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In-vitro and In-silico Evaluation of *Citrus* Peel Flavonoids as Potential Antibacterial Agents against *Streptococcus pneumoniae* Isolated from Pneumonia Patients

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Abstract

Streptococcus pneumoniae is a leading cause of bacterial pneumonia. The continuing global threat of antimicrobial resistance alongside pneumonia being one of the largest infectious diseases worldwide raises the urgent need of novel alternatives to antibiotics using molecular docking, and *Citrus* flavonoids are well-known antibacterial agents. Purpose: In-silico and in-vitro evaluation of the antibacterial activity of 29 flavonoid found *Citrus* peels to identify their activity against *S. pneumoniae* isolated from pneumonia patients and a reference strain. Methods: Conventional methods had been used to identify *Streptococcus pneumoniae* and determine its sensitivity to antibiotics. Well diffusion and microdilution methods were employed for the in-vitro evaluation. The in-silico study was done using two docking programs as modern approaches to identify activity to Toxin-Antitoxin complex (HicBA) as a possible target in *S. pneumoniae*. Results: Binding affinity using iGEMDOCK was lower for glycosidic flavonoids than their respective aglycones. Binding affinity using AutoDock Vina for Quercetin (-6.7 kcal/mole), Rutin (-7.1 kcal/mole) compared to Vancomycin (-6.4 kcal/mole), minimum inhibitory concentration (MIC) range of the of 62.5-500 µg/ml compared to Rutin (-78->500 µg/ml). Conclusions: The docking study implied that HicBA may be a potential target for *Citrus* flavonoids. Rutin had milder effects on *S. pneumoniae* denoting that sugar moiety's role is limited. Quercetin may be a possible antibacterial agent for *S. pneumoniae*.

Keywords: Quercetin; Rutin; Well-diffusion; Microdilution; AutoDock Vina; Toxin-antitoxin Complex

1. Introduction

Bacterial infections are important causes of health problems, physical disabilities and mortalities worldwide [1] Pneumonia is the world deadliest infectious disease, ranking 4th cause of death according to WHO's latest report [2,3]. It accounts for enormous financial and medical burdens and more notably morbidity and mortality particularly among hospitalized patients [4]. Risk factors of pneumoniae include age, antibiotic use, smoking, chronic diseases and viral respiratory infections with subsequent bacterial colonization [5]. Moreover, in the light of the COVID 19 pandemic it was found that 50% of COVID 19 death cases had secondary bacterial infections, and were associated with lengthier hospital stays, ICU admissions, ventilator and broad-spectrum antibiotic use, raising the probability of resistant superinfection occurrence eventually leading to death [6-8].

Antimicrobial resistance is a serious issue that jeopardizes our future, leaving us with less options available to treat communicable diseases [9,10]. Resistance mechanisms have emerged for all classes of antibiotics, and in turn only few novel drugs are being developed and approved for treatment [11]. With continuous spread of resistant bacteria and their acquisition of new types of resistance, serious global action needs to be taken, especially that resistance

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genes can be easily transferred between bacteria [12-14].

S. pneumoniae, a gram-positive bacterium, is well accepted as the most frequent cause of community-acquired pneumonia (CAP) and can cause hospital acquired, health care-acquired pneumonia, meningitis and otitis media. It causes approximately 5 million cases each year worldwide and is considered an important cause of CAP-related deaths in Europe and the US [15-17]. It mostly targets new-borns, elders and people with underlying chronic diseases such as diabetes and heart disease [18]. According to one study *S. pneumoniae* was the most common bacterial species in co-infected COVID 19 patients [19]. Multi-drug resistant *S. pneumoniae* have emerged due to the wide usage of antibiotics in the treatment of pneumococcal disease. Its universal resistance to commonly used antimicrobials has risen in the past decades including beta-lactams, macrolides, and fluoroquinolones [20-22].

The genus Citrus is one the oldest crops cultured by man, with dates of its cultivation back to the 2100 BC [23], [24], with important historic and economic value due to its use in juice, food and cosmetic industries [25,26]. Numerous studies of Citrus peels showed a rich harvest of biologically active compounds including essential oils, flavonoids, coumarins and limonoids [27,28] Large sums of peels are annually discarded as waste from agriculturally based industries causing environmental pollution if left without planned recycling [29,30]. Alternatively, these eco-friendly compounds can be used in food production as preservatives, in biofuel production of methane and promising advances are being explored in its use in modern pharmaceutical and nutraceutical formulations [31,32] such as the green synthesis of antibacterial nanoparticles from Pomegranate peel waste against water borne pathogens [33] and nanoliposomes of Lemon essential oil against food-borne pathogens [34].

Over the recent years, natural products and their structural analogues have played a significant part in drug discovery [35,36] especially in the area of infectious disease due to their safety and efficacy [37,38]. Citrus peel flavonoids are great pharmacotherapy candidates [39-41] especially as antibacterial agents as shown by previous research [42,43]. The main structure consists of (C6-C3-C6) carbon skeleton with varying degrees of oxidation and substitution forming different classes of flavonoids [44]. Therefore, exploiting the abundant sources of phytochemicals can prove vital in finding new antibiotics especially in developing countries like Syria, and successful drug discovery is strongly aided with the use of bioinformatic and drug design [45].

Molecular docking has recently been considered an invaluable, cost- and time-efficient tool in drug discovery, with continuously improving docking potential advances [46]. It includes an automatic protein/ligand interface analysis using thermodynamics where affinity is expressed in a numerical form called the binding energy [47]. It has been largely implemented in the pharmaceutical industry [48]. For example, a novel target (Thioredoxin reductase) for Bacillus anthracis and Bacillus cereus has been newly identified in these bacteria, with the use of AutoDock Vina virtual screening and comparative proteome analysis [49]. iGEMDOCK software was used to study antidiabetic effect [50] and the anticoagulant effect of certain natural flavonoids [51,52].

Consequently, this study was conducted using the latest tools employed by pharmaceutical studies to narrow the scope of finding an effective antibacterial compound found in Citrus peels, with low toxicity, and high possibility of being a potential alternative of traditional antibiotics. *S. pneumoniae* isolated from pneumonia patients in Homs City, was chosen for the in-vitro evaluation of Citrus flavonoids using well-diffusion [53] and microdilution methods [54]. Toxin-antitoxin complex (PDB ID. 5YRZ) [55] was chosen for the in-silico study and two molecular docking software; AutoDock Vina and iGEMDOCK, were used for precise and reliable results. Vancomycin was chosen as positive control [56].

2. Experimental

This Study was done using Rutin and its aglycone Quercetin to demonstrate the role of the sugar moiety on the antimicrobial proprieties, the strains used were clinical and reference *Streptococcus pneumoniae*. Docking study accompanied the experimental testing to elucidate the interactions between selected compounds and the protein toxin-antitoxin complex of *S.pneumoniae.*

2.1. Materials

Vancomycin was obtained from Anhui Biochem United pharmaceutical Co., flavonoid powders were purchased for Sigma Aldrich Chemicals Co., dimethyl formamide, dimethyl sulfoxide (DMSO) (Seelze-Hannover), Mueller Hinton Agar (Titan Biotech Ltd., India), Mueller Hinton Broth (Titan Biotech, India), Fluid Thioglycollate Medium (Abtek Biologicals Ltd., UK), Blood Agar Base (Abtek Biologicals Ltd., UK), Brilliant Green Bile Broth 2% (Titan Biotech Ltd., India).

2.1. Bacterial Strains

A total of 25 patients admitted into two local hospitals in Homs City and diagnosed with pneumonia using a chest radiograph image and clinical criteria employed in those hospitals were enrolled in the study. The study was conducted in compliance with the national code of research ethics at all stages of the project and approved (approval number: 1369) acquired in 15-10-2020 by the Chairman of Research Ethics in Al Baath University – Ministry of Higher Education, Syrian Arab Republic. Non-invasive methods were used to collect samples; endotracheal aspiration in ICU patients who subsequently required ventilator associated respiration, and spontaneous expectoration in cooperative patients after being carefully instructed to obtain a specimen [57]. A reference strain was purchased from the Atomic Energy Commission in Damascus, Syria.

Sputum samples that showed 10-25 polymorphonuclear neutrophils and less than 10 squamous epithelial cells were proceeded to culture [58]. Sputum was then inoculated in thioglycolate broth for 48 hours and positive samples were cultured in Blood, MacConkey and chocolate agar for preliminary identification and latter biochemical tests of Optochin sensitively [59] and bile acid solubility for confirmation [60]. Finally, an antimicrobial sensitivity test was conducted using the Kirby Bauer method as per the Clinical Laboratory Standards Institute (CLSI) guideline using 5% sheep blood added to Muller Hinton Agar (MHA) and incubated in 5% CO2 using a candle jar for 24 hours in 37°C [61].

2.2. Molecular Docking

Molecular docking was performed using AutoDock Vina and GEMDOCK v. 2.1 docking programs as successfully used in previous studies [41], [43], [44], [62], and the target protein used was Toxin-antitoxin HicBA complex (PDB entry 5YRZ), its structure was downloaded from Protein Data Bank (PDB) at (http://www.RCSB.org) [59] The structure consists of a toxin (HicA) which contains a double-stranded RNA binding region vital for RNA recognition. This region is sterically blocked by an antitoxin (HicB). The active site includes a His36 residue necessary for the toxin's ribonuclease activity, and inhibiting HicBA complex formation and toxin release could be one approach to develop novel antimicrobials [55].

Vancomycin was included as control. The 3D structures of the flavonoids and vancomycin were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov) [63] and/or Zinc 15 database (https://zinc15.docking.org) [64] in SDF format. For Vina, the protein and ligands were prepared by adding hydrogens and turned into PDBQT using UCSF Chimera 1.16. The active site was analysed using the virtual tool Protein Plus https://proteins.plus/ [65]. Docking parameters are shown in (Figure 1).

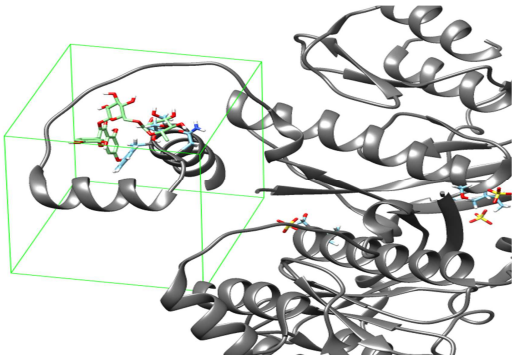

Figure 1: Visuals of docking, conformation within binding pocket in chain A using Chimera software grid box dimensions 21, 26, 26. 2 angstrom and grid center 204. 130, 108 for the X, Y,Z

For GEMDOCK, a built-in binding site preparation tool was used, molecular stable docking and scoring were done via iGEMDOCK (population size 300, generations 80, solutions 10) was used to screen for potential leads and determine the compound with the lowest energy which was then docked using Vina (exhaustiveness 8, binding modes 10, maximum energy difference 3 kcal/mol). Finally, the best conformer was selected and combined with the ligand for further interaction analysis was using Discovery Studio visualizer program, Protein ligand Profiler and Protein Plus websites.

2.3. Antibacterial Activity

Isolated strains were stored in the refrigerator in 4°C using weekly subculture in blood agar until needed [60]. Bacterial assays were done by well diffusion [53] and broth microdilution methods [54] to evaluate to activity of the flavonoid Rutin and its aglycon Quercetin with some modifications made to

culture media and incubation conditions for antimicrobial susceptibility testing methods were prepared as recommended by CLSI, specific to *S. pneumoniae* taken into consideration [66]. Inoculum suspensions equivalent to 0.5 MacFarland were prepared using sterile saline. Antibacterial agents were dissolved in DMSO [67] in concentrations (1000µg/ml Rutin, 1000µg/ml Quercetin) [68], these concentrations were attained in an incremental decrease methodology starting with 50 ml/ml reaching the lowest concentration of each tested component. Vancomycin (512µg/ml) was dissolved in distilled water and used as positive control.

2.3.1. Well diffusion Method

The test was conducted using 5% sheep blood MHA, a chrome well-punching device was fashioned to achieve 8 mm diameter wells. Direct colony method from a 24-hour agar plate was used to prepare the bacterial inoculum. Using a cotton swab, a lawn of bacterial suspension was evenly distributed over the agar plate. Each well was filled with antibacterial solution 100µg/100µl well of individual flavonoids, 51.2µg/100µl positive control Vancomycin, DMSO as negative control. Plates were left in room temperature for 2 hours to allow the tested components to diffuse, then were incubated in 36 °C for 24 hours and inhibition zones (IZ) were measured, attention was paid to differentiate between haemolysis zones and IZs. Each test was done in triplicate and the mean diameter was recorded for 6 strains and the reference strain

2.3.2. Broth Microdilution Method

The MIC value was determined to assess the antibacterial activity using a 96 well microtiter tray. All tests were done in triplicate. Stock solutions were dissolved in DMSO then diluted 1/10 in MHB supplemented with 5% LHB giving a final test concentration of 1 mg/ml in 1% DMSO. 50µl of two-fold diluted antibacterial solution was made into each well to achieve a range of 500, 250, 62.5, 31.25, 15.625. 7.81, 3.9, 1.95 µg/ml. Bacterial inoculum was diluted to 5× 105 CFU/ml by adjustment of MacFarland standard using saline solution and 50 µl was added to each well giving a final volume of 0.1 ml/well. Sterility (no bacteria) and growth control (no antibacterial agent) controls were used in each assay. Finally, wells were sealed and incubated for 18-20 hours, the MIC was recorded as the lowest concentration that inhibits growth detected by the unaided eye.

3. Results

3.1. Bacterial Isolation

A number of six bacterial strains were isolated from sputum samples and used for further testing. Streptococcus pneumonia strains were characterized by gram stain, haemolysis pattern and confirmatory biochemical tests; catalase test, optochin sensitivity and bile acid solubility [60] (Table 1). The antibiotic sensitivity was recorded for isolated strains, results are shown in (Table 2). The MIC test implied sensitivity to Vancomycin for clinical strains, while they expressed intermediate sensitivity to cotrimoxazole and were resistant to macrolides.

3.2. In-vitro Antibacterial Evaluation

Our study was done to assert the highly beneficial qualities of flavonoids found in Citrus peels to aid the already mentioned medicinal use of them and to highlight the much-needed attention of these by-products. Regarding well-diffusion, inhibition zones expressed as mean ± standard deviation are shown in (Table 3). Rutin and Quercetin demonstrated inhibition zones against *S. pneumoniae* at the lowest concentration tested (1mg/ml). Pure components exhibited collectively similar inhibition zones of 1 mm. No recorded inhibition zone was noted for DMSO control.

Turning to the MIC test which was done to determine the lowest inhibitory concentration. The lowest MIC of the Citrus tested components were reached by Quercetin, which had a slightly lower mean MIC of 291 µg/ml compared to Rutin with the MIC of (78.125 - >500 µg/ml). The results of this test shown in (Table 3) confirm the fact that flavonoid aglycones display a stronger in-vitro activity than their unhydrolyzed glycoside derivatives represented by Rutin. Most tested strains were sensitive to the antibiotic Vancomycin with a mean MIC of 1.08 µg/ml, nevertheless, the Kirby Bauer test showed considerable resistance to Penicillin, Azithromycin, Clindamycin, Erythromycin and Sulfamethoxazole among these strains. The reference strain had an MIC of 500µg/ml for Rutin and Quercetin and MIC of 1 µg/ml for Vancomycin, while their inhibition diameter mean was 11 mm for both compounds.

Table 1
Laboratory methods of bacterial clinical strains of *Streptococcus pneumoniae*

Method	Microscopic characteristics		Macroscopic Characteristics (colonies)		Biochemical tests		
S. pneumoniae	Gram Stain	Shape	Hemolysis	Shape	Bile solubility	Optochin Sensitivity	Catalase Test
	Gram+	diplococci or short chains of cocci	α-hemolysis	small, grey and mucoid with a depressed center	soluble in Bile salts	sensitive, IZ >14mm	-ve

Table 2
Results of antibiotic sensitivity Kirby Bauer test to isolated stains

Antibiotic / Strain Num.	VA	AZM	CLM	OXA	ERY	COT
1	S	R	R	R	R	R
2	S	I	R	R	R	S
3	R	R	R	R	R	R
4	S	R	R	R	R	I
5	S	S	S	S	S	S
6	S	R	R	R	R	I

R: Resistant, S: Sensitive, I: Intermediate, VA: Vancomycin 30µg, AZM: Azithromycin 15µg, ERY: Erythromycin 15µg, OXA: Oxacillin 1µg, COT: Cotrimoxazole 25µg, CLM: Clindamycin 15µg

Table 3
Diameter of Inhibition zones and minimum inhibitory concentration values for clinical strains

Test compound	IZ* (mm)	MIC (µg/ml)
	Streptococcus pneumoniae	
Rutin	10.0 ± 4.9	78.125 – >500
Quercetin	10.0 ± 2.7	62.5 - 500
Vancomycin	10.8 ± 0.7	1 – 1.5

*mean ± standard deviation, IZ: inhibition zone, n = 6

3.3. Molecular Docking

Among different classes of flavonoids used in the iGEMDOCK virtual screening, the results of iGEMDOCK molecular docking with HicBA are given in (Table 4). Ranking included glycosidic flavonoids with lowest binding energy, followed by Casticin, Isookanin, Tangeretin (glycoside). Flavonol aglycones include Quercetin (15th highest affinity), Eriodictiol (Flavanone), Kaempferol (Flavonol), Flavones Followed, Diosmetin and Luteolin. Lastly, flavanones include Hesperetin and Naringenin. The minimum binding energy showed that flavonoid glycosides and aglycones were successfully docked with HicBA complex as shown in (Figure 2), Rutin (2nd highest affinity) showed promising binding energy therefore it was chosen to further computational docking to analyse the ligand's best binding mode using Chimera, Quercetin (aglycon) was also selected for Chimera docking, results are given in (Table 5).

In regards to the binding site, the most important interactions with the above-mentioned compounds, Quercetin formed Hydrogen bonds with Asp 125 and Phe 135, hydrophobic interactions including π stacking with Phe 135 and carbon hydrogen and π alkyl bonds with Leu 118, Ile 120, Leu 139. Rutin formed similar interactions with Thr 138, Leu 133, Asn 134, Ser 136, Phe 135, Thr 117, Lys 116, Arg 129, Asp 125, Leu 118, Ile 120, Leu 139. Both ligands showed comparable binding energy to Vancomycin. These results show that both compounds could enter the binding pocket of the HicBA complex. The best poses are given in (Figure 3).

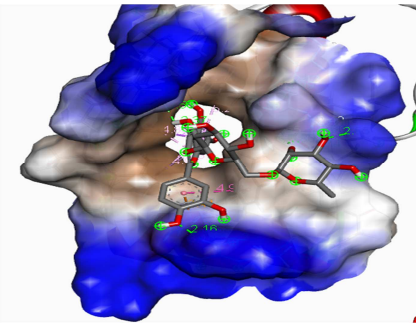

Figure 2: Three- dimensional structure of protein-ligand interaction with surface view using Discovery studio visualizer

Table 4

Docking results analysis with AutoDock vina interfaced with Chimera software

Best Pose	*RMSD* u.b*	*RMSD* l.b*	*Binding energy (kcal/mole)*
Rutin	0	0	-7.1
Quercetin	0	0	-6.7
Vancomycin	0	0	-6.4

*RMSD: Root mean square deviation of binding poses upper and lower bonds

Table 5

Docking Results Analysis with HicBA complex using iGEMDOCK

No.	Ligand	Total Energy	H-Bond	VDW*	Ion Pair
1	Neoeriocitrin	-112.597	-33.4445	-79.152	13.8333
2	Rutin	-112.514	-39.1731	-73.3408	13.2558
3	Hesperidin	-103.55	-24.8519	-78.6985	14.3256
4	Flavanomarein	-101.996	-34.1225	-67.8738	14.3438
5	Hyperoside	-101.46	-37.7782	-63.6819	13.3333
6	Naringin	-97.9182	-21.0216	-76.8966	12.9512
7	Diosmin	-97.1698	-29.2704	-67.8994	13.1628
8	Neohesperidin	-95.9171	-32.3885	-63.5285	13
9	Natsudaidain	-85.9485	-22.1858	-63.7627	14.2333
10	3,3',4',5,6,7,8-Heptamethoxyflavone	-81.1648	-15.6904	-65.4744	13.5484

11	Nobiletin	-78.6966	-15.6237	-63.073	14.1379
12	Casticin	-77.3227	-22.7051	-54.6176	14.2963
13	Isookanin	-76.4918	-23.994	-52.4978	15.3333
14	Tangeretin	-75.3362	-12	-63.3362	14.1481
15	Quercetin	-75.1739	-25.0098	-50.1641	15.0909
16	Retusin	-74.0682	-20.0361	-54.032	14.3077
17	Eriodictiol	-72.9894	-19.2404	-53.7489	16.0952
18	Kaempferol	-72.5177	-21.9373	-50.5804	15.7619
19	Sinensetin	-71.8692	-6	-65.8692	14.5185
20	Diosmetin	-71.1723	-16.7813	-54.391	15.9091
21	Luteolin	-70.6965	-28.4651	-42.2313	14.0952
22	Salvigenin	-69.702	-19.8416	-49.8604	14.1667
23	Hesperetin	-69.555	-14.5	-55.055	15.7273
24	Isosakuranetin	-66.4512	-14.3925	-52.0587	15.619
25	Naringenin	-64.7868	-14.4134	-50.3734	18.8
26	Apigenin	-64.331	-15.1808	-49.1502	18.5

*VDW: Van Der Waals, Energies given in kcal/mole

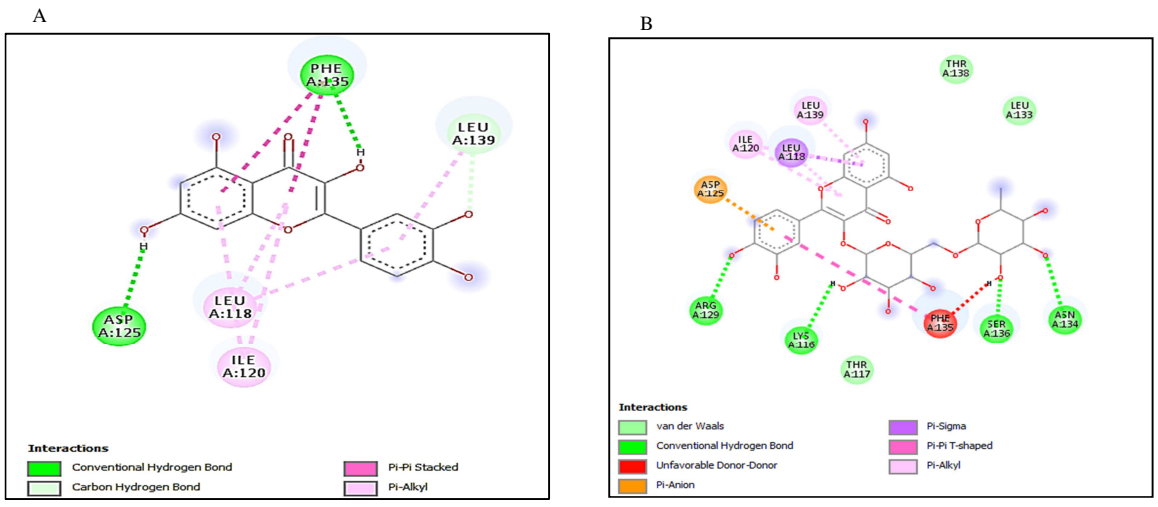

Figure 3: 2D pictures of possible interactions were obtained using DS visualizer. A; Quercetin-5YRZ complex interaction. B; Rutin-5YRZ complex interactions

4. Discussion

This research is part of a master thesis aimed at finding effective, inexpensive antibiotics of natural origin. Attention was put on Citrus peel components as a continuum of group research focused on the exploitation of these natural resources, usually discarded as waste from industrial businesses. One study in the University of Aleppo confirmed the antioxidant activity of Citrus flavonoids [69], another focused on the antibacterial effect against E-coli [53,70] and P. aeruginosa [71]. In this study, flavonoids' antibacterial activity was evaluated against *S. pneumoniae* as the most frequent cause of pneumonia to invest in the affordable sources in face of the recent financial hardship following the crisis that shadowed the last decade in Syria, especially with the recent pandemic resulting in increased pneumonia cases and the stemmed economic burden on medical care, alongside the uncontrolled use of antibiotics furthermore highlighting the need for effective alternatives.

Flavonoids can appear as glycosides or aglycones free of sugars. Previous studies of structure activity relationship (SAR) of Citrus flavonoids relating to their antibacterial effect revealed that the lipophilicity and the balance between polar and hydrophobic groups influence the antibacterial activity, the hydroxyl groups on C5 and C7 on ring A [72-74], the absence of polar groups on ring B. However, possession of certain polar hydroxyl groups may contribute to improved activity as seen in C3' hydroxyl on ring B [72,73] therefore, the flavonoid class may govern the pattern of interaction and consequent SAR for antibacterial activity. The substitution number is not as important as the substitution position where the presence of catechol group on ring C and the higher C3 charges correlates with the best pharmacological results [74]. Lastly, the planar structure of flavones/flavonols vs the perpendicular flavonones/flavononols/flavanonols shows an overall more potent antibacterial activity [73].

S. pneumoniae strains were chosen for antibacterial evaluation, and were obtained from pneumonia patients. According to antibiogram results, most strains were Vancomycin sensitive as shown in the Kirby Bauer test and the MIC test, nevertheless, they were resistant to other antibiotics such as macrolides and cotrimoxazole, this agrees with the latest reports by a study done on the prevalence of resistance in *S. pneumoniae* in the Middle East region[75] due to the misuse of antibiotic prescription among the medical society particularly during the current pandemic raising much threat to pneumonia patients with streptococcal infection resistant to most available treatments [76].

One main aim was comparing the antibacterial activity of the glycoside derivative Rutin and its aglycon represented by Quercetin. Screening included the well-diffusion method, indicating that both compounds inhibited bacterial growth in-vitro at a low of 1mg/ml, surpassing previous results of Quercetin against isolated E-coli [53] Rutin had an effect on some strains but Quercetin showed a lower MIC mean presenting that the latter has a stronger antibacterial effect against isolated *S. pneumoniae* compared to Rutin. This result adds to one study as elucidated by G. Mandalari et al. that aglycones Eriodictiol and Naringenin had a lower MIC ranging between 250-800 µg/ml compared to their conjugated derivatives (Neoeriocitrin and Naringin) against a range of Gram negative and positive bacteria[27], these results also supplement the findings of previous studies led by Amin et al. that Quercetin alone has a more powerful effect than Rutin on clinical isolated MRSA strains[77], proving that Quercetin may be a useful candidate for drug development against *S. pneumoniae*.

Studies have revealed that Rutin possesses an antiviral effect [78] and an antimicrobial effect against Mycobacterium [79]. However, in bacteria, it was shown that the presence of the sugar moiety has no significant effect on them, which indicates that sugar substitution diminishes the antibacterial activity of flavonoids or enhances the antibacterial activity of different flavonoids/antibiotics in synergy studies[77,80] This correlates with a reasearch done by J. Echeverría at al. which illustrated that lipophilicity properties dictate flavonoids' ability to dissolve in cell membranes and reach the bacterial target[73]. Taking into consideration log P values of Rutin 1.3 and Quercetin 1.5 and their molecular weights of 610.5, 302.23 g/mol computed via PubChem, the behaviour of Rutin may be attributed to its higher hydrophilic affinity and weight which may affect its ability to cross the lipid layers of the cell membrane, despite its greater virtual affinity with the target shown by the lower energy mainly attributed by 3 H-Bonds formed within the sugar polar moiety. Therefore, including the Lipinski Rule of 5 into consideration may help overcome the limitation of false positive docking results.

Likewise, similar studies were led by other researchers and presented with comparable results, according to Akroum et al. Quercetin MIC against *S. pneumoniae* was 350µg/ml [81] where they illustrated that Quercetin was the most interesting flavonoid with the least toxicity, while mean MIC in our study was slightly lower at 291 µg/ml, this may

either be attributed to testing method differences including the use of distilled water to dissolve Quercetin or the bacterial strains source which were isolated from pneumonia patients. Quercetin's MIC value ranged between 62.5-500 µg/ml, variable MIC values between strains may be attributed to differing antibiotic susceptibility patterns, where two strains had an MIC of 62.5µg/ml and are well under 100µg/ml the cut-off MIC for an individual compound to be considered for further studies [68]. Genotyping or immunotyping of strains could interpret these differences more accurately in later studies. The reference strain used as control exhibited a similar pattern of effectivity for the aglycon and its glycoside, where Rutin and Quercetin both had a similar MIC of 500µg/ml and comparable inhibition zones indicating that indeed these compounds display anti-infective effects against *S. pneumoniae*.

Turning to docking, virtual screening was performed and it was concluded that among the different classes of Citrus flavonoids, glycosidic flavonoids showed the lowest binding energy followed by flavonols, flavones and lastly flavanones which equates with the previously mentioned SAR of flavonoids [72-74]. In-silico study confirmed that flavonoids had a satisfactory interaction with 5YRZ protein of *S. pneumoniae*, noting the possibility of HicBA complex being a target for novel antibiotic development and that bioinformatics can indeed play a major role in predicting the possible small active molecules orientation within the protein pocket. The more negative the energy, the stronger and more stable interaction can take place with the protein. Rutin had a lower energy than Quercetin and Vancomycin using AutoDock Vina, however by contrast, the in-vitro evaluation showed an inverse pattern of activity indicating the significance of performing in-vitro testing alongside docking studies.

The use of two docking programs implied that AutoDock Vina produced more accurate results with slight energy differences (-6.7 kcal/mole for Quercetin, -6.4 kcal/mole Vancomycin, -7.1 kcal/mole Rutin), in comparison to iGEMDOCK corresponding better with the MIC results. Furthermore, looking at Quercetin's interactions with HicBA, it can be noted that the H-bond of C7 hydroxyl with Asp 125, H-bond of C3 hydroxyl with Phe 135, hydrophobic interactions with C3' and C4' on ring B and the C2=C3 double bond hydrophobic interactions with Leu 120 and Ile 118 seem to perfectly correspond with previous findings of a well-established SAR, reinforcing the antimicrobial role Quercetin plays against *S. pneumoniae*.

As a result, HicBA may be a possible target of flavonoids, which supplements previous findings of research focused on mechanisms of action that has revealed that bacterial exposure to high concentration of flavonoids was accompanied with cellular leakage [72]. Also, the inhibition of peptidoglycan and nucleic acid synthesis, and efflux pumps are some of the reported bactericidal effects of flavonoids [82]. In-depth examination on Quercetin's mechanism of action against *S. pneumonia*, Wang et al. have shown that it inhibits the formation of *S. pneumoniae* biofilms in a dose-dependent manner [83], and its ability to inhibit Suilysin, a secreted cytotoxin by *Streptococcus suis* that is known to involve in the infection [84]. Worthwhile detailed research is further needed to investigate the inhibiting effects of Quercetin as an antibacterial agent against clinically isolated *S. pneumoniae*.

5. Conclusion

This study has shown that Rutin and Quercetin possess an antibacterial effect towards drug resistant *S. pneumoniae* isolated from pneumoniae patients. The use of eco-friendly, affordable sources is beneficial in finding novel antibacterial agents to face the worldwide emerging threat of antimicrobial resistance and its economical and health related complications. Bioinformatics are considered as great tools in drug discovery within the pharmaceutical industry. Rutin and Quercetin showed comparable binding affinity with Vancomycin and Quercetin had the lower minimum inhibitory concentration and higher antibacterial effectivity. In other words, the use of molecular docking programs could give insights into the mechanism of action, but only when supplemented with laboratory assessment to give better prediction of the behaviour of compounds.

6. Conflicts of Interest

There are no conflicts to declare.

7. Formatting of Funding Sources

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