greyish colour |
| Saponins | Foam test | To 1 ml of extract, 1ml of distilled water was added and warmed**.** | Formation of stable foam in |

| | | | |
|---|---|---|---|
| | | | the form of honeycomb. |
| Carbohydrates | Molish Test | To 2 ml of extract, few drops of α-naphthol were added. 1ml of conc. $H_2SO_4$ was added in a drop-wise manner along the sides of the test tube | Formation of a violet ring at the junction of two layers. |
| Proteins | Ninhydrin Test | 2 ml of extract was boiled with 2ml of 0.2% Ninhydrin solution | Formation of violet colour |

**2.1.3.2. 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 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 (1ml) was separated and used for 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:**

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

### 2.1.4. Isolation of the Phytoconstituents

The preliminary phytochemical analysis demonstrated that the Petroleum ether extract of *Aloe vera* gel was abundant in non-polar phytochemicals as compared to other solvents; therefore, all the further studies were conducted using the petroleum ether extract of *Aloe vera* gel. The constituents of the petroleum ether extract were sub-fractionated by column chromatography and identified by thin layer chromatographic technique.

#### 2.1.4.1. Column Chromatography

As shown in Figure 2.3, the column was packed with silica gel (60-120 Mesh) by wet packing method wherein a padding of cotton was placed at the bottom of the column and then it was filled with eluting solvent of the lowest polarity (Benzene). Then 100g of silica gel- 230-400 mesh size (stationary phase) was poured into the column to form a bed of silica. The solvent was added to the top of the bed. Petroleum ether extract of *Aloe vera* gel (10 g) was thoroughly mixed with silica gel (100g) until a non-sticky homogenous powder was form and then loaded into the silica gel column, a layer of cotton covered it again and more amount of solvent was poured over it using solvent reservoir, the column was then eluted gradiently as shown in Table 2.2.

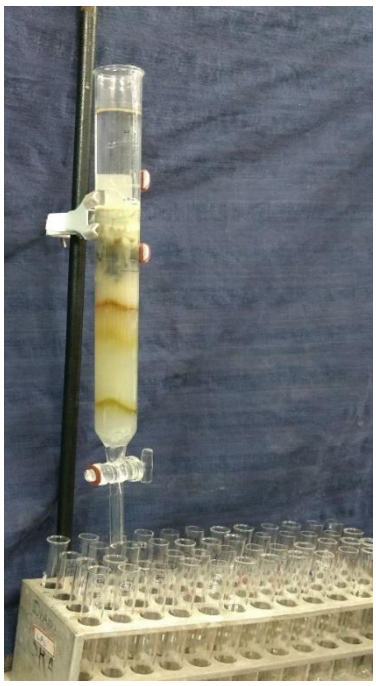

**Figure 2.3** Setup for column chromatography

**Chromatographic Conditions**

Column Dimension: 32 cm in length, 1.6 cm in diameter

Stationary phase – Silica gel (230- 400 Mesh)

Mobile phase – Benzene, Diethyl ether, Ethyl acetate

Charged matter – Petroleum ether extract of *Aloe vera* gel

TLC solvent systems – Toluene: Ethyl acetate: Methanol: Formic acid (7.75:1.5:0.25:0.5)

Volume of each fraction – 20 mL

**Procedure**

The development and elution of the column was carried with successive series of solvents in various combinations, Benzene- Diethyl ether (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, 50:50, 45:55, 40:60, 30:70, 20:80,5:95 and 0:100 v/v), and with Diethyl ether- Ethyl acetate (95:5, 90:10, 80:20 and 70:30 v/v). The completion of elution of the component(s) was ascertained when evaporating a small fraction of eluent left no residue. The eluents were analysed by using pre-coated TLC plate with suitable mobile phase to confirm the homogeneity of eluents. Optimized mobile phase i.e., Toluene: Ethyl acetate: Methanol: Formic acid (7.75:1.5:0.25:0.5) was used. Visualization of spot/band was done both in UV chamber as well as after derivatization with anisaldehyde-sulphuric acid spraying reagent and heated at 90-100°C for 5 minutes in a hot air oven. Over all 110 fractions of 20 ml each were collected; fractions containing the same compound as determined by TLC were combined and allowed to stand for the separation/crystallization of compounds. The dried fractions were then weighed, split into two parts and recovered using Dichloromethane and Methanol for GC-MS and HPLC analysis respectively.

### 2.1.5. 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.

### 2.1.5.1. Sample preparation

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

#### 2.1.5.1.1. 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 partially purified isolates respectively and vortexed till completely soluble. 2 µL of prepared samples were injected into the programmed GC/MS instrument and rest of the sample stored at -20˚ C deep freezer for further study.

#### 2.1.5.1.2. Extraction conditions and sample preparation for rat derived biological matrices for GC/MS analysis

For GC/MS analysis of phytocomponents present in rat derived biological matrices, an aliquot of 200 µL blood plasma, 10% tissue (hypothalamus, pituitary, liver, kidney, skeletal muscles, ovary, adrenal, uterus, kidneys) homogenates (prepared in 1X PBS, pH-7.4), 5 ml of urine and 10% of feces (100mg) homogenate prepared in 1X PBS respectively was mixed thoroughly with 50% KOH (w/v) (200 µl) and 1% ethanolic pyrogallol (w/v) (1.5 ml). The tubes were kept at 70 °C in a water bath for 40 min. Water (0.5 ml) and hexane (3 ml) were added to the tubes after cooling on ice. The tubes were shaken and centrifuged at 400 × g for 10 min. The hexane phase was removed and the extraction repeated with hexane (1 ml). The combined hexane extracts were dried and was redissolved in 100 µL of Dichloromethane. 2 µL of prepared samples were injected into the GC/MS instrument and remaining sample was stored at -20˚ C deep freezer for further study.

### 2.1.5.2. Instrumentation

The GC/MS analysis of the phytocompounds were 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 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, 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 scan 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 areas. 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.

#### 2.1.5.3. Identification of phytocomponents

Interpretation on mass-spectrum GC-MS was conducted using the database of 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 was determined.

### 2.1.6. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis

#### 2.1.6.1. Instrumentation

The chromatographic system (UltiMate™ 3000 HPLC System, Thermo Fisher Scientific) used to perform development and validation of this assay method comprised of Dionex Ultimate 3000 Pump (Thermo Scientific) and Dionex Ultimate™ 3000 VWD Variable Wavelength Detector (Thermo Scientific). It was operated by Chromeleon™ software. Separations were carried out on Acclaim™ 120 (Thermo Scientific) C18 column of 4×250mm, 120 Å pore diameter and 5 µm particle size.

#### 2.1.6.2. Preparation of calibration standards and quality control samples

10 mg of reference standards (Cholesterol, β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene) were accurately weighed and transferred to a standard volumetric flask (10 mL) respectively for HPLC analysis. The content of the flask was initially dissolved in minimum quantity of HPLC grade methanol and sonicated for 10

minutes after which it was diluted up to the mark. The stock solution of 1mg /mL was used to prepare working solutions of 100 µg/mL, 10 µg/mL and 1 µg/mL. Calibration standards were prepared by 20 times dilution of the corresponding working solutions with methanol to obtain the final concentrations of 5, 25, 50, 100, 200, 300, 400, and 500 ng/mL. The quality control samples containing low, medium and high concentrations were prepared at the concentrations of 5, 100 or 350 ng/mL. The Internal standard solution of 5α-Cholestane was prepared in methanol at a concentration of 100 ng/mL. All solutions were stored at 4°C and were brought to room temperature before use.

#### 2.1.6.3. Sample Preparation

##### 2.1.6.3.1. Extraction conditions and sample preparation for plant derived matrices for RP-HPLC analysis

For the estimation of bioactive phytochemicals from petroleum ether extract and partially purified isolates of *Aloe vera* gel, 1 mL of HPLC grade methanol containing 100 ng/mL of 5α-Cholestane (Internal Standard) was added to 1 mg of accurately weighed petroleum ether extract of *Aloe vera* gel and partially purified isolates respectively and vortexed till completely soluble. Methanol had the highest sensitivity of all the chemical reagents examined, especially when it came to eliminating endogenous interference and improving extraction recovery. The mixture was vortexed using vortex mixer. The filtrate obtained was further passed through a nylon micro filter paper (0.45 µm) prior to injection. Volume of 20 µL was kept constant for HPLC injections.

##### 2.1.6.3.2. Extraction conditions and sample preparation for rat derived biological matrices for RP-HPLC analysis

To remove endogenous protein interference and maximize extraction recovery, a selective and sensitive sample preparation approach was used. In the present study, Cholesterol, β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene were extracted from rat plasma and tissue homogenates using a simple liquid–liquid extraction method. Blood was obtained from retro orbital plexus of animals and collected in EDTA coated eppendorf tubes, followed by centrifugation at 2500 g for 15 min. The resulting plasma layer was separated and stored in tubes at −20 °C for further analysis. After samples were thawed at room temperature, 100ng/mL of 5α-Cholestane (Internal Standard) was added to 200 µL blood

plasma, 10% tissue (hypothalamus, pituitary, liver, kidney, skeletal muscles, ovary, adrenal, uterus, kidneys) homogenates (prepared in 1X PBS, pH-7.4), 5 ml of urine and 10% of feces (100mg) homogenate prepared in 1X PBS respectively and this was mixed thoroughly with 50% KOH (w/v) (200 µl) and 1% ethanolic pyrogallol (w/v) (1.5 ml). The tubes were kept at 70 °C in a water bath for 40 min. Water (0.5 ml) and hexane (3 ml) were added to the tubes after cooling on ice. The tubes were shaken and centrifuged at 400 × g for 10 min. The hexane phase was removed and the extraction repeated with hexane (1 ml). The combined hexane extracts were dried and was redissolved in HPLC grade Methanol (200 µl), filtered through a 0.45 µm syringe filter and transferred into a fresh tube for further HPLC analysis. For HPLC injections, a constant volume of 20 µL was used.

#### 2.1.6.4. Method development for RP-HPLC

For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant.

##### 2.1.6.4.1. Detection of wavelength

The spectra of diluted solutions of Cholesterol, β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene were recorded separately on UV spectrophotometer. The peaks of maximum absorbance wavelengths were observed. The spectra of all the standards showed balanced wavelength at 205 nm.

##### 2.1.6.4.2. Choice of Stationary Phase

Preliminary development trials were performed with C18, C8 and C3 with different types of configurations and from different manufacturers. Finally, the expected separation and shapes of peak was succeeded with Acclaim™ 120 (Thermo Scientific) C18 column of 4×250mm, 120 Å pore diameter and 5 µm particle size.

##### 2.1.6.4.3. Selection of the Mobile Phase

In order to get sharp peak and base line separation of the components, the author has carried out a number of experiments by varying the composition of various solvents and its flow rate. To effect ideal separation of the drug under isocratic conditions, mixtures of solvents like water, ethanol and acetonitrile with or without different buffers in different combinations were

tested as mobile phases on a C18 stationary phase. A mixture of Acetonitrile: Ethanol (80: 20 v/v) was found to be the most suitable of all the combinations since the chromatographic peaks obtained were better defined and resolved and almost free from tailing. The Mobile phase was filtered through a 0.45 µm nylon membrane (Millipore Pvt. Ltd. Bangalore, India) and degassed in an ultrasonic bath (Spincotech Pvt. Ltd., Mumbai) prior to use. HPLC grade Methanol was used as a diluent for solubilization of the

#### 2.1.6.4.4. Flow Rate

Flow rates of the mobile phase were changed from 0.5-2.0 mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 1.5 mL/min flow rate was ideal for the successful elution of the analyte. The total run-time was 30 minutes.

### 2.1.6.5. Method Validation for the RP-HPLC analysis

The proposed method was validated as per ICH guidelines. The parameters studied for validation were selectivity, linearity, precision, accuracy, robustness, and limit of detection and limit of quantification.

#### 2.1.6.5.1. Selectivity

The capacity of an analytical method to detect precisely and specifically the analyte of interest in the presence of components that might be present in the sample matrix is referred to as selectivity. The term "selective" refers to an analytical approach that can separate and resolve the numerous components of a mixture while also detecting the analyte qualitatively. The selectivity was determined with the aim of revealing eventual interfering compounds by comparing the Retention time (Rt) values of Cholesterol, β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene from the blank samples, spiked samples, and samples after petroleum ether extract of *Aloe vera* gel administration, with that of the reference standards at absorption spectra of 205 nm.

#### 2.1.6.5.2. Linearity

A series of dilutions in the range of 5 to 500 ng/mL for all the reference standards were prepared from standard stock solution, from which 20 µL was injected into the HPLC system. Before

and after the samples were injected, calibration standards were run. The linear regression equation and coefficient of determination were calculated using both sets of standard peak areas. A graph showing the mean peak area of each standard against the appropriate concentrations was used to create the calibration curve for each standard. Blank samples were included with each set. To determine the linearity of each calibration curve, three calibration curves were created on three different days and studied. The calibration curves were represented by the equation $y = mx \pm c$, where y represents the analyte area and x represents the concentration (ng/mL). Each standard curve's slope, intercept, and correlation coefficient were calculated. The equation of the calibration curve was used to determine unknown concentrations.

#### 2.1.6.5.3. Sensitivity

In case of sensitivity, stock solution of β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene were serially diluted with methanol to prepare the series of solutions of different concentrations ranging from 5-500 ng/mL and injected into the HPLC system. Sensitivity was expressed in terms of limit of detection (LOD) and limit of quantification (LOQ) which were estimated by measuring the signal-to-noise ratio (S/N). LOD and LOQ were considered at S/N of 3:1 and 10:1, respectively. The LOD is a value that represents the lowest concentration in a sample that can be identified but not quantified. The lower limit of detection (LOD) and lower limit of quantitation (LLOQ) were determined with acceptable precision and accuracy (15%).

#### 2.1.6.5.4. Precision and Accuracy

Precision is the degree of repeatability of an analytical method under normal operational conditions. Six duplicates were tested for inter- and intra-precision and accuracy of reference standards- β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene at three concentration levels (5, 100, and 350 ng/mL) respectively. The precision and accuracy tests were completed on the same day. Three analytical lots were examined in three separate days to determine inter-day precision and accuracy. All precision and accuracy experiments employed freshly manufactured calibrators and quality control samples. Relative standard deviation (RSD) was used to assess precision, and relative error was used to assess accuracy (RE).

#### 2.1.6.5.5. Extraction Recovery

By comparing the peak regions of analytes spiked before extraction with those of analytes spiking after extraction, the extraction recoveries of β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene at the three-quality control (5, 100, and 350 ng/mL) concentration levels were determined. The peak area ratio of analyte to internal standard was used as a recovery indicator. The peak regions of analytes spiked after extraction were compared to those of analytes in reconstitution solution to determine the matrix impact. Six samples were analyzed for each level of the three quality control concentration levels. The extraction recovery and matrix effects are generally in the range of 85–115%.

Chromatography with mass spectrometry has been widely employed in recent years to assess the contents of chemical compounds in pharmacological preparations, as well as in pharmacokinetic studies in rats and humans. The capacity to detect a wide range of compounds with high sensitivity and specificity in a single analytical run is the main benefit of mass spectrometry.

### 2.2 Pharmacokinetics and distribution study

#### 2.2.1. Animals

Healthy, 3-4 months virgin female Charles Foster rats weighing 180-200g were chosen for the study which were housed in standard controlled animal care facility, in polypropylene cages (two rats/cage), and maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12: 12-hour dark-light cycle. The animals were provided with commercial food pellets (VRK Nutritional Solutions, Pune, India) with drinking water ad libitum throughout the experiment. All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature. The Nations Control and Supervision of Experiments on Animals (CPCSEA) guidelines were strictly followed and all the studies 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/2016/07).

#### 2.2.2. Pharmacokinetics analysis

Before initiation of the pharmacokinetics study, the animals were randomly divided into two groups, i.e., Vehicle Control (VC) and petroleum ether extract treated group (PE), each group

containing six rats. The animals were fasted overnight, but had free access to water before the experiment. For the PE group, petroleum ether extract of *Aloe vera* gel was dissolved in 0.1% DMSO and administered orally at a dose of 100 mg/kg body weight using oral gavage. The content of n-Hexadecanoic acid, β-Sitosterol, Campesterol acetate, Stigmasterol acetate and Cholesta-3,5-diene in the plant extract were 124.8 $\pm$ 5.8, 11.232 $\pm$ 0.92, 15.6 $\pm$ 0.541, 156 $\pm$ 12.08 and 27.456 $\pm$ 0.941 µg/g, respectively, respectively. Animals from the VC group received equivalent volumes of 0.1% DMSO by oral gavage. After oral administration of the doses, 0.5 mL of blood was collected from the retro orbital plexus of the same animal at 0 min (pre-dose), 30 min, 1, 2.5, 5, 24, 48 and 72 hrs. The plasma was separated by centrifugating the blood samples for 15 minutes at 2500 g. For GC/MS and HPLC analysis, the detailed technique for extracting phytochemicals from plasma is described in sections 2.1.5.1.2 and 2.1.6.3.2, respectively. For preparation of the calibration curve, blank plasma was spiked with 5-500 ng/mL of reference standards- β-Sitosterol, Campesterol acetate, n-Hexadecanoic acid, Stigmasterol acetate and Cholesta-3,5-diene respectively.

For the pharmacokinetic study, the plasma concentrations versus time profiles of individual animals were estimated by a non-compartmental model using Pk solver software (Alsulays et al., 2019). The following parameters were calculated: Maximum plasma concentration (Cmax), area under plasma concentration-time curve (AUC), elimination rate constant (k), total clearance (CL), volume of distribution (Vd), time to maximum concentration (Tmax) and apparent elimination half-life (T1/2).

### 2.2.3. Pharmacodynamic analysis

Pharmacodynamic analysis was performed along with the pharmacokinetic experiment, wherein, the isolated plasma of the rats at 0 min (pre-dose), 30 min, 1, 2.5, 5, 24, 48 and 72 hrs post oral administration of 100 mg/kg body weight petroleum ether extract of *Aloe vera* gel was used as a sample to evaluate the impact of the non-polar phytocomponents present in plant extract on the hormone levels of the animals. Testosterone, Estradiol and Progesterone levels were estimated from the stored plasma using kit-based direct ELISA, procured from Diagnostics Biochem Canada (DBC) as per the manufacturer's instructions. Each sample was assayed in duplicate. Sensitivity of the kits was 0.022ng/ml, 10pg/ml and 0.1ng/ml for testosterone, estradiol and progesterone kits respectively. The working range was 0.08 to 16.7ng/ml, 20 to 3200pg/ml and 0.3 to 60ng/ml of testosterone, estradiol and progesterone

respectively. The intra-assay coefficient of variation (CV) was between 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 between 6.1% to 7.3%, 6.2% to 10.1% and10.2% 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.

### 2.2.4. Analysis of Distribution

Rats were orally administered 100 mg/kg of petroleum ether extract of *Aloe vera* gel, then three rats were sacrificed for each 5, 24, and 48-hour time period in the distribution experiment. Cardiac puncture was used to get blood samples, and centrifugation was used to obtain plasma samples. The hypothalamus, pituitary, liver, kidney, skeletal muscles, ovary, adrenal, uterus, kidneys were all removed and blotted on filter paper to remove blood. The plasma and tissues were stored at -80°C until they were analyzed. The detailed procedure for extraction of phytochemicals from the tissues have been explained in sections 2.1.5.1.2 and 2.1.6.3.2 for GC/MS and HPLC analysis respectively. To understand the conversion of n-Hexadecanoic acid, β-Sitosterol, Campesterol acetate, Stigmasterol acetate and Cholesta-3,5-diene *"in-vivo"* at different time intervals, the weight percentage of each phytochemical was also calculated (% = [amount of phytocompound in plasma or tissue/ amount of phytocompound by oral gavage] × 100).

### 2.2.5. Excretion experiment

For the excretory experiment, six rats were orally administered with 100 mg/kg body weight of petroleum ether extract of *Aloe vera* gel and housed individually in metabolic cages (Figure 2.4). Urine and fecal samples were collected at the following time intervals: 0-10, 10-24, 24-48, and 48-72 hrs after oral administration of plant extract. Before extraction, the volume of urine and the weight of feces samples from each time point were measured. Before pulverization, the fecal samples were lyophilized. The detailed protocol for extracting phytochemicals from plasma for GC/MS and HPLC analysis is described in sections 2.1.5.1.2 and 2.1.6.3.2, respectively. The calibration curve in urine and feces samples was prepared using a process similar to that utilized for plasma samples. The total amount of n-Hexadecanoic acid,

β-Sitosterol, Campesterol acetate, Stigmasterol acetate and Cholesta-3,5-diene expelled from urine and feces was determined using urine volume and feces weight.

**Figure 2.4** Setup for excretion study

After the isolation and characterization of the bioactive non-polar phytocomponents of *Aloe vera* gel, an *"in-silico"* study was performed to identify the potential molecular targets of these isolated phytochemicals.

### 2.3 "*In- silico*" Analysis and its Validation by Steroidogenesis Modulatory Bioassay

#### 2.3.1. Molecular Docking Study

Molecular docking studies have been carried out by using GLIDE (Grid-based Ligand Docking with Energetics) software v5.5 developed by Schrödinger running on Red Hat Enterprise Linux 5 (RHEL5) workstation. Maestro v9.0 Graphical User Interface (GUI) workspace was used for all the steps involved in ligand preparation, protein preparation and Induced Fit Docking (IFD).

#### 2.3.2. Ligand Preparation

The structures of the ligands obtained from the result of GC/MS were drawn by using. LigPrep module of v2.3 of Schrödinger Suite, 2009 and the structures were saved in the MOL format. LigPrep is designed to prepare high quality, all atom 3D structures for large numbers of drug-

like molecules, starting with 2D or 3D structures. The LigPrep process consists of a series of steps that perform conversions, apply corrections to the structures, generate variations on the structures, eliminate unwanted structures, and optimize the structures. Many of the steps are optional, and are controlled by selecting options in the LigPrep panel or specifying command-line options. The steps involved were as follows: structure format was converted, Structures were selected, hydrogen atoms were added, unwanted molecules were removed, charged groups were neutralized, ionization states were generated, tautomers were generated, Structures were filtered, alternative chiralities were generated, low-energy ring conformations were generated and geometry of the ligands were optimized. Ligand structure with minimum potential energy and similar chirality to that of selected ligand IUPAC name was selected for the docking experiment.

### 2.3.3. Retrieval of Target Sequence

The FASTA format of key steroidogenic targets were retrieved from the RCSB Protein Data Bank. The details of their accession numbers and resolution are mentioned in Table 2.2. Amongst the different PDB IDs available at the protein databank site, the files having lowest resolution and Homo sapiens as the source was given preference. Also, the method by which structure was obtained was selected as X-ray diffraction method. The complexes bound to the receptor were removed using discovery studio, and the non-essential water molecule was removed and polar hydrogen was added and the already prepared receptor was saved in PDB format.

**Table 2.2: List of targets, their accession nos., sources, resolution and details of method by which structure was obtained based upon the RCSB protein data bank**

| Protein name (Targets) | PDB Accession no. | Source | Resolution (Å) | Method by which structure was obtained |
|---|---|---|---|---|
| Follicle Stimulating Hormone Receptor | 1XWD | *Homo sapiens* | 2.92 | X-ray diffraction |
| Steroidogenic Acute Regulatory protein | 3H3Q | *Homo sapiens* | 2.0 | X-ray diffraction |

| | | | | |
|---|---|---|---|---|
| 3-beta hydroxysteroid dehydrogenase | 1HXH | *Comamonas testosteroni* | 1.22 | X-ray diffraction |
| Aromatase | 3S79 | *Homo sapiens* | 2.75 | X-ray diffraction |
| Estrogen Receptor alpha | 5KRO | *Homo sapiens* | 2.1 | X-ray diffraction |
| Estrogen Receptor beta | 2J7Y | *Homo sapiens* | 1.8 | X-ray diffraction |
| Androgen receptor | 2AM9 | *Homo sapiens* | 1.64 | X-ray diffraction |
| Progesterone receptor | 1A28 | *Homo sapiens* | 1.8 | X-ray diffraction |
| Phosphorylated Insulin Receptor tyrosine kinase | 1IR3 | *Homo sapiens* | 1.9 | X-ray diffraction |

### 2.3.4. Absorption, Distribution, Metabolism, Elimination and Toxicity (ADMET) Predictions

A good drug candidate should not only have sufficient efficacy against the therapeutic target, but also show appropriate ADME properties at a therapeutic dose (Guan et al., 2019). A lot of *"in-silico"* models are hence developed for prediction of ADME properties of probable drug molecules. The absorption, distribution, metabolism, and excretion properties were calculated by SwissADME web-based tool, which predicts physically significant and physiochemical descriptors of potential drug compounds (Daina et al., 2017). The SwissADME web tool presented here was freely accessible at http://www.swissadme.ch and was used for fast prediction of physicochemical properties (molecular weight, H-bond donors and acceptors, LogP(o/w) values), pharmacokinetics (GI absorption, blood brain barrier permeability, CYP enzymes inhibitors, P-Gp substrate, skin permeation), lipophilicity, water solubility, drug-likeness (Lipinski's rule of five, Bioavailability score), and medicinal chemistry friendliness (lead likeness) of the partially purified non-polar phytochemicals isolated from *Aloe vera* gel.

*"In-silico"* studies underpinned the potential of isolated partially purified non-polar phytocomponents of *Aloe vera* gel as steroidogenic and metabolic modulators. To further validate the targets, *"in-vitro"* steroidogenesis modulatory bioassay was performed in KGN cell-line under normal physiological conditions.

### 2.3.5. Effect of Isolated Partially Purified Phytocomponents on the Cell Viability of KGN cell-line

The effect of isolated partially purified phytocomponents on the cell viability of KGN cell-line was evaluated by MTT assay and the $IC_{50}$ values of the isolates were estimated. KGN cell-line is a steroidogenic human granulose-like tumour cell-line and it possesses properties similar to those of normal granulose cells: 1) Expression of functional Follicle Stimulating Hormone receptor, 2) High Aromatase activity and 3) Production of progesterone and pregnenolone upon luteinization. Cells were seeded in a 96-well flat-bottom microtiter plate at a density of $1 \times 10^4$ cells/well and allowed to adhere for 24 hours at 37°C in a $CO_2$ incubator. After 24 hours of incubation, culture medium was replaced with fresh serum free DMEM/F12 medium for next 24 hours. The isolated semi-purified phytocomponents (LP1 to LP5) were dissolved in DMSO and the final concentration of DMSO was below 0.5% v/v in all experiments. Cells were then treated with various concentrations of isolated phytocomponents and crude petroleum ether extract of *Aloe vera* gel in a dose dependent manner (0.5 to 500ng/ml) for 24 hours at 37°C in a $CO_2$ incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium and 10µL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 2-3 hours at 37°C in a $CO_2$ incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 100µL of DMSO per well for 30 min at 37°C in a $CO_2$ incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 570 nm and 620nm. The IC50 values were expressed as Mean$\pm$SEM.

### 2.3.6. "*In- vitro*" Steroidogenesis Modulatory Bioassay

$1\times10^6$ cells were seeded in 6 –well plates having 1 ml of DMEM/ F12 (1:1) media containing 10% Fetal Bovine serum, 1% Penstrep (100 U/ml Penicillin, 100 µg/ml Streptomycin) and 50 ng/ml human menopausal gonadotropin-HUMOG (LH:FSH::1:1) for 24 hours at 37°C in a CO2 incubator. The cells were serum starved for another 24 hours before addition of the compounds. To determine whether the isolated phytocomponents have an inhibitory or stimulatory steroidogenic modulatory potential, they were compared with 1µM Letrozole (Antagonist) and 10µM Forskolin (Agonist) respectively. The isolates and other chemicals were dissolved in DMSO and the final concentration of DMSO was below 0.5% v/v in all experiments. The cells were treated on the basis of IC50 values—25ng/ml of LP1, 9ng/ml of LP2, 32ng/ml of LP3, 4ng/ml of LP4, 29ng/ml of LP5 and 70ng/ml of petroleum ether extract

of *Aloe vera* gel for 24 hours at 37°C in a CO2 incubator. After 24 hours, 10nM Testosterone was added to the treated and untreated cells and incubated for 24 hours at 37°C. At the end of the treatment period, spent medium was collected at different time intervals and stored at -20°C until assayed for hormonal content. For protein expression analysis, cells were lysed and western blot analysis was performed.

### 2.3.7. Hormone Estimation

Spent media collected after giving treatments and were lyophilized and then dissolved in 1ml of Phosphate buffered saline. It was used as samples to estimate the hormones- testosterone, estradiol and progesterone levels using kit-based direct ELISA, procured from Diagnostics Biochem Canada (DBC) as per the manufacturer's instructions.

*"In-vitro"* steroidogenesis modulatory bioassay was competent in screening the most potential bioactives from the petroleum ether extract of *Aloe vera* gel. However, these phytochemicals need to be tested for their potential to recover the dysregulated steroidogenesis and thereby, pose a potent therapeutic towards management of ovarian pathology like polycystic ovarian syndrome (PCOS). Therefore, an attempt was made to develop *"in-vitro"* and *"in-vivo"* PCO models and evaluate the efficacy of the bioactives on them.

## 2.4. "*In-vitro*" Analysis in PCO-like Ovarian Cellular Models

*"In-vitro"* analysis was performed using two different kinds of cells:

i) primary culture of rodent derived granulosa luteal cell culture and
ii) human derived-KGN cell-line.

Further, an attempt was made to develop an "*in-vitro"* PCO- like ovarian microenvironment in these cells.

### 2.4.1. Primary Culture of Rodent-derived Granulosa Luteal Cells

#### 2.4.1.1. Animals

Forty weaning virgin Charles Foster female rats were chosen for the study which were housed in standard controlled animal care facility, in cages (four rats/cage), and maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12: 12-hour dark-light cycle. The animals were maintained under standard nutritional and environmental conditions throughout

the experiment. All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature. The Control and Supervision of Experiments on Animals (CPCSEA) guidelines were strictly followed and all the studies 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/2016/09).

#### 2.4.1.2. Granulosa Luteal Cell Isolation from Rat Ovaries

The rats were super-ovulated in batches of four animals, by injecting 10 I.U of Pregnant Mare's Serum Gonadotropin (PMSG) subcutaneously. After 48 hours, 50 I.U of human Chorionic Gonadotropin (hCG) was injected to the animals intra-peritoneally. After 24 hours, the ovaries from four super-ovulated rats were excised and processed for isolation of granulosa- lutein cells by the Campbell method (1979). In this procedure, ovaries were initially punctured and then incubated in EGTA followed by hypertonic sucrose at 37°C. The ovaries were then gently squeezed to release the cells into Hank's Balanced Salt solution (HBSS) and further centrifuged over 45% Percoll to remove RBCs. Luteal cells were isolated from the interface, washed 3-4 times with HBSS and resuspended in 1 ml of Modified serum- free DMEM/F12 media (Media Composition: DMEM/ F12 (1:1), 0.1% BSA, Insulin (10 ng/ml), hMG (50 ng/ml), 5 µg/ml Transferrin, 1% Penstrep (100 U/ml Penicillin, 100 µg/ml Streptomycin) and the cell yield was estimated using trypan blue exclusion method.

#### 2.4.1.3. Characterization of Isolated Luteal Cells

Cultures of luteal cells were tested for cell type purity by analysis of the transcript abundance of key molecular targets such as Follicle-stimulating Hormone Receptor (*Fshr*), Luteinizing Hormone Receptor (*Lhr*), Steroidogenic acute regulatory protein (*Star*), and Aromatase (*Cyp19a1*). The crude cell isolates were counted with a hemocytometer and diluted to $1\times10^5$ cells/ ml and cultured in 1.0 ml of modified media in 35-mm polystyrene tissue culture treated dishes. Cells were collected at different time intervals of 24 hours upto 72 hours and their RNA was extracted using RNAiso Plus reagent and reverse transcribed to form cDNA. The relative expression of the molecular targets mentioned above was studied by Real-time quantitative polymerase chain reaction (qPCR). The details of RNA isolation, cDNA preparation, primers and Real time qPCR has been mentioned in the later section.

### 2.4.2. KGN cell-line

$1\times10^6$ cells were cultured in 2.0 ml of DMEM/ F12 (1:1) media containing 10% Fetal Bovine serum, 1% Penstrep (100 U/ml Penicillin, 100 μg/ml Streptomycin) and 50 ng/ml human menopausal gonadotropin-HUMOG in 35-mm polystyrene tissue culture treated dishes and the culture conditions were humidified atmosphere of 5% CO2 and 95% air at 37ºC. The doubling time for KGN cell-line was estimated to be around 46 hours and the plates became confluent in 3-4 days.

### 2.4.3. Culture and development of PCO- like Ovarian microenvironment

$1\times10^6$ primary granulosa luteal cells as well as KGN cells were cultured independently in modified serum- free DMEM/F12 media and DMEM/F12 media containing 10% FBS respectively and the culture conditions were humidified atmosphere of 5% CO2 and 95% air at 37ºC for 24 hours. After 24 hours, the cells were incubated with insulin [0.1 – 2 IU/ml (0.7 – 13.9 nM)] and Dihydro-testosterone (DHT) [10 – 100 ng/ml (34.4 – 344 μM)] treatments separately as well as in combinations for 36 hrs. The concentrations of insulin and DHT used in these experiments are significantly greater than the concentrations present in the normal women. However, the concentrations used in these experiments are within an order of magnitude of the concentrations present in the patients with severe insulin resistance (Abbott et al., 2013). DHT (non-aromatizable substrate) was used as an androgen for assessment of the appropriate AR action. At the end of the treatment period, spent medium was collected at different time intervals and stored at -20°C until assayed for hormonal content. For gene expression analysis, cells were lysed for RNA extraction as per the protocols.

### 2.4.4. Effect of Hyperandrogenic and Hyperinsulinemic conditions on the Cell Viability

Firstly, the effect of hyperandrogenic and hyperinsulinemic conditions on the cell viability of both the types of ovarian cell culture was firstly evaluated independently and then followed by co-incubations of insulin and DHT by performing MTT assay. Cells were seeded in a 96-well flat-bottom microtiter plate at a density of $1 \times 10^4$ cells/well and allowed to adhere for 24 hours at 37°C in a $CO_2$ incubator. After 24 hours of incubation, culture medium was replaced with a fresh medium. Cells were then treated with various concentrations of insulin and DHT, independently as well as in combination for 36 hours at 37°C in a $CO_2$ incubator. After 36 hours of incubation, culture medium was replaced with a fresh medium and 10μL of MTT

working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 2-3 hours at 37°C in a $CO_2$ incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 100µL of DMSO per well for 30 min at 37°C in a CO2 incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 570 nm and 620nm.

### 2.4.5. Validation of *"in-vitro"* PCO- like model

The doses of insulin were fixed as 0.1 mIU/mL and 2.0 mIU/mL for primary granulosa luteal cells and KGN cell-line respectively. However, the doses of Androgen (DHT) were varied (10, 25, 50 and 100ng/mL) when co-administered with the minimum effective dose of insulin in both the cell types and the gene expression of steroidogenic targets (*Lhr, Fshr, Star, Cyp19a1, Cyp17a1, Hsd3b1, Amh and Ar*) were evaluated by real time PCR. Also, the hormone secreted by the cells in the spent media was evaluated by ELISA. The minimum effective dose of insulin and DHT to mimic ovarian microenvironment present in PCO using *"in vitro"* primary culture of rodent derived luteinized granulosa cells and human derived KGN cell-line was evaluated.

After development of an "*in-vitro"* PCO-like ovarian microenvironment in both- primary granulosa luteal cell culture and KGN cell-line, an *"in-vitro"* steroidogenic modulatory bioassay was performed. Both models showed dysregulated steroidogenesis, which is comparable to that seen in PCOS, and may be utilized to explore detailed downstream cellular signaling as well as screen therapeutic candidates for ovarian dysfunctions such as PCOS, ovarian cancer, and infertility. However, the cell- yield of rodent- derived granulosa luteal cells was quite low. In order to obtain sufficient cells for performing the bioassay, large numbers of animals were required to be sacrificed, which is not ethically feasible. Therefore, we opted to use only the hyper androgen and hyper insulin induced *"in-vitro"* PCO model of KGN cell-line for evaluating the bioactivity of LP1 and LP3.

### 2.4.6. Effect of Isolated Partially Purified Phytocomponents on the *"In-vitro"* PCO-like Model

Following the validations, *"in-vitro"* PCO-like model of KGN cell-line were incubated with 25ng/mL of LP1, 9ng/mL of LP3 and 70ng/mL of petroleum ether extract of *Aloe vera* gel. The doses of the phytochemicals were decided based upon the $IC_{50}$ values obtained in the previous chapter. The potential of these bioactives to modulate the steroidogenic targets and

restore the dysregulated steroidogenesis was evaluated by performing transcriptional analysis of key steroidogenic targets like *Lhr, Fshr, Star, Cyp19a1, Cyp17a1, Hsd3b1, Amh, Ar, Pgr, Esr-1* and *Esr-2* in the cells. The hormones (Testosterone, Estradiol and Progesterone) were estimated from the spent media as per the previously mentioned protocol.

As PCOS is a multi-factorial disorder and cross talk between several pathways play an important role towards the etiology of this pathology, therefore, an *"in-vitro"* analysis has certain limitations. Hence, an *"in-vivo"* PCO model was developed and the efficacies of the isolated bioactive compounds of *Aloe vera* gel were studied in it.

### 2.5. "*In-vivo*" Analysis in PCOS Rodent Model

#### 2.5.1. Animal Housing and Maintenance

One hundred twenty-eight adult virgin (3-4 months) Balb/c female mice weighing 20-25g were chosen for the study which were housed in standard controlled animal care facility, in cages (four mice/cage), and maintained in a temperature-controlled room (22-25°C, 45% humidity) on a 12: 12-hour dark-light cycle. The animals were maintained under standard nutritional and environmental conditions throughout the experiment. All the experiments were carried out between 9:00 and 16:00 hours, at ambient temperature. All the studies 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/2018/12).

#### 2.5.2. PCOS Mouse Model

PCOS mouse model was developed using Letrozole, a non-steroidal aromatase inhibitor. 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 4°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 mortar-pestle and the powder was weighed and mixed with 0.5 ml of 1% CMC and administered orally to animal in a dose and time dependent manner. Three doses- 0.1; 0.5 and 1.0 mg letrozole/kg body weight were given orally daily to the animals at two different time points- 11 days and 21 days. The animals were validated for PCOS based upon the Rotterdam criteria (2003). To be diagnosed with PCOS by the Rotterdam criteria, a woman must have two of the following three manifestations: irregular or absent ovulation, elevated levels of androgenic hormones, and/or enlarged ovaries containing at least 12 follicles each. In case of animals, any two of the following indicates PCOS- arrested and extended estrus cycle, elevated serum testosterone levels and presence of multiple peripheral cysts in the ovary (>6) upon ultrasound or ovarian histological analysis.

### 2.5.3. Drug administration and experimental design

Firstly, the animals were categorized into two major groups- Control (n=56) and PCOS (n=72). The Control animals received 1% Carboxymethyl cellulose (CMC) orally every day for 21 days. The PCOS group was given oral dose of Letrozole (0.5mg/kg body weight) daily for 21 days. For validation of PCOS, body weight, oral glucose tolerance test, HOMA-IR, estrus cyclicity, serum hormone profile and ovarian histology was performed after 21 days of Letrozole administration. Further, the animals were distributed into 10 groups of 8 animals/group as following:

**Group I** (C 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.

**Group III** (PCOS +AVG group) PCOS animals treated with oral doses of Aloe vera gel (10 mg/ day) for 60 days (Maharjan et al., 2010).

**Group IV** (PCOS +PE group) PCOS animals treated with oral doses of Petroleum ether extract of Aloe vera gel (25µg/kg/day) for 60 days (Radha and Laxmipriya, 2016a).

**Group V** (PCOS +LP1 group) PCOS animals treated with oral doses of PPNPP- LP1 (5 µg/kg/day) for 60 days.

**Group VI** (PCOS +HA group) PCOS animals treated with oral doses of pure compound-n-Hexadecanoic acid (5 µg/kg/day) for 60 days.

**Group VII** (PCOS +LP3 group) PCOS animals treated with oral doses of PPNPP- LP3 (0.5 µg/kg/day) for 60 days.

**Group VIII** (PCOS +CA group) PCOS animals treated with oral doses of pure compound-Campesterol acetate (0.5 µg/kg/day) for 60 days.

**Group IX** (PCOS + Metformin group) PCOS animals treated with oral dose of Metformin (100 mg/kg/day) for 60 days and served as Positive Control (Zaafar et al., 2014).

**Group X** (PCOS+ DMSO group) PCOS animals treated with oral dose of DMSO (1%, 0.2ml/day) for 60 days and served as Vehicle/ Negative Control

The doses of LP1 and LP3 were decided as 5 µg/kg/day and 0.5 µg/kg/day based upon their abundance in petroleum ether extract of *Aloe vera* gel as per the GC/MS analysis, so that the outcomes are comparable.

After 60 days of the treatments, both metabolic and reproductive features were evaluated. The treatments were continued until the animals were sacrificed. Throughout the whole treatment period, the animals were weighed weekly and their estrus cyclicity was measured daily. Blood samples were collected by cardiac puncture. The plasma was separated and kept in a freezer at − 80 °C for determining the levels of plasma hormones, lipid profile, toxicity parameters. At the end of experiment, the animals were sacrificed in diestrus stage and one of the ovaries were dissected from all groups and stored in RNAiso Plus reagent at -20 °C for the gene expression studies. The remaining ovary from all the groups were dissected and stored in 10% buffered formalin, for the histopathological investigations.

### 2.5.3. Oral Glucose Tolerance Test (OGTT) and HOMA-IR

After 60 days of the treatment; 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: 65-90mg/dl and rat-85-100 mg/dl respectively (Sun et al., 2016; Wang et al., 2010).

The homeostasis model assessment of insulin resistance (HOMA-IR) is a simple and particularly helpful tool in the assessment of insulin resistance and can be calculated using the equations, HOMA-IR = FI × FG/22.5, where FI is fasting insulin (in μU/mL) and FG is fasting glucose (in mmol/L). HOMA-IR<2.60 refers to normal range, HOMA-IR 2.60–3.80 as borderline high" without labelling as insulin resistant, and HOMA-IR>3.80 as "high" having clear correlates of insulin resistance (Qu et al., 2011).

### 2.5.4. Lipid Profile

Plasma lipid profile (Total Cholesterol, HDL-Cholesterol, LDL-Cholesterol and triglycerides) was measured using commercial kits (Reckon Diagnostics P. Ltd., India). The catalogue numbers of the kits were as follows: Total Cholesterol (CC2-CLE.005), LDL-Cholesterol (CC3-LDL.022), HDL-Cholesterol (CC2-HDC.013) and Triglycerides (CC2-TGS.18M) respectively. All the reagents in the kits were ready to use. The reagents hydrolysed the fat in the sample, which was then acted upon by an oxidase and then a peroxidase, which formed a coloured complex that was assayed spectrophotometrically at 505nm for Total Cholesterol, HDL-Cholesterol, LDL- Cholesterol and 520nm for Triglycerides. Assays were performed according to manufacturer's protocol using isolated plasma from the animals.

### 2.5.5. Estrus Cyclicity

Estrus cyclicity was monitored during the whole period of treatment by examining vaginal lavage. Cells on vaginal walls were collected by washing vagina with normal saline (0.9% NaCl) and smeared on glass slides. The slides were examined under microscope for the relative

abundance of different types of cells. Predominant nucleated epithelial cells and some cornified epithelial cells indicated the proestrus stage; predominant cornified squamous epithelial cells indicated the estrus stage; both cornified squamous epithelial cells and leukocytes indicated the metestrus stage; and predominant leukocytes indicated the diestrus stage.

### 2.5.6. Hormone Estimation

Blood plasma was used as samples to estimate the insulin, testosterone, estradiol and progesterone levels using ELISA kits (DiaMetra for insulin and Diagnostics Biochem Canada (DBC)- for testosterone, estradiol and progesterone measurement). All measurements were taken according to the manufacturer's instructions.

**Sample preparation:**

Animals were fasted overnight and blood was collected at diestrus stage in the morning between 8 and 9 AM. Plasma 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 manufacturer's protocol.

### 2.5.7. Histological Examination of the Ovaries

**Reagents:**

i) 10% neutral buffered formalin (NBF):
   Formalin: 10 %
   $NaH_2PO_4.H_2O$: 0.4 %
   $Na_2HPO_4$: 0.65 %

Ovaries from 6 different animals from each group 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 were screened for and micrographs were taken on a Leica DM2500 microscope through a Leica EZ camera.

### 2.5.8. Estimation of Toxicity Markers

The animals were treated as per the experimental design provided in Section 2.5.3. Following which, the animals from control and treatment groups were removed from their cages and examined for any sign of toxic effect on their body weights. The body weight of each animal was weighed before the test, weekly during the study 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, pilorection, breathing rhythm, and reaction to handling) were monitored once daily.

The analysis of biochemical parameters is also very important when evaluating the toxic effect of plant extracts or isolated phytochemicals. At the end of the 81 days of treatment, 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 plasma was isolated and analyzed for the biochemical parameters like total cholesterol, high density lipoproteins (HDL), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine. The total cholesterol, HDL-cholesterol and triglyceride levels were estimated by using the commercial kits (Reckon Diagnostics P. Ltd., India). The liver and kidney are more predisposed to toxic effects of xenobiotics, as it is the major site for their metabolism and excretion (George, 2011). Hence, liver and kidney toxicity key indicators such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine levels were measured in all groups of animals after 81 days of different treatment regimens. The ALT and AST enzymes were determined by the colorimetric technique of Reitman and Frankel (1957). Creatinine level in plasma was estimated by alkaline picrate method (Jaffé, 1886). The detailed procedure is mentioned below:

### 2.5.8.1. Alanine aminotransferase (ALT) activity

(Reitman and Frankel, 1957)

**Principle:** Alanine aminotransferase transfers amino group from alanine to α- keto glutarate & converts it into pyruvate. This reacts with 2,4-Dinitrophenylhydrazine (DNPH). The resulting hydrazone of pyruvate is highly colored and its absorbance at 540 nm is proportional to ALT activity.

ALT

L-Alanine + α-keto glutarate ⟶ Pyruvate + Glutamate

Pyruvate + DNPH ⟶ Brown color in alkaline medium

**Sample Preparation:** The isolated plasma was used for the estimation.

**Reagents:**

I. Phosphate Buffer: 15g of potassium phosphate dibasic anhydrous ($K_2HPO_4$) and 2g of potassium dihydrogen ortho phosphate ($KH_2PO_4$) was dissolved in 200ml of distilled water. The pH was adjusted to 7.4 and the final volume was made upto 1 litre.

II. ALT Substrate: 1.78 g of alanine, 30 mg of α-keto glutaric acid, 20 ml of 1N NaOH in phosphate buffer. The pH was adjusted to 7.4 and finally the volume was made up to 100 ml with distilled water.

III. DNPH Reagent: 20 mg of dinitrophenylhydrazine in 8.5 ml of conc. HCl. Final volume was made up to 100 ml with distilled water.

IV. 0.4 N NaOH

V. 44 mg/dl Sodium Pyruvate std. (Range:100-500 nmoles)

**Procedure:**

| | **Blank** | **Test** |
|---|---|---|
| Plasma | 0.05 mL | 0.05 mL |
| Substrate | - | 0.25 mL |
| Distilled Water | 0.25 mL | - |
| Incubate at 37 ˚ C for 30 min | | |
| DNPH | 0.25 mL | 0.25 mL |
| Incubate at Room Temperature for 20 min | | |
| 0.4 M NaOH | 2.5 mL | 2.5 mL |
| Read at 540 nm | | |

**Calculations:** ALT= $\frac{\text{O.D. } \times 1000 \times \text{ sample dilution}}{\text{Slope} \times 30 \text{ (Incubation time)}}$

**Units:** nmoles of pyruvate formed/min/lit

**Normal Range:** 5-35 IU/dL

**2.5.8.2. Aspartate aminotransferase (AST) activity**

**(Reitman and Frankel, 1957)**

**Principle:** Aspartate aminotransferase transfers amino group from aspartate to α- keto glutarate & converts it into Oxaloacetate. This reacts with 2,4-Dinitrophenylhydrazine (DNPH). The resulting hydrazone of pyruvate is highly colored and its absorbance at 540 nm is proportional to AST activity.

Aspartate + α-keto glutarate $\xrightarrow{\text{AST}}$ Oxaloacetate + Glutamate

Oxaloacetate + DNPH $\longrightarrow$ Brown color in alkaline medium

**Sample Preparation:** The isolated plasma was used for the estimation.

**Reagents:**

I. Phosphate Buffer: 15g of potassium phosphate dibasic anhydrous ($K_2HPO_4$) and 2g of potassium dihydrogen ortho phosphate ($KH_2PO_4$) was dissolved in 200ml of distilled water. The pH was adjusted to 7.4 and the final volume was made upto 1 litre.

II. AST Substrate: 66 g of aspartic acid, 30 mg of α-keto glutaric acid, 20 ml of 1N NaOH in phosphate buffer. The pH was adjusted to 7.4 and finally the volume was made up to 100 ml with distilled water.

III. DNPH Reagent: 20 mg of dinitrophenylhydrazine in 8.5 ml of conc. HCl. Final volume was made up to 100 ml with distilled water.

IV. 0.4 N NaOH

V. 22 mg/dl Sodium Pyruvate std. (Range: 100-500 nmoles)

**Procedure:**

| | **Blank** | **Test** |
|---|---|---|
| Plasma | 0.05 mL | 0.05 mL |
| Substrate | - | 0.25 mL |
| Distilled Water | 0.25 mL | - |
| Incubate at 37 ˚ C for 60 min | | |
| DNPH | 0.25 mL | 0.25 mL |
| Incubate at Room Temperature for 20 min | | |
| 0.4 M NaOH | 2.5 mL | 2.5 mL |
| Read at 540 nm | | |

**Calculations:** AST= $\frac{\text{O.D.} \times 1000 \times \text{sample dilution}}{\text{Slope} \times 60 \text{ (Incubation time)}}$

**Units:** nmoles of pyruvate formed/min/lit

**Normal Range:** 8-38 IU/dL

**2.5.8.3. Estimation of Creatinine**

**(Jaffé, 1886)**

**Principle:** Creatinine reacts with picric acid in alkaline medium to form a reddish yellow complex, intensity of which is directly proportional to the concentration of creatinine in sample and can be measured at 520 nm.

**Sample Preparation:** 0.3 mL of picric acid was added to 0.1 mL of isolated plasma and then centrifuged at 3000 g for 15 minutes. Supernatant was used for the assay.

**Reagents:**

I. Picric acid: 0.91gm/dl (40mM)

II. 0.75 M NaOH

III. Working Creatinine Std: (10-50 µg) in distilled water.

Procedure:

| | **Blank** | **Test** |
|---|---|---|
| Supernatant | - | 0.2 mL |
| Distilled Water | 0.4 mL | 0.2 mL |
| Picric Acid | 0.2 mL | 0.2 mL |
| 0.75M NaOH | 0.2 mL | 0.2 mL |
| Read at 520 nm | | |

**Calculations: Creatinine (mg/dL)** = $\frac{\text{O.D.} \times \text{sample dilution} \times 100}{\text{Slope}}$

### 2.6. Gene Expression Analysis

The details of the methodology opted for transcriptional analysis of both *"in-vitro"* as well as *"in-vivo"* analysis has been duly mentioned below.

#### 2.6.1. RNA Isolation

1. Diethyl pyrocarbonate (DEPC) water

1 ml of DEPC was added to 1 litre of distilled water and kept overnight on magnetic stirrer for complete homogeneous solution. The solution was autoclaved and used for 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.

**RNA isolation protocol**

Isolation: For RNA isolation from cells, $1\times10^6$ cells were a pre-requisite. Firstly, the media was removed and 1 ml of TRIzol reagent was directly added to the culture plates, incubated at cold condition for 5-7 minutes. The cells were scraped and pipetted several times and collected in fresh tubes. Tissues (10-100mg) were homogenized in 1 ml of TRIzol reagent. This was followed by addition of 200µl Chloroform in both the cases. 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 separated with a visible white interphase. The lower organic layer was transferred to a fresh tube and was used further for protein isolation. 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 (overdrying was avoided since it can lead to trouble with dissolution of the RNA in water). After excess ethanol had dried off, the pellet obtained from cells or tissues were dissolved in 10 µl and 30 µl of DEPC water respectively.

### 2.6.2. Quantification: RNA was quantified using UV spectrophotometer

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 spectrophotmeter. The following formula was used to analyse the quantity of RNA:

$RNA$ (µg/µl) = 40 × $OD_{260}$

A ratio of the $OD_{260}$ to $OD_{280}$ was used to check for quality of the RNA preparation. A ratio >1.8 was accepted as indicative of 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 good integrity of the RNA.

### 2.6.3. cDNA SYNTHESIS

Isolated RNA was reverse transcribed using Verso cDNA synthesis kit (Thermo Scientific™, USA). The cDNA reaction was carried out for tissues and cells 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 following components were added | |
| 5X RT Buffer | 4 µl |
| Reverse Transcrptase (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.

### 2.6.4. REAL-TIME PCR

For the current study, all quantitative gene expression analyses were carried out by real time PCR using PowerUp™ SYBR™ Green master-mix (Applied Biosystems, Thermo Fisher Scientific™) on a QuantStudio 5.0 Real Time PCR system (Life Technologies, USA). Primers were procured from IDT (CA, USA). (Table 2.3)

**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 |
| DEPC 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 | 40 cycles |
| 60°C | 1 min | |

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 |

**Data analysis:**

Cq values were obtained for each well; normalized Cq values were calculated by subtracting the Cq values of internal control gene or reference gene (beta-Actin) from those of the target gene. Mean of these normalized Cq values were 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 vertical axis following a logarithmic scale.

## 2.7. STATISTICAL ANALYSIS

Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test, using GraphPad Prism 5 software. For each experiment 6-8 animals were used from both the groups. All values are presented as Mean+SEM). P-values when less than 0.05 were considered to be statistically significant at 95% confidence limit.

**Table 2.3: List of primers used in the study**

| Genes | Accession Number | Sequence (5' →3') |
|---|---|---|
| *Actb* | NM_031144 | CCCGCGAGTACAACCTTCTTG |
| | | GTCATCCATGGCGAACTGGTG |
| *Amh* | NM_007445 | CTGGCTGAAGTGATATGGGA |
| | | CACGGTTAGCACCAAATAGC |
| *Ar* | NM_013476.4 | TATGGGGACATGCGTTTGGA |
| | | AGGTCTTCTGGGGTGGAAAG |
| *Cyp17a1* | NM_012753.2 | ATGATCCAAAACTGACCGCC |
| | | AACCCTTTATCACCTCCAAGCC |
| *Cyp19a1* | NM_017085 | GCTTCTCATCGCAGAGTATCCGG |
| | | CAAGGGTAAATTCATTGGGCTTGG |
| *Esr1* | XM_017313797.1 | AATTCTGACAATCGACGCCAG |
| | | GTGCTTCAACATTCTCCCTCCTC |
| *Esr2* | NM_207707.1 | GACGAAGAGTGCTGTCCCAA |
| | | GGGGTACATACTGGAGTTGAGG |
| *Fshr* | NM_013523 | ACGCCATTGAACTGAGATTTG |
| | | GTTGGAGAACACATCTGCCT |
| *Hsd3b1* | NM_012584 | TCTACTGCAGCACAGTTGAC |
| | | ATACCCTTATTTTTGAGGGC |
| *Insr* | NM_017071 | GCCTGGGCAACTGTTCAGA |
| | | GTTTCGACAGGCCACACACTT |
| *Lhr* | NM_012978.1 | GCTTTACAAACCTCCCTCGG |
| | | GCGAGATTAGAGTCGTCCCA |
| *Pgr* | NM_008829.2 | ATGGTCCTTGGAGGTCGTAA |
| | | CAACACCGTCAAGGGTTCTC |
| *Star* | NM_031558.3 | AGTGACCAGGAGCTGTCCTA |
| | | GCGGTCCACCAGTTCTTCATA |