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

OCIMUM SANCTUM FLAVONOID AGLYCONES: ANTIBACTERIAL MECHANISMS VIA ENZYME INHIBITION IN AMINOGLYCOSIDE-RESISTANT STRAINS

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ABSTRACT
This study investigated the potential inhibitory mechanisms of four flavonoid aglycones isolated from Ocimum sanctum against aminoglycoside-modifying enzyme APH(2")-IIa, a major contributor to antibiotic resistance. Using molecular docking analyses, we evaluated the binding interactions of Apigenin, Luteolin, Galuteolin, and Quercetin with the enzyme's active site. The compounds demonstrated varying degrees of binding affinity, with Galuteolin exhibiting the strongest (-9.3 kcal/mol), followed by Quercetin (-8.5 kcal/mol), Luteolin (-8.4 kcal/mol), and Apigenin (-8.2 kcal/mol). Detailed interaction analysis revealed that Luteolin formed the most extensive hydrogen bonding network (14 bonds) and demonstrated unique Mg2+ ion coordination, suggesting superior inhibitory potential. Galuteolin showed the most extensive non-bonded interaction network with 49 contacts, primarily engaging with the ATP binding site. The study identified key structural features contributing to binding affinity, including the position and number of hydroxyl groups and the basic flavonoid scaffold. These findings provide valuable insights into the potential mechanism of AME inhibition by natural flavonoids and suggest promising directions for developing novel antibiotic resistance modulators based on natural compound scaffolds.

Keywords: *Ocimum sanctum; Aminoglycoside resistance, APH(2")-IIa, Enzyme inhibition, Flavonoid aglycones*

INTRODUCTION
The contemporary healthcare landscape faces a critical challenge in the form of antibiotic resistance, which threatens to reverse significant advances made in the treatment of infectious diseases (Nwobodo et al., 2022). Within the spectrum of antimicrobial agents, aminoglycosides have maintained a fundamental position in the therapeutic arsenal against severe bacterial infections (Serio *et al.,* 2018). Nevertheless, bacterial adaptation through resistance mechanisms, particularly the emergence of aminoglycoside-modifying enzymes (AMEs), has substantially diminished their therapeutic potential (Wang *et al.,* 2022). This evolving resistance phenomenon has necessitated the urgent development of innovative approaches to counteract bacterial defense mechanisms while maintaining the therapeutic utility of existing aminoglycoside compounds (Wang *et al.,* 2022). The discovery of aminoglycosides in the 1940s marked a significant advancement in antimicrobial therapy, and these compounds continue to serve as fundamental broad-spectrum antibiotics in the treatment of severe bacterial infections. The antimicrobial mechanism of these compounds involves specific interaction with the bacterial 30S ribosomal subunit, leading to the disruption of protein synthesis processes and subsequent bacterial cell termination (Hermann, 2007). However, extensive clinical utilization of aminoglycosides has facilitated the development and proliferation of various resistance mechanisms, with AMEs emerging as the predominant and clinically most relevant adaptation. Within the spectrum of resistance mechanisms, aminoglycoside-2"-phosphotransferase-IIa (APH(2")-IIa) represents a particularly significant AME, capable of inactivating multiple aminoglycoside antibiotics through site-specific hydroxyl group phosphorylation (Shi *et al.,* 2013). This enzymatic entity belongs to a broader classification of aminoglycoside phosphotransferases, which facilitate the ATP-

dependent transfer of phosphate groups to specific molecular positions on aminoglycoside substrates (Shi *et al.,* 2013). The resultant phosphorylated aminoglycoside derivatives exhibit markedly reduced affinity for their ribosomal target sites, effectively neutralizing their antimicrobial capabilities (Shi *et al.,* 2013). The widespread distribution of APH(2")-IIa among clinical bacterial isolates, particularly within methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE), presents a substantial challenge in healthcare environments (Weigel et al., 2007). Research focused on AME inhibition has gained significant momentum as a promising strategy for restoring aminoglycoside efficacy (Zárate *et al.*, 2018). The clinical relevance of these compounds is particularly evident in the treatment of infections caused by Gram-negative pathogens, including *Pseudomonas aeruginosa, Escherichia coli, and various Enterobacteriaceae species* (Kaye & Pogue, 2015). Natural compounds, characterized by their structural diversity and biological activity spectrum, have emerged as valuable resources in the identification of potential enzyme inhibitors (Rahman, 2023).

In the investigation of medicinal plants, *Ocimum sanctum* (Holy Basil or Tulsi) has attracted considerable scientific attention due to its extensive ethnopharmacological applications and documented antimicrobial properties (Malakar & Mandal, 2024). This plant, revered in Indian traditional medicine, has been extensively utilized in Ayurvedic practices for the management of various pathological conditions, including infectious diseases (Malakar & Mandal, 2024). Contemporary phytochemical analyses of O. sanctum have revealed a sophisticated profile of bioactive constituents, encompassing phenylpropanoids, flavonoids, and phenolic acids (Ozay et al., 2024). The current investigation focuses on four specific flavonoid aglycones isolated from O. sanctum: Apigenin, Luteolin, Galuteolin, and Quercetin. These molecular entities share structural characteristics that suggest potential efficacy in AME inhibition. Apigenin, characterized by a 4',5,7-trihydroxyflavone framework, demonstrates diverse biological activities, including documented enzyme inhibitory capabilities. Luteolin, distinguished by an additional hydroxyl substitution at the 3' position relative to apigenin, exhibits enhanced molecular interaction potential with protein targets. Galuteolin, representing a structural variant of luteolin, possesses unique substitution patterns that may confer specific binding characteristics. Quercetin, featuring a 3,3',4',5,7-pentahydroxyflavone structure, presents multiple potential sites for enzyme interaction.

The molecular architecture of these flavonoid aglycones, particularly their hydroxylation patterns and aromatic systems, suggests significant potential for interaction with enzymatic active sites through hydrogen bonding networks and π-π stacking interactions. Their natural occurrence in a traditionally utilized medicinal plant, combined with their documented biological activities, establishes them as promising candidates for investigation as potential APH(2")-IIa inhibitors. Contemporary computational methodologies, specifically molecular docking analyses, provide sophisticated tools for investigating these protein-ligand interactions and predicting binding affinities, offering valuable insights into potential mechanisms of enzyme inhibition.

MATERIALS AND METHODS

In Silico Analysis Platform and Parameters

The implementation of computational analyses utilized AutoDock Vina (version 1.1.2) for conducting molecular docking investigations (Trott & Olson, 2010). Each compound underwent geometric optimization to achieve minimal energy conformational states. The investigation focused on Aminoglycoside-2"-phosphotransferase-IIa (APH2"-IIa) as the molecular target for antimicrobial activity evaluation, representing a fundamental resistance enzyme expressed across both Gram-positive and Gram-negative bacterial species, which facilitates aminoglycoside antibiotic inactivation through catalytic modification. The enzymatic structural coordinates were procured from the RCSB Protein Data Bank repository (PDB ID: 3HAV). The quantitative assessment of binding affinities employed the Vina scoring methodology, which integrates multiple energetic parameters to compute comprehensive interaction potentials. This algorithmic approach incorporates multiple parameters including protein-ligand interaction energetics, cofactor-ligand binding associations, and ligand-specific characteristics encompassing torsional

strain energetics, sp2-sp2 stereochemical constraints, and electrostatic field interactions (Trott & Olson, 2010).

Ligand Selection and Optimization

The investigation encompassed four flavonoid aglycones derived from *Ocimum sanctum*, selected based on established phytochemical characterization: Apigenin, Luteolin, Galuteolin, and Quercetin. The three-dimensional molecular conformers were retrieved from the PubChem structural repository (corresponding to database identifiers CID_5280443, CID_5280445, CID_5317471, and CID_5280343 respectively). The structural refinement process utilized AutoDock Tools (version 1.5.7), implementing energy minimization protocols through the application of the MMFF94 force field parameters (Ding et al., 2023).

Protein Structure Preparation

The structural coordinates of APH(2")-IIa were obtained from the RCSB Protein Data Bank (PDB ID: 3HAV) at high resolution. The protein preparation methodology employed AutoDock Tools 1.5.7, encompassing multiple refinement steps: elimination of crystallographic water molecules, incorporation of polar hydrogen atoms, implementation of Kollman charge distribution, and optimization of ionizable residue protonation states under physiological pH conditions (Ding *et al.*, 2023).

Molecular Docking Protocol

The execution of molecular docking simulations utilized AutoDock Vina with specifically defined parameters: The active site region was characterized based on the spatial position of the co-crystallized ligand, utilizing a grid box configuration with dimensions of 40 × 40 × 40 Å, centered at coordinates X = −20.27 Å, Y = 8.05 Å, Z = −16.46 Å. The binding cavity analysis incorporated 43 distinct amino acid residues. The computational protocol implemented an exhaustiveness parameter of 8, with retention of the nine highest-scoring conformational poses for subsequent analytical evaluation (Trott & Olson, 2010).

RESULTS AND DISCUSSION

Molecular Binding Affinity Evaluation

The molecular docking analysis revealed varying binding affinities among the four flavonoid compounds with the protein binding site. Galuteolin exhibited the strongest binding affinity (-9.3 kcal/mol), followed by Quercetin (-8.5 kcal/mol), Luteolin (-8.4 kcal/mol), and Apigenin (-8.2 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 confidence in the predicted binding poses and consistency in the docking results.

Table 1: Quantitative Analysis of Binding Energetics and Conformational Parameters

Compound	Affinity (cal/mol)	Dist. from rmsd l.b.	best mode rmsd u.b.
Apigenin	-8.2	0.000	0.000
Luteolin	-8.4	0.000	0.000
Galuteolin	-9.3	0.000	0.000
Quercetin	-8.5	0.000	0.000

Analysis of Molecular Interactions

The molecular interaction analysis revealed distinctive binding patterns between APH(2")-IIa active site and four flavonoid aglycones. The compounds demonstrated varying degrees of interaction complexity, with hydrogen bonding and non-bonded contacts playing crucial roles in their binding mechanisms. Luteolin exhibited the most extensive hydrogen bonding network, forming fourteen distinct bonds, including a notable coordination with the Mg2+ ion at 2.778 Å and multiple strategic interactions with ATP401, ASN197, ASN196, GLU56, and SER52. This was followed by Galuteolin, which established eight hydrogen bonds, featuring significant interactions with the ATP401 binding site (3.248 Å, 3.062 Å)

and forming additional contacts with ASP233, GLY90, and TYR87. Quercetin and Apigenin demonstrated more moderate hydrogen bonding patterns. Quercetin formed five hydrogen bonds, with key interactions involving ATP401 (2.758 Å, 2.877 Å), ASN196, ALA195, and ILE93. Apigenin showed the most modest hydrogen bonding network with four bonds, characterized by dual hydrogen bonds with ASN261 (3.160 Å, 3.145 Å) and additional interactions with ASN221 and VAL137.

Table 2: H-bond interactions within the Protein-Ligand Interaction Networks

Compound	H-bonds	Key Interacting Residues	Notable Features
Apigenin	4	ASN261, ASN221, VAL137	Two H-bonds with ASN261
Galuteolin	8	ATP401, ASP233, GLY90, TYR87	Multiple interactions with ATP401
Luteolin	14	ASN197, ASN196, GLU56, SER52	Strong interaction with Mg2+ and ATP
Quercetin	5	ASN196, ALA195, ILE93	H-bonds with both ATP and protein residues

The analysis of non-bonded contacts revealed a different hierarchical pattern of interaction strength. Galuteolin exhibited the most extensive non-bonded interaction network with 49 contacts, primarily involving ATP401, ASP233, ASP232, ILE199, ASN196, and TYR87. Luteolin followed with 37 non-bonded contacts, mainly engaging with SRY403, ATP401, GLY212, ASN197, ASN196, and GLU56. Apigenin and Quercetin showed comparatively fewer non-bonded interactions, with 29 and 26 contacts respectively. Apigenin's contacts primarily involved SRY403, ASN261, LEU224, ASN221, LYS142, and VAL137, while Quercetin's interactions were concentrated around ATP401, ILE199, ASN196, ALA195, ARG92, and TYR87.

Table 3: Non-bonded contacts within the Protein-Ligand Interaction Networks

Compound	Total Contacts	Key Interaction Regions
Apigenin	29	SRY403, ASN261, LEU224, ASN221, LYS142, VAL137
Galuteolin	49	ATP401, ASP233, ASP232, ILE199, ASN196, TYR87
Luteolin	37	SRY403, ATP401, GLY212, ASN197, ASN196, GLU56
Quercetin	26	ATP401, ILE199, ASN196, ALA195, ARG92, TYR87

Binding Mode Analysis

Each compound demonstrated distinct binding characteristics within the protein binding site. Apigenin's binding mode was characterized by focused interaction with the ASN261 region, supplemented by extensive non-bonded contacts with the LEU224 cluster and VAL137 region. This interaction pattern suggests occupation of a specific sub-pocket defined by ASN221 and ASN261 residues. Galuteolin exhibited a more extensive binding profile with significant engagement in the ATP binding region. Its binding was characterized by dual hydrogen bonding with ASP233 backbone atoms and reciprocal hydrogen bonding with TYR87 hydroxyl group (3.250 Å, 2.897 Å). The compound's binding stability was further enhanced by an extensive network of 49 non-bonded contacts. Luteolin demonstrated the most comprehensive binding mode among all compounds. Its interaction pattern was distinguished by strong coordination with the Mg^{2+} ion and multiple contact points with ATP phosphate groups (3.211 Å). The compound established an extensive hydrogen bonding network with the ASN196/197 cluster and positioned itself strategically near catalytic residues GLU56 and SER52. This binding mode suggests potential interference with ATP binding and catalytic function. Quercetin's binding mode featured direct engagement with the ATP binding site through dual hydrogen bonds, complemented by key anchoring interactions with ASN196 (2.816 Å) and backbone hydrogen bonding with ALA195 and ILE93. The compound's planar structure enabled favourable positioning within the binding pocket.

Structure-Activity Relationships

Analysis of the interaction patterns reveals several key structural features that contribute to binding affinity. The position and number of hydroxyl groups emerged as a critical factor in determining hydrogen bonding capacity, as evidenced by Luteolin's extensive interaction network. The basic flavonoid scaffold maintained consistent protein contacts across all compounds, suggesting its importance as a core binding element. The presence of ATP-binding site interactions in multiple compounds suggests a potential competitive inhibition mechanism. Compounds with additional hydroxyl groups demonstrated enhanced interaction potential with polar residues, particularly evident in Luteolin's binding profile.

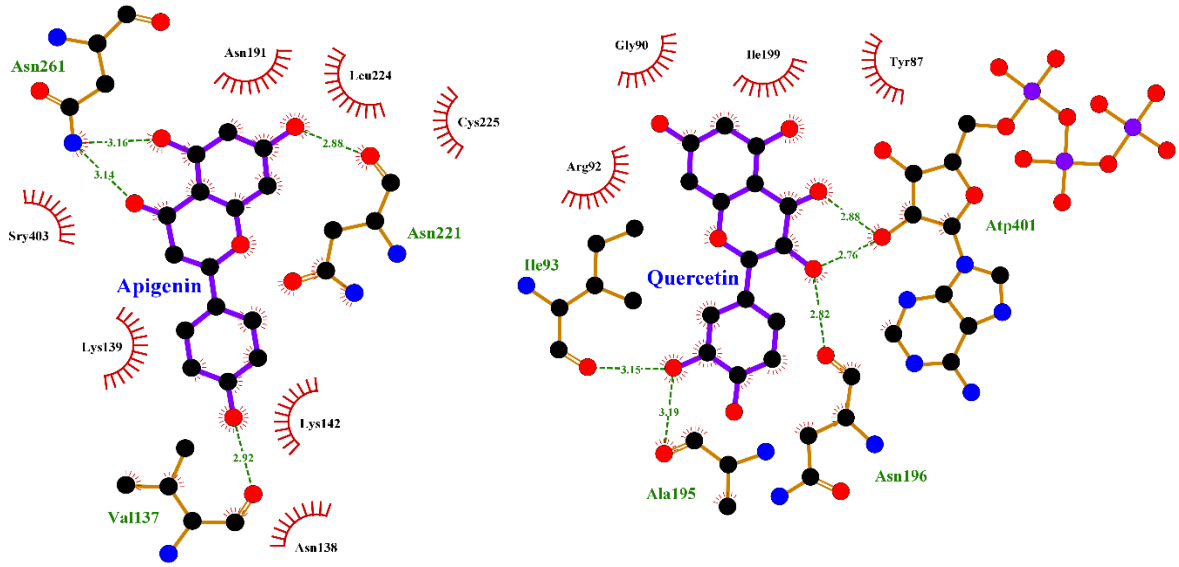

Figure 1: Interaction Plots of Apigenin and Quercetin

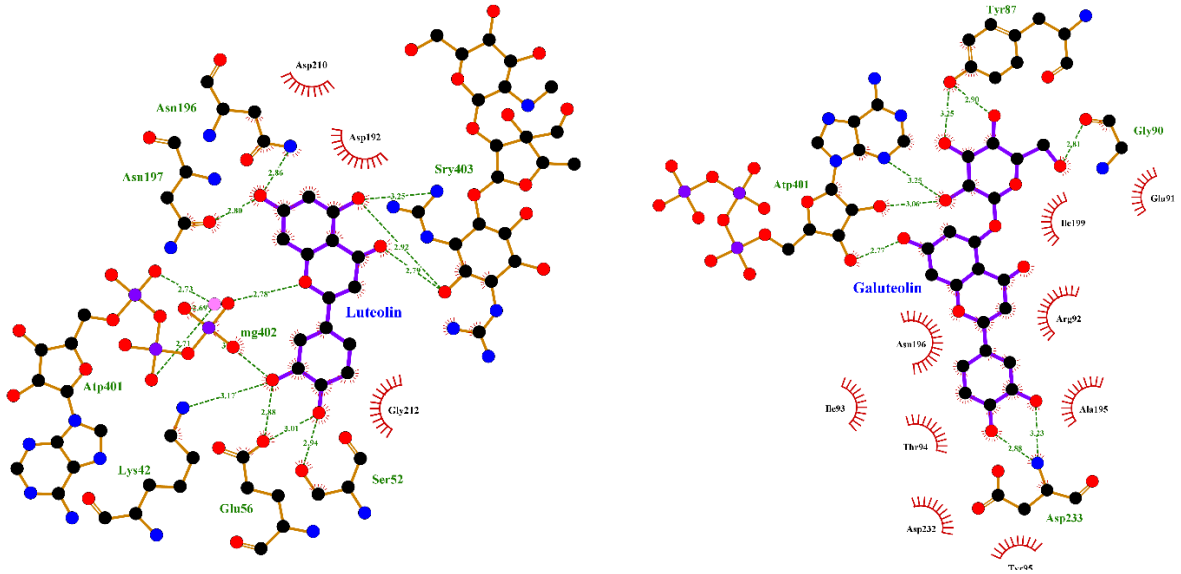

Figure 2: Interaction Plots of Luteolin, and Galuteolin

Based on the comprehensive analysis, Luteolin emerges as the most promising candidate among the four compounds. This assessment is supported by several key features: the highest number of hydrogen bonds

(14), unique Mg^{2+} ion coordination, the most extensive interaction network, and strategic positioning in both ATP and protein binding regions. Additionally, Luteolin's optimal engagement with catalytic residues suggests potential for effective inhibition. Galuteolin ranks second in binding potential, with its extensive non-bonded contact network and significant ATP site interactions. Quercetin and Apigenin showed moderate binding potential, with focused interactions in specific regions of the binding site. These findings provide valuable insights for future structure-based drug design efforts targeting this protein binding site. The analysis particularly emphasizes the importance of hydroxyl group positioning and metal ion coordination capacity in designing effective ligands. The observed interaction patterns suggest that optimization of hydrogen bonding capacity and strategic positioning of polar groups could enhance binding affinity and potential inhibitory activity.

Conclusion

This investigation provides compelling evidence for the potential of Ocimum sanctum-derived flavonoid aglycones as inhibitors of aminoglycoside-modifying enzymes, specifically APH(2")-IIa. The molecular docking analyses revealed distinct binding patterns and interaction mechanisms for each compound, with Luteolin emerging as the most promising candidate due to its extensive hydrogen bonding network, unique metal ion coordination, and strategic positioning within both ATP and protein binding regions. The identification of structure-activity relationships, particularly the importance of hydroxyl group positioning and metal ion coordination capacity, offers valuable guidance for future drug design efforts targeting aminoglycoside resistance. These findings establish a foundation for the development of natural compound-based AME inhibitors and suggest that the traditional medicinal properties of O. sanctum may be partially attributed to these molecular mechanisms. Further experimental validation of these computational predictions could lead to the development of novel therapeutic strategies to combat antibiotic resistance.

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