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***Research Article** (Open Access)*

INHIBITION OF AMINOGLYCOSIDE-MODIFYING ENZYMES BY *OCIMUM SANCTUM* COMPOUNDS: AN IN-SILICO STUDY ON 3,4-DIHYDROXYPHENYLLACTIC ACID, CAFFEIC ACID, AND CHLOROGENIC ACID

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ABSTRACT
Aminoglycoside resistance through modifying enzymes poses a significant challenge in antimicrobial therapy. This study investigated the potential inhibitory effects of three bioactive compounds isolated from *Ocimum sanctum* (Holy Basil) - 3,4-dihydroxyphenyllactic acid, caffeic acid, and chlorogenic acid - against aminoglycoside-2"-phosphotransferase-IIa (APH(2")-IIa), a clinically significant resistance enzyme. Using molecular docking approaches, we analyzed the binding interactions and inhibitory potential of these compounds. The results revealed that chlorogenic acid exhibited superior binding affinity (-8.8 kcal/mol) compared to 3,4-dihydroxyphenyllactic acid and caffeic acid (both -6.1 kcal/mol). Chlorogenic acid formed an extensive network of seven hydrogen bonds with key catalytic residues and demonstrated comprehensive interactions with the SRY403 cofactor interface. The compound established strategic binding within the catalytic domain, particularly with active site residues Asn261 and Asp228, supported by 55 non-bonded contacts. In comparison, 3,4-dihydroxyphenyllactic acid formed three hydrogen bonds, notably with Arg92, while caffeic acid established five hydrogen bonds, including direct interaction with the ATP binding domain. The structural analysis revealed that molecular size, hydroxyl group positioning, and aromatic scaffolds were crucial determinants of binding affinity. These findings suggest that chlorogenic acid from O. sanctum could serve as a promising lead compound for developing AME inhibitors to combat aminoglycoside resistance.

Keywords: *Aminoglycoside resistance; APH(2")-IIa; Ocimum sanctum; Chlorogenic acid; Caffeic acid; 3,4-dihydroxyphenyllactic acid.*

INTRODUCTION

Antibiotic resistance represents one of the most pressing challenges in modern healthcare, threatening to undermine decades of progress in infectious disease treatment (Nwobodo *et al.,* 2022). Among the various classes of antibiotics, aminoglycosides have historically played a crucial role in combating serious bacterial infections (Serio *et al.,* 2018). However, the emergence and spread of resistance mechanisms, particularly through aminoglycoside-modifying enzymes (AMEs), have significantly compromised their therapeutic efficacy (Wang *et al.,* 2022). This concerning trend has sparked an urgent need for innovative strategies to overcome bacterial resistance mechanisms while preserving the utility of existing aminoglycoside antibiotics (Wang *et al.,* 2022). Aminoglycosides, discovered in the 1940s, continue to serve as essential broad-spectrum antibiotics in treating severe bacterial infections. These compounds exert their antibacterial effect by binding to the bacterial 30S ribosomal subunit, disrupting protein synthesis and ultimately leading to bacterial cell death (Hermann, 2007). However, the widespread use of aminoglycosides has led to the evolution and dissemination of various resistance mechanisms, with AMEs emerging as the most prevalent and clinically significant.

Aminoglycoside-2"-phosphotransferase-IIa (APH(2")-IIa) represents one of the most concerning AMEs, capable of rendering multiple aminoglycoside antibiotics ineffective through phosphorylation of specific hydroxyl groups (Shi *et al.,* 2013). This enzyme belongs to the larger family of aminoglycoside phosphotransferases, which catalyze the transfer of a phosphate group from ATP to specific positions on the aminoglycoside molecule (Shi *et al.,* 2013). The resulting phosphorylated

aminoglycosides exhibit significantly reduced binding affinity for their target site on the bacterial ribosome, effectively neutralizing their antibacterial activity (Shi *et al.,* 2013). The prevalence of APH(2")-IIa in clinical isolates, particularly in *methicillin-resistant Staphylococcus aureus* (MRSA) and *vancomycin-resistant enterococci* (VRE), has become a major concern in healthcare settings (Weigel *et al.,* 2007). The search for AME inhibitors has gained considerable attention as a promising strategy to restore aminoglycoside effectiveness (Zárate *et al.,* 2018). Their clinical significance is particularly notable in treating infections caused by Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, and various *Enterobacteriaceae* species (Kaye & Pogue, 2015). Natural products, with their diverse chemical structures and biological activities, have emerged as valuable sources of potential enzyme inhibitors (Rahman, 2023).

Among various medicinal plants, *Ocimum sanctum* (Holy Basil or Tulsi) has garnered particular interest due to its rich ethnopharmacological history and demonstrated antimicrobial properties (Malakar & Mandal, 2024). This sacred plant of Indian origin has been extensively used in traditional Ayurvedic medicine for treating various ailments, including infectious diseases (Malakar & Mandal, 2024). Recent phytochemical investigations of *O. sanctum* have revealed a complex profile of bioactive compounds, including phenylpropanoids, flavonoids, and phenolic acids (Ozay *et al.,* 2024). Of particular interest are three compounds: 3,4-dihydroxyphenyllactic acid, caffeic acid, and chlorogenic acid (Ozay *et al.,* 2024). These compounds share structural features that suggest potential interaction with enzyme active sites, including hydroxyl groups capable of forming hydrogen bonds and aromatic rings that could participate in π-π stacking interactions with aromatic amino acid residues in proteins (Hădărugă & Hădărugă, 2023).

3,4-dihydroxyphenyllactic acid, a phenolic compound found in *O. sanctum*, has demonstrated various biological activities, including antioxidant and antimicrobial properties (Hasan *et al.,* 2023). Its structure, featuring a carboxylic acid group and two hydroxyl groups on a phenyl ring, suggests potential for multiple interaction points with enzyme active sites (Hasan *et al.,* 2023). Similarly, caffeic acid, a hydroxycinnamic acid derivative, has shown promising biological activities, including enzyme inhibition in various contexts (da Silva *et al.,* 2023). Chlorogenic acid, an ester formed between caffeic acid and quinic acid, represents another important phenolic compound with demonstrated biological activities and potential enzyme-inhibitory properties (Dizdar *et al.,* 2024). The structural characteristics of these compounds, combined with their natural occurrence in a traditionally used medicinal plant, make them interesting candidates for investigation as potential AME inhibitors. Modern computational approaches, particularly molecular docking studies, offer powerful tools for investigating protein-ligand interactions and predicting binding affinities. These methods can provide valuable insights into the potential mechanisms of enzyme inhibition and guide the development of more effective inhibitor compounds.

MATERIALS AND METHODS

In Silico Analysis Platform and Parameters

Computational analyses were executed using AutoDock Vina (version 1.1.2) for molecular docking simulations (Trott & Olson, 2010). All compounds were geometrically optimized to their lowest energy conformational state in three-dimensional space. The molecular target selected for antibacterial activity assessment was Aminoglycoside-2"-phosphotransferase-IIa (APH2"-IIa), a critical resistance enzyme expressed in both Gram-positive and Gram-negative bacteria that catalyzes the deactivation of aminoglycoside antibiotics. The enzyme structure was obtained from the RCSB Protein Data Bank (PDB ID: 3HAV). Binding affinity quantification utilized the Vina scoring function, which computes the aggregate of intermolecular and intramolecular energetic components. The scoring algorithm incorporates protein-ligand interaction energies, cofactor-ligand associations, and ligand-specific parameters including torsional strain energetics, sp2-sp2 stereochemical constraints, and electrostatic interactions (Trott & Olson, 2010).

Ligand Selection and Optimization

Three bioactive compounds from *Ocimum sanctum* were selected based on previous phytochemical characterization studies: 3,4-dihydroxyphenyllactic acid, caffeic acid, and chlorogenic acid. Three-dimensional conformer structures were acquired from the PubChem database (CID_439435,

CID_689043, and CID_1794427, respectively). Structural optimization was performed using AutoDock Tools (version 1.5.7) through energy minimization protocols implementing the MMFF94 force field (Ding *et al.*, 2023).

Protein Structure Preparation

The high-resolution crystal structure of APH(2")-IIa was retrieved from the RCSB Protein Data Bank (PDB ID: 3HAV). Protein preparation was conducted using AutoDock Tools 1.5.7, encompassing the removal of crystallographic water molecules, addition of polar hydrogen atoms, Kollman charge assignment, and optimization of protonation states for ionizable residues at physiological pH (Ding *et al.*, 2023).

Molecular Docking Protocol

Molecular docking simulations were executed using AutoDock Vina with the following parameters: The binding site was delineated based on the spatial coordinates of the co-crystallized ligand, with grid box dimensions of 40 × 40 × 40 Å centered at X = −20.27 Å, Y = 8.05 Å, Z = −16.46 Å. The binding cavity encompassed 43 amino acid residues. Docking calculations employed an exhaustiveness parameter of 8, and the nine highest-scoring poses were retained for subsequent analysis (Trott & Olson, 2010).

RESULTS AND DISCUSSION

Molecular Binding Affinity Evaluation

Molecular docking simulations revealed differential binding energetics among the three bioactive compounds. Chlorogenic acid exhibited superior binding affinity (-8.8 kcal/mol), while both 3,4-dihydroxyphenyllactic acid and caffeic acid demonstrated equivalent affinities (-6.1 kcal/mol). All conformations achieved optimal convergence with root-mean-square deviation (RMSD) values of 0.000 for both lower and upper bounds, indicating high docking precision.

Table 1. Quantitative Analysis of Binding Energetics and Conformational Parameters

Compound	Affinity (kcal/mol)	Dist. from rmsd l.b.	best mode rmsd u.b.
3,4-dihydroxyphenyllactic acid	-6.1	0.000	0.000
Caffeic acid	-6.1	0.000	0.000
Chlorogenic acid	-8.8	0.000	0.000

Table 1. Characterization of Protein-Ligand Interaction Networks.

Compound	Hydrogen Bonds	Notable Interacting Residues	Key Interaction Features
3,4-dihydroxyphenyl-lactic acid	3	Thr231, Arg92	• High-affinity H-bond with Arg92 (2.894 Å) • Asp232/233 hydrophobic cluster engagement • Sub-pocket occupancy near Ala195-Tyr95-Thr94
Caffeic acid	5	ATP401, Ala195, Arg92, Gly90, Tyr87	• ATP binding site recognition (3.000 Å) • Bifurcated backbone H-bonding • π-π stacking with Tyr87 (2.902 Å)
Chlorogenic acid	7	SRY403, Asp268, Asn261, Ala258, Asn191, Lys142	• Extensive cofactor interactions • Catalytic residue engagement • Multiple stabilizing non-bonded contacts (n=55)

Intermolecular Interaction Analysis

The molecular docking analysis revealed distinct interaction patterns between APH(2")-IIa and the three bioactive compounds from Ocimum sanctum. A comparative analysis of binding interactions is presented in Table 2.

Structure-Based Binding Mode Analysis

3,4-dihydroxyphenyllactic acid

The compound established a tripartite hydrogen bonding network, with the most significant interaction observed with Arg92 (2.894 Å), suggesting strong anchoring at the binding site. The extensive non-bonded contacts, particularly with the Asp232/233 cluster, indicate stabilization through hydrophobic interactions. The binding topology indicates preferential occupation of a defined sub-pocket bordered by Ala195, Tyr95, and Thr94 residues.

Caffeic acid

Caffeic acid demonstrated unique binding characteristics through five hydrogen bonds, including a direct interaction with the ATP binding domain (3.000 Å). The phenolic hydroxyl groups served as key interaction points, forming hydrogen bonds with both protein backbone and side chain atoms. The compound's planar aromatic structure enabled favourable π-stacking interactions, particularly with Tyr87 (2.902 Å), enhancing binding stability.

Chlorogenic acid

Among the three compounds, chlorogenic acid exhibited the most extensive interaction network, forming seven hydrogen bonds. The compound established an extensive heptavalent hydrogen bonding network throughout the binding pocket, complemented by comprehensive interactions with the SRY403 cofactor interface. Its direct strong interaction with catalytic residues (Asp268, Lys142) suggests potential interference with enzymatic function. The complex is further stabilized by an extensive network of non-bonded contacts (55 interactions) providing additional stabilization, while maintaining strategic positioning within the catalytic domain positioning near the enzyme's active site residues (Asn261, Asp228)that optimizes its inhibitory potential.

Structure-Activity Relationship Analysis

Detailed examination of the molecular interactions has revealed several key determinants governing binding affinity in this system. The strategic vectorization of hydroxyl groups throughout the molecule facilitates optimal hydrogen bond formation with complementary protein residues. The presence of aromatic scaffolds provides essential hydrophobic complementarity within the binding pocket, while the overall molecular size demonstrates a direct correlation with the extensiveness of the interaction network. Additionally, the strategic positioning of carboxylic acid moieties enables the formation of stabilizing salt bridges with basic residues within the binding site, contributing significantly to the overall binding energy. Chlorogenic acid distinguishes itself as the most promising inhibitor candidate through multiple compelling characteristics. Its superior binding energetics, quantified at -8.8 kcal/mol, significantly outperform those of other tested compounds. This enhanced affinity is attributed to its complex and extensive hydrogen bonding network, coupled with optimal engagement with the enzyme's catalytic architecture and strategic occupation of the cofactor binding site. These findings provide critical insights for structure-guided optimization of APH(2")-IIa inhibitors, establishing a robust foundation for rational drug design targeting aminoglycoside resistance mechanisms. The natural occurrence of these compounds in *O. sanctum* suggests favourable therapeutic indices, though comprehensive pharmacological validation studies are warranted to fully establish their clinical potential. These findings provide critical insights for structure-guided optimization of APH(2")-IIa inhibitors. The identified molecular recognition features, particularly those of chlorogenic acid, establish a foundation for rational drug design targeting aminoglycoside resistance mechanisms. The natural occurrence of these compounds in *O. sanctum* suggests favourable therapeutic indices, though further pharmacological validation is warranted.

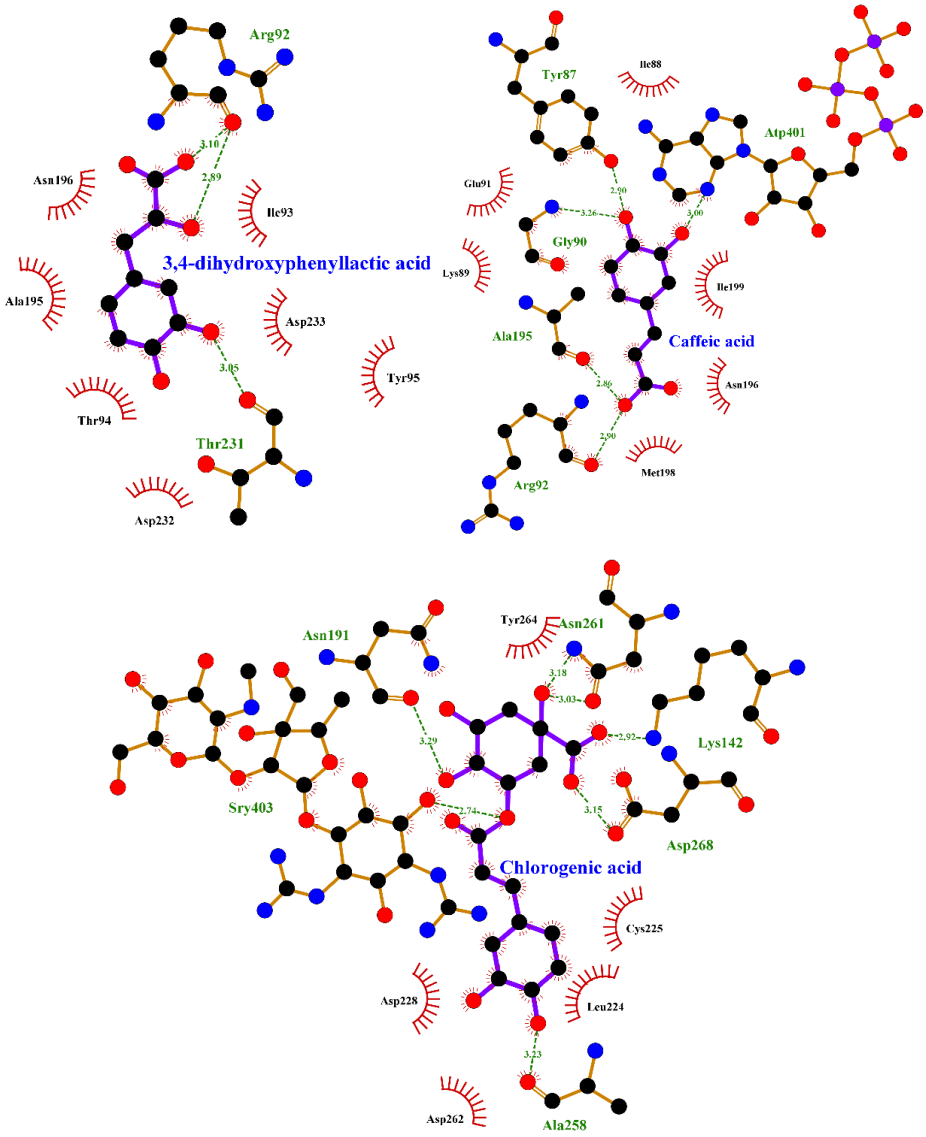

Figure 1: Interaction Plots of 3,4-dihydroxyphenyllactic acid, caffeic acid, and chlorogenic acid.

CONCLUSION

This computational study provides compelling evidence for the potential of Ocimum sanctum compounds as inhibitors of APH(2")-IIa, with chlorogenic acid emerging as the most promising candidate. The molecular docking analysis revealed distinct binding patterns and interaction networks for each compound, with chlorogenic acid demonstrating superior binding affinity and the most extensive interaction network. Its strategic engagement with catalytic residues and comprehensive binding profile, including seven hydrogen bonds and 55 non-bonded contacts, suggests significant potential for enzyme inhibition. The structure-activity relationship analysis highlighted the importance of hydroxyl group vectorization, aromatic scaffolds, and molecular size in determining binding affinity. The natural occurrence of these compounds in O. sanctum suggests potential therapeutic advantages, though further experimental validation is essential. These findings establish a valuable foundation for the rational design of AME inhibitors and offer a promising direction for addressing aminoglycoside resistance. Future research should focus on experimental validation of these computational findings, including in vitro enzyme inhibition assays and determination of minimum inhibitory concentrations in resistant bacterial strains.

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