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# *Caesalpinia bonducella* seeds restore the ovarian functions in mifepristone-induced PCOS rats acting through insulin –insulin-like growth factors

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The prime complications namely hyperandrogenism and hyperinsulinemia, observed in PCOS condition is due to the abnormal signalling pathways triggered by insulin as well as insulin-like growth factor family of genes and proteins. The aim of the present study is to evaluate the downregulating effect of ethanolic seed extract of *Caesalpinia bonducella* (ESECB) on the genes like IGF-1, IGF-2 and PTEN and proteins IRS1 and IRS2 in mifepristone induced PCOS rats. The level expression pattern of genes were studied using *in vivo* methods and the *in silico* method is used to study the proteins. Mifepristone induced PCOS rats were treated with 200 mg and 400 mg/kg b.w. p.o of ESECB extract for 28 days. The ovaries were collected for gene expression study using semi quantitative real time PCR analysis. Molecular docking analysis was performed from the GC-MS phyto-constituents for a antagonistic ligand which can halt the reactions of IRS1 and IRS2 proteins. The fold change of the mRNA expression of IGF-1, IGF-2 and PTEN genes were found to be 1.4, 0.34 and 0.36 respectively in the mifepristone induced PCOS rat ovaries. ESECB treatment decreased the fold change to 0.44, 0.13 and 0, 20 respectively. In docking studies, eight ligands namely, dioxan, propyl acetate, acetic anhydride, methyl butenoic acid, methyl isopropyl carbonate, glycerine, diethanolamine and 2,2, dimethyl propanoic anhydride inhibted the IRS1 and IRS2 proteins by interacting with their amino acid residues. It can be concluded that ESECB extract can be used as a potential drug for the treatment of PCOS as it acts at the molecular level to correct the complications of this disease.

**Keywords**: IGF-1, IGF-2, IRS1, IRS2, PCOS, PTEN

**IPC Code:** Int Cl.[24]: A61K 36/00

Polycystic ovarian syndrome occurs in women of any reproductive age and is characterised by endocrine dysfunction leading to abnormal glucose metabolism due to insulin resistance as well as hyperandrogenism[1]. The ovarian functional abnormalities due to hyperandrogenism lead to the dysregulation of hypothalamic–pituitary–ovarian axis[2]. The action of insulin for the production of androgen in the ovaries is mediated through the binding to insulin-like growth factor (IGF-1) receptors present in the theca and stromal cells[3]. Abnormalities in the insulin-like growth factor-I (IGF-I), signalling system due to insulin resistance and compensatory hyperinsulinemia are considered as an important molecular pathophysiology associated with PCOS condition[4]. The insulin being a peptide hormone binds to its receptor and activates two types of signalling cascade. Primarily, it controls the metabolic pathway through phosphatidylinositol 3 kinase/protein kinase B (PI3K/PKB) pathway and in addition, it regulates the growth through mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway which mainly occurs in the ovary[5].

The insulin binds to its receptor which belongs to the tyrosine kinase family of proteins present on the cell surface thereby initiating the biochemical function. Several ligands like epidermal growth factor, fibroblast growth factor and insulin-like growth factor I (IGF-I) have the ability to bind and activate insulin receptor to phosphorylate the specific tyrosine residues of the corresponding intracellular substrate to activate the signal transduction. This signal transduction is mediated through the intracellular substrate namely insulin-receptor substrate (IRS)-1 and IRS-2 which plays important role in the activation PI3K and MAPK pathways[6]. The intraovarian prominent role of IGF system in regulating the folliculogenesis has been proven by

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many researchers and according to Adashi[7], IGF-1 gene expression and action takes place in the ovary especially in the granulosa cells where they take part in cell proliferation, steroidogenesis and oocyte growth[8]. Moreover, increased circulating levels of insulin and IGF-I synergizes with luteinising hormone (LH) to produce excess androgen in the theca cells of the ovary.

PTEN (Phosphatase and tensin homolog) a lipid phosphatase along with PI3K/Akt pathway regulates the proliferation and differentiation of granulosa cells. Jie-Xiu Ouyang *et al.*[9] in their studies have shown the increased PTEN gene and protein expression in PCOS rats. There are experimental evidences to suggest the close relationship between insulin signaling pathway and PTEN expression in causing altered follicular growth in PCOS patients[10]. From the above facts, it is clear that lowering the insulin resistance as well as downregulating the insulin signalling pathway including the IGF family proteins may be considered a better therapeutic approach in the management of PCOS in women. Currently, insulin sensitizers are prescribed for PCOS patients as they reduce the hyperinsulinemia and androgen production in the ovaries and regulate the follicular growth[11]. However, these drugs have many side effects. Being multifaceted disease, PCOS requires a treatment with multiple therapeutic approaches. At present, there is a paradigm shift observed all over the world towards phyto therapy, in the form of plant and plant products since they harbour many phytochemicals required to treat diseases like PCOS. *Caesalpinia bonducella* is a plant that is generally known as "Bonduc nut" and is a member of the Caesalpiniaceae family. It is found across India, particularly in tropical regions of the world. According to Pandey *et al.*[12] there are numerous folkloric medical benefits associated with the seeds, including antipyretic, anti-periodic, antidiabetic, anthelmintic, antidiuretic, anti-convulsant, anti-diarrheal, anti-amoebic, and anti-inflammatory capabilities. Menorrhagia and leucorrhoea are two gynaecological conditions for which leaf powder is reportedly utilised, according to Vidyasagar and Prashantkumar[13]. Despite having a number of medical properties, the seed has not been clinically proven to be effective in treating PCOS. In this study, we made an attempt to investigate the effect of administration of ethanolic seed extract of *Caesalpinia bonducella* on the down regulation of genes and proteins involved in the insulin signalling pathway in mifepristone-induced PCOS rats as this will correct the dysregulated hormonal pathway to regain the normal ovarian physiology in PCOS condition.

## Materials and Methods

### Collection and preparation of Ethanolic Seed Extract of *Caesalpinia bonducella* (ESECB)

The dried seeds of *Caesalpinia bonducella* were obtained from local market, Chennai, India and were authenticated by Professor, Dr. Sankaranarayanan, Department of Medicinal Botany and Pharmacognosy, Government Siddha Medical College, Chennai, India and for future reference a voucher specimen was deposited at the department. *Caesalpinia bonducella* seeds were shadow dried and made into powder for the extraction process. ESECB was prepared by suspending the powder in 95% ethanol for 3 days at room temperature (cold maceration process). Then the extract was filtrated and concentrated using a rotary evaporator and sample yield was calculated which was about 1.25% w/w and ESECB was used for analysis.

### Animals

The female albino adult rats of Wistar strain were used in the present study. The inbred animals were obtained from the C. L Baid Mehta College of pharmacy animal house Chennai, India. The animals were maintained in a natural day–night cycle of 12±1 h at a well-ventilated room in the polypropylene cages. The animals were fed with a balanced diet in the form of pellets for rodents and pure water *ad libitum*. To make them acclimatize to the laboratory condition they were sheltered in the laboratory prior to one week before the experimental procedure. The present experimental evaluation protocol was approved by the Animal Ethics Committee for the purpose as per CPCSEA (12/321/PO/Re/S/01/ CPCSEA dated 12/10/18).

### Acute toxicity studies

The acute toxicity studies were usually conducted for fixing the efficacy dose level. Kshirsagar *et al*.[14] has already conducted the acute toxicity studies for the ethanolic seed extracts of *Caesalpinia bonducella* as per the OECD guidelines 420, and reported that the seed extract does not show any toxicity up to the dose level of 2000 mg/kg. There was no mortality observed in the mice with normal haematological and biochemical parameters. To study the efficacy of the

extract, one-tenth of the LD50 dose and an additional higher dose of 200 mg/kg b.w. and 400 mg/kg b.w., respectively were selected.

**Mifepristone-induced PCOS in female rats**

PCOS was induced in the female rats according to the method of Sanchez-Criado *et al.*[15]. Briefly Wistar female rats with four day estrous cycle were injected with Mifepristone RU486 subcutaneously (4 mg RU486/0.2 mL oil) for eight consecutive days and their vaginal smear was prepared to check the irregularity in the estrous cycle. As soon as a persistent vaginal cornification (PVC) stage is attained, which is an indicator of follicular cysts in the ovaries the animals were grouped further for the efficacy studies.

**Animal grouping for treatment protocol**

The animals were divided into five groups with each group containing six animals and received the treatment protocol as follows.

Group I - Served as control and received only the vehicle (2% CMC suspension)

Group II – Mifepristone-induced PCOS rats serves as disease control

Group III – PCOS diseased rats received ESECB 200 mg/kg .b.w. p. o. for 28 days

Group IV - PCOS diseased rats received ESECB 400 mg/kg p.o. b.w. for 28 days

Group V - Served as standard and treated with Metformin 20 mg/kg p.o. for 28 days

At the end of the experimental period the fasted animals were anesthetised and sacrificed. The ovaries were dissected out, washed in ice cold saline, blotted to remove the connective tissue or fat. They were stored in the Tris HCl buffer [0.1M pH 7.4] for gene expression studies.

**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 10 mL volume of reaction mixture containing 1 mg 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 pattern of IGF-1, IGF-2 and PTEN genes under study was performed by referring to NCBI/primer-BLAST tool software (Table 1). The thermo cycling 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 60s 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 software. The variation in the fold change gene expression levels of target genes were calculated with normalization to β-actin values using the ΔΔCt comparative cycle threshold method. The experimental procedure was conducted in triplicates and all data regarding the expression pattern of the genes were analysed using Light Cycler 96 SW 1.1 Software.

**GC-MS analysis of ethanolic seed extract of *Caesalpinia bonducella* (ESECB)**

The GC-MS analysis of ethanolic seed extract of *Caesalpinia bonducella* (ESECB) was performed to identify the phyto constituents present in the extract. It was performed in the Agilent technologies 6890 N JEOL GC Mate II GC-MS model with HP-5 column (30 m × 0.25 mm i.d with 0.25 μm film thickness). Briefly the samples were injected into the column by following the chromatographic conditions, the flow rate of 1mL/min with Helium as a carrier gas maintained at the temperature of 200°C and programmed as 50-250°C at a rate of 10°C/min injection mode. As far as MS conditions are concerned an ionization voltage of 70 eV; ion with a sourced temperature of 250°C with the interface temperature of 250°C and mass range of 50-600 mass units.

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 |

**Molecular docking analysis of IRS-1 and IRS-2 Proteins**

To identify a suitable antagonistic ligand for the Insulin receptor substrate proteins (IRS1 and IRS2) from the phyto constituents of *Caesalpinia bonducella* seeds through patch dock analysis, the *in silico* molecular docking analysis were conducted. The 3D structures of the proteins IRS1 (PDB ID: 1K3A) and IRS1 (PDB ID:3BU3) were downloaded from protein data bank (http://www.rcsb. org/pdb/). Nearly 37 phyto components were identified in ethanolic seed extract of *Caesalpinia bonducella* through GC-MS analysis in the present study and the two dimensional structures were obtained for 20 ligands from PUB chemsite based on the docking score values and using corina 3D converter their three dimensional structures were retrieved. To study the protein ligand interaction, "patch dock" server was employed and the docking score was obtained. A critical analysis of the docking score was performed and the ligand exhibiting an appropriate docking score in terms of binding energy were chosen and subjected to further study in terms of their amino acid interaction with the particular proteins to manifesting their antagonistic efficiency by using the "LIGPLOT" analysis.

**Statistical analysis**

All the above mentioned data were expressed as mean ± SEM and one way ANOVA was analysed followed by Dunnets "t" test using Graph pad prism 5. 0 software. The $p<0.05$ was considered to be statistically significant.

## Results

**Semi quantitative real time PCR analysis of expression of IGF-1, IGF-2 and PTEN genes**

Figure 1 depicts the effect of the administration of ESECB extracts on the expression patterns of the IGF-1 gene. The fold change of the mRNA expression of IGF-1 gene was found to be 1.44 fold in mifepristone treated PCOS induced group II rat ovaries. A decrease of 0.44 fold and 0.14 fold was observed in ESECB treated group III and group IV treated rat ovaries with a dosage of 200 mg/kg b.w and 400 mg/kg b.w, respectively. In the present investigation, IGF-1gene expression of control rats was found to be 0.31 fold.

The expression pattern of IGF-2 in the ovarian tissues of the rats treated with the ESECB extracts were shown in the Figure 2. A similar kind of

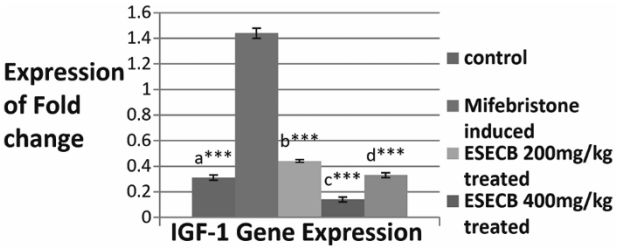


Fig. 1 — The expression pattern of the IGF-1 gene in different experimental groups

Values are mean ± SEM of 3 animals in each group .Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test. Comparison between Group I vs Group II, b-Group II vs Group III, c-Group II vs Group IV and d-Group II vs Group V. p-values- *p<0.05, * *p<0.01, * * *p<0.001, NS-Not Significant.

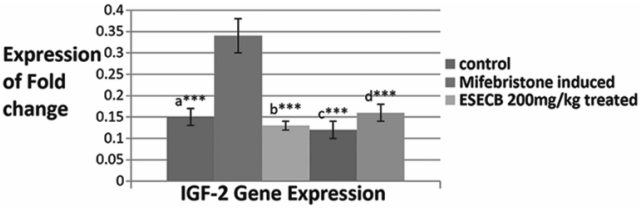


Fig. 2 — The expression pattern of the IGF-2 gene in different experimental groups

Values are mean ± SEM of 3 animals in each group. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test. Comparison between Group I vs Group II, b-Group II vs Group III, c-Group II vs Group IV and d-Group II vs Group V. p-values- *p<0.05, * *p<0.01,* * *p<0.001, NS-Not Significant

expression pattern was observed for the IGF-2 genes in which an increased expression of 0.34 fold is seen for the mifepristone induced PCOS affected group II rats. The 200 mg/kg b.w and 400 mg/kg b.w ESECB drug treatment significantly ($p<0.001$) reduced the level expression to IGF-2 in to 0.13, and 0.12 fold respectively indicates the potential ability of extract in rectifying the PCOS pathophysiology. In align with the IGF-1 and IGF-2 genes, the expression pattern of PTEN gene is also significantly reduced in the ESECB drug treated group III and group IV rats when comparable to the mifepristone induced group II rats (Fig. 3). The PTEN gene expression was 0.36 fold in group II PCOS affected rats which was reduced to 0.20 and 0.06 fold respectively in the group III and group IV rats, revealing the curative nature of the ESECB extract. The reduction in the expression pattern of PTEN gene is more pronounced in the group IV rats which received the extract at the concentration 400 mg/kg b.w when comparable to the group III rats which received 200 mg/kg b.w.

***GC-MS analysis of Ethanolic Seed Extract of Caesalpinia bonducella* (*ESECB*)**

In our present study 37 different phytochemicals were identified in the GC-MS analysis of ethanolic seed extract of *Caesalpinia bonducella* (ESECB). The list of phyto constituents present in the ESECB along with the retention time, molecular formula, molecular weight is shown in the Table 2 and Figure 4.

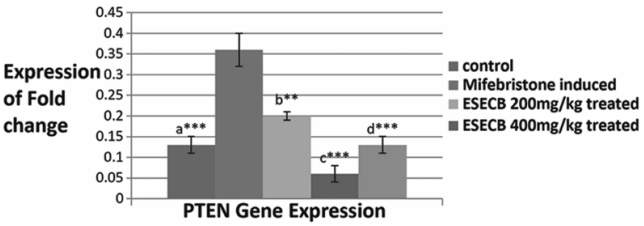


Fig. 3 — The expression pattern of the PTEN gene in different experimental groups

Values are mean ± SEM of 3 animals in each group. Statistical significant test for comparison was done by ANOVA followed by Dunnet's 't" test. Comparison between Group I vs Group II, b-Group II vs Group III, c-Group II vs Group IV and d-Group II vs Group V. p-values- *p<0.05, * *p<0.01,* * *p<0.001, NS-Not Significant

**Molecular docking analysis of IRS-1 and IRS-2 proteins**

The *in silico* docking analysis was performed by molecular docking using patch dock sever to find a suitable antagonistic ligand for the IRS1 and IRS2 proteins. Out the 37 compounds obtained in the GC-MS analysis 20 ligands were selected and docked with IRS1 and IRS2 proteins to obtain the docking score values (Table 3). Based on the docking scores the ligands dioxan ,propyl acetate, acetic anhydride and methyl butanic acid showed a best docking score for the IRS-1 protein and the ligands methyl isopropyl carbonate, glycerine, diethanolamine and

Table 2 — GC-MS chromatogram of ethanolic seed extract of *Caesalpinia bonducella* (ESECB)

| S. No. | RT | Name of the Compound | Molecular formula | Molecular weight | Peak area % |
|---|---|---|---|---|---|
| 1 | 6.36 | Hydroxylamine, O-methyl- | $CH_5NO$ | 47 | 30.26 |
| 2 | 6.41 | Ethyl fluoroformate | $C_3H_5FO_2$ | 92 | 8.38 |
| 3 | 6.45 | Difluorodiazene | $F_2N_2$ | 66 | 11.34 |
| 4 | 6.69 | 1,3-Dioxan-5-ol | $C_4H_8O_3$ | 104 | 37.20 |
| 5 | 7.54 | Dimethyl-t-butylphosphonite | $C_6H_{15}O_2P$ | 150 | 0.27 |
| 6 | 9.93 | Oxazolidine | $C_3H_7NO$ | 73 | 0.15 |
| 7 | 13.89 | Propane, 1,1-dimethoxy | $C_5H_{12}O_2$ | 104 | 0.10 |
| 8 | 16.63 | n-Propyl acetate | $C_5H_{10}O_2$ | 102 | 0.08 |
| 9 | 19.02 | Bioxirane | $C_4H_6O_2$ | 86 | 0.48 |
| 10 | 19.58 | Acetic anhydride | $C_4H_6O_3$ | 102 | 0.19 |
| 11 | 19.69 | DL-2,3-Butanediol | $C_4H_{10}O_2$ | 90 | 0.14 |
| 12 | 20.02 | Hydrogen azide | $HN_3$ | 43 | 0.21 |
| 13 | 20.37 | Glycolaldehyde dimethyl acetal | $C_4H_{10}O_3$ | 106 | 0.15 |
| 14 | 22.53 | 3-Methyl-3-butenoic acid | $C_5H_8O_2$ | 100 | 0.90 |
| 15 | 22.73 | :Methanecarbothiolic acid | $C_2H_4OS$ | 76 | 0.13 |
| 16 | 27.97 | Methyl isopropylcarbamate | $C_5H_{11}NO_2$ | 117 | 0.09 |
| 17 | 30.02 | Glycerin | $C_3H_8O_3$ | 92 | 0.11 |
| 18 | 30.93 | Diethanolamine | $C_4H_{11}NO_2$ | 105 | 2.25 |
| 19 | 32.55 | 2-Hydroxy-gamma-butyrolactone | $C_4H_6O_3$ | 102 | 0.08 |
| 20 | 33.33 | 1,2:5,6-Dianhydrogalactito | $C_6H_{10}O_4$ | 146 | 0.52 |
| 21 | 34.23 | 2,5-Furandione, dihydro-3-methyl | $C_5H_6O_3$ | 114 | 0.19 |
| 22 | 35.80 | Methyl 2-furoate | $C_6H_6O_3$ | 126 | 0.29 |
| 23 | 36.42 | 2-Azaquinuclidone-3 | $C_6H_{10}N_2O$ | 126 | 0.55 |
| 24 | 36.69 | Benzene, (2-methylpropyl)- | $C_{10}H_{14}$ | 134 | 0.09 |
| 25 | 36.92 | Furan | $C_4H_4O$ | 68 | 0.13 |
| 26 | 37.51 | Diethanolamine | $C_4H_{11}NO_2$ | 105 | 0.08 |
| 27 | 37.73 | 1-Trifluoroacetoxy-2-Methylpentane | $C_8H_{13}F_3O_2$ | 198 | 0.03 |
| 28 | 37.83 | 2,2-Dimethylpropanoic anhydride | $CHOH_{18}O_3$ | 186 | 0.11 |
| 29 | 38.08 | dl-Glyceraldehyde | $C_3H_6O_3$ | 90 | 0.55 |
| 30 | 39.92 | 2(3H)-Furanone, dihydro-4-hydroxy | $C_4H_6O_3$ | 102 | 2.24 |
| 31 | 40.11 | Ethanethioamide | $C_2H_5NS$ | 75 | 0.32 |
| 32 | 40.27 | Benzaldehyde, 4-methyl | $C_8H_8O$ | 120 | 0.34 |
| 33 | 40.35 | 4-Hexen-3-one, 4,5-dimethyl | $C_8H_{14}O$ | 126 | 0.07 |
| 34 | 40.97 | 1,2-Ethanediol, 1-(2-furanyl) | $C_6H_8O_3$ | 128 | 0.27 |
| 35 | 41.12 | Furancarboxaldehyde, 5-(hydroxymethyl) | $C_6H_6O_3$ | 126 | 0.26 |
| 36 | 41.21 | (S)-(+)-2',3'-Dideoxyribonolactone | $C_5H_8O_3$ | 116 | 0.89 |
| 37 | 41.39 | 1-Pentene, 5-methoxy | $C_6H_{12}O$ | 100 | 0.54 |

Table 3 — Docking score values of ligands with IRS-1 and IRS -2 proteins

| S. No. | Name of the Compound | IRS-1 | IRS-2 |
|---|---|---|---|
| 1 | Hydroxylamine, O-methyl- | -6.11 | -3.51 |
| 2 | 1,3-Dioxan-5-ol | -2.81 | -3.50 |
| 3 | Dimethyl-t-butylphosphonite | -5.09 | -4.07 |
| 4 | n-Propyl acetate | -2.65 | -4.92 |
| 5 | Acetic anhydride | -1.73 | -3.82 |
| 6 | Hydrogen azide | -3.72 | -3.78 |
| 7 | 3-Methyl-3-butenoic acid | -2.13 | -3.90 |
| 8 | Methyl isopropylcarbamate | -3.24 | -2.42 |
| 9 | Glycerin | -4.20 | -1.89 |
| 10 | Diethanolamine | -3.28 | -2.07 |
| 11 | 2-Hydroxy-gamma-butyrolactone | -3.54 | -3.03 |
| 12 | Benzene, (2-methylpropyl)- | -4.45 | -4.78 |
| 13 | Furan | -6.02 | -3.90 |
| 14 | 2,2-Dimethylpropanoic anhydride | -5.89 | -1.78 |
| 15 | dl-Glyceraldehyde | -4.13 | -4.23 |
| 16 | Ethanethioamide | -3.67 | -3.89 |
| 17 | Benzaldehyde, 4-methyl- | -5.34 | -4.13 |
| 18 | 4-Hexen-3-one, 4,5-dimethyl- | -4.17 | -5.09 |
| 19 | (S)-(+)-2',3'-Dideoxyribonolactone | -5.12 | -6.02 |
| 20 | 1-Pentene, 5-methoxy- | -6.48 | -5.67 |

2,2, dimethyl propanoic anhydride exhibited better docking score for IRS-1 protein.

The amino acid interaction of the above eight ligands with the proteins was studied to analyse the mode of binding and exhibit the existence of their antagonistic nature through the "LIGPLOT" analysis. The mode of binding of the chosen phyto constituent ligand to the receptor protein is evaluated by hydrophilic and hydrophobic nature of amino acid interaction with their numbers. The results obtained through the "LIGPLOT" analysis are explained in Table 4 and Figure 5 & Figure 6. Based on the results obtained through *Insilico* molecular docking it is observed that almost all the phyto ligands selected for the present study binds to the amino acid proline 156 and cysteine 69 in both the IRS-1 and IRS-2 proteins to exhibiting their antagonistic nature.

## Discussion

In this investigation, we have illustrated the ameliorative effects of the ethanolic extract of

Table 4 — Interaction with the proteins IRS-1 and IRS-2 with selected phytochemicals of ESECB

| S. No | Compound | H bonding sites | Hydrophobic contact sites |
|---|---|---|---|
| | | **Amino acid binding site IRS-1 protein** | |
| 1 | Dioxan | 2<br>Gly157, Ala159 | 10<br>Phe 70, Asn71, Ile72, Leu111, His114, Pro156, Gly157, Pro158, Ala 159, Phe 160 |
| 2 | Propyl acetate | 1<br>Cys 69 | 8<br>Leu 66, Glu 67, Cys 69, Phe 70, HIS 114, Val 154, Pro 158, Pro 156 |
| 3 | Acetic anhydride | 2<br>Leu111, Pro 156 | 7<br>Leu 111, His 114, Arg 116, Ala 117, Pro 156,Gly 157, Pro 158 |
| 4 | MethylButenoic acid | 3<br>Cys69,His 114, Val 154 | 7<br>Cys 69, Phe 70, His 114, Asp 153, Val 154, Pro155, Pro 156 |
| | | **Amino acid binding site IRS-2 protein** | |
| 5 | Methyl isopropyl Carbonate | 1<br>Cys 69 | 6<br>Glu67, Cys 69, Phe 70, Val 154, Pro 155, Pro 156 |
| 6 | Glycerine | 2<br>Ala 117, Gly157 | 9<br>Phe 70, Asn 71, Leu 111, His 114, Ala 117, Pro 156, Gly 157, Pro 158, Phe 160 |
| 7 | Diethanolamine | 1<br>Gln 108 | 3<br>Tyr 107, Gln 108, Leu 111 |
| 8 | 2,2, Dimethyl propanoic anhydride | 1<br>Ala 117 | 5<br>His 114, Arg 116, Pro 156, Gly 157,Pro 158 |

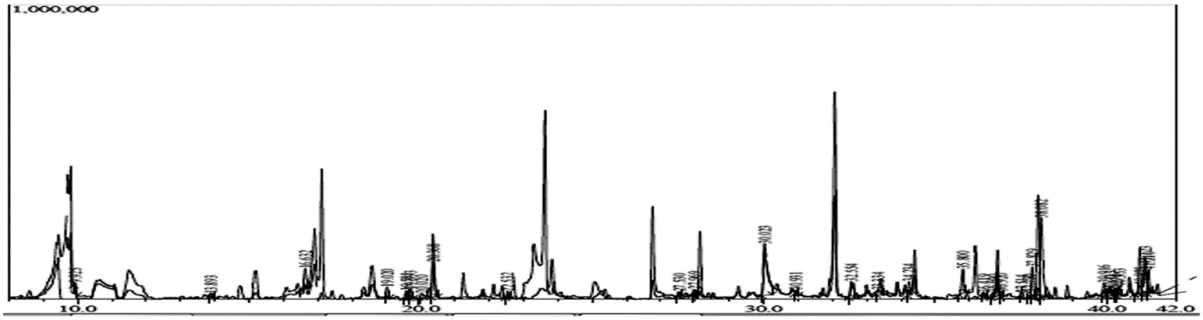


Fig. 4 — GC-MS Chromatogram of Ethanolic seed extract of *Caesalpinia bonducella* (ESECB)

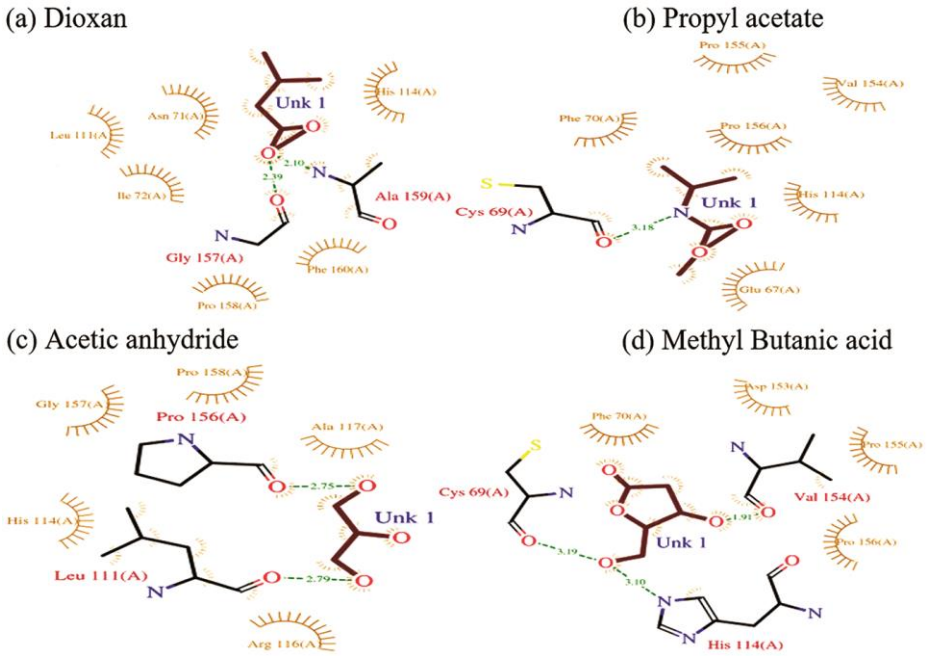


Fig. 5 — Amino acid interaction of IRS-1 protein with specific ligands

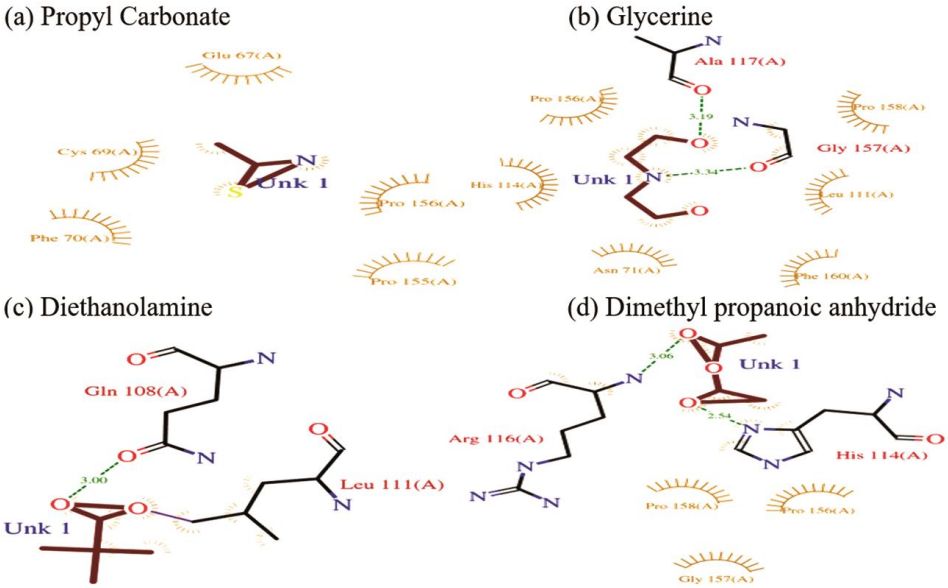


Fig. 6 — Amino acid interaction of IRS-2 protein with specific ligands

*Caesalpinia bonducella* for PCOS through the establishment of mifepristone induced PCOS rat models. The pathology of PCOS includes formation of many number of undeveloped follicles in the ovaries with abnormal androgen production. The state of hyperandrogenism is further triggered by insulin as well as insulin-like growth factors in the ovary. In PCOS women, as per the recent studies, there is strong correlation between insulin resistance, hyperinsulinemia and hyperandrogenism. Human follicle develoment, which is under the influence of ovarian steroids, is not only controlled by hypothalamic-pituitary-ovarian axis but also insulin-like growth factor (IGF) system which comprises of factors like IGF-I, IGF-II, IGF-binding proteins (IGFBPs)[16]. According to Le Roith[17] the IGF-1 is a 70 amino acids containing peptide hormone having more than 60% homology with IGF-II and exerts endocrine,

paracrine and autocrine activities. It also shows partial structural similarity with pro insulin. Because of these structural similarities with insulin, the IGF ligands can bind to insulin receptor with low affinity and vice versa.

The action of IGF family ligands and insulin is mediated through the transmembrane tyrosine kinase receptor IGF1R. These insulin like growth factor receptor (IGF1R) has the ability to bind IGF family ligands as well as insulin due to the structural resemblance observed in these protein hormones. The binding of either insulin or IGF-1 with IGF1R will activate the PI3K signalling pathway controlling the ovarian follicular maturation and steroidogenesis along with the LH from pituitary[18]. It is evident from several studies that increased levels of insulin and IGF-I is in synergy with the higher levels of luteinising hormone (LH) triggers the theca cells of ovary to produce excessive amount of androgens leading to hyperandrogenemia in the PCOS patients[19]. Zhong and Chen[20] also in their studies have shown that the increased concentrations of IGF-II in the follicular fluids of PCOS women. Like IGF-1 excessive amounts of IGF-II also increases the androgen production in the ovaries[21]. As a result, the therapeutic targeting in terms of lowering the IGF family of proteins, may be beneficial in correcting the hyperandrogenism as well as the other complications observed in PCOS. In the present investigation the ethanolic extract of *Caesalpinia bonducella* downregulated the IGF-1 and IGF-2 gene expression considerably which was found to be very high in the mifepristone induced PCOS rats. This lowering potential is attributed to the presence of several phyto components in the ESECB extract which has the capability to modulate the gene expression patterns.

The increase in the IGF-1 and IGF-2 proteins in the PCOS induced rats may be due to the decreased concentrations of IGF-binding protein I (IGFBP-I) produced in liver cells, ovarian granulosa cells and endometrium in response to follicular stimulating hormone[22]. In the presence of hyperinsulinemia and low FSH, the synthesis of IGFBP-1 will be impaired leading to the increased levels of free IGF proteins which automatically stimulate the synthesis of androgens in the ovary[23]. The phyto constituents present in the *Caesalpinia bonducella* downregulates the IGF-1 and IGF-2 gene expression there by lowers the circulating concentrations of free IGF proteins and making the free IGFs to combine with whatever available IGF-binding protein I (IGFBP-I) to reduce the androgen production in the ovarian cells.

Phosphatase and tensin homolog (PTEN) is originally considered as a tumour suppressor gene which is found to be inactivated in several types of cancer such as brain, prostate and endometrial cancer. Recently, in diseases such as chronic obstructive pulmonary disorder, rheumatoid arthritis and pulmonary fibrosis also the altered expression of this gene is reported by Anderson & Bozinovski (2003)[24]. Moreover, Lackey *et al*.[25] have shown a strong relationship between insulin signalling and PTEN in other species and human cancer cell lines and during follicular phase, the expression of PTEN will be increased[26]. In brief the levels of intracellular PIP3 which controls signalling pathway involved in the folliculogenesis of human granulosa cells is mainly decided by PI3K as well as PTEN both are dysregulated in PCOS condition due to hyperinsulinemia and insulin resistance. In our study, the ESECB extract treatment decreased the gene expression pattern of PTEN in mifepristone induced PCOS rats thereby regulating the impaired folliculogenesis in the human granulosa cells.

Several researches have confirmed the role of insulin in regulating the steroidogenesis along with gonadotrophins in the ovarian tissues[27]. Hyperinsulinemia as well as selective insulin resistance in the PCOS ovary leads to the enhanced activity of mitogen-activated protein kinase (MAPK) pathway, specific to insulin target cells of the ovary while impairing the metabolic actions of insulin[28]. According to Makker *et al*.[29] increased androgen produced in the PCOS ovarian tissues also activate MAPK cascade. So the therapeutic agent for the treatment of PCOS is expected to act in a multifaceted manner in reducing the important complications such as hyperinsulinemia and hyperandrogenism as well as the signalling pathway triggered by them. Owing to the presence of phytoconstituents like furanoditerpenes, phytosterinin, β-sitosterol, flavonoids, bonducellin, aspartic acid, arginine, citrulline, and β-carotene as reported by Williamson[30], Murugesan *et al*.[31] reported that the oral administration of *Caesalpinia bonducella* ethanolic extract significantly reduced the levels of insulin and testosterone. In order analyse the effect of our plant extract in reducing the hyperinsulinemia and hyperandrogenism *in silico* docking studies were performed. The action of insulin is mediated through

the activation of insulin receptor substrate (IRS) IRS1and IRS2 after binding with its own receptor through tyrosine residues phosphorylation. Interaction of PI3K with phosphorylated tyrosine residues of IRS proteins leads to the synthesis of the secondary messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidyl inositol-4,5-diphosphate (PIP2). This leads to the activation of mitogen-activated protein kinase (MAPK) activated mitogenic pathway favouring the uncontrolled folliculogenesis as observed in PCOS ovary. So as a therapeutic intervention we wanted to select suitable antagonistic ligand to bind with the IRS1 and IRS2 proteins thereby preventing their interaction with PI3K enzyme. This will inhibit the MAPK kinase pathway by not providing the secondary messenger needed for their activation. In the present investigation through docking studies eight ligands namely dioxan, propyl acetate, acetic anhydride, methyl butenoic acid, methyl isopropyl carbonate, glycerine, diethanolamine and 2,2, dimethyl propanoic anhydride inhibited the IRS1 and IRS2 proteins by interacting with their aminoacid residues.

## Conclusion

It may be concluded that the ethanolic seed extract of *Caesalpinia bonducella* down regulates the expression pattern of genes such as IGF-1, IGF-2 and PTEN as well as phyto chemicals present in the extract act as an antagonistic ligands for the proteins IRS1 and IRS2. These genes and proteins constitute the insulin and insulin like growth factor family and over expression of these signalling molecules are the chief causes for the complications observed in the PCOS condition by deregulating PI3K/AKT pathway. Our ESECB plant extract owing to the presence and synergistic action of several phyto chemicals could inhibit the over expressed genes and proteins to regulate the deregulated signalling pathways and can be used in the treatment of PCOS condition.

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## Conflict of Interests

The authors declare that they do not have any conflict of interest.

## Author Contributions

RH designed the concept and plan of execution of the study. BM and PM conducted the experimental studies and prepared the manuscript. PD and KS performed the bioinformatics work and helped in the *in silico* studies. All authors helped in the preparation of the manuscript. RH and PM helped in correction and approved the final manuscrip**t.**

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