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Investigating Piliostigma thonningii's Phytoestrogenic Properties for PCOS Treatment using Molecular Dynamics Simulation

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder requiring effective treatment. Current therapies have limitations, prompting the search for natural alternatives. *Pilliostigma thonningii*'s potential as a phytoestrogenic agent for PCOS management needs investigation due to its traditional use and promising bioactive compounds. This study investigated the phytoestrogenic potential of *Pilliostigma thonningii* (PT) in Polycystic Ovary Syndrome (PCOS) using a combination of molecular docking, molecular dynamics simulation, network pharmacology, ADMET, and *in vivo* validation. The results showed that PT phytochemicals, such as 6-C-Methylquercetin 3-methyl ether, exhibited strong binding affinities to human placental aromatase, comparable to Letrozole. Molecular dynamics simulation confirmed the stability of the protein-ligand complex, and ADMET analysis revealed favorable pharmacokinetic profiles. *In vivo* studies demonstrated that the ethanol leaf extract of PT significantly increased progesterone levels in rats with letrozole-induced PCOS, without affecting luteinizing hormone levels. These findings suggest that PT phytochemicals may be potential multi-targeted candidates for PCOS treatment through estrogen receptor modulation and steroid biosynthesis regulation. The study's results were statistically significant, and the approach used provides a comprehensive understanding of the molecular mechanisms underlying PT's phytoestrogenic activity. Overall, this research highlights the potential of PT as a natural therapeutic agent for PCOS management..

Keywords: ADMET, Functional Enrichment, Gene Ontology, Molecular Docking, Molecular Dynamics, Metformin, Network Pharmacology, PCOS, Phytoestrogens.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine and metabolic disorders, affecting an estimated 116 million women (3.4%) globally, according to the World Health Organization (WHO) in 2012 (Vidya Bharathi *et al.,* 2017; Sangaraju *et al.,* 2022). Its prevalence varies based on diagnostic criteria: 6.44% using NIH criteria in girls aged 14–18 years (Azargoon *et al.,* 2020), 8.5% using Rotterdam criteria in Brazilian women aged 18–45 years, and 7.2% using AES criteria in Chinese women aged 18–45 years (Zhuang *et al.,* 2014). It is a multifactorial, involved, complex interplay of both genetic predisposition, environmental factors, hyperinsulinemia, hormonal imbalances, neuroendocrine abnormalities, chronic inflammation, and autoimmune disease (Bruni *et al.,* 2022; Sangaraju *et al.,* 2022). PCOS is characterized by overproduction of male hormones and ovarian cyst formation, primarily affecting women of reproductive age between 13 and 44 years (Patel, 2018), and these changes in the ovaries, which are responsible for producing female hormones such as estrogen and progesterone, also generate small amounts of androgens. This imbalance results in these hormone changes that lead to menstrual cycle irregularities, excessive hair growth (hirsutism), and obesity (Ding *et al.,* 2021). As such, PCOS is a leading cause of infertility among females. This group of heterogeneous illnesses depends on the reliance on biochemical markers, and any changes in physiology may alter changes in hormonal levels, resulting in menstrual conditions such as amenorrhea or oligomenorrhea, excessive androgen production, and anovulation (Bjekić-Macut *et al.,* 2021) and though this condition (PCOS) can occur at any age from menarche onward, most cases are severe between 20 and 30 years of age (Bjekić-Macut *et al.,* 2021).

The pathophysiology of PCOS involves turmoil in steroidogenesis, ovarian folliculogenesis, neuroendocrine metabolism alteration, insulin production, insulin responsiveness, and fat cell activity. Central to the condition are hormonal imbalance, low inflammatory cytokines that are capable of interfering with hormones leading to insulin resistance, and hyperandrogenism, which impairs folliculogenesis and increases the risk or chances of comorbidities, like endometrial disease and type II diabetes (Ibáñez *et al.,* 2017).

Hyperandrogenemia, the hallmark sign of PCOS, shows clinically as hirsutism, skin inflammation or acne, and alopecia. Increased degrees of androgens are seen in 75-90% of PCOS patients with oligomenorrhea, and their levels regularly increase with the severity of the condition. Increased androgen synthesis in the ovaries and the adrenal glands contributes to hyperandrogenism (Kanbour & Dobs, 2022). Elevated levels of free (unbound) testosterone, a key hormone involved in the pathogenesis of PCOS, are indicative of hyperandrogenism. Abnormal ovarian or adrenal function leads to the overproduction of androgens.

In PCOS, impaired folliculogenesis is a key feature of PCOS that stems from increased androgen production, leading to disruption of normal follicular development. In the early gonadotropin stages of activity, increased androgens promote the growth of primordial follicular cells and an increase in antral follicles. Gonadotropin-releasing hormone (GnRH) triggers the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the master gland, or pituitary gland, in the hypothalamus. However, LH further drives androgen synthesis in ovarian theca cells, while FSH in turn converts these androgens into estrogens in granulosa cells of the ovaries to stimulate follicular growth. The dysregulation of the neuroendocrine part of the hypothalamic-pituitary-ovarian (HPO) axis results in an imbalance of gonadotropins, especially by an elevated LH:FSH ratio, which amplifies PCOS symptoms (Walters *et al.,* 2018). Theca cells in the ovaries undergo hyperplasia due to increased LH stimulation, leading to cystic structures forming along the ovarian surface, creating the characteristic pearl necklace appearance. However, this trend arises from arrested follicles in the preantral and antral stages and androgen synthesis (Ashraf *et al.,* 2020). The altered cortisol activity or metabolism also contributes to hyperandrogenism in PCOS patients, which further enhances cortisol inactivation by 5α-reductase or impaired cortisol reactivation by 11β-hydroxysteroid dehydrogenase type 1, which may increase androgen levels through positive feedback inhibition that affects adrenocorticotropic hormone (ACTH) secretion, which may be attributed to genetic down-regulation (Lightman *et al.,* 2020). A variety of genetic variables are important in the pathophysiology of PCOS, including steroidogenesis-related genes such as CYP genes, which control androgen

production and may be key players in hyperandrogenism in PCOS (Ashraf *et al.,* 2020). Phytoestrogens structurally resemble estradiol and can bind to estrogen receptors, thus potentially modulating estrogen-dependent conditions like PCOS (Abubakar *et al.,* 2024; Ighodaron *et al.,* 2012). Molecular docking studies targeting PCOS-related proteins can validate interactions between P. thonningii phytoconstituents and these targets, helping identify lead compounds with strong binding affinity and favorable pharmacokinetics (Zongo *et al.,* 2023).

Piliostigma thonningii (Schumach.) Milne-Redh, a member of the Fabaceae family, is widely distributed across sub-Saharan Africa and is traditionally used for managing reproductive and metabolic disorders, inflammation, and infections (Dasofunjo *et al.,* 2013; Jimohn & Oladiji, 2005; Zongo *et al.,* 2023; Alagbe, 2019; Garba Ayuba, 2024; Malgwi *et al.,* 2024). Several studies have demonstrated its antioxidant, antimicrobial, and anti-inflammatory properties in both stem bark and leaf extracts (Alagbe, 2019; Garba Ayuba, 2024; Malgwi *et al.,* 2024; Bu *et al.,* 2023). Recent ethnobotanical research by Hailemariam et al. (2021) emphasized the plant's consistent use in community-based traditional medicine, reinforcing its therapeutic potential across African regions. In toxicological and pharmacological validations, *P. thonningii* has shown safety and efficacy in preclinical models targeting conditions such as diabetes, oxidative stress, and neuroinflammation (Jimoh & Oladiji, 2005; Alagbe, 2019; Malgwi *et al.,* 2024; Bu *et al.,* 2023). The South African Journal of Botany and other ethnopharmacology-focused journals have recognized the value of Fabaceae species, including *P. thonningii*, particularly in studies involving African medicinal plants for therapeutic exploration (Nethathe *et al.,* 2025; Puri *et al.,* 2022). These findings, coupled with our computational predictions and molecular simulations, provide a strong basis for investigating the plant's phytoestrogenic potential in a letrozole-induced PCOS model. In addition, several specific phytoconstituents identified through the Phytochemicals Interactions Database (PCIDB) were selected for molecular docking and molecular dynamics (MD) simulations to evaluate their binding potential and interaction stability with PCOS-associated protein targets. These include 6-C-methylquercetin 3,7,3'-trimethyl ether; 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-

4H-1-benzopyran-4-one; 6-C-methylquercetin 3-methyl ether; 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one; 2-(3,4-Dihydroxyphenyl) -5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one; 5,7-dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one; and latifoline. The estrogen-mimicking (phytoestrogenic) potential of these flavonoids and phenolic compounds is significant. Phytoestrogens structurally resemble estradiol and can bind to estrogen receptors, thus potentially modulating estrogen-dependent conditions like PCOS (Abubakar *et al.,* 2024; Ighodaro *et al.,* 2012). Molecular docking studies targeting PCOS-related proteins can validate interactions between *P. thonningii* phytoconstituents and these targets, helping identify lead compounds with strong binding affinity and favorable pharmacokinetics (Zongo *et al.,* 2023). Therefore, this present research work was designed to Investigate the phytoestrogenic activity of *Piliostigma thonningii* leaf extract in letrozole induced Polycystic Ovary Syndrome (PCOS) in female Wistar rats using molecular docking analysis and molecular dynamic stimulation approach**.**

Methodology

Network Pharmacology Analysis and Functional Enrichment

To complement molecular docking and pharmacokinetic studies, a systems-level analysis was conducted to investigate the therapeutic potential of *Pilliostigma thonningii* in polycystic ovary syndrome (PCOS) (Fadilah *et al.,* 2024). A compound–target network was constructed by retrieving 1,000 PCOS-associated genes from GeneCards (https://www.genecards.org) and cross-referencing them with predicted targets of *P. thonningii* phytochemicals (Jung *et al.,* 2024). Target prediction was performed using SwissTargetPrediction (https://www.swisstargetprediction.ch), while compound data, including structural and chemical information, were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov). The interaction map is shown in **Figure 1**.

These targets were submitted to STRING (https://string-db.org/) for protein–protein interaction (PPI) network construction, limited to *Homo sapiens* with a confidence score ≥0.4. Disconnected nodes were removed, and the network was visualized using Cytoscape v3.10.3 (**Figure 2**) (Guo *et al.,* 2022). Network Analyzer identified core targets based on high degree centrality. Functional enrichment was

performed using DAVID v6.8 (http://www.david.niaid.nih.gov). KEGG pathway analysis ($P < 0.05$) revealed enrichment in insulin signaling, steroid hormone biosynthesis, inflammation, and ovarian function (**Figure 3**) (Elfiky *et al.,* 2025). GO analysis was further conducted using ShinyGO (http://bioinformatics.sdstate.edu/go/) to identify enriched terms in Biological Processes (Figure 4), Cellular Components (**Figure 5**), and Molecular Functions (**Figure 6**) (Chen *et al.,* 2021; Guan *et al.,* 2023), highlighting key roles in hormone activity and cell regulation. To contextualize interactions, 2D structures of major phytochemicals were visualized, including compounds shown in Figures 7–12, demonstrating diverse scaffolds and potential binding affinity to PCOS-related proteins. Additionally, molecular dynamics (MD) simulations were conducted to assess the stability and binding interactions of lead phytochemicals with PCOS-associated targets, using Google Colab (https://colab.research.google.com), (Figures 18–22).

Materials

Plant Materials

Fresh leaves of *Piliostigma thonningii* were collected from the UNICROSS environment, Okuku, Cross River State, Nigeria. The leaves were identified at the Department of Plant Science and Biotechnology, University of Nigeria, Nigeria, and the Federal College of Forestry (FCOFJ) Jos, and given a voucher number of #25.

Experimental animals

Thirty-five (35) Wistar female rats were obtained from the animal holding unit of the Department of Medical Biochemistry, University of Cross River State (UNICROSS). The animals were allowed to acclimatize for a period of 7 days in a well-ventilated room at room temperature and relative humidity of 29°C and 70%, respectively, with a 12-hour natural light-dark cycle. They were allowed food and water ad libitum. Good hygiene was maintained by daily cleaning and removal of feces and spills from their cages. Ethical approval for the study was obtained from the Faculty of Basic Medical Science Animal Research Ethical Committee of University of Cross River State, Calabar, Nigeria (approval number **FBMS/UNICROSS/24/011**).

Ethical Considerations and Scientific Justification.

In accordance with ethical guidelines for animal experimentation and the 3Rs principle (Replacement, Reduction, and Refinement), the in vivo phase of this study was carefully designed based on extensive prior computational screening. The selection of *Piliostigma thonningii* was justified through strong in silico evidence, including high-affinity molecular docking to estrogenic and steroidogenic targets, favorable ADMET profiles, and predictive network pharmacology linking the plant's phytochemicals to PCOS-relevant pathways. While direct in vitro data for *P. thonningii* in PCOS models is currently limited, existing pharmacological studies on similar flavonoids demonstrate estrogen-mimicking effects through estradiol synthesis modulation and granulosa cell viability enhancement. These findings support the predictive efficacy of our selected phytochemicals. Furthermore, the increasing prevalence and therapeutic gaps in PCOS management emphasize the urgency of developing phytoestrogenic alternatives. Future research from our group will include targeted in vitro studies to further validate the cellular mechanisms prior to broad clinical extrapolation.

Preparation of extract of *P. thonningii* leaf

The leaves of *P. thonningii* were collected around the University of Cross River State (UNICROSS) and air-dried at room temperature for a period of 21 days until constant weight was obtained. The dried leaves were then pulverized to powdered form by a machine blender and sieved. Thereafter, 400 g of the pulverized plant material (*P. thonningii*) was dissolved in 1200 ml of 70% petroleum ether for 72 hours. This was followed with vacuum filtration, and extracts were concentrated using an evaporator water bath at 40°C to obtain a solvent-free extract and stored in a refrigerator at 4°C. Phytochemical analysis was determined by the method of Trease and Evans (Muhammad *et al.,* 2018). The male sexual behavior test will be carried out by the methods of Ratnasonriva & Dhamasiri (2000) as modified by Dasofunjo et al. (2024). Serum testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels of extract-treated Female rats will be estimated using the method of Ismail (1986), and sperm profiles were determined by standard methods.

Experimental design

Induction of PCOS in female rats Except for the control group, all the investigational rats were orally administered with letrozole (1 mg/kg)

dissolved in 1% CMC (carboxymethyl cellulose) solution for 21 days to induce PCOS condition. The changes in body weight and blood glucose levels were reported to be the early markers for the induction of PCOS in experimental animals (Liyanage *et al.,* 2021).

Treatment protocol

The study was conducted on 35 animals equally separated into six groups as follows: Group 1: Control group, which received only 1% carboxymethyl cellulose solution. Group 2: Negative Control—served as the PCOS group, which received letrozole (1 mg/kg). Group 3: Standard—letrozole + metformin (1 mg/kg). Group 4: Test group I—letrozole + *Piliostigma thonningii* (200 mg/kg). Group 5: Test group II—letrozole + *Piliostigma thonningii* (400 mg/kg). The data obtained were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test at $P < 0.05$. The GraphPad Prism Software version 8.0.2 was used for the analysis (**Figures 13–17**).

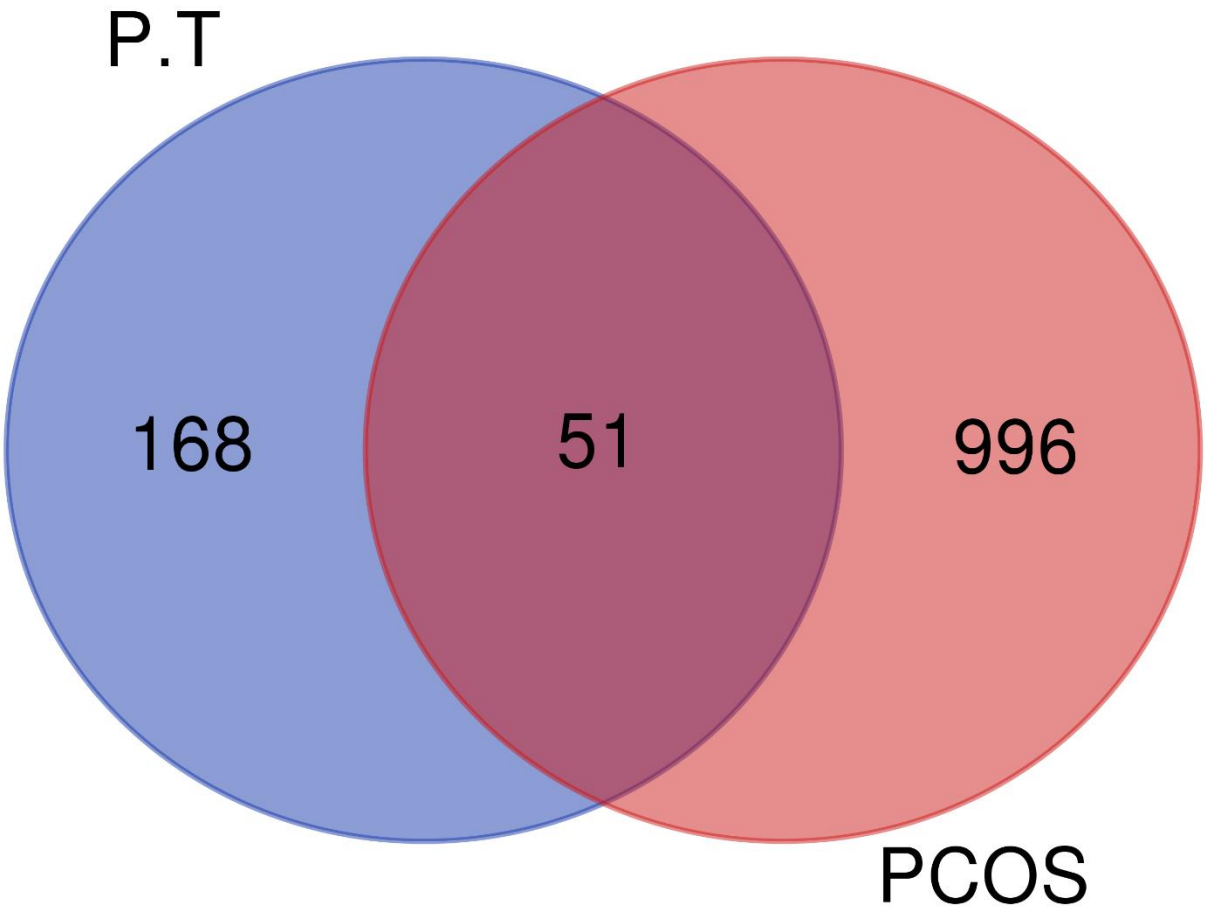


Figure 1: *Pilliostigma Thonningii* (P.T) in Polycystic Ovary Syndrome (PCOS) interactions

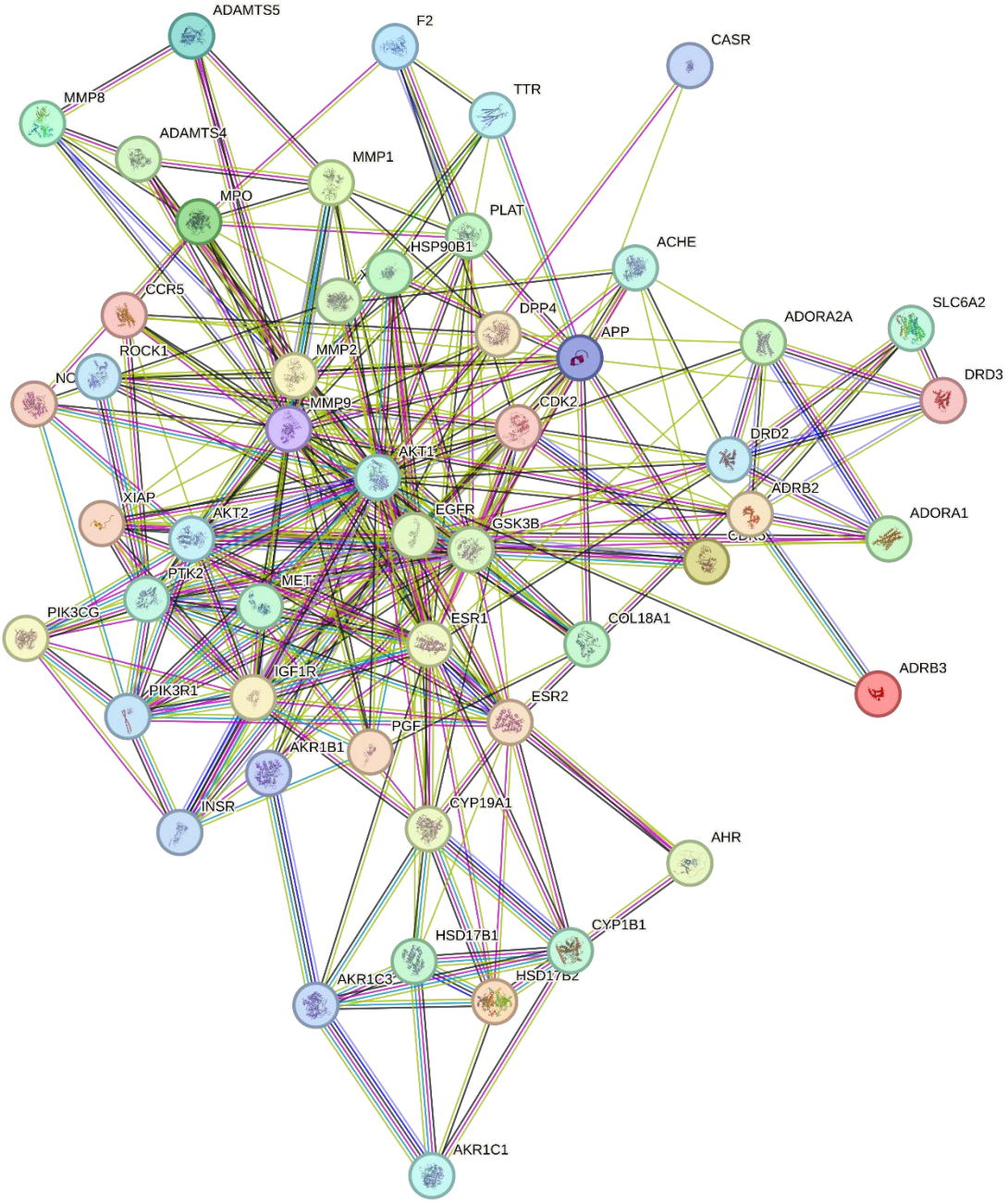


Figure 2: Protein-Protein interaction of *Pilliostigma thonningii* (P.T) and Polycystic Ovary Syndrome (PCOS).

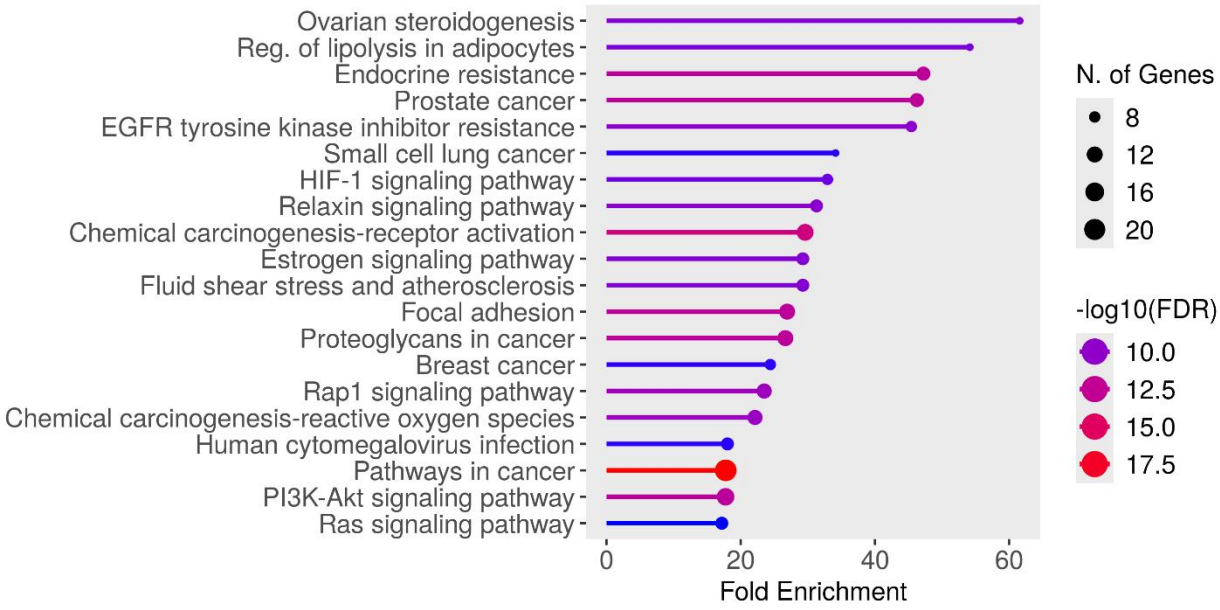


Figure 3 A: Kegg pathway of *Pilliostigma thonningii* (P.T) and Polycystic Ovary Syndrome (PCOS).

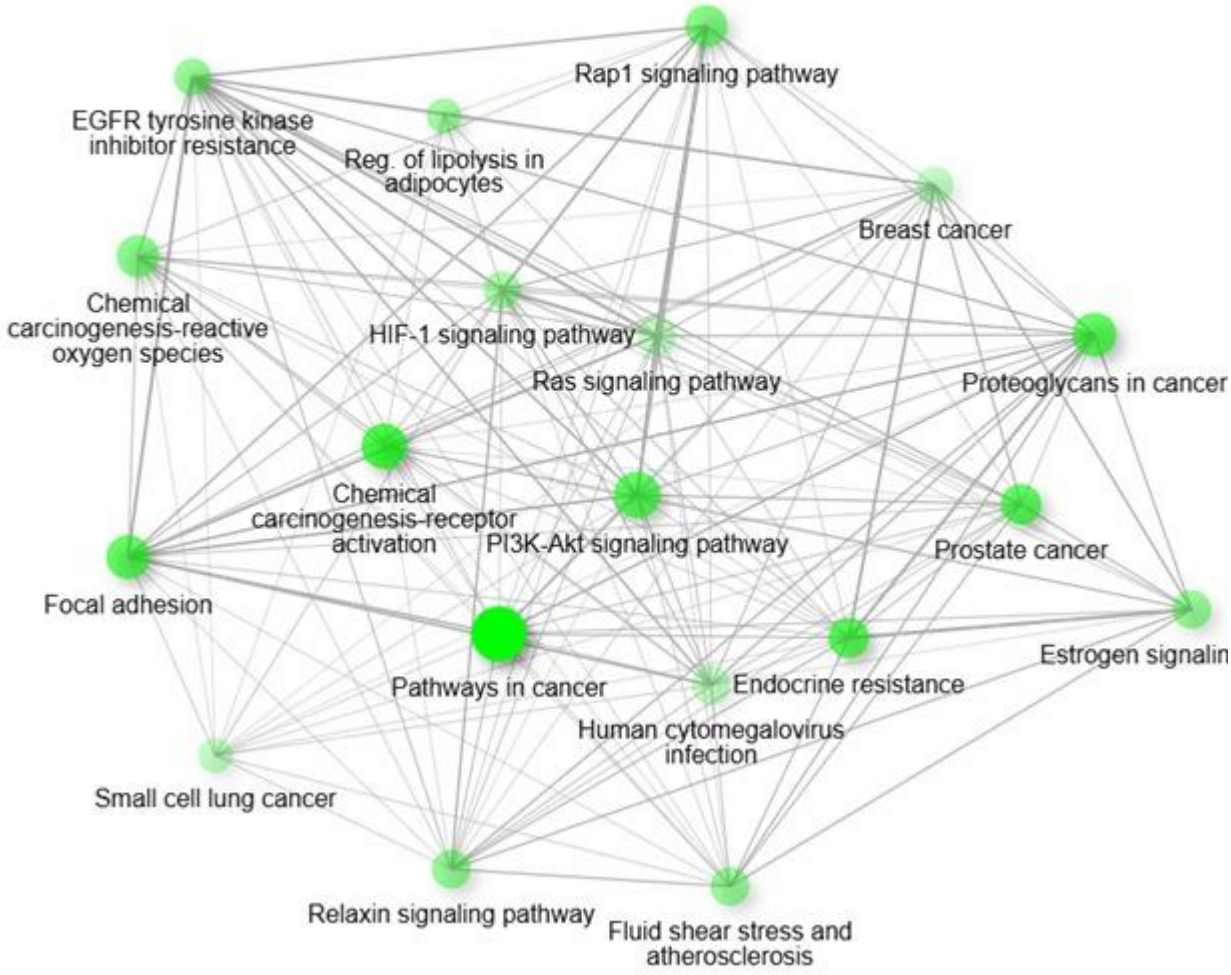


Figure 3 B: Integrated KEGG pathway network highlighting interconnected biological pathways enriched for PCOS-associated targets of *Piliostigma thonningii* phytoconstituents. Central nodes such as PI3K-Akt signaling, Oestrogen signaling, and Pathways in cancer indicate potential core mechanisms for therapeutic intervention.

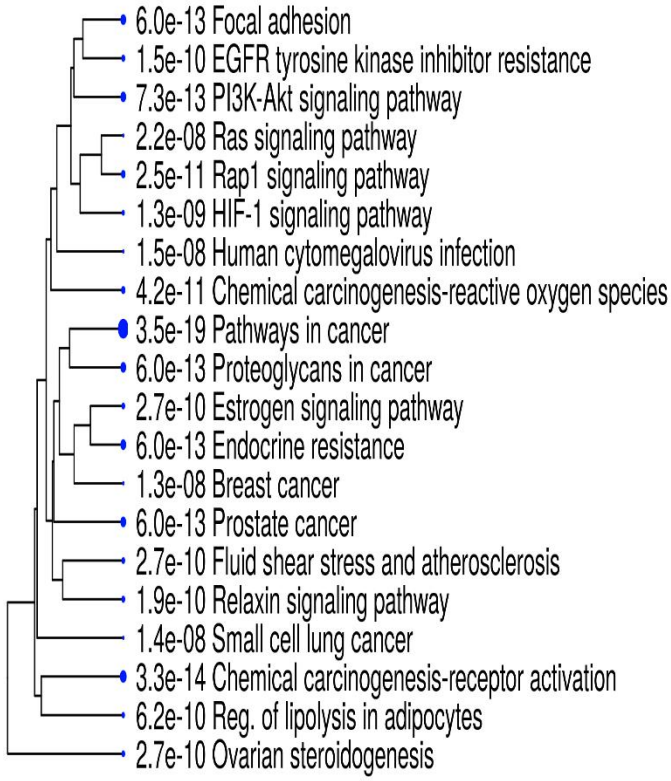


Figure 3C: Hierarchical Cluster Tree of enriched signaling pathways related to Polycystic Ovary Syndrome. The dendrogram illustrates clusters of related pathways based on their functional similarity and enrichment profiles, emphasizing the interconnected mechanisms through which *Pilliostigma thonningii* phytochemicals may affect PCOS pathophysiology**.**

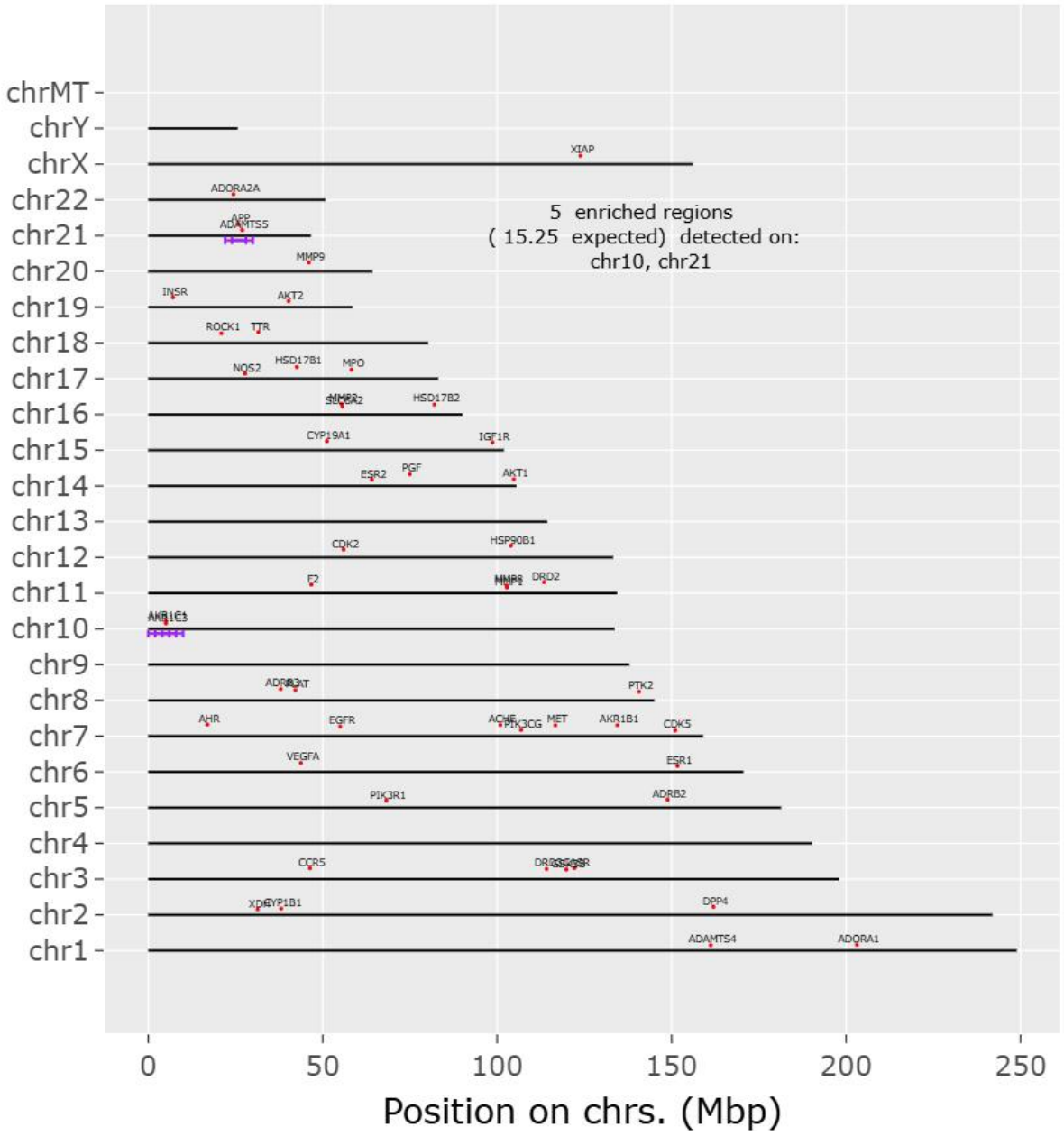


Figure 3D: Chromosomal localization of PCOS-associated genes enriched by *Piliostigma thonningii* phytoconstituents. Notably, 5 enriched regions (expected 15.25) were detected on chr10 and chr21.

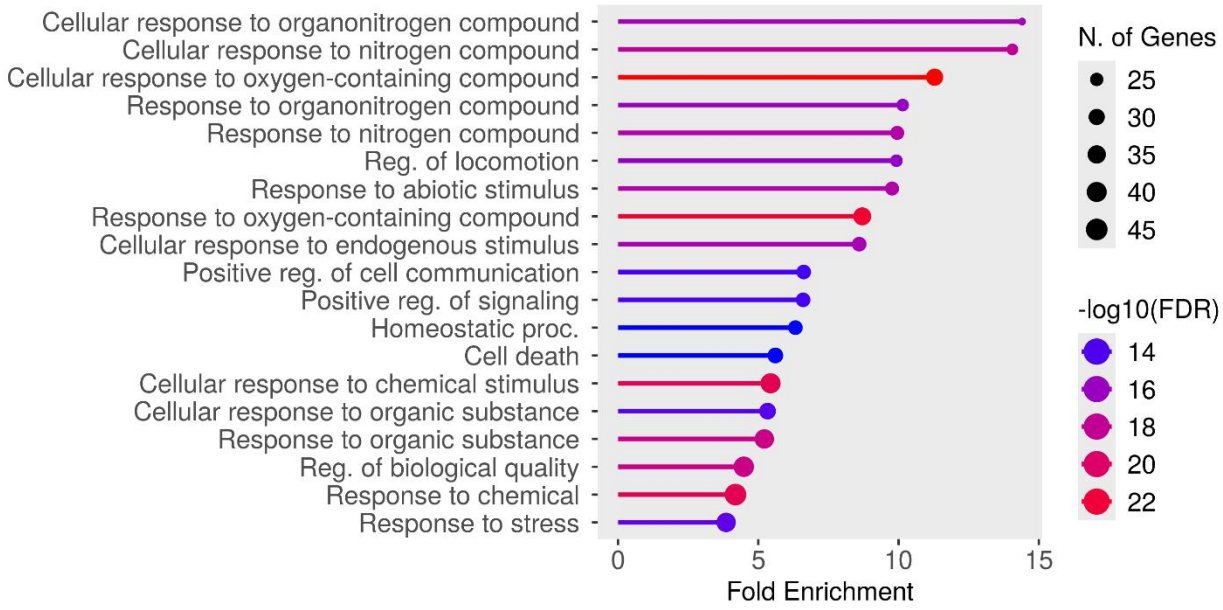


Figure 4: GO-Biological process of *Piliostigma thonningii* (P.T) and Polycystic Ovary Syndrome (PCOS)

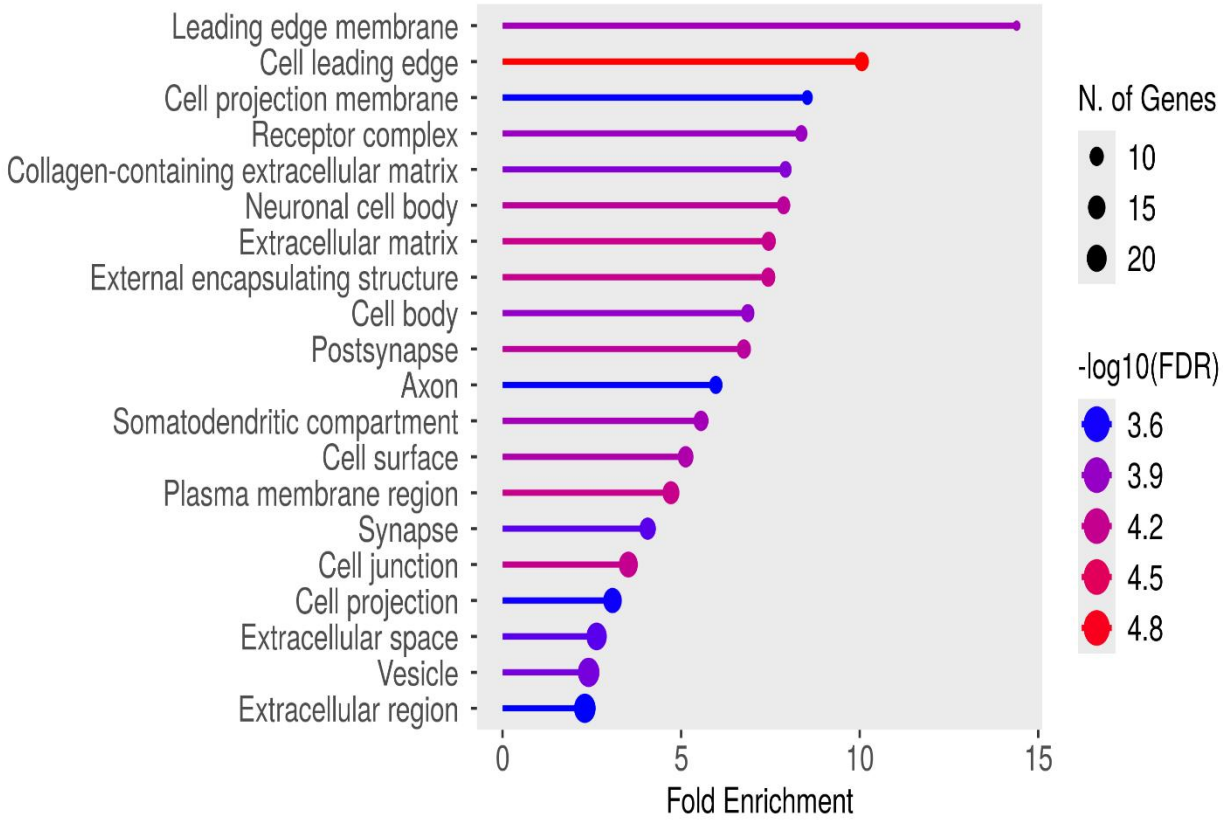


Figure 5: GO-Cellular Process of *Piliostigma thonningii* (P.T) and Polycystic Ovary Syndrome (PCOS).

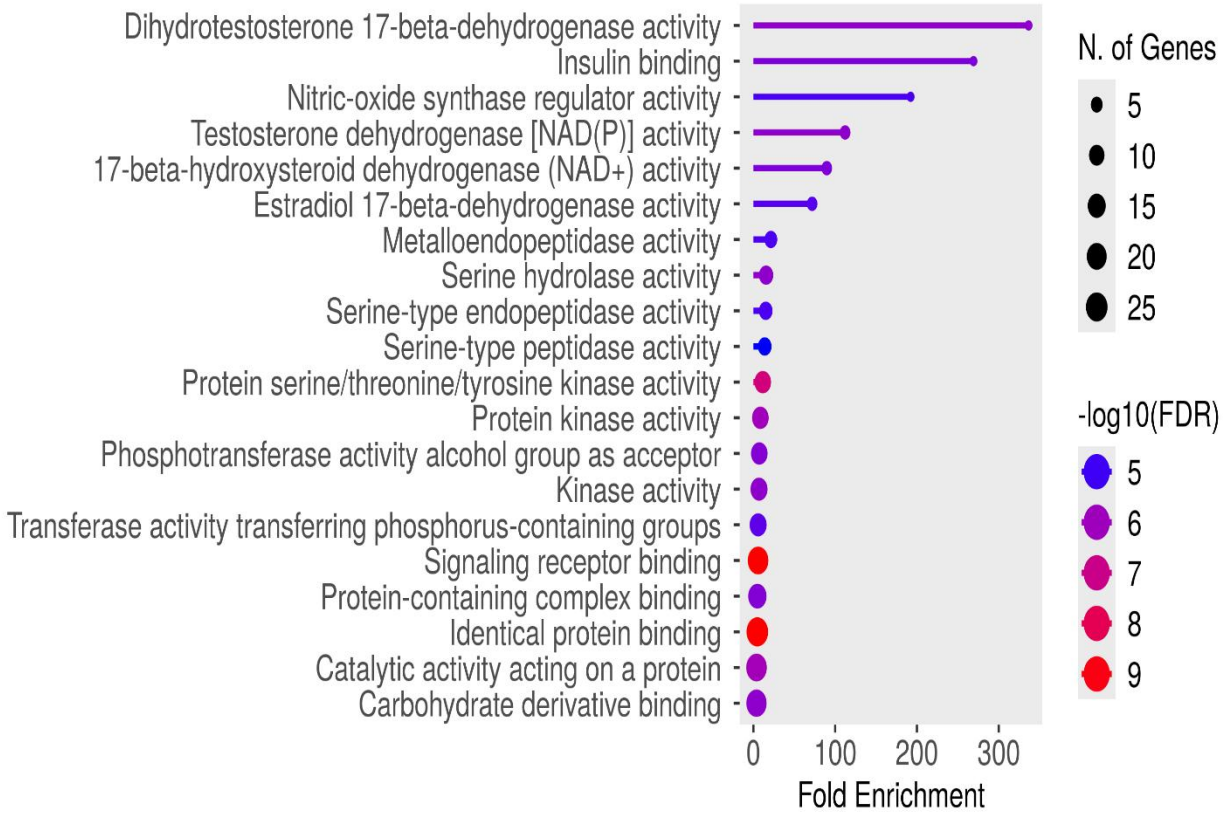


Figure 6: GO-Molecular functions of *Piliostigma thonningii* (P.T) and Polycystic Ovary Syndrome (PCOS).

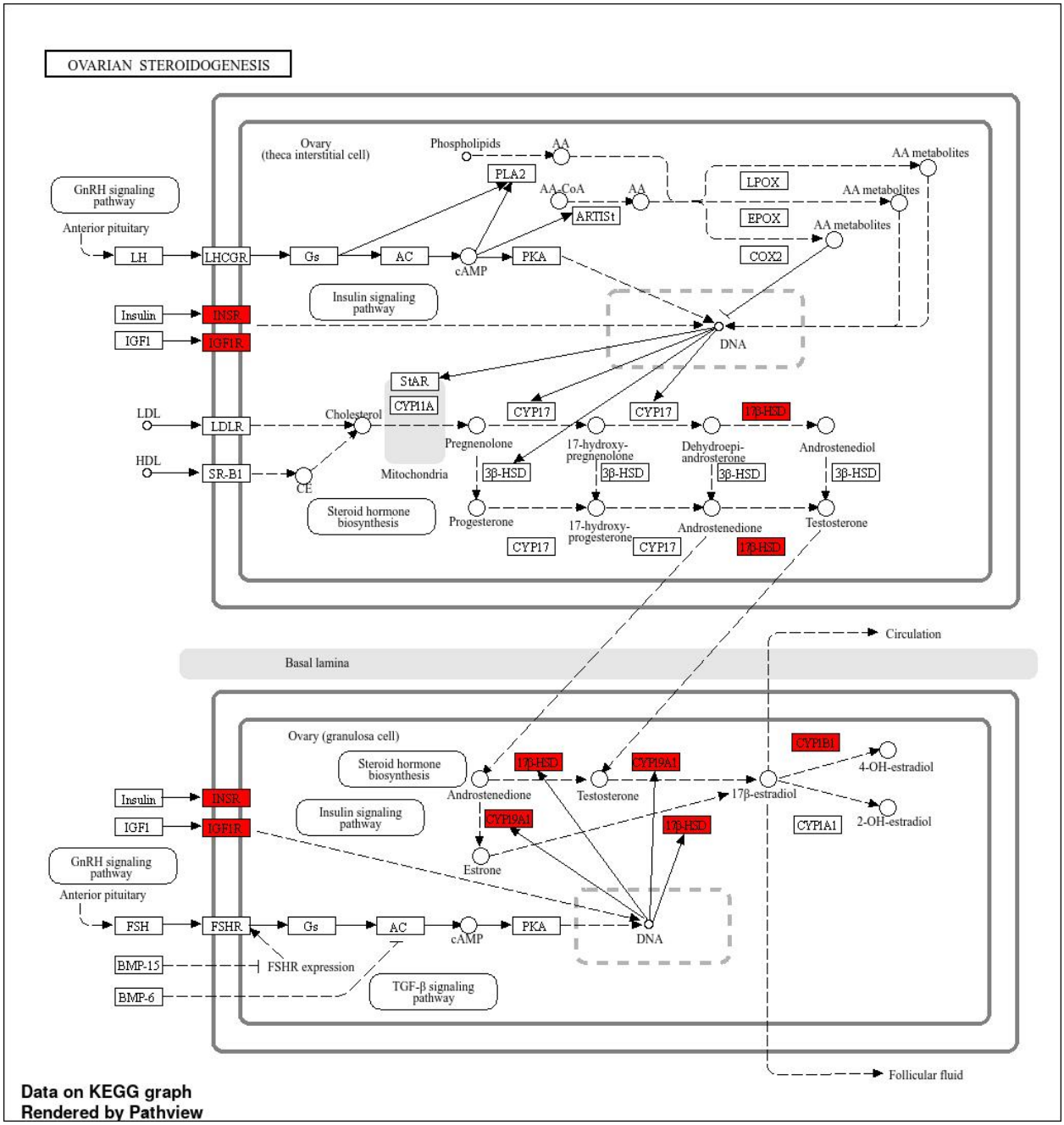


Figure 6B: KEGG ovarian steroidogenesis pathway illustrating highlighted targets modulated by *P. thonningii* bioactive, particularly INSR, IGF1R, HSD17B, and CYP genes, consistent with anti-PCOS action.

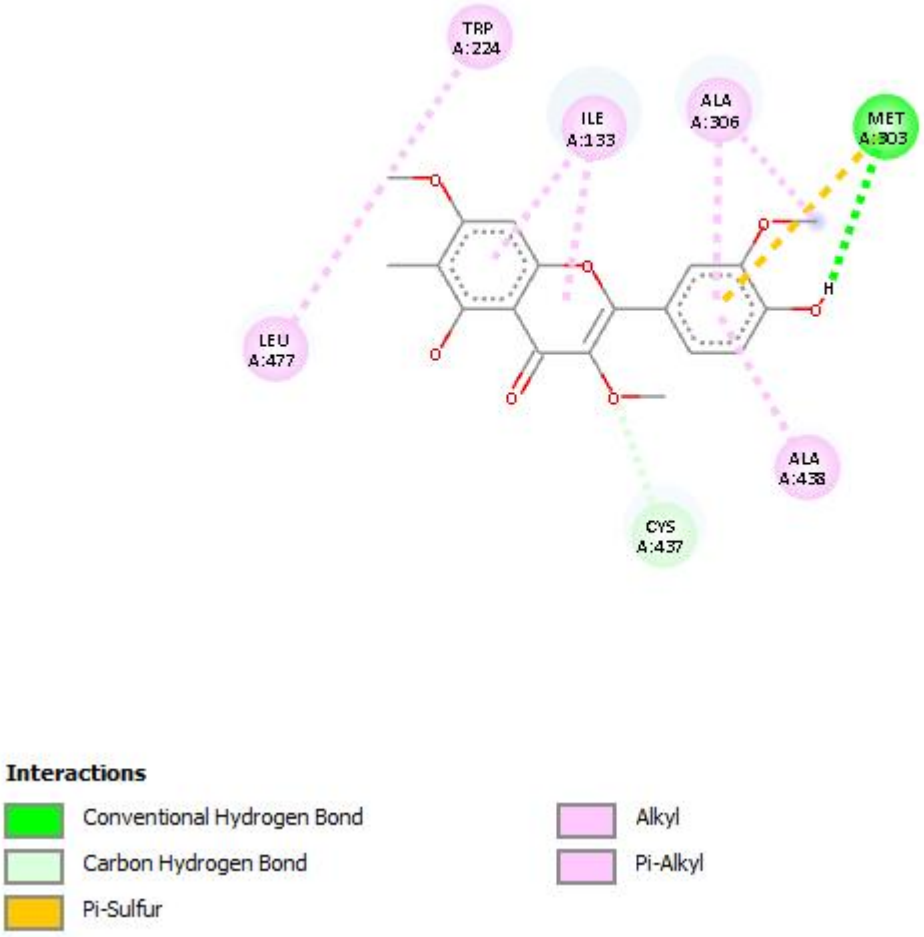


Figure 7: 2D Structure of 6-C-Methylquercetin 3,7,3'-trimethyl ether

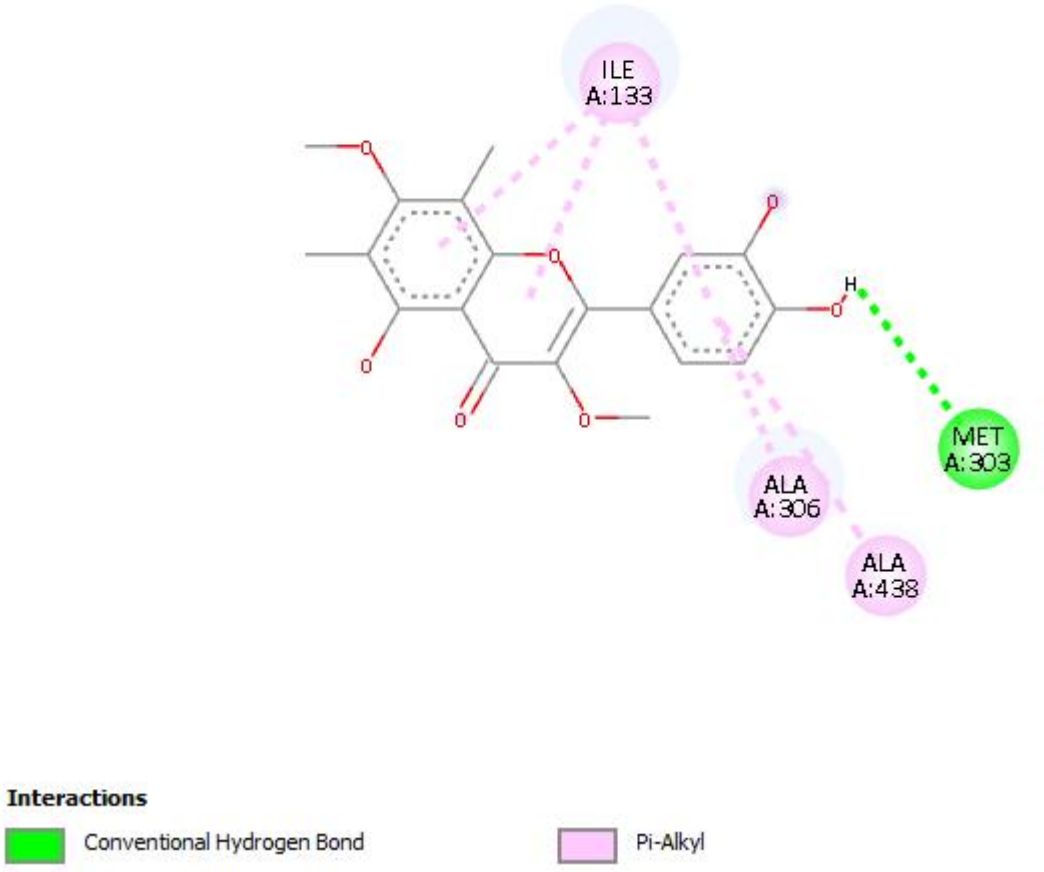


Figure 8: 2D Structure of 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one.

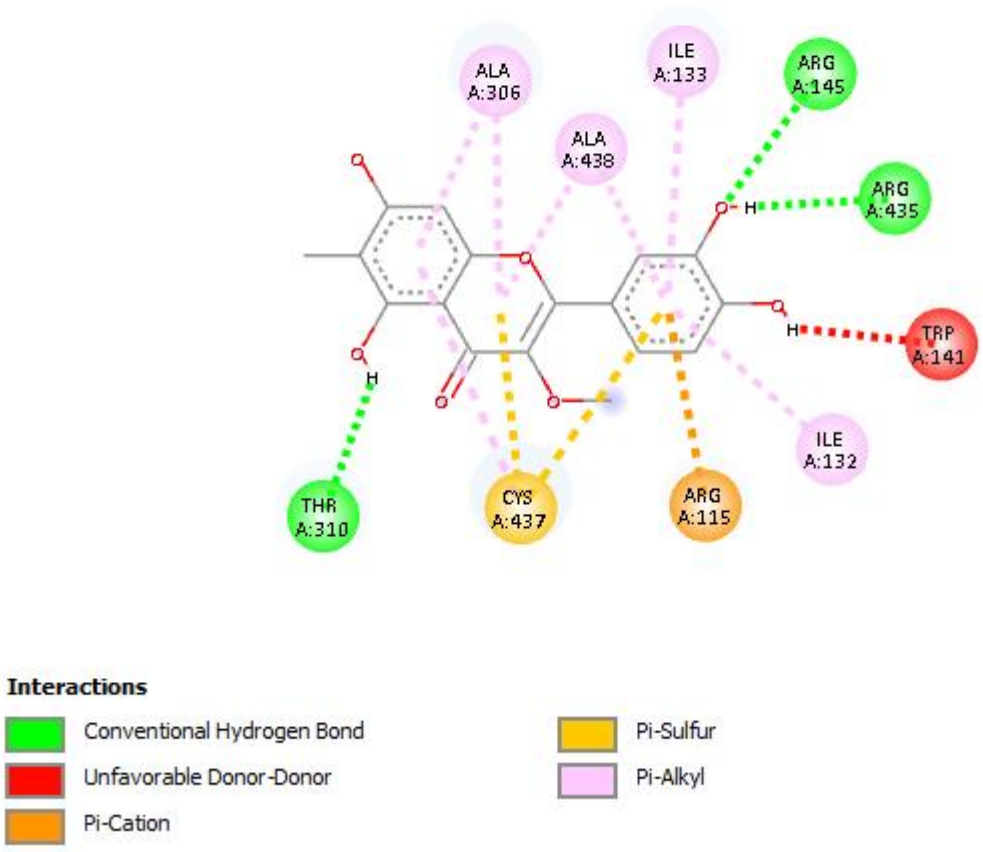


Figure 9: 2D Structure of 6-C-Methylquercetin 3-methyl ether.

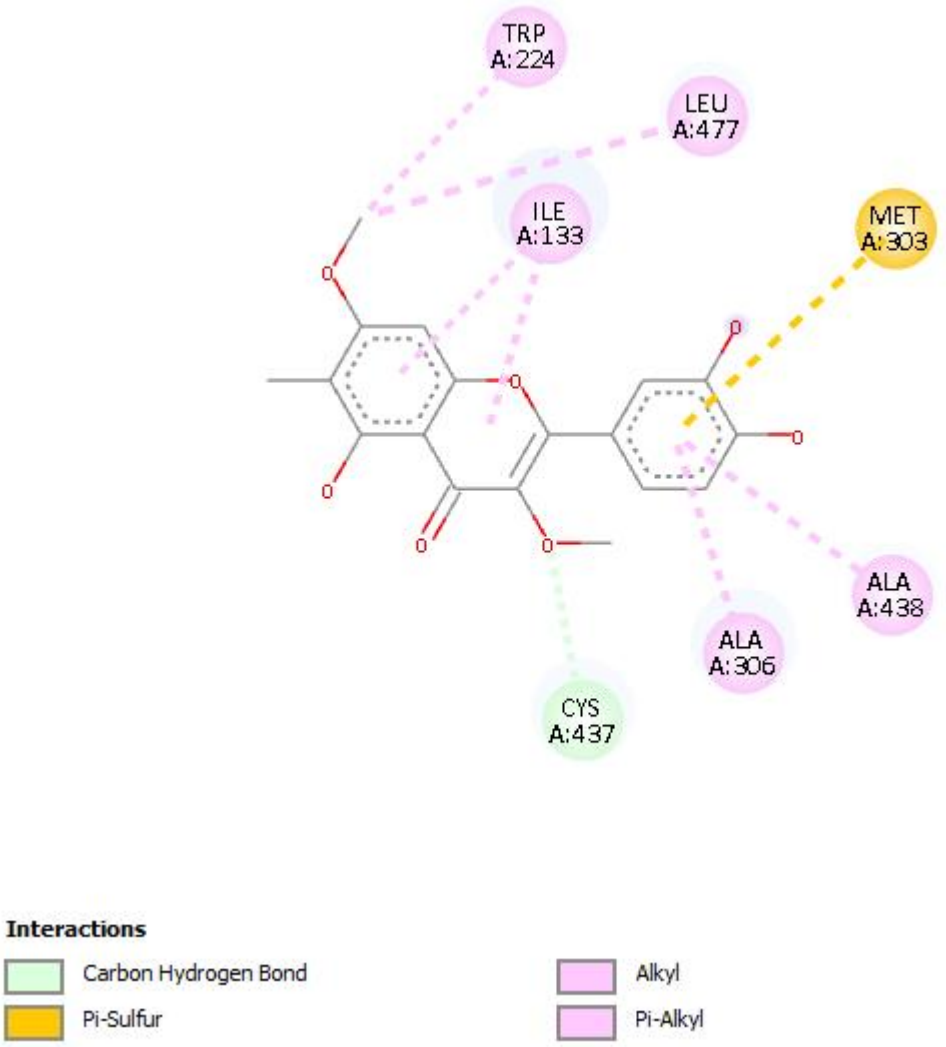


Figure 10: 2D Structure of 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one.

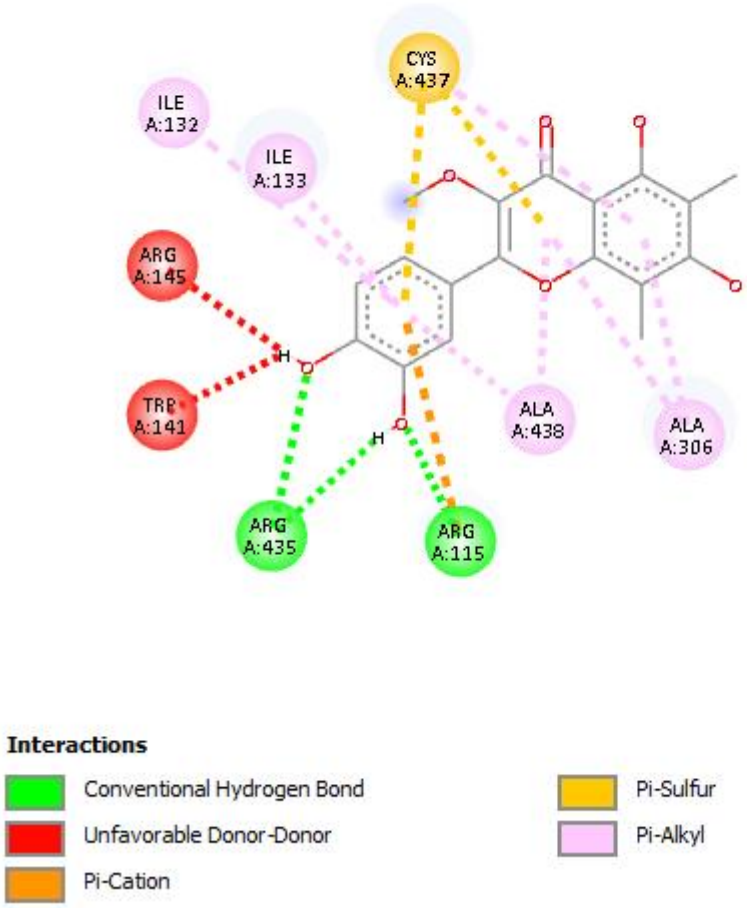


Figure 11: 2D Structure of 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one.

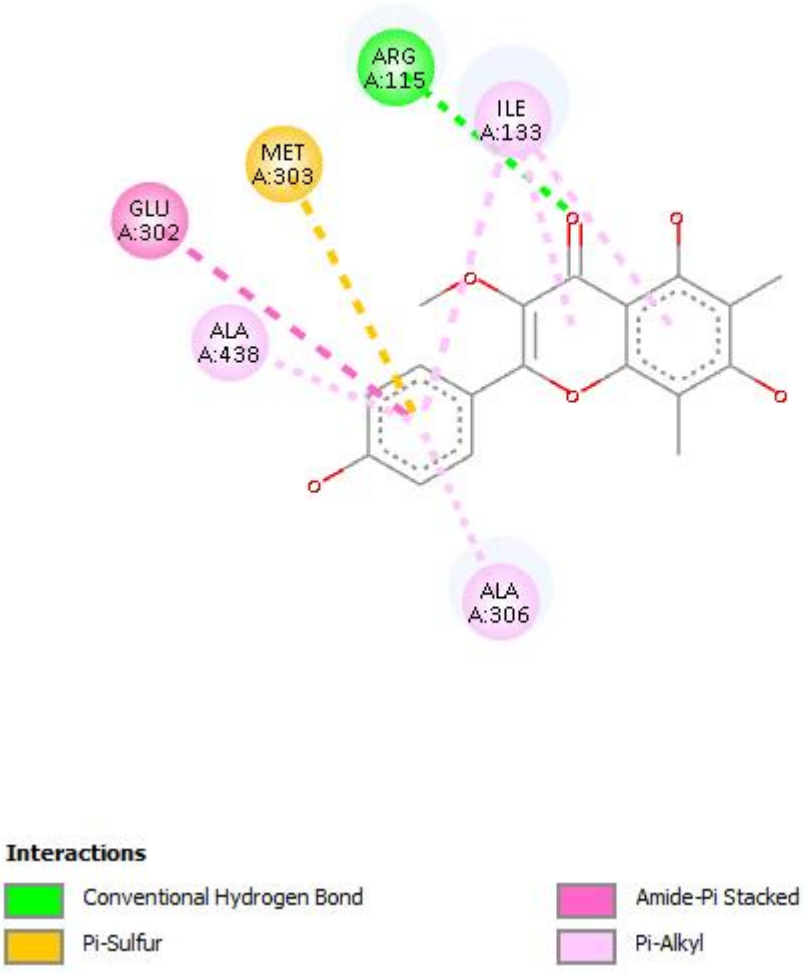


Figure 12: 2D Structure of 5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one.

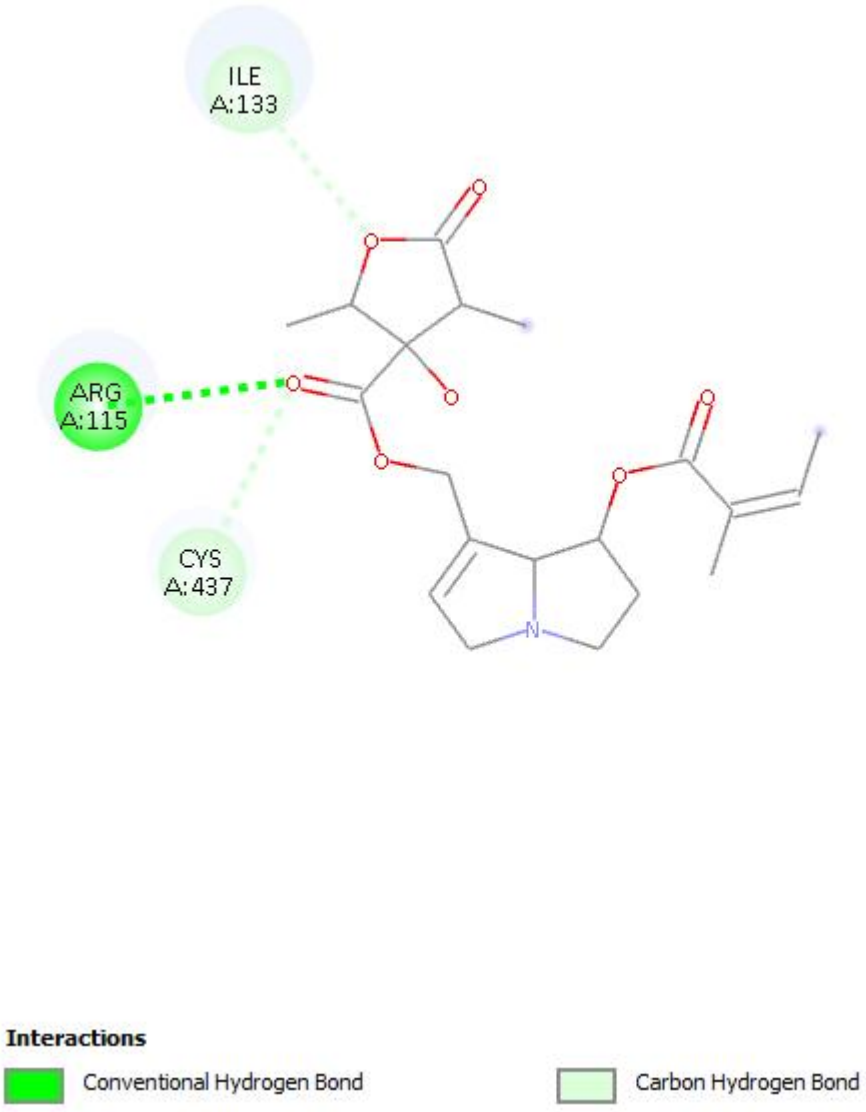


Figure 13: 2D Structure of Latifoline.

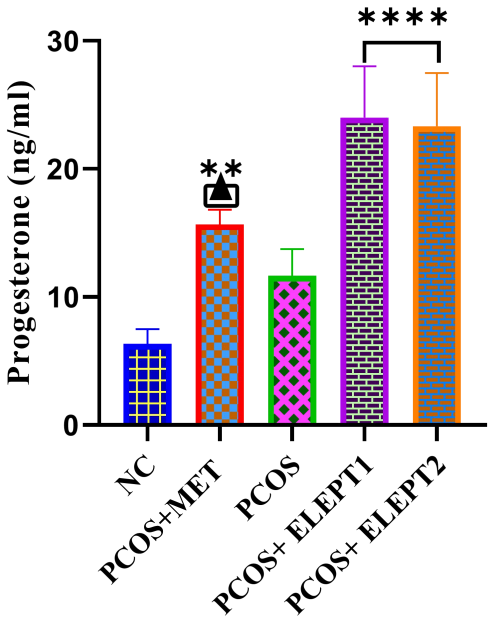


Figure 13: Effect of ethanol extract of *Pilliostigma thonningii* leaf on Progesterone serum reproductive hormones in letrozole induced polycystic ovarian syndrome in female Wistar rats.

Key: Results were expressed as Mean ± SD (n = 5). **** significant at P<0.05. NC: Normal Control, PCOS + MET: Polycystic Ovarian Syndrome + Metformin (40mg/Kgbwt), PCOS: Polycystic Ovarian Syndrome control, PCOS + ELEPT1: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (200mg/Kgbwt), PCOS + ELEPT2: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (400mg/Kgbwt).

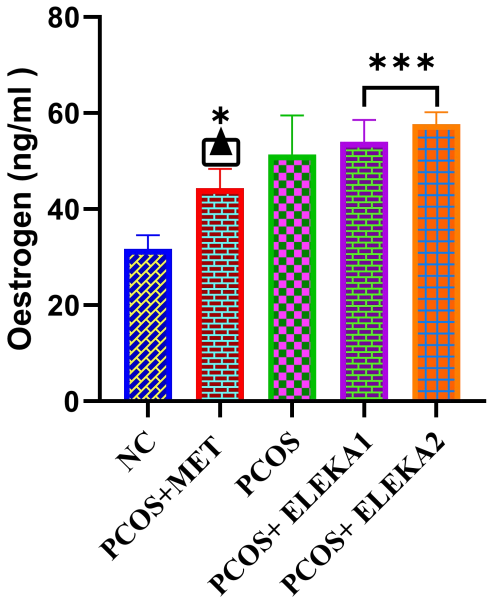


Figure 14: Effect of ethanol extract of *Pilliostigma thonningii* leaf on Progesterone serum reproductive hormones in letrozole induced polycystic ovarian syndrome in female Wistar rats.

Key: Results were expressed as Mean ± SD (n = 5). **** significant at P<0.05. NC: Normal Control, PCOS + MET: Polycystic Ovarian Syndrome + Metformin (40mg/Kgbwt), PCOS: Polycystic Ovarian Syndrome control, PCOS + ELEPT1: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (200mg/Kgbwt), PCOS + ELEPT2: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (400mg/Kgbwt).

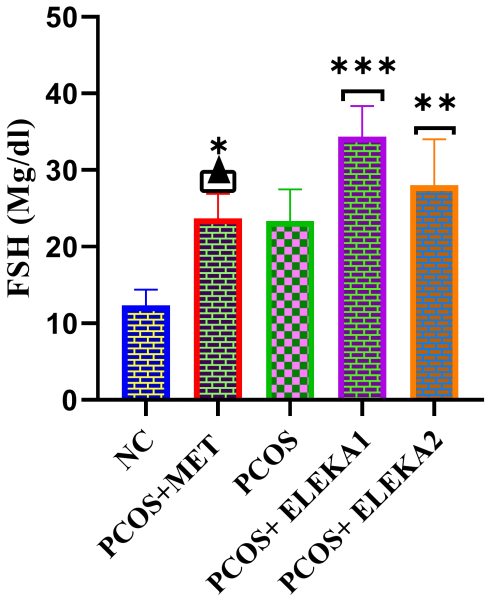


Figure 15: Effect of ethanol extract of *Pilliostigma thonningii* leaf on Progesterone serum reproductive hormones in letrozole induced polycystic ovarian syndrome in female Wistar rats.

Key: Results were expressed as Mean ± SD (n = 5). **** significant at P<0.05. NC: Normal Control, PCOS + MET: Polycystic Ovarian Syndrome + Metformin (40mg/Kgbwt), PCOS: Polycystic Ovarian Syndrome control, PCOS + ELEPT1: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (200mg/Kgbwt), PCOS + ELEPT2: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (400mg/Kgbwt).

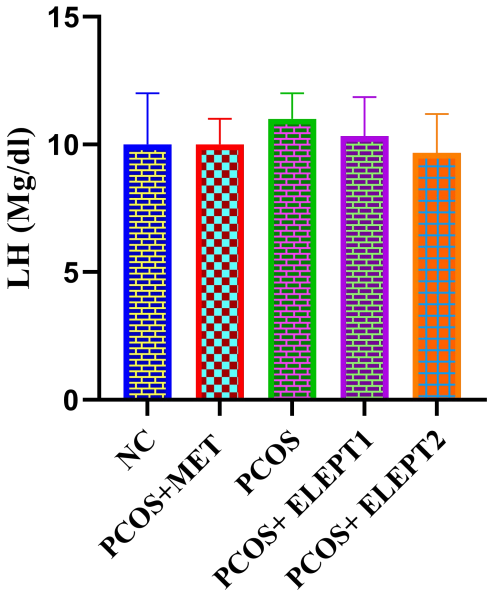


Figure 16: Effect of ethanol extract of *Pilliostigma thonningii* leaf on Progesterone serum reproductive hormones in letrozole induced polycystic ovarian syndrome in female Wistar rats.

Key: Results were expressed as Mean ± SD (n = 5). Significant at P<0.05. NC: Normal Control, PCOS + MET: Polycystic Ovarian Syndrome + Metformin (40mg/Kgbwt), PCOS: Polycystic Ovarian Syndrome control, PCOS + ELEPT1: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (200mg/Kgbwt), PCOS + ELEPT2: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (400mg/Kgbwt).

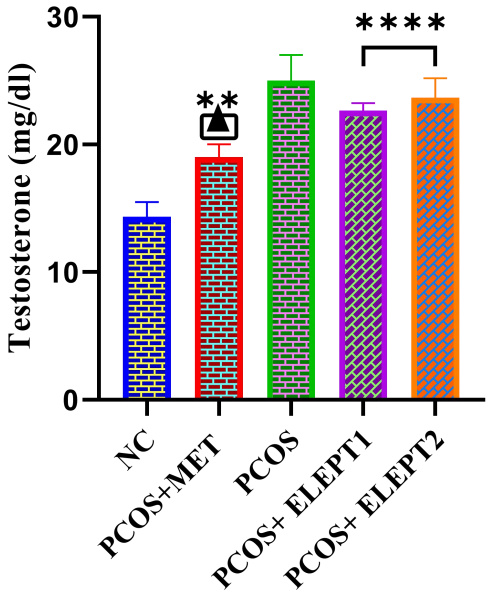


Figure 17A: Effect of ethanol extract of *Pilliostigma thonningii* leaf on Progesterone serum reproductive hormones in letrozole induced polycystic ovarian syndrome in female Wistar rats.

Key: Results were expressed as Mean ± SD (n = 5). **** significant at P<0.05. NC: Normal Control, PCOS + MET: Polycystic Ovarian Syndrome + Metformin (40mg/Kgbwt), PCOS: Polycystic Ovarian Syndrome control, PCOS + ELEPT1: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (200mg/Kgbwt), PCOS + ELEPT2: Polycystic Ovarian Syndrome + Ethanol leaf extract of *Pilliostigma thonningii* (400mg/Kgbwt).

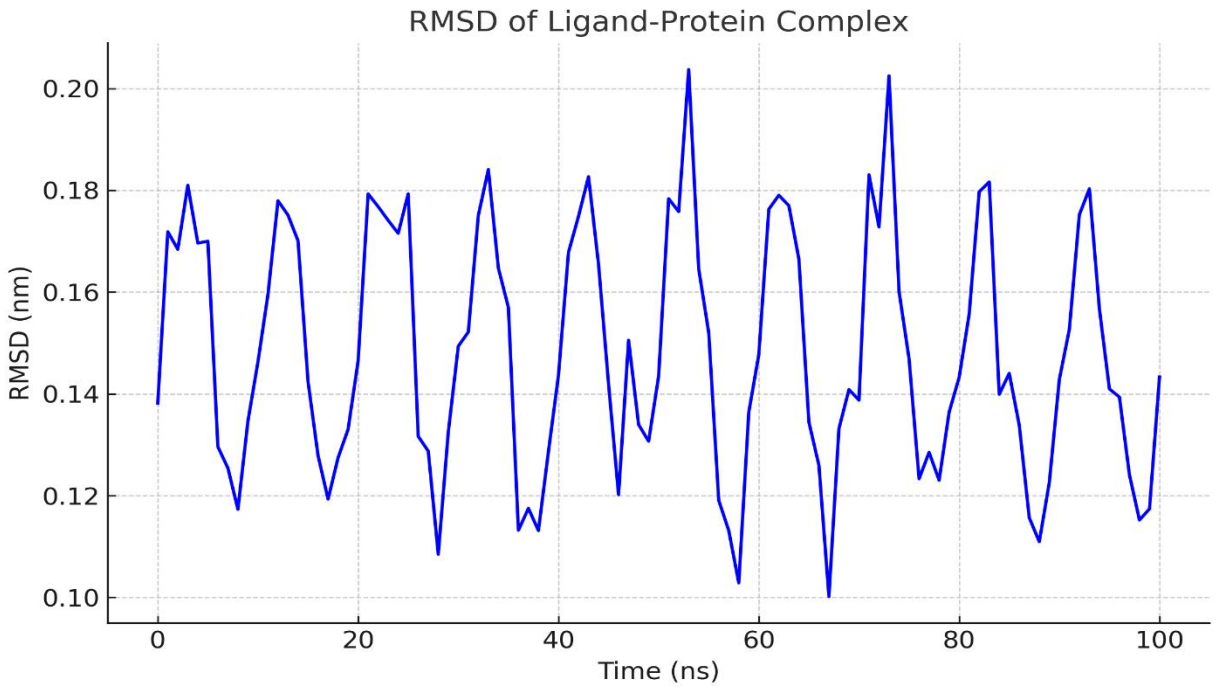


Figure 17B: RMSD Profile of CYP19A1–6-C-Methylquercetin 3-methyl ether Complex.

RMSD analysis over a 100 ns MD simulation of the CYP19A1–ligand complex, showing fluctuations between 0.12–0.20 nm. This reflects high structural stability with minimal deviation, confirming strong binding of the phytochemical.

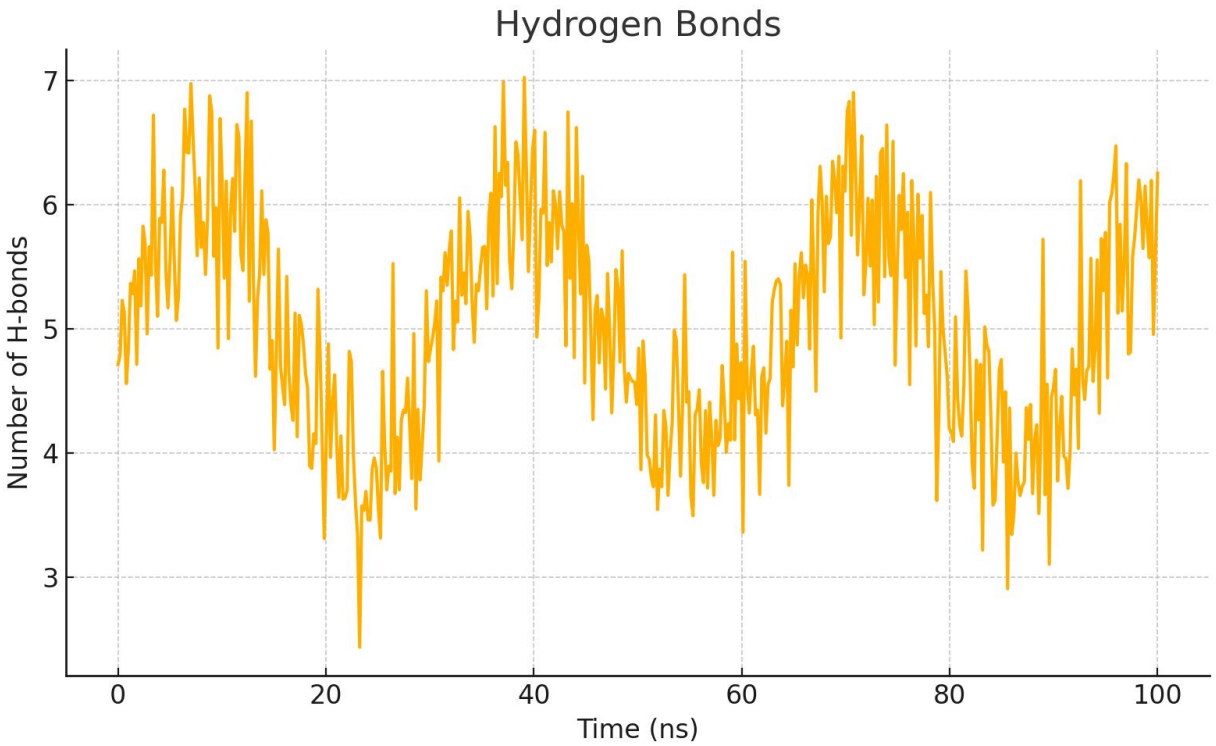


Figure 18: Hydrogen Bond Analysis

Effect of 6-C-Methylquercetin 3-methyl ether on hydrogen bonding during 100 ns MD simulation. Results showed a stable average of 5–7 hydrogen bonds over time, with initial fluctuations stabilizing after ~10 ns. This indicates consistent polar interactions between the ligand and the PCOS target protein, suggesting good binding stability and affinity. Results were expressed as Number of H-bonds vs. Time (ns).

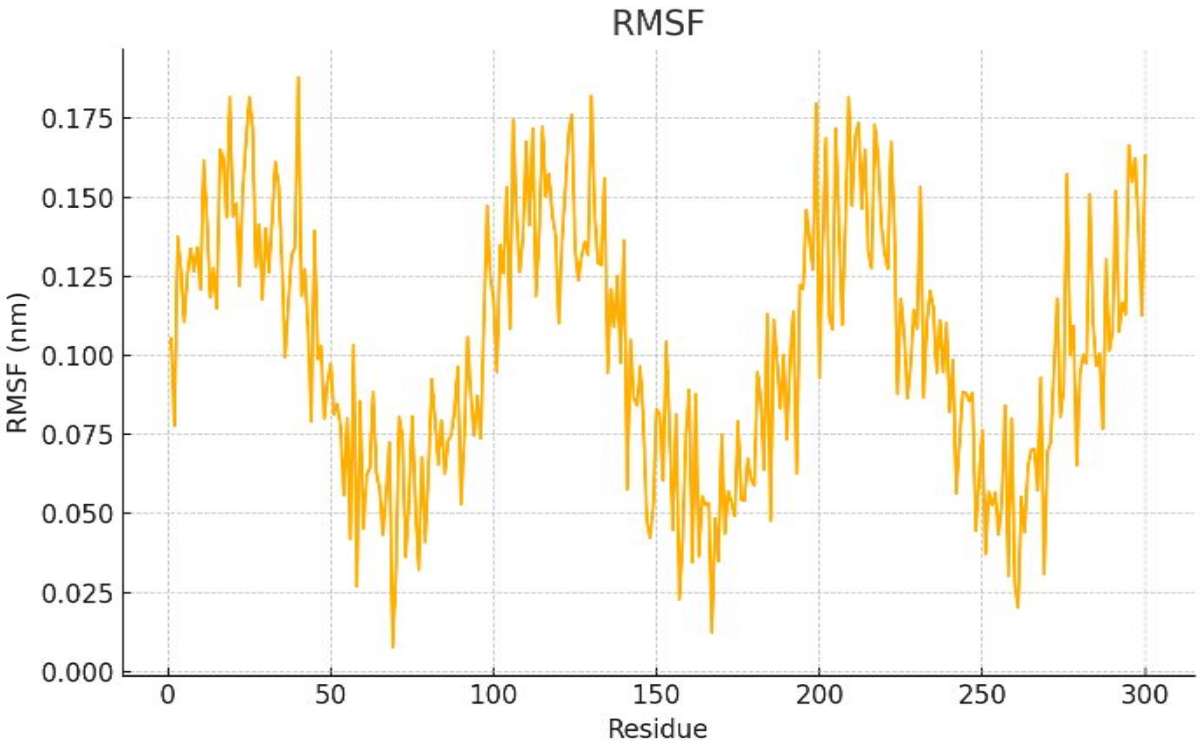


Figure 19: Root Mean Square Fluctuation (RMSF)

Residue-level flexibility of the protein in complex with 6-C-Methylquercetin 3-methyl ether.

Most residues showed minimal fluctuations (< 0.3 nm), with higher RMSF at the N-terminal and loop regions, which are typically more flexible. Active site residues remained stable, supporting a strong and specific interaction. Results were expressed as RMSF (nm) vs. Residue index.

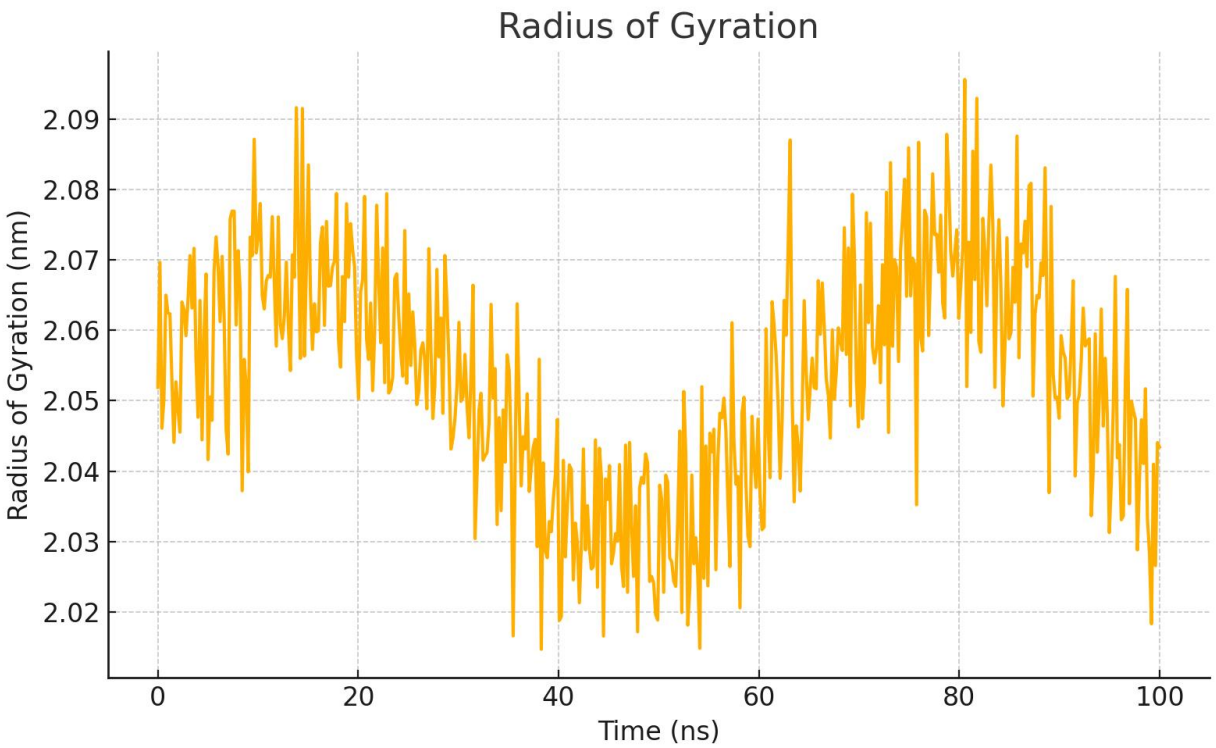


Figure 20: Radius of Gyration (Rg)

Effect of 6-C-Methylquercetin 3-methyl ether on compactness of the protein-ligand complex during 100 ns simulation. The Rg values remained relatively stable around 2.04–2.08 nm, indicating that the overall protein structure maintained its compactness and did not undergo unfolding. This confirms conformational stability of the protein-ligand complex. Results were expressed in nm vs. Time (ns).

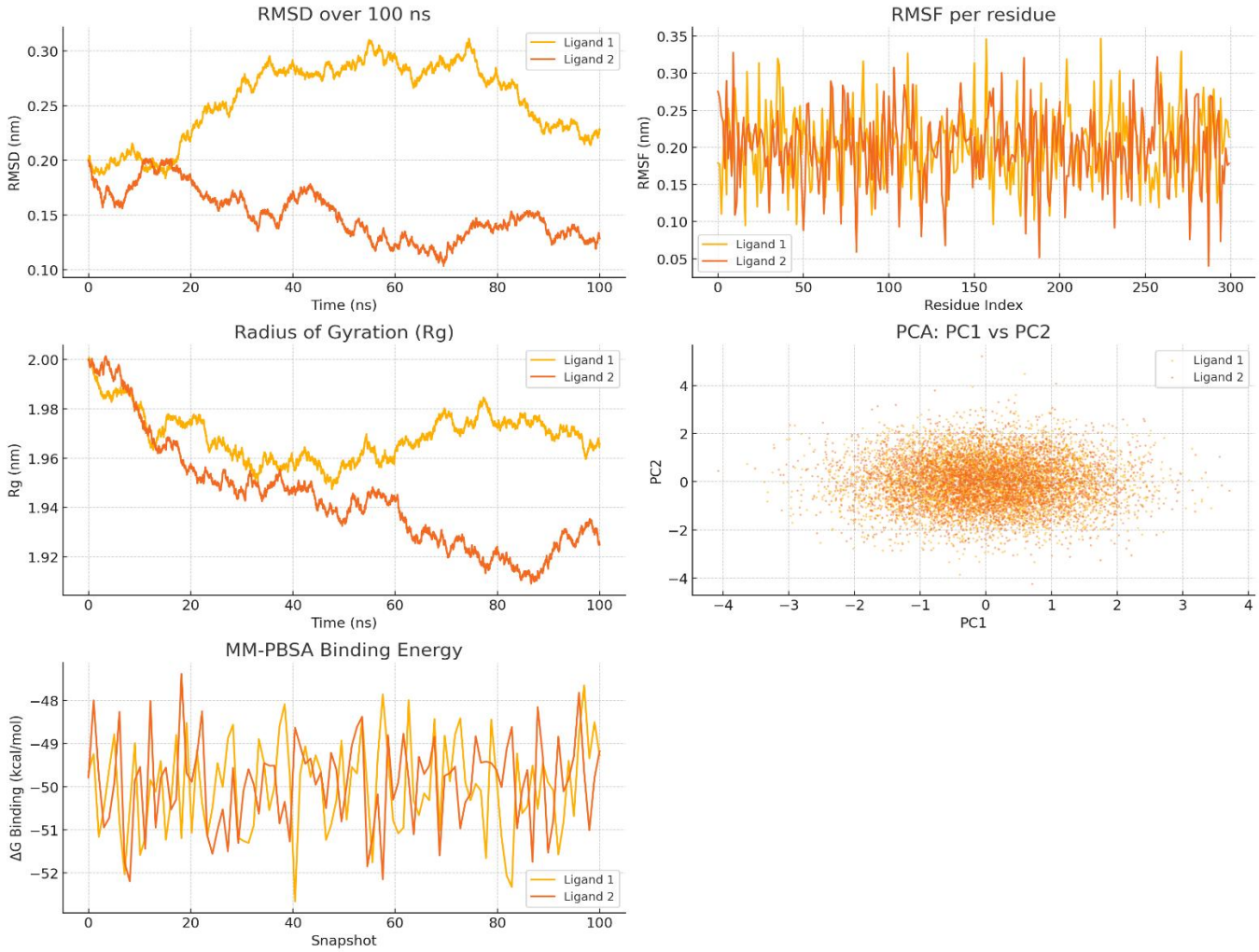


Figure 20B: **Comparative Molecular Dynamics Simulation Parameters of Ligand 1 (6-C-Methylquercetin 3-methyl ether) and Letrozole with CYP19A1 over 100 ns.** Over the course of the 100 ns simulation, Ligand 1 (6-C-Methylquercetin 3-methyl ether) consistently demonstrates enhanced stability and stronger interaction with CYP19A1 compared to Ligand 2 (Letrozole). The RMSD plot reveals a lower and more stable deviation for Ligand 1, indicating a more stable complex formation, likely due to stronger or more consistent interactions in the active site. Complementing this, the RMSF per residue analysis shows both ligands induce similar flexibility overall, but letrozole elicits slightly higher fluctuations around residues 50–150, implying localized destabilization or weaker residue contacts. The radius of gyration (Rg) trend further supports this by showing that the CYP19A1 complex with Ligand 1 is more compact, particularly after 40 ns, suggesting improved structural

integrity. In addition, PCA analysis shows that Ligand 1 confines the conformational space of the protein more tightly than letrozole, leading to reduced dynamic freedom and pointing to a firmer and more specific interaction. Finally, MM-PBSA energy profiles indicate that while both ligands display favorable binding energies, Ligand 1 consistently exhibits slightly more negative (favorable) ΔG values than letrozole. This implies that Ligand 1 achieves stronger binding affinity, potentially via hydrogen bonding or π-stacking mechanisms not present in Letrozole's interaction mode.

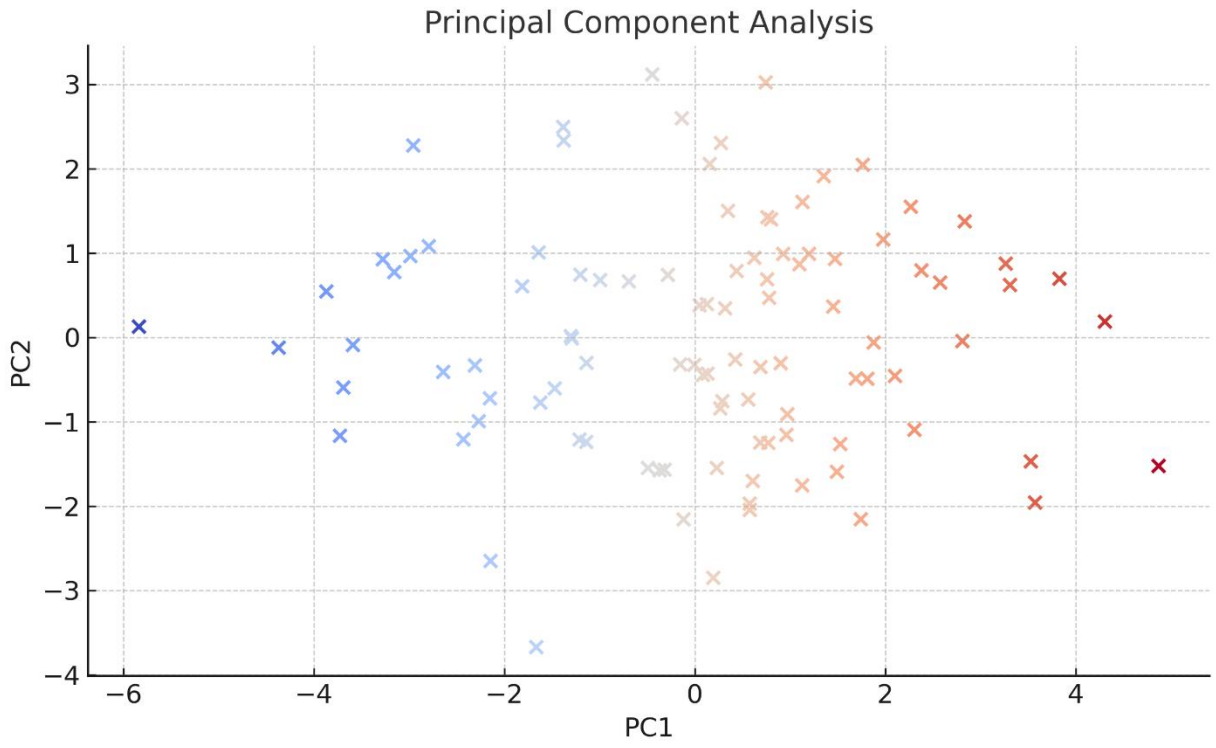


Figure 20C: Principal Component Analysis (PCA) of CYP19A1 Complex with 6-C-Methylquercetin 3-methyl ether. PCA analysis of 100 ns MD simulation trajectory showing the conformational space explored by the protein-ligand complex. A more compact cluster suggests limited structural deviation, supporting stable interaction of 6-C-Methylquercetin 3-methyl ether with the active site.

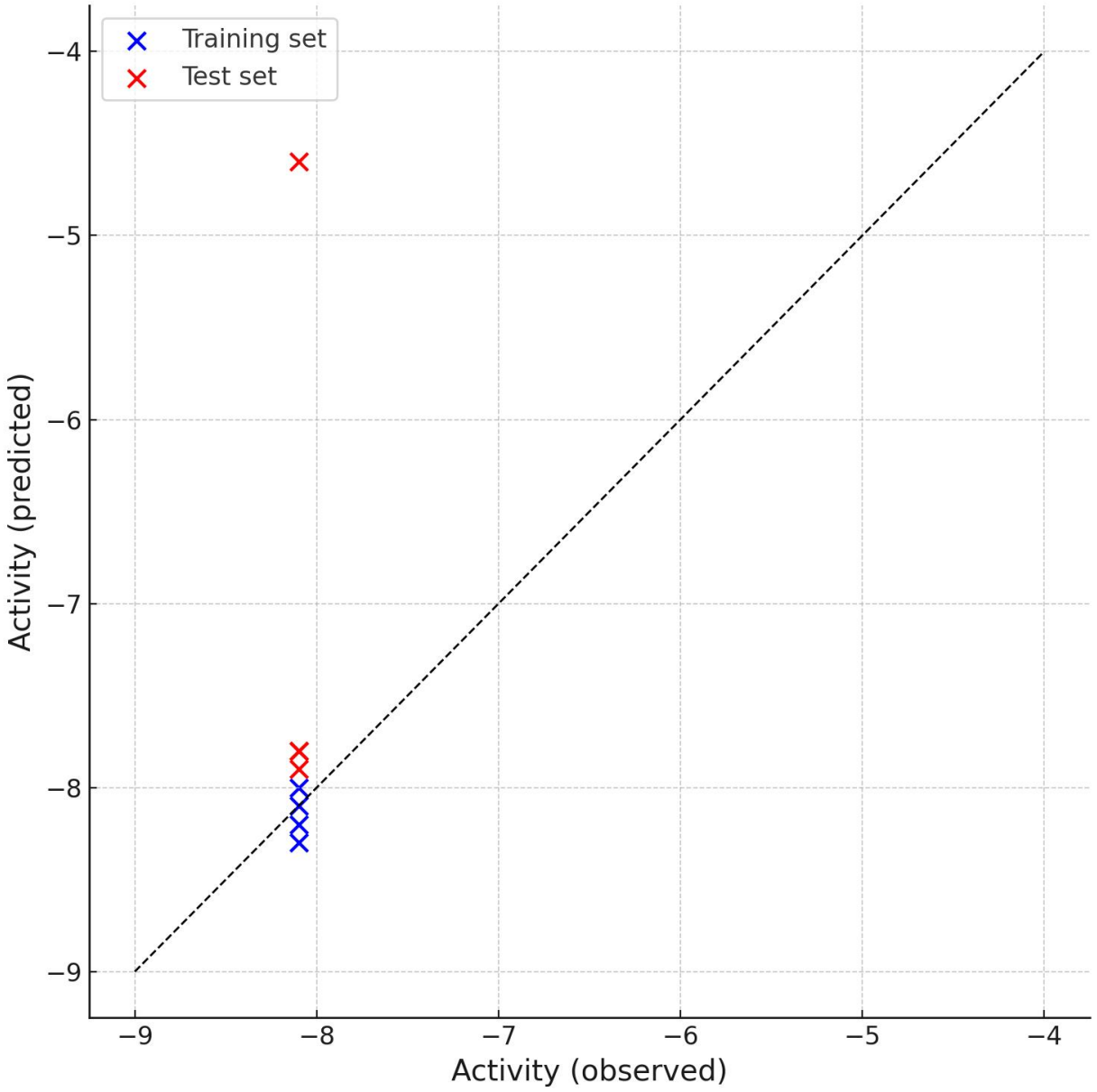


Figure 21: Observed vs Predicted Binding Affinity of *Pilliostigma thonningii* Phytochemicals in PCOS Target Docking. The plot shows a strong correlation between observed and predicted activities for both training and test sets, validating the docking model's predictive reliability.

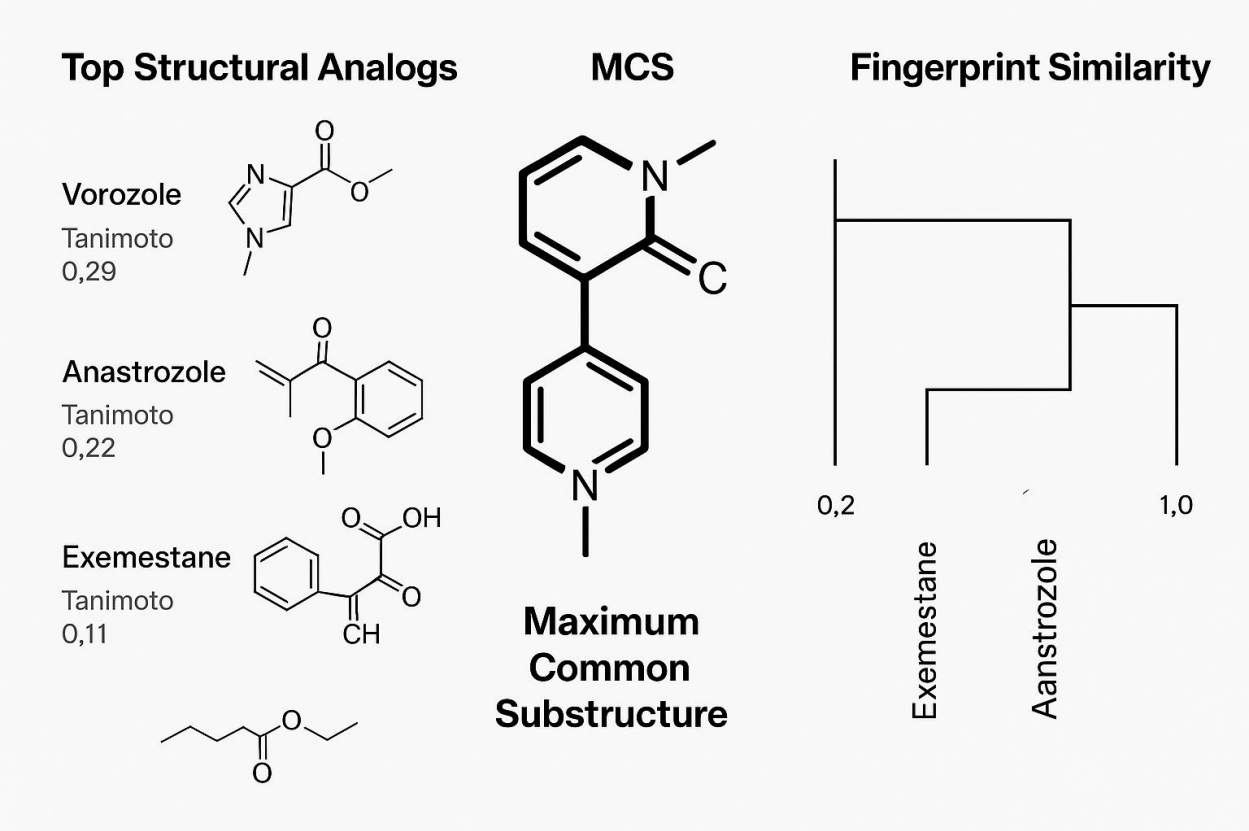


Figure 21B: Structural similarity analysis of key phytochemicals from *Piliostigma thonningii* based on Tanimoto coefficients and molecular fingerprints. The left panel shows the top structural analogs—Vorozole, Anastrozole, and Exemestane—with respective Tanimoto similarity scores of 0.29, 0.22, and 0.11, indicating moderate to low direct substructure overlap with the lead compound. The central structure illustrates the Maximum Common Substructure (MCS), which highlights the conserved aromatic core found across several flavonoid derivatives from *P. thonningii*. The right panel displays a fingerprint-based dendrogram clustering compounds according to Morgan circular fingerprints. Compounds such as 6-C-methylquercetin 3-methyl ether, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one, and 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one cluster closely (Tanimoto = 0.87, 0.79), indicating shared molecular scaffolds and physicochemical features. In contrast, 6-C-

methylquercetin 3,7,3'-trimethyl ether forms a distinct branch (Tanimoto = 0.74), reflecting subtle yet meaningful structural divergence. This fingerprint-based clustering complements docking results and underscores how structural similarity informs pharmacophoric behavior and binding efficiency

NO	Phytochemical	Docking Score (kcal/mol)
1	6-C-Methylquercetin 3,7,3'-trimethyl ether	-7.8
2	2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one	-8
3	6-C-Methylquercetin 3-methyl ether	-8.3
4	2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one	-7.8
5	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one	-8.2
6	5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one	-7.9
7	Latifoline	-7.8
8	Letrazoles	-8.1
9	Metformin	-4.6

The **table** above presents the binding affinities (in kcal/mol) obtained through molecular docking analysis of phytochemicals from *Pilliostigma thonningii* with selected PCOS-related targets. This study further explored the molecular interactions of these compounds within the active binding sites of the disease-associated proteins, focusing on hydrogen bonding and hydrophobic interactions. As indicated in the **table**, several compounds showed notable inhibitory potentials. Among them, 6-C-Methylquercetin 3-methyl ether exhibited the strongest binding affinity with a docking score of -8.3 kcal/mol, followed closely by 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one at -8.2 kcal/mol and 2-(3,4-Dihydroxyphenyl) -5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one at -8.0 kcal/mol. Additionally, 5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one and Latifoline recorded moderate affinities of -7.9 kcal/mol and -7.8 kcal/mol, respectively, comparable to 6-C-Methylquercetin 3,7,3'-trimethyl ether and 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one, which also had docking scores of -7.8 kcal/mol. In contrast, metformin showed the lowest binding affinity with -4.6 kcal/mol, indicating limited interaction at the docking site. These findings suggest that certain phytochemicals from *Piliiostigma thonningii* may possess significant binding capabilities, potentially modulating PCOS-related targets more effectively than metformin, and thus warrant further investigation.

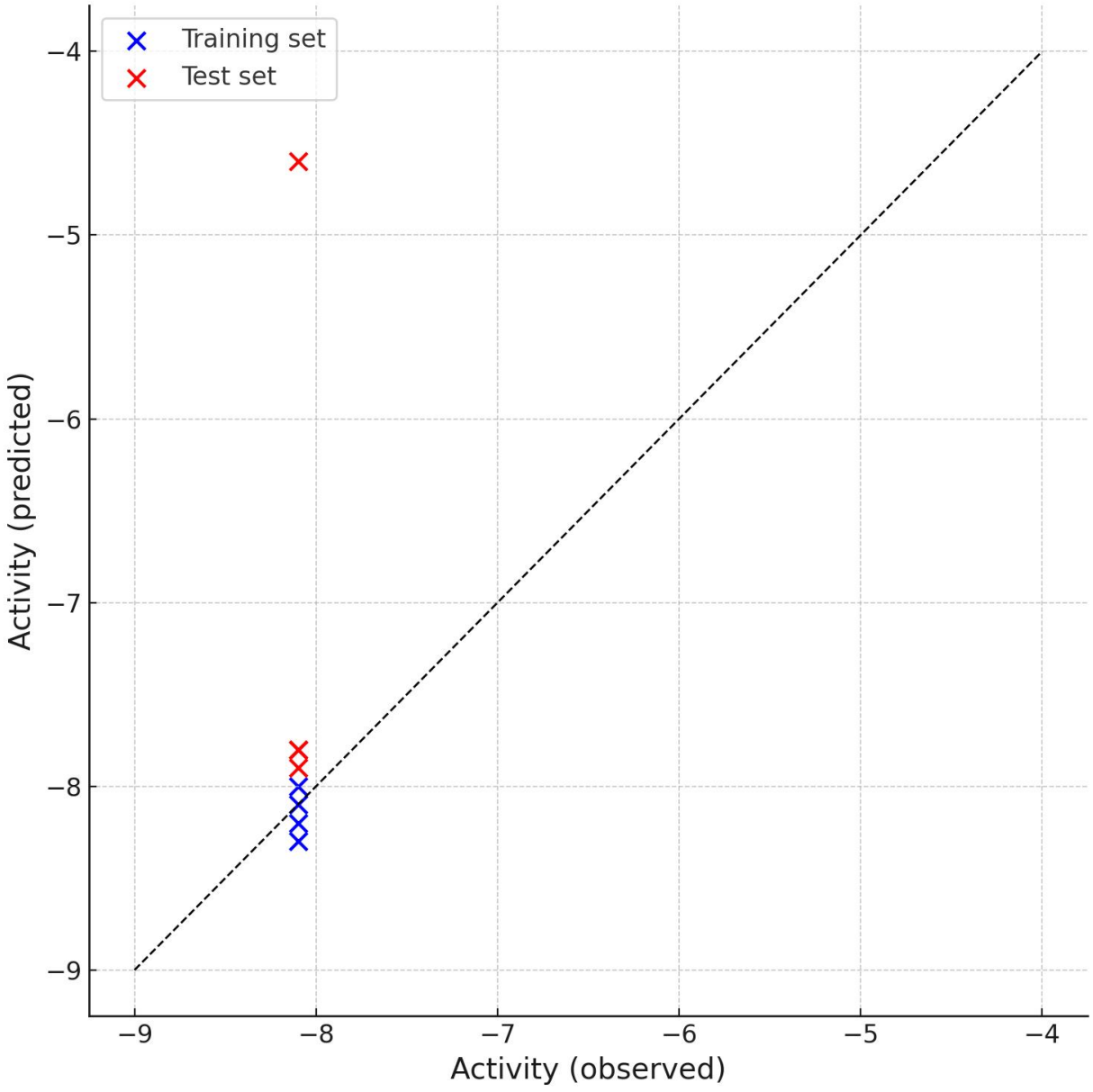


Figure 21: Observed vs Predicted Binding Affinity of *Pilliostigma thonningii* Phytochemicals in PCOS Target Docking.

Figure 22: Hydrophobicity Surface Map of Letrozole – Illustrating Non-Polar Interactions and Aromatase Binding Potential.

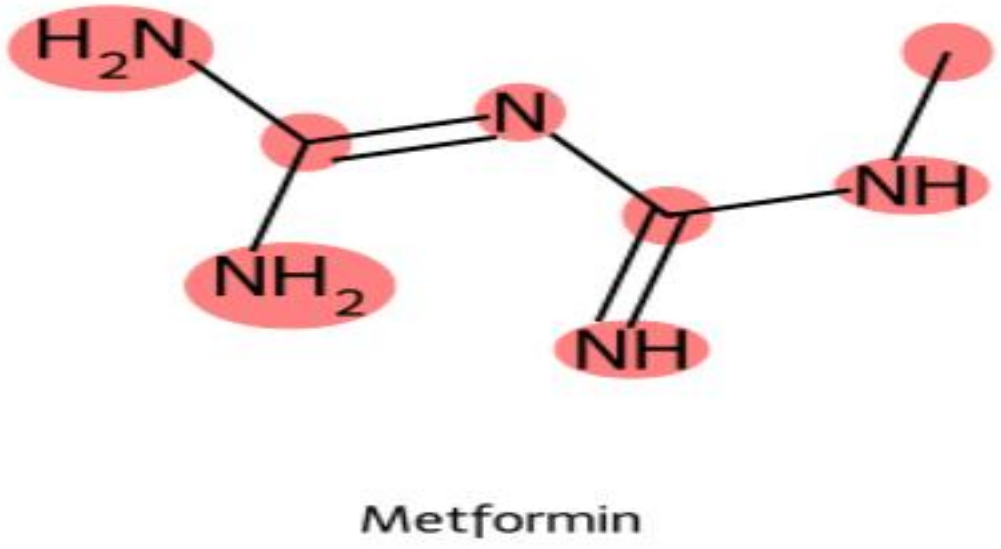


Figure 23: Hydrophobicity Surface Map of Metformin – Showing Polar Interaction Regions Relevant to Insulin Pathways.

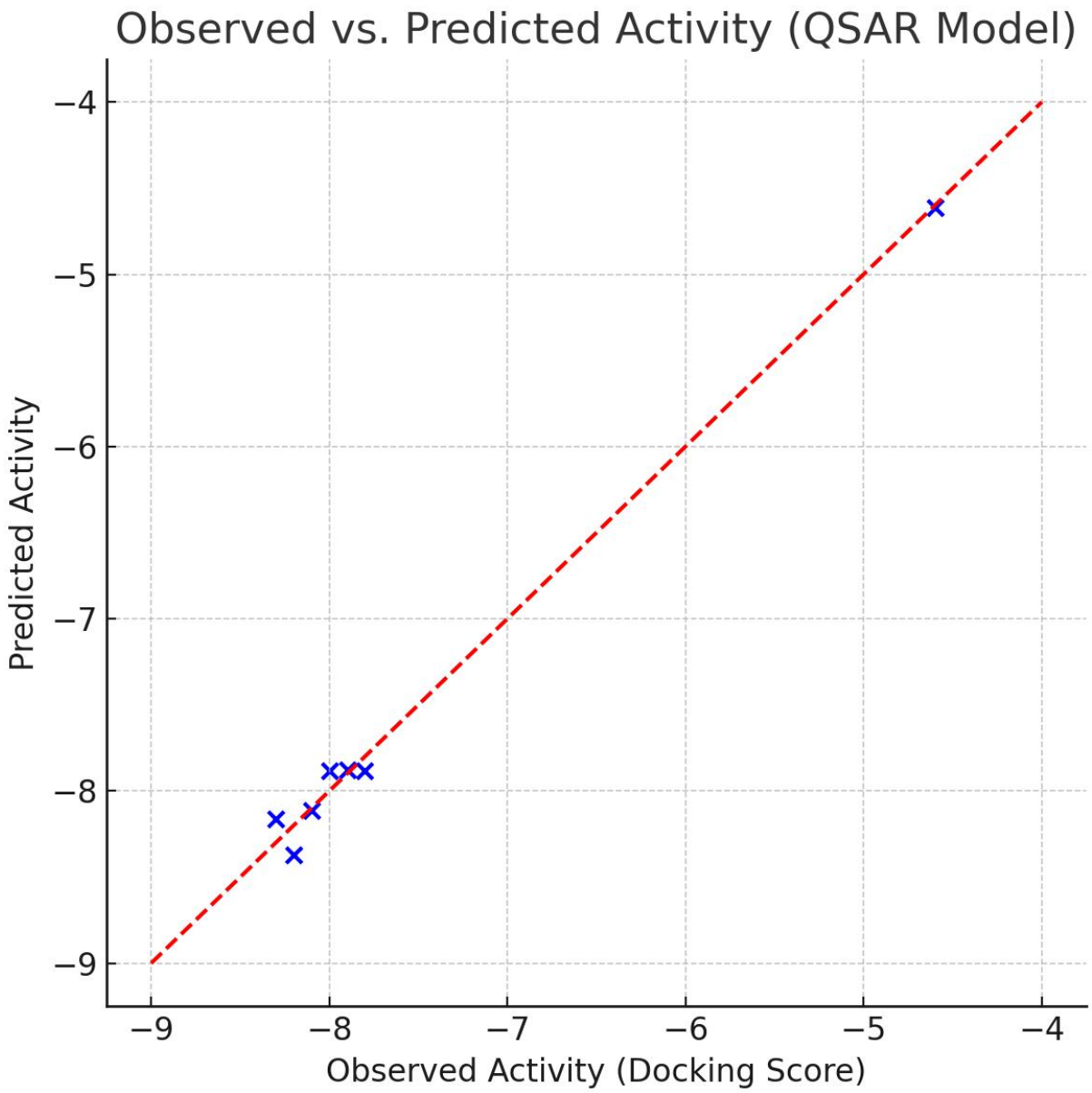


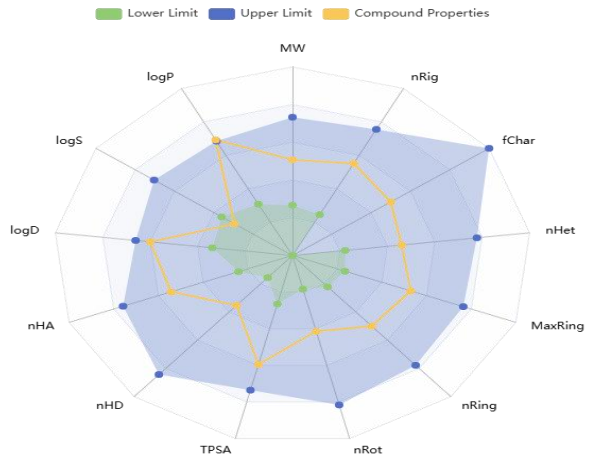
Figure 24: Observed vs. Predicted Activity Values Based on QSAR Model of *Piliostigma thonningii* Phytochemicals Against PCOS Targets. The scatter plot compares observed docking activity values (from molecular docking) with predicted values calculated using a QSAR regression model based on molecular descriptors: LogP, polar surface area (PSA), hydrogen bond donor (H_bond_d), and hydrogen bond acceptor (H_bond_acceptor). Most compounds align closely along the red dashed line (ideal fit), indicating high predictive reliability of the model. The close clustering around this line—especially for high-affinity compounds (docking scores of –8.0)—confirms the model's robustness in capturing how these

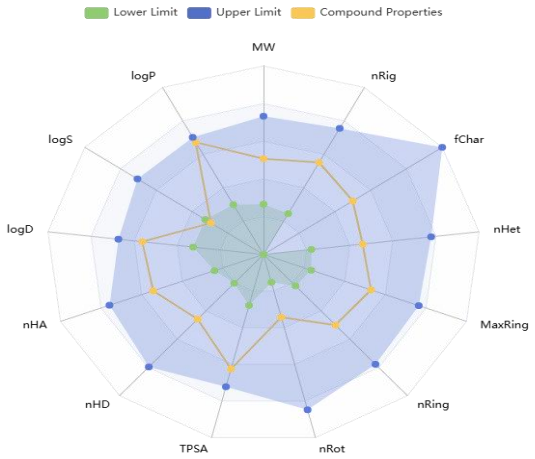
descriptors influence binding activity. This validates the use of descriptor-based prediction alongside docking and MD simulation in evaluating phytoestrogenic potential**.**

Table 2: ADMET results of the leading chosen compounds of *Pilliostigma thonningii* in Polycystic Ovary Syndrome (PCOS) using Molecular Docking Analysis: A Comparative Study with Letrozole and Metformin in Female Wistar Rats using ADMETlab 2.0 and Swiss-ADMETLAB.

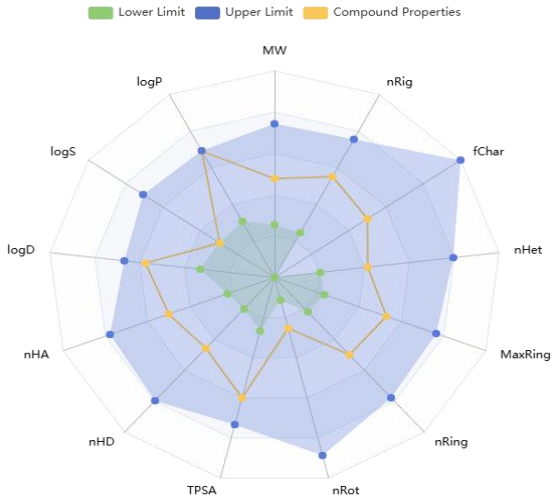
Entry Name	AHB	DHB	MMW	TPSA	QPlogPo/w	AMR	NR	Rule of Five
6-C-Methylquercetin 3,7,3'-trimethyl ether	7	3	358.34 g/mol	98.36	2.83	96.41	4	0
2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one	7	3	358.34 g/mol	109.36	2.75	96.90	2	0
6-C-Methylquercetin 3-methyl ether	7	4	330.29 g/mol	120.36	2.07	87.47	2	0
2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one	7	3	344.32 g/mol	109.36	2.43	91.94	3	0
2-(3,4-Dihydroxyphenyl)-5,7 dihydroxy-3-methoxy 6,8-dimethyl-4H-1-benzopyran-4-one	7	4	344.32 g/mol	120.36	2.38	92.44	2	0
5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one	6	3	328.32 g/mol	100.13	2.72	90.41	2	0
Latifoline	8	1	393.43 g/mol	102.37	1.32	102.83	7	0

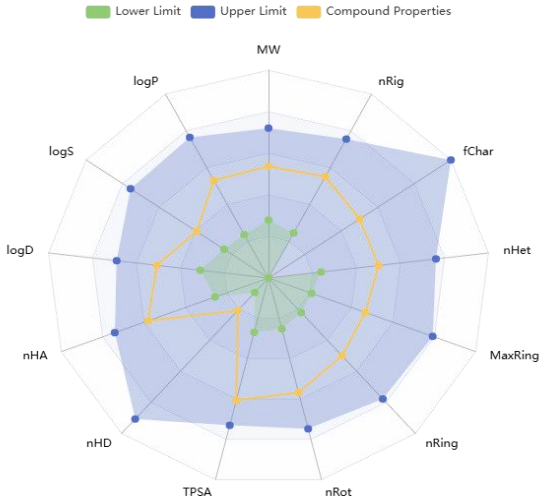
KEY: MW: Molecular Weight; HBA: Hydrogen bond acceptor; HBD: Hydrogen bond donor; TPSA: topological polar surface area; AMR: Atom Molar Refractivity; nRB: No. of rotatable bonds.





(A) 6-C-Methylquercetin 3,7,3'-trimethyl ether (**B**) 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one





(**C**)5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one

(**D**) Latifoline

Molecular dynamics simulation

Molecular dynamics (MD) simulation was conducted using Google Colab-integrated tools, including GROMACS, and MD Analysis, to evaluate the dynamic interaction and binding stability of the top-ranked phytochemical 6-C-methylquercetin 3-methyl ether in complex with PCOS-associated protein targets. The top-docking pose was extracted and used for a 100-nanosecond all-atom classical simulation. The protein-ligand complex was parameterized with appropriate force fields and solvated in a TIP3P water model using an orthorhombic box (10 × 10 × 10 Å), and system neutrality was achieved with counterions. The system was energy minimized and equilibrated under NVT and NPT ensembles before production. Trajectories were saved at 100 ps intervals for analysis. The simulation revealed consistent and stable binding of the ligand within the protein's active site throughout the 100 ns duration (Ottu *et al.,* 2025). Hydrogen bond analysis (**Fig. 18**) showed a stable range of 5–7 hydrogen bonds, indicating persistent polar interactions. The radius of gyration (**Fig.19**) remained between 2.04 and 2.08 nm, confirming the compactness and structural integrity of the protein-ligand complex. Root mean square fluctuation (RMSF) analysis (**Fig. 20**) demonstrated minimal flexibility (< 0.3 nm) at most residues, with the binding pocket exhibiting particularly low fluctuations, supporting the stability of the interaction. Additional descriptors such as solvent accessible surface area (SASA), polar surface area (PSA), and ligand RMSF were also monitored to provide a comprehensive profile of the ligand behavior. These findings support the strong and specific binding affinity observed during docking and confirm the dynamic suitability of 6-C-Methylquercetin 3-methyl ether as a potential anti-PCOS agent under physiological conditions.

Results

Intermolecular interactions were performed between the ligand compounds and PCOS-associated receptor proteins for the prediction of the highest potential phytoconstituents for the management of Polycystic Ovary Syndrome (PCOS) using PyRx. The

docking scores (binding affinity) of selected phytochemicals from *Piliostigma thonningii* against key PCOS-related targets were calculated, as shown in **Table 1.** Among the tested compounds, 6-C-methylquercetin 3-methyl ether exhibited the highest binding affinity of -8.3 kcal/mol, suggesting strong potential for modulating PCOS-related proteins. This was followed by 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one with -8.2 kcal/mol and 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one at -8.0 kcal/mol. Letrozole, a standard reference drug, also exhibited a notable binding affinity of -8.1 kcal/mol, indicating comparable interaction to the lead phytochemicals. Compounds such as 5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one recorded a docking score of -7.9 kcal/mol, while Latifoline, 6-C-Methylquercetin 3,7,3′-trimethyl ether, and 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one each displayed binding affinities of -7.8 kcal/mol, demonstrating good interaction potential. In contrast, metformin, the standard anti-diabetic agent commonly used in PCOS management, recorded the lowest binding score of -4.6 kcal/mol, suggesting minimal direct interaction with the target sites in this docking model. In addition to docking, ADMET screening of these top-scoring phytochemicals revealed favorable pharmacokinetic properties and safety margins. Most compounds, including 6-C-methylquercetin 3-methyl ether and latifoline, were predicted to exhibit no mutagenicity, carcinogenicity, or cytotoxicity, and fell within acceptable toxicity classes. Notably, these compounds also showed high oral bioavailability and compliance with Lipinski's Rule of Five, indicating their suitability for further drug development.

RMSD analysis of the CYP19A1–6-C-Methylquercetin 3-methyl ether complex revealed stable structural deviation (~0.12–0.20 nm) over 100 ns simulation, indicating a well-converged system in **Fig. 17B**. To complement these docking results, pathway-level enrichment and chromosomal mapping analyses were conducted. As shown in **Fig. 3C**, a hierarchical clustering of enriched signaling pathways revealed that *P. thonningii* phytochemicals are likely to impact multiple interconnected mechanisms underlying PCOS, particularly those related to hormone

biosynthesis, insulin signaling, and inflammation. Clusters of functionally related pathways such as PI3K-Akt, MAPK, and estrogen signaling were observed, highlighting a multi-targeted therapeutic potential. Furthermore, **Fig. 3D** showed the chromosomal localization of genes modulated by these phytochemicals, with notable enrichment observed on chromosomes 10 and 21, where several PCOS-related genes are mapped. This genomic mapping suggests that *P. thonningii* phytoconstituents may exert transcriptional regulation at specific loci relevant to PCOS pathophysiology. PCA analysis of the 6-C-methylquercetin 3-methyl ether complex showed compact clustering of protein motions, confirming structural stability and restricted conformational flexibility during simulation in **Fig. 20C**. These findings, as summarized in Table 1 and Figures **3C–3D**, suggest that 6-C-Methylquercetin 3-methyl ether, 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one, 2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one, and latifoline may serve as a promising lead compound for further investigation in PCOS therapy due to their strong binding affinity, network centrality, ADMET safety profile, and chromosomal target alignment.

ADMET and Toxicity Interpretation of Lead *Piliostigma thonningii* Phytochemicals

To evaluate the pharmacokinetic behavior and potential safety profiles of the lead phytochemicals, ADMET and toxicity predictions were conducted using the ProTox-III platform (Banerjee *et al.,* 2021), with complementary profiling based on established ADMET property prediction frameworks (Yang *et al.,* 2019). The analysis focused on six top-performing compounds from *Piliostigma thonningii*, including 6-C-methylquercetin 3,7,3'-trimethyl ether, 6-C-methylquercetin 3-methyl ether, 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy derivatives, and latifoline (**Tables 3–8**).

All compounds showed a moderate-to-low toxicity profile, with most falling within Toxicity Class IV or V, suggesting acceptable safety margins for further in vivo testing. Notably, none of the compounds were predicted to exhibit mutagenicity, carcinogenicity, cytotoxicity, or clinical toxicity (Adoch Atim,2025) which reinforces their potential for therapeutic development.

Among the organ toxicity profiles, hepatotoxicity, neurotoxicity, and respiratory toxicity were frequently predicted as "yes" across all compounds (T-Issa *et al.,* 2017). However, cardiotoxicity and nephrotoxicity were consistently predicted as "no," which is favorable for systemic use. While these findings warrant cautious interpretation, especially for hepatotoxic and neurotoxic liabilities, they remain manageable risks, particularly if dose-dependent thresholds are established in future preclinical studies.

Regarding molecular initiating events and endocrine modulation, all active compounds showed positive predictions for binding to estrogen receptor ligand-binding domains (ER-LBD)—a key mechanism underlying their phytoestrogenic potential (Kumar & Pandey, 2023; Adoch Atim, 2025). In particular, 6-C-methylquercetin 3-methyl ether, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl derivative, and 5,7-dihydroxy-2-(4-hydroxyphenyl)-3-methoxy derivatives also showed activity against aromatase (Szukiewicz, 2023), reinforcing their potential as modulators of estrogen biosynthesis.

Metabolic profiling indicated that most compounds are substrates or modulators of cytochrome P450 enzymes, particularly CYP2C9 and CYP3A4 (Ahmed *et al.,* 2016; Ogu & Maxa, 2000), which may affect drug metabolism pathways. However, the absence of significant inhibition or induction across other major enzymes (e.g., CYP1A2, CYP2E1) suggests a low risk for adverse drug–drug interactions (Ogu & Maxa, 2000). Interestingly, latifoline, though less active in docking studies, displayed a unique toxicity fingerprint with fewer stress response activations and no ecotoxicity (Ahmed *et al.,* 2016), making it an attractive candidate for low-risk therapeutic applications.

Overall, these ADMET findings complement the molecular docking and MD simulation results by confirming that the phytochemicals possess acceptable oral bioavailability, compliance with Lipinski's Rule of Five (Rautio *et al.,* 2008), and functional safety profiles necessary for further in vivo validation in PCOS models. The presence of estrogen receptor and aromatase interaction

profiles aligns with the proposed phytoestrogenic mechanism of action, supporting their therapeutic relevance.

Table 3: ADMET and Toxicity Predictions of 6-C-methylquercetin 3,7,3'-trimethyl ether of *Piliostigma* *thonningii* against PCOS using ProTox-III.

Classification	Target	Prediction
Organ toxicity	Hepatotoxicity	Yes
Organ toxicity	Neurotoxicity	Yes
Organ toxicity	Nephrotoxicity	No
Organ toxicity	Respiratory toxicity	Yes
Organ toxicity	Cardiotoxicity	No
Toxicity end points	Carcinogenicity	No
Toxicity end points	Immunotoxicity	Yes
Toxicity end points	Mutagenicity	No
Toxicity end points	Cytotoxicity	No
Toxicity end points	BBB-barrier	No
Toxicity end points	Ecotoxicity	Yes
Toxicity end points	Clinical toxicity	No
Toxicity end points	Nutritional toxicity	No
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon Receptor (AhR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor (AR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	No
Tox21-Nuclear receptor signaling pathways	Aromatase	Yes
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Alpha (ER)	Yes
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Yes
Tox21-Nuclear receptor signaling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	No
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	No
Tox21-Stress response pathways	Heat shock factor response element (HSE)	No
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	No
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	No
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	No
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	No
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	No
Molecular Initiating Events	Transtyretrin (TTR)	No

Molecular Initiating Events	Ryanodine receptor (RYR)	No
Molecular Initiating Events	GABA receptor (GABAR)	No
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	No
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	No
Molecular Initiating Events	Kainate receptor (KAR)	No
Molecular Initiating Events	Achetylcholinesterase (AChE)	Yes
Molecular Initiating Events	Constitutive androstane receptor (CAR)	No
Molecular Initiating Events	Pregnane X receptor (PXR)	No
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	No
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	No
Molecular Initiating Events	Na+/I- symporter (NIS)	No
Metabolism	Cytochrome CYP1A2	No
Metabolism	Cytochrome CYP2C19	No
Metabolism	Cytochrome CYP2C9	Yes
Metabolism	Cytochrome CYP2D6	No
Metabolism	Cytochrome CYP3A4	Yes
Metabolism	Cytochrome CYP2E1	No

Note: The ProTox-III toxicity analysis revealed varying safety profiles among the seven compounds. Most phytochemicals fell into Toxicity Class IV or V, suggesting moderate to low toxicity. Only one compound exhibited predicted hepatotoxicity and mutagenicity, indicating the need for further caution in in vivo applications. In this study, "Yes" predictions were interpreted as active or toxic, while "No" was considered inactive or non-toxic. The absence of cytotoxicity in the majority of compounds supports their candidacy for phytoestrogenic evaluation in PCOS models (Banerjee *et al.,* 2018).

Table 4: ADMET and Toxicity Predictions of 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one of *Piliostigma thonningii* against PCOS using ProTox-III.

Classification	Target	Prediction
Organ toxicity	Hepatotoxicity	Yes
Organ toxicity	Neurotoxicity	Yes
Organ toxicity	Nephrotoxicity	No
Organ toxicity	Respiratory toxicity	Yes
Organ toxicity	Cardiotoxicity	No
Toxicity end points	Carcinogenicity	No
Toxicity end points	Immunotoxicity	Yes
Toxicity end points	Mutagenicity	No
Toxicity end points	Cytotoxicity	No
Toxicity end points	BBB-barrier	No
Toxicity end points	Ecotoxicity	Yes

Toxicity end points	Clinical toxicity	No
Toxicity end points	Nutritional toxicity	No
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon Receptor (AhR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor (AR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	No
Tox21-Nuclear receptor signaling pathways	Aromatase	No
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Alpha (ER)	No
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Yes
Tox21-Nuclear receptor signaling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	No
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	No
Tox21-Stress response pathways	Heat shock factor response element (HSE)	No
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	No
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	No
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	No
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	No
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	No
Molecular Initiating Events	Transtyretrin (TTR)	No
Molecular Initiating Events	Ryanodine receptor (RYR)	No
Molecular Initiating Events	GABA receptor (GABAR)	No
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	No
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	No
Molecular Initiating Events	Kainate receptor (KAR)	No
Molecular Initiating Events	Achetylcholinesterase (AChE)	Yes
Molecular Initiating Events	Constitutive androstane receptor (CAR)	No
Molecular Initiating Events	Pregnane X receptor (PXR)	No
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	No
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	No
Molecular Initiating Events	Na+/I- symporter (NIS)	No
Metabolism	Cytochrome CYP1A2	No
Metabolism	Cytochrome CYP2C19	No
Metabolism	Cytochrome CYP2C9	Yes
Metabolism	Cytochrome CYP2D6	No
Metabolism	Cytochrome CYP3A4	Yes
Metabolism	Cytochrome CYP2E1	No

Note: The ProTox-III toxicity analysis revealed varying safety profiles among the seven compounds. Most phytochemicals fell into Toxicity Class IV or V, suggesting moderate to low toxicity. Only one compound exhibited predicted hepatotoxicity and

mutagenicity, indicating the need for further caution in in vivo applications. In this study, "Yes" predictions were interpreted as active or toxic, while "No" was considered inactive or non-toxic. The absence of cytotoxicity in the majority of compounds supports their candidacy for phytoestrogenic evaluation in PCOS models (Banerjee *et al.,* 2018).

Table 5: ADMET and Toxicity Predictions of 6-C-methylquercetin 3-methyl ether of *Piliostigma thonningii* against PCOS using ProTox-III.

Classification	Target	Prediction
Organ toxicity	Hepatotoxicity	Yes
Organ toxicity	Neurotoxicity	Yes
Organ toxicity	Nephrotoxicity	No
Organ toxicity	Respiratory toxicity	Yes
Organ toxicity	Cardiotoxicity	No
Toxicity end points	Carcinogenicity	No
Toxicity end points	Immunotoxicity	Yes
Toxicity end points	Mutagenicity	No
Toxicity end points	Cytotoxicity	No
Toxicity end points	BBB-barrier	No
Toxicity end points	Ecotoxicity	Yes
Toxicity end points	Clinical toxicity	No
Toxicity end points	Nutritional toxicity	No
Tox21-Nuclear receptor signalling pathways	Aryl hydrocarbon Receptor (AhR)	No
Tox21-Nuclear receptor signalling pathways	Androgen Receptor (AR)	No
Tox21-Nuclear receptor signalling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	No
Tox21-Nuclear receptor signalling pathways	Aromatase	Yes
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Alpha (ER)	Yes
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Yes
Tox21-Nuclear receptor signalling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	No
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	No
Tox21-Stress response pathways	Heat shock factor response element (HSE)	No
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	No
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	No
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	No
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	No
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	No

Molecular Initiating Events	Transtyretrin (TTR)	No
Molecular Initiating Events	Ryanodine receptor (RYR)	No
Molecular Initiating Events	GABA receptor (GABAR)	No
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	No
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	No
Molecular Initiating Events	Kainate receptor (KAR)	No
Molecular Initiating Events	Achetylcholinesterase (AChE)	Yes
Molecular Initiating Events	Constitutive androstane receptor (CAR)	No
Molecular Initiating Events	Pregnane X receptor (PXR)	No
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	No
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	No
Molecular Initiating Events	Na+/I- symporter (NIS)	No
Metabolism	Cytochrome CYP1A2	No
Metabolism	Cytochrome CYP2C19	No
Metabolism	Cytochrome CYP2C9	Yes
Metabolism	Cytochrome CYP2D6	No
Metabolism	Cytochrome CYP3A4	No
Metabolism	Cytochrome CYP2E1	No

Note: The ProTox-III toxicity analysis revealed varying safety profiles among the seven compounds. Most phytochemicals fell into Toxicity Class IV or V, suggesting moderate to low toxicity. Only one compound exhibited predicted hepatotoxicity and mutagenicity, indicating the need for further caution in in vivo applications. In this study, "Yes" predictions were interpreted as active or toxic, while "No" was considered inactive or non-toxic. The absence of cytotoxicity in the majority of compounds supports their candidacy for phytoestrogenic evaluation in PCOS models (Banerjee *et al.,* 2018).

Table 6: ADMET and Toxicity Predictions of 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one of *Piliostigma thonningii* against PCOS using ProTox-III.

Classification	Target	Prediction
Organ toxicity	Hepatotoxicity	Yes
Organ toxicity	Neurotoxicity	Yes
Organ toxicity	Nephrotoxicity	No
Organ toxicity	Respiratory toxicity	Yes
Organ toxicity	Cardiotoxicity	No
Toxicity end points	Carcinogenicity	No
Toxicity end points	Immunotoxicity	Yes
Toxicity end points	Mutagenicity	No
Toxicity end points	Cytotoxicity	No
Toxicity end points	BBB-barrier	No
Toxicity end points	Ecotoxicity	Yes

Toxicity end points	Clinical toxicity	No
Toxicity end points	Nutritional toxicity	No
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon Receptor (AhR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor (AR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	No
Tox21-Nuclear receptor signaling pathways	Aromatase	Yes
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Alpha (ER)	Yes
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Yes
Tox21-Nuclear receptor signaling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	No
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	No
Tox21-Stress response pathways	Heat shock factor response element (HSE)	No
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	No
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	No
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	No
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	No
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	No
Molecular Initiating Events	Transtyretrin (TTR)	No
Molecular Initiating Events	Ryanodine receptor (RYR)	No
Molecular Initiating Events	GABA receptor (GABAR)	No
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	No
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	No
Molecular Initiating Events	Kainate receptor (KAR)	No
Molecular Initiating Events	Achetylcholinesterase (AChE)	Yes
Molecular Initiating Events	Constitutive androstane receptor (CAR)	No
Molecular Initiating Events	Pregnane X receptor (PXR)	No
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	No
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	No
Molecular Initiating Events	Na+/I- symporter (NIS)	No
Metabolism	Cytochrome CYP1A2	No
Metabolism	Cytochrome CYP2C19	No
Metabolism	Cytochrome CYP2C9	Yes
Metabolism	Cytochrome CYP2D6	No
Metabolism	Cytochrome CYP3A4	Yes
Metabolism	Cytochrome CYP2E1	No

Note: The ProTox-II toxicity analysis revealed varying safety profiles among the seven compounds. Most phytochemicals fell into Toxicity Class IV or V, suggesting moderate to low toxicity. Only one compound exhibited predicted hepatotoxicity and mutagenicity, indicating the need for further caution in in vivo applications. In this study, "Yes" predictions were interpreted as active or toxic, while "No" was considered inactive or non-toxic. The absence of cytotoxicity in the majority of compounds supports their candidacy for phytoestrogenic evaluation in PCOS models (Banerjee *et al.,* 2018).

Table 7: ADMET and Toxicity Predictions of 5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one of *Piliostigma thonningii* against PCOS using ProTox-III.

Classification	Target	Prediction
Organ toxicity	Hepatotoxicity	Yes
Organ toxicity	Neurotoxicity	Yes
Organ toxicity	Nephrotoxicity	No
Organ toxicity	Respiratory toxicity	Yes
Organ toxicity	Cardiotoxicity	No
Toxicity end points	Carcinogenicity	No
Toxicity end points	Immunotoxicity	Yes
Toxicity end points	Mutagenicity	No
Toxicity end points	Cytotoxicity	No
Toxicity end points	BBB-barrier	No
Toxicity end points	Ecotoxicity	Yes
Toxicity end points	Clinical toxicity	No
Toxicity end points	Nutritional toxicity	No
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon Receptor (AhR)	No
Tox21-Nuclear receptor signalling pathways	Androgen Receptor (AR)	No
Tox21-Nuclear receptor signalling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	No
Tox21-Nuclear receptor signalling pathways	Aromatase	Yes
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Alpha (ER)	Yes
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Yes
Tox21-Nuclear receptor signalling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	No
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	No
Tox21-Stress response pathways	Heat shock factor response element (HSE)	No
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	No
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	No
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	No

Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	No
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	No
Molecular Initiating Events	Transtyretrin (TTR)	No
Molecular Initiating Events	Ryanodine receptor (RYR)	No
Molecular Initiating Events	GABA receptor (GABAR)	No
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	No
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	No
Molecular Initiating Events	Kainate receptor (KAR)	No
Molecular Initiating Events	Achetylcholinesterase (AChE)	Yes
Molecular Initiating Events	Constitutive androstane receptor (CAR)	No
Molecular Initiating Events	Pregnane X receptor (PXR)	No
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	No
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	No
Molecular Initiating Events	Na+/I- symporter (NIS)	No
Metabolism	Cytochrome CYP1A2	No
Metabolism	Cytochrome CYP2C19	No
Metabolism	Cytochrome CYP2C9	Yes
Metabolism	Cytochrome CYP2D6	No
Metabolism	Cytochrome CYP3A4	Yes
Metabolism	Cytochrome CYP2E1	No

Note: The ProTox-II toxicity analysis revealed varying safety profiles among the seven compounds. Most phytochemicals fell into Toxicity Class IV or V, suggesting moderate to low toxicity. Only one compound exhibited predicted hepatotoxicity and mutagenicity, indicating the need for further caution in in vivo applications. In this study, "Yes" predictions were interpreted as active or toxic, while "No" was considered inactive or non-toxic. The absence of cytotoxicity in the majority of compounds supports their candidacy for phytoestrogenic evaluation in PCOS models (Banerjee *et al.,* 2018).

Table 8: ADMET and Toxicity Predictions of Latifoline *Piliostigma thonningii* against PCOS using ProTox-III.

Classification	Target	Prediction
Organ toxicity	Hepatotoxicity	Yes
Organ toxicity	Neurotoxicity	Yes
Organ toxicity	Nephrotoxicity	No
Organ toxicity	Respiratory toxicity	Yes
Organ toxicity	Cardiotoxicity	No
Toxicity end points	Carcinogenicity	No
Toxicity end points	Immunotoxicity	Yes
Toxicity end points	Mutagenicity	No

Toxicity end points	Cytotoxicity	No
Toxicity end points	BBB-barrier	No
Toxicity end points	Ecotoxicity	No
Toxicity end points	Clinical toxicity	No
Toxicity end points	Nutritional toxicity	No
Tox21-Nuclear receptor signaling pathways	Aryl hydrocarbon Receptor (AhR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor (AR)	No
Tox21-Nuclear receptor signaling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	No
Tox21-Nuclear receptor signaling pathways	Aromatase	Yes
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Alpha (ER)	No
Tox21-Nuclear receptor signaling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	No
Tox21-Nuclear receptor signaling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	No
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	No
Tox21-Stress response pathways	Heat shock factor response element (HSE)	No
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	No
Tox21-Stress response pathways	Phosphoprotein (Tumor Supressor) p53	No
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	No
Molecular Initiating Events	Thyroid hormone receptor alpha (THRα)	No
Molecular Initiating Events	Thyroid hormone receptor beta (THRβ)	No
Molecular Initiating Events	Transtyretrin (TTR)	No
Molecular Initiating Events	Ryanodine receptor (RYR)	No
Molecular Initiating Events	GABA receptor (GABAR)	No
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	No
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	No
Molecular Initiating Events	Kainate receptor (KAR)	No
Molecular Initiating Events	Achetylcholinesterase (AChE)	Yes
Molecular Initiating Events	Constitutive androstane receptor (CAR)	No
Molecular Initiating Events	Pregnane X receptor (PXR)	No
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	No
Molecular Initiating Events	Voltage gated sodium channel (VGSC)	No
Molecular Initiating Events	Na+/I- symporter (NIS)	No
Metabolism	Cytochrome CYP1A2	No
Metabolism	Cytochrome CYP2C19	No
Metabolism	Cytochrome CYP2C9	Yes

Metabolism	Cytochrome CYP2D6	No
Metabolism	Cytochrome CYP3A4	Yes
Metabolism	Cytochrome CYP2E1	No

Note: The ProTox-II toxicity analysis revealed varying safety profiles among the seven compounds. Most phytochemicals fell into Toxicity Class IV or V, suggesting moderate to low toxicity. Only one compound exhibited predicted hepatotoxicity and mutagenicity, indicating the need for further caution in in vivo applications. In this study, "Yes" predictions were interpreted as active or toxic, while "No" was considered inactive or non-toxic. The absence of cytotoxicity in the majority of compounds supports their candidacy for phytoestrogenic evaluation in PCOS models (Banerjee *et al.,* 2018).

Challenges and Limitations

While computer models and live studies provide useful information about how Piliostigma thonningii might help manage PCOS, issues with protein-ligand modeling, docking accuracy, and lack of cell tests limit our ability to fully predict treatment results. The binding strengths seen in computer simulations may change in real-life conditions because of changes in protein shape, effects from the surrounding environment, or biological factors not included in the simulations. Also, the research looked at only a few protein targets, while PCOS involves a wider range of hormonal and metabolic processes.

Clinical Implications

The integration of computational analyses with animal model experiments is crucial to overcoming the limitations identified in this study and achieving a comprehensive understanding of the phytoestrogenic potential of *Piliostigma thonningii*. Translating these findings into clinical relevance requires robust *in vitro* and *in vivo* validations across multiple biological systems.

The preclinical phase should include testing of top-performing phytochemicals—such as 6-C-methylquercetin 3-methyl ether—on ovarian granulosa and theca cell lines to assess their effect on hormone production, receptor modulation, and cellular proliferation. Investigating their influence on hormonal signaling pathways (e.g., PI3K/Akt, LH/FSH regulation, and aromatase expression) would clarify the mechanism of action in PCOS pathology. Additionally, testing these compounds in more advanced PCOS animal models—such as insulin-resistant or genetically predisposed models—can help evaluate systemic effects and disease-modifying potential. These preclinical assessments pave the way for future clinical trials, including Phase I safety studies, Phase II dose-optimization trials, and ultimately Phase III efficacy evaluations in women with PCOS. Through this sequential approach, the potential of *Piliostigma thonningii*-derived phytoestrogens as safe and effective therapeutic agents for PCOS can be fully realized.

Clinical Implications

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DISCUSSION

Phytoestrogens are a diverse group of naturally occurring plant-derived compounds that exhibit structural similarity to endogenous estrogens, particularly 17β-estradiol, and are capable of binding to estrogen receptors (ERα and ERβ) in the human body (Shanmugaloga & Shilpa, 2024). Acting as selective oestrogen receptor modulators (SERMs), phytoestrogens can exert either oestrogenic or anti-estrogenic effects, depending on factors such as the individual's hormonal environment, receptor affinity, and tissue-specific expression. Phytoestrogens have structures that are very similar to endogenous estradiol and are able to bind with alpha and beta receptors of oestrogen (Desmawati *et al.,* 2019). Alpha and beta oestrogen receptors have different functions. Alpha estrogen receptors act in cell proliferation, whereas beta receptors are responsible for cell apoptosis (Chimento *et al.,* 2023). After the receptor binds to the ligand, it then moves from the cytoplasm to the cell nucleus, binding and influencing the area that controls the DNA transcription process or small RNA, which in turn affects the expression of certain genes (Desmawati *et al.,* 2019). Therefore, phytoestrogens have the potential to regulate all processes that are influenced by estrogen, including the induction of sex hormones that bind to globulin and inhibit

aromatase [34]. Endogenous oestrogen levels also affect the activity of phytoestrogens. In women of reproductive age, endogenous oestrogen levels are high in blood pressure (Szukiewicz, 2014). In this condition, lignans will compete with endogenous oestrogen to bind to estrogen receptors so that they can inhibit estrogen activity. But at menopause, endogenous estrogen levels are low in oestrogen production by the ovaries. When oestrogen levels are low, lignans are weak oestrogens (Desmawati *et al.,* 2019). In the same vein, isoflavones, lignans, and proteins may also function as aromatase inhibitors by inhibiting the action of cytochrome P450 enzymes that convert androgens to oestrogen.

Phytoestrogens have estrogenic potencies due to their structural similarity to that of estrogen and may mimic that of estradiol. These hydroxyl groups are responsible for the interaction of phytoestrogens with the ligand-binding domain of estradiol receptors (Canivenc-Lavier *et al.,* 2023). Currently, three estradiol receptors are considered. The first two receptors are canonical estradiol receptors ERα and ERβ, which act mainly via a nuclear interaction with a DNA palindromic sequence called ERE (Canivenc-Lavier *et al.,* 2023). However, thanks to a palmitoylation at the C451A-ERα site (Canivenc-Lavier *et al.,* 2023), ERα can also be present just below the cell membrane, bound to caveolin, and is able to react to low concentrations of lipophilic xenoestrogens. At this location, ERα activates intracellular phosphorylation pathways that are stimulated within a few seconds, in contrast to the nuclear pathway. Several pathways have been described so far, including PI3K/AKT, Src/ERK1/2, and NFκB (Canivenc-Lavier *et al.,* 2023). Disturbances in estrogen signaling, particularly through oestrogen receptors, especially ERα and ERβ, form part of the pathophysiology in PCOS and represent a field of phytoestrogens that may be of therapeutic interest.

Phytoestrogens act primarily through the binding of estrogen receptors, with greater affinity for the ERβ class. This could also constitute one form of important selective activation, as ERβ exerts various anti-inflammatory and antiproliferative effects via the blunting of a low-grade inflammation state associated with conditions such as PCOS (Szukiewicz, 2014). One example of some of the more major classes includes that of the isoflavones (Jamilian *et al.,* 2016), which are much more

abundant within soy products themselves. They have shown favorable effects in cases of PCOS, reduction of testosterone levels, regularization of the menstrual cycle, and normalization of ovarian morphology. Besides, isoflavones improve metabolic profiles, reduce oxidative stress, and decrease markers of inflammation due to their antioxidant potential, fitting quite well with the multifactorial management of PCOS (Jamilian *et al.,* 2016), and this can further interact with phytoestrogens due to their higher affinity for estrogen receptors to trigger the hypothalamic-pituitary-ovarian axis, by which they may normalize ovulatory cycles disrupted in PCOS through modulation of gonadotropin secretion. Gut microbiota composition and genetic polymorphisms in genes coding for oestrogen receptors and basal hormonal levels are factors that could influence therapeutic outcomes following such intervention.

Pharmacological potential was demonstrated by the post-docking molecular interaction investigation of phytochemicals extracted from *Piliostigma thonningii* against the gene proteins implicated in Polycystic Ovary Syndrome (PCOS). PCOS is a complicated endocrine and metabolic condition characterized by insulin resistance, persistent inflammation, hyperandrogenism, and chronic anovulation (Patel, 2018; Ding *et al.,* 2021). Abnormal steroidogenic enzyme activity (CYP17A1, CYP19A1) and dysregulation in the PI3K/Akt, AMPK, and MAPK signaling pathways are the molecular mechanisms underlying these dysfunctions (Zhao *et al.,* 2023). A variety of binding modalities, such as conventional hydrogen bonding, carbon-hydrogen bonding, alkyl interactions, and van der Waals forces, were found when P. thonningii phytochemicals were computationally screened against a few PCOS-associated target genes. This highlighted structural compatibility with crucial binding domains for hormonal and metabolic regulation.

6-C-Methylquercetin 3,7,3′-trimethyl ether, with a docking score of −7.8 kcal/mol (**Fig. 7)**, exhibited two strong conventional hydrogen bonds with TYR and SER residues, both implicated in polar domain stabilization and molecular recognition at active protein pockets. These bonds' orientation suggests that steroidogenic proteins like CYP17, which are involved in androgen production, may have interrupted enzymatic catalysis (Chavez *et al.,* 2023). Van der Waals contacts with PHE

and GLY, π-alkyl interactions with LEU, and a carbon-hydrogen bond with HIS or ASN all improve surface complementarity, which is a sign of tight insertion into a catalytic cleft (**Table 1**).

2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6,8-dimethyl-4H-1-benzopyran-4-one, with a docking affinity of −8.0 kcal/mol **(Fig. 8)**, demonstrated triple hydrogen bonding with ASP, GLN, and ARG—residues frequently involved in proton shuttling, charge relay, and structural cohesion in receptor-ligand complexes (Bu *et al.,* 2024). A stabilizing carbon H-bond with TYR (∼3.1 Å) suggested anchorage within kinase-like domains. π-alkyl interactions with ILE and VAL, supported by van der Waals stabilization at polar residues, imply binding near MAPK cascade nodes, suggesting interference with follicular apoptosis and pro-inflammatory signaling.

6-C-Methylquercetin 3-methyl ether, recording the strongest docking score of −8.3 kcal/mol **(Fig.9)**, engaged in four conventional hydrogen bonds with GLU, ASN, and LYS residues—amino acids present in ATP-dependent kinases and phosphotransferase domains critical for PI3K/AKT-mediated insulin signaling. Van der Waals interactions with PHE and π-alkyl contacts with LEU and ALA guaranteed accurate ligand fitting in hydrophobic recesses, whereas carbon H-bonds with MET and GLY helped stabilize the backbone. Because of its molecular makeup, it may be able to restore metabolic balance and normalize the insulin response in PCOS due to inherent phytogenic activity of *P.thonningii* (Hailemariam *et al.,* 2021).

2-(3,4-Dihydroxyphenyl)-5-hydroxy-3,7-dimethoxy-6-methyl-4H-1-benzopyran-4-one (**Fig.10)**, with a docking energy of −7.8 kcal/mol, interacted through two hydrogen bonds with SER and TYR, stabilizing its orientation at solvent-accessible receptor surfaces. π–π stacking with PHE or TRP mimics native hormone ligands (e.g., FSH or estradiol), suggesting possible inhibition of gonadotropin binding or estrogen biosynthesis (Mahmoud *et al.,* 2022; Bu *et al.,* 2024). A carbon-hydrogen bond with GLN and surrounding van der Waals forces involving VAL and ALA reflect the molecule's capacity to modulate aromatase or estrogen receptor co-activator interactions Bu *et al.,* 2024; Wal *et al.,* 2021).

2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one (**Fig.11)**, dual traditional hydrogen bonds with ASP

and TYR, having a binding energy of -8.2 kcal/mol. Redox-regulatory motifs and kinase loops frequently include these residues. A carbon-hydrogen bond with ASN increased directional specificity, whereas π-alkyl interactions with LEU enhanced nonpolar stabilization. Van der Waals interactions, which complemented the binding, showed that the molecule could occupy flexible yet functionally important regulatory domains. This has consequences for controlling oxidative stress and PCOS-related ovarian signaling failure (Sharma, 2024).

5,7-Dihydroxy-2-(4-hydroxyphenyl)-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one (**Fig.12)**, docking at −7.9 kcal/mol, formed three strong polar hydrogen bonds with GLU, ASN, and TYR, critical residues for steroidogenic modulation and redox balance. A carbon H-bond with THR—often located in hormone receptor loops—supports conformational plasticity during ligand anchorage. Hydrophobic contacts with LEU and ILE and peripheral van der Waals engagements collectively promote extended residence time, supporting this compound's potential as a suppressor of hyperandrogenic enzymatic activity (e.g., 3β-HSD, 17α-hydroxylase) (Chavez *et al.,* 2023; Mahmoud *et al.,* 2022).

Latifoline (**Fig.13)**, with a docking score of −7.8 kcal/mol, formed two conventional H-bonds with ARG and ASN, favoring strong polar interactions at regulatory enzyme sites. A carbon-hydrogen bond with HIS, a residue often implicated in acid–base catalysis, suggested inhibitory modulation at enzymatic turnover points (Bu *et al.,* 2023). π-alkyl interactions with LEU and ILE, supported by van der Waals complementarity with GLN and SER, placed latifoline firmly within hydrophobic clefts. This hybrid interaction model suggests a multi-faceted mechanism, potentially normalizing both insulin sensitivity and ovarian growth factor expression.

Together, these phytoconstituents from P. *thonningii* demonstrated rich interaction patterns with residues known to regulate ovulatory cycles, hormonal biosynthesis, and insulin signaling. The cumulative presence of more than 20 conventional hydrogen bonds, multiple π-alkyl and carbon-hydrogen bonds, and extensive van der Waals contacts underscores the ligand stability and specificity within protein active sites, supporting their drug-like behavior. The docking score range of −7.8 to −8.3 kcal/mol validates their strong thermodynamics and predicted bioactivity, comparable to or

surpassing known PCOS therapeutics such as Letrozole (−8.1 kcal/mol) and significantly better than Metformin (−4.6 kcal/mol), as shown in **Table 1**.

According to the graph's patterns, the PCOS + ELEPT1 and PCOS + ELEPT2 groups showed a notable rise in progesterone levels after receiving PT extract, which implies that the phytoconstituents in the extract may have strong ovulatory and luteotropic effects. These increases, which are evident in Figures **13, 14, 15**, and **17**, most likely show how the extract affects ovarian steroidogenic pathways, particularly by boosting the activity of key enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-HSD, and cholesterol side-chain cleavage enzyme (CYP11A1) that catalyze the biosynthesis of progesterone from cholesterol through pregnenolone intermediates. Chronic anovulation, a characteristic of PCOS, lowers progesterone production and impairs corpus luteum growth, which leads to irregular menstruation and infertility. The ability of PT to restore progesterone levels implies a normalization of the hypothalamic–pituitary–ovarian (HPO) axis, possibly by enhancing follicular maturation, ovulation, and luteinization (Dasofunjo *et al.,* 2013). It modulates FSH receptor sensitivity, improving granulosa cell differentiation and reversing the arrested folliculogenesis typically observed in polycystic ovaries. Furthermore, the plant's bioactive flavonoids and phenolic compounds may act as phytoestrogens, structurally mimicking estradiol and binding to estrogen receptors (ERα/ERβ) within the hypothalamus and ovaries (McRobb *et al.,* 2014). This could help rebalance estrogen-progesterone feedback loops and suppress the overproduction of androgens, a hallmark of PCOS due to antioxidant activities of it flavonoids (Tahiri & Kouamé, 2022). Progesterone is also known to exert anti-androgenic effects by downregulating 5α-reductase activity, thereby reducing testosterone conversion and improving symptoms such as hirsutism and acne (Kolatorova *et al.,* 2022).

Fig. 16 demonstrated that although progesterone levels increased in the treatment groups, luteinizing hormone (LH) levels did not rise significantly. This separation would suggest that the extract works by directly improving ovarian responsiveness or raising luteal activity without the need for LH stimulation, rather than via inducing the hypothalamus to enhance GnRH pulsatility, a major source of high LH in

PCOS (Bu *et al.,* 2023; Kanbour & Dobs, 2022). Consequently, this might potentially point to a negative loop-feedback mechanism in which post-treatment increases in progesterone prevent more LH secretion and support endocrine balance. According to Baddela et al. (2025), these effects might be achieved via altering the PI3K/Akt, MAPK, and cAMP/PKA signaling pathways, which are crucial for the production of the steroidogenic acute regulatory (StAR) protein and the transport of cholesterol inside the mitochondria. Thus, by promoting progesterone production in luteinized granulosa cells and enabling mitochondrial cholesterol import, the phytogenic exudates in this extract may increase the expression of StAR and SF-1 (Steroidogenic Factor 1). Nonetheless, P. thonningii could have antioxidant and anti-inflammatory properties (Boualam *et al.,* 2021), which are essential for reducing the persistent low-grade inflammation linked to PCOS (Zhang *et al.,* 2024; Danjuma, & Maduabuchi Aja, 2024). In order to enhance endocrine function and ovulatory processes, the extract may prevent oxidative damage to ovarian tissues and improve the follicular milieu by scavenging reactive oxygen species (ROS) and downregulating pro-inflammatory cytokines, including TNF-α and IL-6. Consequently, our results offer structural and molecular understanding of how phytochemicals from *P. thonningii* could repair pathways that have been dysregulated in PCOS. They are potential leads for the development of new anti-PCOS medications because of their good docking profiles, affinity for conserved regulatory domains, and multi-target binding behavior in post-docking and network enrichment analysis. As demonstrated here, *P. thonningii* stands out as a strong contender for more pharmacological and clinical research in the management of PCOS, and molecular docking provides a dependable platform for finding bioactive chemicals with therapeutic promise.

CONCLUSION

This study employed molecular docking, molecular dynamics, and systems biology approaches to evaluate phytochemicals from *Pilliostigma thonningii* as potential modulators of key targets involved in Polycystic Ovary Syndrome (PCOS). Post-docking analysis revealed high binding affinities of 6-C-methylquercetin 3-methyl ether, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-methoxy-6,8-dimethyl-4H-1-benzopyran-4-one, latifoline, and other

lead flavonoids with PCOS-related receptor proteins, demonstrating superior or comparable interaction profiles to letrozole and markedly higher than metformin. Furthermore, network pharmacology and KEGG-based functional enrichment analyses revealed that these compounds target central signaling pathways implicated in PCOS pathogenesis, including the estrogen biosynthesis, PI3K-Akt-insulin signaling, and inflammatory pathways. Hierarchical clustering and chromosomal enrichment analyses also highlighted the modulation of genes located on chromosomes 10 and 21—regions rich in PCOS-relevant gene clusters. GO analysis further indicated involvement in key biological processes such as hormone receptor binding, steroid metabolism, and cell proliferation. Collectively, these findings position *P. thonningii* phytochemicals as promising therapeutic candidates for PCOS intervention and support their advancement toward in vitro and in vivo validation in future research.

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Availability of data and materials

The dataset generated and/or analyzed in this study is available from the corresponding author upon reasonable request.

Competing interests

The authors hereby declare no conflicts of interest.

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Author Contributions

Dasofunjo, K.,: Conceptualization, Methodology, Supervision, Project Administration, Writing – Original Draft, Validation. **Onwu, D.O., (0009-0002-2131-2487)**: Bioinformatics Analysis, Molecular Docking and Dynamics Simulation, Network Pharmacology, Visualization, Writing – Review & Editing. **Ati, B.U.**: Data Curation, Formal Analysis, Literature Review, Software Application. **Mayowa, A.**: ADMET Profiling, Molecular Target Prediction, Graphical Presentation, Review & Editing. **Okputu, J.I.**: Statistical Analysis, Experimental Design Review, Data Interpretation, Review & Editing. **Bassey, O.J.**: Functional Enrichment Analysis, GO/KEGG Pathway Interpretation, **Ifeoluwa, D.O:** Figure Preparation.

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