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Gene Expression Analysis in Polycystic Ovary Syndrome (PCOS) Treated with *Tinospora cordifolia*: Insights into Therapeutic Mechanisms

M. Sri Devi, Rajeswary Hari* and Karthikeyan Sekar

Department of Biotechnology, Dr MGR Educational and Research Institute, Maduravoyal, Chennai - 600095, Tamil Nadu, India; rajihar@gmail.com

Abstract

Background: Polycystic Ovary Syndrome (PCOS) is an ailment with ovarian dysfunction accompanying cardinal features of hyperinsulinemia and hyperandrogenism. Ayurvedic and traditional medical practises frequently use the plant *Tinospora cordifolia* due to its numerous therapeutic benefits, as it contains alkaloids, terpenoids, flavonoids, phenolic chemicals, tannins, and steroids. Niosomes have recently attracted increased interest as a new drug delivery mechanism because of their biodegradability, decreased toxicity, enhanced therapeutic effectiveness, and augmentation of absorption. **Aim:** In this study, an effort has been made to determine the effects of giving mifepristone-induced PCOS rats an Ethanolic Leaf Extract of *T. cordifolia* (ELETC) and *T. cordifolia* loaded phytoniosomes (nELETC) on the downregulation of geneson the genes, such as Insulin-like Growth Factor-1 (IGF-1), IGF-2 and Phosphatase and Tensin (PTEN) involved in the insulin signalling pathway. **Methods:** PCOS was induced by administering mifepristone (4mg/kg b.w.) daily for a period of 8 days to the female rats and confirmed by a persistent oestrous cycle. The rats that developed PCOS were treated with 200mg /kg b. w. each ELETC and nELETC per oral for 28 days. The ovaries were collected for gene expression study using semi-quantitative real-time Polymerase Chain Reaction (PCR) analysis. **Result:** ELETC and nELETC extract treatment on the IGF-1, IGF-2 and PTEN gene showed a good impact on the expression patterns. In PCOS-induced group 2 rats, there was a 1.72-fold change in the mRNA expression of the IGF-1 gene, a 0.6-fold increase in the IGF-2 gene and a 0.64-fold increase in the PTEN gene. The expression of group 3 and group 4 rats when treated with ELETC and nELETC was reduced to 0.72 fold and 0.42 fold, respectively, for the IGF-1 gene, 0.38 and 0.36 fold, respectively, for the IGF-2 gene and 0.45 and 0.31 fold, respectively, for the PTEN gene. The reduction in the expression pattern of these genes is more pronounced in the nELETC extract-receiving group 4 rats compared to the group 3 rats, which received ELETC. **Conclusion:** It may be concluded that the *T. cordifolia* ethanolic leaf extract-loaded phytoniosome can be a useful medication for treating PCOS and helping women to regain their fertility.

Major Findings: The phytoniosome extract of the plant *T. cordifolia* proved to reduce the gene expression and suppress overexpressed genes and proteins, and also regulate unregulated signalling pathways.

Keywords: IGF-1, IGF-2, Phytoniosomes, PTEN, *T. cordifolia*

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1. Introduction

PCOS is an ailment with ovarian dysfunction accompanying cardinal features of hyperinsulinemia and hyperandrogenism[1]. In addition to this, due to endocrine dysfunction, it results in improper glucose metabolism in women during their reproductive period of their age[2]. The functional abnormalities identified in the ovaries as a result of hyperinsulinemia, followed by hyperandrogenism, dysregulate the hypothalamic-pituitary-ovarian axis[3]. As a peptide hormone, insulin attaches to its receptor and initiates two different types of signalling cascades. It predominantly regulates the metabolic process via the phosphatidylinositol 3 kinase/protein kinase B (PI3K/PKB) system, and it additionally affects the development via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, which is primarily

*Author for correspondence

found in the ovary[4]. When the insulin ligand binds to the IGF-1 receptors found in the stromal and theca cells of the ovary, androgen synthesis is triggered[5]. Despite hyperinsulinemia, the primary molecular pathophysiology of PCOS is insulin resistance, which results in excessive IGF-I signalling system expression and elevated androgen levels[6].

The tyrosine kinase family of protein receptors, which are found on the cell surface, are in charge of binding insulin to start the biochemical process. Numerous ligands, such as IGF-I, fibroblast growth factor, and epidermal growth factor, can phosphorylate the specific tyrosine residues of the corresponding intracellular substrate and initiate signal transduction by binding and activating the insulin receptor. Intracellular substrates like Insulin-receptor substrate IRS-1 and IRS-2 are crucial in the activation of the PI3K and MAPK pathways and mediate this signal transduction[7]. Numerous studies have demonstrated the intra-ovarian IGF system's important role in controlling the folliculogenesis, and according to Meng[8], the ovary, specifically the granulosa cells, is the site of IGF-1 gene expression and action. Here, they participate in cell proliferation, steroidogenesis, and oocyte growth. Furthermore, elevated levels of insulin and IGF-I combine with Luteinizing Hormone (LH) to cause an excess of androgen to be produced in ovarian theca cells[9].

The PI3K/Akt pathway and the lipid phosphatase PTEN control granulosa cell proliferation and differentiation. Sezginer *et al* investigations have demonstrated that PCOS rats had higher PTEN gene and protein expression[10]. Experimental data point to a strong connection between the insulin signalling system and PTEN expression in PCOS patients that results in altered follicular growth[11]. The aforementioned information makes it evident that a better therapeutic strategy for the management of PCOS in women may involve reducing insulin resistance as well as suppressing the insulin signalling pathway, which includes the IGF family proteins. Insulin sensitisers are currently given for PCOS patients as they lower hyperinsulinemia, androgen production, and regulate follicular development in the ovaries[12]. However, there are numerous negative effects associated with these medications. PCOS needs to be treated using a variety of therapeutic modalities because it is a complex condition. The world is currently shifting its focus toward using plants and plant products as medicine since they contain the phytochemicals needed to treat conditions like PCOS.

Ayurvedic and traditional medical practises frequently use the plant *T. cordifolia*, due to its numerous therapeutic benefits, including its ability to treat diabetes, fight cancer, prevent inflammation, protect the liver, and treat allergies, that it is regarded as a divine bush[13]. It contains alkaloids, terpenoids, flavonoids, phenolic chemicals, tannins, and steroids[14]. Niosomes - because of their biodegradability, decreased toxicity, enhanced therapeutic effectiveness, and augmentation of absorption, a new drug delivery mechanism, have recently attracted increased interest. They are tiny, have a lamellar structure, are composed of cholesterol molecules and nonionic surfactants, and are hydrated with water[15]. They also contain the appropriate medication. Phytoniosomes are the term used to describe the integration of plant-based medicinal substances into the noisome. In this study, an effort is made to determine the effects of giving mifepristone-induced PCOS rats an ELETC and nELETC on the downregulation of genes involved in the insulin signalling pathway. This will help to correct the dysregulated hormonal pathway and restore normal ovarian physiology.

2. Materials and Methods

2.1 Reagents and Chemicals

Mifepristone procured from Merck Millipore was used for the assessment of biochemical parameters. Ranbaxy kits were used to carry out the biochemical tests. Analytical grade compounds were utilised in the current investigation.

2.2 Preparation of *T. cordifolia* Ethanolic Leaf Extract (ELETC)

Tinospora cordifolia leaves were acquired from Cuddalore, Tamil Nadu. Dr Mythreyi, Professor, K.K. College of Pharmacy's Pharmacognosy Department in Chennai, India, identified and verified the leaves and the same was kept for reference as a specimen voucher. The extraction of leaves was done using 90% ethanol, using cold maceration and stored in a refrigerator until they were used. A Rota flash evaporator was used to evaporate the solvent. The yield of the sample was 1.25 %w/w.

2.3 Preparation of *T. cordifolia* Loaded Phytoniosome Ethanolic Leaf Extract (nELETC)

The film hydration approach was used to create the *T. cordifolia* leaf ethanolic extract-loaded phytoniosome (nELETC)[16]. *T. cordifolia* ethanolic leaf extract (1.0 mg/ml) was added to a round-bottom flask containing cholesterol and Tween 60 (10mM) dissolved in chloroform (1:1 M). To obtain a thin layer of nELETC on the flask, the excess chloroform was eliminated using a rotary evaporator set at 55°C. It was hydrated with phosphate buffer saline and then placed in a water bath at 55°C for two hours. To create fine-natured vesicles, the resulting solution was next subjected to bath sonication for 20 minutes. Overnight dialysis against distilled water was used to remove phytoniosomes from the untrapped components.

2.4 Animals

Wistar strain adult albino female rats weighing between 190 and 200g and 12 weeks of age were used in the current pharmacological investigations. The animals were purchased from Chennai, India's Saveetha University Animal House. The animals were housed in well-ventilated rooms with a natural day-night cycle. They were fed a well-balanced rodent diet in the form of pellets and tap water in a regulated environment (24 ± 2℃). During a meeting at Saveetha Dental College and Hospitals in Chennai, the Institutional Animal Ethics Committee gave the project their clearance number BRULAC/SDCH/SIMATS/IAEC/3-2021/065.

2.5 Studies on Acute Toxicity

According to OECD guidelines 420, research on the acute harmful nature of *T. cordifolia's* ethanolic leaf extract has already been completed[17]. No deaths were found till the last dosage of 2000 mg/kg, and the biochemical and haematological indicators were normal. 200 mg/kg b.w. of the extracts, or one tenth of the LD50 dose, were chosen for the current study in order to examine their effectiveness in treating PCOS.

2.6 Mifepristone-induced PCOS in Female Rats

Beginning with the oestrous cycle and continuing for eight consecutive days, Wister female rats were injected with RU486 grade mifepristone (4 mg/0.2 ml oil)[18]. A vaginal smear test was performed every day to track and observe any irregularities or differences in the oestrous cycle following the mifepristone injection, and the animals were grouped for treatment when Persistent Vaginal Cornification (PVC) was attained, a sign of the formation of follicular cysts in the ovaries.

2.7 Animal Groupings and Treatment Methods

Five groups were arbitrarily created from the twenty adult female rats, consisting of four rats in each group. The treatment was carried out for a period of 28 days.

Group 1: Control rats received only the vehicle (CMC suspension 2%)

Group 2: Negative control administered with Mifepristone RU486 alone

Group 3: Rats induced with PCOD and received 200 mg/kg b.w. of ELETC

Group 4: Rats induced with PCOD and received 200 mg/kg b.w. of nELETC

Group 5: Positive control rats induced with PCOD and received 20 mg/kg b.w. of Metformin (standard)

2.8 Semi-quantitative Real-time PCR Analysis of Expression of IGF-1, IGF-2 and PTEN Genes

The total RNA present in the ovaries of the treated experimental groups was isolated by Trizol reagent by employing the kit procedure (Invitrogen, USA). Briefly, a 10ml volume of reaction mixture containing 1mg of cDNA template, 10 pmol/ml concentration of each primer and Prime Taq Premix (GeNet Bio, Nonsam, South Korea) of 2X solution. The process started with the RNA reverse transcription with the help of using higher capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA). The corresponding primer design for the expression patterns of IGF-1, IGF-2 and PTEN genes under study was performed by referring to the NCBI/primer-BLAST tool software (Table 1). The thermocycling program was initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 30s and annealing or extension of 60 seconds at 60°C. The quantification of resultant amplified mRNA expression was analysed using Quanti tect SYBR1 PCR kit (QIAGEN, Valencia, CA, USA). The data pertaining to the expression patterns

of IGF-1, IGF-2 and PTEN genes in the ovaries of the experimental animals were expressed in terms of fold change by analysing the resultant data using the MxPro Software from Agilent Technologies. The variation in the fold change gene expression levels of target genes was calculated with normalisation to β-actin values using the ΔΔCt comparative cycle threshold method. The experiments were performed in triplicate, and all data regarding the expression pattern of the genes were analysed using Light Cycler 96 SW 1.1 Software.

2.9 Statistical Analysis

The values are described as Mean±SE. ANOVA method was used to carry out the statistical analysis, which was followed by Dunnett's t-test. Significant *p-value* is < 0.05.

3. Results

3.1 Entrapment Efficiency

Entrapment efficiency for the phytoniosome was 92.6%, calculated from the given formula.

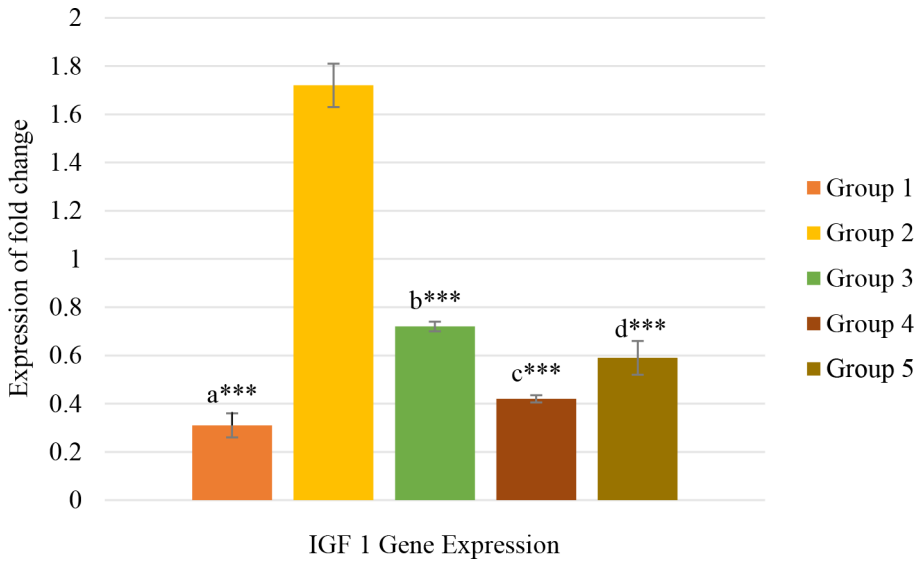
Entrapment efficiency = (3.068/3.311) *100 = 92.6%.

3.2 Semi-quantitative Real-time PCR Analysis of Expression of IGF-1, IGF-2 and PTEN Genes

Figure 1 displays the impact of ELETC and nELETC extract treatment on the IGF-1 gene expression

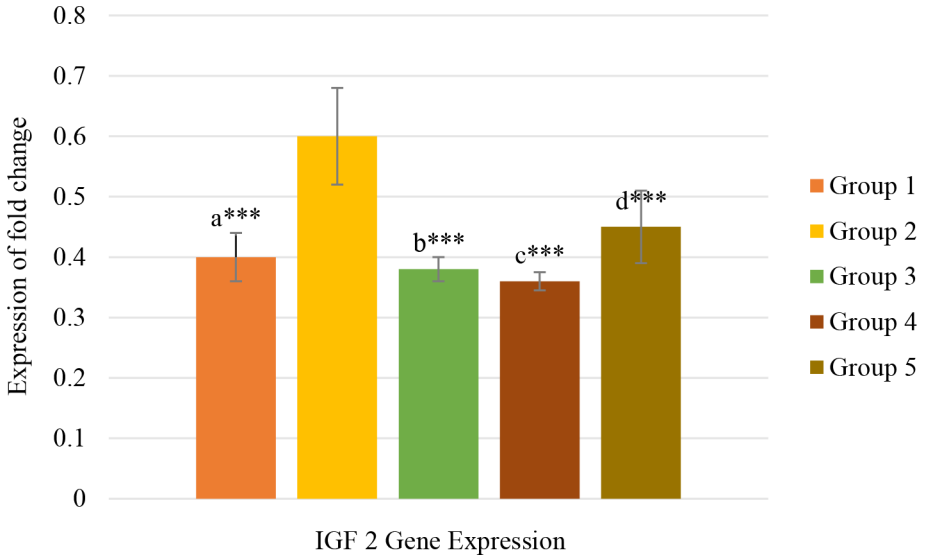
Table 1. The primer sequences used for IGF-1, IGF-2 and PTEN gene expression

Gene	Primer	Primer Sequence
IGF-1	Forward	CCGCTGAAGCCTACAAAGTC
	Reverse	TGTTTTGCAGGTTGCTCAAG
IGF-2	Forward	TGGGGTCCTCTTGAGACATC
	Reverse	AAGCACCAACATCGACTTCC
PTEN	Forward	ACACCGCCAAATTTAACTGC
	Reverse	TTTAAAAACTTGCCCCGATG
β-actin	Forward	CCACCATGTACCCAGGCATT
	Reverse	GAGCCACCAATCCACACAGA



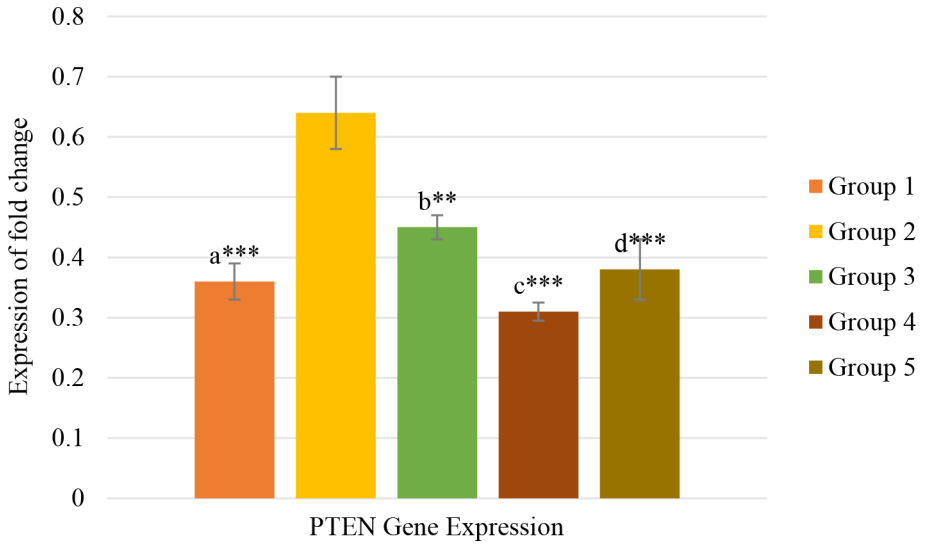
The mean (SEM) values for each group's four animals are presented. Dunnett's "t" test was employed after the ANOVA approach was used to determine statistical significance. There was a comparison between Group 1 and Group 2 (a), Group 2 and Group 3 (b), Group 2 and Group 4 (c), and Group 2 and Group 5 (d). Important p-values: *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Not Significant (NS).

Figure 1. The expression pattern of the IGF-1 gene in different experimental groups.



The mean (SEM) values for each group's four animals are presented. Dunnett's "t" test was employed after the ANOVA approach was used to determine statistical significance. There was a comparison between Group 1 and Group 2 (a), Group 2 and Group 3 (b), Group 2 and Group 4 (c), and Group 2 and Group 5 (d). Important p-values: *$p<0.05$, **$p<0.01$, and ***$p<0.001$. Not Significant (NS).

Figure 2. The expression pattern of the IGF-2 gene in different experimental groups.



The mean (SEM) values for each group's four animals are presented. Dunnett's "t" test was employed after the ANOVA approach was used to determine statistical significance. There was a comparison between Group 1 and Group 2 (a), Group 2 and Group 3 (b), Group 2 and Group 4 (c), and Group 2 and Group 5 (d). Important p-values: * $p<0.05$, **$p<0.01$, and ***$p<0.001$. Not Significant (NS).

Figure 3. The expression pattern of the PTEN gene in different experimental groups.

patterns. In PCOS-induced group 2 rats, there was a 1.72-fold change in the mRNA expression of the IGF-1 gene. In group 3 and group 4 rats, when treated with ELETC and nELETC, the expression was reduced to 0.72-fold and 0.42-fold, respectively. In the current study, it was discovered that control rats had a 0.31-fold higher expression of the IGF-1 gene.

Figure 2 depicts the pattern of IGF-2 expression in the ovarian tissues of rats with ELETC and nELETC extract treatment. The IGF-2 genes showed a similar pattern of expression, with the group 2 rats showing a 0.6-fold increase. The group 3 and group 4 rats showed ($p<0.001$) reduced levels of expression for IGF-2 into 0.38 and 0.36 fold, respectively, which suggests that the extract may be able to rectify the pathophysiology of PCOS.

The PTEN gene exhibited a 0.64-fold expression in the group 2 PCOS-affected rats, which was decreased to 0.45 and 0.31 fold in the group 3 and group 4 rats, respectively, confirming the extract's therapeutic function as shown in Figure 3.

When compared to group 3 rats, which received ELETC extract, the PTEN gene expression pattern reduction is more apparent in group 4 rats, who received nELETC (phytoniosome) extract. When compared to the mifepristone-induced group 2 rats, the expression pattern of the PTEN gene is likewise dramatically diminished in group 3 and group 4 rats treated with the ELETC and nELETC medication.

4. Discussion

This study has demonstrated the ameliorative effects of ELETC and nELETC for PCOS through the development of mifepristone-induced PCOS rat models. The creation of many immature follicles in the ovaries, together with aberrant androgen production, is part of PCOS pathophysiology. The ovary's insulin and insulin-like growth factors further stimulate the hyperandrogenism state. According to recent studies, insulin resistance, hyperinsulinemia, and hyperandrogenism are strongly correlated in PCOS women. In addition to the hypothalamic-pituitary-ovarian axis, the IGF system, which is made up of components including IGF-I, IGF-II, and IGFBP, also regulates the development of human follicles, which is influenced by ovarian steroids[19]. IGF-1, a 70-amino acid peptide hormone with more than 60% homology to IGF-II, has endocrine, paracrine, and autocrine actions[20]. Additionally, it exhibits some structural similarities to pro-insulin. IGF ligands and insulin receptors can bind to one another with low affinity because of these structural similarities.

The transmembrane tyrosine kinase receptor IGF1R mediates the effects of insulin and IGF family ligands. Because of the structural similarities between insulin and the IGF family of protein hormones, the IGF1R can bind both insulin and its ligands. The PI3K signalling pathway, which controls ovarian follicular maturation and steroidogenesis together with pituitary LH, is activated by the binding of either insulin or IGF-1 with IGF1R[21]. Numerous studies have shown that elevated levels of insulin and IGF-I, when combined with elevated levels of LH, cause theca cells of the ovary to overproduce androgens, resulting in hyperandrogenaemia in PCOS patients[22]. Zhang *et al.*[23] have also demonstrated that PCOS women had higher levels of IGF-II in their follicular secretions. Similar to IGF-1, high quantities of IGF-II boost the ovaries to synthesise androgen[24]. Therefore, therapy targeting the reduction of the IGF family of proteins may help treat both the hyperandrogenism and other issues associated with PCOS. In the current study, ELETC and its loaded phytoniosomes (nELETC) significantly reduced IGF-1 and IGF-2 gene expression, which was shown to be high in rats treated with mifepristone. The presence of various phyto components in the ELETC and nELETC extract has the ability to modify the patterns of gene expression, which is credited with this reducing potential.

The decrease in IGF-binding protein I (IGFBP-I) concentrations produced in liver cells, ovarian granulosa cells, and endometrium in response to follicular-stimulating hormone may be the cause of the rise in IGF-1 and IGF-2 proteins in the PCOS-induced rats[25]. The synthesis of IGFBP-1 will be inhibited in the presence of hyperinsulinemia and low FSH, resulting in elevated amounts of free IGF proteins, which automatically induce the synthesis of androgens in the ovary[26]. The phytochemicals in *T. cordifolia* inhibit IGF-1 and IGF-2 gene expression, which lowers the levels of free IGF proteins in the blood and causes the free IGFs to bind to any IGFBP-I that may be present to inhibit testosterone synthesis in ovarian cells.

PTEN, which was once thought to be a tumour suppressor gene, has been discovered to be inactive in several cancer types, including endometrial, prostate, and brain cancer. Recently, it has been shown by Wang *et al.*,[27] that the expression of this gene is changed in conditions such as pulmonary fibrosis, rheumatoid arthritis, and chronic obstructive pulmonary illness. In addition, Shi *et al.*,[28] have demonstrated a close correlation between insulin signalling and PTEN in various species and human cancer cell lines. Furthermore, PTEN expression will rise during the follicular phase[29]. In short, PI3K and PTEN are both dysregulated in the PCOS condition due to hyperinsulinemia and insulin resistance, which affects the signalling pathway involved in the folliculogenesis of human granulosa cells. In our findings, treating

mifepristone-induced PCOS rats with ELETC and nELETC extract reduced the gene expression patterns of PTEN, which in turn controlled the defective folliculogenesis in human granulosa cells.

5. Conclusion

It can be concluded that ELETC and nELETC inhibit the expression of genes, including IGF-1, IGF-2, and PTEN. The insulin and insulin-like growth factor family are made up of several genes and proteins, and the overexpression of these signalling molecules is mostly to blame for the problems of PCOS by downregulating the PI3K/AKT pathway. Our plant extract ELETC and nELETC can be utilised to treat PCOS because it contains numerous phytochemicals that work synergistically to suppress overexpressed genes and proteins and regulate unregulated signalling pathways.

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Author Contribution

Study Conception and/or Design were done by Dr Rajeswary Hari and Dr M Sri Devi. Data Processing, collection, and Performing Experiment was done by Dr M Sri Devi. Analysis and Interpretation of Results were performed by Dr M Sri Devi and Dr S Karthikeyan, followed by Draft Manuscript Preparation, Visualisation by Dr M Sri Devi and Dr S Karthikeyan. The Critical Revision or Editing of the Article was done by Dr Rajeswary Hari and Dr M Sri Devi. The Final Approval of the Version to be Published was done by Dr Rajeswary Hari, Dr M Sri Devi, and Dr S Karthikeyan under the Supervision of Dr Rajeswary Hari. All authors have read and agreed to the published version of the manuscript.