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RESEARCH

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Decoding the molecular cascade of embryonic-uterine modulators in pregnancy loss of PCOS mother- an "*in vivo*" study

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Abstract

Background: Polycystic ovary syndrome is associated with an increased rate of spontaneous abortion/early pregnancy loss and pups delivered to PCOS animals were abnormal. Currently, assisted reproductive technology has been used to help numerous infertile couples to have their babies. However, there is a low implantation rate after the transfer of embryos. Till now, it could not be concluded whether the reduced pregnancy rates observed were due to abnormal embryos or endometrial modification. Further, transgenic mouse models have been used to find out the molecular deficits behind early pregnancy complications. But, the deletion of crucial genes could lead to systemic deficiencies/embryonic lethality. Also, pregnancy is a complex process with overlapping expression patterns making it challenging to mimic their stage-specific role. Therefore, the motive of the current study was to investigate the probable molecular cascade to decipher the early pregnancy loss in the letrozole-induced PCOS mouse model.

Methods: PCOS was induced in mice by oral administration of letrozole daily for 21 days. Following, the pregnancy was established and animals were sacrificed on the day 6th of pregnancy. Animals were assessed for early pregnancy loss, hormonal profile, mRNA expression of steroid receptors (*Ar, Pr, Esr1/2*), decidualization markers (*Hox10/11a*), adhesion markers (*Itgavb3, Itga4b1*), matrix metalloproteinases and their endogenous inhibitor (*Mmp2/9, Timp1/2)* and key mediators of LIF/STAT pathway (*Lif, Lifr, gp130*, *stat3*) were analyzed in the embryo implanted region of the uterus. Morphological changes in ovaries and implanted regions of the uterus were assessed.

Results: Mice treated with letrozole demonstrated significant increases in testosterone levels along with a decline in progesterone levels as compared to control animals. PCOS animals also exhibited decreased fertility index and disrupted ovarian and embryo-containing uterus histopathology. Altered gene expression of the steroid receptors and reduced expression of *Hox10a*, integrins, *Mmp9, Timp1/3*, *Gp130* & *Stat3* was observed in the implanted region of the uterus of PCOS animals.

Conclusion: Our results reveal that majority of the molecular markers alteration in the establishment of early pregnancy could be due to the aberrant progesterone signaling in the embryonic-uterine tissue of PCOS animals, which further translates into poor fetal outcomes as observed in the current study and in several IVF patients.

Keywords: Polycystic ovary syndrome, Pregnancy loss, Progesterone, Letrozole, Mice

Introduction

Polycystic Ovary Syndrome, a term of infertility has been now recognized as one of the major infertility disorders around the world [1]. Etiopathology of this disorder is mainly linked to hyperandrogenism, hyperinsulinemia, infrequent ovulation, and the presence of numerous

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peripheral cysts in ovaries [2]. This multi etiological pathology is associated with clinical pregnancy complications, an increased rate of spontaneous abortion/early pregnancy loss, and preterm delivery [3, 4]. However, molecular alterations in PCOS pregnancy that originates from the mother, embryo, or both are still in debate.

To achieve a successful pregnancy, the first step is embryo implantation, wherein two-way communication between a competent blastocyst and receptive uterus gives rise to attachment and invasion of the embryo to the uterine epithelium, following the decidualization of the uterine stroma [5]. Each step of the initial pregnancy involves an interplay of the various signaling pathways in which synchronized production of ovarian estrogen and progesterone mediates structural and functional changes in the uterus [6]. These gonadal hormones exert their effect through their receptors, progesterone receptor (*Pgr*) and estrogen receptor (*Esr1* & *2*) respectively, and regulate cell proliferation, differentiation, and secretory protein production in the uterus [7]. In addition, androgen and its receptor (*Ar*) could modulate uterine growth, antagonize the expression of estrogen-regulated genes, and also helps in the decidualization of the uterine stroma [8]. During the adhesion phase of early pregnancy, integrins (e.g., α4, αv, β1, β3) are considered to be endometrial markers, and their expression is synchronized with the blastocyst attachment to the endometrium [9]. As a sequel to blastocyst-uterine attachment, transcription factors such as homeobox (*Hox10a* and *Hox11a*) genes are known to involve in the proliferation and differentiation of stromal cells surrounding the implanting blastocyst into a decidual cell [10]. Further, invasion starts with penetration of the embryo to the uterine wall which involves degradation of extracellular matrix (ECM) through matrix metalloproteases (MMP-2 & 9). Activities of MMPs are tightly controlled by their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs). The elaborated balance between the activation of MMPs and their inhibition by TIMPs is important for the regulation of embryo implantation [11]. Latterly, leukemia inhibitory factor (LIF), is a pleiotropic cytokine of the IL-6 family that is considered to influence ranges from embryo adhesion to the regulation of stromal cell proliferation [12]. LIF transduces its signal through the formation of a heterodimer with specific LIFR and the common co-receptor for the IL-6 family (gp130). The binding of the LIF to its receptor leads to activation of STAT3, which further has an impact on the modulation of embryo-uterine functions during embryo implantation [13].

These key modulators of the implanting embryo and uterus try to establish an appropriate milieu that is crucial for the development and survival of the fetus during pregnancy. However, ethical restrictions and a lack of mechanistic studies have excluded studies on embryo-endometrium interlinkage in PCOS patients. Transgenic mouse models have been used to understand the mechanistic roles of many key determinants in uterine biology and implantation. Even so, the role of crucial genes remains undetermined because their constitutive deletion could lead to systemic deficiencies and embryonic lethality [14]. Additionally, the implantation phase of pregnancy is complex, and overlapping expression patterns make it challenging to mimic their stage-specific roles. Hence, understanding the signaling mechanisms is central to implantation, and deciphering these pathways would help us to potentially alleviate many problems associated with infertility like PCOS. In this line, to study the early pregnancy stage, rodent models have been employed as they exhibit similar anatomical and physiological features of pregnancy as humans [15].

Our previous lab study has shown that oral administration of letrozole (0.5 mg/kg of body weight) daily for 21 days successfully induced PCOS in adult female Balb/c mice [16]. Also, the number of fetuses born to PCOS mothers was less compared to that of control animals along with defects in fetal growth and development have been observed. This suggests that there could be some alteration in the early window of pregnancy. Therefore, the present study was undertaken to investigate the probable regulatory mechanism for the organization of the embryonic-uterine network in the establishment of the early pregnancy events in a letrozole-induced PCOS mouse model.

Materials and methods

Reagents

Letrozole tablets-2.5 mg, marketed under the brand name letronat were procured from Natco Pharma Ltd. Ethanol was procured from HiMedia Laboratories Pvt. Ltd. All other reagents of analytical grade were purchased from Sisco Research Laboratories Pvt. Ltd., India. Hormones-testosterone, estradiol, and progesterone were assayed using ELISA kits (DBC Canada). RNAiso Plus was procured from Takara Inc. High-Capacity cDNA Reverse Transcription Kit was procured from Applied Biosystems. SYBR Green (Power SYBR Green PCR Master Mix Life Technologies, USA). Primers used in the study were designed by the primer blast tool of NCBI and synthesized by INTEGRATED DNA TECHNOLOGIES (IDT).

Animal housing and maintenance

Thirty adult virgin (2–3 months) female Balb/c mice weighing 20-25 g were chosen for the study which was housed in a standard controlled animal care facility, in cages (four mice/cage), and maintained in a

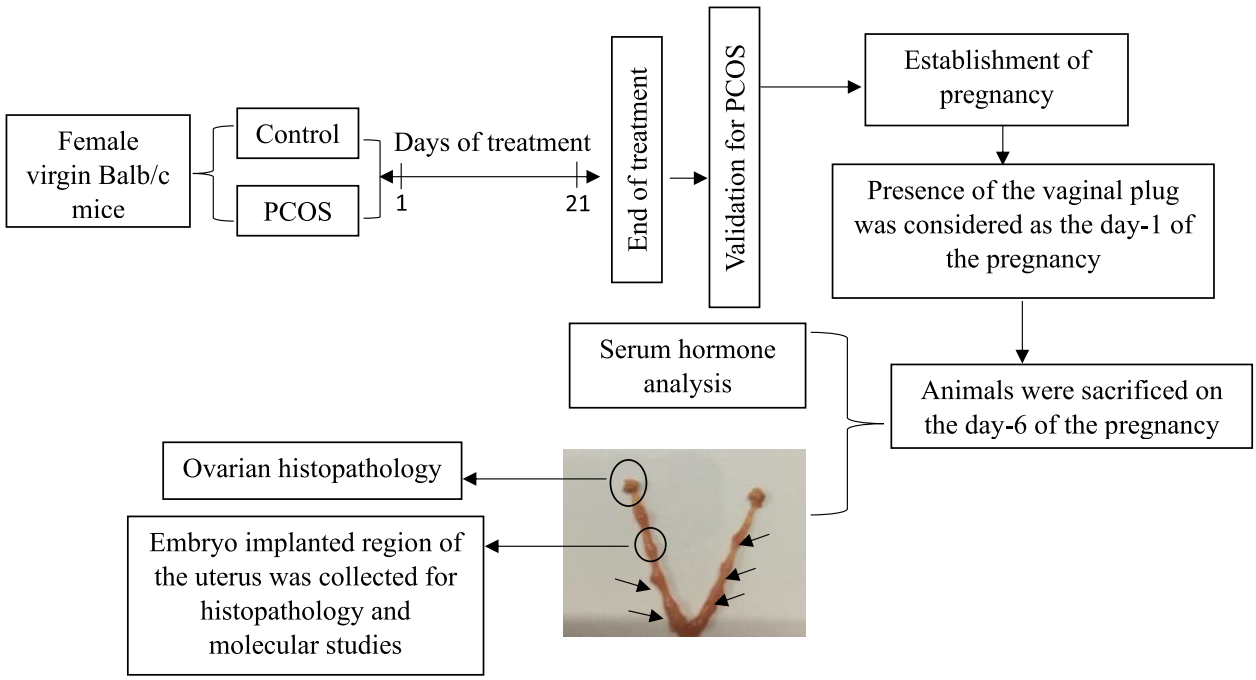

Fig. 1 Plan of work for evaluating the pregnancy loss of letrozole-induced PCOS mouse model

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. Experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara (Ethical Approval Number - MSU/BIOCHEMISTRY/IAEC/2019/4). Also, experiments were performed in compliance with the ethical standards of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Drug administration and experimental design

Firstly, the animals were categorized into two groups-: Group I (Control group $n=15$) received 1% Carboxymethyl cellulose (CMC) orally daily for 21 days and served as untreated Control while Group II (PCOS group $n=15$) received letrozole (0.5 mg/kg body weight) daily for 21 days and served as PCOS. After 21 days of the treatment, PCOS validation parameters bodyweight, estrus cycle profile, hormone analysis, and ovarian histology were analyzed. Next, female mice from both groups were allowed to mate with male mice of the same strain (2:1). The following morning, females were checked for the presence of a vaginal plug. The day of vaginal plug was considered day 1 of pregnancy. On the morning of day 6 (Adhesion of embryo to uterus), animals were sacrificed. Blood samples were collected by cardiac puncture. The serum was separated and kept in a freezer at −80 °C for determining serum hormones levels. At the end of the experiment, animals were sacrificed on the morning of day 6 of pregnancy, and the embryo containing region of the uterus was excised, trimmed, and appropriate parts were separated. One part is stored in RNAiso Plus reagent at −20 °C for the gene expression studies and, the remaining implanted site and ovary from both the groups were dissected and stored in 10% buffered formalin, for histopathological investigations. The plan of work is provided in Fig. 1.

Hormone estimation

Serum from blood was used as a sample to estimate the testosterone, estradiol, and progesterone levels using ELISA kits (Diagnostics Biochem Canada (DBC)- for testosterone, estradiol, and progesterone measurement). All measurements were taken according to the manufacturer's instructions. Each sample was assayed in duplicate. The sensitivity of the kits was 0.022 ng/mL, 10 pg/mL, and 0.1 ng/mL for testosterone, estradiol, and progesterone kits respectively. The working range was 0.08 to 16.7 ng/mL, 20 to 3200 pg/mL, and 0.3 to 60 ng/mL of testosterone, estradiol, and progesterone respectively. The intra-assay coefficient of variation (CV) was 6.6 to 9.6%, 4.6 to

9.3%, and 10.2 to 10.6% for testosterone, estradiol, and progesterone kits respectively. The inter-assay coefficient of variation (CV) was 6.1 to 7.3%, 6.2 to 10.1% and 10.2 to 12.6%, for testosterone, estradiol and progesterone kits respectively. The recovery range was between 80.5 to 110.1%, 90.3 to 116.2% and 78 to 124% for testosterone, estradiol and progesterone kits respectively.

Histological examination of the ovaries and implanted region of the uterus

The ovaries and implanted region of the uterus were fixed in 10% buffered formalin, processed, embedded in paraffin, and cut into 5-µm-thick sections. The ovarian and implanted region of the uterus sections were stained with hematoxylin and eosin and assessed microscopically. In the ovary, Graafian follicles, peripheral cystic follicles, and corpus luteum were identified. Observations and documentation were made on a DM2500 microscope (Leica, Germany) with Leica EZ digital camera under 4X magnification. In the implanted region of the uterus, attached embryo and decidual uterine cells were identified. The examination was carried out on a Nikon Ti2E microscope under 10X magnification.

Gene expression analysis

According to previous lab protocol, relative quantification of gene expression was carried out using real-time PCR [17]. Total RNA was obtained from the implanted region of the uterus using RNAiso plus reagent as per the manufacturer's instructions. The quantification was performed using the NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) with a wavelength of 260 nm. RNA integrity was assessed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. Purity was assessed through absorption rate OD260/OD280 and samples showing a value less than 1.8 were discarded. The reverse transcription reaction to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufactures instructions. Real-time quantitative polymerase chain reaction (qPCR) was performed using QuantStudio 5 Real-Time PCR System using SYBR Green (Power SYBR Green PCR Master Mix – Life Technologies, USA). All samples were run in duplicate and accompanied by a non-template control. Thermal cycling conditions included initial denaturation in one cycle of 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and 1 min at 72 °C. After amplification, the melting curves were analyzed to verify the amplification of only one product. The relative mRNA expression and fold change were calculated based on the amplification of the reference gene β-actin (ACTB). The primers used for the amplification are given in supplementary Table 1. The fold changes in expression levels of less than 0.5 and greater than 2 were considered to be biologically significant.

Gelatin zymography

Matrix metalloproteases activity was measured as described by sohail and group [18] with modifications. All the modifications are mentioned in the procedure. A 10% tissue homogenate (Implanted region of the uterus) was prepared in PBS buffer (NaCl 0.137 M, KCL 0.0027 M, Na_2HPO_4 0.01 M, KH_2PO_4 0.0018 M, pH 7.1) followed by centrifugation at 10000 g for 20 minutes at 4 °C, the supernatant was collected, and the protein was estimated by lowry's method. 80 µg of total protein samples were subjected to electrophoresis in a 10% polyacrylamide gel containing 1% gelatin. After electrophoresis, the gels were treated twice for 30 min each in 2.5% Triton X-100 and incubated for 18 h in a calcium buffer (0.05 M Tris–HCl, 0.2 M NaCl, 0.01 M $CaCl_2$, 1% Triton X-100, and 1 µM $ZnCl_2$, pH 7.5). Gels were stained with 0.1% Coomassie brilliant blue R-250. MMPs activities were visualized as clear bands against a dark background after distaining. The gels were then photographed and the band intensities were quantified using ImageJ software.

Statistical analysis

The values are presented as mean ± standard error mean in all the experiments. Statistical analysis was done using student's t-test (For Control and PCOS group) using (GraphPad Prism 5 software, La Jolla, CA). *P*-values when less than 0.05 were considered to be statistically significant at the 95% confidence limit.

Results

Early pregnancy loss (day-6th of pregnancy) in PCOS mice

PCOS is a complex hormonal disorder with a high risk of first-trimester miscarriage. (To validate the PCOS pathology after the 21 days of treatment, body weight, estrus cyclicity profile, hormone profile, and ovarian histology were analyzed (Supplementary Fig. 1).

Further, blood steroid (Testosterone, progesterone, and oestradiol) levels and ovarian histology were analyzed on day-6 of pregnancy. As shown in Table 1, the serum

Table 1 Serum hormone levels on the day 6th of pregnancy

	Control	PCOS
Testosterone (ng/ml)	0.7020 ± 0.04903	1.240 ± 0.1435 **
Progesterone (ng/ml)	58.25 ± 4.029	16.00 ± 1.826 ***
Estradiol (pg/ml)	73.50 ± 5.377	83.00 ± 4.655 ns

The values are represented as Mean ± SEM. $N = 6$ per group. $^{**}P < 0.01$, $^{***}P < 0.001$, ns-not significant as compared to Control

testosterone in the PCOS model group was significantly higher than that in the control group (**$P < 0.01$), which can be correlated with hyperandrogenaemia in PCOS. Also, a decrease in progesterone content was observed in letrozole-treated mice (***$P < 0.001$) whereas estradiol levels remained unchanged in both groups. Since it is known that maintenance of the steroid milieu is vital for the ovarian structure and function, the histology profile of the ovary using haematoxylin-Eosin stain was analyzed. The control group showed normal ovarian morphology with mature follicles (tertiary and graafian follicles) and corpora lutea. PCOS animals demonstrate

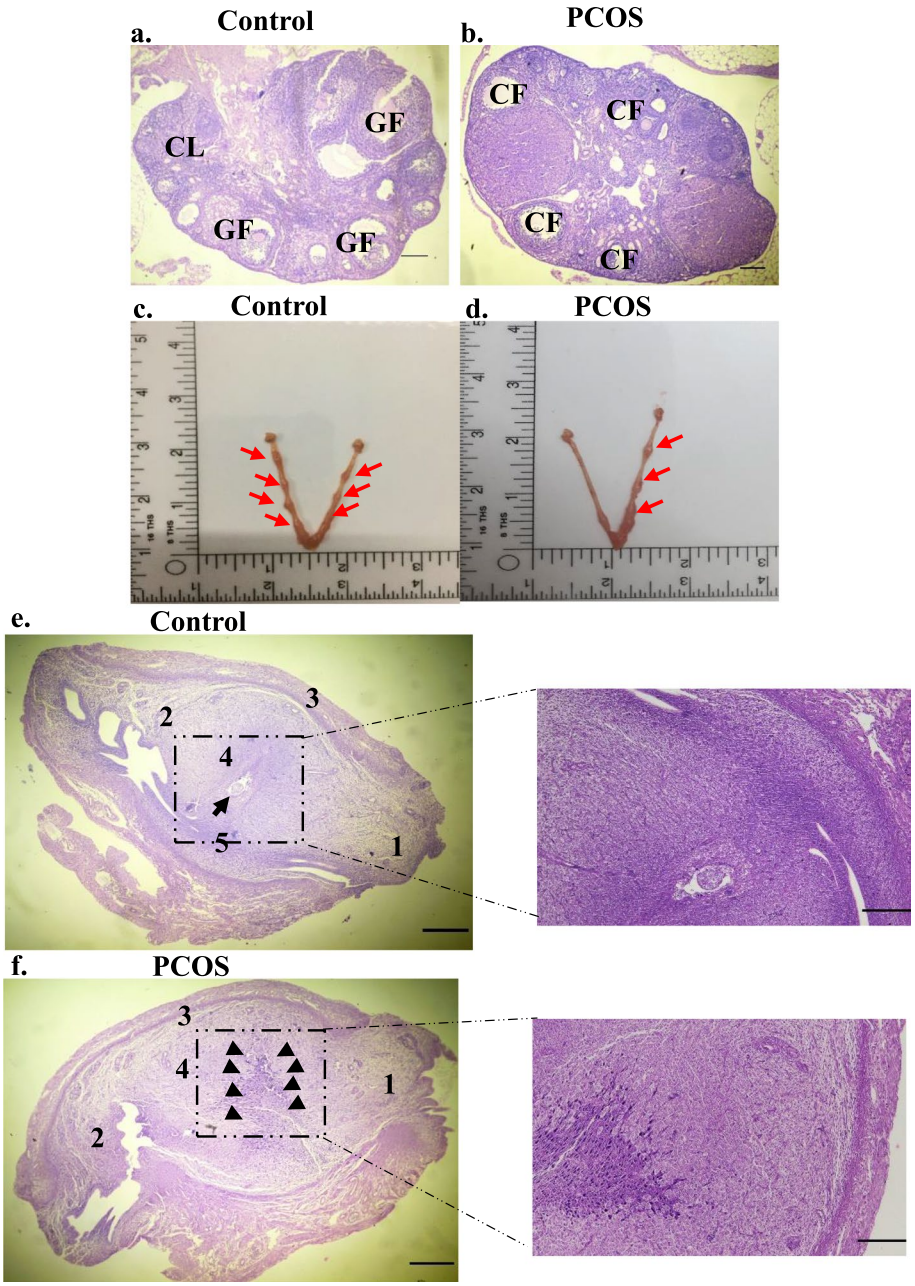

Fig. 2 Hematoxylin and eosin-stained sections of the ovary and implanted region of the uterus. **a.** Control group **b.** PCOS group. CL: corpus luteum; CF: cystic follicle; GF- Graafian follicle, magnification 4X. Pictorial representation of a number of implanted sites. **c.** Control group **d.** PCOS group. Arrows indicate the implanting embryo. Hematoxylin and eosin-stained sections of embryo implanted region of the uterus **e.** Control group, black arrow indicates embryo **f.** PCOS group, black arrows indicate accumulation of erythrocytes. 1. Mesometrium. 2. Mesometrial endometrium. 3. Myometrium. 4. Anti-mesometrial decidua. 5. Embryo. Scale bar = 100 µm

multiple large peripheral cysts, fewer corpus luteum, and reduced mature follicles as compared to control animals (Fig. 2). Further, to study the implantation loss, reproductive performance, number, and histology of implanted region of the uterus were analyzed. It was observed that the number of pregnant females was reduced in letrozole induced PCOS mouse model Table 2. Also, a fewer number of implants were observed in PCOS animals compared to control animals (**$p < 0.01$). Figure 2 demonstrates the pictorial representation of implanted embryos in the uterus of both groups. Histology of implanted region of the uterus demonstrates the embryo has attached to the antimesometrial uterine lumen epithelium (marked by black arrows) and is surrounded by developing decidual cells in the control group. However, the PCOS group exhibited an accumulation of erythrocytes (marked by a black arrow) caused by a gain in vascular permeability was observed.

Altered steroid hormone receptor expression in the implanted region of the uterus in PCOS mice

To accomplish a sequential event of pregnancy, endometrium requires to undergo steroid-dependent changes. Steroids like estrogen, progesterone, and testosterone mediate their effect through their receptor's estrogen receptor α & β (*Esr-1* & *2*), progesterone receptor (*Pgr*), and androgen receptor (*Ar*), respectively. Hence, In the implanted region of the uterus, steroid receptor transcript level was done using quantitative real-time PCR (Fig. 3). Transcriptional downregulation of *Pgr* (***$P < 0.001$), *Esr1* (**$P < 0.01$) and *Esr2* (***$P < 0.001$) was observed in PCOS animals. On the contrary, mRNA levels of *Ar* (* $P < 0.05$) were found markedly high in the implanted site of the uterus in PCOS animals when compared with control tissues.

Table 2 Reproductive performances for female fertility

	Control	PCOS
Females (n)	14	14
Mated females (n)	13	12
Pregnant females (n)	12	8
Not pregnant females (n)	1	4
Time required for conception (in days)	3.769 ± 0.2809	3.308 ± 0.3279 ns
Mating index* (%)	92.85714 (~93)	85.7248 (~86)
Fertility index** (%)	85.71429 (~86)	57.14286 (~57)
Total number of implants	9.000 ± 0.3162	5.800 ± 1.020 *

The values are represented as Mean ± SEM. * $P < 0.05$, ns = not significant as compared to the control group. *Mating index = Mated females/ Total females kept for mating × 100. **Fertility index = Pregnant females/Total females kept for mating females × 100

Impairment of feto-maternal interaction in the implanted region of the uterus in PCOS mice

Subsequently, numerous integrins - αvβ3 (*Itgav, Itgb3*), α4β1 (*Itga4, Itgb1*) are known to involve in embryo-endometrium interaction in the establishment of a healthy pregnancy. Hence, the transcript levels of these markers were evaluated in the implanted region of the uterus in the letrozole-induced PCOS mouse model. When analyzed for gene expression, *Itgav, Itgb3* (**$P < 0.01$), *Itga4,* and *Itgb1* (***$P < 0.001$) were declined in the PCOS group compared to the control group (Fig. 4).

Modulation of decidualization events of embryo-uterine tissue in PCOS mice

Transcription factors, such as homeobox genes (*Hox10a*, and *Hox11a)* are known to involve in the decidualization of uterine stroma to complete the early stage of pregnancy. Hence, the transcript levels of these markers were evaluated in the implanted site of the uterus in the letrozole-induced PCOS mouse model. When analyzed for gene expression, *Hox10a* (**$P < 0.01$) were declined in the implanted region of the uterus in the PCOS group compared to the control group with no difference in *Hox11a* in both the groups (Fig. 5).

Imbalance in the expression of matrix metalloproteinases and their endogenous inhibitor in the implanted region of the uterus in PCOS mice

Invasiveness of embryo to receptive endometrium required extensive degradation and remodeling of the extracellular matrix (ECM). Matrix metalloproteinases (*Mmp2* & *9*) are responsible for the breakdown of ECM during the implantation process. The activity of MMPs is tightly controlled by their endogenous inhibitors, the tissue inhibitors of MMPs (*Timp*). When analyzed for the transcript levels and activity of MMPs, gene expression (**$P < 0.01$) and activity (*$P < 0.05$) of MMP-9 were decreased in the implanted region of the uterus in PCOS animals compared to the control group. However, the activity of MMP-2 did not show any change in both groups. Gene expression of *Timp1* (**$P < 0.01$) and *Timp3* (***$P < 0.001$) were reduced in the implanted site of the uterus in the PCOS group compared to the control group (Fig. 6).

Disruption of the LIF-STAT3 signaling pathway in the implanted region of the uterus in PCOS mice

A tightly regulated rhythm between embryonic development and uterine maturation is essential for a successful pregnancy. This function is mediated through the cytokine on their receptors, mainly by the LIF-STAT pathway. When analyzed for key mediators of the

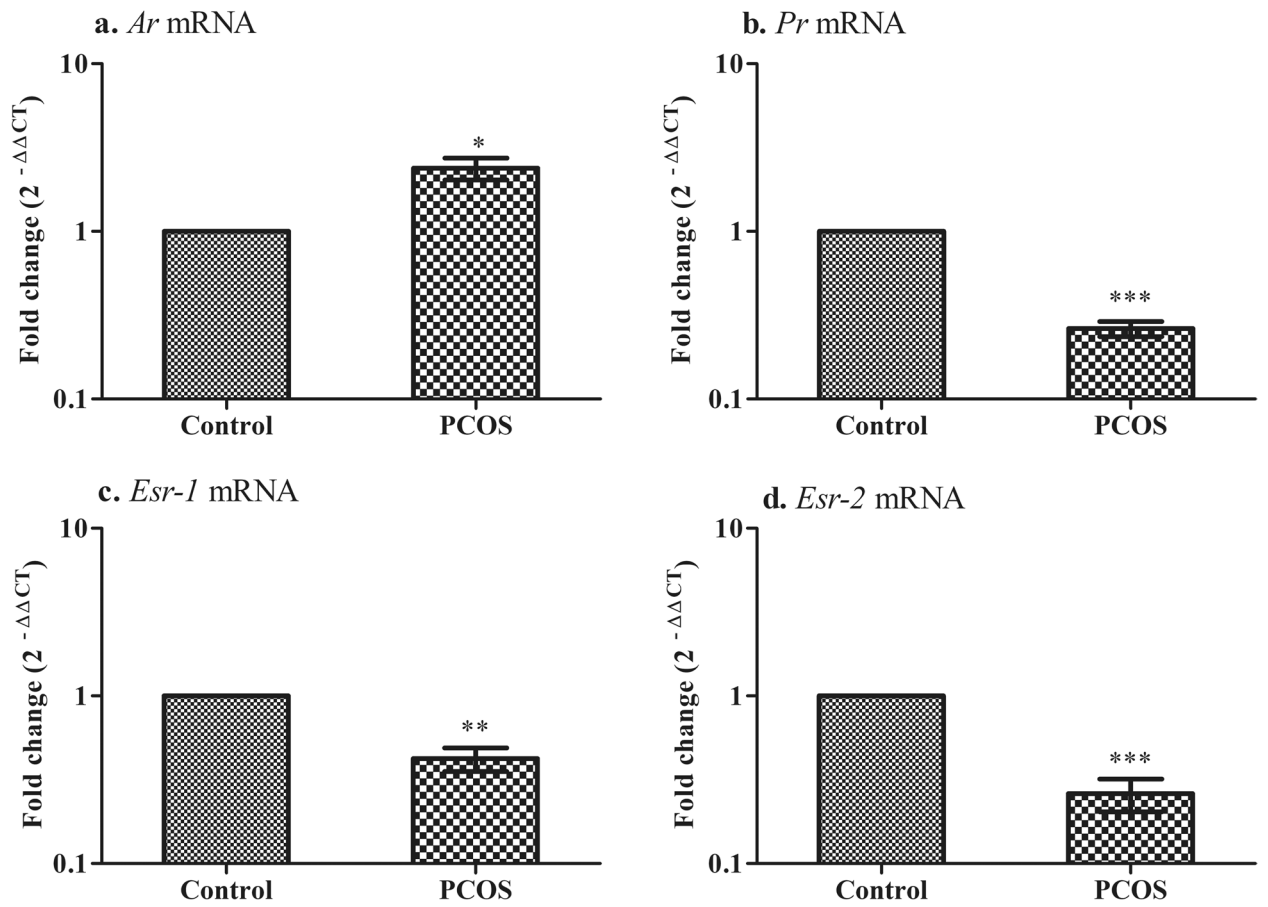

Fig. 3 Steroid hormone receptors in the implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Androgen receptor **b.** Progesterone receptor **c.** Estrogen receptor-α **d.** Estrogen receptor-β in the letrozole-induced PCOS mice model. Error bars represent SEM; $N = 6$ per group. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ as compared to control group

pathway, the transcript level of glycoprotein 130 (*gp130*) (** $P < 0.01$) and Signal transducer and activator of transcription 3 (*Stat3*) (*** $P < 0.001$) were declined in the implanted region of PCOS animal as compared to control group; however, no difference was observed in leukemia inhibitory factor (*Lif*) and leukemia inhibitory factor levels (*Lifr*) (Fig. 7).

Discussion

The current study exhibited retarded fetal growth and a lesser number of pups were born to PCOS animals (Supplementary Fig. 2). In this context, early gestational events are found to be the important window that directs proper fetal growth by the numerous molecular cascade pathways. Currently, a great deal has been brought out in the domain of assisted reproductive technology (ART) and its approach could assist numerous infertile couples to have their babies. However, a major issue with this approach is the low implantation rate after several transfers of good-quality of embryos [19]. Also, it was examined that alterations in oocytes and embryos could be contributing to unsuccessful outcomes in patients with PCOS who are undergoing assisted reproduction [20]. Still, it could not be concluded whether reduced pregnancy rates seen are due to abnormal embryos which fail to implant or whether there are some modifications in the endometrium which do not allow implantation [21]. Hence, it could be noted that abnormalities in embryos and uterine independently may lead to a reduced pregnancy rate in women with PCOS. However, there are not sufficient pieces of evidence in the context of molecular alterations in the embryo containing uterine microenvironment. Thereby, the present study attempted to decode the complex mechanistic signaling of early pregnancy in a letrozole-induced PCOS mouse model.

In a previous lab study, it was reported that oral administration of letrozole (0.5 mg/kg body weight) daily for 21 days exhibits reproductive and metabolic alteration signs similar to the human PCOS condition [16]. In the current experiment, letrozole-induced female Balb/c

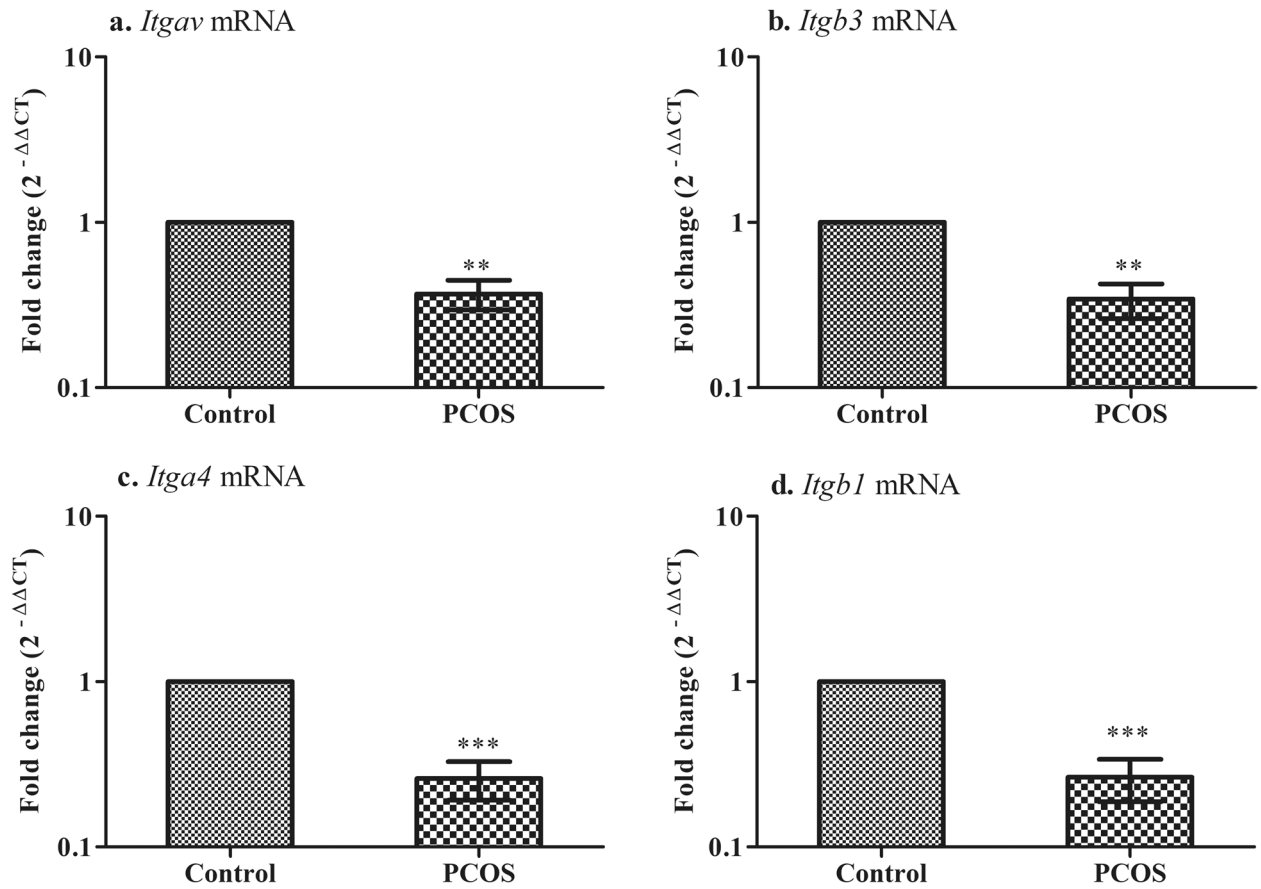

Fig. 4 Integrin gene expression in the implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Integrin- αv **b.** Integrin- β3 **c.** Integrin- α4 **d.** Integrin- β1 in the letrozole induced PCOS mice model. Error bars represent SEM; $N=6$ per group. $^{**}P<0.01$, $^{***}P<0.001$ as compared to the control group

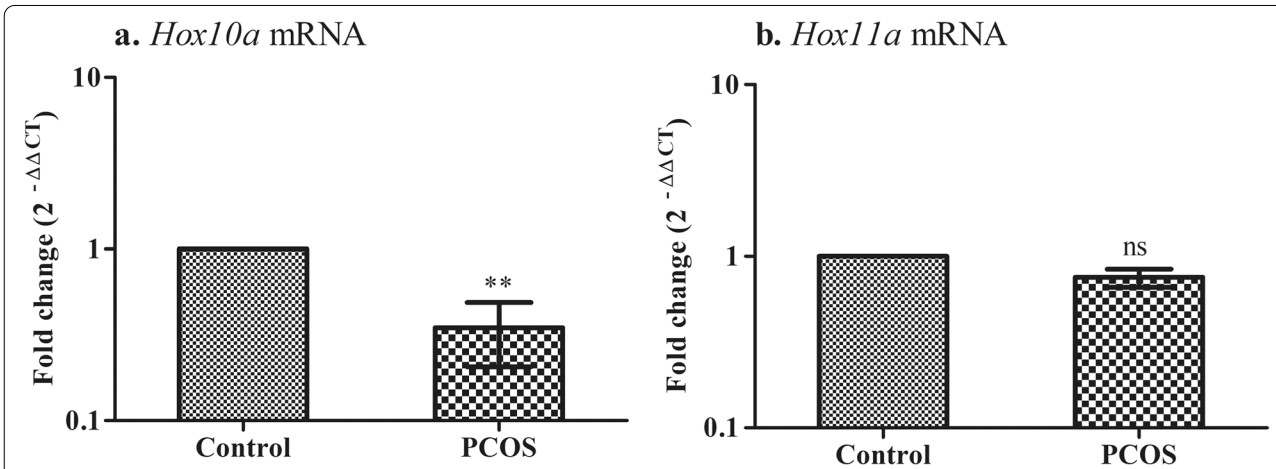

Fig. 5 Transcription factor gene expression in the implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Homeobox-10A **b.** Homeobox-11A in the letrozole induced PCOS mice model. Error bars represent SEM; $N=6$ per group. $^{**}P<0.01$ and ns-non-significant as compared to the control group

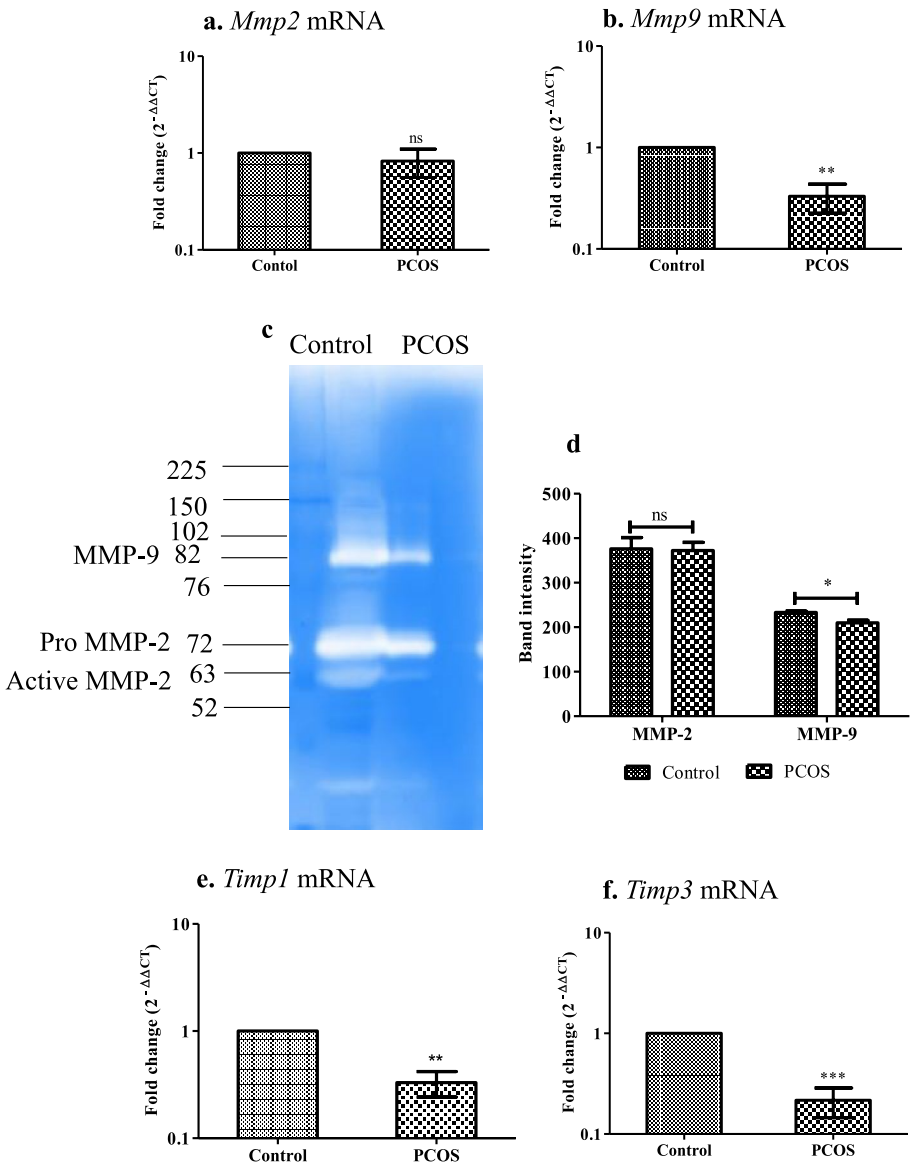

Fig. 6 Matrix metalloproteinases and their inhibitors in the implanted region of the uterus. Values are mean fold changes in gene expression, **a.** MMP-2, and **b.** MMP-9. **c.** Gelatin gel zymograms showing pro-MMP2, active MMP2, and active MMP9 activity (arrows) in the letrozole induced PCOS mice model (represented gel picture was cropped from the same gel- uncropped/original gel picture is given in supplementary fig. S3) **d.** Quantification of total (Pro and active) MMP9 and MMP2 by computer-based densitometry analysis. **e.** & **f.** Values are mean fold change in gene expression of tissue inhibitor of metalloproteinase TIMP-1 & TIMP-3 respectively. Error bars represent mean ± SEM; $N = 6$ per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns-non-significant as compared to the control group

mice showed elevated testosterone levels during early pregnancy. It could be correlated with the efficacy of letrozole (an aromatase inhibitor) in the establishment of PCOS in rodents wherein excessive accumulation of androgens occurs in the ovary [22]. In addition, low levels of progesterone were observed, which can be associated with the disruption of corpus luteum formation in PCOS animals. Similar observations were noted wherein PCOS patients are not able to form corpus luteum and generate a low level of progesterone, thus leading to abnormal ovulation [23]. Interestingly, circulating estrogen did not change in both groups, which is in accordance with studies reported in the letrozole-induced mouse model [24]. The altered hormone profile in PCOS animals might influence ovarian structure. When examined for ovarian histology, the number of peripheral cysts was observed, which is one of the characteristic features of PCOS [25, 26]. Reports have shown that hyperandrogenism and low

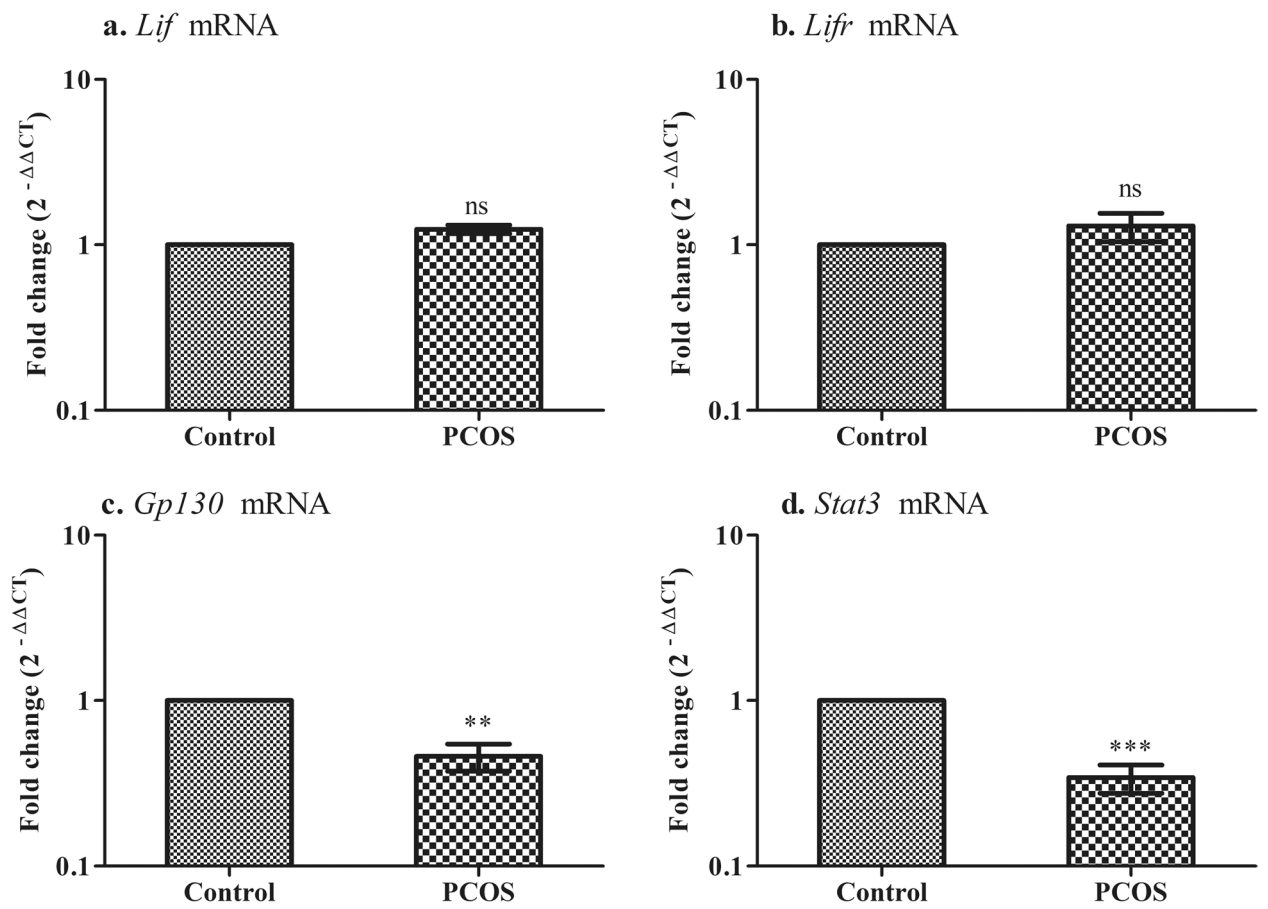

Fig. 7 Key mediators of LIF-STAT3 related genes in the implanted region of the uterus. Values are mean fold changes in gene expression, **a.** Leukemia inhibitory factor **b.** Leukemia inhibitory factor receptor **c.** Glycoprotein 130 **d.** Signal transducer and activator of transcription 3 in the letrozole-induced PCOS mice model. Error bars represent SEM; $N = 6$ per group. $^{**}P < 0.01$, $^{***}P < 0.001$, ns-non-significant as compared to control group

progesterone content in women with PCOS have a lower probability of childbirth, decreased pregnancy rates, and higher miscarriage rates [27–29]. Indistinguishable, results were noticed in our study, the number of pregnant females and the number of implanting embryos in the uterus were significantly reduced in the PCOS animals. It is interesting to note that the above-cited reports have not clearly defined molecular interplay in the reduced pregnancy outcomes seen in PCOS infertility. Thereby, the present study attempts to further narrow down molecular deficits of pregnancy loss in PCOS pathology.

Progesterone signaling is known to have an inhibitory effect on the E/*Esr* signaling pathway in stromal cells of the endometrium for the establishment of pregnancy [30]. In our experimental model, the decline P/*Pgr* signaling did not show an inhibitory effect on estrogen, resulting in no difference in the estrogen-responsive gene (leukemia inhibitory factor (*Lif*)) has been observed in embryonic-uterine tissue. Furthermore, it was reported that progesterone signaling inhibited androgen receptor (*Ar)* expression, whereas estrogen dramatically elevated *Ar* abundance in the stroma of ovariectomized mouse uteri during early pregnancy [31]. In this direction, the current study revealed that altered P/*Pgr* signaling in PCOS animals did not have a prohibited influence on *Ar* expression, as the overexpression of *Ar* was observed in the implanted site of the uterus. Hence, the reduced progesterone signals in letrozole-treated animals which could be leading to dysregulated downstream targets in the implanted region of the uterus during the early pregnancy window.

Further, the study reported that when ovariectomized rats were treated with a sex-steroid regime to mimic the hormonal changes of early pregnancy, their findings have shown that progesterone is likely responsible for the regulation of αvβ3 integrin levels in the uterus [32]. Apart from steroidogenic control, LIF and its receptor are known to increase the expression of integrin αvβ3

and αvβ5 during the adhesion of the blastocyst implantation [33]. Results from the current study exhibited no difference in the gene expression of LIF & its receptor in the implanted region of the uterus in PCOS animals. This suggests that the declined expression of integrins was not mediated via LIF & LIFR. Hence, we can conclude that progesterone could be one of the contributory reasons in the reduced integrin expression, causing an impaired embryo-uterine attachment during implantation in the letrozole-induced PCOS animals.

Blastocyst attachment with the uterine epithelium is followed by the decidualization of the stromal cellsand the homeobox transcription factors are known to regulate this process [10]. Also, it was observed that progesterone and its receptor signaling upregulate the HOX10a in the isolated human endometrial stromal cells [34]. Based on this, and the above-cited references, low serum progesterone concentration in the PCOS group could not induce the HOX10a expression that is required for decidualization. As a consequence, aberrant early embryonic-uterine communication may alter the pregnancy outcomes in the PCOS phenotype.

Matrix metalloproteases (MMPs) and their inhibitors (TIMPs) have a significant role in tissue remodeling, and homeostasis of the MMPs & TIMPs is thought to be crucial during normal early gestation [35] And the importance of these proteases has been described whereas in women with an imbalance in the serum levels of MMPs

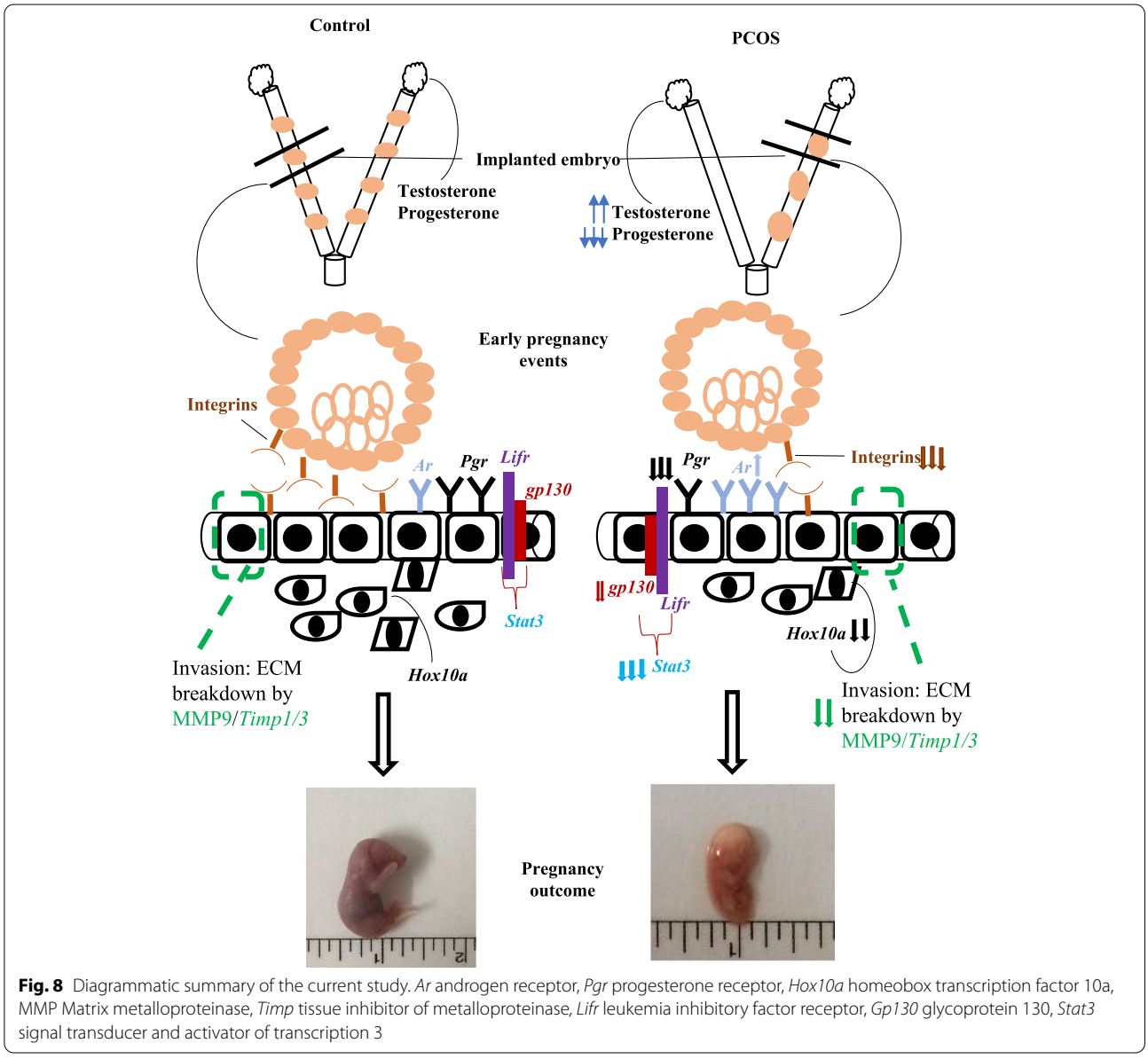

Fig. 8 Diagrammatic summary of the current study. *Ar* androgen receptor, *Pgr* progesterone receptor, *Hox10a* homeobox transcription factor 10a, MMP Matrix metalloproteinase, *Timp* tissue inhibitor of metalloproteinase, *Lifr* leukemia inhibitory factor receptor, *Gp130* glycoprotein 130, *Stat3* signal transducer and activator of transcription 3

and TIMPs has been associated with spontaneously terminated pregnancy in the first trimester [36]. There are reports indicating that proteases and TIMPs in the uterus have been controlled by the action of estrogen. In contrast, MMP-2 and TIMP-3 expression was not changed by steroidal treatment [11, 37]. However, in the current study, PCOS animals did not exhibit any change in estradiol content as compared to the control animals. Also, results revealed that the expression of MMP-9, TIMP-1 & 3 was significantly reduced in the letrozole-treated animals. Hence, the imbalance in the expression of the proteases and their inhibitors may be attributed to improper blastocyst invasiveness during early gestation in PCOS pathology.

Furthermore, emerging evidence suggests that ovarian steroids are reported to play a critical role in regulating the key LIF signaling markers (LIF, LIFR, and GP130) in the uterus throughout the implantation window period [12]. This is supported by the observation wherein exogenous administration of estrogen and estrogen/progesterone both can induce LIF, LIFR and GP130 expression respectively in the endometrium of ovariectomized mice [38, 39]. In addition, uterine conditional ablation of STAT3 leads to dysregulation of PR mediated pathways and decreased PR protein expression in utero, suggesting that STAT3 has a critical role in PR-dependent pathways during implantation in mice [40]. In this study, disrupted LIF-STAT signaling was observed in the PCOS animals. However, it couldn't be confirmed whether the unbalanced LIF signaling could be because of altered progesterone signals or whether declined STAT3 does not activate the PR mediated pathways in the implanted region of the uterus.

Based on all of the above molecular deficits in the PCOS pregnant uterine, it was speculated that the changes observed might be originated from the modification of the histological architecture structure of the implanted region of the uterus on the day 6^{th} of pregnancy. Moreover, healthy growing implanted embryos were found in the untreated animals, whereas in the letrozole treated animals, the appearance of vascular permeability was observed in the implanted region of the uterus. These inherent changes in the structure area of the uterus could be implicated in the endometrial dysfunction in the pregnant PCOS mice.

Conclusion

A significant strength of this study is to explore the potential mechanism by which PCOS may alter the embryonic-uterine microenvironment thereby preventing the establishment of a healthy pregnancy (Fig. 8). The effect of the letrozole on the implanted region of the uterus suggests that the majority of the molecular alterations were due to the aberrant PR expression and it signaling dysregulates the expression of the genes that are involved in the uterine-embryonic cross-talk during the early gestation period. Further, the abnormal expression of key markers of early gestation in PCOS could be the reason for the early pregnancy complications/early fetal loss associated with PCOS women. As evidence of the poor fertility index and reduced number of implanted embryos were observed in the PCOS animals. Thus, the current study gives insight into the regulation of intracrine molecules to improve uterine-embryonic functions and potential medicinal targets to expand the conceptive outcome of the PCOS patient.

Abbreviations

PCOS: Polycystic ovary syndrome; AR: Androgen receptor; PR: Progesterone receptor; ESR1/2: Estrogen receptor α/β; HOX10/11a: Homeobox 10/11a; ITGAVB3: Integrin αvβ3; ITGA4B1: Integrin α4β1; MMP2/9: Matrix metalloproteinases 2/9; TIMP: Tissue inhibitor of metalloproteinase; LIF: Leukemia inhibitory factor; LIFR: Leukemia inhibitory factor receptor; GP130: Glycoprotein 130; STAT3: Signal transducer and activator of transcription 3.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-022-01041-x.

Additional file 1: Supplementary Table 1. List of primers used in the study.

Additional file 2: Supplementary Fig. 1. Validation of PCOS phenotype in mice.

Additional file 3: Supplementary Fig. 2. Defects in fetal growth and development on the day 18th of pregnancy in letrozole induced PCOS animals.

Additional file 4: Supplementary Fig. 3. Original/uncropped full-length gel of Fig. 6 (in the main manuscript).

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Authors' contributions

SD wrote the manuscript, designed the analysis, collected the data, performed the data analysis, and review and edited the manuscript. LN designed analysis, reviewed, and edited the manuscript. Both the authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article [and its supplementary information files].

Declarations

Ethics approval and consent to participate

All the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC), Department of Biochemistry, The M. S. University of Baroda, Vadodara (Ethical Approval Number (MSU/BIOCHEMISTRY/IAEC/2019/4). Also, Also, experiments were performed in compliance with the ethical standards of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Partially purified non-polar phytocomponents from *Aloe barbadensis* Mill. gel restores metabolic and reproductive comorbidities in letrozole-induced polycystic ovary syndrome rodent model- an *"in-vivo"* study

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ABSTRACT

Ethnopharmacological relevance: In India, Kumaryasava, a popular *Aloe barbadensis Mill. gel* preparation has therapeutic value in treatment of female reproductive disorders like menstrual disturbances and menopausal problems. Despite their widespread use, only a limited number of studies have probed into the scientific evidence for their varied bioactivities. In this regard, studies have demonstrated that *Aloe vera* gel has the potential to modulate steroidogenic activity in letrozole induced polycystic ovary syndrome (PCOS) rat. However, isolation and identification of the bioactive molecule/s from *Aloe vera* gel and studying their molecular targets will underpin the treatment regime for PCOS.

Material and methods: The Partially Purified Non-Polar Phytocomponents (PPNPP)- LP1 and LP3 were isolated from the petroleum ether extract of *Aloe vera* gel by column chromatography. Based upon the GC-MS analysis, LP1 and LP3 comprised of n-Hexadecanoic acid and Campesterol acetate with an abundance of 97.07%, and 96.07% respectively. For evaluation of their bioactivities, eighty 3–4 months female Balb/c mice were classified as 10 groups with 8 animals in each group. Groups were control (C), PCOS (0.5 mg/kg/day Letrozole orally for 21days), PCOS treated orally for 60 days with *Aloe vera* gel (AVG) (10 mg/kg/day) (PCOS + AVG), PCOS treated orally for 60 days with petroleum ether extract (PE) of *Aloe vera* gel (25 μg/kg/day) (PCOS + PE), PCOS treated orally for 60 days with LP1 (0.5 μg/kg/day) (PCOS + LP1), PCOS treated orally for 60 days with commercially available pure compound-n-Hexadecanoic acid (HA) (0.5 μg/kg/day) (PCOS + HA), PCOS treated orally for 60 days with LP3 (0.01 μg/kg/day) (PCOS + LP3), PCOS treated orally for 60 days with commercially available pure compound- Campesterol acetate (CA) (0.01 μg/kg/day) (PCOS + CA), PCOS treated orally for 60 days with Metformin (100 mg/kg/day) (PCOS + Metformin) and PCOS treated orally for 60 days with DMSO (Vehicle) (PCOS + DMSO). Body weight, Oral glucose tolerance test, lipid profile, fasting glucose, insulin, estrus cycle, hormonal profile, gene expression of gonadotropin receptors *(Fshr and Lhr),* steroid receptors *(Ar, Esr1, Esr2 and Pgr)* and steroidogenic markers (*Star*, *Hsd3b1*, *Cyp19a1* and *Amh*) were analysed in the ovaries. Polycystic ovarian morphology was assessed through histopathological changes of ovary. Toxicity markers- SGOT, SGPT and creatinine were also measured at the end of the study.

Results: Mice treated with letrozole demonstrated significant increase in body weight, glucose intolerance, fasting insulin levels, HOMA-IR, triglycerides levels as well as testosterone levels, and a significant decline in the progesterone levels as compared to the control animals. PCOS animals also exhibited arrested estrus cyclicity, disrupted ovarian histopathology with the presence of multiple peripheral cysts and abnormal gene expression of gonadotropin receptor, steroid receptor and steroid markers. Oral administration of AVG, PE extract of AVG, LP3 and metformin greatly alleviated these complications in PCOS animals.

Conclusion: The above findings indicate the effectiveness of LP3, isolated from *Aloe vera* gel against letrozole induced PCOS in mice and may be used in the treatment of PCOS as an alternative to metformin.

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1. Introduction

Abbreviations:

3βHSD	3 beta-hydroxysteroid dehydrogenases
AVG	*Aloe vera* Gel
CA	Campesterol Acetate
CYP19A1	Cytochrome p450 family 19 subfamily A member 1
FSHR	Follicle-stimulating hormone receptor
GC/MS	Gas Chromatography- Mass Spectrometry
HA	n-Hexadecanoic acid
HDL-C,	High-density lipoprotein cholesterol
HOMA-IR	Homeostasis model assessment of insulin resistance
HPLC	High performance Liquid Chromatography
LDL-C,	Low-density lipoprotein cholesterol
LHR	Luteinizing hormone receptor
OGTT	Oral Glucose Tolerance Test
PCOS	Polycystic ovary syndrome
PE	Petroleum ether extract of *Aloe vera* gel
PPNPP	Partially purified non-polar phytocomponents
Rf	Retention Factor
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
StAR	Steroidogenic Acute Regulatory Protein

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine disorders affecting 4–26% women belonging to reproductive age and is characterized by hyperandrogenism, hyperinsulinemia, oligo-ovulation and multiple peripheral cysts in the ovaries-due to dysregulated steroidogenesis and its signalling (Chatterjee and Bandyopadhyay, 2020; Dunaif and Book, 1997; Abbott et al., 2002; Doi et al., 2005; Wickenheisser and McAllister, 2007). Although the cause of PCOS is unknown, it is thought to be a multifaceted condition involving environmental, genetic, metabolic and endocrine abnormalities (Franks et al., 2006). Almost 30–40% of women affected with PCOS have impaired glucose tolerance, insulin resistance, dyslipidaemia and other complications associated with metabolic syndrome (Chandrasekaran and Sagili, 2018). Recent animal studies have demonstrated that insulin resistance may play a greater role in the pathogenesis and development of PCOS, and it has been implicated as one of the causes of the onset and progression of hyperandrogenism (Goodman et al., 2015). Biosynthesis of sex steroids is modulated by insulin and insulin-like growth factor-1 (IGF-1), which are considered as most notable extra-ovarian factors stimulating androgen production. Excessive production of androgenic hormones in the ovaries caused by either an excessive amount of luteinizing hormone (LH) from the anterior pituitary gland or a high level of insulin in the blood, alone or in combination, is often linked to the pathogenesis of PCOS (Rosenfield and Ehrmann, 2016). Previous studies have found that obesity, hyperandrogenism, and insulin resistance contributed to the development of hypertriglyceridemia in PCOS (Diamanti- Kandarakis et al., 2007).

Conclusively, PCOS is a polygenic and multifactorial syndromic disorder. Many genes have been associated with PCOS, which affect fertility either directly or indirectly. Many candidate genes have been studied along the HPG axis like luteinizing hormone receptor (*Lhr*), follicle-stimulating hormone receptor (*Fshr*), Steroid receptors such as androgen receptor (*Ar*), estrogen receptor-alpha (*Esr*-1), estrogen receptor-beta (*Esr*-2) and progesterone receptor (*Pgr*) and also several steroidogenic proteins such as Steroidogenic acute regulatory protein (*Star*), 3-beta hydroxysteroid dehydrogenase (*Hsd3b1*) and Aromatase (*Cyp19a1*) (Thathapudi et al., 2015; Zhao and Chen, 2013). Transcriptional regulation of these key genes are implicated in the mechanism of follicle arrest and anovulation in PCOS (Owens et al., 2019). While advancements have been made in the management and diagnosis of PCOS, little is known about the molecular players and signalling pathways that underpin it.

The treatment of PCOS involves the use of several drugs such as metformin and clomiphene citrate, but they are commonly associated with serious side effects, for instance, increased risk of vascular thromboembolism, digestive complications such as nausea, diarrhea and dizziness, and Vitamin B12 deficiency upon prolonged usage (Mokaberinejad et al., 2019; Saha et al., 2012). The use of safe and natural therapeutic agent to modulate ovarian activity and enhance reproductive function is an alternative approach, and many herbs have been tested and found to improve function of the female reproductive system (Fugh-Berman and Kronenberg, 2001; Maharjan et al., 2010; Yang et al., 2018a; Yang et al., 2018b; Yang et al., 2020). Traditional knowledge of Ayurveda and Siddha has several evidences which substantiate the effectiveness of *Aloe barbadensis*, also called as kattrali, kani or kumari towards management of female reproductive system and its associated disorders like PCOS (Nadkarni, 1976; Risvan et al., 2017; Sahu et al., 2013). PCOS, being a metabolic syndrome, is characterized by glucose intolerance, insulin resistance and dyslipidaemia. There are several evidences that have proved *Aloe vera* gel is an efficient modulator of metabolic status (Desai et al., 2012; Tanaka et al., 2006; Misawa et al., 2008, 2012). The varied pharmacological properties of *Aloe vera* gel is due to its abundant phytochemicals such as polysaccharides, glycosides, flavonoids, carbohydrates, coumarins, tannins, chromones, alkaloids, anthraquinones, organic compounds, pyrones, phytosterols, anthrones, fatty acids, sterols, terpenoids, hormones, vitamins, proteins, and mineral constituents (Nalimu et al., 2021; Kar and Bera, 2018; Radha and Laxmipriya, 2015).Though its ethnopharmacological use has been documented in traditional medicine system, its thorough scientific evidence is lacking. In this context, data from our lab demonstrated that *Aloe vera* gel (10 mg dry weight daily for 60 days) could restore ovarian structure-function and decrease co-morbidities like hyperglycaemia and dyslipidaemia in PCOS rat model (Maharjan et al., 2010; Radha et al., 2014; Desai et al., 2012; Radha and Laxmipriya, 2015). Further, it is interesting to note that PCOS rats treated with *Aloe vera* gel (AVG) before conception could increase implantation rate, leading to healthier pups with few or no resorptions, suggesting that AVG is a good pre-conceptive agent and help in management of complications associated with women (Radha and Laxmipriya, 2016b). Further, solvent based extraction of AVG demonstrated that oral administration of non-polar petroleum ether extract (NPE) (25 μg/kg body weight for 60 days) in Letrozole induce PCOS rat model could affectively improve the reproductive and metabolic complications associated with PCOS. The observed efficacy was attributed to the presence of fatty acids, phytosterols and terpenoids in the NPE, which acted at various molecular targets leading to improve the ovarian structure-function along with metabolic modulation (Radha and Laxmipriya, 2016a).

Since plant extracts are typically a mixture of different types of bioactive compounds or phytochemicals with different polarities, separating them remains a significant challenge in the identification and characterization of bioactive compounds. The varied phytochemicals present in the extract may potentiate undesirable side-effects. Moreover, certain plant-derived compounds are effective in combination with others, while others are active as single entities. The current advancement in science has made it possible for the isolation of phytochemicals and studying their therapeutic potential individually or in combination. Therefore, an attempt was made to isolate the Non-Polar Phytochemicals present in the petroleum ether extract of AVG by column chromatography and characterize them using GC-MS and HPLC. The partially purified non-polar phytocompounds (PPNPP) of *Aloe vera* gel (LP1-LP5) demonstrated strong interaction and good docking score with key steroidogenic and metabolic regulatory proteins (Supplementary Table S4; Supplementary Fig. S6). For further validation of *"in-silico"* data, LP1-

LP5 were incubated with KGN cell-line (ovarian cell-line) and it was observed that amongst all the isolates, LP1 and LP3 exhibited maximum potential to modulate the molecular targets associated with PCOS (Supplementary Fig. S7; Supplementary Fig. S8; Supplementary Fig. S9; Supplementary Fig. S10 and Supplementary Fig. S11). Though, cell-based bioassays are the most promising tool for screening of numerous phytochemicals simultaneously (Moore et al., 2014) but PCOS is a multi-organ pathology, testing of efficacy of bio-isolates in *"in-vivo"* system becomes essential. Therefore, the present study was undertaken to investigate the effects of partially purified non-polar phyto-compounds (PPNPP) of *Aloe vera* gel- LP1 and LP3 in Letrozole induced PCOS mice model, with the context to hormonal and metabolic pathways for management of PCOS pathology.

2. Materials and methods

2.1. Chemicals and solvents

Letrozole tablets-2.5 mg, marketed under the brand name Letronat were procured from Natco Pharma Ltd. Dimethyl sulfoxide (DMSO), Petroleum ether 40–60 °C, HPLC grade solvents-methanol, Acetonitrile, Ethanol was procured from HiMedia Laboratories Pvt. Ltd. n-Hexadecanoic acid and Campesterol acetate were procured from Sigma Aldrich India. All other reagents of analytical grade were purchased from Sisco Research Laboratories Pvt. Ltd., India. 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 ELISA kits (DBC Canada). RNAiso Plus was procured from Takara Inc. High-Capacity cDNA Reverse Transcription Kit was procured from Applied Biosystems. Primers for key steroidogenic and metabolic genes were designed by primer blast tool of NCBI and synthesized by INTEGRATED DNA TECHNOLOGIES (IDT).

2.2. Plant material and isolation of non-polar phytocompounds

Commercially available freeze-dried *Aloe vera* [*Aloe barbadensis*] gel powder *Aloe vera*was obtained from M/s. Aum Agrifresh Foods, Vadodara [Product Code- 066; Batch No-06510; Manufactured in Sept'17; ISO 22000, HALAL certified]. It is manufactured without the use of matrix, preservatives and additives. The authentication certificate and the quality control parameters are provided in Supplementary Fig. S1. The plant material (500g) was further subjected to Soxhlet extraction at a temperature of 40 °C for 6–8 h using petroleum ether (1 L) as the solvent. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40–50 °C) in a rotavapor apparatus. The yield of the extract was 4.95%. The Petroleum ether extract of the plant (10 g) was applied to silica gel (230–400 mesh size) column and eluted gradiently 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 overall summary of column elution, % yield and nature of the isolates are presented in Supplementary Fig. S2. The eluents were analysed by using pre-coated TLC plate with suitable mobile phase to confirm the homogeneity of eluents. The developed chromatogram was observed under UV or with detecting agent. 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. After this procedure, the obtained fractions, were grouped in 5 partially purified non-polar phyto-compounds (PPNPP)- LP1, LP2, LP3, LP4 and LP5 according to their chemical profiles assayed by thin layer chromatography (TLC) (Supplementary Fig. S3)

2.3. Identification and quantification of isolated phytocompounds

The partially purified isolates were identified based on comparison of their retention time (min), peak area, peak height and mass spectral patterns with the spectral database of authentic compounds stored in the National Institute of Standards and Technology (NIST) library (Supplementary Fig. S4). On the basis of GC/MS data, the retention times, molecular weight, molecular formula, name of compound, similarity, and peak area (%) of components in each isolated fraction were identified (Supplementary Table S2; Supplementary Table S3) and quantified using reverse-phase HPLC (Supplementary Fig. S5). The HPLC method was validated as per ICH guidelines for parameters like specificity, linearity, precision, accuracy, and limit of detection and limit of quantification (Supplementary Table S5; Supplementary Table S6; Supplementary Table S7; Supplementary Table S8). Cell-based bioassay using an ovarian cell-line- KGN demonstrated that LP1 and LP3 exhibited maximum steroid modulatory properties (Supplementary Fig. S8; Supplementary Fig. S9; Supplementary Fig. S10; Supplementary Fig. S11). For the quantitative analysis of the partially purified non-polar phytocompounds of *Aloe vera* gel, 1 mg of LP1, LP3, HA and CA were dissolved in 1 mL of HPLC grade-methanol and filtered through a 0.45 μm syringe filter and run-on reverse-phase HPLC respectively. The HPLC system (UltiMate™ 3000 HPLC System, Thermo Fisher Scientific) consisted of Dionex Ultimate 3000 Pump (Thermo Scientific) and Dionex Ultimate™ 3000 VWD Variable Wavelength Detector (Thermo Scientific). Separations were carried out on a Acclaim™ 120 (Thermo Scientific) C18 column of 4 × 250 mm, 120 Å pore diameter and 5 μm particle size. Acetonitrile: Ethanol: 80:20 (HPLC grade) was used as the mobile phase which were subjected to filters (5.0 μm). A programmed isocratic flow rate of 1.5 ml/min for 30 min at room temperature was followed. The injection volume was 20 μL and wavelength was set to 205 nm.

2.4. Biological activity studies

2.4.1. 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-h 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 h, 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).

2.4.2. 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 (0.5 mg/kg body weight) daily for 21 days. For validation of PCOS phenotype, 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:

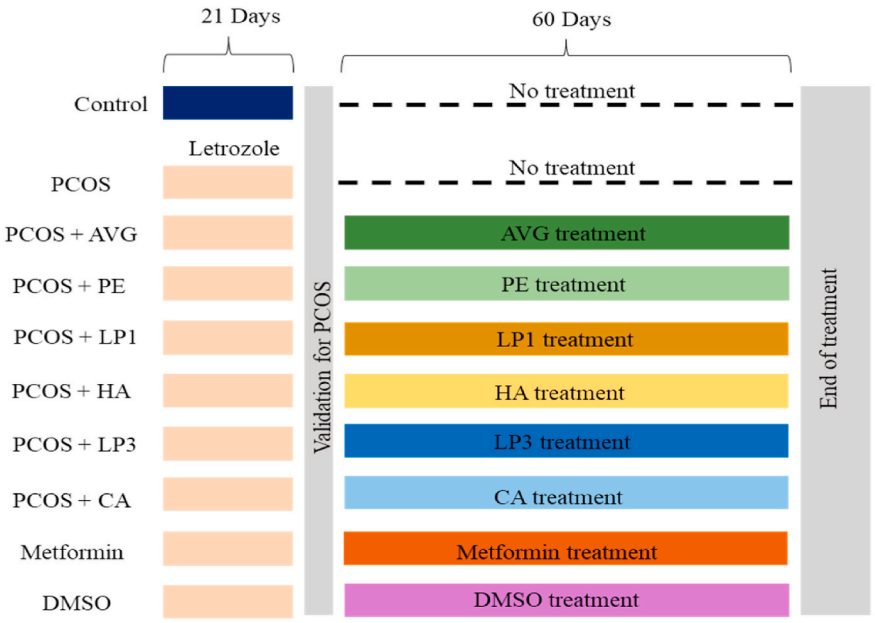
Group I (C group) received 1% CMC orally daily for 21 days and served as Untreated Control.
Group II (PCOS group) received Letrozole (0.5 mg/kg body weight) daily for 21 days and served as Untreated PCOS.

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 (25 μg/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%, 0.2 ml/day) for 60 days and served as Vehicle/ Negative Control

The plan of work and the treatment schedules are provided in Fig. 1.

2.4.2.1. Dose selection criteria for LP1, LP3 and PE. Screening of different doses of chemical components for their toxicity in animal models requires larger number of animals. Hence, we opted for an alternative *"in-vitro"* screening technique using KGN cell-line (ovarian 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. IC_{50} value of the drug indicates the toxicity of the drug candidate. The cells were incubated with LP1, LP3 and Petroleum ether extract of *Aloe vera* gel in a dose dependent manner (0.5–500 ng/ml) for 24 h at 37 °C in a CO2 incubator. Data from Supplementary Fig. S7 depicts the IC_{50} values obtained for LP1, LP3, and PE as 25.3, 8.9, and 70.45 ng/ml respectively. We hypothesize that LP1 and LP3 elicit their bioactivity by reaching the ovaries and modulating steroidogenesis at the ovarian level. Considering the IC_{50} values and the bioavailability of phytosterols in the body (Sanders et al., 2000; Salehi et al., 2021), the doses of LP1 and LP3 were decided as 5 μg/kg/day and 0.5 μg/kg/day. Also, the selected dose could be correlated with their abundance in petroleum ether extract of *Aloe vera* gel as per the GC-MS analysis (Supplementary Table S3).

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



Female virgin Balb/c mice (3-4 months, 20-25 g) were used for the study

Control: Received 1% carboxymethyl cellulose (CMC) daily for 21 days
PCOS: 0.5 mg/kg body weight of Letrozole daily for 21 days
PCOS+AVG: PCOS animals treated with Aloe vera gel (10mg/day) for 60 days
PCOS+PE: PCOS animals treated with Petroleum ether extract of Aloe vera gel (25μg/ kg/day) for 60 days
PCOS +LP1: PCOS animals treated with LP1 (5μg/ kg/day) for 60 days
PCOS+HA: PCOS animals treated with pure compound- n-Hexadecanoic acid (5μg/ kg/day) for 60 days
PCOS +LP3: PCOS animals treated with LP3 (0.5μg/ kg/day) for 60 days
PCOS+CA: PCOS animals treated with pure compound- Campesterol acetate (0.5μg/ kg/day) for 60 days
Metformin: PCOS animals treated with Metformin (100mg/ kg/day) for 60 days and served as Positive Control
DMSO: PCOS animals treated with (1%; 0.2ml/day) for 60 days and served as Vehicle/ Negative Control

All treatments were given orally by gavages daily at 9:00am; n=8 for each group

Fig. 1. Plan of work for evaluating the bioactivity of phytocompounds in Letrozole induced mouse model.

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.

2.4.3. Quantitative analysis of isolated phytocompounds in the plasma and ovaries

Blood plasma (200 µl) and 10% ovarian homogenate (prepared in 1X PBS, pH-7.4) were 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 Methanol (200 µl), filtered through a 0.45 µm syringe filter and transferred into a fresh tube for further HPLC analysis. The values of % recovery was within the range of 85%–110% for the phytochemicals under study (Supplementary Table S7), suggesting that almost all the non-polar molecules were dissolved in methanol.

2.4.4. Oral glucose tolerance test and HOMA-IR

After 60 days of the treatment; oral glucose tolerance test (OGTT) was performed. 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. Data were expressed as the absolute values of blood glucose concentrations.

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).

2.4.5. Lipid profile

Plasma lipid profile (Total Cholesterol, HDL-Cholesterol, LDL-Cholesterol and triglycerides) was measured using commercial kits (Reckon Diagnostics P. Ltd., India). The reagents of the kit hydrolyse the fat in the sample, which is then acted upon by an oxidase and then a peroxidase, which forms a coloured complex that can be assayed spectrophotometrically at 505 nm for Total Cholesterol, HDL-Cholesterol, LDL- Cholesterol and 520 nm for Triglycerides.

2.4.6. Estrus cycle determination

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.

2.4.7. 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. Each sample was assayed in duplicate. Sensitivity of the kits was 0.25 µIU/mL, 0.022 ng/mL, 10 pg/mL and 0.1 ng/mL for insulin, testosterone, estradiol and progesterone kits respectively. The working range was 3.0–200.0 µIU/mL, 0.08–16.7 ng/mL, 20–3200 pg/mL and 0.3–60 ng/mL of insulin, testosterone, estradiol and progesterone respectively. The intra-assay coefficient of variation (CV) was ≤5.0%, 6.6%–9.6%, 4.6%–9.3% and 10.2%–10.6% for insulin, testosterone, estradiol and progesterone kits respectively. The inter-assay coefficient of variation (CV) was ≤10.0%, 6.1%–7.3%, 6.2%–10.1% and 10.2%–12.6%, for insulin, testosterone, estradiol and progesterone kits respectively. The recovery range was between 96.94% and 101.02%, 80.5%–110.1%, 90.3%–116.2% and 78%–124% for insulin, testosterone, estradiol and progesterone kits respectively.

2.4.8. Histological examination of the ovaries

The ovaries were fixed in 10% buffered formalin, processed, embedded in paraffin, and cut into 5-µm-thick sections. The sections were stained with haematoxylin and eosin and assessed microscopically according to the methods described by Kafali et al. (2004). Graafian follicles, peripheral cystic follicles and corpus luteum were identified. Observations and documentation were made on a DM2500 microscope (Leica, Germany) with Leica EZ digital camera under 4X magnification.

2.4.9. Gene expression analysis

Relative quantification of gene expression was carried out using real-time PCR. Total RNA was obtained from the ovaries using RNAiso Plus reagent as per the manufacturer's instructions. The quantification was performed using NanoVue Plus spectrophotometer (GE Healthcare Life Sciences) with a wavelength of 260 nm. RNA integrity was assessed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. Purity was assessed through absorption rate OD260/OD280 and samples showing a value less than 1.8 were discarded. The reverse transcription reaction to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufactures instructions. Real-time quantitative polymerase chain reaction (qPCR) was performed using QuantStudio 5 Real Time PCR system using SYBR Green (Power SYBR Green PCR Master Mix - Life Technologies, USA). All samples were run in triplicate and accompanied by a non-template control. Thermal cycling conditions included initial denaturation in one cycle of 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C and 1 min at 72 °C. After amplification, the melting curves were analysed to verify the amplification of only one product. The relative mRNA expression and fold change was calculated based on the amplification of the reference gene beta actin (ACTB). The primers used for the amplification are given in Supplementary Table S1. The fold changes in expression levels of less than 0.5 and greater than 2 were considered to be biologically significant.

2.4.10. Estimation of toxicity markers

The animals were treated as per the experimental design provided in Fig. 1. 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, piloerection, 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 analysed 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 et al., 2017). 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).

2.5. Statistical analysis

The values are presented as mean ± standard error mean in all the experiments. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test (GraphPad Prism 5 software, La Jolla, CA). p-values when less than 0.05 were considered to be statistically significant at 95% confidence limit.

3. Results

3.1. Quantitative analysis of major compounds in PE extract of Aloe vera gel

Based upon the TLC analysis, the Rf values of the spots were found to be LP1-0.83, LP2-0.78, LP3-0.72, LP4-0.58 and LP5-0.50 and they were matched with the spots of petroleum ether extract of *Aloe vera* gel (Supplementary Fig. S3). Data from GC/MS analysis demonstrates that n-Hexadecenoic acid (HA) and Campesterol acetate (CA) are present in the petroleum ether extract of *Aloe vera* gel with an abundance of 20.1%, and 2.5% respectively (Supplementary Table S2). PPNPP of AVG- LP1 and LP3 contains HA and CA with an abundance of 97.07%, and 96.07% respectively (Supplementary Fig. S4; Supplementary Table S3). GC/MS data (Supplementary Fig. S4) reveals that the major phytocomponent of LP1 was n-Hexadecanoic acid. It exhibited molecular formula- $C_{16}H_{32}O_2$, molecular weight- 256.42, retention time-12.490. GC/MS fragment: The peak at 12.490 min had a mass [M+] 256. The daughter ion spectra of these compounds (inserts) revealed the characteristic fragments m/z 40, 41, 43, 60, 73, 85, 98, 115, 129, 143, 157, 171, 185, 199, 213, 227, 239 and 256 in EI pattern. On the other hand, the major phytocomponent of LP3 was Campesterol acetate. It exhibited molecular formula- $C_{30}H_{50}O_2$, molecular weight- 442, retention time-19.795. GC/MS fragment: The peak at 19.795 min had a mass [M+] 442.7. The daughter ion spectra of these compounds (inserts) revealed the characteristic fragments m/z 27, 41, 43, 57, 81, 95, 107, 120, 147, 159, 173, 199, 213, 228, 247, 255, 274, 297, 314, 353, 368 in EI pattern.

Quantification by HPLC revealed LP1 and LP3 were detected at 12.020 and 20.272 min of retention time at 205 nm (Supplementary Fig. S5). As shown in Table 1A, the equation for linearity and correlation coefficients (r2) were obtained with the calibration curve of HA and CA. The concentrations of HA and CA were found to be 124.8 ± 5.8 and 15.6 + 0.541 μg/g in Petroleum ether extract of *Aloe vera* gel respectively.

3.2. Quantitative analysis of LP1 and LP3 in plasma and ovaries of letrozole induced PCOS mice

Bioaccumulation of the compound at the target site is the critical factor relating to its efficacy. Data from Table 1[B] clearly demonstrates that the concentration of LP1 and LP3 were found to be 106.22 ± 7.39 (ng/mL) and 49.4 ± 3.928 (ng/mL) in the plasma and 25.04 ± 0.009 (ng/mg tissue weight) and 4.22 ± 0.016 (ng/mg tissue weight) in the ovaries of Letrozole induced PCOS mice that have been orally administered LP1 (5 μg/kg/day) and LP3 (0.5 μg/kg/day) for 60 days respectively.

Table 1 [A]
The linear ranges, regression equations, and correlation coefficients of the partially purified non-polar phytochemicals (PPNPP) from *Aloe vera* gel by HPLC.

PPNPP	Retention Time (min)	Linear Range (μg/mL)	Regression Equation	Correlation Coefficient (r^2)	Amount (μg/g) present in Petroleum ether extract of *Aloe vera* gel *
LP1	12.02	5–500	y = 0.7471x-0.0036	0.9932	124.8 ± 5.8
LP3	20.3	5–500	y = 0.0521x+0.0431	0.9876	15.6 + 0.541

* Mean + SD (SD is the standard deviation); y: peak area (mAU) of compounds; x: concentration (μg/mL) of compounds.

Table 1 [B]
Concentration of partially purified non-polar phytochemicals (PPNPP) from *Aloe vera* gel in the plasma and ovaries of Letrozole induced mice.

PPNPP	Conc. in Plasma (ng/mL)	Conc. in Ovaries (ng/mg tissue weight) *
LP1	106.22 ± 7.39	25.04 ± 0.009
LP3	49.4 ± 3.928	4.22 ± 0.016

* Mean + SD (SD is the standard deviation).

3.3. Effects of different treatments on metabolic parameters

3.3.1. Effects of different treatments on body weight

First, the effect of different treatments on the body weight of all groups of animals were evaluated. Results from Fig. 2A demonstrate that the animal body weight before PCOS induction was similar in all groups. Following a 21-day administration of letrozole, animal body weight increased in PCOS group ($P < 0.001$) as compared to the control group of animals. The administration of metformin reversed the effect of letrozole as it decreased animal body weight as compared with the PCOS group ($P < 0.05$). The different phytochemical treatments also induced a similar effect, as they decreased the animal weight as compared to PCOS group ($P < 0.05$), suggesting that phytochemical treatments potentially decreased the adipose tissue deposition in the abdominal and peri-ovarian regions of PCOS animals.

3.3.2. Effects of different treatments on oral glucose tolerance, fasting insulin, HOMA-IR and gene expression of Insulin Receptor

Oral Glucose Tolerance Test was performed after 60 days of different treatment procedures. Results demonstrated that the Untreated Control animals exhibited normal profile to glucose tolerance upon oral administration of glucose, whereas the letrozole induced PCOS animals showed an increase in the glucose intolerance when compared to untreated control (Fig. 2B). Blood glucose levels at 30, 60, and 120 min were higher in the PCOS group compared to the control group ($P < 0.001$, $P < 0.01$). AVG and PE extract of AVG treatment to the PCOS animals exhibited significant improvement in the glucose tolerance in all time point of the test except fasting ($P < 0.001$). Further, PPNPP (LP1 and LP3) treated animals as well as their corresponding standards showed significant reduction in glucose levels at different time points over the period of 120 min when compared to the untreated PCOS group ($P < 0.01$). The observed changes were comparable to that of Metformin treatment ($P < 0.01$), which is the standard drug prescribed for

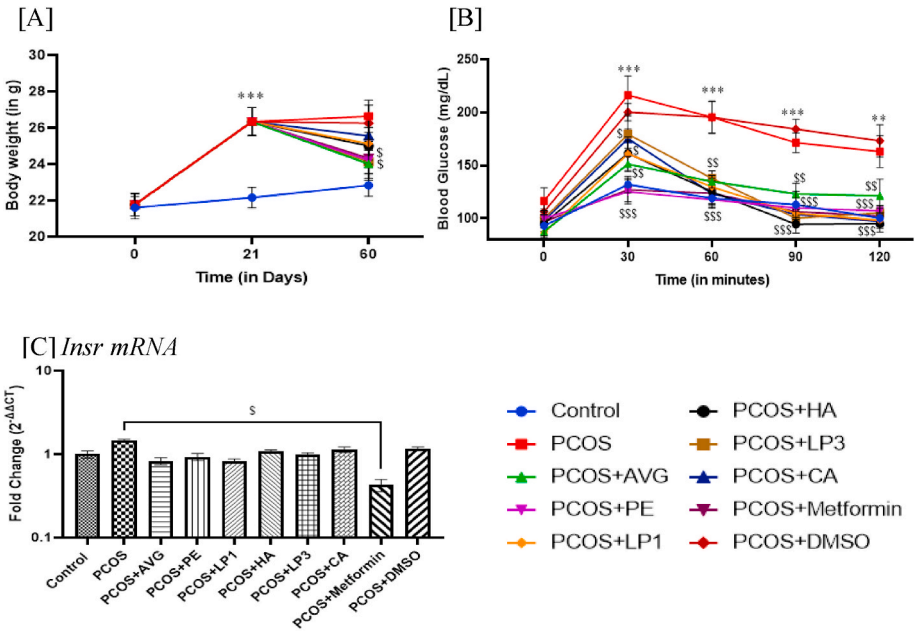


Fig. 2. Effect of different treatments on the [A] Body weight, [B] Oral glucose tolerance test and [C] Fold change in gene expression levels of Insulin Receptor in ovaries of Letrozole induced PCOS mice model. All values are presented as Mean ± SEM; N = 8 per group. **P < 0.01 and ***P < 0.001 as compared to control group; $P < 0.05, $$P < 0.01 and $$$P < 0.001 as compared to PCOS group.

management of PCOS.

Non-significant changes were observed in the fasting glucose levels amongst all the groups of animals. However, PCOS group demonstrated a significant high level of insulin (***p < 0.001) as compared to control group (P < 0.001), while it reverted back to normal levels after treatment with AVG, PE extract of AVG, PPNPP (LP1 and LP3) and their corresponding commercial standards as well as Metformin (P < 0.01) (Table 2). At the molecular level, there was no significant difference in the gene expression of *Insr* mRNA in the ovaries amongst all groups of animals (Fig. 2C), in spite of improved insulin profile in the PCOS animals after different treatments, suggesting that the phytochemicals act at post-receptor level of insulin signalling.

Furthermore, HOMA-IR was substantially higher in the PCOS group as compared to the control (P < 0.001), metformin (P < 0.05), AVG (P < 0.01), PE extract of AVG (P < 0.01), LP1 and HA (P < 0.05) and LP3 and CA (P < 0.01) groups (Table 2). PCOS animals exhibited insulin resistance, which reverted back to normalcy after the treatment with different phytochemicals. Results demonstrated that LP3, derived from *Aloe vera* gel is a potential glucose and insulin sensitizing agent.

Table 2
Effect of different treatments on Fasting Glucose, Fasting Insulin and HOMA-IR in plasma of Letrozole induced PCOS mice model.

	Fasting Glucose (mg/dL)	Fasting Insulin (μIU/mL)	HOMA-IR
Control	93 ± 9.72	7.83 ± 0.14	1.49 ± 0.18
PCOS	106.25 ± 9.42	14.36 ± 0.67 ***	4.76 ± 0.09 ***
PCOS + AVG	87.25 ± 5.6	7.74 ± 0.09 $$	1.27 ± 0.11 $$
PCOS + PE	99.75 ± 7.5	7.68 ± 0.03 $$	1.3 ± 0.13 $$
PCOS + LP1	86.33 ± 6.1	8.25 ± 0.06 $$	1.76 ± 0.14 $
PCOS + HA	95.2 ± 4.1	8.28 ± 0.15 $	1.95 ± 0.08 $
PCOS + LP3	99.8 ± 9.13	7.65 ± 0.06 $$	1.18 ± 0.17 $$
PCOS + CA	97.8 ± 4.58	7.875 ± 0.10 $$	1.2 ± 0.06 $$
PCOS + Metformin	96.8 ± 9.16	7.60 ± 0.12 $$	1.81 ± 0.18 $
PCOS + DMSO	111 ± 8.6	13.01 ± 0.90	3.24 ± 0.17

All values are presented as Mean ± SEM; N = 8 per group. ***P < 0.001 as compared to control group; $ P < 0.05 and $$ P < 0.01 as compared to PCOS group.

3.3.3. *Effects of different treatments on lipid profile*

As seen in Fig. 3, upon administration of letrozole for 21 days, PCOS animals demonstrated a marked increase in the plasma triglyceride levels (P < 0.01), whereas plasma Cholesterol, HDL-C and LDL-C levels remained almost the same as compared to the various control groups. Treatment of PCOS animals with AVG, PE extract of AVG, LP3 and its corresponding commercial standard- CA exhibited a reduction in the triglyceride levels (P < 0.05), suggesting that LP3, derived from *Aloe vera* gel has excellent property to reduce the circulating elevated triglycerides (suggestive marker of metabolic syndrome).

3.4. *Effects of different treatments on reproductive parameters*

3.4.1. *Effects of different treatments on estrus cyclicity*

The estrus cycle was evaluated in all groups of animals as an index of ovarian function. The stage of the estrus cycle was confirmed by vaginal smear. On day 0 (before letrozole treatment), all groups had a regular estrus cyclicity. After 21 days of Letrozole treatment it was observed that PCOS rats exhibited arrested estrus cyclicity as compared to control animals, that had regular estrus cyclicity throughout the study period (Table 3). Remarkably, treatment of PCOS mice with AVG and PE completely reverted the estrus cyclicity to normalcy. Similar results could be observed upon LP3 as well as CA treatment groups. However, treatment with LP1 and HA showed restoration of estrus cyclicity in only 60–75% of animals. The animals treated with Metformin had prolonged estrus and shorter metestrus and diestrus stage. The DMSO treated group demonstrated irregular and arrested estrus cycle as compared to control animals. The estrus cycle is related to alterations in the circulating steroid hormones, which controls ovarian structure and function.

3.4.2. *Effects of different treatments on ovarian histology*

Ovarian sections of the control group exhibited normal ovarian morphology with mature follicles (tertiary and Graafian follicles) and corpora lutea which is an indicator of ovulation. On the contrary, ovarian histology of Letrozole treated mice showed extremely thin layer

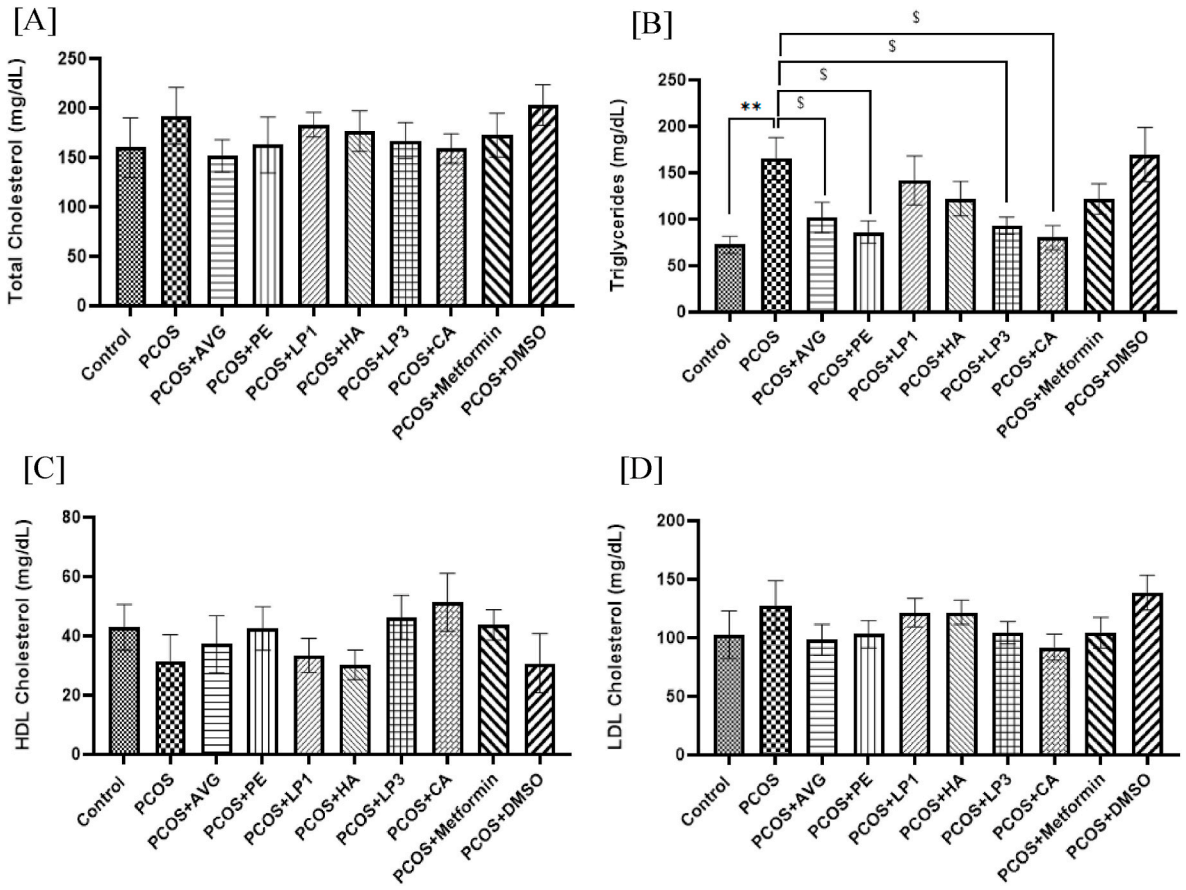


Fig. 3. Effect of different treatments on the Lipid Profile [A] Total Cholesterol, [B] Triglycerides, [C] HDL-Cholesterol and [D] LDL- Cholesterol levels in Letrozole induced PCOS mice model. All values are presented as Mean ± SEM; N = 8 per group. **P < 0.01 as compared to control group; $P < 0.05 as compared to PCOS group.

Table 3
Effect of different treatments on the estrus cyclicity of Letrozole induced PCOS mice model.

	Normal cycle	Extended Proestrus	Extended Estrus	Extended Metestrus	Extended Diestrus
Control	100%	–	–	–	–
PCOS	–	–	–	5%	95%
PCOS + AVG	100%	–	–	–	–
PCOS + PE	100%	–	–	–	–
PCOS + LP1	75%	–	–	15%	10%
PCOS + HA	60%	–	–	30%	10%
PCOS + LP3	90%	–	5%	3%	2%
PCOS + CA	98%	–	–	–	2%
PCOS + Metformin	10%	–	80%	5%	5%
PCOS + DMSO	–	–	–	3%	97%

of granulosa cells, multiple large subcapsular peripheral cysts, fewer corpus luteum and reduced mature follicles as compared to control animals. The appearance of multiple peripheral ovarian follicular cysts represented the pivotal clinical characteristic during PCOS progression. Treatment of PCOS animals with metformin, AVG, PE and LP3 exhibited mature follicles, corpora lutea and few cystic and atretic follicles (Fig. 4).

3.4.3. *Effects of different treatments on hormone profile*

Next, we evaluated steroidogenic function in all groups of animals. The levels of testosterone, estradiol and progesterone were determined using commercially available kits. After induction of PCOS, testosterone levels were significantly increased while progesterone levels were decreased compared with control (Fig. 5). However, there was no significant difference observed in estradiol levels between control and PCOS animals. Metformin and plant treatment (AVG, PE, LP1, LP3 and their corresponding commercial standards) in PCOS animals significantly reduced ($P < 0.05$) testosterone levels. On the contrary, progesterone levels significantly improved upon Metformin ($P < 0.01$) and plant treatment (AVG, PE, LP1, LP3 and their corresponding commercial standards) in PCOS animals ($P < 0.05$). Both LP1 and LP3 demonstrated anti-androgenic and progestogenic potential in Letrozole induced PCOS mice.

This study clearly demonstrates that the PPNPP of *Aloe vera* gel have the potential to restore the metabolic and reproductive parameters of Letrozole induced PCOS mice at physiological level. However, it would be interesting to study the effect of these phytochemicals on the ovary of PCOS mice at molecular level. Hence, the effect of these treatments on gene expression of gonadotropin receptors, steroid receptors and key steroidogenic markers were evaluated.

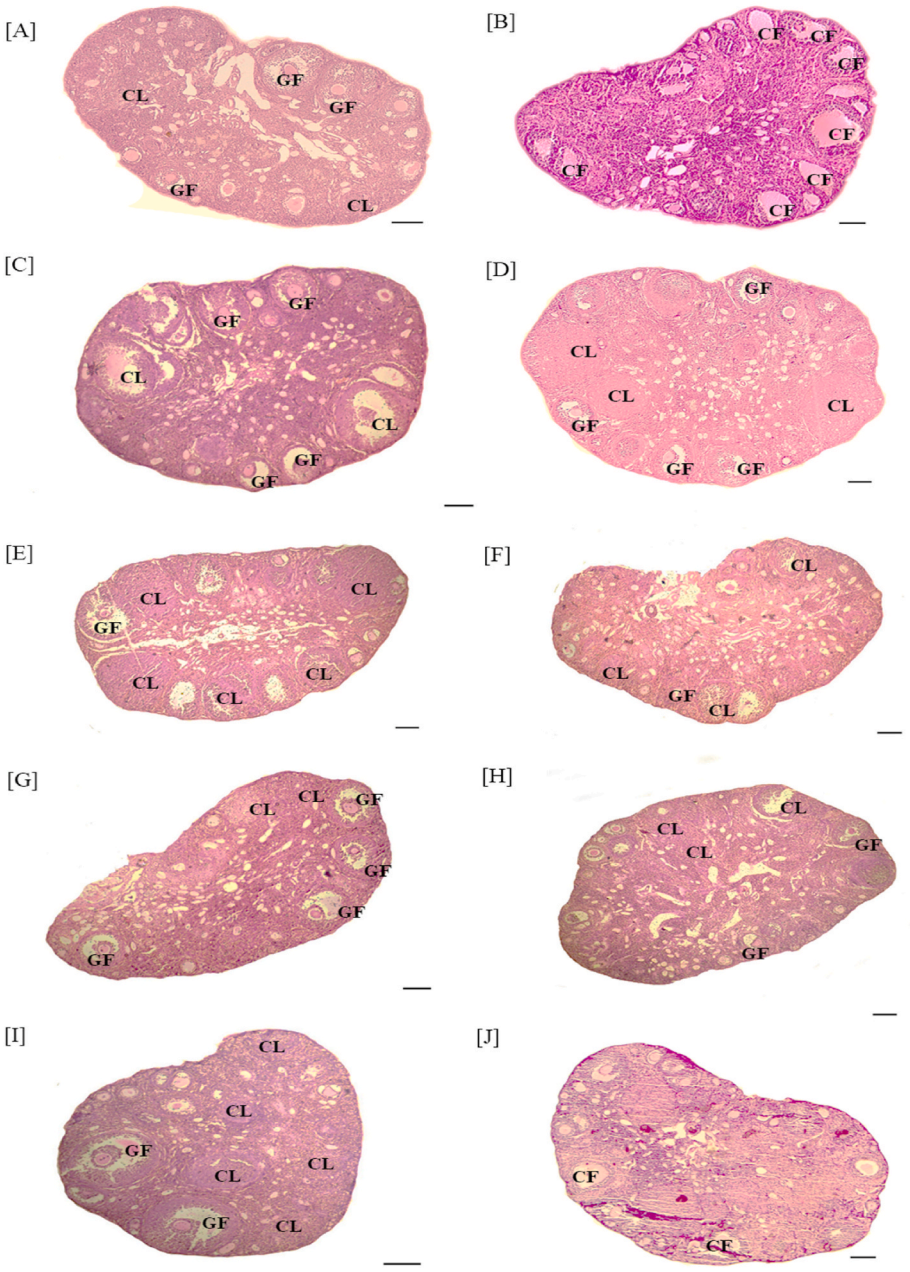


Fig. 4. Effect of different treatments on ovarian histology. [A] Control; [B] PCOS; [C] PCOS + AVG; [D] PCOS + PE; [E] PCOS + LP1; [F] PCOS + HA; [G] PCOS + LP3; [H] PCOS + CA; [I] PCOS + Metformin and [J] PCOS + DMSO. CL: corpus luteum; CF: cystic follicle; GF- Graafian follicle. Magnification 4X. Calibration bar = 100 μm.

3.4.4. Effects of different treatments on gonadotropin receptors

To estimate the transcript levels of gonadotropin-receptors, we performed real-time RT-PCR using specific primers for *Fshr* and *Lhr*. Data from Fig. 6 [A] demonstrates that the mRNA levels of *Lhr* were increased in the ovaries of Letrozole-induced mice ($P < 0.001$), however, its induction was effectively decreased upon treatments with Metformin, AVG and PE. It is to be noted that, PPNPP of AVG (LP1 and LP3) did not affect *Lhr* mRNA expression as shown in Figure. There was no significant change in the mRNA level(s) of *Fshr* amongst all the groups (Fig. 6 [B]).

3.4.5. Effect of different treatments on steroid receptors

To assess the expression levels of mRNAs encoding steroid hormone receptors, we performed real-time RT-PCR using specific primers for *Ar, Pgr*, *Esr*-1 and *Esr*-2. Results from Fig. 7[A] demonstrate that the mRNA levels of ovarian Androgen Receptor (*Ar*) were significantly upregulated in Letrozole induced PCOS animals ($P < 0.001$) as compared to control animals. However, treatment with Metformin and other phytochemicals significantly reduced the expression of *Ar* ($P < 0.05$), suggesting anti-androgenic potential. On the other hand, the mRNA levels of Progesterone Receptor (*Pgr*), Estrogen Receptor-alpha (*Esr*-1) and Estrogen Receptor-beta (*Esr*-2) significantly decreased in Letrozole induced PCOS ovaries ($P < 0.01$, $P < 0.001$ and $P < 0.05$ respectively) as compared with control group (Fig. 7[B], [C] and [D]). Treatment with Metformin, AVG and PE significantly increased the mRNA levels of PR ($P < 0.05$). The PPNPP of AVG (LP1 and LP3) did not influence the mRNA levels of *Pgr*, *Esr*-1 and *Esr*-2. However, administration of pure compound- CA significantly increased the mRNA expression of *Pgr* and *Esr-1* ($P < 0.05$) in the PCOS ovaries. Metformin treatment in PCOS animals showed induced transcription of *Esr-2* in the ovaries ($P < 0.05$).

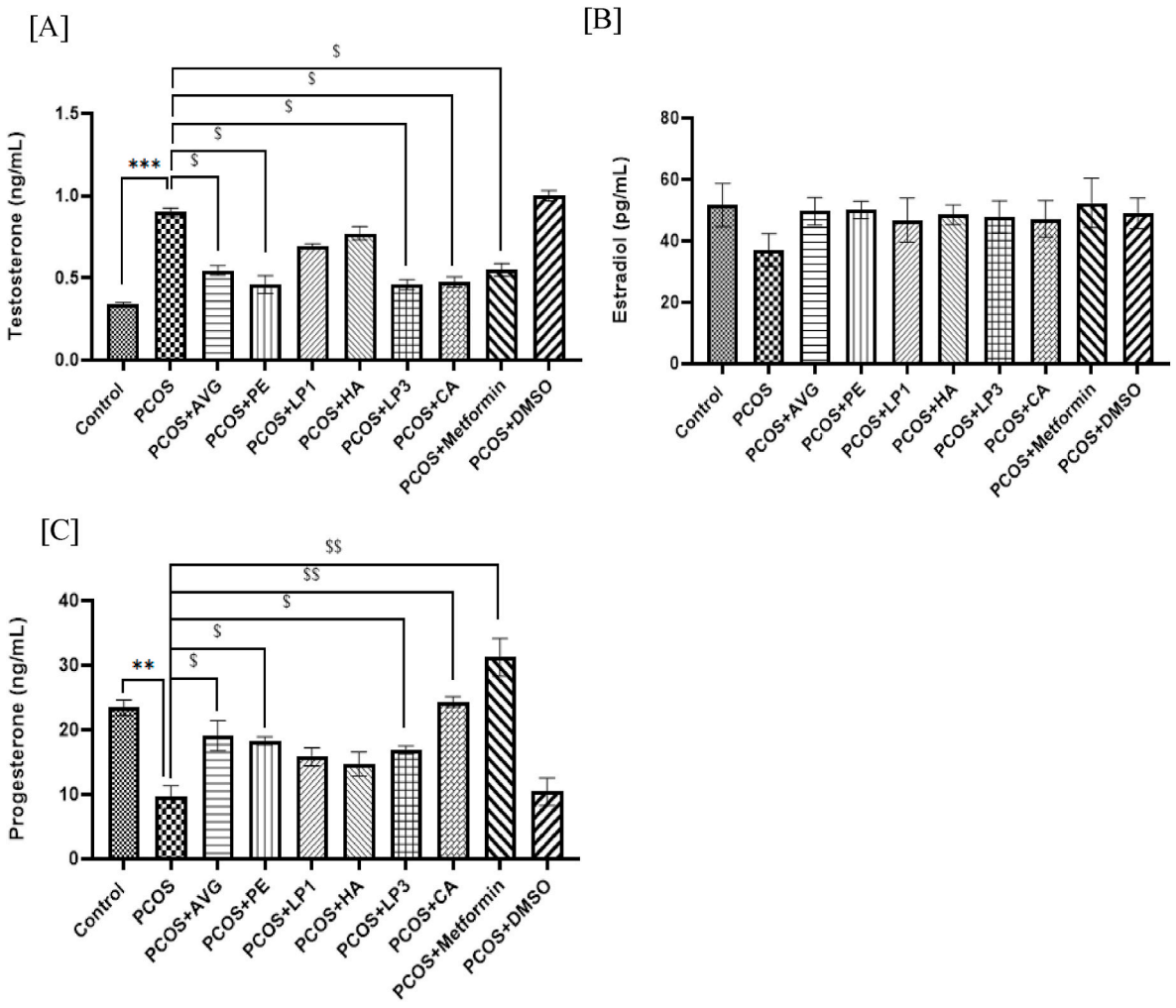


Fig. 5. Effect of different treatments on the Hormone Profile [A] Testosterone, [B] Estradiol and [C] Progesterone levels in Letrozole induced PCOS mice model All values are presented as Mean ± SEM; N = 8 per group. **P < 0.01, ***P < 0.001 as compared to control group; $ P < 0.05, $$ P < 0.01 as compared to PCOS group.

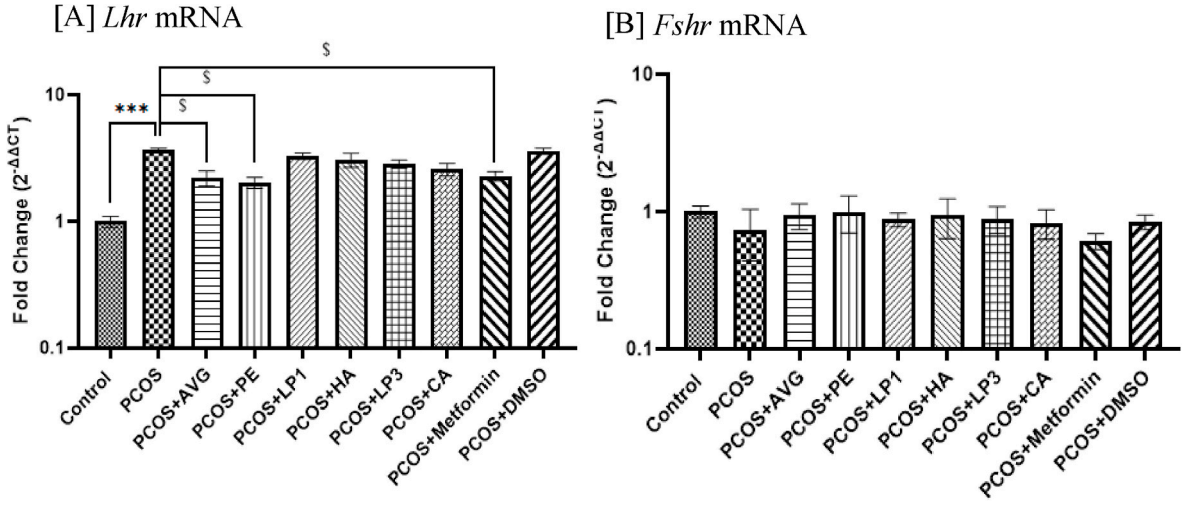


Fig. 6. Comparison of the fold changes in gene expression levels of gonadotropin receptors [A] Luteinizing hormone receptor and [B] Follicle-stimulating hormone receptor in ovaries of Letrozole induced PCOS mice model upon different treatments. Values are mean fold change in gene expression. Error bars represent SEM; N = 8 per group. ***P < 0.001 as compared to control group; $ P < 0.05 as compared to PCOS group.

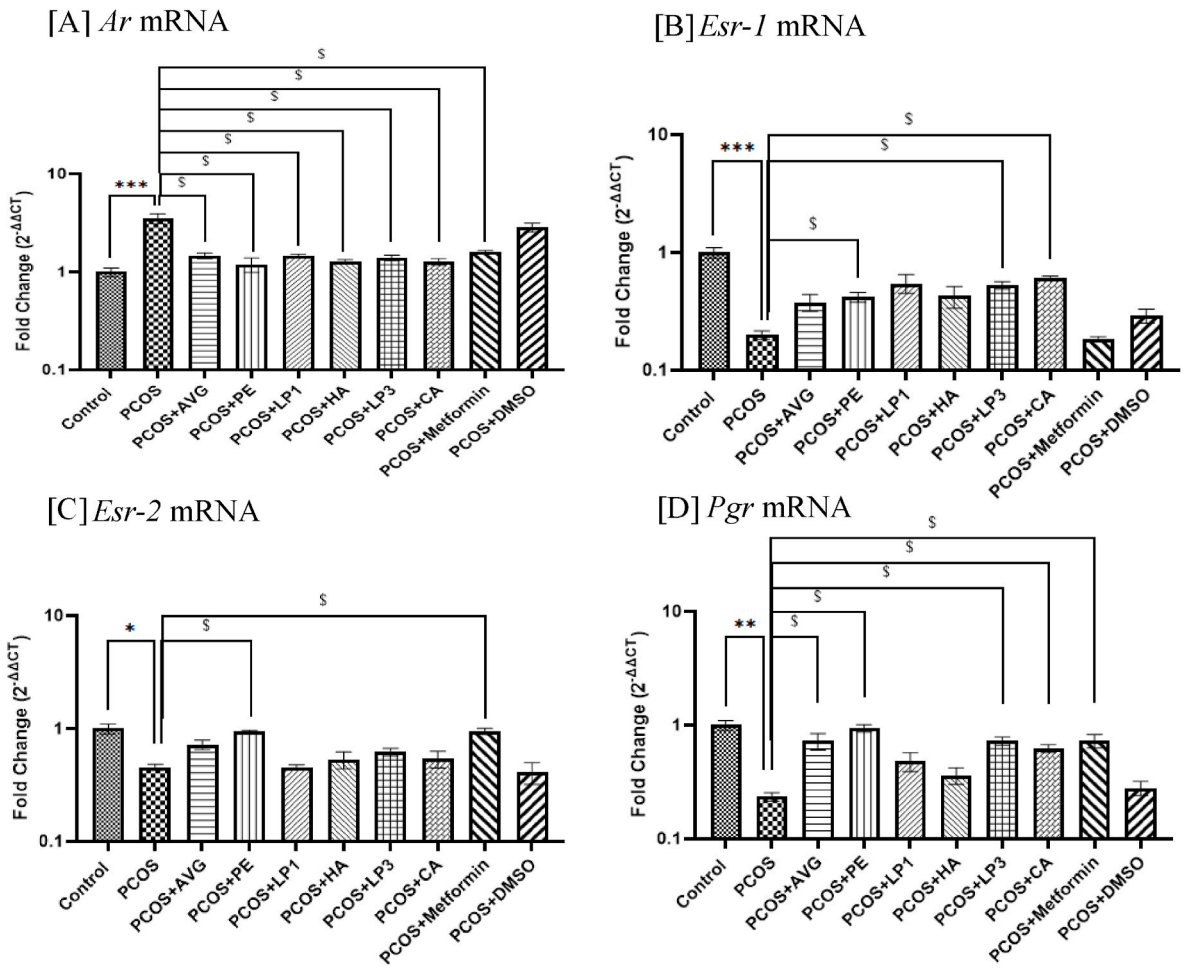


Fig. 7. Comparison of the fold changes in gene expression levels of steroid receptors [A] Androgen Receptor, [B] Estrogen Receptor-alpha, [C] Estrogen Receptor-beta and [D] Progesterone Receptor in ovaries of Letrozole induced PCOS mice model upon different treatments. Values are mean fold change in gene expression. Error bars represent SEM; N = 8 per group. *P < 0.05, **P < 0.01, ***P < 0.001 as compared to control group; $ P < 0.05, $$ P < 0.01 as compared to PCOS group.

3.4.6. *Effect of different treatment on key steroid markers*

Estimation of the expression levels of mRNA encoding enzymes/proteins involved in steroidogenesis, including anti-mullerian hormone (*Amh*), steroidogenic acute regulatory protein (*Star*), 3-beta (β)-hydroxysteroid dehydrogenase (*Hsd3b1*) and cytochrome P450, family 19, subfamily a, polypeptide 1 (*Cyp19a1*) was done using real-time PCR with their specific primers. As shown in Fig. 8, mRNA levels of ovarian *Amh* and *Star* were induced by the Letrozole treatment ($P < 0.01$ and $P < 0.001$ respectively). Oral administration of Metformin, AVG and PE significantly decreased the mRNA levels of *Amh* ($P < 0.01$, $P < 0.05$ and $P < 0.05$ respectively). On the other hand, treatment of PCOS animals with AVG, PE, LP3 and CA significantly reduced the mRNA levels of *Star* ($P < 0.05$). Interestingly, the mRNA levels of *Hsd3b1* and *Cyp19a1* was found to be reduced in Letrozole treated PCOS ovaries ($P < 0.001$). Oral administration of LP3 and CA to PCOS mice significantly increased the mRNA levels of *Hsd3b1* ($P < 0.05$). On the contrary, treatment of Letrozole induced PCOS mice with AVG, PE, LP3, CA ($P < 0.01$) and Metformin ($P < 0.05$) significantly upregulated the transcription of ovarian *Cyp19a1*.

3.5. *Effect of different treatment on toxicity parameters*

No lethal effects or mortality was observed in animals throughout the test period amongst all the treatment groups. The body weight changes serve as a sensitive indication of the general health status of animals. Data from Fig. 2[A] demonstrates that the body weight of the animals initially increased ($P < 0.001$) upon Letrozole administration for 21 days as compared to the control animals, which is a characteristic of PCOS phenotype. Upon oral administration of different phytocompounds, the body weight reduced significantly ($P < 0.05$) in the PCOS animals, however, there was no statistical significance between the body weight of the treated and control animals. In addition, the food and water intakes were found to be unaltered during the entire treatment period, when compared to a control group in this study (data not shown). During the observation period, the animals' appearance did not change in any way. The morphological features (fur, skin, eyes, and nose) remained the same. Tremors, convulsions, salivation, diarrhea, lethargy, or odd behaviours such as self-mutilation or walking backwards were not seen in the treated animals (Supplementary Table S9). The gross observations of the animals' anatomy disclosed that the vital organs such as the heart, liver, spleen, kidneys, lungs and brain were not adversely impacted and did not display clinical symptoms of toxicity, over the course of treatment.

Supplementary Table S10 summarizes the results of the effect of different treatments on various biochemical parameters. No significant differences were observed in plasma glucose levels of the treated groups as compared to the control group. The letrozole induced PCOS animals showed significantly ($P < 0.01$) increased triglyceride levels when compared to the control. However, upon oral administration of different treatments, the triglyceride levels of the PCOS animals significantly reduced ($P < 0.05$) and were comparable with that of control animals. The was no significant difference in the total cholesterol and the HDL-

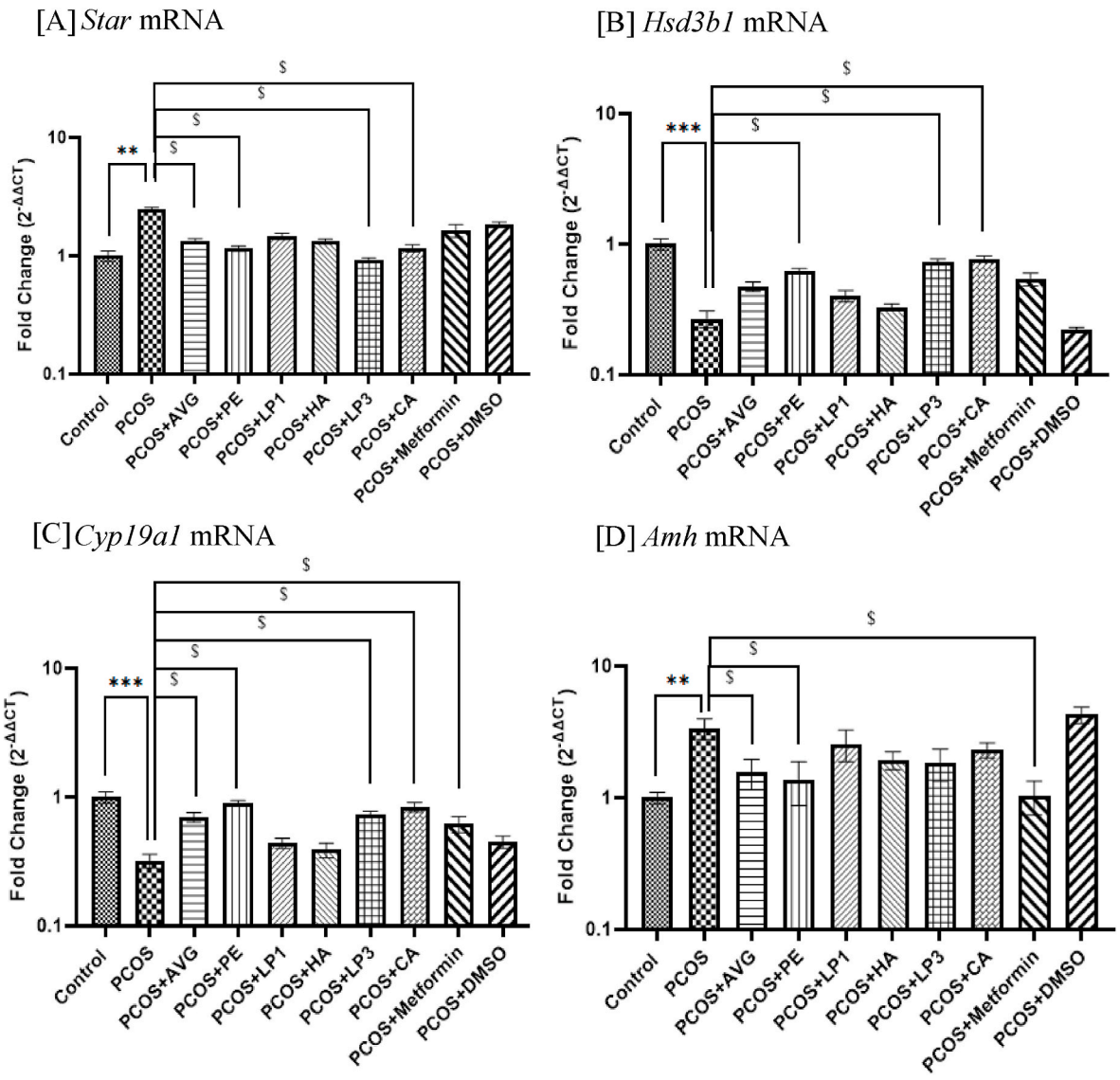


Fig. 8. Comparison of the fold changes in gene expression levels of steroidogenesis regulators [A] Steroidogenic acute regulatory Protein, [B] 3-beta-hydroxysteroid dehydrogenase, [C] Aromatase and [D] Anti-Mullerian Hormone in ovaries of Letrozole induced PCOS mice model upon different treatments. Values are mean fold change in gene expression. Error bars represent SEM; N = 8 per group. **P < 0.01, ***P < 0.001 as compared to control group; $ P < 0.05, $$ P < 0.01 as compared to PCOS group.

cholesterol levels amongst all the different treatment regimens. AST in combination with ALT are considered as good maker of liver disease (Friedman et al., 1996). High levels of these enzymes are implicated in liver diseases or hepatotoxicity (Ramaiah, 2011). The present study reveals no significant changes in ALT as well as AST activities amongst all treated groups. Abnormally high levels of creatinine indicate kidney malfunction or renal toxicity (Davis and Berndt, 1994). No significant change in creatinine level has been observed in treated animals as compared to control. This may indicate that the different treatments did not influence the liver and renal function. As already clear from our results, one of the key organs that is influenced by the treatment of the PPNPPs isolated from *Aloe vera* gel is ovaries and it was found from our data that the ovarian structure-function was improved upon the treatment with the phytochemicals (Fig. 4), suggesting no reproductive toxicity. All the above parameters suggest that the standardised dosage of the non-polar phytocompounds of *Aloe vera* gel are safe for oral consumption, when taken regularly for 60 days.

4. Discussion

Aloe barbadensis has been documented in Ayurveda and Siddha for treatment of female reproductive ailments (Nadkarni, 1976; Risvan et al., 2017). However, its bioactive phytochemical and molecular targets still remains to be elucidated. In this study, we systematically investigated the therapeutic potential of partially purified isolated non-polar phytocomponents (PPNPP) of *Aloe vera* gel against PCOS in Letrozole induced mouse model. The efficacy of letrozole (an aromatase inhibitor) in establishing PCOS in rodents is well documented (Kafali et al., 2004). It acts by inhibition of aromatase, leading to low conversion of androgens to estrogens, resulting in an excessive accumulation of androgens in the ovary (Garcia-Velasco et al., 2005). Oral administration of Letrozole (0.5 mg/kg body weight) daily for 21 days successfully induced PCOS in adult female Balb/c mice (Dey et al., 2017). The success in establishing this disease model was experimentally confirmed in our study. The continuous monitoring of body weight revealed a significant induction of body weight gain in the PCOS group. It has earlier been reported that an increase in accumulation of fat in the abdominal

area is associated with increase in body weight and might induce adipocyte dysfunction and insulin resistance like state in PCOS (Goodarzi et al., 2011). In addition to this, letrozole treated animals exhibited glucose intolerance, elevated fasting insulin levels, increased HOMA-IR values and increased circulating levels of triglycerides. Similar indicators of metabolic dysregulation have been observed in previous studies (Kauffman et al., 2015; Kelley et al., 2016). The insulin resistance and low glucose tolerance created by letrozole are mainly due to elevated androgen concentrations as reported by Desai et al., 2012. Along with the metabolic disruptions, letrozole treatment also induced reproductive abnormalities which was evident by arrested estrus cycle at diestrus stage, elevated testosterone levels and decreased progesterone levels. Additionally, histological examination of ovarian sections showed multiple peripheral cystic follicles, which is characteristic features of PCOS. These findings are similar to other reports (Yang et al., 2018a, 2018b; Rajan and Balaji, 2017).

Women with PCOS have an increased risk of developing metabolic syndrome in later life (Gunning and Fauser, 2017). The effectiveness of metformin in the treatment of PCOS by restoring ovulation, reducing weight, improving insulin sensitivity and glucose tolerance, reducing circulating androgen levels, reducing the risk of miscarriage and reducing the risk of gestational diabetes mellitus has been proven (Lashen, 2010; Johnson, 2014), nevertheless, it is associated with the high incidence of adverse effects including nausea, vomiting and gastro-intestinal disturbances. As reported in the literature (Jahan et al., 2016; Mvondo et al., 2020), we observed in the current study that metformin-treated PCOS animals, displayed a significantly decrease in body weight, blood glucose level, fasting insulin levels and HOMA-IR values compared with untreated PCOS animals. Remarkably, treatment of PCOS animals with AVG, PE, LP3 and CA significantly reduced the body weight, improved glucose tolerance, decreased the fasting insulin levels and HOMA-IR values. We could therefore suggest that non-polar phytocompounds present in *Aloe vera* gel reduces glucose resistance by controlling glucose homeostasis, improving insulin secretion and potentiating the insulin-mediated uptake of glucose. This further strengthens that isolated bioactives of *Aloe barbadensis* is a potential metabolic modulator (Desai et al., 2012). Additionally, these treatments could effectively reduce the circulating triglycerides levels in PCOS animals. These findings are similar to those of Maharjan et al., 2010; Desai et al., 2012; Alinejad-Mofrad et al., 2015). The observed efficacy can be attributed to the presence of plant sterols and fatty acids in the above-mentioned treatments. Several studies from the past have highlighted on the efficacy of phytosterols present in *Aloe vera* gel in regulating glucose and lipid metabolism in rats (Tanaka et al., 2006; Misawa et al., 2008, 2012). Also, combination of omega-3 fatty acids and plant sterols was found to regulate glucose and lipid metabolism in individuals with impaired glucose regulation (Wang et al., 2019). These results suggest that LP3 derived from Petroleum ether extract of *Aloe vera* gel may be a potent therapeutic for the treatment of metabolic disturbances associated with PCOS.

Current evidence suggests that insulin-resistance and secondary hyperinsulinemia play an important role in hyperandrogenism, amenorrhoea or oligomenorrhea in both lean and obese PCOS patients (Laganà et al., 2016). In the present study, PCOS animals had irregular estrus cyclicity, elevated testosterone levels and reduced progesterone levels as compared to control animals. Interestingly, treatment with AVG and PE restored the estrus cyclicity, decreased the circulating free testosterone, increased progesterone levels. Steroid hormones in the follicular fluid play an important role in the physiology of follicular growth, oocyte maturation and ovulation (Walters et al., 2008). Treatment with LP3 and CA was found to be more potent than LP1 and HA in restoration of the estrus cyclicity. However, both LP3 and CA significantly improved the hormone milieu. Of great interest, oral administration of AVG, PE, LP3 and CA alleviated hyperandrogenism in PCOS animals and enhanced progesterone levels. The improved hormone milieu in turn might be responsible for promotion of follicular development and induction of ovulation. Surprisingly, treatment of PCOS animals with metformin (first-line treatment for patients with PCOS), could not revert back the estrus cyclicity as efficiently as AVG, PE, LP3 and CA treatment, even though it improved the hormone levels in the animals. To substantiate our results, previous studies from our lab have demonstrated that non-polar phytochemicals of *Aloe vera* gel improved metabolic phenotype as well as ovarian function in letrozole induced PCOS rats (Radha and Laxmipriya, 2016a). Similar results were found by Demirel (2016) where they demonstrated that α-tocopherol, γ-tocopherol, squalene, β-sitosterol, campesterol and stigmasterol present in *Corylus avellana* seed oil induced a significant reduction in testosterone level and restored ovarian function in PCOS rats. *Vitex agnus-castus* fruit extract also exhibited an antiandrogenic effect in PCOS rats by increasing aromatization leading to low testosterone level (Jelodar and Askari, 2012). Because abnormal increased in testosterone level contributes to the pathogenesis of PCOS, the downregulation of this hormone after LP3 treatment may have beneficial effects on reproductive disorders in PCOS.

PCOS has been reported to be associated with ovarian damage. As observed in the current study, disturbed hormone milieu in untreated PCOS rats is correlated with multiple cysts formation and low number of corpus luteum in the ovary (Dewailly et al., 2016). Moreover, the size of cystic follicles was larger than that of other follicles which can be correlated to the increase levels of intraovarian androgens (Mahesh et al., 1987). Thickness of granulosa layer was lowered while that of theca layer was increased in different follicles of untreated PCOS rats. These detrimental effects of letrozole on ovary architecture were corrected by AVG, PE, LP3 and CA probably due to antiandrogenic properties. These treatments significantly reduced the number of peripheral cysts and increased the number of graafian follicles and corpus luteum, which might be attributed to the restored hormone levels in PCOS animals upon these treatments for 60 days. Additionally, the beneficial effects of these treatments could be mediated through their anti-hyperglycaemic, anti-lipidemic and insulin sensitizing properties. Studies on similar line have demonstrated that polyunsaturated fatty acids (PUFAs) regulate the menstrual cycle, lower testosterone and insulin levels, and improve metabolic health (Prabhu and Abilash, 2021). Also, phytosterols and its metabolites may act as GnRH modulators (Dey and Nampoothiri, 2019). Recent studies are highlighting on the implication of campesterol and stigmasterol as precursors of progesterone (Tarkowská, 2019; Janeczko, 2012), suggesting that plant sterols have the potential to regulate the hormone metabolism. The improvement in hormone levels by metformin corroborated previous studies which demonstrated that this drug improves ovarian related markers and induces ovulation in mice with PCOS (Sander et al., 2006), although the conception rate remains disappointing (Legro et al., 2007).

Ovaries represent one of the primary steroidogenic organs, producing estrogen and progesterone under the regulation of gonadotropins during the estrus cycle. Gonadotropins fluctuate the expression of various steroidogenesis-related genes, such as those encoding gonadotropin receptors, steroid receptors and steroidogenic marker proteins. The gonadotropin receptors, *Fshr* and *Lhr* play a significant role in folliculogenesis and ovulation respectively. Although androgens and AR have known roles in the positive regulation of *Fshr* (Sen et al., 2014), its ovarian expression was not significantly modulated in any of the groups. However, the transcriptional level of *Lhr* was altered in the ovaries of PCOS animals, and these levels were restored to the normal range by AVG, PE and Metformin treatment. *Lhr* is also located on the surfaces of theca cells and granulosa cells, and *Lhr* transcript levels also influence ovulation, corpus luteum formation and the production of other steroids, i.e., estrogen, progesterone and androgen (Edson et al., 2009). The potential of AVG and PE to modulate the gene expression of *Lhr* may be attributed to the non-polar phytochemicals present in them (Nampoothiri et al., 2015). It is to be noted that LP1 and LP3 did not have any significant effect on the transcriptional regulation of gonadotropin receptors.

Ovarian steroid hormones perform several important actions related to ovarian function and their effects are mediated through interaction with specific receptors and steroidogenic enzymes (Salvetti et al., 2009). In this study we have selected most important genes that required for the developmental follicle, oocyte maturation, and regulation of steroidogenesis in the ovaries. Findings from this study provide evidence that letrozole has differential effects on the expression of all the genes studied. A significant increase in *Ar* expression was found in Letrozole treated animals. This supports the suggestion of Manneras et al. (2007), that the ovarian alteration observed in this model is mediated by the accumulation of endogenous testosterone, which also results in pronounced activation or upregulation of the *Ar*. Consistent with this finding, administration of testosterone propionate to prepuberal rats at 5 days of age also increased ovarian nuclear *Ar* expression (Bukovsky et al., 2002). All the different treatments exhibited reduced transcriptional levels of *Ar*, suggesting anti-hyperandrogenic effect.

On the other hand, the expression of *Pgr*, *Esr-1* and *Esr-2* were significantly decreased in PCOS animals, however, this downregulation was reversed by different treatments in the current study. *Pgr* is required specifically for LH-dependent follicular rupture leading to ovulation (Lydon et al., 1995) and transcripts of *Esr*-1 and *Esr*-2 have been reported to play a proliferative role during follicular development (Fitzpatrick et al., 1999). Results from our studies is in line with the results from previous studies, reporting regulation of hormonal imbalance and the expression of steroid biosynthesis-related genes and/or steroid receptor genes by herbal medicines in Letrozole induced rat model (Yang et al., 2018a, 2018b, 2020; Pyun et al., 2018).

Increasing ovarian steroidogenesis and thereby improving female reproductive function is the main treatment technique for female infertility. IVF success rates have improved in many treatment regimens that use gonadotropin or analogues to improve ovarian function (Shrestha et al., 2015). In the current study, the alteration in hormones could be correlated with changes observed in key steroidogenic markers such as *Star*, *Hsd3b1*, *Cyp19a1* and *Amh*. Also, high insulin levels have direct effect on ovarian steroidogenesis and stimulate thecal androgen production (Diamanti-Kandarakis and Dunaif, 2012). Insulin mediated modulation in steroidogenic enzymes like steroidogenic acute regulatory protein (StAR), 3b-HSD and androgen receptor (AR) are also well documented in case of PCOS. Results from the present study revealed that the expression of *Star* and *Amh* was significantly elevated whereas, expression of *Hsd3b1* and *Cyp19a1* was significantly reduced in letrozole-treated animals. Disturbed steroidogenesis was observed as a result of altered enzyme activity, which may be attributable to a shift in StAR expression profile. StAR expression was found to be high in the PCOS community, possibly due to the synergistic effect of high LH and insulin levels, which increase StAR expression by co-binding to the StAR promoter region (Sekar et al., 2000). In the present study, the elevated transcript levels of *Star* upon Letrozole treatment, was significantly reduced when treated with AVG, PE, LP3 and CA. On the contrary, PE, LP3 and CA significantly increased the transcriptional levels of *Hsd3b1*, which was reduced upon Letrozole treatment. 3 beta-hydroxysteroid dehydrogenase is an important enzyme, encoded by the gene *Hsd3b1*, and is responsible for the biosynthesis of progesterone. The reduced capacity of PCO luteinizing granulosa cells to synthesize progesterone in vitro may be due to reduced 3 beta-HSD gene expression (Doldi et al., 2000). The non-polar phytochemicals present in *Aloe vera* gel effectively improved the hormone levels by modulating the transcriptional levels of these key steroidogenic markers. Data from Radha and Laxmipriya (2016a) substantiate our results of present study.

While StAR and aromatase work together to control estradiol production, their effects differ in time and mode of action. An increase in StAR protein normally causes an acute increase in steroid production, while an increase in aromatase levels can cause a later increase in steroidogenesis (Huang et al., 2004). Previous studies have reported dysfunctional aromatase activity in PCOS women, and *Cyp19a1* plays a key role in the normal progression of the menstrual/estrus cycle in rats with PCOS (Kafali et al., 2004). In the present study, reduced expression of *Cyp19a1* transcript was observed in the ovaries of Letrozole treated PCOS animals, which was found to be significantly increased upon treatment with AVG, PE, LP3, CA and metformin.

Another key steroidogenic modulator, which is influenced by hyperandrogenism as well as insulin resistance is Anti- Mullerian Hormone (Wiweko et al., 2018). *Amh* mRNA is produced by the granulosa cells surrounding preantral and antral follicles and has an important role in the development and maturation of follicles. AMH production by granulosa cells in the polycystic ovary is 75 times higher compared to healthy women. AMH, a surrogate marker for hyperandrogenism, is associated with the severity of morphological and hormonal changes in PCOS patients (Wiweko et al., 2014). In the current study, elevated levels of *Amh* mRNA in the ovaries of Letrozole induced PCOS animals was significantly restored to normalcy upon treatment with AVG, PE and Metformin. The partially purified isolates of *Aloe vera* gel did not modulate the *Amh* at transcriptional level.

The present study has demonstrated that LP1 exhibited only anti-androgenic effect. On the other hand, LP3 was found to be more potent than LP1 in regulating the steroidogenic as well as metabolic parameters associated with PCOS. The possible mechanism of LP3, a partially purified non-polar phytochemical derived from *Aloe vera* gel may be due to its modulatory effect on the transcription of steroid receptors and key steroidogenic marker proteins, mainly be acting as an anti-androgenic and progestenic agent. It helped in restoration of ovarian structure-function by regularizing the hormonal milieu in Letrozole induce PCOS mice. In addition to this, the metabolic dysfunction which is associated with PCOS due to glucose intolerance, insulin resistance and dyslipidaemia was improved upon treatment with LP3, AVG and PE. Remarkably, LP3 was found to be equally potent as AVG and PE towards management of reproductive and metabolic complications associated with Letrozole induced PCOS mouse, suggesting that oral administration of LP3 (0.5 μg/kg/day) for 60 days is the adequate dose for treatment of PCOS. Interestingly, the observed therapeutic potential of LP3 was better than metformin, which is the standard prescribed drug for PCOS.

5. Conclusion

The efficacy of LP1 and LP3, partially purified non-polar phytocompounds from *Aloe barbadensis* Mill. gel in the letrozole-induced PCOS mouse model was investigated in this study. Body weight, glucose intolerance, HOMA-IR, triglyceride levels, estrus cyclicity, hormonal profile, ovarian histopathology, and gene expression of gonadotropin receptors, steroid receptors and key steroidogenic markers were all restored after oral administration of LP3. The results were comparable to those obtained with *Aloe vera* gel, Petroleum ether extract of *Aloe vera* gel, and Metformin therapy, suggesting that LP3 is a possible bioactive isolate of *Aloe vera* gel that can be used to treat PCOS and its comorbidities. Also, it is interesting to note that it is first time we have clearly shown that this bioactive with two potential as ovarian modulator as well as metabolic modulator and can be refined for management of PCOS pathology. Further, detailed molecular elucidation of the bioactive needs to be explored which could pave way for herbal formulation in management of the multi etiological endocrine pathology with minimum side effects. Also, the study attempts to add a new facet with scientific explanation to the *Aloe vera* that has been traditionally used in Indian system of ancient medicine.

Conflict of interest declaration and author agreement form

We have no conflict of interest to declare.

This statement is to certify that all Authors have seen and approved the manuscript being submitted. We warrant that the article is the Authors' original work. We warrant that the article has not received prior publication and is not under consideration for publication elsewhere. On

behalf of all Co-Authors, the corresponding Author shall bear full responsibility for the submission.

This research has not been submitted for publication nor has it been published in whole or in part elsewhere. We attest to the fact that all Authors listed on the title page have contributed significantly to the work, have read the manuscript, attest to the validity and legitimacy of the data and its interpretation, and agree to its submission to the **Journal of Ethnopharmacology.**

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No additional authors will be added post submission, unless editors receive agreement from all authors and detailed information is supplied as to why the author list should be amended.

CRediT authorship contribution statement

Arpi Dey: Conceived, Formal analysis, Conceptualized the research work and experimental design, Collected the data: Yes, Performed phytochemical analysis and animal experimentation, Contributed data or analysis tools: Yes. Performed the analysis: Yes. Performed the statistical analysis, Wrote the paper: Yes. Data compilation and wrote the entire manuscript, Other contribution: Reviewed and edited the manuscript. **Shivani Dhadhal:** Formal analysis, Collected the data: Yes. Helped with the animal experimentation, Contributed data or analysis tools: Yes. Performed the microscopic analysis, Performed the analysis: Yes, Wrote the paper, NA, Other contribution: Proof-reading of manuscript. **Radha Maharjan:** Conceived and designed analysis: NA, Data curation, NA, Contributed data or analysis tools: Yes. Provided preliminary data for the animal experimentation, Performed the analysis: NA, Wrote the paper, NA, Other contribution: Proof-reading of manuscript. **Padamnabhi S. Nagar:** Formal analysis, Helped in designing the experiments, Collected the data: NA, Contributed data or analysis tools: NA, Performed the analysis: Yes. Helped with the phytochemical analysis, Wrote the paper: NA, Other contribution: NA. **Laxmipriya Nampoothiri:** Formal analysis, Helped in designing the experiments, Collected the data: NA, Contributed data or analysis tools: NA, Performed the analysis: NA, Wrote the paper: NA, Other contribution: Reviewed and edited the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115161.

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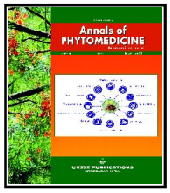

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Fruit juice of *Garcinia indica* Choisy modulates dyslipidemia and lipid metabolism in cafeteria diet based rat model

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Abstract

There is a significant rise in the incidences of dyslipidemia, leading to obesity. The therapeutics available for dyslipidemia are limited and associated with major side-effects. Thereby, researchers are shifting towards nutraceuticals compounds. In the current study, *Garcinia indica* Choisy, which is an endemic species of Western Ghats of India was evaluated for its anti-dyslipidemic properties in cafeteria diet fed obese rat model. Firstly, cafeteria diet fed rat model was developed and validated. After successful development of the model, the rats were orally fed with 1 ml *G. indica* fruit juice for 4 weeks and parameters such as OGTT, lipid profile, hormone levels of insulin and leptin, HMG CoA reductase and LCAT enzyme activities and toxicity parameters were evaluated. Identification and quantification of the hydroxycitric acid in *G. indica* fruit juice was done by HPLC method. Toxicity parameters like SGPT and creatinine were performed to evaluate the toxicity of the dose. Results showed that cafeteria diet fed animals exhibited increased body weight, increased food intake, decreased water intake, increased glucose intolerance and dyslipidemia at 10 weeks. Treatment with *G. indica* fruit juice for 4 weeks, reduced the body weight, improved the metabolic parameters like glucose sensitivity, dyslipidemia, insulin and leptin levels and lipid metabolizing levels without causing toxicity. Oral dosage of *G. indica* fruit juice for 4 weeks exhibits antiobesity potential in cafeteria diet fed dyslipidemic rats. The results obtained were better than orlistat, which is a standard mode of chemotherapy for management of dyslipidemic obesity.

1. Introduction

The prevalence of dyslipidemia leading to obesity is rapidly increasing, however, limited medications are presently available in the market (Birari and Bhutani, 2007). Obesity is a dyslipidemic disorder, wherein, derangement in lipid metabolism has been seen along with abnormal lipid levels (Bays *et al.*, 2013), often associated with higher storage of lipid in adipocytes (Arner *et al.*, 2011). It is interesting to note that dyslipidemia is associated with a cluster of diseases thereby, being a central player in development of metabolic syndrome (Jung and Choi, 2014). The manifestation of syndrome has decreased life expectancy and its quality (Katz *et al.*, 2000; Pimenta *et al.*, 2015; Taylor *et al.*, 2013). Several nutritional theories have implicated diet as an important contributor in development of abnormal lipid levels (Kamran *et al.*, 2016). It is stated that over nutrition with low metabolic output is major cause of developing dyslipidemia (Grundy and Barnett, 2004). In this context, cafeteria based diet which is enriched with high refined sugar and high fat serves as a powerhouse of excessive calories and when used in animal models mimics all features of metabolic syndrome (Gomez-Smith *et al.*, 2016; Sampey *et al.*, 2011). Data from several reports also indicate that people increase the intake of high energy snack foods when stressed, thereby leading to dyslipidemic obesity (Anderson *et al.*, 2011; Shori *et al.*, 2017). This abnormal lipid status has affected immensely other metabolic pathways leading to cardiovascular risk. This has led to urgent need for developing the new antiobesity drugs that could manage dyslipidemia with fewer side effects. Currently, potential use of nutraceuticals for the management of dyslipidemia is not fully explored and could be an outstanding substitute approach for developing safe and effective dyslipidemic drugs. India, especially north-eastern region is a rich source of various medicinal plants. In this context, several herbs have been explored, amongst which *Garcinia* is of utmost importance due to its several important biological properties. Plants from the genus *Garcinia* have been reported from Asia, Africa and Polynesia. Anti-inflammatory, antinociceptive, antioxidant, antitumoral, antifungal, anticancer, antihistaminic, antiulcerogenic, antimicrobial, antiviral, vasodilatory, hypolipidemic, hepatoprotective, nephroprotec-tive and cardioprotective properties of plants from this genus have been reported (Santo *et al.*, 2020). *G. indica* commonly known as “Kokum” is one of the *Garcinia* species used in traditional medicine in Asian countries as folk medicine to treat various ailments. In Ayurveda, it is known as Vrikshamla. Juice obtained from Kokum fruit or extracts prepared from aril or rind is used in the preparation of drugs in

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Indian, Chinese, Thai and Malaysian systems of medicine. Kokum finds its place in Ayurvedic systems of medicine and is considered to be beneficial for health (Swamy *et al.*, 2014). Also, infusion prepared from the Kokum is used to treat piles, dysentery and infections. Furthermore, Kokum is known to strengthen the cardiovascular system and stabilize liver function (Braganza *et al*., 2012). Prophylactic potential of *G. indica* fruits for ailments as varied as rheumatism, rickets, enlargement of spleen, uterine complaints and in animal disorders have been described in *Ayurveda*. Its fruit juice or syrup is used as a coolant and helps to reduce body weight (Braganza *et al*., 2012). It is also used for getting relief in stomach and liver disorders (Bhat *et al*., 2005; Krishnamurthy, 1984; Krishnamurthy *et al.*, 1982; Mishra *et al.*, 2006). The present investigation was undertaken to investigate the efficacy of fruit juice of *G. indica* as a hypolipidemic agent in cafeteria based diet dyslipidemic rodent model.

2. Material and Methods

2.1 Chemicals

Hydroxycitric acid (HCA), calcium salt was purchased from Natural Remedies, Bengaluru, India. HPLC grade solvents (acetonitrile, trifluroacetic acid, TFA, and water) were purchased from Merck, Mumbai, India. Cholesterol (HiMedia Laboratories) and Orlistat (German Remedies) were purchased locally. Metabolic profile involving oral glucose tolerance test, lipid profile were performed using GOD-POD kit and lipid profile test kit was purchased from Enzopak (Rankem). Trizol, cDNA kit was procured from Takara Inc (PrimeScript 1st strand cDNA Synthesis Kit). Primers for metabolic enzymes were designed by primer express and synthesized by integrated DNA technologies. Serum hormones-insulin and leptin were assayed using ELISA kits (DBC Canada).

2.2 *G. indica* fruit juice

Mature fruits of *G. indica* were collected. Fruit pulp was compressed to get the juice. Collected juice was stored in glass bottles under refrigerated conditions till further use.

2.3 Apparatus and chromatographic conditions for profiling of HCA in *G. indica* fruit juice

Quantification of hydroxycitric acid was carried out using HPLC (Waters, USA) system consisting of quaternary pumps, an in-line vacuum degasser, and a photodiode array detector (PDA). The instrumentation was controlled by using Empower 3.0 software (Waters). The chromatographic separation was achieved using Sunfire™ C18 Column (4.6 x 250 mm, 5 µm, Waters, Milford, MA, USA) at ambient temperature. The mobile phases consisted of a mixture of solvents: 0.1 % trifluoroacetic acid in water (A) and 0.1 % trifluoroacetic acid in acetonitrile (B). The optimised HPLC condition for gradient elution mode is as follows: elution was initially started with 90 % of solvent (A) and 10 % solvent (B) with a flow rate of 0.8 ml/min. Further, after 10 min solvent (A) was decreased to 80 % and solvent (B) was increased to 20 %. After 25 min of solvent (A) was decreased to 70 % and solvent (B) was increased to 30 %. Solvent (A) was gradually decreased to 60 % and solvent (B) was increased to 40 % after 30 min. At 31 min composition of solvent (A) and solvent (B) was brought back to 90 % and 10 %, respectively. The flow rate through out the run was 0.8 ml/min and run time was 35 min (Kureshi *et al.*, 2018). Detection wavelength was 211 nm. Injection volume was 20 µl (Kureshi *et al.*, 2018).

2.4 Animals

Forty adult virgin female Charles Foster rats (3-4 months) showing regular estrous cyclicity were chosen for the study. Animals were housed in a standard controlled animal care facility (22-25^{o}C and 45% humidity) in cages (one rats/cage) under equal dark and light cycle (1:1). Standard nutritional and environmental conditions for animals were maintained throughout the experiment (Chidrawar *et al*., 2011). 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) (Protocol Number: BC/11/2017).

2.5 Induction of dyslipidemia through diet

The cafeteria diet was as described by Chidrawar *et al*. (2012). It consisted of 3 different diets: diet 1-condensed milk (8 g) + bread (8 g); diet 2-chocolate (3 g) + biscuits (6 g) + dried coconut (6 g); diet 3-cheese (8 g) + boiled potatoes (10 g) for each animal. The three cafeteria diets were fed to each rat of the group that had 6 animals on days one, two, and three, respectively with repetition in the same succession for 10 weeks along with standard pellet diet. Cholesterol rich high fat diet (Kumar *et al*., 2008) was given to each rat four weeks prior to the *G. indica* fruit juice treatment.

2.6 Treatment with *G. indica* fruit juice

Animals were divided into six major groups, Group I (C) had animals that received lab chow diet and were considered as control. Further, animals fed with cafeteria diet and high-fat diets were divided into five groups, Group II (CD) consisted of animals which were fed with cafeteria diet and were considered to be dyslipidemic animals; Group III (CD + O) consisted orlistat (standard antiobesity drug) treated animals fed with cafeteria diet; Group IV (CD + G) were *G. indica* fruit juice treated animals fed with cafeteria diet; Group V(HF) animals were high fat diet treated animals, which served as positive control for dyslipidemia and Group VI (HF + G) were high-fat diet fed animals, which received *G. indica* fruit juice treatment. All *G. indica* fruit juice treatment was given daily orally for 30 days at a dose of 1 ml.

2.7 Parameters analysed

During 30 days of *G. indica* fruit juice treatment, all animals were continuously monitored for body weight and food intake. Oral glucose tolerance test (OGTT) was performed after 12 h fasting in all rats (Buchanan *et al.*, 1991). Glucose (2g/kg body weight) was orally fed to the rats and blood samples was collected in sodium fluoride (NaF) and EDTA coated tubes considered as 0 minute. After that blood sample was collected at different time intervals (30, 60, 90, and 120 minutes) and plasma was used for the estimation of glucose. Analysis of the lipid profile was done by Enzopak kits which estimated total cholesterol, triglycerides, HDL-C, and LDL-C from serum. Serum hormones like insulin and leptin were assayed by ELISA method. Homeostatic model assessment, Insulin Resistance (HOMA-IR) was calculated based on the fasting blood glucose and fasting insulin values using the following formula HOMA IR = (Fasting insulin x Fasting glucose)/405. Normal insulin resistance: < 3; moderate insulin resistance: between 3-5; severe insulin resistance: > 5. In addition to

this, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) activity was carried out according to the protocol described by (Rao and Ramakrishnan, 1975). Also, plasma lecithin cholesterol acyl transferase (LCAT) activity was assayed by the method described by Hitz (Hitz *et al.*, 1983). In addition to this, gene expression of cholesterol biosynthetic enzymes like Acetyl-CoA carboxylase (ACC) was evaluated by RT-PCR. RNA was isolated using trizol reagent following which 2 microgram of RNA was subjected to cDNA preparation. cDNA was later used for expression studies. Primer details are indicated in Table 1. Serum glutamate pyruvate transaminase (SGPT) and creatinine were analyzed to understand the toxicity of *G. indica* fruit juice.

Table 1: Sequences of target gene specific primers

Gene name	Accession number	Sequence of primers
Acetyl-coenzyme A carboxylase	NM_022193.1	F-5'ATGGTCTACATTCCCCCACA 3'
		R-5'ATCACAACCCAAGAACCACC 3'
GAPDH (internal control)	NM_017008	F-5'CAAGGTCATCCATGACAACTTTG 3'
		R-5'GTCCACCACCCTGTTGCTGTAG 3'

2.8 Statistical analysis

Comparison of different groups was done using analysis of variance (ANOVA) and Student *t*-test. The analysis was carried out using GraphPad version 5.0. $P \geq 0.05$ was considered significant. All results are expressed as the mean ± SEM for 6-7 animals in each group.

3. Results

3.1 HPLC analysis of *G. indica* fruit juice

Identification and quantification of the HCA in *G. indica* fruit juice was carried out with the comparison of retention time and PDA spectra of the peak of standard HCA (Figure 1A). Peak of HCA eluted at 5.3 min. Furthermore, identity of peak of HCA was also confirmed by spiking studies. Quantification of HCA was carried out using a calibration curve prepared from the different concentration of standard HCA and the content of (-)-hydroxycitric acid (HCA) in *G. indica* juice was 16.57 % (Figure 1B).

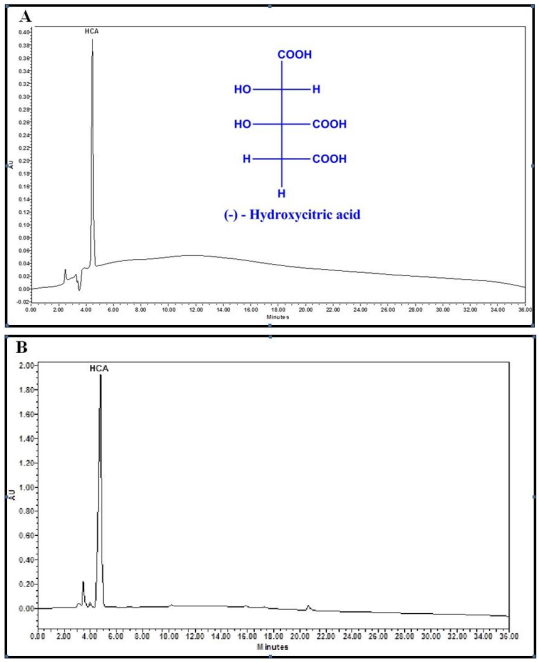
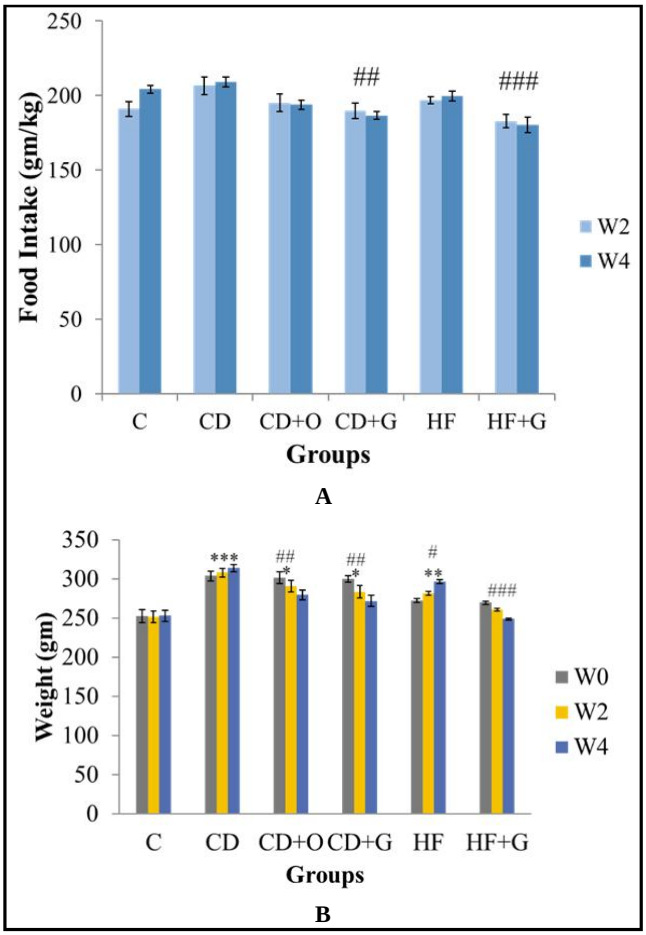


Figure 1: HPLC analysis of *G. indica* fruit juice. A peak of standard HCA B. Quantification of HCA in *G. indica* fruit juice.

3.2 Effect of *G. indica* fruit juice on body weight, food and water intake

Figure 2A. represents the effect of *G. indica* fruit juice on the food consumption of the animals. There was significant increase in body weight observed after 10 weeks of treatment in cafeteria group as compared to control group as shown in Figure 2B. After 4 weeks of treatment with *G. indica* fruit juice, it was observed that there was significant decrease in body weight as compared to non-treated cafeteria group as well as orlistat treated cafeteria group. Thereby, treatment of *G. indica* fruit juice for 4 weeks was effective in reduction of body weight in the animals.



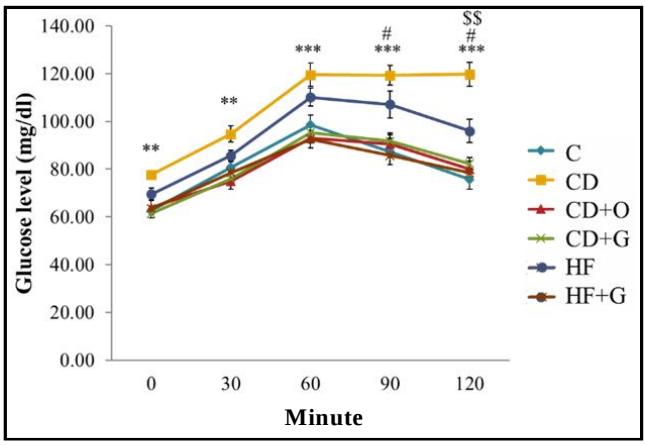


Figure 2: Effect of *G. indica* fruit juice on A. food intake, B. body weight. * comparison with control, # comparison with cafeteria diet, N=5, values are Mean ± SEM, # $p<0.05$, ## $p<0.01$, ### $p<0.001$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, W0, W2, W4 represents week 0 (before treatment), week 2 and week 4 (after treatment) C. glucose tolerance. * comparison with cafeteria diet, # comparison with high-fat diet, $ comparison between cafeteria diet and high-fat diet N=5, values are Mean ± SEM, # $p<0.05$, ** $p<0.01$, *** $p<0.001$, $$ $p<0.01$.

3.3 Effect of *G. indica* fruit juice on metabolic parameters

High fat affects insulin sensitivity. Thereby, oral glucose tolerance test was performed after *G. indica* fruit juice treatment. Figure 2C demonstrated that the treatment with *G. indica* fruit juice for 4 weeks significant decrease in glucose intolerance was recorded. This data was comparable to group treated with orlistat, thereby, suggesting that *G. indica* fruit juice has potential to improve glucose sensitivity. However, cafeteria group and high fat group were glucose intolerant. In addition to this, serum hormone profile for insulin and leptin along with HOMO-IR demonstrated that there was significant increase in serum insulin level in cafeteria treated group (Table 2). Upon treatment with *G. indica* fruit juice, there was significant decrease in serum insulin level. Similar results were observed in orlistat treated group. In case of high-fat group, no significant change was observed as compared to control group. Serum leptin levels were significantly very high as compared to control group in cafeteria treated group. *G. indica* fruit juice treatment showed significant decrease in its level. While calculating HOMO-IR, it was observed, there is severe insulin resistance in cafeteria treated group. After treatment with *G. indica* fruit juice and orlistat, its value decreased but still it was showing moderate insulin resistance similar to control group. Thereby, it could be possible that long term treatment may improve glucose sensitivity.

Table 2: Effect of *G. indica* fruit juice on hormone profile

Groups	Insulin (mIU/ml)	Leptin (ng/ml)	HOMO-IR
C	24.09	0.74	3.73
CD	36.80**	1.17***	7.06
CD+O	32.10#	0.84###	5.07
CD+G	28.47##	0.76###	4.31
HF	29.37*##	0.98**##	5.04
HF+G	27.36	0.81	4.29

* Comparison with Control, # Comparison with cafeteria diet. n=5, Values are represented as Mean ± SEM, # $p<0.05$, ## $p<0.01$, ### $p<0.001$* $p<0.05$,** $p<0.01$,*** $p<0.001$.

Table 3 represents lipid profile after administration of *G. indica* fruit juice for 30 days. There was significant decrease in serum total cholesterol, serum tryglycerides and LDL-cholsterol and increase in HDL-cholesterol. It suggested that dyslipidemic rat model was reverting back to normal phenotype. These data when compared to standard drug shows similar result. Cafeteria treated group and high-fat treated group both shows significant alteration in lipid profile.

Table 3: Effect of *G. indica* fruit juice on lipid profile.

Groups	Total cholesterol (mg/dl)	HDL-cholesterol (mg/dl)	Triglycerides (mg/dl)	LDL-cholesterol (mg/dl)
C	55.55 ± 3.54	32.94 ± 1.09	80.83 ± 4.05	6.445 ± 4.63
CD	95.12 ± 2.90 ***	22.06 ± 0.86 **	141.9 ± 6.36 ***	44.69 ± 4.03 ***
CD + O	78.65 ± 3.75 #	32.16 ± 0.57 ##	97.98 ± 6.64 ##	26.9 ± 4.47 ##
CD + G	80.32 ± 1.58 #	31.3 ± 0.43 ##	95.3 ± 2.33 ##	29.96 ± 1.37 ##
HF	86.16 ± 2.10 ***	28.72 ± 0.78 *	118.1 ± 7.61 **	33.83 ± 1.88 **
HF+ G	82.92 ± 2.37	30.29 ± 0.96	107.5 ± 6.90	31.14 ± 1.23

* Comparison with Control, # Comparison with cafeteria diet n=5, Values are represented as Mean ± SEM, ** $p<0.01$, *** $p<0.001$, # $p<0.05$, ## $p<0.01$.

3.4 Effect of *G. indica* fruit juice on lipid metabolizing enzymes

Figure 3A represents the activity of key lipid metabolizing enzyme HMG-CoA reductase. Upon treatment of *G. indica* fruit juice, HMG-CoA reductase activity was decreased, thus indicated a decreased cholesterol biosynthesis. Ideal decrease in activity was seen on treatment with orlistat. High-fat group does not show significant change suggesting no change, in HMG-Co reductase activity. Figure 3B shows the effect of *G. indica* fruit juice on LCAT activity. On administering *G. indica* fruit juice, a significant increase in LCAT activity was observed as compared to the cafeteria group. This activity was not increased in case of orlistat treated group. While high-fat treated groups showed decrease in LCAT activity. No significant change was observed in *G. indica* fruit juice treated group as compared to high-fat treated group. Figure 3C represents transcript level of enzyme acetyl Co-a carboxylase, a lipid metabolizing enzyme. As seen in the figure, cafeteria diet treated group shows significant increase in ACC level as compared to control.

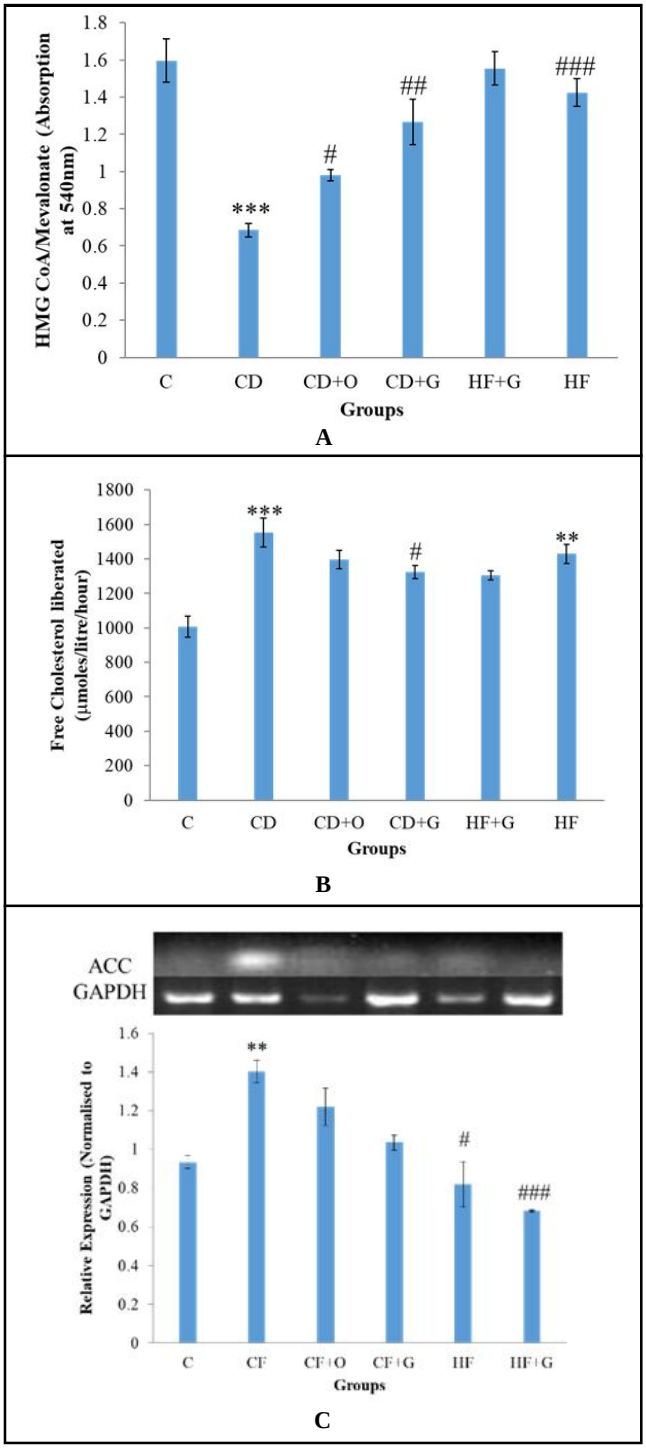


Figure 3: Effect of *G. indica* fruit juice on lipid metabolizing enzymes A. HMG CoA reductase activity B. LCAT activity and C. gene expression level of ACC enzyme. * comparison with control, # comparison with cafeteria diet, N=5, Values are Mean ± SEM, # <0.05, # *p*<0.01, ## *p*<0.001, ** *p*<0.01, * *p*<0.001, C. relative gene expression of ACC.* comparison with control, # comparison with cafeteria diet. n=5, values are Mean ± SEM, # *p*<0.05, ### *p*<0.001, ** <0.01.**

After treatment of *G. indica* fruit juice for 30 days, there was marked decrease in its level. The data is similar to the orlistat treated group. High-fat diet treated group does not show significant change in ACC level as compared to control group.

3.5 Effect of *G. indica* fruit juice on toxicity parameters

Figure 4 describes the toxicity parameters of liver and kidney upon administration with the *G. indica* fruit juice. As seen in Figure 4A, there was significant increase in SGPT activity, suggesting liver toxicity due to cafeteria diet treatment as compared to control. Upon the administration of *G. indica* fruit juice, there was significant decrease in its activity. However, there was no significant change in high-fat treated group. Figure 4B represents serum creatinine level and as seen in the Figure, there was significant increase in serum creatinine level in cafeteria treated group and the level decreased in *G. indica* fruit juice treated group. In case of high-fat treated group, significant increase in serum creatinine level was recorded.

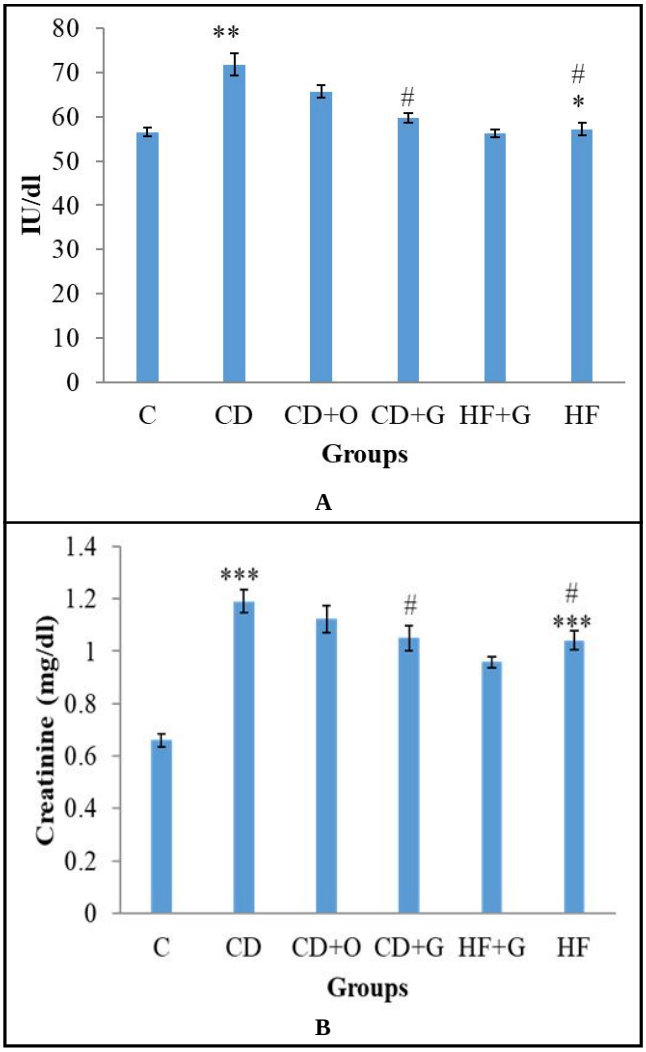


Figure 4: Effect of *G. indica* fruit juice on toxicity parameters of A. liver by SGPT activity and B. kidney by creatinine level. *comparison with control, # comparison with cafeteria diet. N=5, values are Mean ± SEM, ** *p*<0.01, * *p*<0.001, # *p*<0.05.**

4. Discussion

Obesity is a one of major dyslipidemia associated metabolic syndrome. Dyslipidemia is one of the initial steps for the development of obesity. In present study, cafeteria diet was used to generate a metabolic syndrome model for dyslipidemia to study initial stages for obesity. During dyslipidemia, there is increase in glucose intolerance, which is one of the important factor of metabolic syndrome and this may be due to increase in free fatty acid (Nambi *et al.*, 2002). OGTT profile was observed upon cafeteria diet administration, suggesting that enriched fat diet for 10 weeks is sufficient to cause dyslipidemia. In dyslipidemia, lipoprotein metabolism is altered which results in either overproduction or deficiency in certain lipoprotein molecule (Meisinger *et al.*, 2006). There may be alteration in the levels of either one or more lipoprotein molecules followed by elevated levels of total cholesterol, low density lipoprotein and triglycerides along with decreased levels of high density lipoprotein which later on can be developed in obesity (Mishra *et al.*, 2005; Misra *et al.*, 2006; Snehalatha, 2003; Vikram *et al.*, 2003). In our model, it was observed that there was significant increase in total cholesterol, LDL-cholesterol and triglycerides. Also, significant decrease in HDL-cholesterol was also observed. These all parameters are major characteristics of dyslipidemia (Nambi *et al.*, 2002).

HMG-CoA-Reductase catalyses the rate limiting step of liver cholesterol biosynthesis (Bucher *et al.*, 1960; Siperstein,. 1966). In dyslipidemia, it was observed that HMG-CoA-reductase activity was higher as compared to control group (Wu *et al.*, 2013). In our study, it was observed that there was significant decrease in substrate to product ratio suggesting an increase in HMG-CoA-reductase activity. Increase in its activity has resulted in increase in serum cholesterol levels after 10 weeks of treatment. LCAT is an enzyme that catalyses transfer of fatty acids from phosphatidylcholine to the hydroxyl residue (Assmann *et al.*, 1978). Acetylation of cholesterol is helpful for clearance of excess cholesterol. LCAT promotes maturation of HDL particles in plasma and transport cholesterol maintaining a concentration gradient for the diffusion of cellular un-esterified cholesterol to HDL-cholesterol (Shigematsu *et al.*, 2001). In diet induced obese rats, it was observed that there was significant decrease in LCAT activity which correlates with decrease in HDL-cholesterol (Subash and Augustine, 2014). Current study demonstrates that in dyslipidemia, rats there was significant decrease in LCAT activity which can be correlated with low HDL-cholesterol.

Dyslipidemia is a major symptom in development of metabolic syndrome (Mishra *et al.*, 2007). There are several medications available for dyslipidemia. Two major classes are statins and fibrtaes (Wierzbicki *et al.*, 2003). But, due to high cost and hazardous side-effects, there is need for new and better therapy in management of dyslipidemia. Plant derived products or nutraceuticals can be potential targets for the development of new drugs (Chidrawar *et al.*, 2012), as it renders few side-effects. In this context, several plants have been explored for the hypolipidemic effects like *Aloe barbadensis* Mill., *Enicostemma littorale*, *G. cambogia* (Desai *et al.*, 2012; Oluyemi *et al.*, 2002; Vasu *et al.*, 2005). *G. indica* is an indigenous plant however, medicinal properties of this plant are not well studied. Thereby, our present study, involves evaluating efficacy of *G. indica* for its hypolipidemic property.

After the treatment of *G. indica* fruit juice, there was significant decrease in body weight as compared to non-treated groups, suggesting that the *G. indica* fruit juice could be used for the development of anti-dyslipidemic drugs. Similar result was observed in the rats treated with standard drug. Similarly glucose intolerance was reduced in the *G. indica* fruit juice treated group as compared to non-treated group. In case of positive control (high-fat) group, no significant change in the body weight was observed as compared to control group, suggesting that dyslipidemic rat model was not developed. Glucose intolerance is a characteristic feature of insulin resistance, therefore, serum insulin levels were measured (Vikram *et al.*, 2003). There was significant increase in serum insulin level in cafeteria group as compared to control group. After the treatment with *G. indica* fruit juice, there was significant reduction in insulin level. It can be correlated with serum triglycerides level. HOMO-IR is used for evaluation of insulin resistance. While calculating HOMO-IR, it was found that the animals treated with cafeteria diet showed significant increase in index suggesting severe insulin resistance. While treatment with *G. indica* fruit juice resulted in decrease in index but the values were still in the range of moderate insulin resistance. During dyslipidemic obesity, there is increased level of circulating leptin level (Dobrian *et al.*, 2000). Serum leptin levels were elevated in cafeteria treated and high-fat diet treated groups as compared to control. After treatment with *G. indica* fruit juice, it was observed that there was significant reduction in serum leptin levels.

Lipid profile showed significant change in the *G. indica* fruit juice treated groups as compared to non-treated ones. Serum total cholesterol, triglycerides and LDL-cholesterol were back to normal in *G. indica* fruit juice treated group as compared non-treated group. It suggests that *G. indica* fruit juice is altering the lipid metabolism pathway resulting in normal phenotype.

HMG-CoA-reductase and LCAT activity were measured to check effect of *G. indica* fruit juice on lipid metabolising enzymes. After treatment, it was observed that in there was significant increase in the ratio of substrate to product resulting in decreased HMG-CoA-reductase activity. This might be the reason for decreased cholesterol level. Group treated with standard drug also showed the similar result, but to a lower extent. LCAT activity was significantly increased in *G. indica* fruit juice treated group as compared to non-treated group. In case of group treated with standard drug, no significant change was observed. mRNA transcripts levels of acetyl-coenzyme A carboxylase (ACC) were also checked. ACC is an enzyme responsible for the production and regulation of fatty acid. It is predominately expressed in the liver and it is inducible in response to feeding. Transcript level of ACC is increased in obesity resulting in production of free fatty acid (Jeffery *et al.*, 2013). mRNA levels of ACC were increased in cafeteria treated group. As a result of administration of *G. indica* fruit juice, significant reduction in its level was observed, suggesting decrease in free fatty acid levels. However, other transcriptional factors need to be evaluated for further confirmation. Thereby, present study has clearly shown the *G. indica* fruit juice could be a strong candidate for a hypolipidemic drug. Further, investigations needs to be done to mark the product as a dyslipidemic nutraceutical. Liver and kidney function tests showed that after 10 weeks of treatment with cafeteria diet there was significant increase in SGPT activity and serum creatinine, respectively, suggesting that prolong dose of cafeteria diet can alter liver and kidney function (Chidrawar *et al.*, 2012). After treatment with *G. indica* fruit juice, it was observed that there was significant

decrease in SGPT activity and serum creatinine levels. It suggests that *G. indica* fruit juice may have potential to restore liver and kidney functions. These results were not observed in the group treated with standard drug.

5. Conclusion

Increasing evidences are suggesting the significant impact of plant derived phytocomponents in the regulation of different aspects of human physiology and thereby, aid in the treatment and management of cardiovascular diseases and metabolic syndrome like obesity and dyslipidemia. In the current study, cafeteria diet induced obesity model was successfully developed which exhibited the associated complications of metabolic syndrome such as increased body weight, dyslipidemia, and insulin resistance after 10 weeks. Also, the potential of *G. indica* fruit juice to ameliorate dyslipidemia was evaluated in cafeteria diet fed obese rat model. It was observed that treatment with *G. indica* fruit juice at a dose of 1 ml for 4 weeks could restore the body weight, dyslipidemia, glucose sensitivity, insulin resistance and expression and activities of key lipid metabolizing enzymes in cafeteria diet induced dyslipidemic rat model. The observed therapeutic potential was better than orlistat, which is the standard drug used for treatment of dyslipidemia and obesity. Thereby, suggesting that phytocomponents such as HCA present in *G. indica* fruit juice is a potent anti-dyslipidemic agent without inducing toxicity. However, phytocompounds from *G. indica* can be further analysed for understanding its molecular targets towards amelioration of dyslipidemia.

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Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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