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In Silico Design and Molecular Docking of Flavone Compounds as Potential Enzyme Inhibitors Targeting *Pseudomonas aeruginosa* RmlA Enzyme in Fish Pathogenesis

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ABSTRACT

Pseudomonas aeruginosa was found to be an opportunistic pathogen in aquaculture. It can cause severe diseases in fish and thus economic losses. The RmlA enzyme is essential for the biosynthesis of dTDP-L-rhamnose, and this compound plays a critical role in the bacterial virulence and its survival. Our study aimed to identify potential inhibitors of RmlA through molecular docking of 12 plant-derived flavonoid compounds. The three-dimensional crystal structure of RmlA was retrieved from the Protein Data Bank, and binding interactions were estimated using computational tools. The results of molecular docking of plant-derived flavonoid compounds showed strong binding affinities, and the most promising inhibitors were querciturone, isoquercetin, and spiraeoside, with energy affinities of –9.60kcal/ mol, –9.20kcal/ mol, and –8.60kcal/ mol, respectively. These compounds demonstrated interactions with key residues amino acids in the enzyme's active site, including ASP110, GLU161, and TYR145, forming diverse bonds such as hydrogen bonds, hydrophobic interactions, and electrostatic bonds. The inhibitors prevent the conformational transitions necessary for RmlA's ordered bi-bi mechanism, thereby blocking the biosynthesis of bacterial dTDP-L-rhamnose. This study highlighted the potential of using flavonoid-derived compounds as effective natural inhibitors against *P. aeruginosa* to help develop novel therapeutic strategies against bacterial infections in aquaculture.

INTRODUCTION

Due to the increasing consumption of fish and other marine products worldwide, their microbiological quality must be improved **(Ćirković *et al.*, 2002)**. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes serious infections in both humans and animals including fish. It is considered one of the most dangerous types of bacteria that infect fish since it is linked to hemorrhagic sepsis and ulcer syndrome **(Eissa *et al.*, 2010)**. Fish *Pseudomonas* is similar to a lot of other bacterial illnesses. The broad symptoms are typical of any bacterial fish illness and are not unique to a *Pseudomonas*

infection **(Algammal *et al.*, 2020)**. Different bacterial diseases are known to afflict many aquatic species, causing prominent financial losses globally. Up to 50% of fish farms are thought to experience losses due to bacterial infections before the fish reach the market **(El-Sayed *et al.*, 2019)**. Poor growth, high mortality, and low fish quality are key factors driving remarkable economic losses in the aquaculture industry. These issues stem from inadequate nutrition, poor water quality, diseases, and environmental stress **(Algammal *et al.*, 2020; Alzobaidy *et al.*, 2024)**. *P. aeruginosa* is recognized as an extremely dangerous and opportunistic organism, associated with catastrophic illnesses such as gill necrosis, hemorrhagic septicemia, splenomegaly, friable liver, and congested kidney **(Ardura *et al.*, 2013)**. Specifically, due to the active effect of antibiotics and the permeability of its outer membrane, *P. aeruginosa* may develop distinctive and acquired resistance to a variety of antimicrobial drugs **(Mesquita *et al.*, 2013)**. To prevent the formation of antibiotic-resistant strains that pose a threat to world health, the majority of inherited antibiotic-resistance genes should be molecularly typed **(Dalmasso *et al.*, 2009)**. The process of creating dTDP-L-rhamnose increases the virulence of *P. aeruginosa* by assisting in the production of lipopolysaccharides, which are necessary for the bacteria to attach to surfaces, avoid immune systems, and help them survive in different environments **(Williams *et al.*, 2017)**. The enzyme RmlA (dTDP-glucose-4,6-dehydratase) catalyzes the first step, which determines the rate at which this process occurs, therefore this enzyme is considered a target for the development of antimicrobial drugs **(Zhang *et al.*, 2024)**. Since the bacterial cell wall is essential to the survival of the bacteria and is made up of peptidoglycans and lipopolysaccharides that are absent from eukaryotic cells, it is a target for numerous antibiotics **(Gou *et al.*, 2018)**. Instead of binding at RmlA's active site, the inhibitors bind to a different location that is further away from the active site. Nevertheless, the compounds exhibit strong cooperativity while acting as competitive inhibitors of Glucose 1-Phosphate. Structural analysis was used to investigate this unusual behavior, and the results indicate that the inhibitors function by stopping RmlA from going through the conformational transition necessary for its ordered bi-bi process **(Alphey *et al.*, 2013)**. Flavonoids are among the most commonly consumed substances by humans and animals and are found in many plants such as onions and hawthorn **(Petersen *et al.*, 2016; Wang *et al.*, 2020)**. Plant flavonoids reduce oxidative stress and enhance immune responses, classifying them as immunopotentiators **(Li *et al.*, 2019; Mousa & Kareem, 2023)**. Flavonoids have various biological effects in mammals, including acting as antiestrogens **(Santini *et al.*, 2009)**. Modern computer techniques, such as molecular docking, provide new approaches to identify these compounds and to study their effects by predicting their affinity and inhibitory capacity against target enzymes **(Agamah *et al.*, 2020)**. The present study aimed to perform *in silico* design and molecular docking of some flavonoid-derived compounds targeting the RmlA enzyme in *P. aeruginosa*. Thus, disrupting the synthesis of dTDP-L-rhamnose, which in turn reduces the ability of the bacteria to cause diseases

in fish. The binding interactions of 12 flavonoid compounds to the RmlA enzyme were studied using computational tools with the aim of identifying compounds that could be used in the future. This study not only addresses the identification of antimicrobial agents in the field of aquaculture but also contributes to finding new ways to combat antibiotic-resistant pathogens in general.

MATERIALS AND METHODS

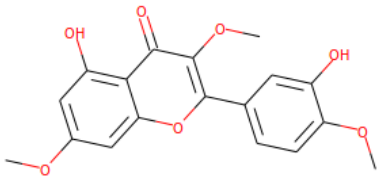
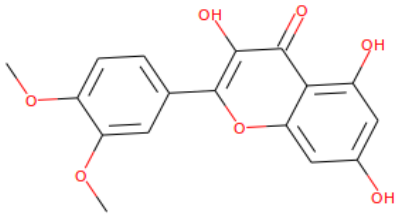
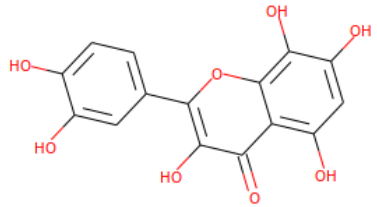
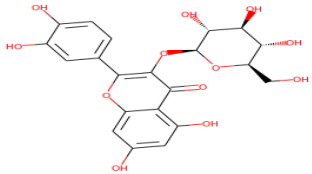
Pre-docking screening

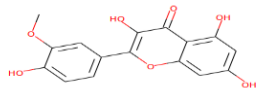
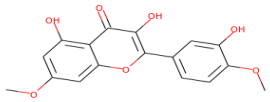
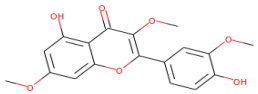
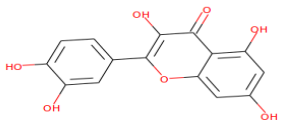
A diverse range of X-ray crystal structures has been identified in the Brookhaven protein data bank (http://www.rcsb.org/pdb). The model evaluation method, which included stereochemistry and overall quality factor analysis, was used to determine which Amrl protein had the best distribution of crystallographic structure of proteins **("PROCHECK: a program to check the stereochemical quality of protein structures - Laskowski - 1993 - Journal of Applied Crystallography - Wiley Online Library", n.d.)**. The RCSB Protein Data Bank was used to obtain the RmlA dimensional structure (PDB ID: 4LOV). Software called Discovery Studio 2021 was used to exclude the water molecules from the protein structure, which had a resolution of 1.95 Å **(Schrödinger, 2021)**. Polar hydrogen atoms were added, and then Kollman and Gasteiger charges were added to the protein structure's amino acid residues using the AutoDockTools4 program **(Morris *et al.*, 2009)**.

Ligand preparation

The 3D ligand coordinates were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The Open Babel 3.1.1 program was then used to convert each ligand into a 3D SDF file ("Open Babel: An Open Chemical Toolbox - Springer", n.d.). Table (1) displays the names and structures of twelve flavonoid compounds from the citrus family, selected for their diverse biological activities. The flavonoid ligands were identified in the PubMed literature as having various pharmacological and anticancer effects, as shown in Table (2) ("S41598-020-72264-4.pdf", n.d.).

Table 1. Phytochemical details of some flavones

Number	Flavonoid compound	PubChem CID	Molecular formula	Molecular weight	Structure
1	Ayanin	5280682	C18H16O7	344.3 g/mol	
2	Dillenetin	5487855	C17H14O7	330.29 g/mol	
3	Gossypetin	5280647	C15H10O8	318.23 g/mol	
4	Isoquercetin	5280804	C21H20O12	464.4 g/mol	

5	Isorhamnetin	5281654	C16H12O7	316.26 g/mol	
6	Ombuin	5320287	C17H14O7	330.29 g/mol	
7	Pachypodol	5281677	C18H16O7	344.3 g/mol	
8	Quercetin	5280343	C15H10O7	302.23 g/mol	

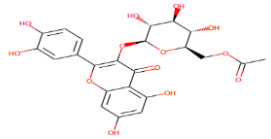
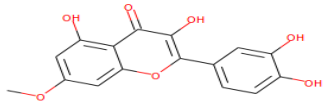
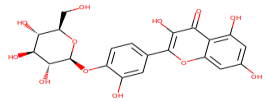
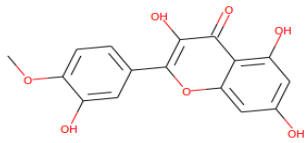
9	Querciturone	5274585	C21H18O13	478.4 g/mol	
10	Rhamnetin	5281691	C16H12O7	316.26g/mol	
11	Spiraeoside	5320844	C21H20O12	464.4 g/mol	
12	Tamarixetin	5281699	C16H12O7	316.26 g/mol	

Table 2. Phytochemical constituents of some plant flavones and reported biological activities

Drug/Compound	Class of compound	Activities
Ayanin	3',5-dihydroxy-3,4',7-trimethoxyflavone	Antioxidant
Dillenetin	3',4'-Di-O-methylquercetin	Antioxidant
Gossypetin	7-hydroxyflavonol and a hexahydroxyflavone	Antioxidant
Isoquercetin	3-O-beta-D-glucopyranoside	Antineoplastic, antioxidant
Isorhamnetin	monomethoxyflavone	Anti-inflammatory, metabolite
Ombuin	dimethoxyflavone	Anti-inflammatory, metabolite
Pachypodol	trimethoxyflavone	Metabolite, antiemetic
Quercetin	pentahydroxyflavone	Antibacterial, antioxidant
Querciturone	quercetin O-glycoside	Metabolite, antioxidant and antidepressant
Rhamnetin	quercetin methylated	Metabolite, an antioxidant and an anti-inflammatory
Spiraeoside	quercetin O-glucoside	Metabolite, antioxidant, antineoplastic
Tamarixetin	quercetin methylated	Metabolite and antioxidant

Molecular docking

Molecular docking of the candidate was performed using Pyrex software connected with AutoDock 4.2, using the limited co-crystalline binding site as a chemical search area **(Dallakyan & Olson, 2015)**. For ligands docked inside the Grid Box, the scoring function was developed using the Lamarckian genetic process. In this work, molecular docking was performed using AutoDock 4.2. grid box.

RESULTS AND DISCUSSION

Results shown in Table (3) reveal that 12 chemicals derived from plant flavonoids were docked with the protein responsible for RmlA. The docked ligand molecules were selected based on docking energy and good interaction with the active site residues and bond category.

Table 3. The interactions of ligands with the Amrl gene from the docking evaluation

Ligand	Energy affinity (kcal/mol)	Chemical interaction position		
		Hydrogen bond	Hydrophobic bond	Electrostatic
Ayanin	-7.70	GLU161, ASP110, ASP225, VAL172, ASN111	TRP223, LEU108, TYR145, VAL172	ARG144
Dillenetin	-8.10	ASP110, ASP225, LEU224, ASN111	TYR145, LEU108, VAL172, LEU88, ILE199	ASP110
Gossypetin	-8.70	GLU161, VAL172, LEU224, THR226, ASN111	TYR145	ASP225
Isoquercetin	-9.20	HIS119, GLU120, SER41, TYR38, LYS249, GLU255, GLN246	ALA251, VAL250, LEU45	
Isorhamnetin	-8.30	TYR31, LEU232, GLN237	ALA240	
Ombuin	-8.10	ASP110, GLU161, ASN111, TYR145, TYR176	TRP223, TYR145, LEU108, VAL172, ILE199	
Pachypodol	-8.20	ARG144, TYR176, THR200, GLU161, TYR145, LEU224, ASN111, THR226	VAL172, TRP223, TYR145	ASP225
Quercetin	-8.50	ASP110, THR226, LEU224, ASN111, TYR176	TYR145, LEU108	ARG144
Querciturone	-9.60	LYS249, GLY115, TYR114, GLN246, ASP117	HIS116	ASP117
Rhamnetin	-8.30	GLU255, TYR113, THR42, SER41, TYR38, GLY115	LEU248, TYR114, VAL250, ALA251	
Spiraeoside	-8.6	GLN237, LEU233, TYR31	ALA240, PRO29, TYR31	
Tamarixetin	-8.20	GLU120, TYR113	ALA251, LEU45	

Molecular docking results revealed that ayanin interacted with the target proteins at the inhibitor binding sites. The interaction between ayanin and *amrl* gene exhibited a

binding affinity score of –7.70kcal/ mol. Complex formed two conventional hydrogen bonds (ASP110, GLU161), one carbon hydrogen bond (VAL172), and two hydrogen bond interactions with ayanin, involving residues (ARG144, LEU108, TRP223); these results are displayed in Fig. (2) and Table (2). The binding affinity score of the dillenetin-AMRL gene complex was –8.1 kcal/mol. Dillenetin formed two conventional hydrogen bonds (with ASP110 and LEU224), two carbon-hydrogen bonds (with ASP225 and ASN111), and interactions with several residues, including TYR145, LEU108, VAL172, LEU88, and ILE199.

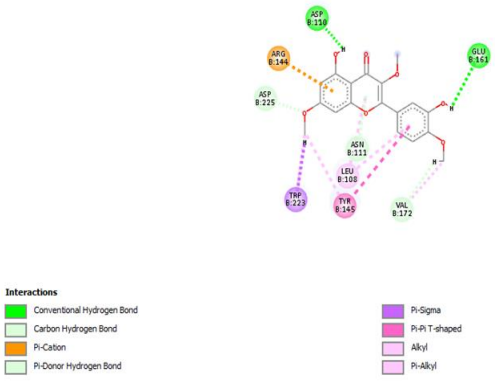
Gossypetin and *amrl* gene inhibitor complex was the binding affinity (–8.70kcal/ mol). Gossypetin formed four conventional hydrogen bonds (GLU161, VAL172, LEU224, THR226), one hydrogen bond (ASN111), involving residues (TYR145, ASP225). Isoquercetin and *amrl* gene inhibitor complex exhibited a high binding affinity (–9.20 kcal/mol) foring seven conventional hydrogen bonds (LYS249, GLU255, GLN246, HIS119, GLU120, SER41, TYR38) and involving residues (ALA251, VAL250, LEU45). The interaction between isorhamnetin and *amrl* gene had binding affinity of –8.30kcal/ mol. Isorhamnetin formed three conventional hydrogen bonds (TYR31, LEU232, GLN237) and one involving residue (ALA240).

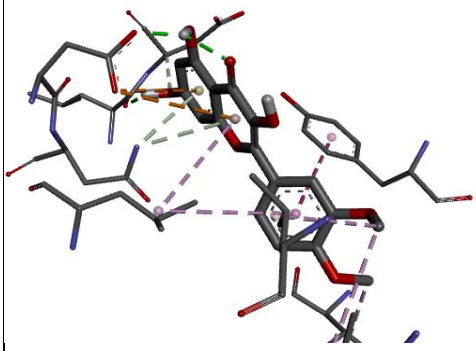
Ombuin formed complexes, exhibiting a binding affinity value of –8.10kcal/ mol including ASP110 and GLU161 that were conventional hydrogen bond; ASN111, TYR145, and TYR176 were carbon hydrogen bond; LEU108, VAL172, ILE199, and TRP223 involving residues. Ligand pachypodol and *amrl* gene had binding affinity of –8.20kcal/ mol. Pachypodol formed four conventional hydrogen bonds (with ARG144, TYR176, THR226, and LEU224) and two carbon-hydrogen bonds (with THR200, TYR145, and GLU161). It also interacted with three residues (ASP225, TRP223, and VAL172). The interaction between quercetin and the AMRL gene had a binding affinity of –8.50 kcal/mol. Quercetin formed four conventional hydrogen bonds (with ASP110, THR226, LEU224, and TYR176) and one hydrogen bond (with ASN111), along with three interacting residues (LEU108, ARG144, and TYR145). The interaction between querciturone and the AMRL gene had a high binding affinity of –9.60 kcal/mol. Querciturone formed five conventional hydrogen bonds (with LYS249, GLY115, TYR114, GLN246, and ASP117) and interacted with two residues (HIS119 and HIS116). The interaction between rhamnetin and the AMRL gene had a binding affinity of –8.30 kcal/mol. Rhamnetin formed four conventional hydrogen bonds (with GLY115, TYR38, THR42, and TYR113), two hydrogen bonds (with GLU255 and SER41), and interacted with three residues (ALA251, VAL250, and LEU248). The interaction between spiraeoside and the AMRL gene had a binding affinity of –8.60kcal/ mol. Spiraeoside formed three conventional hydrogen bonds (with GLN237, LEU233, and TYR31) and interacted with two residues (ALA240 and PRO29). The interaction between tamarixetin and the AMRL gene had a binding affinity of –8.20 kcal/mol. Tamarixetin formed one

conventional hydrogen bond (with GLU120) and one hydrogen bond (with TYR113), along with two interacting residues (ALA251 and LEU45), which was slightly similar to the VEGFA-inhibitor complex (–7.2 kcal/mol). The compound interacted with VEGFA at the same residues as the inhibitor, including Ser50, Gly59, and Asp63 (Fig. 2D & Table 2). The presence of similar interacting residues between quercetin and the inhibitors suggests that quercetin occupies a similar binding position as the control inhibitors, indicating its potential as an inhibitor of the target proteins.

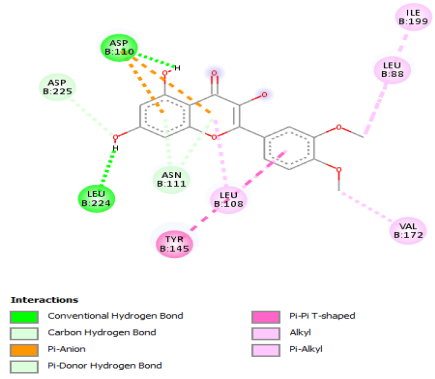
The docking evaluation of 12 flavonoid-derived compounds with the RmlA enzyme revealed notable binding interactions and varying binding affinities, ranging from –9.60kcal /mol to –7.70kcal/ mol. Querciturone showed the highest binding affinity (–9.60 kcal/mol) among the ligands tested, interacting with amino acid residues such as LYS249, GLY115, and ASP117, supported by hydrogen bonds, hydrophobic interactions and electrostatic forces. This high affinity is in line with previous studies such as those of **Saragusti *et al.* (2010)** and **Gilbert and Liu (2010)**, who emphasized the potential of flavonoids as enzyme inhibitors due to their structural diversity and ability to form multiple interactions within the active site. Isoquercetin (–9.20kcal/ mol) and spiraeoside (–8.60kcal/ mol) also showed promising binding profiles, consistent with the results of **Nile *et al.* (2021)**, who confirmed that hydrogen bonds and Pi-Pi stacking are critical factors in the efficiency of flavonoids. Key amino acid residues such as ASP110, GLU161, TYR145, and ASN111 were also found to be consistently involved in multiple interactions, confirming their essential role in the stability of the ligand-enzyme complex. Compounds like quercetin and pachypodol demonstrated diverse bonding, including Pi-Cation and Alkyl interactions, consistent with **Oluwafisayo's *et al.* (2024)** findings on the importance of multi-type bonding for strong inhibitory action. The structure-activity relationship analysis revealed that the binding strength is significantly influenced by the flavonoid structure and functional group positioning, which enhance their ability to form specific bonds. These results reinforce previous reports on the utility of flavonoids as inhibitors in microbial systems

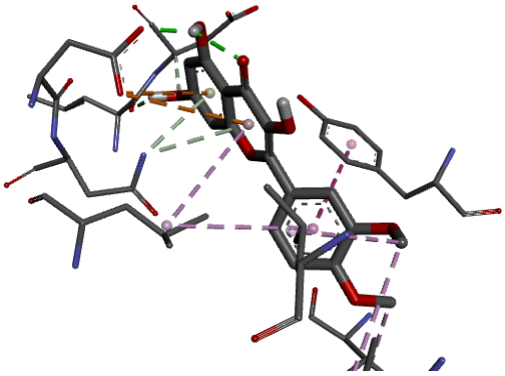
Ayanin Ligand





Dillenation Ligand





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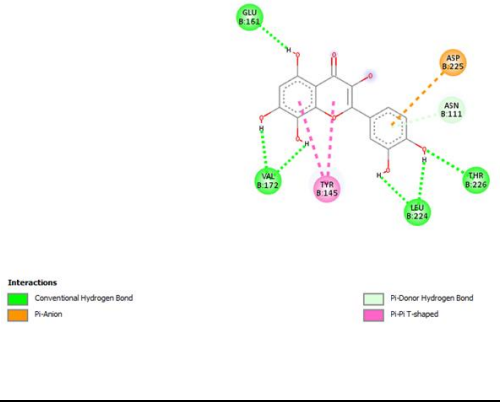

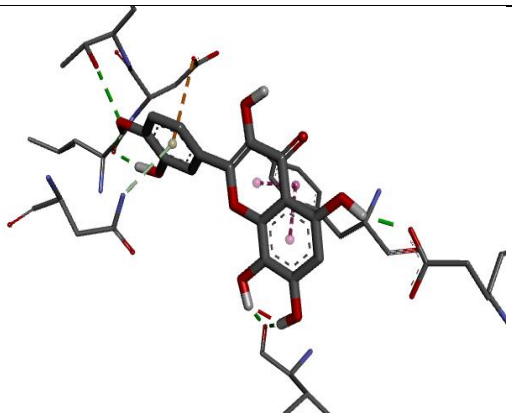

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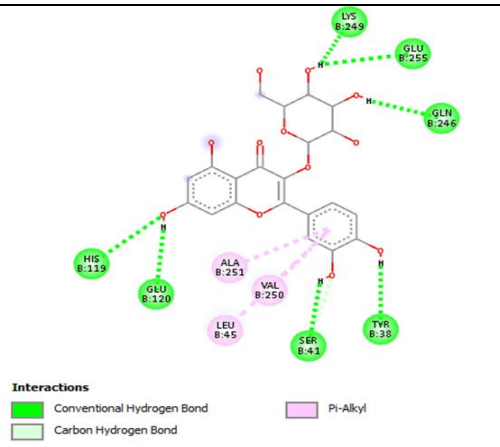

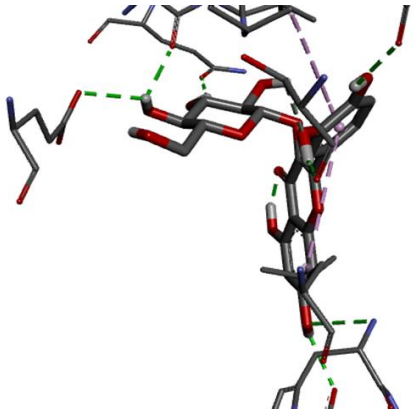

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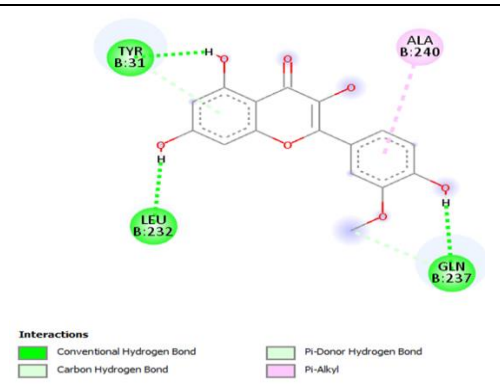

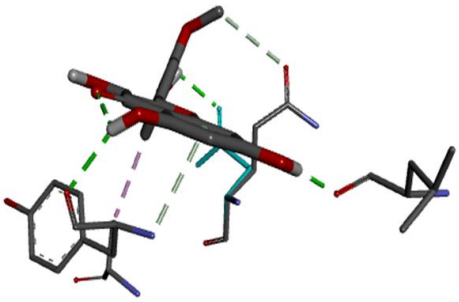

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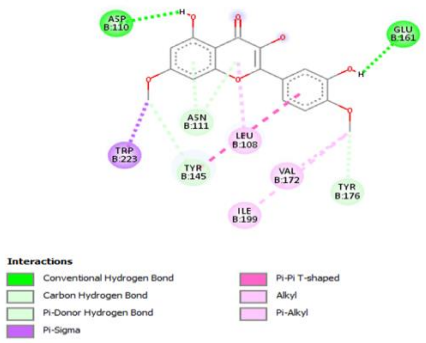
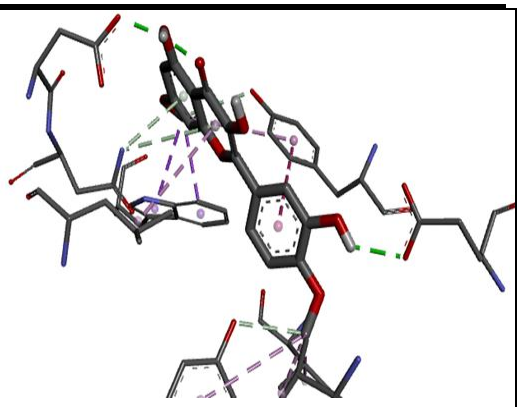
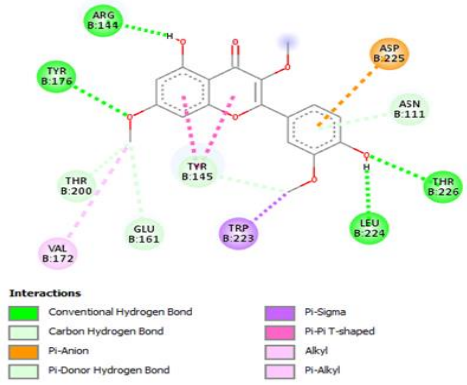
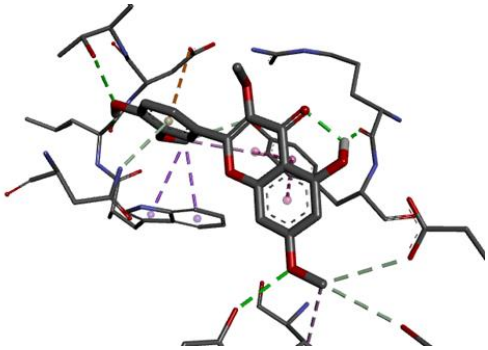


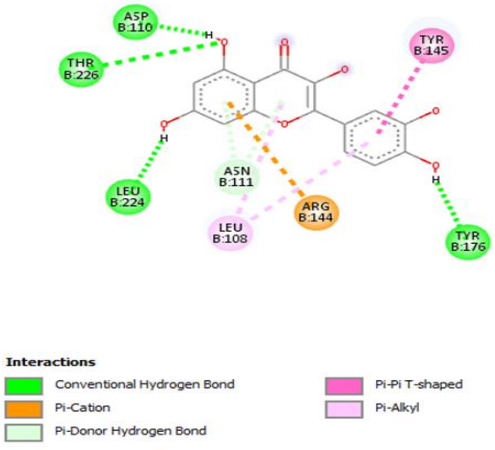


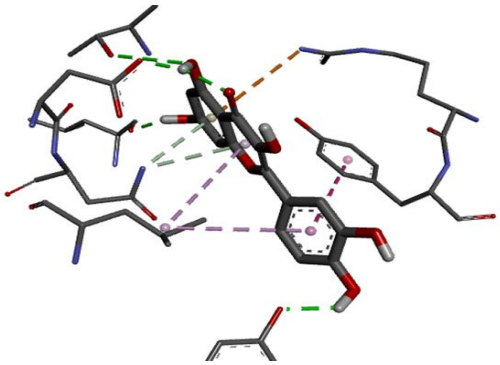
Pachypodol Ligand



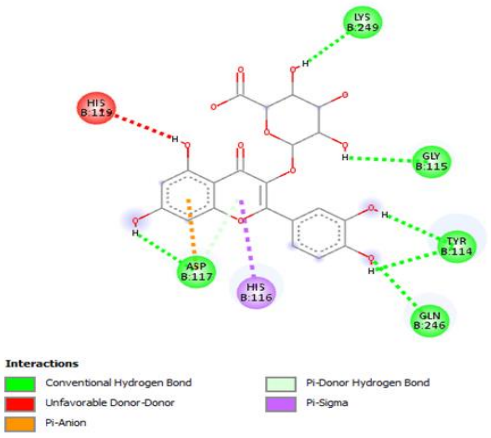


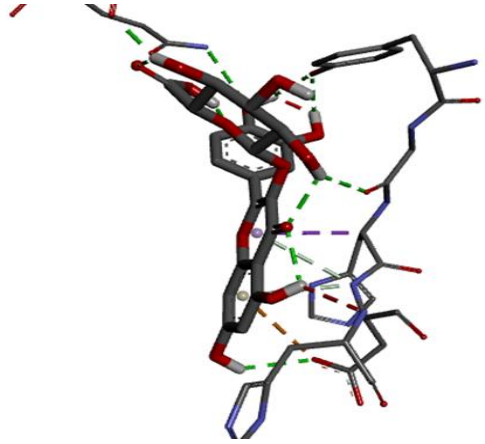
Quercetin Ligand





Querciturone Ligand





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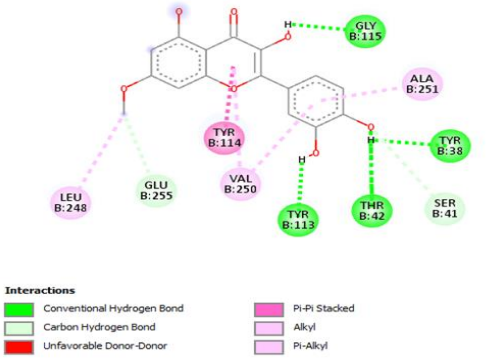
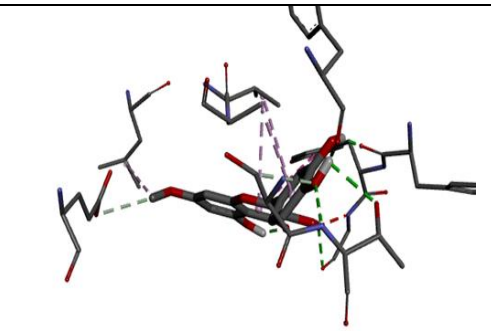
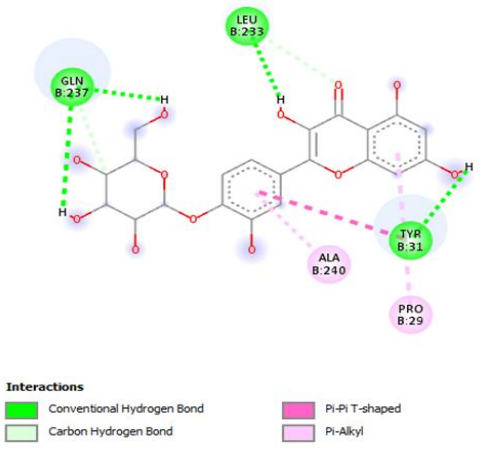
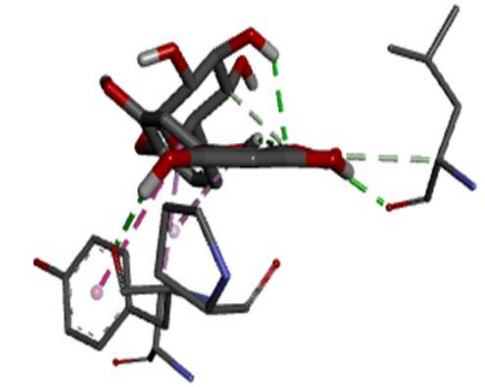




Spiraeoside Ligand





Tamarixetin Ligand

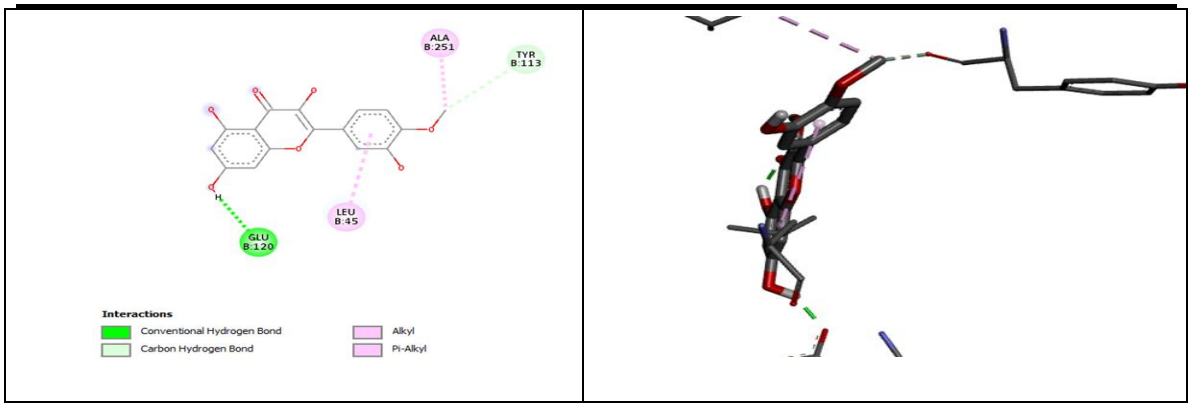


Fig. 1. Interaction between flavones and target proteins

CONCLUSION

Flavones are rich natural phenolic compounds of medicinal value. These compounds are ecofriendly, safer, and cheaper for application. In order to employ flavonoid chemicals as a viable therapeutic alternative for a variety of illnesses and disorders, this article provides an overview of them. The results obtained from this study will be useful in understanding the inhibition mechanism of compounds extracted from trees and in predicting the effectiveness of designed inhibitors based on their binding degrees as well. Here we concluded that these compounds derived from flavonoids could be novel chemical inhibitors for *Pseudomonas aeruginosa* preventing the uncontrolled cell division. There is a need for more research to study the mechanism of improving the activity of the ligand and the mechanism of destruction of the RmlA protein, especially in animal systems, in addition to studying the determination of safe dose levels of these inhibitors.

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