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Investigating Dapagliflozin's Effect on Purified Aromatase from Poly Cystic Ovary Syndrome (PCOS) Women's Serum, with Molecular Docking Study

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ABSTRACT

Polycystic Ovary Syndrome (PCOS) is associated with altered gonadotropin and steroid hormone profiles, including elevated LH, testosterone, and AMH, with normal or slightly reduced FSH. Aromatase, a key enzyme in androgen-to-estrogen conversion, was partially purified from PCOS patients undergoing metformin therapy to investigate its kinetic properties and concentration. The purification involved ammonium sulfate precipitation, dialysis, ion exchange, and gel filtration chromatography, with ELISA used for quantification. HPLC confirmed enzyme purity (~84.91 kDa). The enzyme exhibited optimal activity at pH 7.2 and 37°C, with Vmax = 8.199 µmol/min·ml and Km = 0.2719 mM (no inhibitor). Inhibition reduced Vmax to 3.1918 µmol/min·ml and Km to 0.1299 mM. Aromatase levels were significantly higher in metformin-treated PCOS patients versus controls ($P < 0.05$). Dapagliflozin acted as an aromatase inhibitor, and the study provides insight into molecular interactions between metformin derivatives and this enzyme.

Keyword: PCOS, Aromatase enzyme, Dapagliflozin, Purification.

Introduction

The ovaries are key reproductive organs responsible for producing oocytes and secreting sex hormones, primarily estrogen and progesterone, along with other hormones [1–2]. Polycystic Ovary Syndrome (PCOS) is a prevalent endocrine disorder in reproductive-age women, characterized by hyperandrogenism [3], insulin resistance, ovulatory dysfunction [4], menstrual irregularities, and hirsutism [5]. A hallmark feature is the presence of multiple ovarian follicles, leading to enlarged polycystic ovaries. FSH stimulates follicular growth, while LH supports follicular maturation, ovulation, and corpus luteum formation, which secretes progesterone [6]. In PCOS, hormonal imbalance—especially reduced luteal-phase progesterone—

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results in impaired endometrial shedding and menstrual cycle disruption [7–8]. Ovarian cysts are fluid-filled sacs with thin walls within ovarian tissue[9]. Aromatase, encoded by CYP19A1, is a cytochrome P450 enzyme catalyzing the conversion of androgens (androstenedione, testosterone) to estrogens (estrone, estradiol) [10–11]. It plays a critical role in hormonal balance; its overactivity is linked to conditions such as estrogen-dependent breast cancer and PCOS [12]. Aromatase inhibitors are used therapeutically to reduce estrogen synthesis, particularly in hormone-dependent breast cancer[13]. Partial purification of aromatase from PCOS serum involves removal of non-protein impurities to ensure accurate kinetic analysis. Purification efficiency is assessed by enzyme yield and purification fold [14], using methods such as protein precipitation, ion exchange, gel filtration, and advanced techniques like HPLC [13–15]. Crystallization offers the highest purity for structural studies via X-ray crystallography [16–17]. Purification progress is monitored by increases in specific activity until maximal purity is achieved [18,19].Dapagliflozin, an SGLT2 inhibitor, lowers glucose by reducing renal glucose reabsorption in the proximal tubules, promoting glycosuria and decreasing blood glucose independently of insulin [20]. Benefits include modest weight loss, reduced blood pressure, and improved cardiovascular and renal function [21]. In PCOS, dapagliflozin improves insulin resistance [22], promotes 2–3 kg weight loss, lowers androgens, and restores hormonal balance. It also improves triglycerides, blood pressure, and hepatic fat content [23]. Although not a first-line PCOS therapy, it may be used adjunctively in cases with insulin resistance, obesity, or high diabetes risk, under medical supervision due to potential adverse effects such as diabetic ketoacidosis and genitourinary infections [24].

Materials and methods

Cases of Polycystic Ovary Syndrome (PCOS) in women were collected during the period from November 2024 to March 2025.

2.1- Samples Collection:

In this study, 90 blood samples were collected from women between November 15, 2024, and March 15, 2025, at Al-Gazalia Laboratory and Life Medical Laboratory in Tikrit, Salah al-Din Governorate. The samples included 60 from PCOS patients (30 on metformin and 30 without medication) aged 18–49 years, and a control group aged 15–50 years. Venous blood (5 ml) was drawn into additive-free plastic tubes, allowed to clot for 10 minutes, and centrifuged at 4000 rpm for 10 minutes to separate serum. The clear serum was transferred into tubes for analysis. Aromatase (CYP19A1) activity was determined using a commercial ELISA kit (e.g., My BioSource, Bioassay Technology, or Abcam) according to the manufacturer's instructions. This method relies on antigen–antibody binding, with enzyme concentration quantified colorimetrically at ~450 nm, proportional to the intensity of color developed.

Separation and Purification of Aromatase Enzyme: Aromatase was purified from the serum of PCOS patients through a series of steps:

- Enzyme precipitation using ammonium sulfate: Under cold conditions with continuous stirring for 60 minutes, 9.75 g of ammonium sulfate was gradually added to 14 mL of crude enzyme. The mixture was centrifuged at 2000 × g for 30 minutes at 4°C, achieving enzyme precipitation at 75% ammonium sulfate saturation ($(NH_4)_2SO_4$). The precipitate was dissolved in Tris-HCl solution (0.8 g Tris in 100 mL distilled water), and total protein, enzyme activity, and specific activity were measured. Dialysis against 10 mM Tris-HCl buffer (pH 7.2) at 4°C for 24 hours was performed to remove residual salts, and specific activity was calculated [25].
- Dialysis: This step removed remaining ammonium sulfate. The protein solution was placed in a dialysis bag and submerged in 10 mM Tris-HCl buffer (pH 7.2), with the buffer refreshed every 4 hours over 24 hours at 14°C.

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- Ion-exchange chromatography: Chromatography was used for molecular separation based on size and charge differences [26]. Protein fractions from gel filtration were collected and analyzed via absorbance at 280 nm or a protein assay kit. The DEAE-Sepharose column, equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$, was loaded with the serum sample, and angiotensin-converting enzyme (ACE) was eluted at 0.5 mL/min [27].
- Gel filtration: Fifteen pooled fractions from ion-exchange chromatography were subjected to size-exclusion chromatography using Sephadex G-100 resin, producing 15 additional fractions. This technique is also called gel filtration chromatography [28].
- Total protein determination: The Biuret assay was used, where cupric ions (Cu^{2+}) in alkaline conditions react with peptide nitrogen atoms to produce a violet complex. Color intensity is proportional to total protein concentration [29].
- Molecular weight determination by SEC-HPLC: Size-exclusion chromatography coupled with high-performance liquid chromatography (SEC-HPLC) was used to separate proteins based on molecular weight, providing a rapid and versatile method for analyzing protein molecules [30].

Table 1: Operational conditions for finding molecular weight by HPLC

Col. Manufacturer	Col. Type	Col. Length	Col. Diameter	Col. Particle size
Zrorbax PSM 60 S	BIMODAL	250 mm	4.6 mm	5 µm

- Statistical analysis: Data analysis was performed using the statistical software SPSS version 29, with normality of distribution assessed by Student's t-test. A p-value less than 0.001 was considered highly significant, less than 0.05 significant, while values greater than 0.05 were regarded as non-significant. Additionally, diagnostic accuracy and receiver operating characteristic (ROC) curves were evaluated using MedCalc version 23.1.7.

Results and Discussion

A. FSH, LH, Testosterone levels

The (mean ± SD) of FSH, LH, and Testosterone ng/ml of patients and control groups levels in the serum were shown in table (2) and figure (1). The results showed the levels in the patient PCOS group are normal when without significant difference (P>0.05) when compared to control group.

Table 2: Serum Level of FSH, LH, Testosterone

Parameters	**Control**	**PCOS With drug**	**PCOS With out drug**	**P value**
FSH (ng/ml)	6.77±1.340	6.81±1.71	6.98±1.8	>0.05
LH (ng/ml)	5.12±1.00	5.81±1.85	7.15±1.73	>0.05
Testosterone (ng/ml)	1.58±0.421	1.60±0.21	2.33±0.50	>0.05

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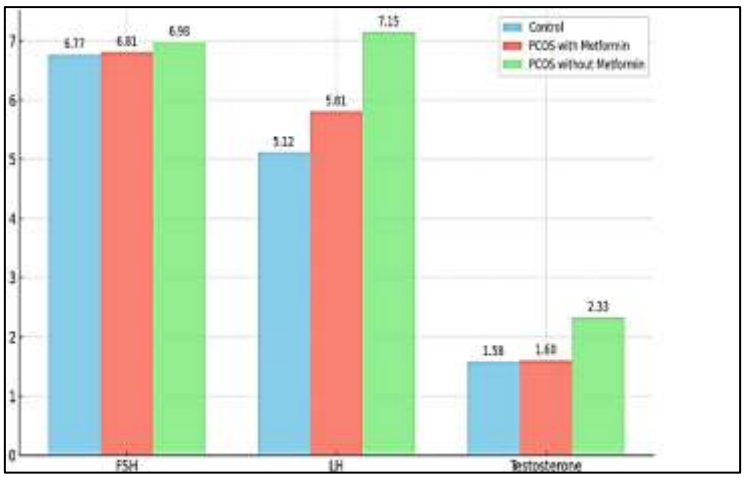


Fig.1: serum level of FSH,LH and Testosterone

FSH, LH, and Testosterone in PCOS: FSH levels were similar between controls (6.77 ng/ml) and metformin-treated PCOS patients (6.81 ng/ml), with a slight increase in untreated PCOS (6.98 ng/ml), consistent with evidence that metformin may modestly affect FSH without significant overall change [31][32]. LH was moderately elevated in untreated PCOS (7.15 ng/ml) but reduced in the metformin group (5.81 ng/ml), reflecting metformin's role in improving the LH/FSH ratio [31][33]. Testosterone was highest in untreated PCOS (2.33 ng/ml) and substantially lower with metformin (1.60 ng/ml), approaching control levels (1.58 ng/ml), supporting clinical findings of metformin's significant reduction of total testosterone, including rapid LH-induced decreases independent of insulin sensitivity [34][35].

B. **Aromatase activity**

The (mean ± SD) of Aromatase activity ng/ml of patients and control groups levels in the serum were shown in table (3) and figure (2). The results showed the levels in the patient PCOS group are normal when without significant difference (P<0.05) when compared to control group.

Table 3: Serum Aromatase activity

Parameters	Control	With	With out	P value
Aromatase (ng/ml)	5.19±1.12	7.07±1.52	4.27±0.95	<0.05

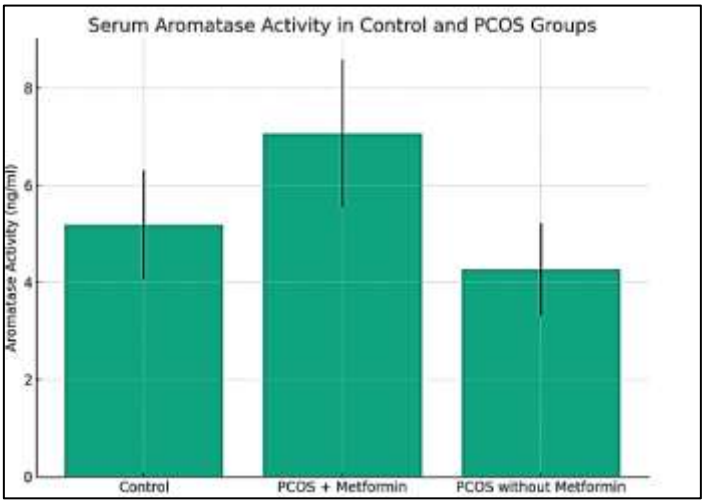


Fig.2: Serum level of Aromatase enzyme

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The results indicate that metformin enhances aromatase activity in PCOS patients, as levels were significantly higher in the metformin-treated group compared to controls. Conversely, untreated PCOS patients showed reduced aromatase activity, suggesting that metformin may help restore hormonal balance by promoting the conversion of androgens to estrogens. This is supported by a recent study demonstrating that metformin increases aromatase activity in ovarian granulosa cells, improving hormonal regulation in PCOS women [36].

C. Molecular docking of some Metformin Derivatives with Aromatase enzyme:

Comp.1:

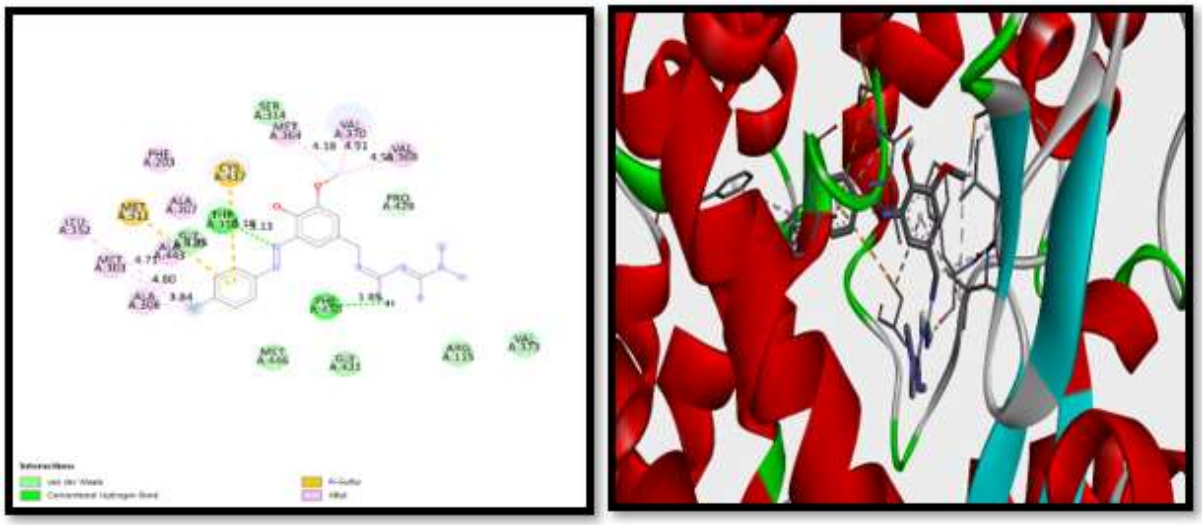


Fig.3: The reaction between comp.1 with Aromatase enzyme

The molecular docking study of the synthesized compound (1) revealed the number and types of bonds through which this compound interacts with amino acid residues present in the active site. The study showed that the compound interacts with the active site residues by forming three types of bonds: **One hydrogen bond:** Formed between the residue TYR310 in the active site and the lone pair electrons of the azo group in the compound, with a bond length of 3.13 Å. **Six alkyl interactions:** Three of them are formed between the residues LEU152, MET303, and ALA306 in the active site and the lone pair electrons of the chlorine atom substituted on the aromatic ring of the compound, with bond lengths of 4.71 Å, 4.80 Å, and 3.84 Å, respectively; the other three are formed between the residues VAL369, VAL370, and MET364 in the active site and the methoxy group substituted on the aromatic ring of the compound, with bond lengths of 4.51 Å, 4.41 Å, and 4.18 Å, respectively.

Two Pi-Sulfur interactions: Formed between the π-electrons of the aromatic ring and the residues CYS437 and MET311 in the active site, with bond lengths of 5.18 Å and 4.33 Å, respectively.

Comp. 2:

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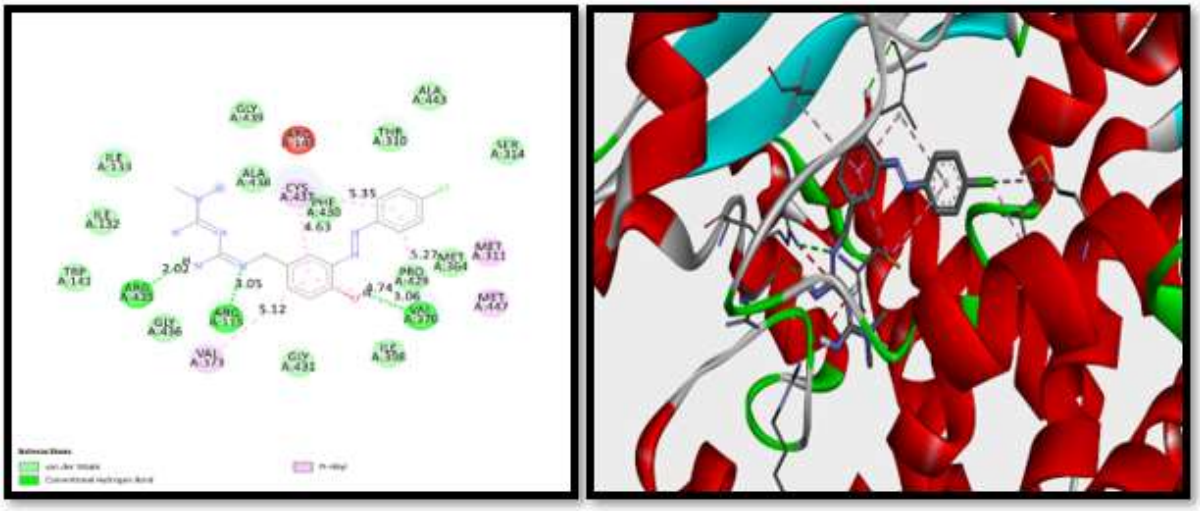


Fig. 4: The reaction between comp.2 and Aromatase enzyme

The molecular docking study of the synthesized compound (2) revealed the number and types of bonds through which this compound interacts with amino acid residues present in the active site. The study showed that the compound interacts with the active site residues by forming two types of bonds: **Three hydrogen bonds:** The first bond is formed between the residue ARG435 located in the active site and the amino hydrogen atom of the compound, with a bond length of 2.02 Å. The second bond links the residue ARG115 with the lone pair of the nitrogen atom in the azo group, with a bond length of 3.05 Å. The third bond is formed between the residue VAL370 and the lone pair of the oxygen atom of the hydroxyl group substituted on the ring, with a bond length of 3.06 Å. **Three Pi-Alkyl interactions:** These are established between the aromatic ring's π-electrons of the compound and the residues VAL373, VAL370, and CYS437 in the active site, with bond lengths of 5.12 Å, 4.74 Å, and 4.63 Å, respectively.

Comp.3:

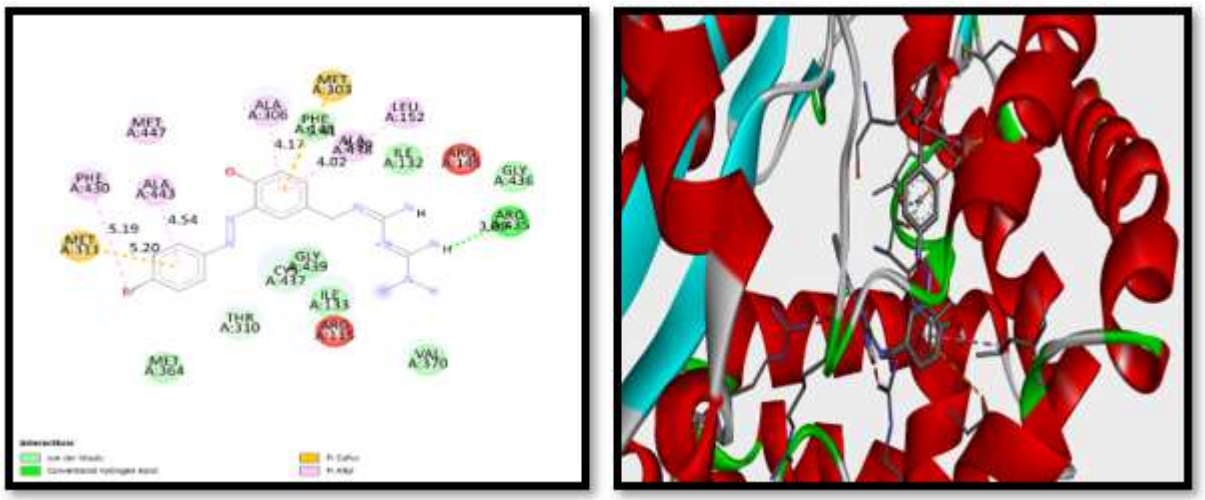


Fig. 5: The reaction between comp.3 and Aromatase enzyme

The molecular docking study of the synthesized compound (3) revealed the number and types of bonds through which this compound interacts with amino acid residues present in the active site. The study showed that the compound interacts with the active site residues by forming three types of bonds: **One**

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hydrogen bond: Formed between the residue ARG435 in the active site and the hydrogen atom of the amino group in the compound, with a bond length of 3.09 Å. **Five Pi-Alkyl interactions:** Formed between the π-electrons of the aromatic ring of the compound and the amino acid residues PHE430, ALA443, ALA306, LEU152, and ALA438 in the active site, with bond lengths of 5.19 Å, 4.54 Å, 4.17 Å, 5.39 Å, and 4.02 Å, respectively. **Two Pi-Sulfur interactions:** Formed between the π-electrons of the aromatic ring and the amino acid residues MET311 and MET303 in the active site, with bond lengths of 5.20 Å and 5.41 Å, respectively.

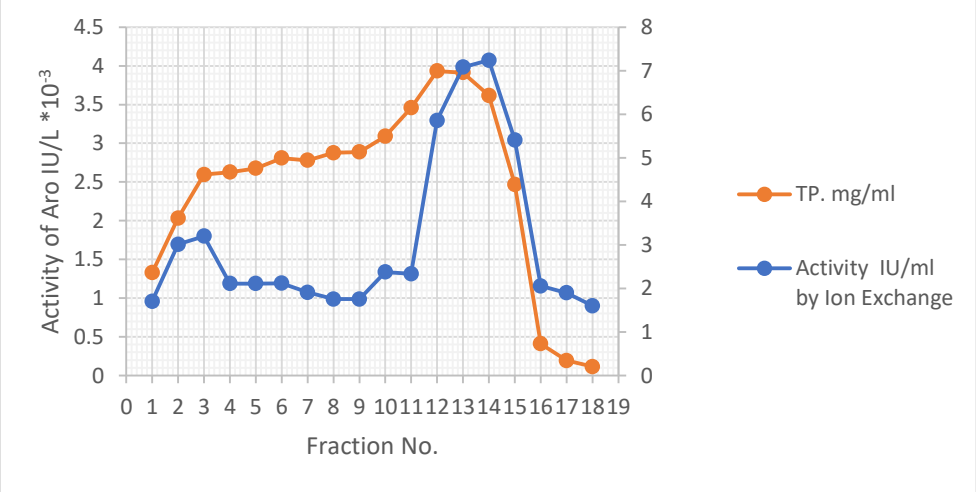
D. Partial purification of aromatase enzyme in the serum of patients with polycystic ovary syndrome:

Most proteins precipitated at 75% ammonium sulfate saturation, increasing aromatase specific activity from 0.025 IU/mg to 0.028 IU/mg and raising the purification fold from 1 to 1.121. This improvement results from ammonium sulfate concentrating the enzyme and removing interfering molecules without affecting activity. Controlled "salting-out" reduces protein solubility, promotes selective precipitation, and enhances purification efficiency by eliminating major impurities [37].

Table 4: Stages of purification of the enzyme Aromatase in patients with Polycystic ovary syndrome

Purification Steps	Total Volume mL	Activity IU/mL	Total Activity	Protein mg/mL	Protein	Specific Activity U/mg	Recovery Yield %	Fold of Purification
Crude	18.0	2.669	48.049	108.592	6.033	0.025	100.000	1.000
Ammonium sulfate precipitation	12.0	2.010	24.122	72.921	6.077	0.028	50.204	1.121
Membrane separation/dialysis	14.0	2.061	28.857	70.205	5.015	0.029	60.058	1.194
ion exchange	7.0	4.239	29.671	25.315	3.616	0.167	61.752	6.812
gel filtration	5.0	6.002	30.011	18.040	3.608	0.333	62.459	13.535

Ione-Exchange chromatography: This chromatographic method enables fractionation and purification of biomolecules such as proteins, enzymes, hormones, antibiotics, and nucleic acids. In this study, it was applied to enzyme isolation, combining ammonium sulfate precipitation with DEAE-cellulose ion-exchange chromatography.



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Fig.6: Purification of the enzyme Aromatase by ion exchange chromatography

The elution profile showed a single, symmetrical peak, indicating successful aromatase purification. Purified enzyme activity reached 3.616 IU/L, with specific activity of 0.167 IU/mg, representing a twofold increase in purity and a 61.752% yield, consistent with previous studies [38].

Gel filtration chromatography: Gel filtration chromatography using a Sephadex G-100 column further purified aromatase, yielding a single symmetrical peak. The purified enzyme showed activity of 6.002 IU/mL and specific activity of 0.333 IU/mg, representing a 13.535-fold purification and 62.459% yield, demonstrating significant enhancement in purity and specific activity [39].

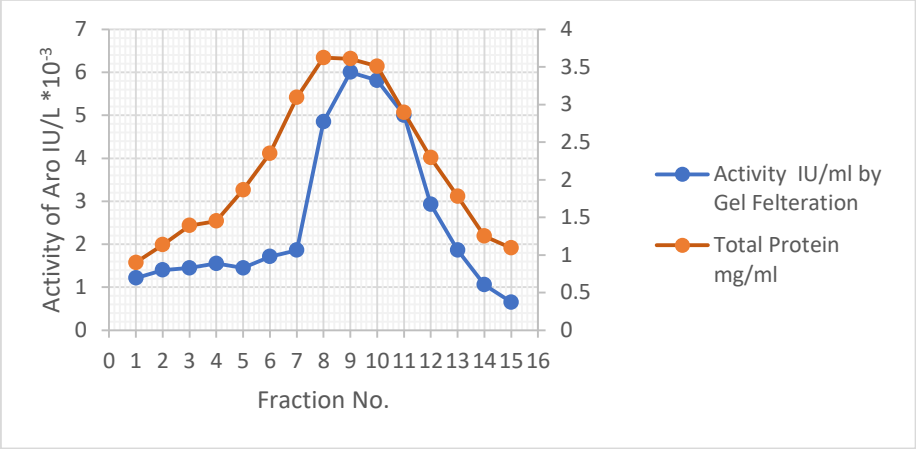


Fig.7: Purification of the enzyme Aromatase by gel filtration chromatography

Investigation Of the Kinetic Characteristics of The Partly Purified Enzyme Aromatase from Polycysic Ovary Syndrome Patients' Serum:

Aromatase activity was measured at substrate concentrations of 0.2–1 mM (Chromogen A&B), with maximal activity (6.952 IU/L) at 0.6 mM. The reaction velocity increased with substrate concentration, displaying typical Michaelis-Menten kinetics.

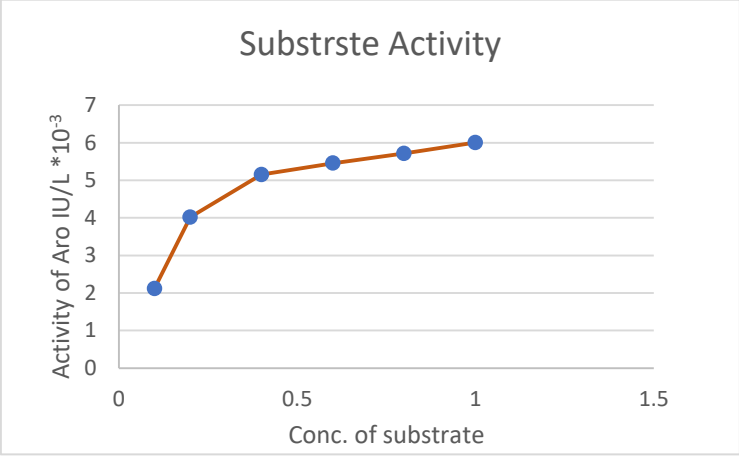


Fig. 8: velocity vis substate concentration on purified Aromatase activity

Effect of Temperature on Aromatase Activity

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The optimal temperature for aromatase activity was 37 °C, with activity increasing slightly from 10–30 °C and rising sharply between 30 °C and 37 °C, then declining at higher temperatures [40]. This decrease is attributed to enzyme denaturation, structural alterations, and changes in ionization of functional groups, consistent with the influence of enzyme three-dimensional structure on catalytic efficiency [41].

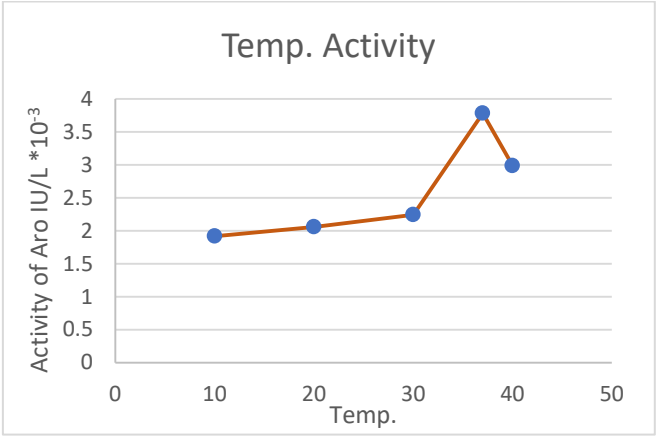


Fig.9: Effect of temperature on the activity of the purified Aromatase enzyme

Effect of pH on enzyme activity

A study was conducted to investigate the effect of pH on Aromatase enzyme activity. Various pH values (3, 5, 7.2, 9, and 11) were tested to determine the optimal pH for maximal enzyme activity, as illustrated in Figure (10). The highest enzyme activity was observed at pH 7.2 in the purified Aromatase sample.

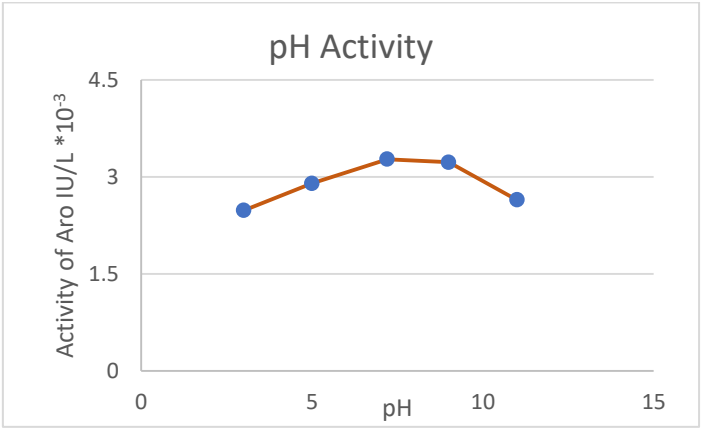


Fig. 10: Effect PH on the activity of the purified Aromatase enzyme.

Determination of Enzyme Molecular Weight by High-Performance Liquid Chromatography – Size Exclusion Chromatography (HPLC-SEC).

The chromatogram shown in Figure (11) displays distinct peaks corresponding to Aromatase, allowing the determination of its molecular weight.

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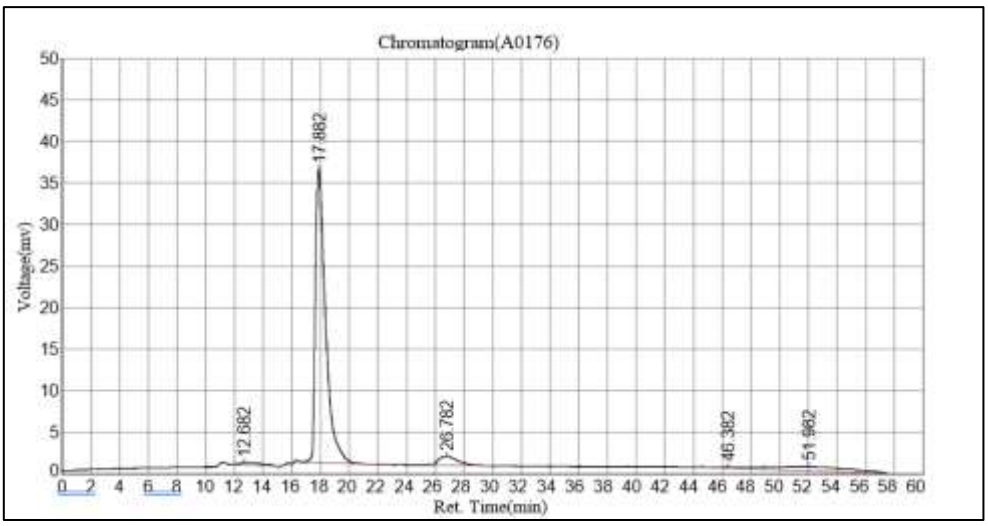


Fig 11: Chromatogram showing the peak of Aromatase enzyme determined by HPLC-SEC for molecular weight estimation

The chromatogram displayed five major protein peaks, representing components with different molecular weights according to their retention times. The molecular weights were estimated using a linear calibration equation ($R^2 = 0.9659$). The peaks most relevant to the Aromatase enzyme are as follows:

Table 5: Summary of Protein Peaks from HPLC-SEC Analysis of Aromatase Enzyme

peek n.	Peek RT	MW Kda	%
1	12.68	174.18	2.59
2	17.88	84.91	80.16
3	26.78	36.49	4.48
4	46.38	11.57	3.42
5	51.98	9.12	9.36

Study of the effect of some Metformin Derivatives on the Aromatase enzyme:

The effect of certain metformin derivatives on the aromatase enzyme has been investigated, and the findings revealed that these derivatives act as activators of the enzyme's activity, based on the following results:

Table 6: The effect of different concentrations of stimulants on the Aromatase enzyme

Conc.	Without Comp.	COMP.1	COMP.2	COMP.3
-	6.002144	-	-	-
Stock(1Mm)	-	8.35	16.46	15.57
Conc.1 (0.6mM)	-	20.42	23.03	14.04
Conc.2 (0.2mM)	-	28.48	20.65	22

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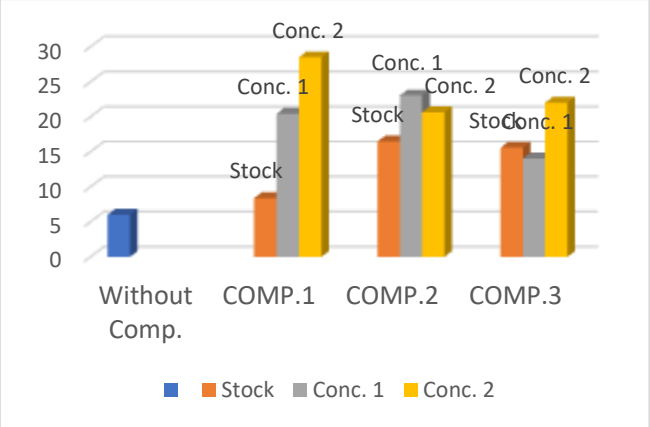


Fig.12: Investigation of the impact of various concentrations of metformin derivatives on the activity of the aromatase enzyme.

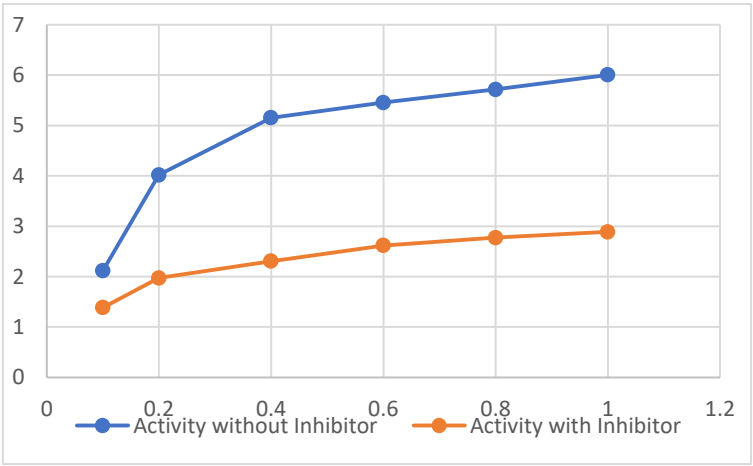
The results demonstrate that all three metformin derivatives act as activators of aromatase enzyme activity, supporting the hypothesis that chemical modifications to the metformin structure may enhance its hormonal properties. Among these derivatives, Compound 1 exhibited the highest enzyme activation efficacy, followed by Compound 2, and then Compound 3 with a lower degree of activity.

Study of the effect of Dapagliflozin treatment on the Aromatase enzyme:

The effect of dapagliflozin treatment on the aromatase enzyme has been investigated, and findings demonstrated that this drug acts as an inhibitor of the enzyme's activity. According to the following results:

Table 7: The effect of substance concentration on the activity of Aromatase.

Substrate	Activity without Inhibitor	Activity with Inhibitor	1/s	1/v	1/I
Stock(1mM)	6.002144	2.885834	1	0.166607132	0.346520278
Conc.1 (0.8mM)	5.7137	2.772227	1.25	0.175017939	0.360720821
Conc.2 (0.6mM)	5.45247	2.618826	1.666666667	0.186829632	0.381850493
Conc.3 (0.4mM)	5.15247	2.305219	2.5	0.194081673	0.433798264
Conc.4 (0.2mM)	4.016775	1.972227	5	0.248955941	0.507041025
Conc.5 (0.1mM)	2.115443	1.385834	10	0.472714226	0.721587145



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Fig. 13: The effect of the inhibitor on the reaction rate

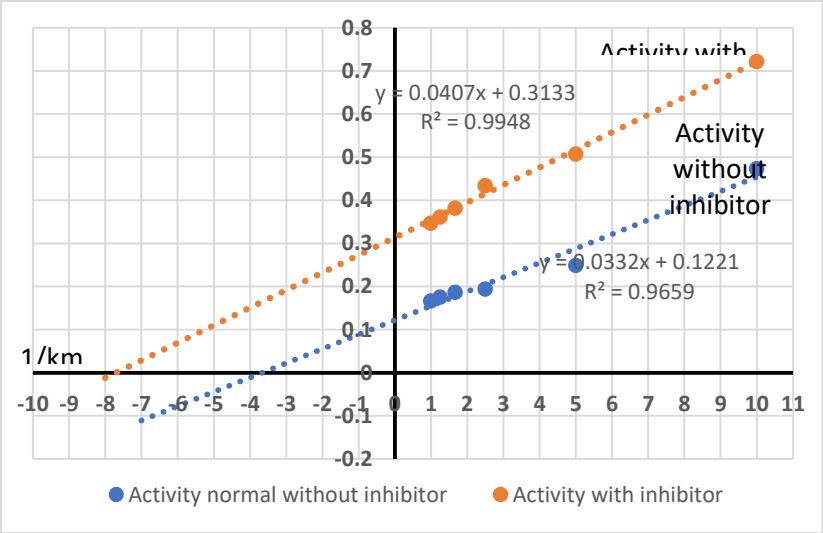


Fig. 14: Line Weaver–Burk plot for partially purified Aromatase.

Activity without inhibitor: The enzymatic activity decreases progressively with the reduction of substrate concentration. **Activity with inhibitor:** In the presence of dapagliflozin, the enzymatic activity is markedly lower compared to the inhibitor-free condition at all substrate concentrations. These findings confirm that dapagliflozin significantly inhibits aromatase activity.

Table 8: KM and Vmax values with and without inhibitor

	Without inhibitor	With inhibitor
Km	0.271908272	0.12990744
Vmax	8.19000819	3.191828918

Decrease in Km and Vmax, That is, the inhibition is Uncompetitive.

The concurrent decrease in Km and Vmax indicates that dapagliflozin inhibits aromatase through uncompetitive inhibition, binding to the enzyme–substrate complex and reducing its maximum capacity to convert the substrate into product.

Table 9: inhibition ratio

C0NC (PPM) ligand complex	Activity IU/L	Inhibition %	Recovery
Control	6.002144	0	100
Inhibitor (Stock)(10mM)	2.885834	58.78642	41.21358
(8 mM) Conc.1	2.772227	60.40888	39.59112
(6 mM) Conc.2	2.618826	62.59966	37.40034
(4 mM) Conc.3	2.305219	67.07838	32.92162
(2 mM) Conc.4	1.972227	71.83396	28.16604
(1 mM) Conc.5	1.385834	80.20843	19.79157

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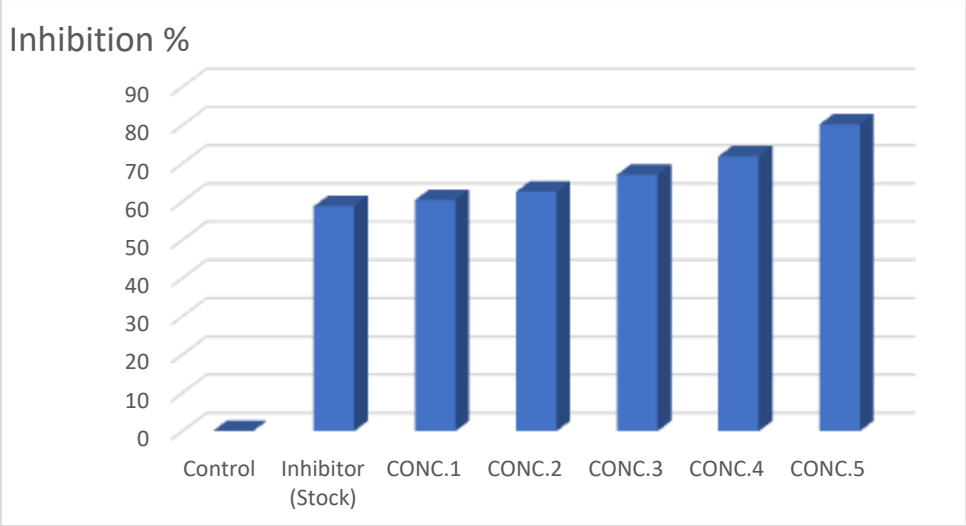


Fig.15: Enzyme inhibition rate at different concentrations of inhibitor

At the highest tested concentration (CONC.5), dapagliflozin achieved approximately 80% inhibition of aromatase activity, leaving only about 20% residual activity. Notably, even at the lowest tested concentration (Stock), inhibition remained substantial at around 59%, highlighting dapagliflozin's potency as an aromatase inhibitor, with significant effects observed even at low concentrations.

Conclusions

The study successfully purified aromatase enzyme from PCOS patients and determined its optimal conditions and kinetic parameters. Dapagliflozin treatment was found to act as an inhibitor of the aromatase enzyme, and understanding the molecular interactions between certain metformin derivatives and the aromatase enzyme.

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