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Original Article

Structure-Activity Relationship of Flavonoids as Antibacterial Agents: Insights from *in vitro* and *in silico* Evaluation

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ABSTRACT

Background: Flavonoids, a large class of polyphenols, have wide structural diversity which can be exploited to design potential leads for treating bacterial infections. However, the effect of the functional groups present in these flavonoids on their antibacterial actions remains largely unexplored. **Objectives:** To evaluate the antibacterial activity of various flavonoid classes against gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli* bacteria, as well as to validate the results using *in silico* molecular docking studies. **Materials and Methods:** The Minimum Inhibitory Concentration (MIC) of 21 flavonoids was determined by the microbroth dilution method. Their structures were docked against proteins of both bacterial strains using Python Prescription (PyRx) software and visualized via Biovia Discovery Studio. **Results:** Different flavonoids exhibited stark differences in MIC values across both strains. Molecular docking suggested binding preferences based on the type, number, and positions of functional groups. **Conclusion:** The study established the Structure-Activity Relationship (SAR) of these natural compounds, providing insights into structural modifications like glycosylation and hydroxylation, alongside physicochemical properties like lipophilicity.

Keywords: Antibacterial activity, Flavonoids, Molecular docking, Natural products, Structure-activity relationship.

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INTRODUCTION

Development of antibiotic resistance remains one of the major hurdles in the battle against bacterial infections (Odonkor and Kennedy, 2012). A contributing factor associated is the extensive usage of antibiotics over time (Sengupta *et al*., 2013) due to which bacteria have become resistant to these drugs by employing use of strategies such as genetic mutation (Tenover, 2006). The upsurge of bacterial infections poses a risk to the healthcare systems globally. It leads to increased financial burden on the patient and there has been an unprecedented rise in the morbidities and mortalities associated with antibiotic resistance worldwide (Lin *et al*., 2015). It is estimated that there are approximately 1.27 million cases of deaths associated with antimicrobial resistance in bacteria every year globally (Marino *et al*., 2025) and could result in 10 million fatalities annually by 2050 if the current trends continue. The major microbes linked to these ailments are *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Murray *et al*., 2022). Substantial efforts must be made for the development of new and alternative antibacterial drugs which will be beneficial for tackling these diseases as compared to the conventional antibiotics.

Shedding light on the mechanisms through which bacteria develop resistance to antibiotics will help to design better drug candidates for the treatment (Munita and Arias, 2016). The mechanisms of antibiotic resistance are varied such as changes in the bacterial enzymes and ribosomes that the antibacterial drugs target, production of enzymes that inactivate the antibiotics, altering the permeability of the cell membranes, by active pumping systems and the adaptation of alternate biochemical pathways (Hasan and Al-Harmoosh, 2020). *E. coli* has been known to develop resistance to quinolones and fluoroquinolones by mutations in the genes of antibiotic targets such as enzymes topoisomerase IV and DNA gyrase (Poirel *et al*., 2018). *S. aureus* has exhibited resistance against β-lactam antibiotic drugs such as penicillin by synthesis of penicillinase enzyme that hydrolyses the β-lactam ring which is crucial for the antibacterial action of penicillin (Pantosti *et al*., 2007).

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There is an emerging interest in the use of natural products as antimicrobial agents. They have also found applications in the treatment of cancer (Demain and Vaishnav, 2011), diabetes (Shapiro and Gong, 2002), cardiovascular disease (Dong *et al*., 2025), psychiatric illnesses (Küpeli Akkol *et al*., 2021) and arthritis (Khanna *et al*., 2007). Plants are rich sources of naturally occurring substances such as polyphenols, terpenoids, alkaloids, essential oils and tannins (Cowan, 1999a). In general, the rate of absorption of these compounds are better than their synthetic counterparts (Harvey, 2008). They offer wide spectrum of diverse chemical structures which can be exploited for drug discovery and development (Shu, 1998). Besides they have numerous advantages such as low toxicity and promising drug-like characteristics (Domingo-Fernández *et al*., 2024). However one major limitation is their low bioavailability and recent advancements in nanotechnology have helped to address these problems more effectively (Watkins *et al*., 2015).

Flavonoids are phytochemicals having a common benzo-γ-pyran skeleton (Kim *et al*., 2007) and are divided into six subclasses namely flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanins based on the difference in their chemical structures as depicted in Figure 1 (Cui *et al*., 2016). They are broadly classified based on the presence or absence of structural features such as C2=C3 double bond, hydroxyl group at 3 position and oxo group at position 4 in the C ring (Shamsudin *et al*., 2022). They belong to the family of polyphenols which have been reported to possess numerous health benefits (Rasouli *et al*., 2017). They have a multitude of functions such as anti-inflammatory activity, anti-microbial, antioxidant as well as anti-carcinogenic activity (Panche *et al*., 2016) and offer protection against biotic and abiotic stress (Samanta *et al*., 2011). They also modulate aspects related to plant growth and development (McClure, 1975), provide protection against UV light and attract insects for pollination (Falcone Ferreyra *et al*., 2012). They impart pigmentation to plant parts (Harborne *et al*., 2017) and have cardioprotective and anti-allergic effects (Prithviraj Karak, 2019).

Previous reports have linked the antibacterial activity of flavonoids to mechanisms such as suppression of nucleic acid production, dysfunction of the cytoplasmic membrane and inhibition of energy supply for the microbes (Xie *et al*., 2015). *S. aureus*, a cocci-shaped gram-positive bacterium is the causative agent of skin infections such as scale skin syndrome, food poisoning, pneumonia, toxic shock syndrome, bacteremia and cutaneous infections (Ghalehnoo *et al*., 2018). It is commonly found in the nostrils, nasopharynx, skin and other mucous membranes in humans as well as animals (Foster *et al*., 2002, Haag *et al*., 2019). It can tolerate wide range of temperatures (4 to 44°C) and pH (4.5-8) (Villanueva *et al*., 2018). This pathogen, a member of class Bacilli is a facultative anaerobe (Gulzar *et al*., 2018). It can be spread by direct contact with infected organism (Bihan *et al*., 2017) or contaminated surfaces (Wang *et al*., 2024). The transmission route can be airborne carried by aerosols (Liu *et al*., 2012). Lack of hygiene in households can also contribute to the spread of *S. aureus* (Mork *et al*., 2020). Methicillin Resistant *Staphylococcus aureus* (MRSA), a strain of *S. aureus* responsible for various hospital acquired infections and community acquired infections poses potential health hazard due to its resistance to antibiotics and ability to spread quickly (Turner *et al*., 2019). Epigallocatechin, a flavonoid belonging to flavan-3-ol subclass has exerted antibacterial activity by inhibiting the synthesis of nucleic acids such as DNA in *Proteus vulgaris* and RNA in *S. aureus* (Mori *et al*., 1987). Kaempferol, a flavonol found in saffron, kale, broccoli and cauliflower has the potential to suppress the biofilm formation, a mechanism developed for antibiotic resistance in *S. aureus* (Ming *et al*., 2017).

E. coli, a gram-negative bacterium normally found in gut of humans and animals as well as in water and soil is responsible for large number of urinary tract infections and gastrointestinal disorders (Savageau, n.d.). This rod-shaped organism is one of the extensively studied microbes among researchers (Vollmer and Höltje, 2001). It acts as a facultative aerobe, exhibiting both aerobic and anaerobic mode of respiration and cannot survive at high temperature and pH (Blount, 2015). Some strains of *E. coli* are beneficial acting as probiotics for the host whereas some are pathogenic leading to kidney and respiratory ailments, anaemia (Basavaraju *et al*., 2022) as well as inflammatory bowel disease (Rhodes, 2007). The transmission of this pathogen can occur through intake of raw or undercooked meat, food or water contaminated with the microbe, unprocessed dairy products and direct contact with any infected organism (Ferens and Hovde, 2011). Some flavonoids such as morin and epicatechin have shown inhibitory action on ATP synthase of *E. coli* thereby affecting the energy production (Chinnam *et al*., 2010). However these phytochemicals have disadvantages such as poor bioavailability and varying sensitivities across bacterial strains (Liu *et al*., 2025).

The difference in the structural characteristics of gram-positive and gram-negative bacteria can significantly impact the antibacterial activity of the compounds. The structure of gram-negative bacteria is more complicated having outer membrane of lipopolysaccharide, the cell membrane and a thin peptidoglycan layer located between the membranes. Gram-positive bacteria have cytoplasmic membrane and a thick layer of peptidoglycan. The lipopolysaccharide rich outer membrane in the gram-negative bacteria acts as a barrier making penetration difficult for the antibacterial agents (Tavares *et al*., 2020).

The present paper provides a comprehensive understanding of the structure-activity relationship of flavonoids using *in vitro* and *in silico* approaches, offering valuable insights that can aid in drug discovery and development of future antibacterial agents.

MATERIALS AND METHODS

The bacterial strains, gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*, were procured from the Department of Microbiology, The Maharaja Sayajirao University of Vadodara.

Nutrient broth, ampicillin and dimethyl sulphoxide were purchased from Sisco Research Laboratories Pvt. Ltd., (SRL).

The flavonoids for the required study were purchased from certified suppliers. Hesperidin, quercetin, naringenin, diosmetin, catechin hydrate, morin, taxifolin, epigallocatechin gallate hydrate, acacetin, orientin, galangin, isorientin, and isorhamnetin were procured from Tokyo Chemical Industry (TCI). Vitexin and gossypetin were purchased from Merck. Rhamnetin and tricin were obtained from the Laboratory of the Government Chemist (LGC), and apigenin, luteolin, myricetin, and kaempferol from GLR Innovations.

Preparation of stock solution of the flavonoids

Stock solutions of 1 mg/mL of the flavonoids were prepared by dissolving them in 0.2% Dimethyl Sulphoxide (DMSO) and making up the volume to 1 mL with sterile distilled water.

Microbroth dilution assay

The assay was performed in triplicates in 96-well plate. Ampicillin was used as positive control for the assay. 200 µL of nutrient broth was used as growth medium for bacteria in 96-well plate. 1% standard inoculum of fresh *E. coli* or *S. aureus* bacterial culture was inoculated in the growth medium. Followed by overnight incubation at 37°C, dosing of flavonoids was done in the concentration range 10 to 100 µg/mL from 1mg/mL stock solution of the flavonoids. After incubation at 37°C for 24 hr, the absorbance values were taken on Biotek Synergy H1 microplate reader at 600 nm after spinning for 10 sec.

The graph of % cell survival versus log concentration was used to determine the MIC of the flavonoid. The % cell survival was calculated using the formula:

$$\%\ cell\ survival = \frac{(absorbance\ of\ sample)}{(absorbance\ of\ control)} \times 100$$

Statistical Analysis

The results of Minimum Inhibitory Concentration (MIC) have been determine after analysis in triplicate. Statistical comparisons using the one-way analysis of variance, and Tukey's multiple comparisons were performed using GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, California, USA, http://www.graphpad.com/).

Molecular docking with antibacterial proteins

Preparation of protein and ligand

The crystal structures of the following *S. aureus* and *E. coli* proteins were downloaded from Protein Data Bank (PDB) in.pdb file format:

S. aureus proteins

Quorum-sensing pathway proteins

AgrA (PDB ID: 4G4K), AgrC (PDB ID: 4BXI).

Antimicrobial resistance proteins

mecA (PDB ID: 1VQQ), PBP4 (PDB ID: 6C3K), BlaR1 (PDB ID: 6O9W), DHFR (PDB ID: 3FRB).

E. coli proteins

Quorum sensing pathway proteins

LuxS (PDB ID: 5E68), LsrB (PDB ID: 1TJY), LsrK (PDB ID: 5YA0).

LsrR (PDB ID: 4GO1), LsrF (PDB ID: 3GLC), LsrG (PDB ID: 3QMQ), SdiA (PDB ID: 4LGW).

Antibiotic efflux proteins

AcrA (PDB ID: 2F1M), AcrB (PDB ID: 1IWG), TolC (PDB ID: 1EKG).

Reduced permeability proteins

OmpF (PDB ID: 3POQ), OmpC (PDB ID: 2J1N), OmpA (PDB ID: 1QJP).

Antibiotic target replacement proteins

MrdA (PDB ID: 6G9S), FtsI (PDB ID: 4BJP).

Protein preparation was done by using AutoDock Tools version 1.5.7 by deletion of water molecules, addition of polar hydrogens and assignment of Kollmann charges. The cleaned protein was saved in pdbqt format for docking.

Molecular docking and visualization

The structures of the 21 flavonoids were retrieved from Pubchem in.sdf format and the energy was minimised by PyRx software. Molecular docking of the flavonoids was performed with the cleaned target proteins and their binding energies were calculated using PyRx software. The docked flavonoid-protein complexes were further visualized with the help of Biovia Discovery Studio Visualizer and the 2D diagrams of the complexes were analysed for the flavonoid-protein interactions.

RESULTS

The minimum inhibitory concentrations of each tested flavonoid against both gram positive as well as gram negative bacterial strains have been depicted in Table 1. The effect of the variation in functional groups among the compounds are diverse and noteworthy.

The molecular docking study of the tested compounds against six proteins in *S. aureus* as well as thirteen proteins in *E. coli* revealed the binding affinities, hydrogen bonding and hydrophobic interactions as shown in supplementary data. The results of the best three flavonoid complexes with each of the *S. aureus* proteins have been highlighted in Table 2, while those with *E. coli* proteins have been shown in Table 3. The top scoring flavonoid-protein complexes and their 2D interaction diagrams predicted by docking have been depicted in Figures 2-9.

DISCUSSION

Natural compounds have attracted much attention lately in the battle against bacterial infections. Flavonoids, a large group of polyphenolic natural compounds offer a wide variety of chemical structures which can be potential substitutes for the conventional antibiotics. They are promising candidates that may help to address the problem of antibiotic resistance which remains a pressing need of the hour. Considerable progress has been made to understand the structure-activity relationship of the flavonoids in the recent years. An in-depth understanding of the structure-activity relationship is essential for designing more effective leads for antimicrobial therapy.

Several reports in previous literature have linked the chemical structure of the flavonoid with the antibacterial efficacy. The antibacterial action of the flavonoids depends on the chemical structure, type of substitutions on the rings (Montenegro *et al*., 2017) and the hydroxylation pattern (Resende *et al*., 2015). Hydroxyl groups at 5,7 positions in the A ring and 3',4' positions in the B ring are favourable for the antibacterial action of flavonoids irrespective of the subclass (Shamsudin *et al*., 2022). Methoxyl groups at various positions reduced the bacterial inhibition by flavonoids (Xie *et al*., 2015). The antibacterial action is significantly enhanced by polyhydroxylation (Shamsudin *et al*., 2022) as observed in the case of myricetin, luteolin, morin, quercetin, rhamnetin, isorhamnetin and kaempferol. Balance between hydrophobic and hydrophilic moieties is crucial for the antibacterial activity of the flavonoids (Farhadi *et al*., 2019).

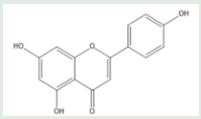
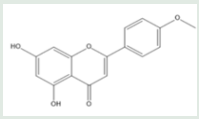
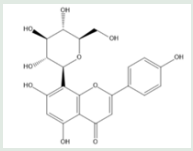
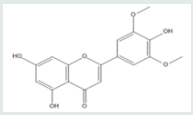
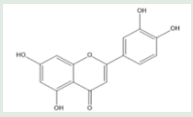
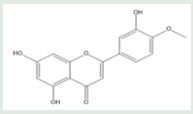
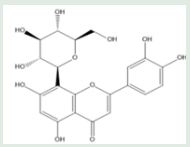
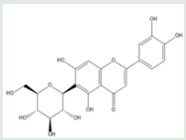
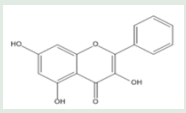
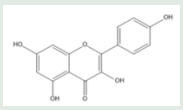
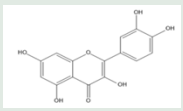
Luteolin (3',4',5,7 tetrahydroxyflavone) exhibited lower MIC than apigenin (4',5,7 trihydroxyflavone) against both the bacterial strains. This difference in the activity can be attributed to the additional hydroxyl group at the 3' position in the B ring in case of luteolin. This finding aligns with previous research which states that polyhydroxylation imparts better activity and flavones having more hydrophilic nature can inhibit the bacterial growth much better (Xu and Lee, 2001). Acacetin (4'-methoxy 5,7 dihydroxyflavone) has similar structure as that of apigenin with the only difference in the substitution at the 4' position in the B ring. Apigenin has a 4' hydroxyl group whereas acacetin has a 4' methoxyl group. The significant decrease in the antibacterial activity of acacetin against *S. aureus* as compared to that of *E. coli* can be attributed to the methoxylation at 4' position. Methoxylation at 4' position was found to impart better activity in gram-negative bacteria whereas in gram-positive bacteria it had the opposite effect (Osorio-Olivares *et al*., 2024). Acacetin having methoxyl group is more lipophilic than apigenin and therefore can penetrate the outer lipopolysaccharide-rich membrane of gram-negative bacteria exerting better activity. Our results exhibited similar patterns against both the bacterial strains.

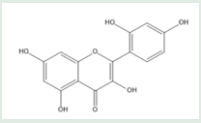
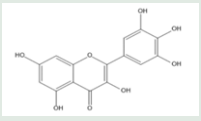
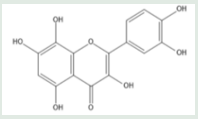
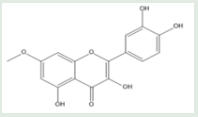
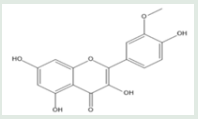
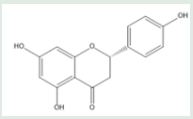
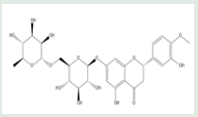
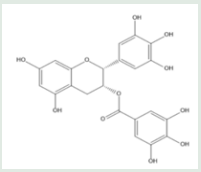
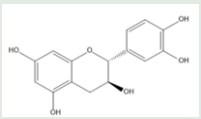
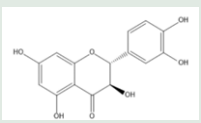
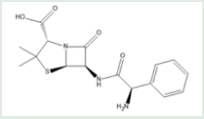
Diosmetin (4'-O-methylated luteolin) had higher MIC values than luteolin which suggested that hydroxyl substituents at various positions in the B ring contribute to activity whereas methoxylation reduces it (Xie *et al*., 2015). Our observations suggested that orientin (luteolin-8-C-glucoside) and isorientin (luteolin-6-C-glucoside) have better antibacterial activity as indicated by their lower MIC values than those of luteolin. Glycosylation at the 6 and 8 positions improved the antibacterial activity significantly. This contrasts with previous studies which suggest that they have comparable activities and requires further validation (Adamczak *et al*., 2019). Gram-negative bacteria were more susceptible to luteolin and its glycosylated derivatives than gram-positive bacteria (Karpiński *et al*., 2020). The MIC values obtained for the aglycone luteolin and its glycosides express the same fact.

Vitexin (apigenin-8-C-glucoside) also demonstrated much better antibacterial activity than its aglycone apigenin which is contrary to what has been reported previously (Karpiński *et al*., 2020). Such inconsistencies can be attributed to the variation in the experimental methods used for antibacterial testing, bacterial strains and the purity of the flavonoids used. Tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) was found to be more effective against *E. coli* than *S. aureus*. Methoxylation at 3' and 5' in B ring in tricin makes it a weak antibacterial agent against *S. aureus*. This observation is consistent with previous studies carried out against gram-positive bacteria which states that methoxylation reduces the antibacterial activity of flavonoids (Shamsudin *et al*., 2022). The methoxyl groups in B ring increase the lipophilicity of tricin facilitating penetration in gram-negative bacteria, thereby exhibiting potent antibacterial action.

Hydroxylation at 4' position in the B ring was significant for antibacterial activity against *S. aureus* (Tsuchiya *et al*., 1996). Galangin lacking 4'-OH group exerted much less antibacterial activity against *S. aureus* as compared to kaempferol, quercetin and morin possessing 4'-OH group. Quercetin with 3',4' hydroxylation (catechol moiety) has more hydrophilic nature which reflects its ability to penetrate the gram-positive bacteria

Table 1: Minimum inhibitory concentrations of flavonoids and standard antibiotic by microbroth dilution assay.

Sl. No.	Flavonoid	Structure	Subclass	MIC against *S. aureus* (µg/mL)	MIC against *E. coli* (µg/mL)
1	Apigenin		flavone	31.40	52.95
2	Acacetin		flavone	174.8	39.17
3	Vitexin		flavone	8.348	2.033
4	Tricin		flavone	34.60	1.794
5	Luteolin		flavone	29.43	16.72
6	Diosmetin		flavone	56.59	60.83
7	Orientin		flavone	21.10	1.794
8	Isorientin		flavone	13.99	2.035
9	Galangin		flavonol	65.51	15.21
10	Kaempferol		flavonol	29.15	47.91
11	Quercetin		flavonol	12.99	35.48

Sl. No.	Flavonoid	Structure	Subclass	MIC against *S. aureus* (µg/mL)	MIC against *E. coli* (µg/mL)
12	Morin		flavonol	35.18	24.03
13	Myricetin		flavonol	1.676	1.876
14	Gossypetin		flavonol	Inactive	927.9
15	Rhamnetin		flavonol	25.93	21.50
16	Isorhamnetin		flavonol	2.281	15.62
17	Naringenin		flavanone	88.38	19.02
18	Hesperidin		flavanone	68.90	32.97
19	Epigallocatechin gallate hydrate		flavan-3-ol	2.015	21.25
20	Catechin hydrate		flavan-3-ol	173.4	12.88
21	Taxifolin		flavanonol	33.80	47.90
	STANDARD ANTIBIOTIC				
22	Ampicillin		-	10.87	31.17

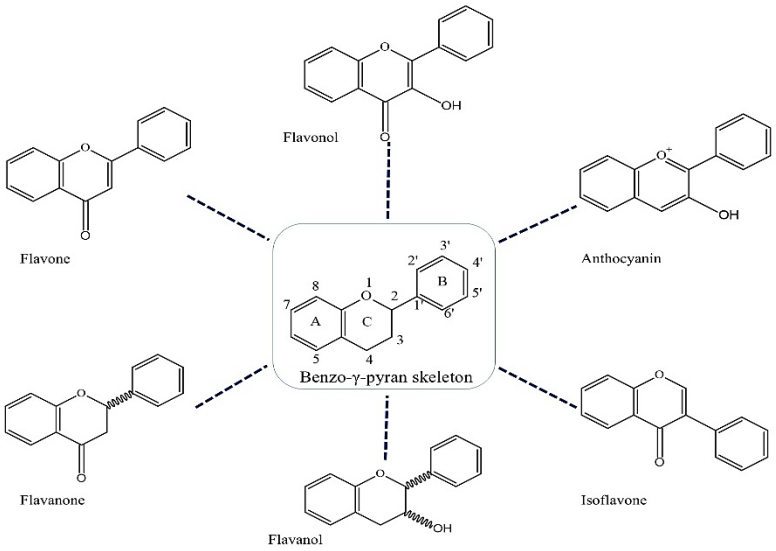


Figure 1: Different subclasses of flavonoids having common benzo-γ-pyran core.

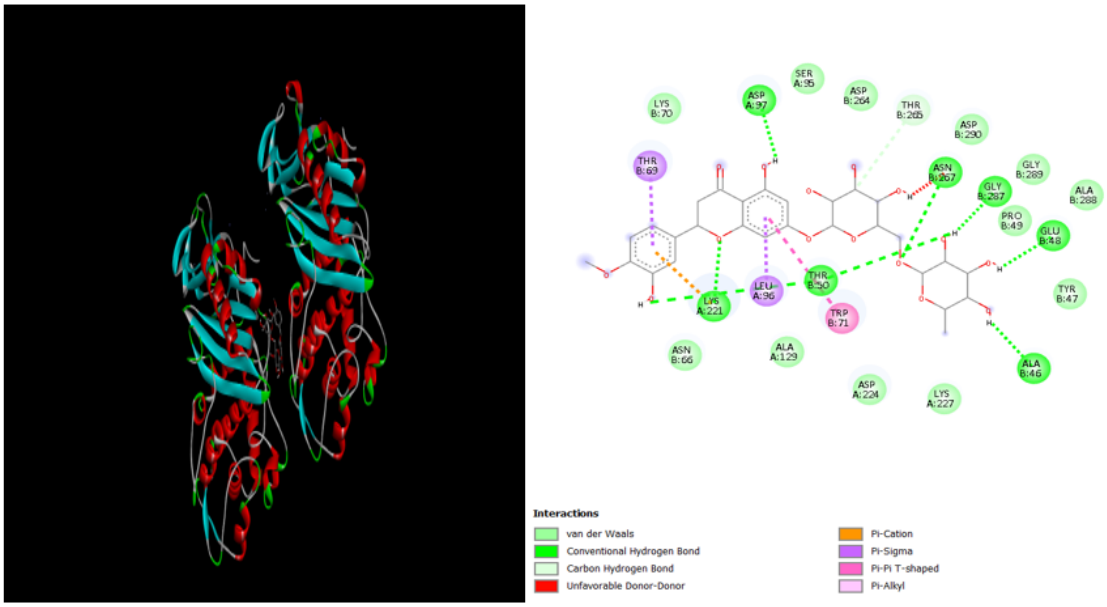


Figure 2: Docked complex and 2D interaction diagram of hesperidin with PBP4 protein of *S. aureus.*

like *S. aureus* easily than morin having more hydrophobic nature with 2',4' hydroxylation (Resende *et al.*, 2015). On the other hand, morin with more hydrophobic nature is more potent against gram-negative bacteria like *E. coli* than quercetin. This is indicative of the fact that hydrophilic and hydrophobic nature of the compounds is an important point to be taken into consideration for the antibacterial activity (Rafał *et al.*, 2018).

The antibacterial efficacy of the compounds against gram-positive and gram-negative bacteria differs owing to the differences in the structure (Beveridge, 1999). Even slight changes in the hydroxylation patterns in flavonoids can lead to differences in the activity. Galangin with no substituent on the B ring and 5,7 dihydroxylation in the A ring exerted significant antibacterial activity against *E. coli* which is in accordance with previous reports (Echeverría *et al.*, 2017). This can be attributed to the amphiphilic balance created by the polar hydrophilic groups on the A ring and the unsubstituted B ring. Some authors have mentioned the fact that flavonoids not having any hydroxylation on the B ring exert stronger antimicrobial activity than those with hydroxylation (Wu *et al.*, 2013). Myricetin, a flavonol with pyrogallol moiety in its structure, was found to be a potent inhibitor of both the bacterial strains, *S. aureus* and *E. coli*, with minimum inhibitory concentrations 1.676 µg/mL and 1.876 µg/mL respectively. Our finding is consistent with previous study by Xie *et al.*, which indicates that having a pyrogallol like structure with trihydroxylation at 3',4' and 5' in the B ring gives remarkable antibacterial activity (Xie *et al.*, 2015).

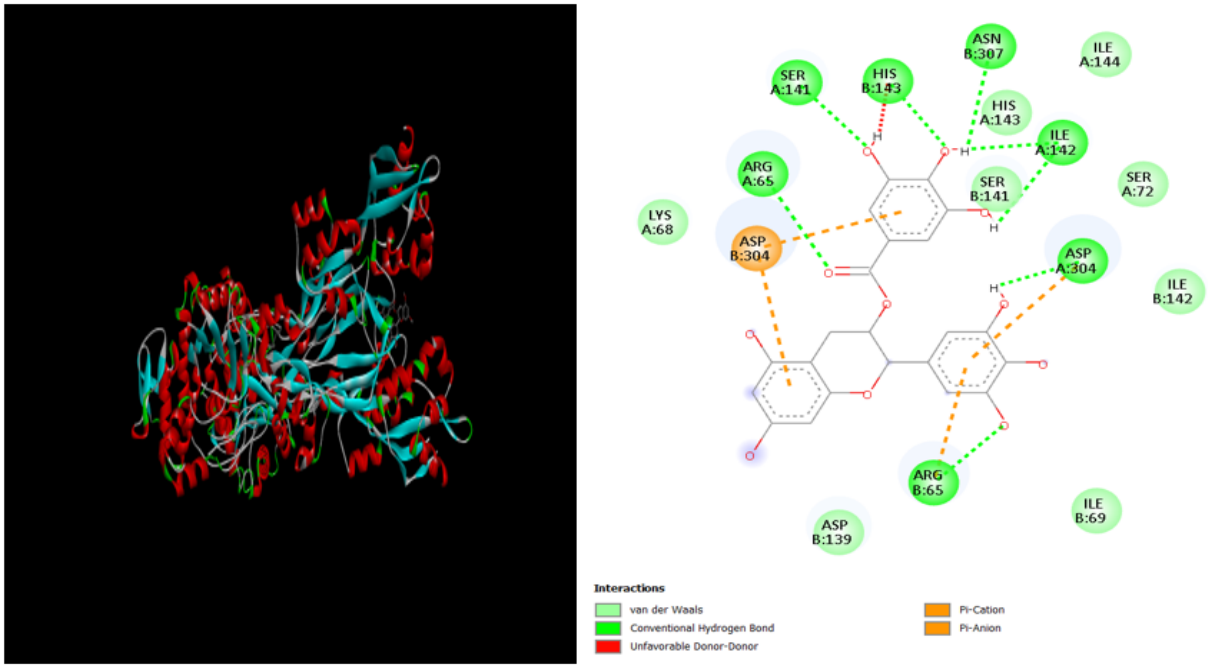


Figure 3: Docked complex and 2D interaction diagram of epigallocatechin gallate with mecA protein of *S. aureus*.

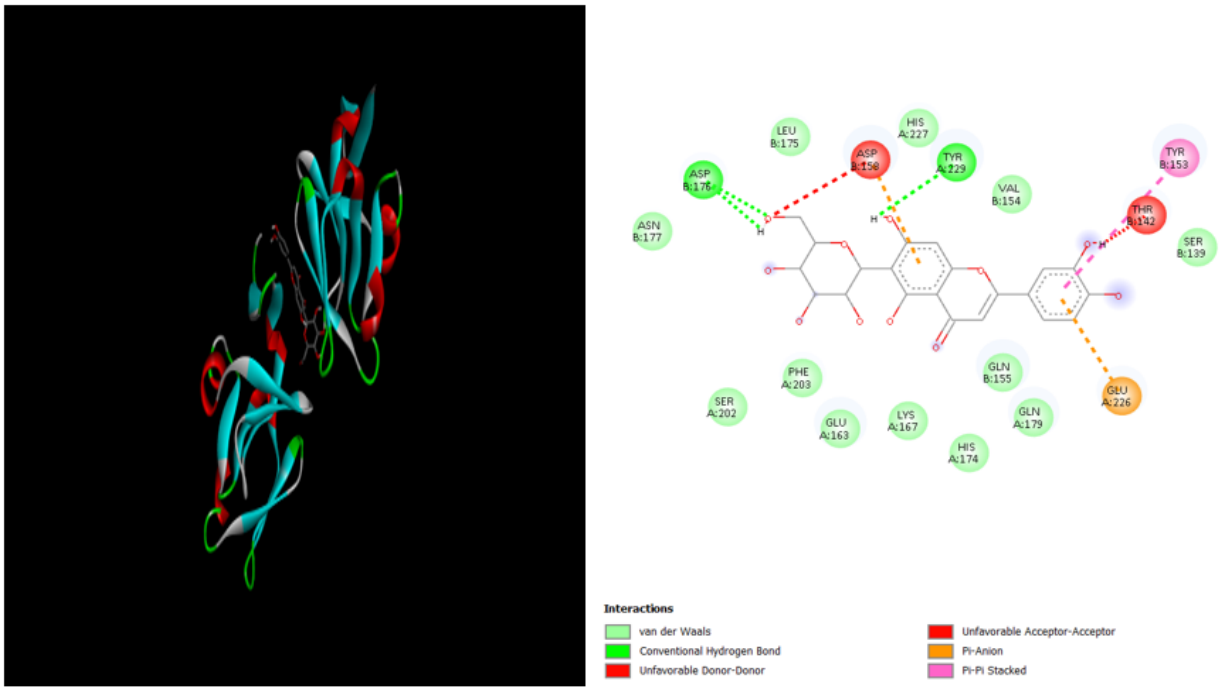


Figure 4: Docked complex and 2D interaction diagram of isorientin with AgrA protein of *S. aureus.*

Gossypetin had the least antibacterial activity, being almost inactive against *E. coli* and entirely ineffective against *S. aureus*. It is a richly hydroxylated flavonol with polar hydroxyl groups at the 3,5,7,8 positions in A ring and the 3' and 4' positions in the B ring. The lack of hydrophobic groups coupled with the high hydrophilicity disturbs the amphiphilic balance for the membrane penetration of the flavonoid to exert antibacterial activity (Cowan, 1999b). The MIC values obtained for gossypetin align well with this observation.

As reported in earlier studies, hydroxylation at 5 and 7 position in A ring and 4' position in the B ring is significant for antibacterial activity against *S. aureus* (Shamsudin *et al.*, 2022). Rhamnetin and isorhamnetin are O-methylated derivatives of quercetin having methylation at the 7 and 3' positions respectively. Comparing

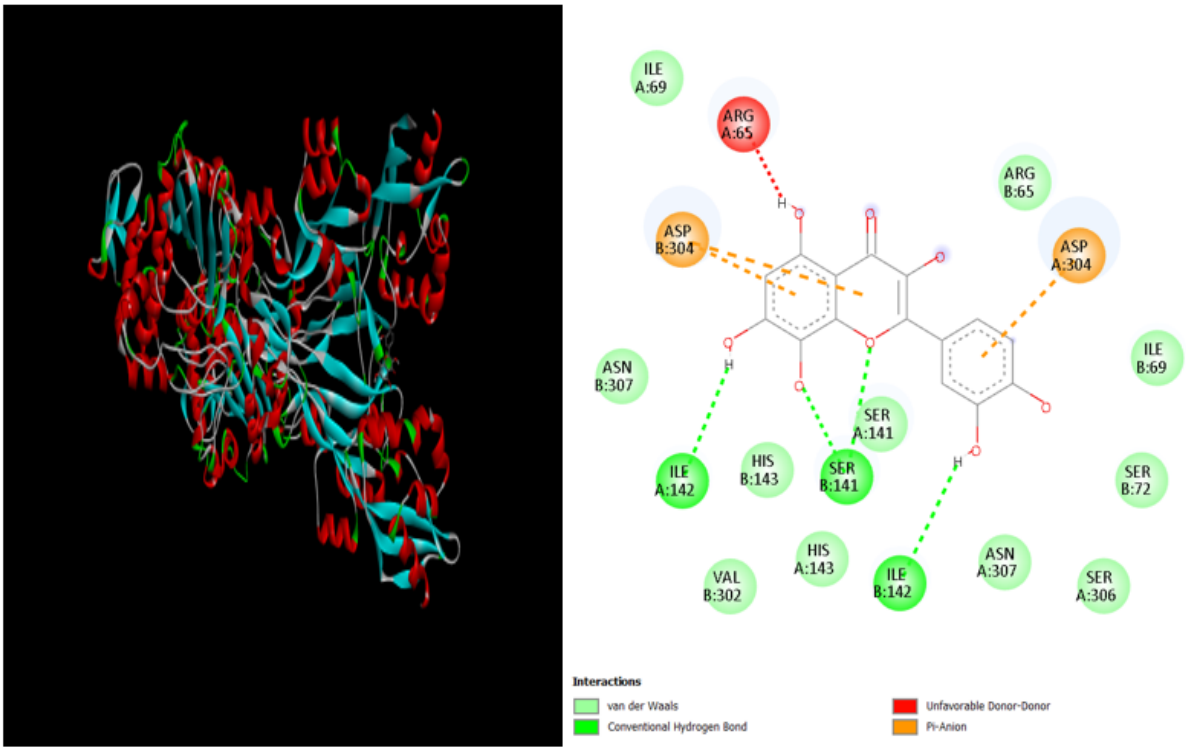


Figure 5: Docked complex and 2D interaction diagram of gossypetin with mecA protein of *S. aureus.*

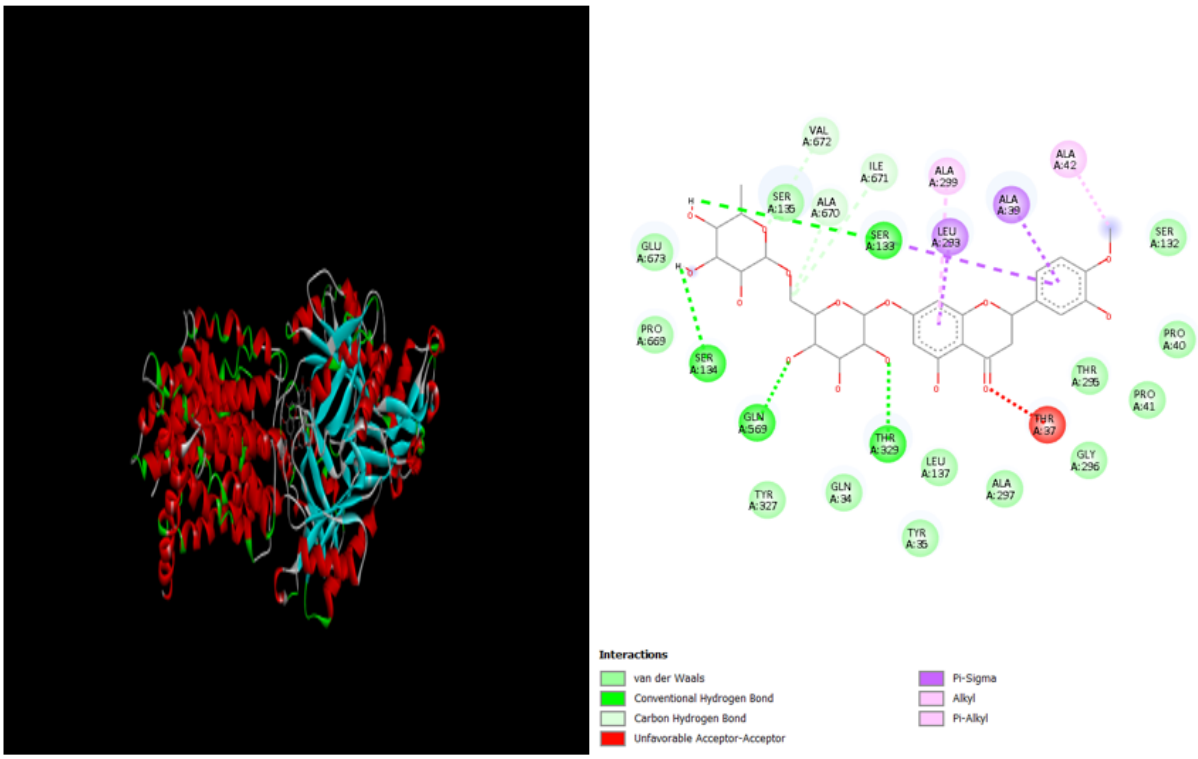


Figure 6: Docked complex and 2D interaction diagram of hesperidin with AcrB protein of *E. coli.*

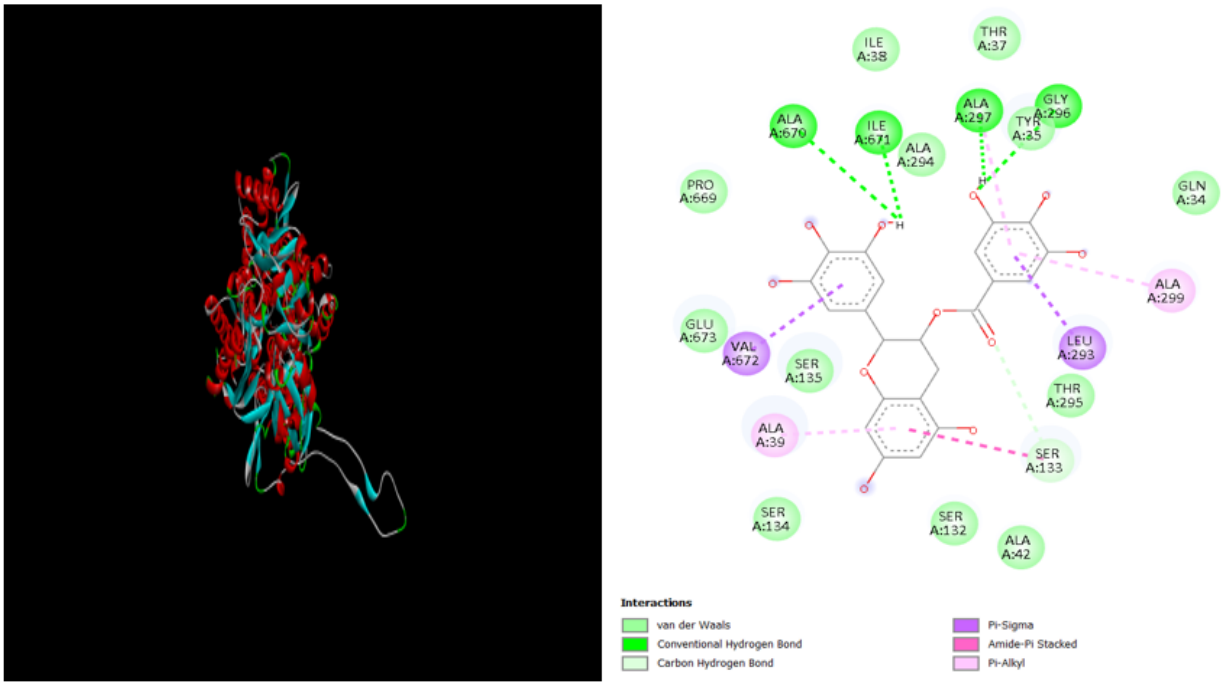


Figure 7: Docked complex and 2D interaction diagram of epigallocatechin gallate with AcrB protein of *E. coli*.

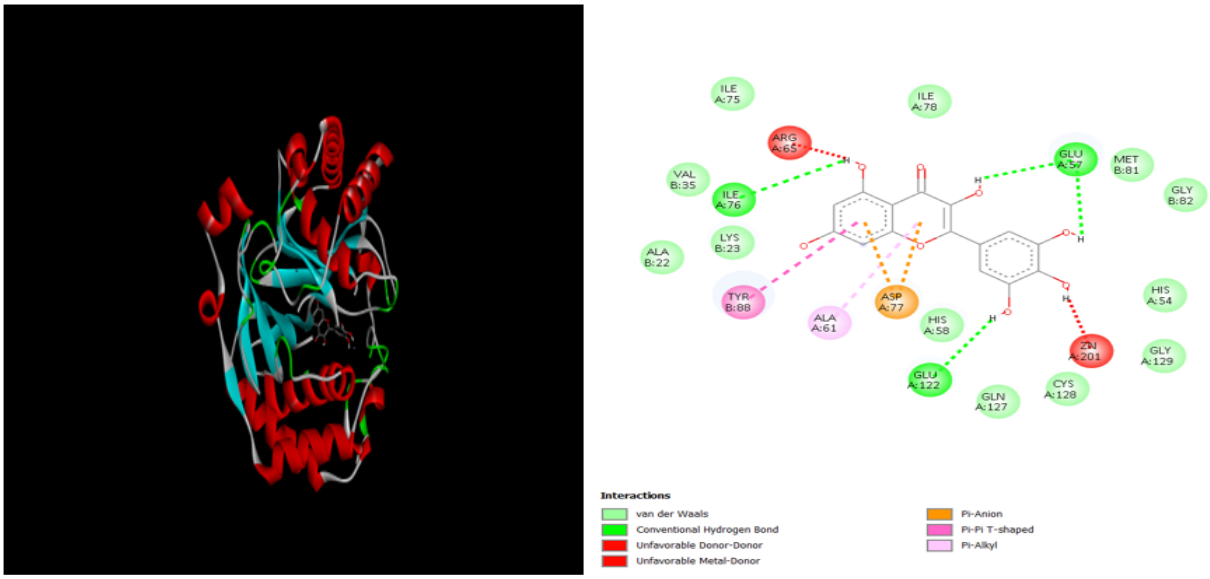


Figure 8: Docked complex and 2D interaction diagram of myricetin with LuxS protein of *E. coli*.

the MIC values of these flavonols quercetin, rhamnetin and isorhamnetin, rhamnetin lacking free hydroxyl group in the 7 position because of O-methylation is the least potent against *S. aureus*. Methylation at 3' position instead of free hydroxyl group yields isorhamnetin promising antibacterial activity against *S. aureus* as compared to quercetin likely due to increased lipophilicity needed for membrane penetration. However, there is insufficient data regarding this, and it requires further investigation.

Hydrophobicity has been positively correlated to the antibacterial activity against *E. coli* as per QSAR studies carried out previously (Wu *et al.*, 2013). The antibacterial activities of quercetin, rhamnetin and isorhamnetin follow the order: isorhamnetin >rhamnetin >quercetin which can be correlated to their order of lipophilicity: isorhamnetin>rhamnetin> quercetin. This also highlights the fact that the position of the substituent can also affect the hydrophobicity of the compound and as a result, the antibacterial activity.

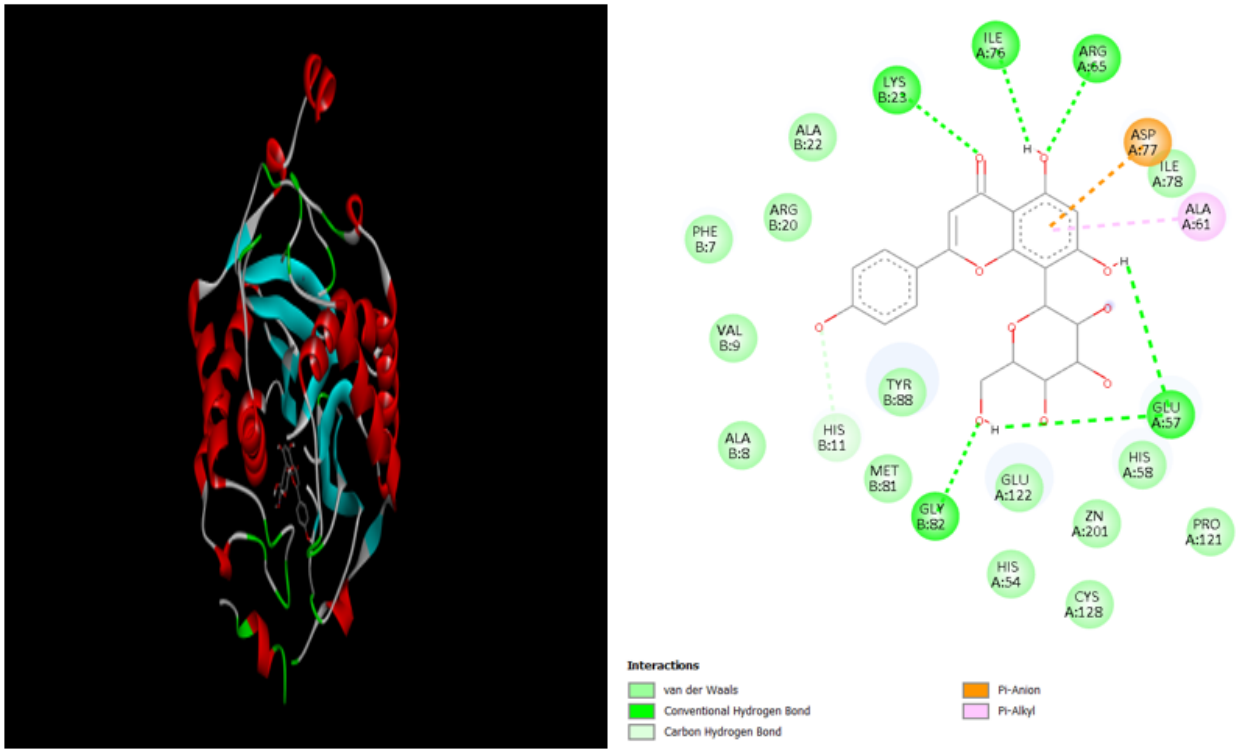


Figure 9: Docked complex and 2D interaction diagram of vitexin with LuxS protein of *E. coli*.

Table 2:Binding affinities, hydrogen bonding and hydrophobic interactions of best three flavonoid complexes with *S. aureus* proteins.

Sl. No.	Flavonoid	Protein	Binding affinity	Hydrogen bonding	Hydrophobic Interactions
1	Hesperidin	AgrA	-10.2	LYS A:167, TYR B:153	GLN A:179, GLU A:226
2	Hesperidin	PBP4	-10.5	ASP A:97, ASN B:267, GLY B:287, GLU B:48, ALA B:46, THR B:50, LYS A:221	THR B:69, LEU A:96, TRP B:71, THR B:265
3	Hesperidin	BlaR1	-10	GLU B:569, THR B:527, ASN A:533	VAL B:438, VAL A:532, TRP B:424, NXL A:601, MET A:476, GLN B:435

Naringenin had lower MIC against *E. coli* than apigenin with the only structural difference being the absence of C2=C3 double bond in the C ring which implies that the absence of C2=C3 double bond in flavanones was significant for antibacterial activity particularly against gram-negative bacteria (Xie *et al.*, 2017). Conversely, apigenin showed better activity than naringenin against gram-positive bacteria *S. aureus* which is in accordance with the prior work where flavones showed better antibacterial activity against gram-positive bacteria than flavanones due to the C2=C3 double bond in the structure which makes them planar in nature. The inhibition of bacterial growth in gram-positive bacteria was more when the number of hydroxyl groups were less in flavanones (Echeverría *et al.*, 2017) which supports our observations where hesperidin with two hydroxyl groups has

Table 3: Binding affinities, hydrogen bonding and hydrophobic interactions of best three flavonoid complexes with *E. coli* proteins.

Sl. No.	Flavonoid	Protein	Binding affinity	Hydrogen bonding	Hydrophobic Interactions
1	Hesperidin	AcrB	-10.6	SER A:133, SER A:134, GLN A:569, THR A:329	ALA A:299, ALA A:42, ALA A:39, LEU A:293, SER A:133
2	Hesperidin	OmpC	-10.4	LEU C:107, TYR C:35, ARG C:37, LYS C:317, GLN C:59, GLN C:61	GLU C:109, VAL C:29, TYR C:22, ASP C:105
3	Hesperidin	LuxS	-10.3	PRO A:121, HIS B:11, GLN A:127, ARG B:20, PHE B:7 GLU A:122, LYS B:23	LYS B:23, HIS A:58, PHE B:7, ALA B:8, GLU A:122, TYR B:88

lower MIC (MIC=68.90 µg/mL) against *S. aureus* than naringenin with three hydroxyl groups (MIC=88.38 µg/mL). Previous studies indicate that the hydroxyl groups at the 5 and 7 position in the A ring impart antibacterial activity in the flavonols and flavanones subclass which is in alignment with our findings as seen in quercetin, morin, myricetin, galangin, isorhamnetin, kaempferol and naringenin (Xie *et al*., 2017).

In case of flavan-3-ols, catechin hydrate and epigallocatechin gallate exhibited differences in their potencies against *S. aureus* and *E. coli*. Epigallocatechin gallate having polyhydroxylated structure owing to hydroxyl groups in the A as well as B ring and the galloyl moiety exhibited potent antibacterial activity against *S. aureus.* Some authors have reported that galloylated catechins have better antibacterial activity against *S. aureus* than non-galloylated counterparts which is in line with our findings (Gibbons *et al*., 2004). Reports from previous literature have also mentioned the fact that gram-positive bacteria have higher sensitivity to epigallocatechin gallate in comparison to gram-negative bacteria (Steinmann *et al*., 2013). The hydrophilic nature of epigallocatechin gallate along with its bulkiness owing to the galloyl moiety hinders the membrane penetration in gram-negative bacteria which is an indispensable factor for antibacterial activity. Catechin hydrate being more hydrophobic and smaller in size than epigallocatechin gallate shows much better antibacterial activity against *E. coli.* This in in alignment with earlier reports relating the hydrophobicity and antibacterial activity against *E. coli* (Wu *et al*., 2013).

Hydroxylation at positions 5,7 in the A ring and 3',4' in the B ring imparts antibacterial effect to taxifolin against *S. aureus* as reported previously (Shamsudin *et al*., 2022). However, the high hydrophilicity imparted by the multiple hydroxyl groups in taxifolin limits its ability cross the outer membrane of *E. coli* resulting in a higher MIC value compared to *S. aureus.* The C4 carbonyl group in the C ring increases the hydrophilicity of taxifolin thereby exhibiting higher potency against *S. aureus* as compared to catechin hydrate which lacks this carbonyl group. The higher hydrophobicity of catechin hydrate compared to taxifolin can be attributed to its low MIC value against *E. coli* (Wu *et al*., 2013).

Based on the data obtained by *in vitro* studies, flavonols showed the best antibacterial activity followed by flavones. The enhanced antibacterial action of flavonols in comparison to flavones can be attributed to the presence of hydroxyl group at 3 position in the C ring (Xie *et al*., 2015). The C2=C3 double bond in conjugation with the 4-oxo group are vital structural features for antibacterial activity as observed in the most potent flavonols and flavones (Shamsudin *et al*., 2022). Flavanones were less potent due to absence of C2=C3 double bond and less planar nature. Flavan-3-ols such as epigallocatechin gallate showed excellent activity against *S. aureus* and catechin against *E. coli* which implies that the antibacterial activity of flavan-3-ols depends on the impact of functional groups in the overall structure. Flavononols such as taxifolin exhibited moderate antibacterial potency against both the bacterial strains. The C2=C3 double bond in conjugation

with the 4-oxo group are key structural features for antibacterial activity as observed in the most potent flavonols and flavones.

Myricetin was the most potent antibacterial agent amongst all the flavonoids against both the bacterial strains. Myricetin, epigallocatechin gallate, isorhamnetin and vitexin surpassed the antibacterial activity of the standard antibiotic ampicillin against *S. aureus*. (MIC=10.87µg/mL) In case of *E. coli*, a large number of flavonoids such as orientin, tricin, isorientin, vitexin, myricetin, catechin hydrate, luteolin, naringenin, epigallocatechin gallate, rhamnetin, morin, isorhamnetin and galangin exhibited better antibacterial potential than the reference antibiotic ampicillin (MIC=31.17 µg/mL). Flavonoids with broad spectrum activity which show strong antibacterial potency against both the bacterial strains are myricetin, isorhamnetin, epigallocatechin gallate, vitexin, isorientin, orientin and luteolin.

In silico docking data against *S. aureus* proteins showed that the flavonoids have variable binding affinities for the proteins based on their subclasses. Flavonones have better binding affinities against AgrA, PBP4, BlaR1 and mecA proteins indicating their capability to inhibit quorum sensing as well as antimicrobial resistance pathways. Catechins have high binding affinities for mecA which may prove beneficial for sensitising the resistant antibacterial strains to β-lactam antibiotics. Flavones displayed good binding energies for AgrA, PBP4 and BlaR1 proteins suggesting that they are inhibitors of quorum sensing and antimicrobial resistance mechanisms. Flavonols are better inhibitors of antimicrobial resistance pathways as shown by their binding energies against mecA and BlaR1 proteins. Flavononols showed moderate binding across all proteins.

Glycosylation was found to enhance the antibacterial activity against *S. aureus* in flavanones as in the case of hesperidin which exhibited strong binding affinities against quorum sensing pathway protein AgrA and antimicrobial resistance proteins mecA, PBP4 and BlaR1. This can be attributed to the presence of rutinoside group at the C-7 position of hesperidin having free hydroxyl groups which form many hydrogen bonding interactions with the amino acids of the protein. Naringenin, devoid of the sugar moiety had weak binding against the proteins as compared to hesperidin. The free hydroxyl groups in the glycosyl unit of hesperidin form hydrogen bonds with amino acids ASN B:267, GLY B:287, GLU B:48, THR B:50 and ALA B:46 of PBP4 protein in addition to hydrogen bonds formed by other hydroxyls on the structure. Hydrophobic interactions such as Van der Waals, carbon-hydrogen bond, pi-sigma, pi- alkyl and pi-pi T-shaped bonds also contribute to the strong binding affinity of hesperidin (-10.5 kcal/mol) against PBP4 as shown in Figure 2. Naringenin forms only two hydrogen bonds with THR B:265 and SER B:75 in addition to hydrophobic interactions such as van der Waals, carbon hydrogen and pi sigma bond which explains its low binding affinity (-8.3 kcal/mol) against PBP4. This is in line with previous reports which suggest that glycosylated flavanones exhibit better biological activity than their aglycone counterparts. (Mohamed Yusof *et al*., 2022) Glycosylation has also been known to increase the solubility and bioavailability of the flavonoid for better antibacterial activity (Dahiya *et al*., 2023).

Flavan-3-ols, also known as catechins are the major flavonoids present in green tea. Galloyl group in epigallocatechin gallate significantly increases the binding affinities against the *S. aureus* proteins, particularly against mecA as compared to catechin hydrate which lacks the galloyl group. The free hydroxyl groups in the galloyl group of epigallocatechin gallate form hydrogen bonds with amino acids such as SER A:141, HIS B:143, ASN B:307 and ILE A:142 of mecA which contributes to the strong binding affinity (-9.7kcal/mol) as shown in Figure 3. Previous studies have shown that the galloylated catechins form interactions with the cytoplasmic membrane of *S. aureus* (Bernal *et al*., 2010). Flavononol such as taxifolin have average binding affinities among the flavonoids which can be explained by their non-planar nature which decreases the π-stacking interactions with the proteins.

C-glycosylation was found to improve the binding affinities in the flavones as observed in orientin, isorientin and vitexin, specifically against AgrA, PBP4 and BlaR1 proteins in *S. aureus*. This may be explained by the increased hydrogen bond interactions by the hydroxyl groups in the glycosyl unit attached to the flavone. For example, the hydrogen bond interactions of isorientin with residues ASP B:176 and TYR A:229 of AgrA protein contributes significantly to the high binding affinity (-8.2kcal/mol) as shown in Figure 3. The increased hydroxylation of the B ring makes luteolin better inhibitor of the protein targets than apigenin.

The hydroxyl group at the 3 position of C ring in flavonols and other hydroxyls on the skeleton form hydrogen bonding interactions with the proteins which translates to strong binding affinities of the flavonols against the *S. aureus* proteins, specifically against mecA. Flavonols with multiple hydroxyl groups such as gossypetin and myricetin exhibited best binding affinities across the proteins. However, the reason why gossypetin was found to be inactive against *S. aureus* in our experimental studies despite its high binding affinity predicted by *in silico* studies needs to be investigated. The probable factor for the same can be the lack of balance between hydrophilic and hydrophobic groups required for effective membrane penetration.

Flavonones were most potent against *E. coli* proteins as per *in silico* studies owing to their high binding affinities across quorum sensing, antibiotic efflux, reduced permeability and antibiotic target replacement proteins. Glycosylation and hydroxylation in flavanones caused strong binding to the protein targets. Catechins exhibited high binding affinities especially for antibiotic efflux proteins. Epigallocatechin gallate with gallate moiety had strong affinities for efflux proteins AcrA, AcrB and TolC suggesting that galloylation can improve the binding potential significantly. They also had comparable binding affinities to quorum sensing

as well as permeability proteins and average values for antibiotic target replacement proteins. Increased hydroxylation in the structure improved the binding potential of the flavonols subclass especially against LuxS, LsrK, AcrB and OmpC proteins for morin, myricetin and quercetin. Methoxylation in flavonols such as rhamnetin and isorhamnetin caused minor decrease in the binding affinities.

Flavones had the weakest binding affinities for quorum sensing proteins among all the subclasses which can be attributed to their planar structure and lack of flexibility to interact with the residues of proteins like LuxS and LsrB. C-glycosylated flavones like orientin, isorientin and vitexin had better binding affinities in comparison to non-glycosylated flavones like apigenin, luteolin and diosmetin against quorum sensing proteins. This can be explained by the increased number of hydrogen bond interactions by the glycoside unit with the residues of proteins such as LsrK and LsrR. Isorientin shows strong binding with LuxS protein (-8.8 kcal/mol) by hydrogen bond formation of free hydroxyls in glycoside with HIS B:58, GLU B:122, LEU A:3, ASP A:5 and ARG B:65 residues. C-glycosylated flavones also outperform their non glycosylated counterparts in binding against permeability and antibiotic target replacement proteins. Luteolin had better binding affinities for the *E. coli* proteins as compared to apigenin due to additional hydroxyl group in the structure. Flavononols like taxifolin had weak binding to quorum sensing proteins and average values for permeability proteins.

In general, the binding affinities obtained by docking against *S. aureus* and *E. coli* proteins exhibited some similarities and suggested that structural modifications such as incorporation of glycosyl and galloyl moieties may help to improve the binding potential of the flavonoids. Increasing the hydroxyl groups may help to bind strongly with the target proteins through hydrogen bonding and methoxylation reduces the binding affinities.

CONCLUSION

There has been significant work in flavonoid research in the recent years. This paper highlights the structure-activity relationship of flavonoids identifying pharmacophores important for the antibacterial activity such as 5,7 hydroxylation of A ring and hydroxyl group at 3 position in C ring which can help in the development of more effective and potent leads in antimicrobial treatment. Hydroxyl substituents increased the inhibitory effect whereas methoxy groups decreased it. The most potent flavonoids exhibited a balance between hydrophilic and lipophilic substituents implying that the amphiphilic nature is favourable for antibacterial potential. *In silico* analysis revealed that flavonoids show subclass-specific binding preferences for target proteins. Extensive screening of such phytochemicals can help to establish a comprehensive overview of the relation between chemical structure and the antimicrobial activity. More efforts must be directed towards the *in vivo* studies of these natural compounds as they are very scarce. The low bioavailability of flavonoids can hamper its antibacterial potential in living systems. Improving drug delivery systems by using techniques like nanotechnology can help to overcome this limitation.

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ABBREVIATIONS

MIC: Minimum Inhibitory Concentration; **PyRx:** Python Prescription; **2D:** Two-Dimensional; **SAR:** Structure-Activity Relationship; **UV:** Ultraviolet; **DNA:** Deoxyribonucleic acid; **RNA:** Ribonucleic acid; **ATP:** Adenosine triphosphate; **DMSO:** Dimethyl Sulphoxide; **SRL:** Sisco Research Laboratories Pvt. Ltd.; **TCI:** Tokyo Chemical Industry; LGC: Laboratory of the Government Chemist; **PDB:** Protein Data Bank; **PDB ID:** Protein Data Bank Identification; **MecA:** Methicillin-Resistance Gene A; **PBP4:** Penicillin-binding protein 4; **DHFR:** Dihydrofolate reductase; **QSAR:** Quantitative Structure-Activity Relationship.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

Rachael Ashok: Methodology, Investigation, Formal Analysis, Writing- original draft, Data curation, Foram Patel: Procurement of bacterial strains, Methodology, Formal Analysis, Writing- review and editing, Denni Mammen: Conceptualization, Formal Analysis, Writing- review and editing, Supervision, Darshee Baxi: Formal Analysis, Writing- review and editing, Supervision

SUMMARY

The present research work has been undertaken to study the effect of variation in structure on the antibacterial potential of different flavonoid classes. Though these compounds are well known to exhibit good action against growth of bacteria, there are sparse studies on how the type and number of functional groups on the aromatic rings of flavonoids can govern their activity. Interclass as well as intraclass comparison of the activity against both gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli* strains yielded a better overview on how the structure affects the activity. Hydroxylation, gallolylation and glycosylation in the aromatic rings exhibit enhanced antibacterial

activity, whereas methoxylation in the same positions are observed to show decline in the same. The studies have also been supported by using *in silico* docking studies, which are in line with all previously reported data. This is one major comprehensive work done to establish the structure-activity relationship in flavonoids.

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