CHAPTER 3

MATERIALS & METHODOLOGY

3.1 MATERIALS

3.1.1 ANIMALS

Adult Charles foster female rats weighting 200 ±15 g were used for the study. All rats were housed in cages and maintained in ambient temperature of 25±1°C and 45.5% relative humidity, with a photoperiod cycle of 12 h:12 h (light and dark) with access to standard pellet food (Sai Durga Feeds and Foods, Bangalore, India) and water. Each experiment was priorly approved by the Institutional Animal Ethics Committee, according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

3.1.2 CHEMICALS

Bovine serum albumin (BSA), Folin & Ciocalteu's (FC) phenol reagent Nicotinamide adenine dinucleotide reduced (NADH), Ethylenediaminetetraacetic acid (EDTA), n-butanol, Sodium dihydrogen phosphate dehydrate (NaH2PO4.2H2O), Copper sulphate (CuSO4.5H2O), Sodium hydroxide (NaOH), Di-potassium Hydrogen Phosphate (K2HPO4), p-Nitrophenol (PNP), Sodium Dodecyl Sulphate (SDS), 1-chloro-2,4dinitrobenzene (CDNB), Tris-base, Ethidium bromide, Ethylene Glycol Tetra Acetic acid (EGTA), Trypan blue, Ammonium persulphate (APS), Tween-20, Phenyl Methane Sulfonyl Fluoride (PMSF), Stigmasterol, para-Nitrophenylphosphate (pNPP) from SRL, Mumbai.

Diethyl pyrocarbonate (DEPC), Agarose, Glycine, β-mercaptoethanol, Acrylamide, Bis-acrylamide, Tetramethylethylenediamine (TEMED), Triton-X 100 from Himedia.

β-Sitosterol, Lupeol, PCR Master Mix purchased from Sigma Aldrich, India. Analytical grade solvents like Methanol, Petroleum Ether, Toluene, Ethyl acetate, Formic acid, Glacial Acetic acid, were obtained from Qualigens (Mumbai, India.). Precoated silica gel aluminium plate 60 F254, (10 cm × 10 cm with 250 μm thickness was procured from Merck, Mumbai, India. Primers were designed by PRIMER BLAST software from National Centre for Biotechnology Information (NCBI) and purchased from Sterling Biotech Ltd., Mumbai, India. Primary antibodies were purchased from Cell Signalling Technology (CST) and Santa Cruz Biotechnology (SCBT). Secondary antibody was

purchased from Merck Millipore, Germany. cDNA synthesis kit was procured from Thermo Fisher Scientific, Mumbai, India.

3.2 METHODOLOGY

3.2.1 INDUCTION OF PCOS RAT MODEL

Charles foster virgin female rats (2-3months) exhibiting regular estrus cyclicity, weighing 180-225 g were maintained under controlled conditions of light and temperature; with having free access to diet and water. Animals were treated orally with Letrozole (0.5 mg/kg body weight) (Kafali et al., 2004) daily for 21 days, while control group received Carboxymethylcellulose (1% CMC) as vehicle. After 21 days of treatment, PCOS rat model was validated by various biochemical parameters as follows:

3.2.2 ORAL GLUCOSE TOLERANCE TEST (OGTT)

(Buchanan et al., 1991)

OGTT was performed after 12 hours of fasting. The blood sample was collected in fasting condition and following which glucose was fed (1gm/kg body weight) orally to the rats. After glucose load, blood was periodically collected at the time interval 30', 60', 90', 120' for blood glucose estimation. Glucose was assayed at all the specified time points by kit (Enzopak, India) based on GOD POD principle.

Glucose (GOD-POD method)

Principle:

The method is an enzyme coupled reaction for the estimation of blood glucose. Glucose Oxidase (GOD) catalyses the oxidation of aldehyde group of glucose in the presence of oxygen to form gluconic acid with liberation of H_2O_2. Peroxidase (POD) converts H_2O_2 into water and active oxygen which reacts with chromogen 4-amino anti-pyrine to form a pink colored complex which can be estimated colorimetrically at 505nm.

Sample Preparation: Blood was collected in fresh vial coated with Sodium Flouride (NaF) (6mg/ml). NaF is an anticoagulant and it inhibits glycolytic pathway. The blood was centrifuged at 1200 g for 15 minutes for plasma separation. The plasma was collected into fresh vials and immediately processed for glucose estimation.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{4-amino antipyrene} + \text{Phenol} \xrightarrow{POD} \text{Red colored quinol} + H_2O$$

Reagents:

i) Enzyme GOD-POD

ii) Glucose std. (100mg/ml)

iii) Chromogen

Procedure:

REAGENT	BLANK	STANDARD	TEST
Enzyme	1.0 ml	1.0 ml	1.0 ml
Std.	-	0.01 ml	-
Sample	-	-	0.01 ml

Mix well and incubate for 5 Min at 37°C. Mix & read absorbance at 505nm against blank.

Calculation: Glucose concentration (mg/dl) $= \dfrac{\text{Absorbance of Sample x 100}}{\text{Absorbance of STD}}$

Unit: mg/dl

Normal Values:
- Fasting: - 70-110 mg/dl
- Two hour post prandial :- up to 140 mg/dl

3.2.3 ESTRUS CYCLE

Cyclicity was checked in all rats by vaginal smear wherein saline dipped ear bud was inserted into vagina of the rat slowly and uniform smear was made on glass slide. These slides were observed under light microscope (10x). Each stage of the cycle was confirmed by types of cells were observed under microscope given below.

Estrus cycle with vaginal changes:

- **Pro-estrus:** Thickening of epithelium, Leucocytes no longer migrate through the epithelium. Cells were small, round, nucleated epithelial.
- **Estrus:** Outer layer of epithelial cells become cornified and sloughed into lumen. In the beginning of phase, cell retain nuclei, but in later stage no nuclei visible. These cells were irregular, flat and cornified.

- **Metaestrus:** Deeper layers of estrus epithelium line the lumen, superficial layer become cornified & sloughed off with reduction of mitotic activity in epithelium. Many Leucocytes & few cornified cells were seen.
- **Diestrus:** Thinning of epithelium & leucocytes of stroma migrate through the epithelium in the vaginal lumen. Stringy mucous with many leucocytes a few nucleated epithelial cells are seen.

3.2.4 HORMONAL PROFILE

Sample Preparation: Blood samples were collected in fresh vials and centrifuged at 1200 g for 15 minutes to separate serum from whole blood. Separated serum sample then transferred into another fresh vial.

Serum was collected from all the rats used for hormonal profile. Serum hormones levels were checked in all animal groups using ELISA kits procured from Diametra Inc, Germany. Sensitivity of the kits was as follows: Insulin -2μIU/ml; Testosterone -0.075 ng/ml; Progesterone -0.05 ng/ml and 17 β-estradiol (8.68pg/ml) at 95% confidence limit. The variability within run replicate is 2% for Insulin, 4.6% for Testosterone; < 5.9% (Progesterone), < 9% (17 β-estradiol) whereas between assay variability was 6% (Insulin), 7.5% (Testosterone), < 10.5% (Progesterone), < 10% (17 β-estradiol).

Procedure: Samples were processed according to Kit (Meril Diagnostics, Canada) protocol. In this method, Serum samples were added [For Insulin 100μl, Progesterone 20μl, Testosterone 25 μl, Estradiol 25 μl] in pre-coated ELISA plate. The plate was incubated for 2 hours in case of insulin and 1hour for rest of steroids. After incubation, the plate was washed 3 times with 300 μl wash buffer and added 100μl of TMB (3,3',5,5'-Tetramethylbenzidine) substrate with 15 minutes incubation in dark. Then 100μl of Stop solution was added to each well to stop the reaction. Shake gently and read at 450 nm.

3.2.5 HISTOLOGICAL ANALYSIS

Ovaries were removed and were fixed in 4% aqueous solution of formaldehyde, often called 10% formalin. Histological examinations of ovary from all groups were

carried wherein trans-section of 5 µm thickness cut and stained with Hematoxylin- Eosin stain and images were taken for each group under Leica light microscope under 4 x magnifications.

3.2.6 3 BETA- AND 17 BETA-HYDROXY STEROID DEHYDROGENASE

(Shivanandappa and Venkatesh, 1997)

Principle:

3β HSD converts DHEA to Androstenedione & 17β HSD converts 17 Estradiol to Estrone. In both reactions, NAD is reduced to NADH, which is coupled to the Iodo Nitro Tetrazolium Chloride (INT) and gives formazan, a pink coloured chromophore that absorbs light at 490nm.

$$\text{DHEA} + \text{NAD} \xrightarrow{3\ \beta\ \text{HSD}} \text{Androstenedione} + \text{NADH}$$

$$\text{17Estradiol} + \text{NAD} \xrightarrow{17\ \beta\ \text{HSD}} \text{Estradiol} + \text{NADH}$$

Sample Preparation: Make 10% homogenate of tissues (ovary, uterus, adrenal, hypothalamus, pituitary) in 0.1 M Tris-Cl buffer (pH- 7.8) and centrifuge 10,200 g for 30 min at 4ºC. Supernatant was used as a source of enzyme assay.

Reagents:

i) Phthalate buffer (50mM, pH-3.0): To 51 ml of N/10 (0.1 N) HCl add 2.5 ml of Tween 20. Mix well & add 2.55 g Potassium Hydrogen Phthalate to the mixture. Adjust to pH 3.0 and make up to volume to 250 ml with distilled water.

ii) 0.1M Tris-HCl buffer (pH-7.8)

iii) 0.5mM NAD (Freshly Prepared)

iv) Colour reagent: Dissolve 40 mg INT in 50 ml of distilled water and add 0.5 of Tween 20. For the Standard solution add 10 mg PMS. (Store in Dark)

v) Substrate: (Freshly Prepared)

- DHEA (1mM): Dissolve 1mg of DHEA in 50-100 µl of DMFO. Make up the volume to 35 ml with distilled water.
- 17Estradiol (1mM): Dissolve 1 mg of Estradiol in 50-100 µl of 100% Ethanol. Make up the volume to 36.8 ml with distilled water.

vi) Standard (Range: 25 -175 nmoles): 1mM stock NADH solution

Reagents I, II and IV are stored at 4ºC and are stable for 3 months.

Procedure:

	Reagents	TEST	BLANK
1	0.1 M Tris-Cl (pH-7.8)	1.0 ml	1.0 ml
2	NAD	1.0 ml	1.0 ml
3	Substrate (Estradiol/Testosterone)	1.0 ml	1.0 ml
4	INT	0.1 ml	0.1 ml
5	Enzyme source	0.1 ml	0.1 ml
Incubate at 37°C for 1 hour			
6	Phalate buffer (pH-3.0)	2.0 ml	2.0 ml

Mix well the tubes and read at 490 nm.

Calculation: Calculation was done according to the standard graph.

Unit: nmoles of NADH formed/min/mg protein.

3.2.7 PROTEIN ESTIMATION

(Lowry et al., 1951)

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

Reagents:

i) Lowry's Reagent:

Reagent A—2 % Sodium Carbonate dissolved in 100 ml of 0.01N NaOH

Reagent B—1 % Copper Sulfate

Reagent C—2 % Sodium Potassium Tartarate in 100 ml of Distilled Water

To make 100 ml of Lowry's reagent (Reagent-D) of reagent A with 1 ml of reagent C & 1 ml of reagent B.

ii) Folin Ciocalteau reagent (FC reagent): [Freshly prepared]

Mix equal volume of FC reagent and D/W (1:1 ratio).

iii) BSA: [Range: 5-50µg] Dissolve BSA (10 mg) in 10 ml of D/W (Standard concentration 1mg/ml).

Procedure:

REAGENT	BLANK	TEST
Aliquot	-	0.01 ml
Distilled Water	0.1 ml	0.09 ml
Reagent-D	1.0 ml	1.0 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 Dark)		
Read absorbance at 660 nm		

Calculation: Calculation was done according to the standard graph.

Unit: mg/ml

3.2.8 ALKALINE PHOSPHATASE (ALP)

(Bowers and McComb, 1975)

Principle: The enzyme reacts with p-Nitrophenyl Phosphate (PNNP) & converts it into p-nitrophenol (PNP), which is yellow in colour. PNP in alkaline medium gives bright yellow which can be measured at 405nm.

Sample preparation: Implanted area from uterus was excised and 10 % homogenate was made in glycine buffer and centrifuged at 900 g for 10 min. Supernatant was used as enzyme source. Serum was also source of enzyme for Toxicological parameters.

Reagents:

1. 0.4% p-Nitrophenyl phosphate (PNPP) in distilled water.
2. 0.05 M NaOH
3. Glycine buffered substrate: Mix 5.7g of glycine and 0.095 g of anhydrous $MgCl_2$ in 750 ml of distilled water, add 85ml of 1N NaOH and make upto 1L.
4. Working substrate: Mix equal volume of glycine buffer substrate and stock PNPP. Adjust pH to 10.3-10.4.
5. Standard PNP solution (Range: 0.04-0.16 µmole): stock 1mM in 0.05N NaOH (Prepared freshly)

Procedure:

	TEST	BLANK
1. Buffered substrate	1.0 ml	-
Place tubes in water bath at 37°C for 5 minutes.		
2. Sample (Tissue/Serum)	0.05 ml	-
Incubate at 37°C for 39 minutes.		
3. 0.05 N NaOH	10 ml	10 ml
Read at 405 nm.		

Calculation: Calculation was done according to the standard graph.

Unit: µmoles of PNP formed/min/mg of protein

3.2.9 CATHEPSIN– D

(Anson, 1937)

Principle:

Cathepsin D is a lysosomal endopeptidase and known to have role in physiological & pathological breakdown of intracellular & extracellular protein. Cathepsin – D activity is measured by degradation of Haemoglobin (Hb) which leads to liberation of acid soluble tyrosine & tryptophan containing peptides. These peptides are quantitatively measured by sensitive reaction of Folin's Ciocalteau reagent & giving the absorbance at 660 nm.

Sample preparation: Implanted area from uterus was excised and 10 % homogenate was made in Acetate buffer and centrifuged at 100 g for 5 min. The supernatant was separated in a fresh vial and centrifuge at 10,200 g for 30 min. The supernatant was again centrifuged at 10,200 g for 30 min. Supernatant was separated in fresh vial and used as enzyme source.

Reagents:

i. Acetate Buffer (pH- 3.5):
   -0.1 M Acetic acid
   - 0.1 M Sodium acetate
ii. Haemoglobin (Hb) Substrate solution (10g/100 ml)
iii. 0.3 M TCA
iv. 0.5 M NaOH

v. Folin Ciaceau's reagent: (1:2) dilution

vi. Std. Solution: 1mM tyrosine in 2.5 %TCA. Use it after 10-20 minutes after it is dissolved properly.

Standard: Standard graph for Cathepsin D was prepared in range 4-40 µmole

	TEST	BLANK
1. Sample	0.2 ml	-
2. Distilled water	-	0.2 ml
3. Substrate	0.1 ml	0.1 ml
Shake vigorously and keep at 37°C for 10 minutes		
4. 0.3 M TCA	2.0 ml	2.0 ml
Shake and stand tubes at RT for 30 minutes and filter through Whatman filter paper no.3		
5. Filtrate	1.0 ml	1.0 ml
6. 0.5 M NaOH	2.0 ml	2.0 ml
7. FC reagent	0.6 ml	0.6 ml
Mix well and wait for 5 minutes & Read at 750 nm		

Calculation : Calculation was done according to the standard graph.

Units : µmole of tyrosine formed /min/ mg protein

3.2.10 UDP-GLUCURONYL TRANSFERASE:

(Gorski and Kasper, 1977)

Principle: UDP-Glucoronyl transferase catalyses transfer of D-glucoronic acid from UDP α- glucoronic acid to a compound with inversion at the C-1 carbon of the sugar to yield the β-glucoronide. The degree of conjugation was based on the disappearance of p-nitrophenol absorbance in the visible region. The ratio of glucoronide formation was calculated directly from the absorbance change.

Sample Preparation: 10% homogenate of liver tissue was made in 0.25 M sucrose solution. and centrifuged at 100 g for 5 min.. The supernatant was separated in a fresh

vial and centrifuged at 10,200 g for 30 min. The supernatant was again centrifuged at 10,200 g for 30 min. The supernatant was separated in fresh vial and used as enzyme source.

Reagents:

i) 0.4 M Phosphate Buffer (pH-7.4)

ii) 40mM $MgCl_2$

iii) 1.6 mM p-Nitrophenol (PNPP)

iv) 20 mM UDP-glucoronic acid

v) 0.2 N TCA

vi) 0.5 N NaoH

vii) Standard PNP (p-Nitrophenol) solution (Range: 20-200 µmoles): 1mM stock solution

Reagent	Test	Blank
Buffer	0.09 ml	0.130 ml
UDPGA	0.02 ml	-
Mgcl2	0.02 ml	.02 ml
PNPP	0.02 ml	-
Enzyme	0.05 ml	0.05 ml
Incubate 37ºC for 10 minutes		
TCA	0.2 ml	0.2 ml
Centrifuge at 2000 g for 20 minutes and take 0.250 ml of supernatant		
0.5M NaOH	0.750 ml	0.750 ml
Measure absorbance at 450 nm		

Calculation : Calculation was done according to the standard graph

Unit: µmoles of PNP conjugated/min/mg protein

3.2.11 Hydroxy Steroid Oxidoreductase (HSOR)

Principle:

17β HSD converts 17β -Estradiol to Estrone. In both reactions, NAD is reduced to NADH, which is coupled to the Iodo Nitro Tetrazolium Chloride (INT) and gives

formazan, a pink coloured chromophore that absorbs light at 490 nm.

Sample Preparation: 10% homogenate of liver was made in 0.25 M sucrose solution and centrifuge at 100 g for 5 minutes and the supernatant was separated. The supernatant was again centrifuged at 10,200 g for 30 min at 4ºC. Supernatant was use as a source of enzyme assay.

Reagents:

i) Phthalate buffer (50mM, pH-3.0): To 51 ml of N/10 (0.1 N) HCl add 2.5 ml of Tween 20. Mix well & add 2.55 gm Potassium Hydrogen Phthalate to the mixture. Adjust to pH 3.0 and make up to volume to 250 ml with distilled water.

ii) 0.1M Tris-HCl buffer (pH-7.8)

iii) 0.5mM NAD (Freshly Prepared)

iv)Colour reagent: Dissolve 40 mg INT in 50 ml of Distilled Water & to which, add 0.5 of Tween 20. For the Standard solution add 10 mg PMS. (Store in Dark)

v) Substrate: (Freshly Prepared): 17β- Estradiol (1mM): Dissolve 1 mg of Estradiol in 50-100 µl of 100% Ethanol. Make up the volume to 36.8 ml with distilled water.

vi) Standard solution (Range: 25- 175 nmoles): 1 mM stock solution of NADH

Reagents I, II and IV are stored at 4ºC and are stable for 3 months.

Procedure:

	Reagents	TEST	BLANK
1	0.1 M Tris-Cl (pH-7.8)	1.0 ml	1.0 ml
2	NAD	1.0 ml	1.0 ml
3	Substrate (Estradiol)	1.0 ml	1.0 ml
4	INT	0.1 ml	0.1 ml
5	Enzyme source	0.1 ml	0.1 ml
Incubate at 37°C for 1 hr			
6	Phalate buffer (pH-3.0)	2.0 ml	2.0 ml

Mix well the tubes and read at 490 nm.

Calculation : Calculation was done according to the standard graph

Unit: nmol of NADH formed/min/mg protein.

3.2.12 GLUTATHIONE-S-TRANSFERASE (GST)

(Habig et al., 1974)

Principle: The reaction is measured by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). This is analysed by an increase in absorbance at 340nm. One unit of enzyme will conjugate 10.0 nmol of CDNB with reduced glutathione per minute at 25°C.

Sample Preparation: 10% homogenate of liver tissue was made in 0.25 M sucrose solution. and centrifuged at 100 g for 5 min.. The supernatant was separated in a fresh vial and centrifuged at 10,200 g for 30 min. The supernatant was again centrifuged at 10,200 g for 30 min. The supernatant was separated in fresh vial and used as enzyme source. Serum was also source of enzyme for toxicological parameters.

Reagent:

i) 1mM Na_2EDTA in 0.1 M Sodium phosphate buffer

ii) 20mM GSH

iii) 20mM CDNB (1-chloro-2,4 Di nitro benzene) dissolved in 100% ethanol

REAGENTS		VOLUME
1	Buffer	0.850 ml
2	20 mM Reduced glutathione	0.050 ml
3	20 mM CDNB	0.050 ml
	Auto zero & reaction was initiated by adding enzyme	
4	Sample	0.050 ml

The increase in absorbance was monitored for about 2 minutes at 340 nm.

The enzymes activity was calculated using the extinction coefficient. E_{412}=9.6 ($mmol^{-1}cm^{-1}$)

Change in absorbance (DA340)/minute, in the linear range of the plot, for the sample and for the blank using the following equation:

$$(DA340)/min = \frac{A_{340}(\text{final read}) - A_{340}(\text{initial read})}{\text{reaction time (min.)}}$$

$$\text{GST activity} = \frac{\Delta A_{340}\ \text{min-1 x dilution factor}}{0.0096\ \mu\text{mol-1 cm-1 x mg of protein}}$$

Unit: Amount of Enzyme that catalyzes the conjugation of 1 µmole of CDNB per min per mg protein.

3.2.13 LIPID PROFILE

3.2.13.1 TRIGLYCERIDES

Principle:

Lipoprotein lipase hydrolyses triglycerides sequentially into Di and Monoglycerides and finally to glycerol. Glycerol Kinase (GK), using ATP as phosphate source, converts Glycerol liberated to Glycerol-3-Phosphate (G-3-Phosphate). G-3-Phosphate Oxidase (GPO) oxidizes G-3-Phosphate formed to Dihydroxy acetone phosphate (DHAP) and hydrogen peroxide is formed. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidize 4- minoantipyrine and DHBS (3,5 Dicloro-2-hydroxy benzene sulphate) to form a red coloured complex. The absorbance of the coloured complex is measured at 520nm.

Triglycerides + H2O $\xrightarrow{\text{Lipase}}$ Glycerol + fatty acids

Glycerol + ATP $\longrightarrow$ Glycerol-3-Phosphate + ADP

Glyccerol-3-Phosphate + O2 $\xrightarrow{\text{GPO}}$ Dihydroxyacetone phosphate + H2O2

H2O2 + 4-Aminoantipyrine + DHBS $\xrightarrow{\text{POD}}$ Quinoneimine + H2O

Sample Preparation: Blood was collected in fresh vial without any anticoagulant and centrifuged at 1200 g for 15 minutes. The clear supernatant comprising of serum was transferred into fresh vial and used for the estimation.

Reagents:

i) Lipase Reagent

ii) Triglycerides Standard (200mg/dl)

iii) Triglycerides (buffer)

REAGENT	BLANK	STD	TEST
Working Reagent	1.0 ml	1.0 ml	1.0 ml
Std.	-	0.02 ml	-
Sample	-	-	0.02 ml

Mix and incubate at 37°C for 5minutes and read absorbance of test and standard against reagent blank at 520nm (Green filter).

Calculation:

$$\text{Triglycerides (mg/dl)} = \frac{\text{Abs of Sample}}{\text{Abs of Standard}} \times 200$$

Unit: mg/dl ; **Normal Values**: Females: 45 -145mg/dl

3.2.13.2 CHOLESTEROL

Principle:

Cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (CE). Free cholesterol is then oxidized by cholesterol oxidase (CO) to cholesten-4-en-3-one. H2O2 is also produced simultaneously which reacts with 4-aminoantipyrine and phenol in the presence of peroxidase and forms pink-colored quinonemine dye which is read at 505nm.

$$\text{Cholesterol esters} \xrightarrow{CE} \text{Cholesterol + fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CO} \text{Cholesterol-4-en-One} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AAP} \xrightarrow{POD} \text{Quinoneimine dye} + 4H_2O.$$

Sample Preparation: Blood was collected in fresh vial without any anticoagulant and centrifuged at 1200 g for 15 minutes. The clear supernatant comprising of serum was transferred into fresh vial and used for the estimation.

Reagents:

i) Cholesterol L (ready to use)

ii) Cholesterol Standard (200mg/dl)

Procedure:

REAGENT	BLANK	STD	TEST
Working Reagent	1.0 ml	1.0 ml	1.0 ml
Std.	-	0.01 ml	-
Sample	-	-	0.01 ml

Mix and incubate at 37°C for 10 minutes and read absorbance of test and standard against reagent blank at 520nm at room temperature.

Calculation:

$$\text{Cholesterol concentration (mg/dl)} = \frac{\text{Abs of Sample X 200}}{\text{Abs of Standard}}$$

Unit: mg/dl

Normal Values: 100 - 250mg/dl.

3.2.13.3 HDL-CHOLESTEROL

Principle:

High density lipoproteins (HDL) are separated from other lipoprotein fractions by treating serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipitated; cholesterol content of which is estimated by enzymatic method.

Serum + Phosphotungstic acid (PTA) ⟶ Supernatant + Precipitates (other fractions).

Sample Preparation: Blood was collected in fresh vial without any anticoagulant and centrifuged at 1200 g for 15 minutes. The clear supernatant comprising of serum was transferred into fresh vial and used for the estimation.

Reagents:

i) HDL – Cholesterol (Precipitating Reagent)
ii) HDL – Cholesterol standard (50mg/dl)
iii) Standard- 50 mg/dl

Procedure:

REAGENT	BLANK	STD	TEST
Working Reagent	1.0 ml	1.0 ml	1.0 ml
Std.	-	0.05ml	-
Sample	-	-	0.05 ml

Mix well and incubate at 37°C for 10minutes and read absorbance of test and standard against reagent blank at 520nm.

Calculation:

$$\text{Serum HDL Cholesterol (mg/dl)} = \frac{\text{Abs of Sample X 50 (Dilution Factor) X 2}}{\text{Abs of Standard}}$$

Unit: mg/dl

Normal Values:

Females: - 45 -65mg/dl

3.2.13.4 LDL CHOLESTEROL

Estimated by calculation:

LDL Cholesterol = [Total Cholesterol - HDL Cholesterol – Triglycerides/5] mg/dl

3.2.14 SERUM GLUTAMATE PYRUVATE-TRANSAMINASE (SGPT) ACTIVITY

(Reitman and Frankel, 1957)

Principle: Glutamate pyruvate transaminase transfers amino group from alanine to α-keto glutarate (α-KG) & converts it into pyruvate. Thus formed is reacted with 2, 4 Di Nitro Phenyl Hydrazine (DNPH). The resulting hydrazone of pyruvate is highly colored and its absorbance at 540 nm is proportional to GPT activity.

L-Alanine + α-keto gluterate $\xrightarrow{\text{SGPT}}$ Pyruvate + Glutamate

Pyruvate + DNPH $\longrightarrow$ Brown colour in alkaline medium

Sample Preparation: Blood was collected in fresh vial and allowed to stand for half n hour. Later, samples were centrifuged at 1200 g for 15 minutes. The clear supernatant comprising of serum was transferred into fresh vial and used for the estimation.

Reagents:

I. SGPT 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.
II. DNPH Reagent: 20 mg of dinitrophenylhydrazine in 8.5 ml of conc. HCl. Final volume was made up to 100 ml with distilled water.
III. 0.4 N NaOH
IV. 44 mg/dl Sodium Pyruvate std. (Range: 22-110μg)

Procedure:

	Test	Blank
1. Serum	0.05 ml	0.05 ml
2. Substrate	0.25 ml	-
3. Distilled Water	-	0.25 ml
Incubate at 37°C for 30 min		
4. DNPH	0.25 ml	0.25 ml
Incubate at RT for 20 min		
5. 0.4M NaOH	2.5 ml	2.5 ml
Read at 540 nm		

Normal Range: 6-72 IU/dl

$$\text{SGPT} = \frac{\text{O.D. x1000 x sample dilution}}{\text{Slope x 30 (Incubation time)}}$$

Unit: µmoles of pyruvate formed/min/lit

3.2.15 SERUM CREATININE

(Bonsnes and Taussky, 1945)

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: Blood was collected in fresh vial and allowed to stand for half an hour. Later, samples were and centrifuged at 1200 g for 15 minutes. The clear supernatant comprising of serum was transferred into fresh vial.1.5 ml of Picric acid was added to 0.5 ml of Serum and then centrifuged at 3000 g for 15 minutes. Supernatant 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:

Reagent	TEST	BLANK
1. Supernatant	1.0 ml	-
2. Distilled Water	1.0 ml	2.0
3. Picric acid	1.0 ml	1.0 ml
4. 0.75 M NaOH	1.0 ml	1.0 ml

Read at 520 nm.

$$\text{Creatinine (mg/dl)} = \frac{\text{O.D. x sample dilution x 100}}{\text{Slope}}$$

3.2.16 GRANULOSA CELL ISOLATION

(Campbell, 1979)

Sample: Animals were sacrificed in Proestrus stage of estrus cyclicity and the ovaries were excised and placed in tubes containing cold sterile HBSS.

Reagent:

i) Hank's Balanced Salt Solution (HBSS)[250 ml]:

-2 g NaCl

- 0.1 g KCl

-0.015 g Na_2HPO_4

-0.015g $KH2PO_4$

-0.0625 g $NaHCO_3$

-0.25 glucose

Mixture was dissolved in minimum volume. Adjust pH at 7.2 and later volume was made up to 250 ml.

ii) EGTA (6.8mM) containing BSA (0.2%) solution

iii) 0.5M Sucrose with 1.8mM

iv) HBSS containing EGTA (2mM)

v) Trypan Blue (0.18%)

Procedure:

i) Ovaries were centrifuged at 100 g for 5 minutes to remove fat in 2 ml of HBSS solution at 4°C.
ii) Ovaries were suspended in 2 ml of EGTA solution and incubated for 15 minutes at 37°C and later centrifuge at 100 g for 5 minutes.
iii) These ovaries were suspended in 2 ml of hypertonic sucrose solution and incubated for 5 minutes in ice and centrifuge at 100 g for 5 minutes.
iv) Ovaries were expressed for cells using blunt forceps in 2 ml of HBSS solution in cold condition. After expressing cells, cells were again centrifuged at 1500 rpm for 8 minutes at 4°C.
v) Cells were suspended in 2 ml of HBSS containing EGTA & centrifuge at 100 g for 5 minutes.
vi) Pellet was washed with HBSS solution 3-4 times to remove fat and cell debris.
vii) Final pellet was suspended in 100-200 µl of HBSS solution.
viii) 10 µl of cells were stained with Trypan blue & counted in16 small squares of 4 corner squares of the Hematocytometer.
ix) No. Of cells were calculated by taking the average no. of cells presented per square.
x) No. Of cells= Avg of no. of cells in all 4 Squares X 10^4 X dilution Factor
Expression Unit= No. of cells/ mg ovary.
Sonication protocol: Ampliture 45 %, Total Time : 20 sec, On 2 sec, Off 2 sec

3.2.17 RNA ISOLATION BY TRIZOL METHOD

1. For 50-100 mg of tissue, 1.0 ml of TRIZOL reagent was added and the tissues were homogenized in cold condition.
2. Homogenized sample was incubated at room temperature for 5 minutes.
3. 200 µL of chloroform (per 1mL Trizol) was added and shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes.
4. Samples were centrifuged at 1250 g at 4°C for 15 minutes to separate phases.
5. Upper (clear) aqueous layer was transferred to a fresh tube.

6. 0.5mL of isopropanol was added to the aqueous layer, mixed gently and incubated in -20°C for 1 hr.
7. The samples were centrifuged at 1250 g at 4°C for 15 minutes to pellet RNA.
8. The supernatant was carefully removed and 1ml of 75% DEPC-ethanol was added to wash the pellet thoroughly.
9. Samples were centrifuged at 7500 g at 4°C for 5 minutes to re-pellet RNA.
10. Carefully remove the supernatant and air-dry the pellet at room temperature for 5-10 minutes.
11. Dissolve the pellet in sterile DEPC distilled water (10-100uL, depending on yield) by gentle pipetting and quantitate the concentration by 260/280 nm ratio.

3.2.18 RT PCR (REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION)

- All reagents were thawed and stored on ice.
- RNAse and DNAse free work environment was maintained.
- The individual reagents were thoroughly mixed and spinned down.

i) **cDNA preparation:**

RNA 2µL (2µg) was added into fresh PCR tube along with 3 µL DEPC-treated water. It was incubated at 65°C for 10 minutes and kept in ice. To that, 3µL of 5X 1st strand buffer was added along with 1.0µL dNTPs, 0.5µL oligo dT primer, 0.5µL reverse transcriptase and incubated at 42°C for 45 minutes. The cDNA was stored in -20°C for further use.

ii) **PCR conditions:**

1µL of cDNA (transcribed from 2 µg of RNA) was added to fresh PCR tube and 5µL Ready to use Master Mix was added along with 1µL dNTPs, 1µL forward primer, 1µL reverse primer, 1µL sterile distilled water. Mix the tubes in microfuge and PCR program appropriate to the primers was run.

iii) **PCR program:**

Step	Time
Initial denaturation Time	94°C, 2 min
Denaturation	94°C, 2 min
Annealing (According to primer Tm value	30 sec – 2 min
Extension	72°C, 30 sec- 2 min
Repeat 25-35 cycles	
Final extension	72°C, 10 min
Hold	4°C , 2 min then end the program

iv) Gel Electrophoresis: Run the PCR amplicon on 1.2 % agarose gel with 100 bp ladder.

v) 50 X TAE Buffer [100 ml]

Tris-base 40mM - 24.2 g

Glacial acetic acid - 5.71 ml

0.5 M EDTA -10 ml

Make upto 70 ml with distilled water, set the pH 8.4 and Make upto volume 100 ml with distilled water.

vi) 5X TBE Buffer [200 ml]

Tris base (44mM)- 10.8 g

Boric acid - 5.5 g

0.5 M EDTA - 4.0 ml

Make upto 150 ml with distilled water, set the pH 8.4 and Make upto volume 200 ml with distilled water.

vii) RNA Loading Dye (2x)

500mM EDTA (pH-8.0)

37% Formaldehyde

20% Glycerol

1% Formamide

0.1% Bromophenol blue

10% Electrophoresis buffer

viii) 6x Loading Dye (1ml)

0.2 % Bromopheol buel

20 % Sucrose

ix) Primers of rat targeted genes

Targeted Genes	Primer sequence	Annealing temperature (^{0}C)	Reference
Aromatase	F: 5'GCTTCTCATCGCAGAGTATCCGG 3' R: 5'CAAGGGTAAATTCATTGGGCTTGG 3'	60	NM_017085
FSH β	F: 5' AGGAAGAGTGCCGTTTCTGC 3' R: 5' GCTGTCACTATCACACTTGC 3'	53	Schirman-Hildesheim et al.2008
LH β	F: 5' CTGTCCTAGCATGGTTCGAGT 3' R: 5' AGTTAGTGGGTGGGCATCAG 3'	58.4	Schirman-Hildesheim et al.2008
FSHR	F: 5' CTCATCAAGCGACACCAA 3' R: 5' GGAAAGGATTGGCACAAG 3'	54	Cavalcante et al. 2013
LHR	F: 5' GCTTTTACAAACCTCCCTCGG 3' R: 5' GCGAGATTAGAGTCGTCCCA 3'	55	NM_012978
ER α	F: 5' CCAGTCGAGCATCACTTA 3' R: 5' TGGCGTCGATTGTCAGAA 3'	53	Suzuki M and Nishihara, 2002
ER β	F: 5' TAGACAACCGCCATGAGT 3' R: 5' CACAACTGCTCCCACTAA 3'	54	Suzuki M and Nishihara, 2002
AR	F: 5' GGAAGCACTGGAACATCT 3' R: 5' GTAGTCGCGATTCTGGTA 3'	53	Suzuki M and Nishihara, 2002
PR	F: 5' CGCCCTACCTCAACTACCTG 3' R: 5' TTCTCAGACGACATGCTGGAC 3'	56	NM_022847
GAPDH	F:5'CAAGGTCATCCATGACAACTTTG 3' R:5'GTCCACCACCCTGTTGCTGTAG 3'	58.1	NM_017008

3.2.19 SDS PAGE AND WESTERN BLOT (WB)

Sample preparation: Make 10 % homogenate in Lysis buffer, centrifuge aat 900 g for 10 minutes to pellet down cell debris then sonicate the sample twice (Ampliture 45 %, Total Time : 20 sec, On 2 sec, Off 2 sec)

Sample used: Ovary, Placenta

Reagents:

i) 1.5M Tris-Cl buffer (pH-8.8)

ii) 0.5 M Tris-Cl buffer (pH-6.8)

iii) SDS Electrophoresis buffer (1L)
-3 g Tris base (25 mM)
-14 g Glycine (1.92mM)
-1 g SDS (1%)

iv) 30% acrylamide solution (100 ml):
-Acrylamide: 30 g
- Bisacrylamide: 0.9g

v) Coomassive stain (100 ml):
-0.1% Commassie blue
-40% Methanol
-10% Acetic acid
- 50% distilled water

vi) Lysis Buffer (For 50 ml)
- Tris-Cl (pH-7.5) 50 mM
- NaCl 200mM
- $CaCl_2$ 20mM
- Triton-X 100%
- Protease Inhibitor 1mM

vii) 2x SDS Loading dye:
10mM Tris-Cl (pH -6.8)
4% SDS
0.2% Bromophenol blue
20% Glycerol
10 % β-mercaptoethanol

viii) Phosphate buffer saline (10X) (1 lit):
- 80g of NaCl
- 2.0g of KCl
- 14.4g of Na_2HPO_4
- 2.4g of KH_2PO_4

Adjust pH to 7.4, adjust volume to 1L with additional distilled H_2O and sterilize by autoclaving.

ix) PBS-T: (PBS containing 0.1 % Tween 20)[Add 0.5 ml of tween 20 into 100 ml of 10x PBS and volume make upto 1 litre]

x) Blocking buffer/PBSMT (5% non-fat dry milk in PBS-T buffer)

xi) Transfer Buffer(10x):[Tris-base - 15.14 g, Glycine - 72.06 g]

To make 1x transfer buffer, add 100 ml of 10x Transfer buffer into 200 ml of Methanol and volume make upto 1 litre with distilled water. Make fresh 1x transfer buffer prior transfer and keep in cold condition.

xii) Primary antibody: Primary antibodies dilutions made according to

standardized protocol in PBS-MT along with 1mM NaN_3 (Final concentration) and kept in 4°C.

xiii) Secondary antibody: 1:2500 dilution in PBS-MT

Preparation of SDS PAGE gel: [20 ml]

Reagents	12% (ml)	10% (ml)	6% (ml)
Distilled water	6.6	7.9	10.6
30% Acrylamide	8.0	6.7	4.0
Running gel buffer (pH-8.8, 1.5M)	5.0	5.0	5.0
10% SDS	0.2	0.2	0.2
10% (APS)	0.2	0.2	0.2
TEMED	0.02	0.02	0.02

Preparation of stacking gel: [10 ml]

Reagents	(ml)
Distilled water	6.9
30%Acrylamide	1.66
Running gel (pH-6.8, 0.5M)	1.26
10% SDS	0.1
10% (APS)	0.1
TEMED)	0.01

Antibodies used in the present study:

Primary antibodies dilutions			
No.	Antibodies	Source	Dilution
1	Androgen Recepter (AR)	Rakesh Tyagi, JNU, India	1:1000
2	StAR	Stocco, Texas Tech University, Texas, USA	1:2000
3	3β-HSD	Prof. Ian Mason, University of Edinburgh, France	1:2000
4	β-actin	CST, # 4967	1:10,000
5.	P450arom	CST , #8799	1:1000
Secondary antibody dilution			
	Conjugated Anti-rabbit IgG	Puregene, GX1202E-3	1:2500

Procedure:

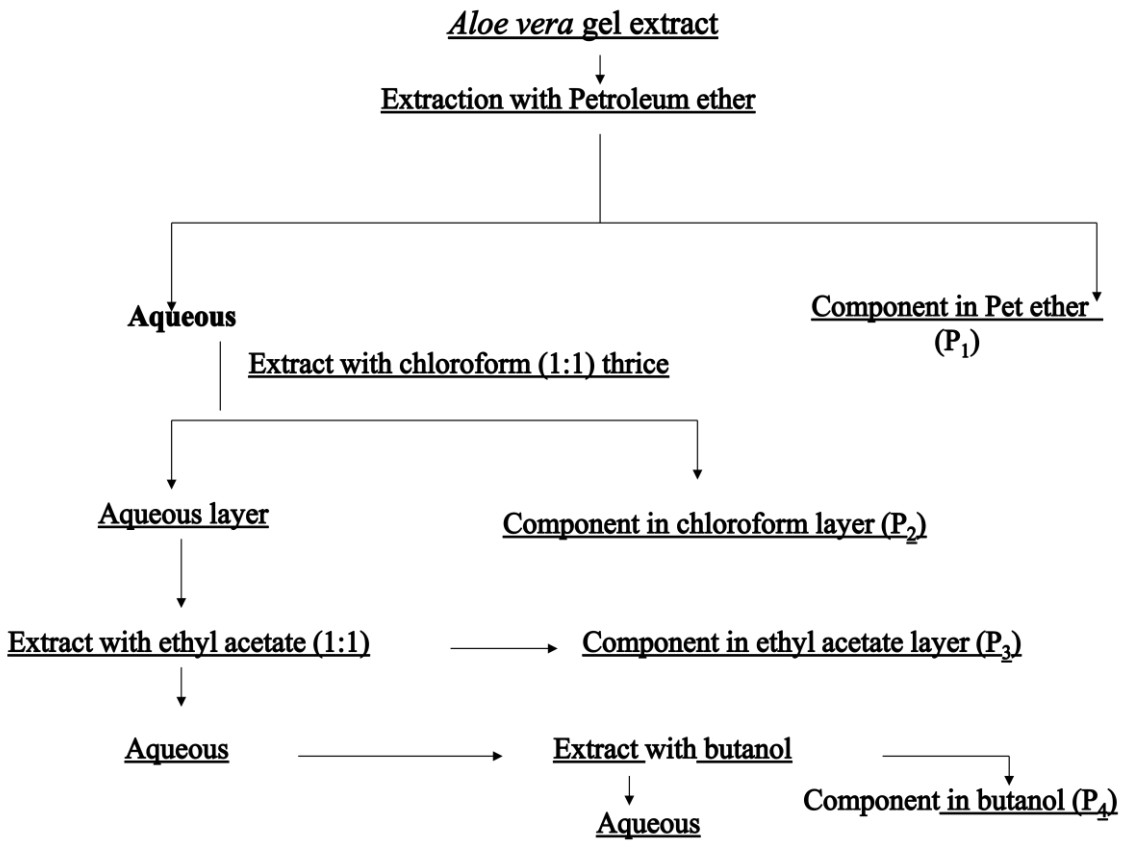
1) The protein molecular marker (14-97.3 kDa) and 40 µg of samples were loaded on SDS PAGE wells and run at constant voltage (100 V, 50 current) until the dye front of the samples reach the bottom of the gel.
2) The proteins were transferred from the gel to a nitrocellulose or PVDF membrane in transfer unit containing transfer buffer at 4°C, at 100 V for 1.5 hours.
3) After the transfer is completed, the membrane was stained with Ponceau-S to identify the mark of the molecular weight.
4) 20 ml of blocking buffer was added and membrane was incubated for 1 hour at room temperature with shaking.
5) The primary antibody was diluted in PBSMT as mentioned in table.
6) The membrane was incubated with the diluted antibody overnight (~16 hrs) at 4°C with shaking.
7) The membrane was washed 4 times with PBST for 15 minutes each.

8) The secondary antibody conjugate was diluted 1:2500 in PBSMT.
9) The membrane was incubated with the diluted antibody for 1 hour at room temperature with shaking.
10) The membrane was washed 3 times with PBST for 15 minutes each. This was followed by 2 washes of PBS for 10 minutes each.
11) Finally, an appropriate detection method utilizing DAB (3, 3'-diaminobenzidine) was used. Horseradish peroxidase (HRP), which is bound to the secondary antibody, catalyzes the oxidation of DAB in the presence of peroxidase. The oxidation product of DAB polymerizes to form a brown, sharply localized insoluble product, which can be easily detected by visible light.

3.2.20 Preparation of Aloe vera extract

***Aloe barbadensis* Mill.** (Voucher no. PSN 723) was compared with the specimen (Bhatt 2486, 653, 279, JVJ 448) lying with the nationally recognized BARO Herbaria of the Department of Botany, The M.S. University of Baroda, Vadodara, Gujarat, India. Fresh mature *Aloe vera* leaves (3.5 years old) were taken and washed with water. Later, the leaves were incised with the sterilized knife and allowed to stand for 2 hours in order to remove the aloin. Later, the gel was removed by separating the epidermis and later gel was sonicated to get a homogenous gel.

3.2.21 PREPARATION OF THE PETROLEUM ETHER EXTRACT (P1)



3.2.22 PHYTOCHEMICAL SCREENING

Major groups of phytochemical constituents	Test	Reagents and assay procedure	Inference
Steroidal Glycosides	Baliget test	Solution A: 1% Picric acid in Ethanol Solution B: 10% NaOH Solution A and Solution B were mixed in 1:1 ratio and added drops by drop to 1.0 ml of sample.	Orange to deep red colour

	Killer-Killani Test	1ml of extract was mixed with 2ml of Glacial acetic acid containing 1-2 drops of 2% $FeCl_3$.To this 2ml 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	Solution: 1 ml chloroform + 1 ml acetic anhydride cooled to 0°C and few drops of conc. H_2SO_4 was added. This reagent was added to the sample.	Blue, Green, Red, Orange colour change with time
	Salkouski Test	1mL Chloroform + 1ml conc. H_2SO_4 was added to 1ml of the sample.	Formation of Red colour
Alkaloids	Mayor's test	Solution A: Mercuric chloride (1.358 g) + 60 mL distilled water. Solution B: KI (5.0g) + 10 mL of distilled water. Both solutions were mixed and the final volume was made up to 100 mL with distilled water. To this reagent few drops of Sample was added.	A white or creamy precipitate

	Wagner's test	Solution: Iodine (1.27g) + KI (2 g) + 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 the filtrate.	Reddish brown precipitate
	Dragendroffes's test	Solution A: 0.85 g bismuth nitrate in a solution of 10 mL acetic acid + 40 ml water Solution B: 8 g KI in 20 mL water. Both solutions were mixed in equal proportion and this reagent was added to the filtrate.	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 Blue colour: Tannins
	Gelatin test	To the extract, few ml of distilled water and 1% solution of gelatin containing NaCl (10 %) were added.	Formation of white precipitate
Flavonoids	Lead acetate test	To 1 ml of extract, 1ml 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.
Protein	Ninhydrin Test	The extract was boiled with 2ml of 0.2% Ninhydrin solution	Formation of violet colour

3.2.23 QUANTIFICATION OF MAJOR PHYTOCHEMICAL CONSTITUENTS OF ALOE VERA GEL

3.2.23.1 ESTIMATION OF TOTAL FLAVONOIDS

(Slinkard and Singleton, 1977)

Quercetin (standard) was used to make a calibration curve. Quercetin (10 mg) was dissolved in 10 ml of 80% ethanol.Range of standard: 10-100 µg/ml.

TEST: Diluted standard solution (0.5ml) + (1.5ml) 95% ethanol.

	BLANK	STD	SAMPLE
95% Ethanol	2.0	2.0	2.0
10% $AlCl_3$	0.1	0.1	0.1

1 M CH_3COOK	0.1	0.1	0.1
D/W	2.8	2.8	2.8

Incubate at room temperature for 30' & take absorbance at 415 nm.

3.2.23.2 ESTIMATION OF TOTAL PHENOLICS

(Zheng and Wang, 2001)

p- Coumaric acid (standard) was used to make a calibration curve. p- Coumaric acid (10 mg) was dissolved in 10 ml of 95% ethanol.

Range of standard: 10-100 µg/ml.

TEST: Dilute standards upto 200µl.

	BLANK	STD	SAMPLE
95% Ethanol	0.2	-	-
Folin-Ciocalteu's reagent	1.0	1.0	1.0
7.5% Na_2CO_3	0.8	0.8	0.8

Incubate at 30 °C for 1.5 h. and take absorbance at 765 nm.

3.2.23.3 ESTIMATION OF TOTAL STEROLS

(Sabir et al., 2003)

Total 5 ml of sample was added to 5 ml of chloroform (1:1). The organic phase (1ml) was separated and used for estimation of total sterols.

Standard stigmasterol solution: 10 mg of standard stigmasterol was dissolved in 10 ml chloroform and mixed well. Range of standard: 10-100 µg/ml.

Libermann-Burchard reagent (LB Reagent): 0.5 ml of sulfuric acid cholesterol is dissolved in 10 ml of acetic anhydride. Covered and kept in ice bucket

	BLANK	STD	SAMPLE
Chloroform	1.0	1.0	-
LB Reagent	1.0	1.0	1.0

Incubate at room temperature for 15' & take absorbance at 625 nm.

3.2.24 THIN LAYER CHROMATOGRAPHY (TLC)

1) Preparation of GLASS TLC plates:

- Take silica gel and make approx 50% solution with d/w
- Clean TLC glass plates with methanol and pour the silica gel slowly

homogeneously.

- Allow to dry the plate at RT.
- Before run the plate keep the TLC plate in oven at high temperature.

2) Determine an appropriate solvent system: Various compounds will travel different distances up the plate depending on the solvent have chosen. In non-polar solvents like pentane and hexane, most polar compounds will not move, while non-polar compounds will travel some distance up the plate. In contrast, polar solvents will usually move non-polar compounds to the solvent front and push the polar compounds off of the baseline. A good solvent system is one that moves all components of your mixture off the baseline, but does not put anything on the solvent front – R_f values between 0.15 and 0.85.

Polarity	Solubility
Very Polar	Methanol > Ethanol > Isopropanol
Moderately Polar	Acetonitrile > Ethyl Acetate > Chloroform > Dichloromethane > Diethyl Ether > Toluene
Non-polar	Cyclohexane > Petroleum Ether > Hexane > Pentane

3) Common solvent combinations:

Ethyl Acetate/Hexane: 0–30% most popular combination, sometimes tough to remove solvents completely on rotary evaporator

Ethanol/Hexane or Pentane: 5–30% useful for very polar compounds

Dichloromethane/Hexane or Pentane: 5–30% sometimes useful when other mixtures fail

4) Fill TLC chamber with 1–2 mL of the desired solvent system. Place a large piece of cut filter paper in the chamber as well. .

5) Run the TLC. Let the solvent go about 90% of the way up the plate.

6) Remove the plate from the chamber and mark the solvent front immediately with a pencil and calculate the Rf value.

7) Let the solvent dry off of the plate. .

8) Visualize the TLC using non-destructive technique(s): The best non-destructive method is the UV lamp wherein keep the plate under the UV lamp and circle any UV active spots with pencil.

3.2.25 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

3.2.25.1 INSTRUMENTATION

Chromatographic separation was achieved on HPTLC plates precoated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminium sheet support. Samples were spotted using CAMAG Linomat IV Automatic Sample Spotter (Camag Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). Plates were developed in a glass twin trough chamber (CAMAG, 20 X 10 cm) pre-saturated with the mobile phase for 30 minutes Scanning device used was CAMAG TLC Scanner 2 equipped with WinCATS 3 software. The experimental condition was maintained at 22 ± 2° C.

3.2.25.2 CHROMATOGRAPHIC CONDITION

Parameters	Description
Stationary Phase	Merck Silica gel 60 F_{254} HPTLC pre-coated plates
Mode of separation	Normal phase
Mobile phase	Toluene: Ethyl acetate: Methanol (7.5:5:0.5)
Development chamber	Camag twin trough chamber
Chamber saturation	30 minutes
Sample applicator	Camag Linomat IV
Band width	10 mm
Space between the bands	10 mm
Rate of sample application	15 secs/µL
Development distance	90 mm
Densitometric scanner	Camag Scanner II with WinCats 3 Software
Lamp	Tungsten
Wavelength	550 nm

Pre-coated silica gel on aluminum plate 60 F_{254}, (10 cm ×10 cm, prewashed by methanol and activated at 60° C for 5 min prior to chromatography)

3.2.25.3 SAMPLE PREPARATION

Aloe vera gel (100 mL) was extracted with 100 ml of - Methanol by mixing - vigorously. The mixture was kept in a shaker - for 24 hrs -. The organic phase was separated and

concentrated to 1 ml with methanol. This concentrated extract was analyzed using HPLTC.

3.2.25.4 Preparation of Standard working solutions for Assay

Preparation of Standard Stock Solutions: Standard Stock solution of Lupeol (1000 µg/mL) was prepared by dissolving accurately weighed standard (10 mg) -in - methanol (10 mL). From the -standard stock solution, calibrant and quality control samples were prepared in methanol. Six different calibrant samples: 2, 4, 6, 10, 15, 25 µg/mL, respectively.

Three quality control samples: 3, 8, 20 µg/mL, respectively

3.2.25.5 Sample Application

The standard and Aloe extract sample were spotted on pre-coated TLC plates in the form of narrow bands (6 mm). Samples were applied under continuous drying stream of nitrogen gas at constant application rate of 150 nL/s.

3.2.25.6 Method development

After completion of TLC run, plate was developed by 10% Methanolic sulphuric acid (H_2SO_4) wherein plate was dipped in solution and kept for 10 seconds. Then, allowed it to air dry and kept in oven at high heat for 10 minutes. The developed bands were observed in visible light.

3.2.25.7 Method validation

Linearity and Calibration Curve:

Linearity of the method was determined by analyzing six different calibrant samples using HPTLC. Calibration curves were plotted over a concentration range of 2-25 µg/mL by plotting the respective peak area values versus concentrations (n = 6).

Photodocumentation was carried out using Camag Reprostar III, before derivatization under UV Light 254 nm, 366 nm and after derivatization (Fig.1). The plate was derivatized with Anisaldehyde-Sulphuric Acid reagent followed by heating at 110 °C for 10 minutes. The dried plate was scannedimmediately using Camag TLC Scanner III at wavelength 455 nm. WinCats an integrated Software 4.02 was used for the detection as well as for the evaluation of data. It was observed that Lupeol appeared at Rf. 0.64 (brown colour).

Limit of detection and Limit of quantitation:

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using the following formulae. LOD = 3 (SD)/S, LOQ = 10 (SD)/S Where, SD = Standard Deviation of response

Precision:

Precision was evaluated in terms of Intraday and Interday precisions. Intraday precision was determined by analyzing quality control samples of Lupeol (n=3) at three levels covering low, medium, and higher concentrations (3, 8, 20 µg/mL) on the same day. Interday precision was determined by analyzing standard solutions of Lupeol over a period of three days (n = 3). The peak areas obtained were used to calculate mean and %RSD (relative SD) values.

Accuracy (%Recovery):

Accuracy of the method was evaluated by carrying out the recovery study at three levels. Recovery experiments were performed by adding three different amounts of standard lupeol, that is, 80%, 100%, and 120% of the drug, to the pre-analyzed Aloe extract and the resultant was reanalyzed three times.

3.2.26 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

3.2.26.1 CHROMATOGRAPHIC CONDITION FOR HPLC:

INSTRUMENT	Jasco HPLC-PU 980 pump fitted with AS-1555-10 Auto-sampler
Mobile Phase	Acetonitrile : Ethanol 50:50 (v/v)
Flow-rate	1.0 mL / minute.
Injection Volume	20 µL.
Analytical Column	Cosmosil C18 (150 mm x 4.6 mm) Type: Waters
Detector	Jasco UV– 970, UV-Visible
Wavelength	210 nm
Integrator	Borwin Integrator Software, version 1.21.

3.2.26.2 Preparation of Standard and Sample solution

Standard stock solution of β-Sitosterol, Stigmasterol and Lupeol (1000 µg/mL each) were prepared by mixing the accurately weighed standards (10 mg each) in Methanol (10 mL). Working solution of the standards (10 µg/mL) was further prepared using methanol as a diluents. The petroleum ether extracts of Aloe vera gel was evaporated and reconstituted in 1 ml of methanol and filtered through 0.45 µm nylon membrane filter paper.

3.2.26.3 Sample application

The standards and sample (20 µL each) were injected into the HPLC system and the run length was kept constant runtime (30 minutes) while the mobile phase was delivered at -1ml/min -.

3.2.27 Gas chromatography and mass spectroscopy (GC-MS)

3.2.27.1 Instrumentation

Analyzer: Quadrupole with prefilter

Mass Range: 20-620 Daltons (amu)

Mass Stability: ± 0.1 m/z mass accuracy over 48 hours

Ionization Modes: EI, Positive / Negative Chemical Ionization.

Vacuum Pump: Turbo molecular pump 250L/Sec.

Software: Turbo Mass

Volatile compound from the peel of Aloe vera (L.) was analyzed AutoSystem XL Gas Chromatograph and the TurboMass Mass Spectrometer under the following condition: column used were PE-JMS [Column: 30 m x 0.250 mm x 1.0 µm PE-1]. The initial column temperature was 75 °C and final temperature was 220 °C (5°C/minute), while the injector temperature was 250 °C with split mode injector and split ratio of 72.6 and pressure of 14.0 kPa. The flow rate was 1 mL/minute and the flow within the column was 0.50 mL/minute. The detector temperature was 300 ° C and using Helium as the carrier gas with EI (Electron Impact). Detection: PerkinElmer TurboMass MS.

3.2.27.2 Sample application

Samples (1 µL) were injected in to the GC-MS/MS system and the compounds were identified by comparing their respective retention indices or mass spectra of - with those of the authentic samples or library.

3.2.27.3 CHARACTERIZATION OF UNKNOWN COMPOUND

Interpretation on mass spectra of GC-MS/MS was conducted using the database of National Institute of Standard and Technology (NIST). The name, molecular weight and structure of the components of the test samples recorded.

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