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NETWORK PHARMACOLOGY AND INTEGRATED MOLECULAR DOCKING REVEAL BIOACTIVE COMPONENTS AND POTENTIAL TARGETS OF *TRIFOLIUM PRATENSE* AGAINST PCOS.

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ABSTRACT

Aim: To study some phytoconstituents for the treatment of PCOS (polycystic ovarian syndrome) using Molecular docking, Network pharmacology, PPI, and Gene ontology approach. **Objective:** PCOS (Polycystic Ovary Syndrome) affects millions of women worldwide, causing hormonal imbalance and group of symptoms like hirsutism, obesity, irregular periods and infertility. There are limited drugs are available in market to overcome this symptom, and they have number of side effects. Herbal treatment is alternative way with potentially fewer side effects. So, we try to find new herbal treatment to overcome PCOS symptoms by using molecular docking, network pharmacology and analysis through PPI (Protein-Protein interaction) and GO (Gene Ontology) approach. These botanical remedies may help to regulate hormones, improve insulin sensitivity, and reduce inflammation, also give contribution to managing PCOS symptoms and try to improving overall quality of life. **Material and Method** We apply molecular docking criteria for screening the desired targets, this study was conducted on 2 different targets, which are estrogen and androgen protein, to find out the potential effect of 70 active phytoconstituents obtained from two plants such as Raktakanda and Pudina. From these criteria, we screened out 6 potential targets based on their affinities, who may be more effective against PCOS. Detailed information about these phytochemicals was gathered from the PubChem database, which provides valuable information regarding chemical structure and properties. To identify the potential targets and genes of respected phytoconstituents related to PCOS, we use bioinformatic tools such as Binding DB and Swiss

Target Prediction databases. these tools helped in the detection of genes that are expressed on stimulation of targeted by the selected phytoconstituents. Furthermore, the Kegg Mapper database is used to find the gene ids and list of pathways through which these genes get expressed. In the next step, we collect the targets and genes related to PCOS by using the Gene Card database and screen out common targets between PCOS and phytoconstituents.to find more effective information also compared with hyperandrogenic and hypoestrogenic targets and genes. Further performed protein-protein interaction using the STRING database to identify strong interactions between targets, this information helps to find out potential synergistic effects. Then to understand the functional implication of the identified target genes, Gene Ontology functional enrichment analysis was conducted with the help of the Metascape database. Finally, to visualize the interaction between compounds, targets, genes, pathways, and disease we use cytoscape 3.10.1. **Results:** The result shows that out of 6 phytoconstituents, a pratensein is express more genes and the targets are associated with PCOS potentially they are P11511(aromatase), P03372(estrogen receptor), Q97731(estrogen receptor beta) with expression of genes has:1588(CYP19A), has:2099(ESR1), has:2100(estrogen receptor beta.

KEYWORDS: Polycystic ovary syndrome (PCOS), Calycosin, Irilone, Pratensein, Pseudobaptigenin, molecular docking, network pharmacology.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a condition characterised by oligoovulation and polycystic ovarian morphology. It affects about 7% of women in their adult age and is costly to treat. It was first identified by Stein and Leventhal in the 1930s.[1, 2] It mainly causes symptoms through obesity, acne, and infertility and increases the risk of heart disease, and endocrine cancer as well. Hyperandrogenism which leads the acne, hirsutism, and alopecia is a hallmark of PCOS. Out of all about 75-90% of patients are increasing their androgen level.[3, 4] The disturbance in the FSH-LH ratio raises androgen synthesis. Aberrant steroidogenesis is partly caused by genetic factors, especially CYP genes. PCOS hypoestrogenism is caused by abnormal hypothalamus-pituitary signalling, which impacts oocyte development and estrogen production. The estrogen in both theca and granulosa cells maturing the follicles. PCOS alters the ovarian expression, so in that condition, ER alpha and ER beta play a crucial role.[5–7]

The red clover (*Trifolium pratense*) is relevant for treating PCOS symptoms because it contains the highest isoflavones concentration. Which plays an important role in the balancing estrogen level and plays a vital role against PCOS. Its constituents have antioxidant and anti-inflammatory qualities, which also improve insulin sensitivity. Red clover is also a useful natural treatment for PCOS because of its capacity to control the menstrual cycle and enhance fertility.[8–10]

Network pharmacology is gaining attention in Indian medicine for its ability to unravel the complexities of disease and their mechanism. By combining pharmacology and bioinformatics to create "disease-target gene-drug" networks, network pharmacology emphasizes the beneficial effects of phytochemicals. In silico theories that predict ligand-protein interactions to aid in lead optimization and drug development are supported by molecular docking.[11–13]

Red clover is valuable ingredient, mainly due to its isoflavone content. Howere, other group of compounds may influence the pleiotropic biological effects of raw material. It is used to reduce menopausal symptoms, but since there are many varieties of this plant that can be grown, the taste and muscle phytochemical content of the plant need to be compared. Also interesting is the difference between the leaves and flower of the plant. The aim of this study is to evaluate the characteristics of leaves and flower of six clover cultivars: Tenia, Atlantis, Milena, Magellan, Lemmon and Lucrum. Principal components analysis (PCA) determines the relationship between activity and active compound content. The result show that antioxidant activity and active compound content. The result show that antioxidant activity, inhibition of glucosidase, collagenase and lipase are independent of isoflavone content. Red clover flowers are higher in total polyphenol, while the leaves of almost all species are higher in isoflavones. The exception is the Lemon variety, which in tests is characterized by a high content of isoflavones and high activity.

a) Calycosin

Natural sources of Calycosin include red clover and Astragalus membranaceus. It is full of beneficial ingredients that plays an important role in reducing inflammation, inhibiting the growth of bacteria, and controlling diabetes. Researchers have discovered that Calycosin has a variety of interesting anti-cancer properties. It can instruct cancer cells to self-destruct, stop growing, and even block the formation of new blood vessels that would otherwise feed the tumor. It can also increase the effectiveness of standard cancer medications when taken with

them. Calycosin is quite easy for the body to utilize and safe. It can be found in naturally occurring plant sources, and researchers are working to improve how well our bodies absorb it.Even though research on Calycosin and cancer has been done, much more has to be discovered. Thus, researchers are eager to continue studying Calycosin in order to determine how it can aid in the battle against cancer and other diseases even further.

b) Irilone

Red clover, or Trifolium pratense L., is a well-liked herbal supplement used for women's health. Red clover isolate irilone has been shown to have progestogenic potentiation effect in the past. Some studies found that irilone increased PR protein levels in T47D breast cancer cells, which could be inhibited by estrogen receptor (ER) antagonists, indicating an ER dependent effect. Instead of increasing progesterone signalling through post-translational phosphorylation or by lowering progesterone receptor (PR) protein expression. Furthermore, in a cell line that contained the glucocorticoid receptor (GR) but lacked ER and PR, irilone boosted the luciferase activity from a hormone responsive element. In Ishikawa PR-B endometrial cancer cells, GR was knocked down using siRNA, which decreased irilone's capacity to boost progesterone signalling Irilone failed to cause proliferation of the uterine epithelium in a model of CD-1 mice with ovariectomies. Irilone's mode of action sheds light on PR interaction with other steroid hormone receptors, which is relevant to our comprehension of botanical remedies for women's health.

c) Pratensein

Pretensesin is a flavone compound and present in several plant sources, most notably in clover (Trifolium pratense). Pharmacognostically, it has a variety of therapeutic effects. Because of its capacity to scavenge free radicals and suppress inflammatory pathways, studies indicate that it may have potential as an antioxidant, anti-inflammatory, and anticancer drug. Because of its antioxidant properties, it helps lower oxidative stress, which benefits cardiovascular health and neuroprotection. Its potential benefits for ailments including inflammatory bowel disease and arthritis also stem from its anti-inflammatory qualities. By causing apoptosis and disrupting the growth of tumor cells, proteansein also demonstrates anti-cancer properties. Trifolium genus members, such as red clover, are substantial producers of pratensein. To fully realize its medicinal potential, more research is needed to understand its workings and maximize its therapeutic uses

d) Pseudobaptigenin

Pseudobaptigenin, a flavonoid compound, is found in various plant sources, notably in species like Crotalaria pallida, Crotalaria mucronate and clover. Pharmacognostically, it exhibits promising medicinal properties. Research indicates its potential as an anti-inflammatory, antioxidant, and anticancer agent.Pseudobaptigenin's anti-inflammatory properties are attributed to its ability to inhibit inflammatory mediators, offering potential in conditions such as rheumatoid arthritis and inflammatory bowel diseases. Additionally, its antioxidant activity contributes to cellular protection against oxidative stress, thus implicating its role in preventing chronic diseases. Furthermore, pseudobaptigenin shows anticancer effects by interfering with tumor cell proliferation and inducing apoptosis. The plant sources containing pseudobaptigenin hold promise for further pharmaceutical exploration, necessitating extensive research to unravel its mechanisms and therapeutic potential.

MATERIAL METHOD

Selection of plant and screening of phytoconstituents.

Based on the literature review, the plant was chosen for its potential to overcome PCOS symptoms, the same searching revealed the list of active phytoconstituents. Performing molecular docking, appropriate phytoconstituents were selected, focusing on key proteins including estrogen and androgen receptors. The aim was to inhibit the androgen and activation of estrogen receptors. We chose the phytoconstituents with the highest binding affinity based on the docking data.

Targets of Phytoconstituents

Using a methodical approach, the targets of the chosen phytoconstituents were determined. Firstly each compound was searched on PubChem database and canonical smiles notation was replicated. Next on Binding DB, the selected “Find my compound targets” from Special tools. After pasting the smiles into the relevant window and setting the similarity to 0.7, the search was started by clicking ‘GO’. On the upcoming window click on each “Hits” and collect final data like Uniproat ID, Uniproat protein, Uniproat gene. Provide the Uniproat ID on the Kegg Mapper database to find the respected gene ID and Pathway ID, with the prior set organism ‘homosepian’.

Targets of Disease

By using the Gene Card database find targets of diseases, after login place the disease names, and simply export an Excel sheet containing Uniproat ID, Gene ID, and other disease-related

information. In our study, 3 diseased conditions were analyzed: PCOS, Hyperandrogenism and Hypoestrogenism. The inclusion of these two additional conditions supports a comprehensive investigation.

Venn diagram construction

To find common targets from Phytoconstituent and Disease we constructed a Venn diagram from 'Interactive Venn'. On this site add group group-wise target list of phytoconstituents and diseases. Further 'Submit' and simply download.

Network construction:

Common target network: In this, we provide Uniproat IDs to Cytoscape(3.10.1) of all selected targets. the constructed network clears the outline of the study and also builds a connection between them.

Pathway-oriented network construction: On cytoscape provide information like Uniproat ID, Gene ID, Pathway ID, and constructed network for each selected phytoconstituent.

Disease-oriented network: The network of each phytoconstituent was constructed by providing Uniproat ID, Gene ID, Pathway ID, and common disease ID to Cytoscape.

PPI network construction and analysis: For PPI network construction, To STRING database provides a list of proteins collected from Binding DB and Submit. Appeared the network got exported in image and 'tsv' format. On Cytoscape open this 'tsv' file and apply Cytohubba by following the steps first select network then choose 'calculate' further 'Top 10' and 'Shortest path' and save the network with a colour scale.

Cluster analysis: A cluster analysis tool in Cytoscape called MCODE was utilized to examine the PPI network's sub-regions. Based on nodes that had comparable or equal goals, clusters were created to investigate deeper details within the PPI network. The settings for the analysis were as follows: degree cutoff =2, K-core =2, and node score cutoff = 0.2.

GO analysis

Provide a list of genes associated with selected phytoconstituents to the Shiny GO 0.8 database for GO analysis. Select Kegg pathway enrichment analysis, and set a p-value cutoff 0.05 also adjusting parameters to concentrate on the top 20 pathways based on statistical

significance. After that Shiny Go finds and prioritizes the pathways and displays the result along with statistical metrics. Download chat in a suitable form

RESULT

Common compound-target network

The four phytoconstituents in the network are irilone, pseudobaptigenin, calycosin, and pseudotensein, along with the corresponding targets that were retrieved from Binding DB. The interactions with biological systems are revealed by the investigation of the compound-target network. With 49 nodes representing the phytoconstituents and their targets, the network's 111 edges show the connections between them.Subsequent examination reveals that the network has a characteristic path length of 2.733 and a diameter of 4, indicating effective interactions between the targets and phytoconstituents. The network density, which measures how crowded the network is, is 0.094. The results of this research indicate that P03372, Q92731, and P11511 are shared targets. [Fig.1] This suggests that our drugs work in concert to activate these specific targets, which include the estrogen, estrogen beta and aromatase receptor respectively.

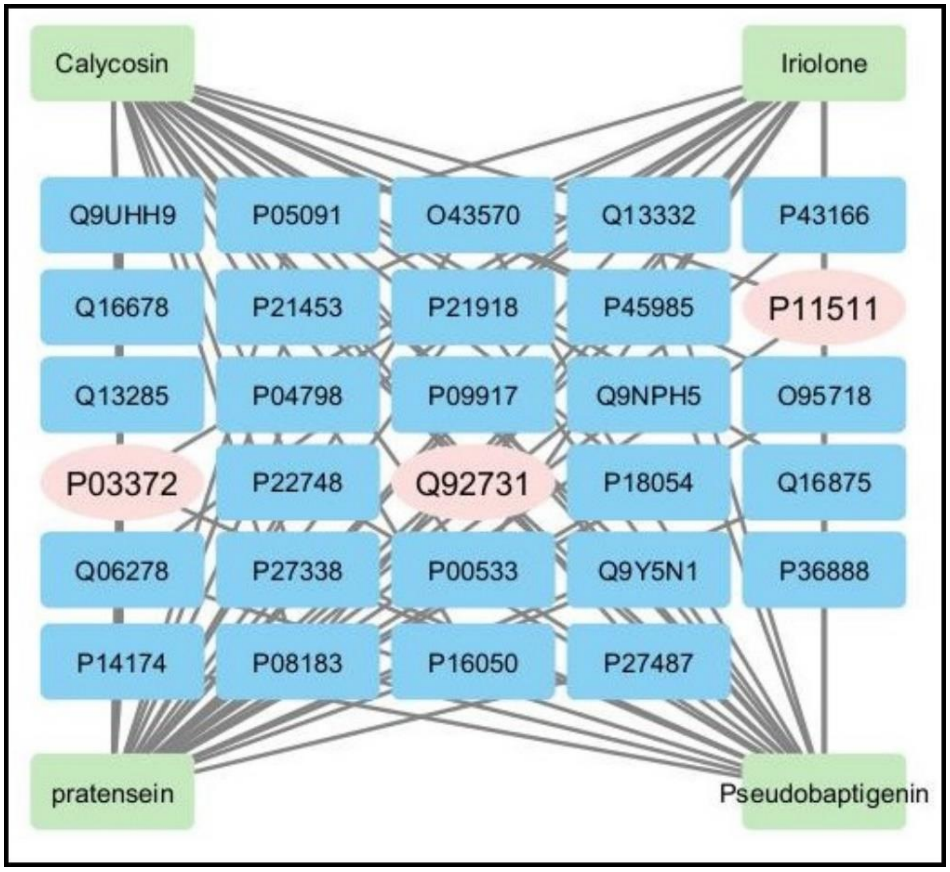

Fig. 1: Common compound target network.

Pathway oriented network

We analyzed networks one by one, The calycosin shows interaction with 24 body proteins and express further 24 genes through 116 pathways; the topological analysis as network density 0.008 indicate crowdiness, characteristics path length 1.653 indicate efficient interaction, and network diameter 3. The network visual analysis shows that the genes hsa:1956, hsa:5416, hsa:217, hsa:4129, hsa:316, hsa:2099, hsa:2100, hsa:246, hsa:239, hsa:50507 are more expressed. In case of irilone it activates 18 proteins further express 18 genes through 102 pathways. Analysis shows network diameter 3, characteristics path length 2.167 and network density 0.010; also it expresses mainly hsa:1956, hsa:6416, hsa:217, hsa:1543, hsa:316, hsa:2099, hsa:2322, hsa:2100, hsa:5209, hsa:1816. The pratensein shows interaction with 29 body proteins and express the 29 genes through 121 pathways. The network consists network diameter 3, characteristic path length 1.699, and network density 0.008; The visual analysis shows the more expressed genes are hsa:1956, hsa:6416, hsa:217, hsa4:129, hsa:1543, hsa:2099, hsa:316, hsa:1545, hsa:2100, hsa:246. After investigation of Pseudobaptigenin network it was found that, it activate 21 protein and express further 21 genes through 79 pathways; topologically network density 0.009, network diameter 3, characteristic path length 1.849; and expresses mainly hsa:1956, hsa:6416, hsa:217, hsa:4129, hsa:2099, hsa:316, hsa:240, has:2100, hsa:5243, and hsa:1901. Overview of this realise that the genes hsa:2099 and gene hsa:2100 are commonly expressed by compounds, namely ESR1 and ESR2. [Fig.2]

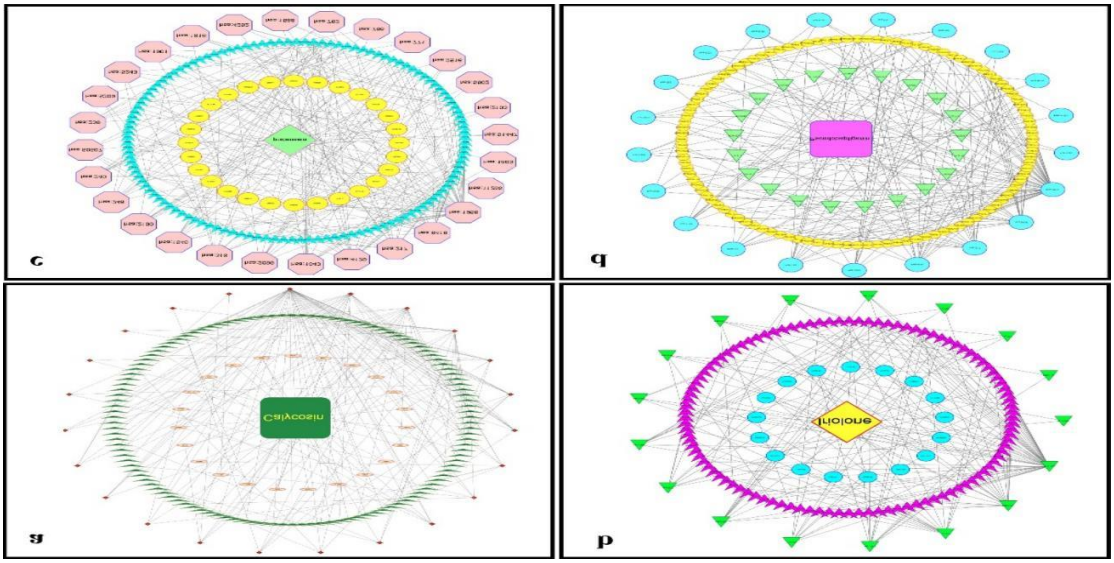

Fig. 2: Pathway oriented network constructed between 'Phytoconstituent-targets-genes-pathways'. a) Calycosin network. b) Irilone network. c) Pratensein network. d) Pseudobaptigenin network.

Disease oriented network

Network results that Calycosin act on targets Q92731, P11511, Q13285, P03372 and expresses genes has:2100, has:1588, has:2516 and has:2099 respectively; through pathways like hsa04915, hsa05224, hsa05200, hsa04917, hsa01522, hsa05207, hsa0429, hsa04913, hsa00140, hsa01100, hsa04927, hsa04934, hsa04919, hsa04961, hsa05205; and cure the PCOS symptoms. Network results that Irilone act on targets Q92731, P11511, P03372 and expresses genes has:2100, has:1588, has:2516 and has:2099 respectively; through pathways like hsa04915, hsa05224, hsa05200, hsa04917, hsa01522, hsa0429, hsa04919, hsa04961, hsa05205; and cure the PCOS symptoms. Network results that Pratensein act on targets Q92731, P11511, Q13285, P03372 and expresses genes has:2100, has:1588, has:2516 and has:2099 respectively; through pathways like hsa04915, hsa05224, hsa05200, hsa04917, hsa01522, hsa05207, hsa0429, hsa04913, hsa00140, hsa01100, hsa04927, hsa04934, hsa04919, hsa04961, hsa05205; and cure the PCOS symptoms. Network results that Pseudobaptigenin act on targets Q92731, P11511, P03372 and expresses genes has:2100, has:1588, has:2516 and has:2099 respectively; through pathways like hsa04915, hsa05224, hsa05200, hsa04917, hsa01522, hsa0429, hsa04919, hsa04961, hsa05205; and cure the PCOS symptoms. Overview of this study we obtained that there are 3 targets Q92731, P11511, and P03372 are common in all compounds which means all of our compounds act on estrogen beta receptor, estrogen receptor, and aromatase and express key genes like ESR2, CYP19A1, ESR1 through respected pathways. [Fig.3]

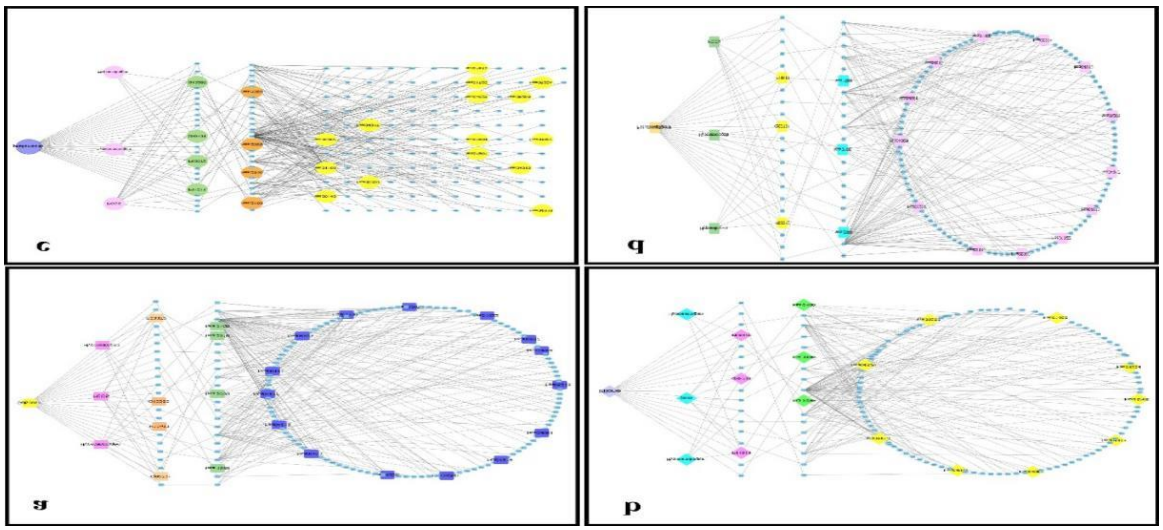

Fig. 3: Disease Oriented Network constructed between 'Bioactive compound-disease-targets-genes-pathways'. a) Calycosin network. b) Irilone network. c) Pratensein network. d) Pseudobaptigenin network.

Protein-protein interaction network and analysis

The PPI network gives an idea about how hub proteins interact with each other. In the case calycosin network, it consists of 20 nodes connected with different colored lines. This indicates the strength of interaction more lines represent stronger interaction between them. Further cytohubba analysis of this network screens the top 10 genes that show stronger interaction, and the result found that the genes are EGFR, ESR1, ESR2, CYP19A1, MOAB, ESRRB, CA4, ALDH2, AOX1, ABCB1. On comparing the disease-oriented network results with cytohubba results it was found that the ESR1, ESR2 and CYP19A1 are common. This suggests that these three targets are more potent targets of Calycosin. In the case irilone network, it consists of 16 nodes connected with different colored lines. This indicates the strength of interaction more lines represent stronger interaction between them. Further cytohubba analysis of this network screens the top 10 genes that show stronger interaction, and the result found that the genes are EGFR, ESR1, ESR2, CYP19A1, MOAB, ESRRB, CA4, ALDH2, AOX1, ABCB1. On comparing the disease-oriented network results with cytohubba results it was found that the ESR1, ESR2, and CYP19A1 are common. This suggests that these three targets are more potent targets of Irilone. In the case Pratensein network, it consists of 24 nodes connected with different colored lines. This indicates the strength of interaction more lines represent stronger interaction between them. Further cytohubba analysis of this network screens the top 10 genes that show stronger interaction, and the result found that the genes are EGFR, CYP1A1, ESR1, ESR2, CYP19A1, CYP1B1, ABCB1, MAOB, ALDH2, ESRRB. On comparing the disease-oriented network results with cytohubba results it was found that the ESR1, ESR2, and CYP19A1 are common. This suggests that these three targets are more potent targets of Pratensein. In the case Pseudobaptigenin network, it consists of 19 nodes connected with different colored lines. This indicates the strength of interaction more lines represent stronger interaction between them. Further cytohubba analysis of this network screens the top 10 genes that show stronger interaction, and the result found that the genes are ESR1, EGFR, ESR2, MAOB, ESRRB, CYP19A1, CA12, CA4, ALDH2, AOX1. On comparing the disease-oriented network results with cytohubba results it was found that the ESR1, ESR2, and CYP19A1 are common. This suggests that these three targets are more potent targets of Pseudobaptigenin.[Fig.4]

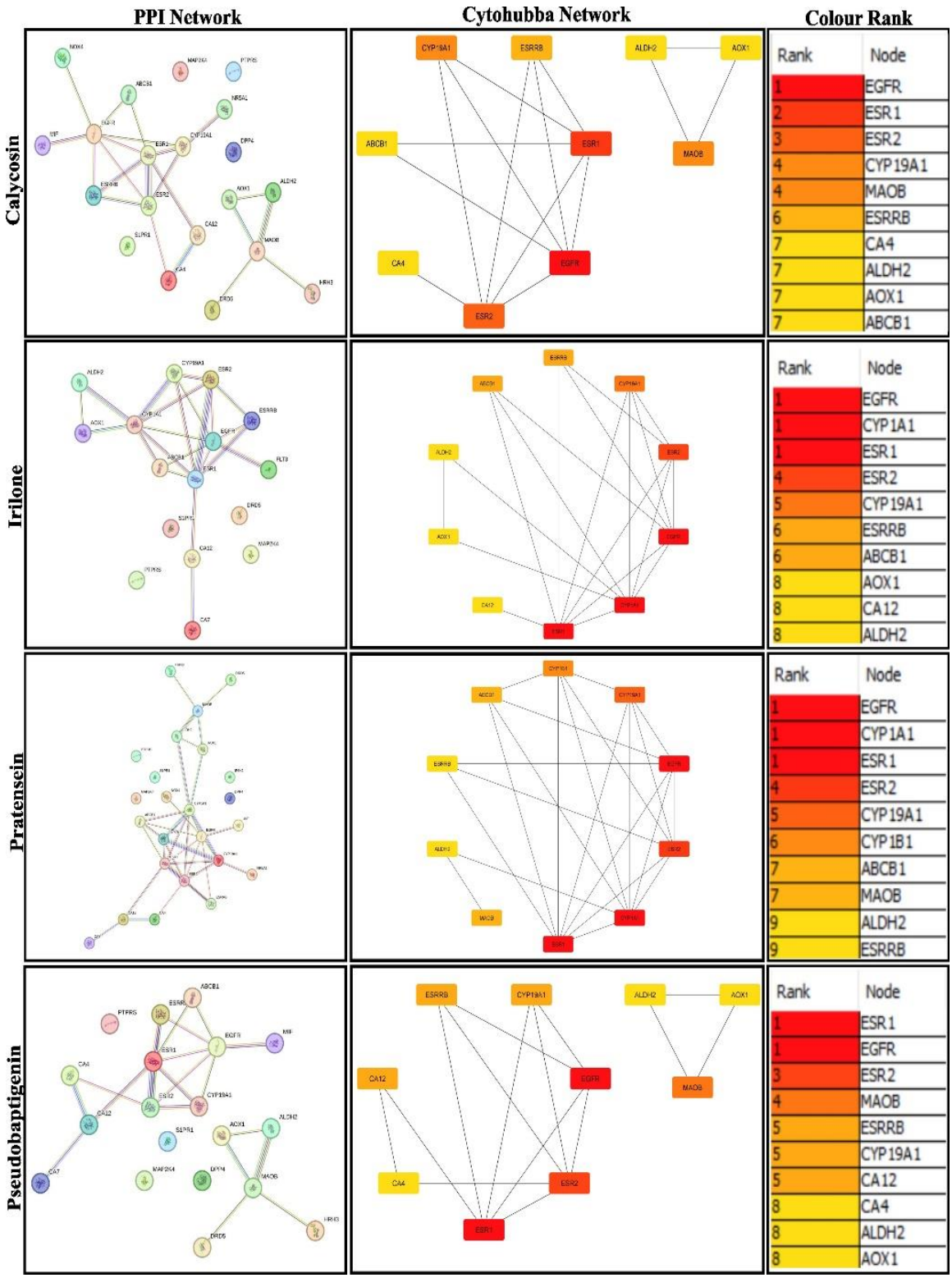

Fig. 4: Protein-Protein interaction network with cytohubba analysis and their respective colour ranking, orange to red increases the interaction intensity.

Cluster analysis

In MCODE analysis criteria, the MCODE pick up the strong interaction nodes with their supporting targets nodes in the form of clusters. After MCOD analysis of Calycosin network

we found that two clusters first cluster with 5 nodes and 9 edges containing CYP19A1, ESRRB, ESR1, ESR2, and EGFR. Another has 3 nodes and 3 edges with targets ALDH2, MAOB, AOX1. On analysis of MCODE result we found that the targets ESR1, ESR2, CYP19A1 who are finalized by cytohubba are also present in cluster and show strong interaction with each other and give expression on action of calycosin. In MCODE analysis criteria, the MCODE pick up the strong interaction nodes with their supporting target nodes in the form of clusters. After MCOD analysis of the irilone network we found that two clusters first cluster with 5 nodes and 10 edges containing CYP19A1, CYP1A1, ESR1, ESR2, and EGFR on analysis of MCODE result we found that the targets ESR1, ESR2, CYP19A1 who are finalized by cytohubba are also present in cluster and show strong interaction with each other and give expression on action of irilone. In MCODE analysis criteria, the MCODE picks up the strong interaction nodes with their supporting target nodes in the form of clusters. After MCOD analysis of Pratensein network, we found that two clusters first cluster with 6 nodes and 14 edges containing CYP19A1, CYP1B1, ESR1, ESR2, CYP1A1and EGFR. Another has 3 nodes and 3 edges with targets ALDH2, MAOB, and AOX1. On analysis of MCODE result, we found that the targets ESR1, ESR2, and CYP19A1 who are finalized by cytohubba are also present in clusters and show strong interaction with each other and give expression on action of Pratensein. In MCODE analysis criteria, the MCODE picks up the strong interaction nodes with their supporting target nodes in the form of clusters. After MCOD analysis of Pseudobaptigenin network, we found that two clusters first cluster with 5 nodes and 9 edges containing CYP19A1, ESRRB, ESR1, ESR2, and EGFR. Another has 3 nodes and 3 edges with targets ALDH2, MAOB, and AOX1. On analysis of MCODE result, we found that the targets ESR1, ESR2, and CYP19A1 are finalized by cytohubba are also present in cluster and show strong interaction with each other and give expression on the action of Pseudobaptigenin. [Fig.5]

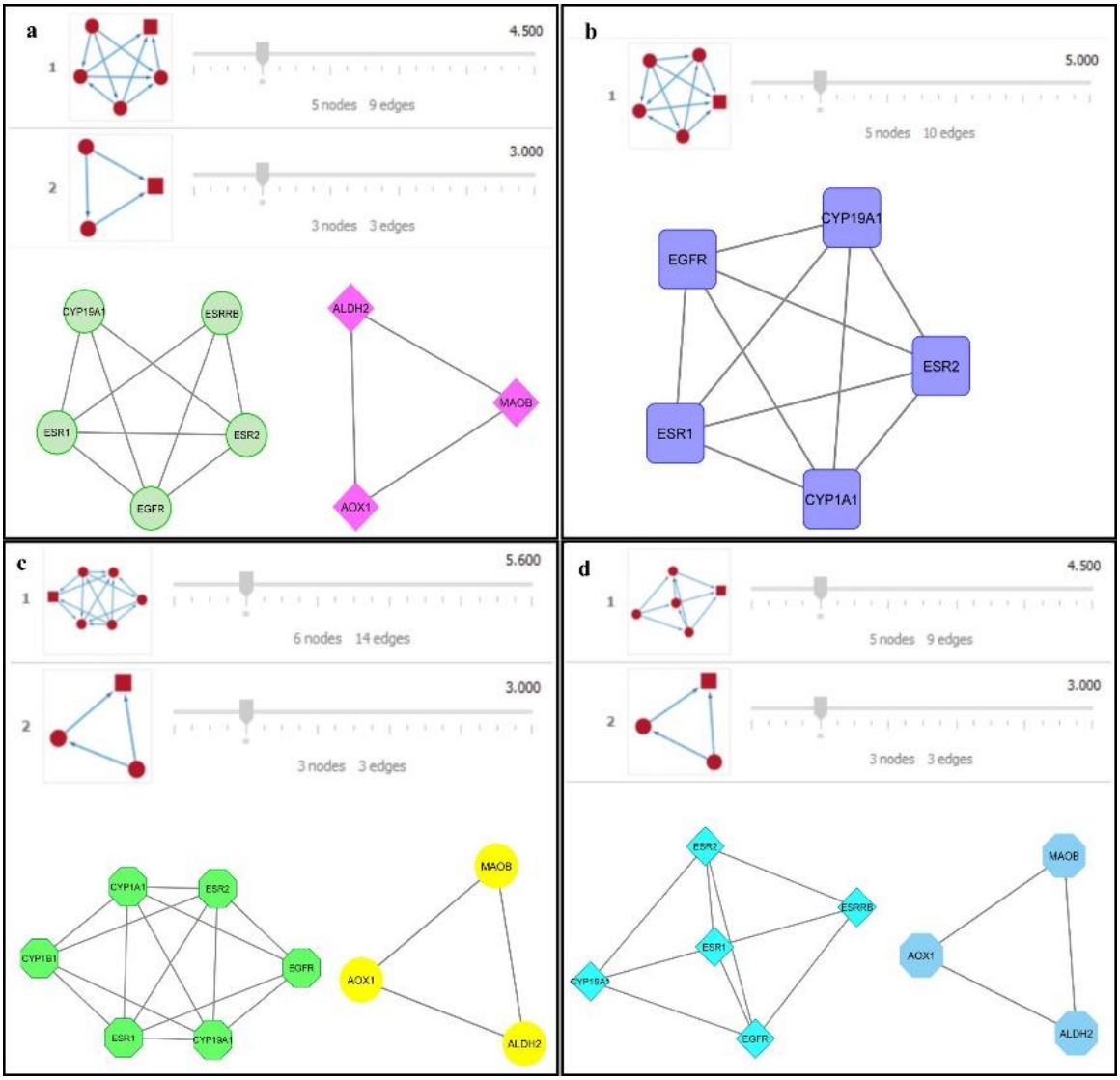

Fig. 5: MCODE Cluster analysis of bioactive compounds. a) Calycosin cluster. b) Irilone cluster. c) Pratensein cluster. d) Pseudobaptigenin cluster.

Gene Ontology analysis

The GO analysis of calycosin is shows that our phytoconstituent have more significant impact on pathways like prolactin signalling pathway and estrogen signalling pathway. Important genes, including ESR1, and ESR2 are modulated in order to provide this therapeutic effect. By targeting this genes phytoconstituent help to regulate hormonal imbalance and reproductive functions associated with PCOS. Whereas the expression of ESR1 and ESR2 is essential for estrogen signalling. We examined how our phytoconstituent, pratensein, interacts with effective targets to treat PCOS using Gene Ontology (GO) analysis. The results showed that the pathways involved in ovarian steroidogenesis, steroid hormone production, oestrogen signalling, and endocrine resistance are the main mechanisms via

which pratensein functions. These pathways play a critical role in controlling the reproductive dysfunction and hormone abnormalities that characterise PCOS. Pratensein is a promising multimodal treatment for PCOS because it affects the expression of important genes such as CYP19A1, ESR1, and ESR2. This helps the body respond better to hormonal cues and normalise hormone levels. Using GO analysis we found that the mechanism by which our phytoconstituent interact with potent targets. The findings of the investigation shows that the primary metabolic pathways through which irilone acts includes ovarian steroidogenesis, prolactin signalling pathway, estrogen signalling pathway, and endocrine resistance. Through which irilone treat the PCOS on expression of ESR1, ESR2 and CYP19A1. Using GO analysis we found that the mechanism by which our phytoconstituent interact with potent targets. The findings of the investigation shows that the primary metabolic pathways through which irilone acts includes prolactin signalling pathway, estrogen signalling pathway, and endocrine resistance. Through which irilone treat the PCOS on expression of ESR1, and ESR2. [Fig.6]

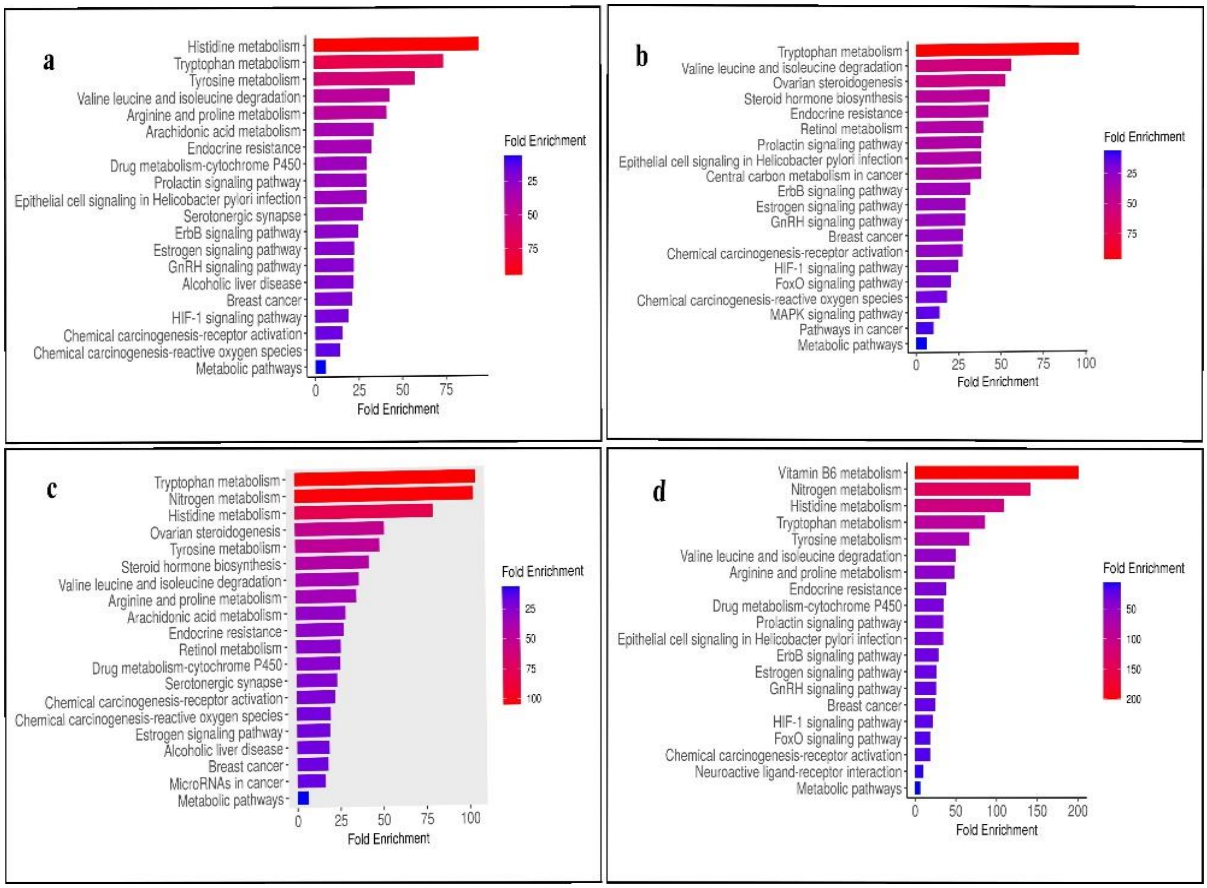

Fig. 6: Gene Oncology analysis of bioactive compounds. a) Calycosin GO. b) Irilonr GO. c) Pratensein GO. d) Pseudobaptigenin GO.

Docking results for screening of phytoconstituents

By performing literature survey, we identified about 14 active phytoconstituents from red clover namely Fisetin, Naringenin, Pratensein, 4-hydroxycoumarin, Calycosin, Prunetin, Quercetin, Irilone, Formononetin, Genistein,Pseudobaptigenin, Diadzein, Biochanin. On literature review, these compounds have phytoestrogen properties. To verify this, we performed molecular docking studies of these compounds with the estrogen receptor (PDB:2IOG) and the androgen receptor (PDB:2AXA). [Table1] Surprisingly we found that listed compound shows good affinity not only for estrogen receptor but towards androgen receptor also. From these results, we shortlisted four compounds i.e. Calycosin, Irilone, Pratensein, Pseudobaptigenin based on their higher affinity scores. For androgen receptor the binding affinities were -8.2, -8.4, -8.4, and -8.2 respectively. While for estrogen receptor affinities were -9.1, -9.4, -9.3, and -9.6 respectively. We have shortlisted these four compounds for further studies. [Table1]

Table 1: Molecular docking results for screening of compounds.

Phytoconstituents	Androgen receptor (2AXA)	Estrogen receptor (2IOG)
Fesetin	-6.5	-8.8
Naringenin	-7.2	-8.8
Pratensein	-8.4	-9.3
4-Hydroxycoumarin	-7.3	-7.3
Calycosin	-8.2	-9.1
Prunetin	-8.6	-8.8
Quercetin	-5.5	-8.6
Irilone	-8.4	-9.4
Formononetin	-5.6	-8.5
Genistein	-7.6	-8.7
Pseudobaptigenin	-8.2	-9.6
Daidzein	-8.0	-8.4
Biochanin	-5.2	-8.3

Validation Through Molecular Docking

The amino acids ARG:A115, ALA :A306 are common binding sites for both Calycosin and the standard compound Androstenedione for CYP19A1, the amino acids GLU A:353,LEU, A:387, ALA,A:350, PHE A:404,LEU A:346, MET A:421, ILE A:424 are common binding sites for both Calycosin and the standard compound Estradiol for ESR1,and the amino acids HIS A:475, LEU A:298, LEU A:476, LEU A:339, PHE A:356, LEU A:343, MET A:340, ALA A:302 are common binding sites for both Calycosin and the standard compound Diarylpropionitrile for ESR2. The amino acids ALA A:306 are common binding sites for

both Irilone and the standard compound Androstenedione for CYP19A1, the amino acids LEU A:387, MET A:388, LEU A:346, MET A:421, ILE A:424 are common binding sites for both Irilone and the standard compound Estradiol for ESR1.[Fig.7] The amino acids ARG:A115, ALA :A306 are common binding sites for both Pratensein and the standard compound Androstenedione for CYP19A1, the amino acids LEU A:387, PHE A:404, LEU A:346, ALA A:350, ILE A:424, MET A:421, GLU A:353 are common binding sites for both Pratensein and the standard compound Estradiol for ESR1,and the amino acids HIS A:475, LEU A:298, LEU A:476, LEU A:339, PHE A:356, LEU A:343, MET A:340, ALA A:302 are common binding sites for both Pratensein and the standard compound Diarylpropionitrile for ESR2. The amino acids ALA :A306 are common binding sites for both Pseudobaptigenin and the standard compound Androstenedione for CYP19A1, the amino acids GLU A:353, LEU A:387, LEU A:346, ALA A:350, ILE A:424, MET A:388 are common binding sites for both Pseudobaptigenin and the standard compound Estradiol for ESR1,and the amino acids LEU A:335, HIS A:475, LEU A:343, MET A:340, PHE A:356, LEU A:298, ALA A:302, LEU A:476 are common binding sites for both Pseudobaptigenin and the standard compound Diarylpropionitrile for ESR2.[Fig.8] Overview of study, Indicating that the compound and standard are bind to the same pocket of the protein. Additionally, the compound forms more hydrogen bonds than the standard, suggesting that our compound has greater stability. [Table2] [Table3]

Table 2: Results of molecular docking between the standard compound and the predicted targets.

Standard	Protein	Residue	Hydrogen bond
17 β-oestradiol	2IOG	MET A:388, PHE A:404, LEU A:387, LEU A:391, LEU A:346, LEU A:525, CYS A:530	GLU A:353
Flutamide	2AXA	TRP A:224, VAL A:370, ALA A:306	MET A:374, ARG A:115
Androstenedione	CYP19A1	TRP A:224, VAL A:370	MET A:374, ARG A:115, ALA A:306
Estradiol	ESR1	LEU A:387, ALA A:350, ARG A:394, MET A:388, PHE A:404, LEU A:391, LEU A:346, MET A:421, ILE A:424	GLU A:353
Diarylpropionitrile	ESR2	HIS A:475, LEU A:298, LEU A:476, LEU A:339, PHE A:356, LEU A:343, MET A:340, ALA A:302	-

Table 3: Results of molecular docking between the bioactive compound and the predicted targets.

Ligand	Protein	Residue	Hydrogen bond
Calycosin	2IOG	ALA A:350, LEU A:391, PHE A:404, LEU A:346, LEU A:384, MET A:338, MET A:528, ILE A:424, LEU A:525	ARG A:394, LEU A:387
	2AXA	MET A:749, PHE A:764, LEU A:704, LEU A:701, PHE A:891, MET A:780, LEU A:880, ASN A:705	ARG A:752, GLN A:711, MET A:895, MET A:745
	CYP19A1	TRP A:141, ILE A:133, CYS A:437, ALA A:306, ALA A:307	ARG A:435, ARG A:115, ILE A:132, ALA A:438
	ESR1	LEU A:349, LEU A:387, PHE A:404, LEU A:346, ALA A:350, LEU A:525, ILE A:424, MET A:421	GLU A:353
	ESR2	LEU A:343, MET A:340, PHE A:356, LEU A:298, ALA A:302, LEU A:476, MET A:295, MET A:479	ARG A:346, LEU A:339, GLU A:305, HIS A:475, GLY A:472
Irilone	2IOG	PHE A:404, LEU A:391, LEU A:346, ILE A:424	LEU A:387, ARG A:394
	2AXA	MET A:749, PHE A:746, LEU A:704, MET A:780, MET A:895	MET A:745, ARG A:752, GLN A:711
	CYP19A1	MET A:311, CYS A:437, ALA A:306, ALA A:438, ALA A:443, GLY A:439	SER A:314
	ESR1	ILE A:424, MET A:421, LEU A:525, MET A:388, LEU A:387, LEU A:387 LEU A:391, GLU A:353, PHE A:404	MET A:528, LEU A:346
	ESR2	GLY A:342, PRO A:277, GLU A:305, ARG A:346, HIS A:279	TYR A:397, LYS A:401
Pratensein	2IOG	LEU A:525, ILE A:424, MET A:388, LEUA:384, LEU A:391, ALA A:350, PHE A:404	LEU A:387, ARG A:394, LEU A:346
	2AXA	LEU A:701, LEU A;880, PHE A:891, PHE A:764, MET A:749, MET A:780	MET A:745, ARG A:752, GLN A:711, MET A:895, MET A:787, LEU A:704, ASN A:705
	CYP19A1	TRP A:141, ILE A:133, CYS A:437, ALA A:306, ALA A:307, ILE A:132, ALA A:438, ARG A:435	ARG A:310, ARG A:115
	ESR1	LEU A:349, LEU A:387, PHE A:404, LEU A:346, ALA A:350, LEU A:525, ILE A:424, MET A:421, GLU A:353	-
	ESR2	LEU A:343, MET A:340, PHE A:356, LEU A:298, ALA A:302, LEU A:476, MET A:295, MET A:479	ARG A:346, LEU A:339, GLU A:305, HIS A:475, GLY A:472
Pseudobaptigenin	2IOG	MET A:388, ALA A:350, PHE A:404, LEU A:391, LEU A:346, ILE A:424	LEU A:387, ARG A:394
	2AXA	LEU A:707, PHE A:746, LEU A:704, MET A:780, MET A:895	MET A:745, GLY A:708, GLN A:711
	CYP19A1	MET A:311, MET A:447, CYS A:437, ALA A:306, ALA A:307, ALA A:443	SER A:314
	ESR1	LEU A:346, ALA A:350, LEU A:525, ILE A:424, MET A:388	GLU A:353, LEU A:387, ARG A:394
	ESR2	LEU A:343, MET A:340, PHE A:356, LEU A:298, ALA A:302, LEU A:476	ARG A:346, LEU A:335, GLU A:305, HIS A:475

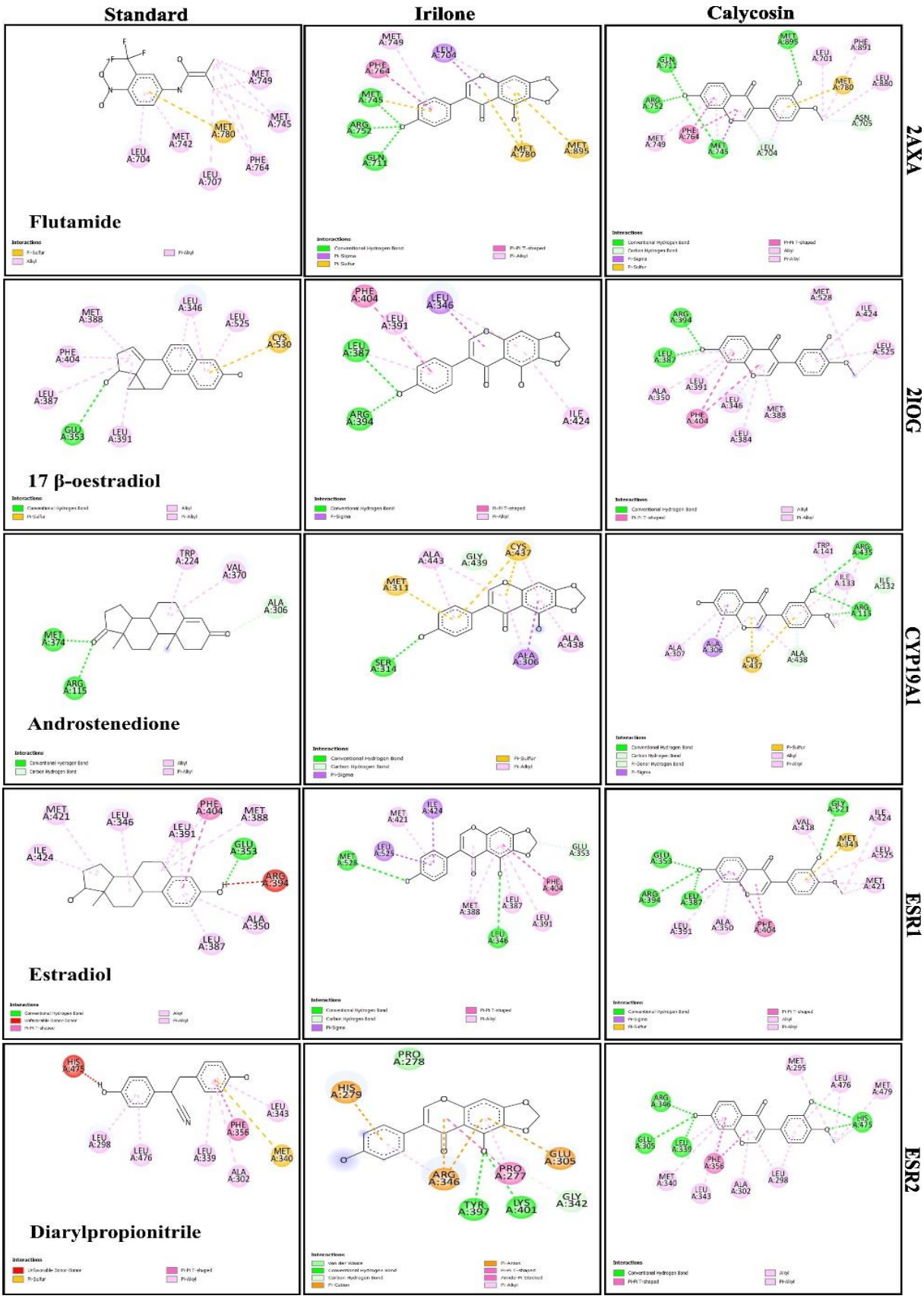

Fig.7: Molecular docking 2D interaction between Calycosin and Irilone with their predicted targets.

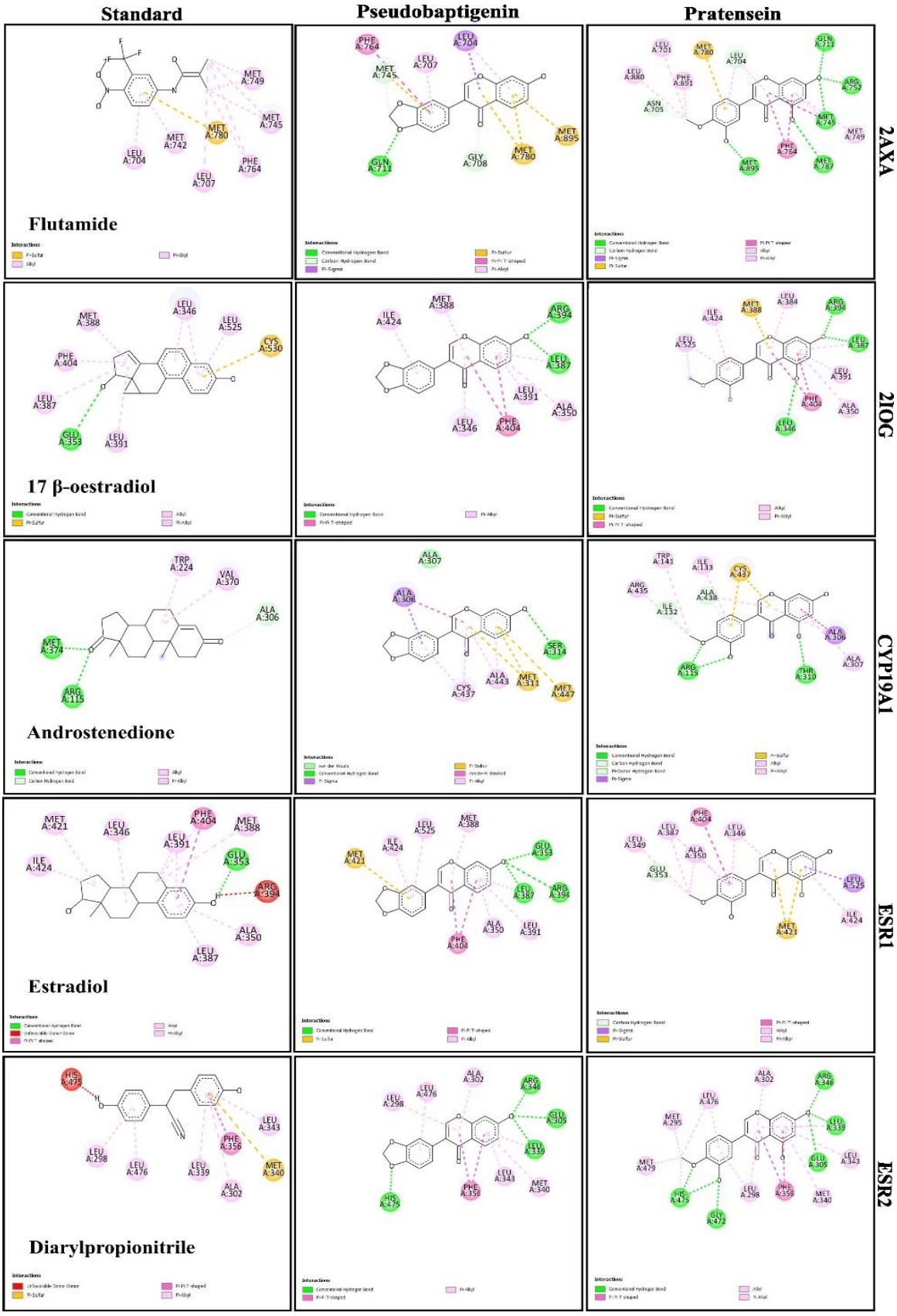

Fig. 8: Molecular docking 2D interaction between Pseudobaptigenin and Pratensein with their predicted targets.

RMSD validation

The RMSD values for CYP19A1, ESR1, and ESR2 with Calycosin are 1.240, 0.441, and 0.812, respectively. These values are significantly less than those of the standards (2.827, 1.949, and 1.806) and co-crystal structures (2.592, 3.241, and 3.086). The RMSD values for CYP19A1, ESR1, and ESR2 with Irilone are 1.887,1.223 and 1.624 respectively. These values are significantly less than those of the standards (2.827,1.949 and 1.806) and co-crystal structures (2.592,3.241 and 3.086). The RMSD values for CYP19A1, ESR1, and ESR2 with Pratensein are 1.139,0.540 and 0.707 respectively. These values are significantly less than those of the standards (2.827,1.949 and 1.806) and co-crystal structures (2.592,3.241 and 3.086). The RMSD values for CYP19A1, ESR1, and ESR2 with Pseudobaptigenin are 2.047,1.704 and 2.216 respectively. These values are significantly less than those of the standards (2.827,1.949 and 1.806) and co-crystal structures (2.592,3.241 and 3.086). According to these findings, Calycosin, Irilone, Pratensein, and Pseudobaptigenin interacts with these proteins more steadily with less deviation, preserving their structural integrity with little change from their starting shape. The higher binding efficiency of Calycosin, Irilone, Pratensein, and Pseudobaptigenin, as seen by its greater stability, may make it a more potent therapeutic agent for targeting these proteins.[Fig.9]

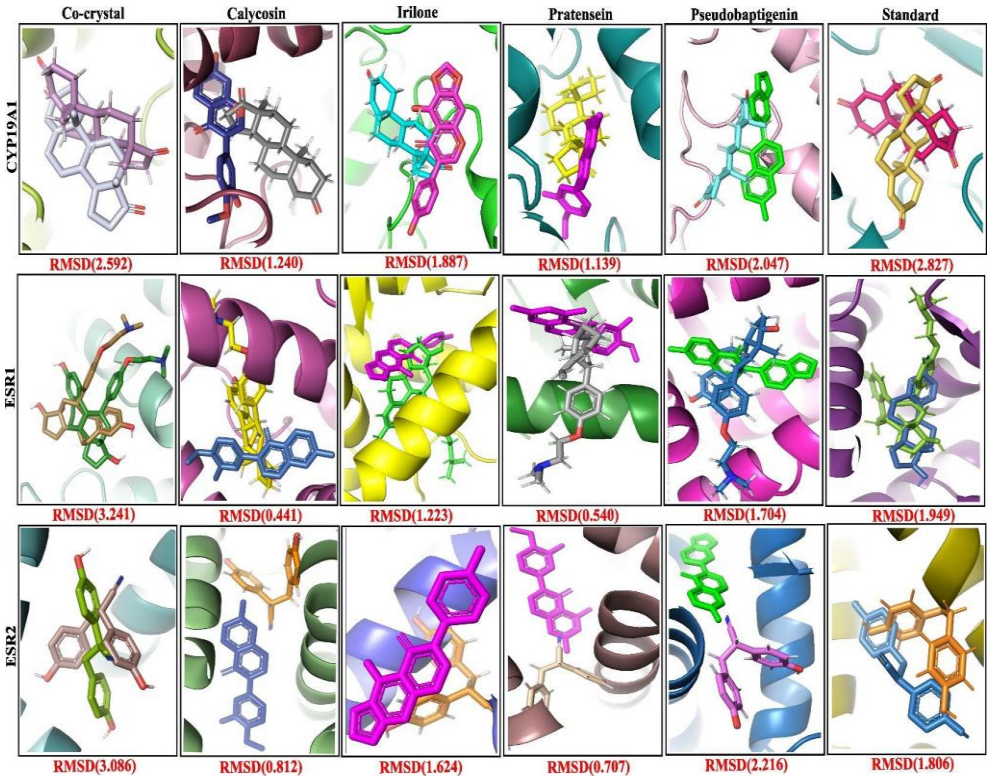

Fig. 9: RMSD validation of docking results, and comparison between Co-crystal, bioactive compounds and respected standards.

DISCUSSION

This study gives an idea about the unexplored properties of red clover (Trifolium pratense) against PCOS symptoms by using a new approach which is network pharmacology. Prior studies say that worldwide approximately 7% of women are affected particularly in their reproductive age, and due to significant treatment costs, new therapeutic approaches are essential. Red clover is abundant in isoflavones, which have estrogen-balancing quality, and also have antioxidant, and anti-inflammatory properties.[9, 14]

The plant and its phytoconstituents were selected and screened through a literature survey. Phytoconstituents were chosen according to their highest binding affinities towards the androgen and estrogen receptors. Using PubChem and Binding DB, targets for these phytoconstituents were found. Canonical smiling notation was reproduced and processed to locate compound targets. Using the Gene Card database, disease targets for PCOS, hyperandrogenism, and hypoestrogenism were found. A Venn diagram was used to identify common targets, and Cytoscape was used to build networks for common targets, pathway-oriented interactions, and disease-oriented interactions.[15, 16] Additionally, MCODE cluster analysis and networks of protein-protein interactions (PPIs) were carried out. The Shiny GO 0.8 database was used to perform gene ontology (GO) analysis with an emphasis on the KEGG pathway.

On molecular analysis found that from 14 phytoconstituents there are 4 that have the highest binding affinity namely Calycosin, Irilone, Pratensein, and Pseudobaptigenin. So, by constructing a common network of phytoconstituents with their targets realize that the ESR1, ESR2, and CYP19A1 are common in all i.e. they show synergic effect. The pathway-oriented network shows the interaction between targets, genes, and pathways related with only phytoconstituents analysis indicating that ESR1 and ESR2 are key gens. While the disease-oriented network confirms that the targets estrogen receptor, estrogen bets receptor, and aromatase are potent targets for PCOS also they further express ESR1, ESR2, and CYP19A1 genes. PPI network gives idea about how proteins interact with each other and in which intensity, after cytohubba and MCODE analysis results that the genes ESR1, ESR2, and CYP19A1 are hub genes that show more interaction with each other. GO analysis found that some pathways for each phytoconstituent like the estrogen signaling pathway and endocrine resistance. Finally checked out the affinity of our phytoconstituents for hub genes, and higher affinities as compare to standard indicate that our compounds are potent for particular genes.

The RMSD validation was also performed to check and compare the binding pocket position of co-crystal, compound, and standard, the lowest RMSD value of the compound shows less deviation than the standard; overall they perfectly bind with receptors with higher affinity.

COCLUSION

This study shows *In silico* evaluation of potential targets of Red clover (Trifolium pratense) against PCOS symptoms, by using a novel network pharmacological approach. This research focuses on identifying and evaluating key phytoconstituents from red clover plant, specifically Calycosin, Irilone, Pratensein, Pseudobaptigenin from Red clover, and to identify their efficacy in managing PCOS symptoms. Including hyperandrogenism and hypoestrogenism, key conditions related to PCOS symptoms, broadens the study's scope. These conditions are central to the hormonal imbalance observed in PCOS, thereby enhancing the relevance of the findings. By performing extensive literature survey and molecular docking phytoconstituents was selected according to their binding affinities to androgen and estrogen receptors which was also validated through RMSD calculation. Network analysis studies found that Calycosin, Irilone, Pratensein, and Pseudobaptigenin significantly affects the estrogen receptors and express ESR1 and ESR2 genes, also affect aromatase and express CYP19A1. While Eriodictyol only affects aromatase and express CYP19A1. PPI analysis by using Cytohubba and MCODE, it confirms that ESR1, ESR2 and CYP19A1are hub genes and show strong interaction and interconnection. Gene Ontology study reveals that compouds follows key metabolic pathways such as estrogen signaling pathway, prolactin signaling pathway, ovarian steroidogenesis, and endocrine resistance. Each compound docked with respected hub genes and cross-examined there binding stability also performed RMSD validation to support the docking study. This study demonstrates the potential of compounds from red clover in treating PCOS. These plant compounds can effectively target key hormonal pathways and receptors, laying the groundwork for developing new, affordable treatments. To fully confirm these benefits, further research, especially clinical trials, is essential to explore the complete potential of these compounds in managing PCOS.

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