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EFFICACY OF NON POLAR EXTRACT (NPE) OF *ALOE BARBADENSIS* MILL. IN POLYCYSTIC OVARIAN SYNDROME (PCOS) RODENT MODEL- AN "*IN VIVO*" STUDY

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Keywords:

Aloe vera, Phytosterol, Polycystic ovarian syndrome, Steroids.

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ABSTRACT: Aim of the current study was to evaluate the efficacy of non polar extract (NPE) of *Aloe vera* gel (AVG) for 60 days (~25µg/daily/orally) in rodent model of polycystic ovarian syndrome (PCOS). After treatment, steroid hormones and insulin levels were estimated by ELISA, serum lipid profile along with ovarian steroidal enzyme activity. Also, transcript as well as protein level of key receptors and steroidogenic enzymes were evaluated. Parallely, pairs of ovaries were excised and stained with Hematoxylin and Eosin (HE) to monitor the structural changes from group of animals. During course of study, stability of non polar extract (NPE) of AVG was evaluated by High Performance Thin Layer Chromatography (HPTLC) during study (15, 30, 45, 60 days). PCOS rats after treatment with NPE demonstrated a decrease in serum testosterone and insulin level with improved estradiol and progesterone levels. Also, decrease in transcripts level of steroid receptors while increased aromatase expression were observed after NPE treatment. These genes expression study was correlated with relative changes were observed with protein expressions. Non polar phytosterol rich fraction treated PCOS rats improved metabolic phenotype as well as ovarian function. These modulations ameliorate the PCO condition suggesting that these could possible novel targets in management of PCOS.

INTRODUCTION: Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder among women of reproductive age [1]. It is characterized by hyperandrogenism, insulin insensitivity and chronic an ovulation [2]. In this regard, also has shown that PCOS follicle demonstrated high levels of LH, Steroidogenesis Acute Regulatory Protein (StAR), CYP17 and CYP11A mRNA expression when compared with size-matched control follicles [3]. Also, muscles biopsied from PCOS obese women exhibited impaired insulin-stimulated samples association of IRS-1 with PI-3K, concomitant with a decrease in glucose transport [4].

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As central core of PCOS is insulin resistance, current available mode of treatment is use of insulin sensitizers like metformin [5]. But these drugs have their own side effects upon prolong usage [6]. Hence, currently researchers are exploring alternative therapy to treat and manage the infertility disorders [7]. In this context, many scientists have demonstrated the role of medicinal plants in the control of hyperglycemic condition [8]. One such plant, that has been explored, recently is *Aloe vera* Mill. which has shown to have several medicinal effects including hypoglycemic effect [9]. Our previous reports also suggested that whole extract of Aloe vera improves steroid status and lower hyperlipidemic condition in PCOS phenotype [10, 11].

Moreover, *Aloe vera* gel contains 75 potentially active constituents: Polysaccharides, vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acids and amino acids [12]. Several reports

have suggested various efficacies such as hypoglycaemic, antitumor, anti-inflammatory, antioxidant and laxative effects of *Aloe vera* gel [12] could be attributed to the various phyto-compounds found in the inner leaf parenchymatous tissue [12, 13]. One of the well studied phyto-component in *Aloe vera* gel is phytosterol, which has been reported for various biological properties [14, 15]. Phytosterols (plant sterols) are secondary plant metabolites which are structural and biological counterparts of cholesterol. Isolated phytosterols have modulatory action that can prevent visceral fat by improving hyperglycemia, hyperlipidemia and insulin resistance in metabolic syndrome [16]. Major phyto-component, β sitosterol is known to regulate key molecules that is involved in inflammation, the immune response, anti-cancer defences, and apoptosis [17, 18].

"In vitro" study suggested that phytosterols also affect cell cycle kinetics wherein β-sitosterol can cause cell cycle arrest at the G2/M transition [19]. It clearly indicates that *Aloe vera* gel (AVG) has several phytosterols (β sitosterol, lupeol, stigmasterol), have insulin sensitizing and glucose lowering properties. Also, it is important to note that PCOS pathology is associated with insulin resistance and ovarian dysfunction. This suggests that non polar extract (NPE) of AVG could be active phyto-components in modulation of ovarian steroidogenesis. Thereby, current study aimed to understand the efficacy of non polar extract (NPE) from AVG on steroidogenesis with special reference to ovary.

MATERIALS AND METHODS:

Non polar extract (NPE) fraction preparation from *Aloe vera* gel: Initially, epidermis had removed from the leaf and homogenate of the *Aloe vera* gel was prepared. After that equal volume of *Aloe* gel and petroleum ether was taken in volumetric flask (1:1) ratio, vigorously stirred and kept in shaker for 24 hrs at room temperature. Then, organic layer evaporated at room temperature. Quantification of total sterols content was assayed from dried extract of petroleum ether (Non polar extract) [20]. Previous study evident that form HPLC analysis that 10 mg weight contained ~25 µg phytosterols. Thereby, ~25 µg of non polar fraction of AVG was used for "*in vivo"* study wherein it was resuspended in olive oil (Vehicle) according to dry weight of *Aloe vera* gel (10 mg).

HPTLC analysis: A Camag TLC system (Muttens, Switzerland) comprising of Camang Linomate V was used for analysis. Pre-coated silica gel on aluminium plate 60 F 254, (Prewashed by methanol and activated at 60° C for 5 min prior to chromatography). Mobile Phase: Proportion of Toulene: Ethyl Acetate: Methanol (7.5:1.5:0.5), (v/v/v/v). TLC Chamber saturation Time: 30 min at room temperature (30 ± 1° C). Application rate: 0.1µL/s. Scanning speed: 10 mm/s. Detection: Deep the TLC plate in Ethanolic 10% H_2SO_4 and allow it to dry. Further keep in oven at high heat for 10 min and observed the plate in visible light. The standard and NPE sample was spotted on pre-coated TLC plate.

HPLC analysis: Jasco HPLC-PU 980 pump fitted with AS-1555-10 was used for analysis. Mobile phase Acetonitrile: Ethanol (80:20) (v/v). Flow rate: 1.0 ml/minute. Injection Volume: 20 µl. Analytical Column: C18 (150x 4.6mm). Detector: Jasco UV-970, UV-visible. Wavelength: 210nm. Integrator: Borwin Integrator Software, Version 1.21. Standard stock solution of β-Sitosterol, Stigmasterol and Lupeol (1000µg/mL each) were prepared by mixing the accurately weighed standards (10 mg each) in Methanol (10mL). Working solution of the standards (10µg/mL) was further prepared using methanol as a diluents. The NPE was evaporated and reconstituted in 1 ml of methanol and filtered through 0.45 µm nylon membrane filter paper. 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.

Identification phyto-components by GC-MS/MS analysis:

Analyzer: Quadrupole with prefilter. Mass Range: 20-620 Daltons (amu). 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 Auto System XL Gas Chromatograph and the Turbo Mass 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 Turbo Mass MS.

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. 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.

Animal treatment: Animals were initially divided into 2 groups wherein one group received 1% CMC (carboxymethylcellulose) daily for 21 days orally and served as vehicle control (C). The other groups of animals were treated orally with letrozole daily for 21 days (0.5 mg/kg body weight). Letrozole treated animals demonstrated insulin resistance with disturbed estrus cyclicity and were considered as PCOS group (P). Further, these PCO animals treated with partially purified NPE (~25 µg/ml) (P-NPE) daily for 2 months.

Other group of PCOS animals received Olive oil as vehicle group (V-PCOS). In addition to this, untreated animals receiving NPE was designated to be herbal control (C-NPE) during the course of experiments.

Oral Glucose Tolerance Test (OGTT): OGTT was performed after 12 hrs fasting in all rats and blood samples were collected in fluoride coated bulb. Later, glucose (1 gm/Kg body wt) orally fed to the rats and blood samples were collected at the different time interval (30', 60', 90', 120'). These samples were subjected to centrifugation at 300 g for 10 min and plasma was separated [21]. Glucose was estimated using GOD-POD based kit.

Toxicity study: Serum Glutamate Pyruvate Transaminase (SGPT) [22] and serum creatinine [23] were assayed as both are toxicity markers.

Lipid Profile:
Lipidemic status was assessed in serum of all animals in all groups using enzymes based kits.

Hormone profile: Serum insulin level and steroid hormones levels were checked in all animal groups using ELISA kits procured from Diametra Inc, Germany. Sensitivity for method for Insulin is 2µIU/ml; for Testosterone is 0.075 ng/ ml; for Progesterone is 0.05ng/ml and for 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 6% (Insulin), 7.5% (Testosterone), ≤ 10.5% (Progesterone), ≤ 10% (17 β estradiol).

Enzyme assays: 10% ovarian homogenate was prepared in 0.1 M Tris-HCl buffer (pH-7.8) and centrifuged for 10,020 g for 30 min at 4°C. The supernatant was used as a source of enzyme. The key steroidogenic enzymes - 3 β hydroxy steroid dehydrogenase (3β HSD) and 17 β hydroxy steroid dehydrogenase (17β HSD) were assayed [24].

Total RNA isolation and Reverse transcriptase-Polymerase chain reaction (RT-PCR): Total RNA was extracted from ovarian tissue by TRIZOL method and amplified by cDNA synthesis kit wherein 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 the mixture, 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 preceded for further PCR reaction.

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 for gene expression of StAR, Aromatase, Androgen receptor (AR), Insulin receptor (IR), Luteinizing hormone receptor (LHR), Follicle

stimulating hormone receptor (FSHR) by Reverse transcriptase Polymerase Chain Reaction (RT-PCR) method and were normalized using internal control-GAPDH (Primer sequences as mentioned in **Table 4**).

Western Blot analysis: In addition to this, ovarian tissue was excised, kept in lysis buffer and stored at -80°C. Later, tissue was processed for western blot analysis to check the key protein expression of StAR, 3β-Hydroxysteroiddehydrogenase (HSD), Aromatase and Androgen receptor (AR) and β actin as internal control (Primary antibodies dilution as mentioned in **Table 5**).

Histological study: To analyze changes that occurred in ovarian structure after treatment of NPE, animals were sacrificed and ovaries were removed and kept in 10 % formalin for 24 hrs and tissues were further processed for sectioning with 5µm thickness. Following which tissues were fixed with Hematoxylin-eosin stain and were observed in light microscope. After observation, images were taken with 4 x magnifications.

RESULTS: A time-dependent profiling of phyto-components present in the NPE were performed by HPTLC qualitative fingerprint to check their stability during study period wherein results demonstrate that the NPE was stable up to 60 days (**Fig. 1**). Quantitative analysis of phytosterols in *Aloe vera* gel exhibited high phytosterols in petroleum ether fraction. After analysis, the spectra confirmation of the representative peak of phytosterols in sample was carried out with the phytosterols standards (β sitosterol, stigmasterol, lupeol). NPE exhibited presence of all three main sterols wherein majorly present sterol content was β sitosterol in the analysis. Stigmasterol and lupeol present in extract were very less concentration in fraction (**Fig. 2**).

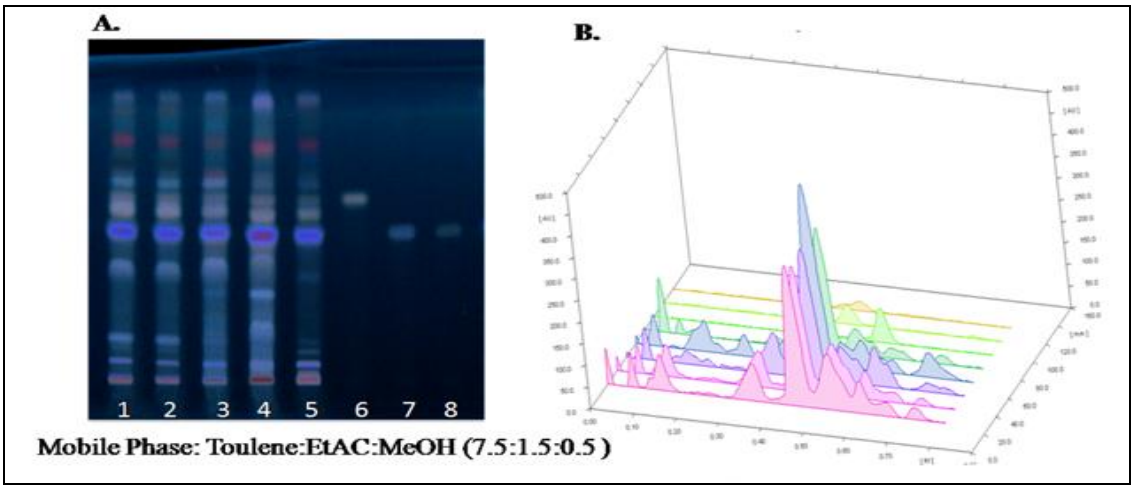
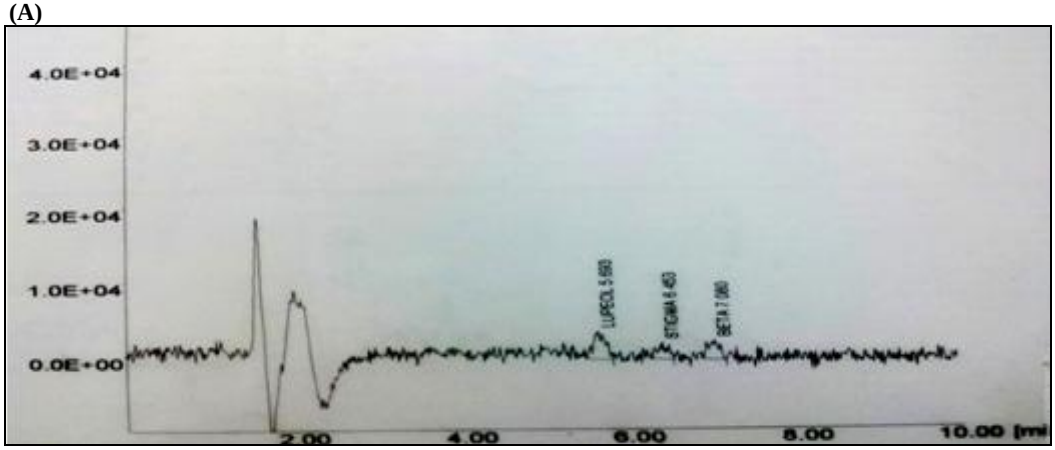


FIG. 1: CHROMATOGRAPHIC ANALYSIS OF NPE. (A) HPTLC FINGERPRINT OF NON POLAR EXTRACT (NPE) OF *ALOE VERA* AT VARIOUS TIME POINT. 1: 15 DAYS, 2: 30 DAYS, 3: 45 DAYS, 4: 60 DAYS, 5: FRESH *ALOE VERA* GEL, 6: LUPEOL 50 PPM, 7: β SITOSTEROL 25 PPM, 8: STIGMASTEROL 10 PPM (B) FRONT VIEW OF HPTLC DENSITOGRAM WAS DISPLAYED IN 3D WITH VARIOUS PEAK INTIGRATIONS.

(A)



(B)

	Lupeol	Stigmasterol	β-Sitosterol
Concentration in mg/ml	0.027	0.410	0.511
Retention Time (Minute)	5.693	6.453	7.080

FIG.2: HPTLC ANALYSIS OF NON POLAR EXTRACT (A). HPLC CHROMATOGRAM OF NON POLAR EXTRACT (NPE) OF *ALOE VERA* AND UV SPECTRA OF IDENTIFIED PHYTOSTEROL COMPOUNDS. 1: LUPEOL, 2: STIGMASTEROL, 3: B SITOSTEROL. (B). RETENTION TIMES (RT) AND UVMAX ABSORPTION AND CONTENT OF PHYTOSTEROLS IN NON POLAR EXTRACT (NPE) OF *ALOE VERA* GEL

Under standard optimized condition, peak of β Sitosterol was detected at 35.16 minutes in GC chromatogram (**Fig. 3a**) and mass spectra of the same is depicted in figure 3b. On the basis of RT value and fragmentation pattern of some high intensity peaks in the mass spectra, the presence of β sitosterol (m/z = 414) in the P1 fraction was confirmed. Fragmentation pattern (mass spectra) of some other phytochemical constituents separated as peak during GC-MS/MS analysis of the P1 fraction of *Aloe vera* gel were matched with the data from the NIST library. The detailed information on the other probable phytochemical constituents detected in the P1 fraction of *Aloe vera* gel (name of the compound, molecular formula and molecular weight) is summarized in the **Table 1**.

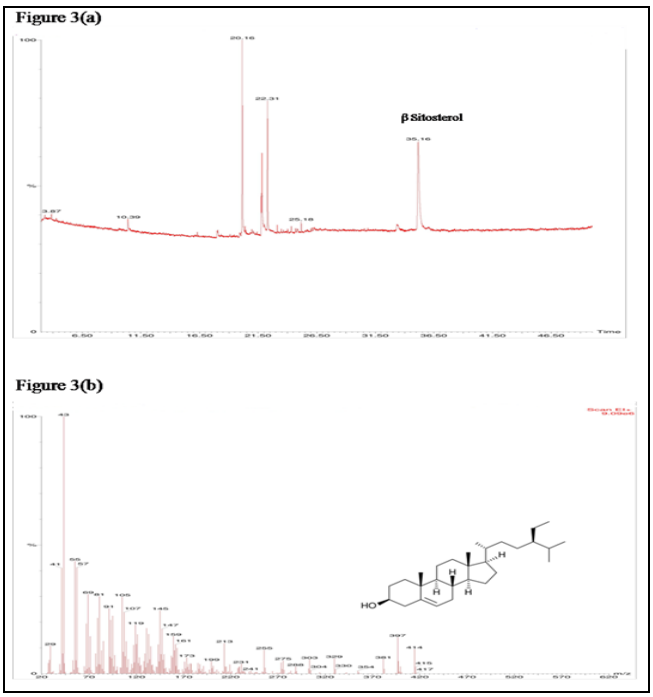


FIG. 3: GC MS ANALYSIS OF NPE. (a) MASS SPECTRA OF THE SAME IS DEPICTED IN FIGURE 3.18. ON THE BASIS OF RT VALUE AND FRAGMENTATION PATTERN OF SOME HIGH INTENSITY PEAKS IN THE MASS SPECTRA OF THE NPE OF *ALOE VERA* GEL SHOWING THE PEAK CORRESPONDING TO B SITOSTEROL AT RT 35.16 MIN. FIGURE 3(b) ON THE BASIS OF RT VALUE AND FRAGMENTATION PATTERN OF SOME HIGH INTENSITY PEAKS IN THE MASS SPECTRA, THE PRESENCE OF B SITOSTEROL (M/Z = 414) IN NPE WAS CONFIRMED.

The probable phytochemical constituents detected in the P1 fraction of *Aloe vera* gel:

TABLE 1: FRAGMENTATION PATTERN (MASS SPECTRA) OF SOME PROBABLE PHYTO-COMPONENTS DETECTED IN NPE OF *ALOE VERA* GEL SEPARATED AS PEAK DURING GC-MS/MS ANALYSIS AND MATCHED WITH THE DATA FROM THE NIST LIBRARY.

S.no.	Compound Name	Formula	Molecular Weight
1	3-acetoxy-bisnor-5-cholenic acid	C24H36O4	388
2	20-ethynyl-5-pregnen-3,20-diol	C23H34O2	342
3	Cholesta-3,5-diene	C27H44	368
4	26-nor-5-cholesten-3.beta.-ol-25-one	C26H42O2	386
5	26-nor-5-cholesten-3.beta.-ol-25-one	C26H42O2	386
6	Stigmastan-3,5-diene	C29H48	396
7	Beta- sitosterol	C29H50O	414
8	Gamma.-sitosterol	C29H50O	414
9	Cholest-5-en-3-ol (3.beta.)-, acetate	C29H48O2	428
10	Cholest-8-en-3.beta.-ol, acetate	C29H48O2	428
11	Cholest-5-en-3-ol (3.beta.)-, acetate	C29H48O2	428
12	Cholest-5-en-3-ol (3.beta.)-, propanoate	C30H50O2	442
13	Cholest-5-en-3-ol (3.beta.)-, propanoate	C30H50O2	442
14	Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	C28H45O2Cl	448
15	Beta.-sitosterol acetate	C31H52O2	456
16	Beta.-sitosterol acetate	C31H52O2	456
17	Cholest-5-en-3-ol (3.beta.)-, tetradecanoate	C41H72O2	596
18	22, 23-dibromostigmasterol acetate	C13H50O2Br2	612

After letrozole treatment, PCOS rats exhibited elevation in glucose level (**p<0.01, **p<0.01, ***p<0.001) at 60, 90 and 120 minutes of the test as compared to control group. PCOS-NPE groups exhibited significant change in all time point of the test except fasting while V-PCOS group also showed significant change at 90 and 120 minute

(@p<0.05). C-NPE depicted similar results to control rats (**Fig.3**).

In current study, PCOS group demonstrated a significant high level of insulin (***p<0.001) while it reverted back to normal level after treatment of NPE (@@@p<0.001). PCOS-NPE group demonstrated a change in insulin level as compared to PCOS group (@p<0.05). HOMA-IR index also has been evaluated to indicate insulin resistance condition. PCOS group demonstrated an increased insulin resistance condition (HOMA IR- 4.2) (***p<0.001) whereas NPE treatment caused a reduction in an insulin resistance in all group as similar to control group (@@@p<0.001) (HOMA IR < 3) (**Table 1**).

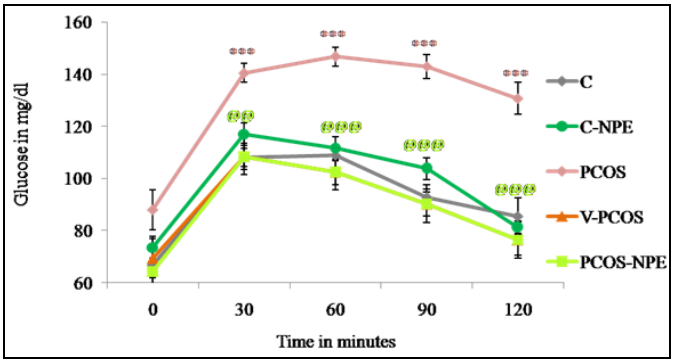


FIG. 4: MEAN PLOT OF ORAL GLUCOSE TOLERANCE TEST (OGTT) OVER THE 60 DAYS TREATMENT OF NON POLAR EXTRACT (NPE) IN PCOS RATS. C: CONTROL; C-NPE: NPE TREATED CONTROL; V-PCOS: VEHICLE (OLIVE OIL) TREATED PCOS; PCOS-NPE: NPE TREATED PCOS. N=4, THE VALUES REPRESENTED AS MEAN±SEM. *P<0.05, **P<0.01, ***P<0.001 VS CONTROL. @P<0.05. @@P<0.01, @@@P<0.001 PCOS. ϕP<0.05,ϕϕP<0.01 VS. PCOS.

During the course of experiment, toxicity markers like Serum Glutamate Pyruvate Transaminase (SGPT) and creatinine were evaluated. All groups of animals demonstrated no change in the above parameters upon treatment indicating no toxic effects of NPE during course of study (**Fig. 4**).

Lipid profile was evaluated in PCOS group wherein it exhibited elevation in triglycerides level (***p<0.001) whereas no change was observed in total cholesterol level. In PCOS rats, LDL level was also elevated (***p<0.001) while no significant changes were observed in HDL and VLDL levels. Elevated TG levels implicated the presence of metabolic syndrome in PCOS (**Table 2**). Hypertriglyceridemia of PCOS rats has been normalized with NPE treatment (@@@p<0.001) and demonstrated more significant change as compared to V-PCOS group; this (#p<0.05) suggested that phytosterols of AVG may have lipid lowering efficacy. In PCOS rats, LDL levels were also reduced after NPE treatment (@@@p<0.001) whereas no significant changes were observed in HDL and VLDL levels. V-PCOS also showed significant change in LDL level (@@@p<0.01) but NPE again exerted more significant change as compared to V-PCOS group (###p<0.001) (**Table 2**). Letrozole induced PCOS rats demonstrated an elevation in activity of both enzymes in ovary (*p<0.05, ***p<0.001). PCOS-NPE group exhibited significant reduction in both enzymes activities (@p<0.05, @@@p<0.001) in ovary whereas vehicle group demonstrated a change in 3β HSD activity (@p<0.05) as compared to PCOS rats (**Fig. 5**).

TABLE 2: COMPARISON BETWEEN SERUM INSULIN AND HOMA IR IN NON POLAR EXTRACT TREATED PCOS RATS. OVER 60 DAYS TREATMENT. DATA ARE SHOWN AS AS MEAN±SEM OF 4 ANIMALS. **P<0.01, ***P<0.001 VS CONTROL, @@P<0.01, @@@P<0.001 VS PCOS. HOMA: HOMEOSTATIC MODEL ASSESSMENT, IR: INSULIN RESISTANCE. NORMAL INSULIN RESISTANCE <3, MODERATE INSULIN RESISTANCE BETWEEN 3 – 5, SEVERE INSULIN RESISTANCE >5.

Groups	Insulin (μIU/ml)	HOMA-IR
C	12.3±0.6	1.8±0.1
C-NPE	13.2±1.2	2.5±0.1
PCOS	21±1.0**	4.5±0.3***
V-PCOS	13.5±0.76@	2.5±0.2@@
PCOS-NPE	9.1±1.2@@@	1.4±0.1@@@

Serum steroid hormones levels were estimated in all groups of animals wherein letrozole induced PCOS group demonstrated elevated level of serum testosterone level (*p<0.05) which reverted back to normal after NPE treatment (@p<0.05). PCOS rats also exhibited a decrease in level of progesterone (**p<0.01) and estradiol level (*p<0.05) which returned to normal values after NPE treatment (@p<0.05). V-PCOS group exhibited no significant changes in steroid hormones level (**Table 3**). Thereby, indicating no role of olive oil in steroid metabolism.

Histological changes were evaluated in current study wherein peripheral cysts with follicular fluid and arrested antral follicles were observed in letrozole induced PCOS rats as compared to control. These PCOS rats after treatment of NPE demonstrated a reduction in peripheral cysts with increased growing follicles in ovary. Herbal control

(C-NPE) demonstrated a normal follicle growth similar to control rats. V-PCOS also exhibited reduced peripheral cysts in ovary as compared to PCOS rats (**Fig. 6**).

Fig. 6 elucidated the effect of NPE on transcript level of key proteins of steroidogenesis wherein PCOS rat demonstrated a elevated gene expression of StAR (***p<0.001), insulin receptor (INSR) (**p<0.01), Luteinizing hormone receptor (**p<0.01) and androgen receptor (AR) (***p<0.001) whereas decrease was observed in gene expression of aromatase (*p<0.05) as compared to control group. However, there was no significant change was observed in gene expression of follicle stimulating hormone receptor (FSHR) in PCOS group. After NPE treatment, there were significant changes observed in these key steroidogenic protein wherein substantial decrease in StAR (***p<0.001), insulin receptor (IR) (*p<0.05), LHR (**p<0.01) and AR (**p<0.01) transcripts level as compared to PCOS rats whereas significant increase was observed in transcript level of aromatase (***p<0.001) in NPE treatment. However, no significant changes were observed in all transcript level in V-PCOS group (**Fig. 7**).

TABLE 3: LIPID PROFILE OF PCOS RATS TREATED NON POLAR EXTRACT (NPE) OF *ALOE VERA* GEL. DATA ARE SHOWN AS MEAN±SEM. ***P<0.001 VS CONTROL. @P<0.05, @@@P<0.001 VS PCOS. #P<0.05, ###P<0.001 VS VEHICLE.

	Triglycerides	Total Cholesterol	LDL	HDL	VLDL
C	70.8±2.9	48.3±1.7	51.9±3.6	31.1±2.0	13.8±0.69
C-NPE	71.6+4.9	52.6±5.7	49.6±3.1	27.6±2.8	16.0±1.5
PCOS	96.0±6.3***	55.8±3.4	108.0±10.2***	24.5±2.3	22.6±2.0
V-PCOS	78.7±2.3$^{@}$	43.6±3.7	79.11±5.5$^{@@@}$	34.1±1.4	13.7±1.3
PCOS-NPE	64.2±2.4$^{@@@\#}$	45.3±4.3	45.8±2.2$^{@@@\#\#\#}$	28.7±1.4	13.2±0.4

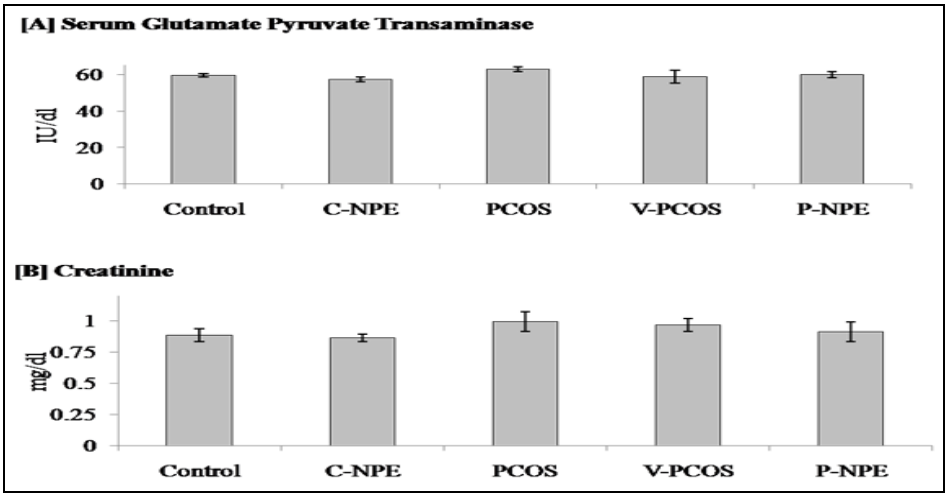


FIG. 5: EFFECT OF NON POLAR EXTRACT OF *ALOE VERA* GEL ON LIVER TOXICITY MARKERS. [A] SERUM GLUTAMATE PYRUVATE TRANSAMINASE. [B] CREATITINE. DATA ARE SHOWN AS MEAN±SEM OF 4 ANIMALS.

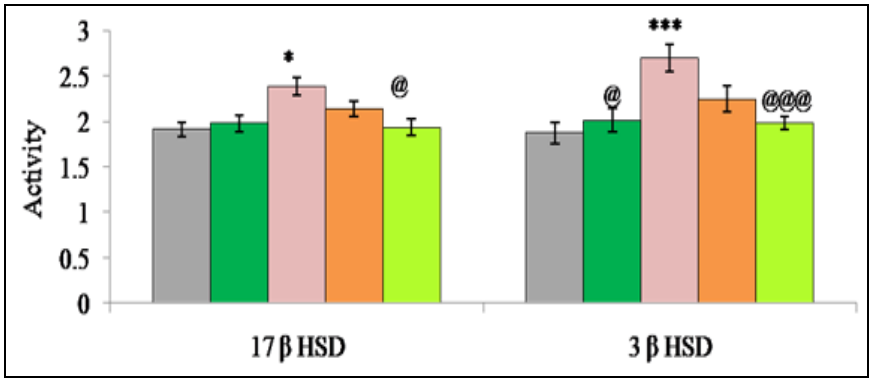


FIG. 6: 17 B HYDROXYSTEROID DEHYDROGENASE (17 β HSD) AND 3 B HYDROXYSTEROID DEHYDROGENASE (3 β HSD) ACTIVITIES WERE MEASURED IN MICROSOMAL FRACTION OF OVARIAN TISSUE. DATA ARE SHOWN AS MEAN±SEM OF 4 ANIMALS. *P<0.05, **P<0.01, ***P<0.001 VS CONTROL. @P<0.05, @@@P<0.001 VS PCOS. RESULTS ARE EXPRESSED PER INDIVIDUAL OVARY.

TABLE 4: COMPARISON OF STEROID HORMONES IN NON POLAR EXTRACT (NPE) TREATED PCOS RATS OVER THE 60 DAYS OF TREATMENT. DATA ARE SHOWN AS MEAN±SEM OF 3 ANIMALS. *P<0.05, **P<0.01 VS CONTROL. @ P<0.05 VS PCOS.

Groups	Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
C	0.42±0.04	72±2.5	14±1.5
C-NPE	0.48±0.26@	70.6± 17.1	7.7± 1.6
PCOS	1.13±0.15*	49±6.5*	5.5±1.2**
V-PCOS	0.96±0.24	56±6.5	7.9±1.2
P-NPE	0.48±0.21@	75.3±10.7@	11.5±3.5@

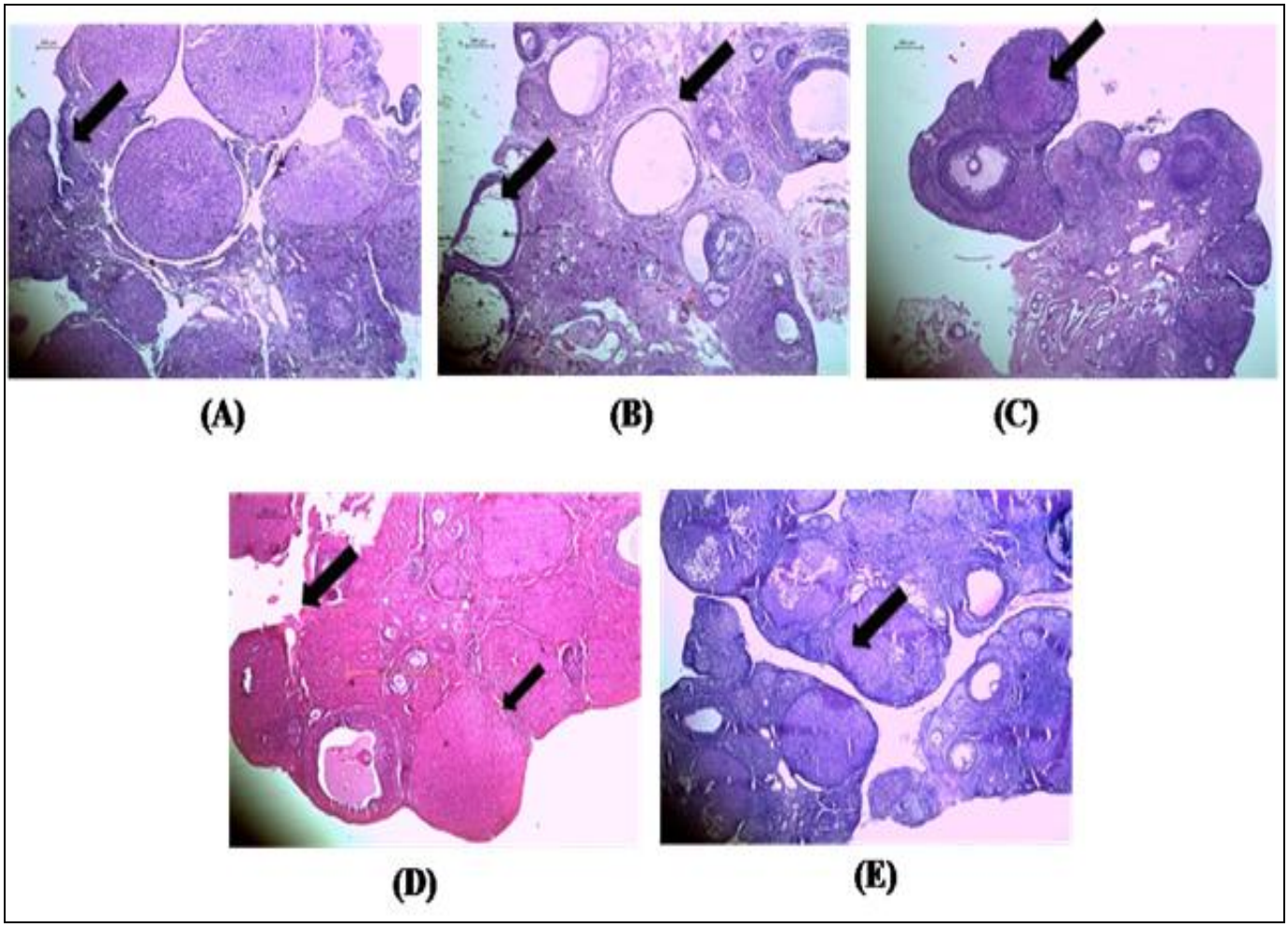


FIG. 7: REPRESENTIVE HEMATOXYLIN AND EOSIN (H&E) STAINING (40X) OF OVARY SECTION FROM NON POLAR EXTRACT (NPE) TREATED PCOS RATS. (A) SECTION OF OVARY FROM CONTROL RAT SHOWING FOLLICLES WITH FRESH CORPORA LUTEA. (B) SECTION OF OVARY FROM PCOS RAT SHOWING NUMEROUS PERIPHERAL CYSTS AND ATRETIC SECONDARY FOLLICLES ALONG WITH CYSTIC DEGENERATING FOLLICLES SHOWING THIN GRANULOSA CELL LAYER. (C) SECTION OF OVARY FROM CONTROL RAT TREATED WITH NPE SHOWING NORMAL FOLLICULAR GROWTH WITH CORPORA LUTEA. (D) SECTION OF OVARY FROM PCOS RAT TREATED WITH OLIVE OIL (VEHICLE) SHOWING NORMAL CORPORA LUTEA WITH SMALL SUBCAPSULAR CYSTS. (E) SECTION OF OVARY FROM PCOS RAT TREATED WITH NPE SHOWING NORMAL SMALL GROWING FOLLICLES ALONG WITH CORPORA LUEA.

Relative protein expression of key regulatory proteins involved in steroidogenesis was evaluated wherein PCOS group demonstrated an elevation in relative protein expression of StAR (***p<0.001), androgen receptor (AR) (***p<0.001) while significant decrease in expression of aromatase (**p<0.01) as compared to control group (*p<0.05); while it reverted back as similar to control StAR (**p<0.01), androgen receptor (AR) (***p<0.001), aromatase (***p<0.001) when treated with NPE. However, no significant change was observed in protein expression of 3β hydroxy steroid dehydrogenase (3β HSD) in all groups of animals. Vehicle group also showed significant

change in StAR (*p<0.05) whereas no change was observed in AR and aromatase protein expression. In addition, NPE group exhibited more significant change in protein expression of aromatase as compared to vehicle group (**p<0.02), which elucidated efficacy of NPE.

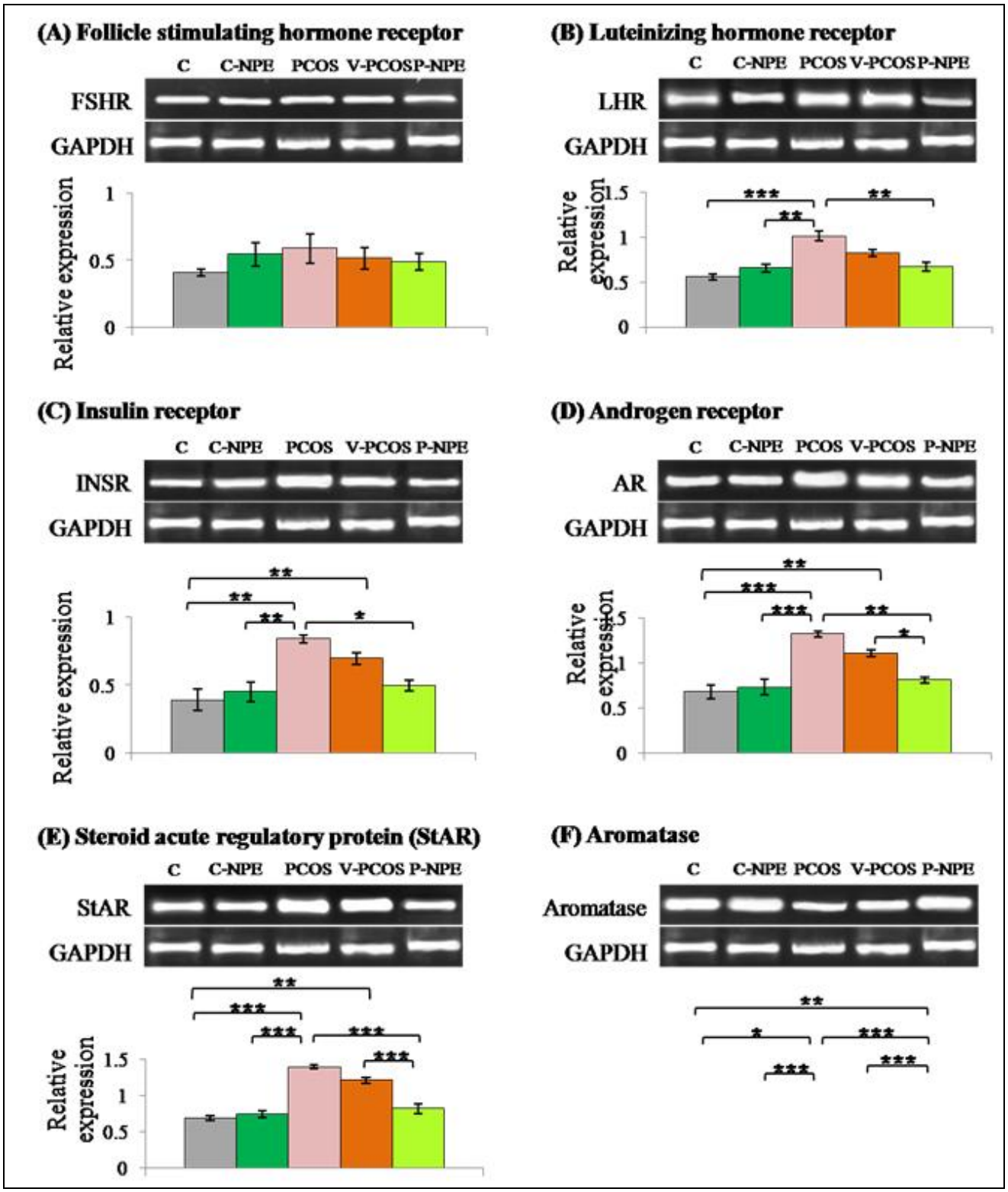


FIG. 8: mRNA EXPRESSION OF STEROID RECEPTORS, STEROIDOGENIC ENZYMES AND INSULIN RECEPTOR IN NPE TREATED PCOS RATS. (A) FOLLICLE STIMULATING HROMONE RECEPTOR (FSHR), (B) LUTEINIZING HORMONE RECEPTOR (LHR). (C) INSULIN RECEPTOR (INSR), (D) ANDROGEN RECEPTOR (AR), (E) STEROID ACUTE REGULAOTRY PROTEIN (STAR), (F) AROMATASE. DATA ARE SHOWN AS MEAN ±SEM OF 3 ANIMALS. *P<0.05; **P<0.01; ***P<0.001

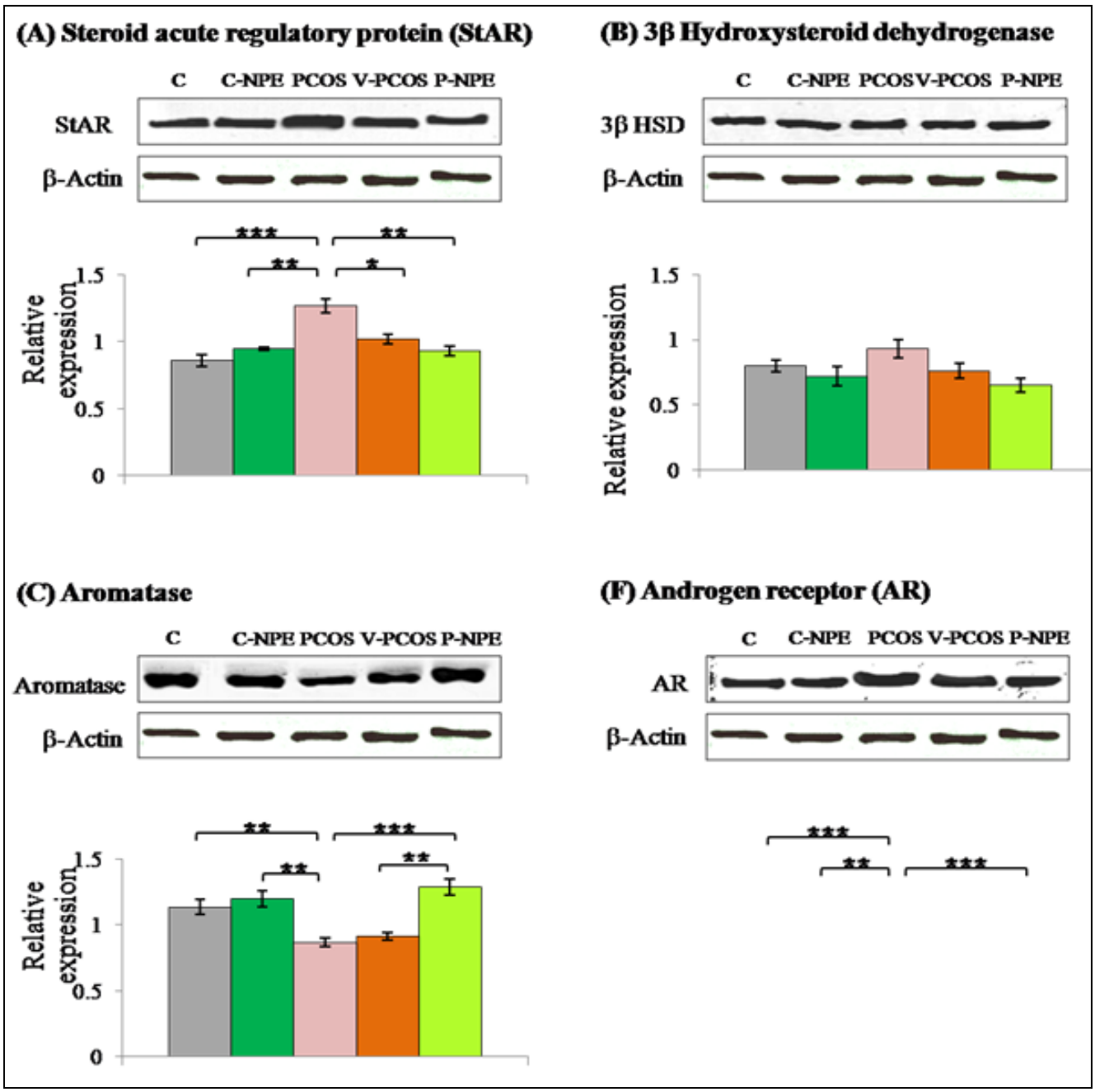


FIG. 9: WESTERN BLOT. REPRESENTATIVE IMAGES WITH DENSITOMETRIC ANALYSIS SHOWING PROTEIN EXPRESSION OF STEROID RECEPTORA AND OVARIAN STEROIDOGENIC ENZYMES WITH B ACTIN AS INTERNAL CONTROL. (A) STEROID ACUTE REGULATORY PROTEIN (STAR), (B) 3β HYDROXYSTEROID DEHYDROGENASE (B HSD), (C) AROMATASE, (D) ANDROGEN RECEPTOR (AR). DATA ARE SHOWN AS MEAN ±SEM OF 3 ANIMALS. *P<0.05; **P<0.01; ***P<0.001

TABLE 5: OLIGONUCLEOTIDE PRIMERS USED IN RT-PCR ANALYSES

Targeted Genes	Primer sequence	Annealing temperature (^{0}C)	Reference
Aromatase	F: 5'GCTTCTCATCGCAGAGTATCCGG 3' R: 5'CAAGGGTAAATTCATTGGGCTTGG 3'	60	NM_017085
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
AR	F: 5' GGAAGCACTGGAACATCT 3' R: 5' GTAGTCGCGATTCTGGTA 3'	53	Suzuki M and Nishihara, 2002
GAPDH	F: 5' CAAGGTCATCCATGACAACTTTG 3' R: 5' GTCCACCACCCTGTTGCTGTAG 3'	58.1	NM_017008

TABLE 6: ANTIBODIES DILUTION USED IN WESTERN BLOT ANALYSIS

Primary antibodies dilutions			
S.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

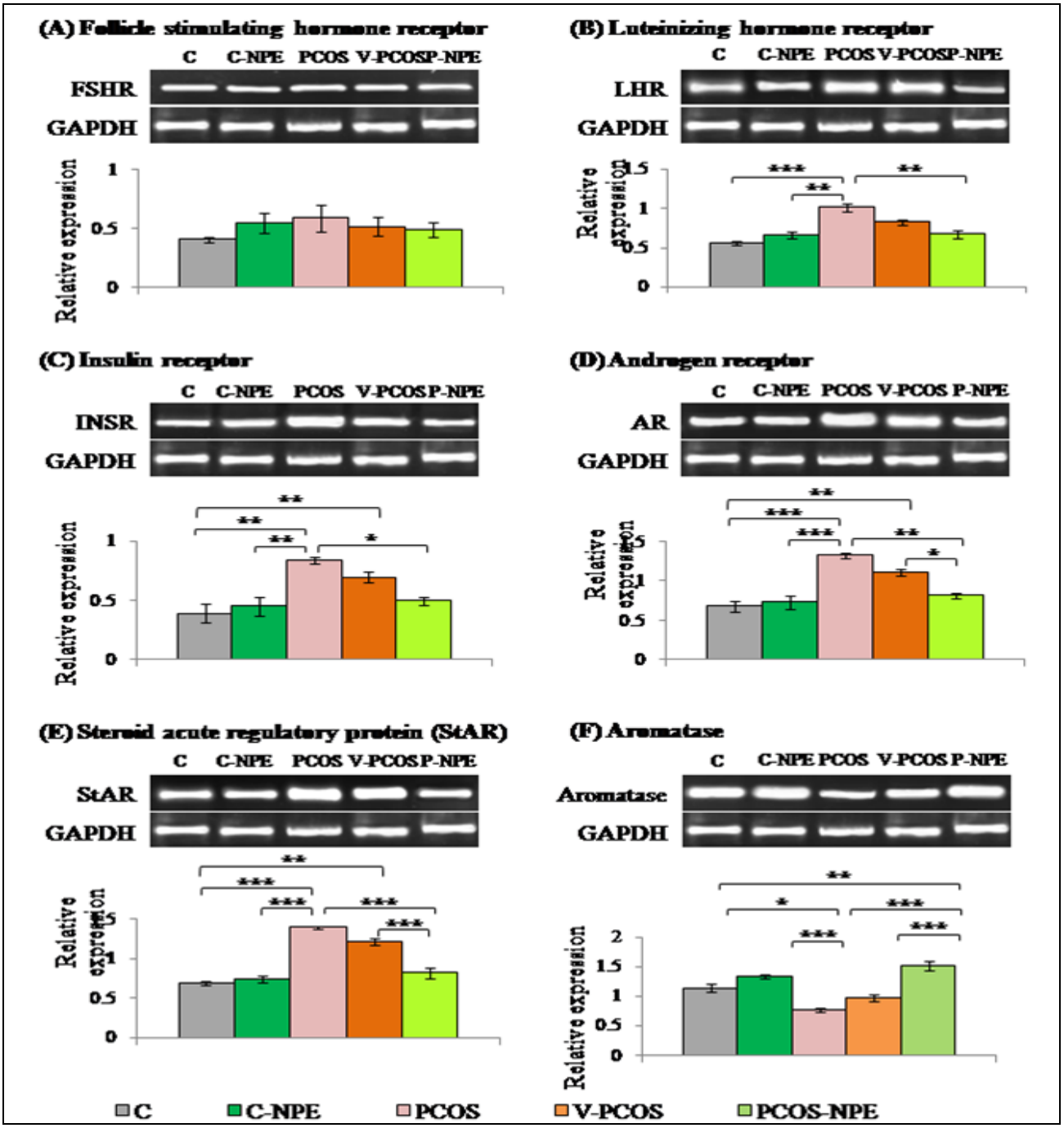


FIG. 10: mRNA EXPRESSION OF STEROID RECEPTORS, STEROIDOGENIC ENZYMES AND INSULIN RECEPTOR IN NPE TREATED PCOS RATS. (A) FOLLICLE STIMULATING HROMONE RECEPTOR (FSHR), (B) LUTEINIZING HORMONE RECEPTOR (LHR). (C) INSULIN RECEPTOR (INSR), (D) ANDROGEN RECEPTOR (AR), (E) STEROID ACUTE REGULAOTRY PROTEIN (STAR), (F) AROMATASE. DATA ARE SHOWN AS MEAN ±SEM OF 3 ANIMALS. *P<0.05; **P<0.01; ***P<0.001

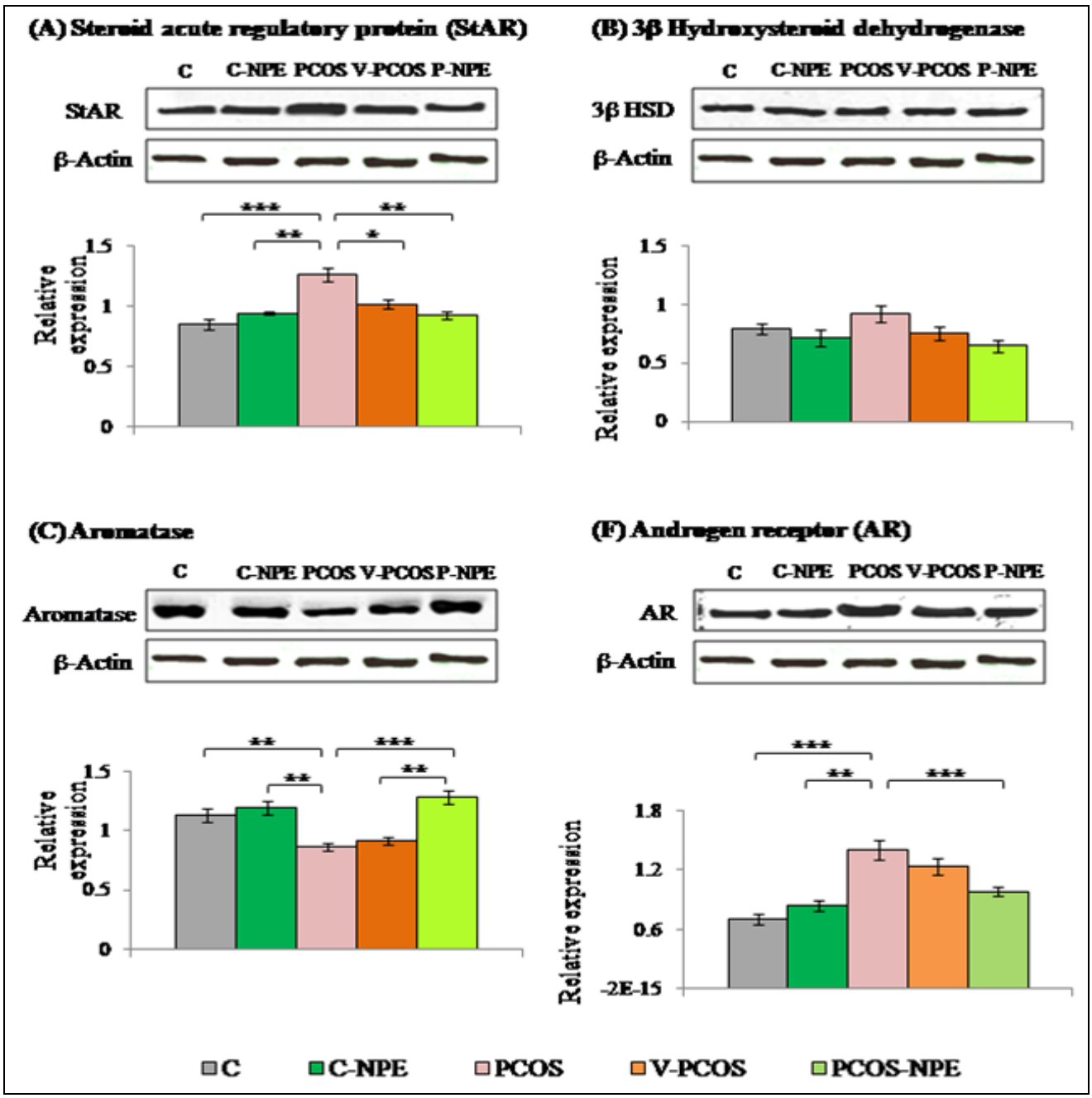


FIG. 11: WESTERN BLOT. REPRESENTATIVE IMAGES WITH DENSITOMETRIC ANALYSIS SHOWING PROTEIN EXPRESSION OF STEROID RECEPTORA AND OVARIAN STEROIDOGENIC ENZYMES WITH B ACTIN AS INTERNAL CONTROL. (A) STEROID ACUTE REGULATORY PROTEIN (STAR), (B) 3βHYDROXYSTEROID DEHYDROGENASE (B HSD), (C) AROMATASE, (D) ANDROGEN RECEPTOR (AR). DATA ARE SHOWN AS MEAN ±SEM OF 3 ANIMALS. *P<0.05; **P<0.01; ***P<0.001

DISCUSSION: PCOS has many clinical manifestations, which include oligomennorhea and hyper-androgenism, leading to metabolic dysfunction[25]. Letrozole (a non-steroidal aromatase inhibitor) induced PCOS rat model that exhibited increase in androgens, leading to hyperandrogenism, which is a hallmark of PCOS. Also, significant weight gain was observed in PCOS rat as compared to control rats which could be attributed to deposition of abdominal fat [26]. The model created as similar as shown by [24]. Apart from above feature rats were hyperglycemic and insulin resistance [27] indicating PCO model has been developed [24]. Present study was an attempt to investigate the efficacy of partially purified NPE (Non polar extract) of *Aloe vera* gel with reference to its modulation of ovarian structure-function in letrozole induced PCOS rat model.

Our earlier published data suggested that treatment of whole *Aloe vera* gel restored glucose intolerance and dyslipidemia along with cyclicity and has modulatory effect on ovarian steroidogenesis [10, 11]. This efficacy could be attributed to phyto-components present in gel. It is clear from reports that AVG also has several phytosterols having insulin receptor sensitizing and glucose lowering properties [12].

Out of them well studied phytosterol is β sitosterol that has cholesterol lowering property and direct effect on Steroid Acute regulatory protein- StAR protein that plays a key role in ovarian steroidogenesis [28]. During experimental regime, stability of NPE as various phyto-components (volatile in nature) present in gel was evaluated. HPTLC data suggested phytosterols were stable up to 60 days. Also, quantification of HPLC data from current study also demonstrated that NPE was rich in various phytosterols mainly β sitosterol, stigmasterol and lupeol which known to have anti-hyperglycemic properties. It is known that phytosterols are volatile in nature. Stability of phytosterols when evaluated it showed upto 60 days as demonstrated in present study of HPTLC and HPLC. NPE treated PCOS rats exhibited restored the glucose sensitivity as mediated by improved HOMA-IR implicating that phytosterols of NPE has good glucose lowering property [29]. Also, "*Ex vitro"* study wherein NPE incubated with ovarian cells showed an increased steroidogenic enzyme activity. These suggest that phytosterols of AVG may be as component that restores ovarian structure function in PCOS as evident from our previous report [11].

Present study showed that PCOS rats with elevated insulin level exhibited high HOMA-IR index suggesting hyperinsulinemia with insulin resistance. The values returned to normal level when treated with NPE (phytosterols rich) suggesting that it has potential to sensitize the insulin receptor and reduce insulin level in PCO condition; thereby reverting insulin resistant state to sensitive status as indicated by improved HOMA-IR change. This could be evident from that reports which indicate the potentiality of phytosterols of AVG as insulin sensitizer [30]. Phytosterols isolated from different plants also reported to have anti hyperglycaemic effect and improved insulin sensitivity [30].

Apart from glucose metabolism, dyslipidemia also restored after treatment of NPE indicating lipid lowering property. In this content, clinical study also suggested that consumption of phytosterol-β-sitosterol helps to decrease lipoproteins; although there was usually no change was observed in high-density lipoprotein (HDL) [31]. In addition to above, β sitosterol exerts hypo-cholesterolemic effects by inhibiting the absorption of cholesterol in the intestine as well as competing by LDL-cholesterol and also decreases in total, along with LDL-cholesterol concentrations 5–15% as evident from several studies [18]. This mechanism may be exerted by phytosterols of AVG in PCOS rodent model as demonstrated in our earlier report [10].

Our earlier study has shown a potential role of AVG has on ovarian steroidogenic enzyme activity in PCO phenotype [11, 24]. Further, NPE treated PCOS model demonstrates a reduction in both enzymes activities. Reduced steroidogenic enzyme-3 β HSD activities is also correlated with decreased testosterone production and improved progesterone production in NPE treated PCOS rats. Data from literature shows that β-Sitosterol has estrogen-like effects in fish [32] and modulates the steroidogenic pathway at its first step, the conversion of cholesterol to pregnenolone (P5) by cytochrome P450 side-chain cleavage (P450scc) [33]. Thus, suggesting that NPE has potential to modulate steroidogenesis by affecting the various targets.

Regulation of steroidogenesis governed by level of gonadotropin receptors and steroid receptors expressed on ovarian cells wherein expression of LHR and Androgen receptor is shown to be elevated on the stimulation by high insulin level in PCOS rats which further lead to high theca androgen [34]. Also, it has been demonstrated that insulin acts directly via its own insulin receptor at physiological concentrations in cultured polycystic ovary theca cells [35]. Thereby, increased expression level of insulin receptor (INSR) in PCO rats could also contribute to rise in androgen content as seen in present study. Insulin alone can also increase androstenedione production and can synergize with LH to increase androgen biosynthesis in PCO condition [34, 36].

This elevated IR expression was reduced when treated with NPE, suggesting efficacy of NPE as an insulin sensitizer [37].

A direct modulation of steroidogenesis is could be due to increase in gonadotropin receptor-LHR. In addition to this, transcript level of LHR was decreased while treated with NPE. This may be because of the estrogenic effects of β-sitosterol on the pituitary suggesting role of phytosterols in feedback regulation. Also, studies in mammals have demonstrated that genistein (phytoestrogen) decreases GnRH-induced luteinizing hormone (LH) in rats [38] and that coumesterol (phytosterol) decreases the amplitude of LH pulses in ewes. Thereby, NPE may act at GnRH/LH. However, this fact needs to be examined. It is that known that, aromatase also plays a crucial role in management of steroid status wherein it converts androgen to estrogens. In present study, transcript of aromatase level as well as relative protein expression level was decreased in PCOS rats suggesting disturbed testosterone: estrogen flux due to lower conversion and thus leading to excess androgen production though ovarian steroidogenesis.

Also, decreased aromatase expression is well correlated with low level of estradiol as seen in PCOS rats. However, altered expression level observed in PCO phenotype was reverted back to normal when treated with NPE. This may be attributed to the fact that StAR protein has been normalized by phytosterols, which reorients its flux where androgen is converted into estrogens efficiently by aromatase [39]. During course of experiments, levels of toxicity markers were estimated wherein no significant changes were observed in Serum Glutamate Pyruvate Transaminase (SGPT) and creatinine level. This suggests that dose used doesn't cause any toxic effects.

Any functional modulation is mainly reflection of structural aberrations. Structurally, PCO rats demonstrated the presence of multiple fluid filled peripheral cysts in the ovary. Several studies have shown similar results [40]. NPE treated PCOS rats demonstrated a reduction in peripheral cysts in ovary and increased growing follicles with presence of corpus luetum that mainly indicates successful ovulation and release of dominant mature graffian follicle. The restoration in the ovarian structure and function can be attributed to phytosterols that lead to modulation in the HPO axis. This modulation helped in maturation of follicles and release matured ova during ovulation. However, role of individual phytosterol of NPE at molecular level needs to be studied.

CONCLUSION: "*In vivo"* effect of non polar extract (NPE) of *Aloe vera* gel in letrozole induced PCOS rat model ameliorates PCO condition that altered glucose sensitivity and regular cyclicity. It also restored lipid profile and disturbed hormonal profile that again helped to repair ovarian dysfunction in PCO phenotype. Apart from organ level, NPE also significantly affects transcripts level of StAR, LHR, androgen receptor (AR), aromatase and Insulin receptor (IR) as well as relative protein expression of StAR, 3β HSD and aromatase in PCOS rats, which may be one of possible reason for modulated steroidogenesis. This implies that NPE rich in β sitosterol, stigmasterol, lupeol and other sterol derivatives that has potential to act at various targets for restoration of ovarian function in PCO phenotype. These phyto-components might help us to design future drug that can improve abnormal ovarian structure-function in pathology of PCOS.

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CONFLICT OF INTEREST STATEMENT: We declare that we have no conflict of interest.

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