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Antibiofilm Activities of Quercetin on ESKAPE Pathogens: An *in-silico* Analysis

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ABSTRACT

The treatment of ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.) is difficult due to their antibiotic resistance, which is mostly attributed to the biofilm matrices produced by these bacteria. These biofilms are notoriously difficult to counter though some natural compounds show promise. Quercetin, a plant-derived flavanol is found to have antibiofilm activities against these ESKAPE pathogens. In this study, molecular docking experiments demonstrated a strong affinity between quercetin and biofilm forming proteins of ESKAPE pathogens, namely 3TIP, 3ZYB, 5KED, 5FCE, 5D6H and 6YF6, which indicates that quercetin could be effective for eradicating ESKAPE pathogens. Optimization of parameters for best molecular interaction was studied using Box Behnken Response Surface Methodology.

Keywords: Biofilm; ESKAPE pathogen; Molecular docking; Quercetin; Response surface methodology

1. Introduction

Among pathogenic microorganisms, ESKAPE pathogens, namely *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp. are responsible for various biofilm mediated diseases [1]. Biofilm is a syntrophic, complex, and sessile microbial population contained in a self-created biopolymer matrix that can shelter and protect pathogenic microbes from adverse conditions. These diseases become more dangerous and challenging to treat since these pathogens are mostly multidrug resistant and common antibiotics are ineffective against them. Hence some alternative is required that can replace conven-

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tional antibiotics. Scientists are trying to exploring plants as a source of bioactive compounds that have the potential to act as potent antimicrobial and antibiofilm compounds able to eradicate both the planktonic as well as sessile forms of theses pathogens. The pathogenicity of ESKAPE bacteria is acquired by their ability to adopt multiple mechanisms of self-defence such as synthesis of virulence factors and biofilm formation.

In recent years, several antibiofilm compounds have been reported from plants and microbes, many of them can eradicate biofilm through direct disintegration of the extracellular polymeric substance (EPS) matrix of the biofilm [2]. Some of these phytocompounds can significantly affect biofilm formation by preventing microbial adherence through lowering the hydrophobicity of the surfaces [3].

But some compounds can eradicate biofilm through preventing quorum sensing (QS), a process of intercellular communication dependent on cell population density; after attaining a certain cell concentration, the bacterial population synchronizes the expression of related genes to initiate biofilm formation and allied processes leading to bacterial pathogenesis [4]. Hence, QS inhibition is one of the effective strategies to prevent biofilm formation. Considering the rapidly growing trend of antimicrobial resistance, QS inhibition is increasingly preferred as it does not generate drug resistance [5].

Quercetin (3,3′,4′,5,7-pentahydroxyfavone) is a bioflavonoid found in nature and is present in various plant parts that possesses medicinal attributes. It has been found to prevent inflammation, reduce high blood pressure and blood cholesterol, and can reduce obesity and tendency of atherosclerosis [6]. Due to these therapeutic activities, this major polyphenolic flavonoid is used for the treatment of various physiological disorders including metabolic problems, allergic and inflammatory problems, prevention of optic and cardiovascular diseases, and osteo arthritis. Pharmacologically, quercetin has been tested to combat various microorganisms, including pathogenic bacteria, viruses, and protozoan parasites like *Plasmodium, Theileria* and *Babesia*, [7]. Quercetin was found to influence several stages of biofilm growth by blocking different cellular targets or pathways [8] and many researchers have suggested the use of quercetin as an antibiofilm agent against ESKAPE pathogens [9, 10].

Although a few studies have demonstrated the antibiofilm efficacy of quercetin by in silico modelling [11-13], the affinity between quercetin and biofilm proteins of ESKAPE pathogens analysed from their molecular interactions are not available.

The present study employed molecular docking in order to evaluate the affinity between quercetin and biofilm forming proteins of ESKAPE pathogens and evaluated their effectiveness in biofilm eradication.

2. Materials and Methods

2.1 Pre-processing of the proteins

For the in silico study, Schrodinger Maestro's protein preparation wizard (Protein Preparation Wizard; Epik, Schr□dinger, LLC, New York, NY, 2021; Impact) was used to pre-process the selected proteins. The Protein Preparation Wizard automates and integrates the most frequently used technologies and tools inside the structure preparation process. The CCD database was used to determine correct ordering for bond numbering. The optimization of the hydrogen bond network was carried out in addition to the formation

of the missing disulphide bonds. The molecules of water which were over 5Ao from het groups were eliminated and the het states were created by the usage of Epik pH 7±2. After that, the side chains that were missing were identified and added.

2.2 Molecular docking study

Molecular docking was done using the software Schrodinger. Each of the biofilm forming and QS proteins underwent molecular docking against quercetin (ligand), adding up to a total of eight molecular docking operations. Schrodinger is the leading chemical and biological simulation software in the field of bioinformatics, biotechnology, pharmaceuticals, chemical engineering and material sciences. Maestro is the portal to all the computational technologies of Schrodinger. It also helps in the analysis and the organisation of data. Throughout the entire process of molecular docking, the prediction of the binding site of the selected protein receptor was firstly done using SiteMap [14]. The generation of the Receptor grid was done on the site of the prediction. This receptor grid was chosen for undergoing the process of molecular docking with the respective ligands. The docking was performed by Glide, a protein-ligand docking tool.

2.3 Determination of binding energy

Glide docking (Glide, Schr□dinger, LLC, New York, NY, 2021.) generates a pose viewer file which is then taken for the determination of the binding energies using Molecular Mechanics, General Born Surface Area (MMGBSA). This was carried out with the help of the OPLS2005 force field and the VSGB solvation model. The two-dimensional interactions of the ligands and their interaction diagrams with the respective docked protein structures were closely observed and then compared with the other structures.

2.4 Analysis by response surface methodology

The parameters used for regulation of molecular docking interactions were analysed by standard response surface methodology design called Box-Behnken design. Response surface methodology (RSM) is used to fit the quadratic surface suitably by optimizing the process parameters using a minimum number of experiments after analysing the interactions between the parameters [15]. RSM being a combination of mathematical and statistical techniques, is required to develop the empirical model by improving and optimizing process parameters through the interaction with several affecting factors. The qualitative data from related experiments are used by the statistical method of RSM in order to determine the regression model and also to optimize the output variable called 'response', influenced by several independent variables (input variables).

Box-Behnken is one of the methods used in RSM, which finds the optimum response by optimizing input variables. Box-Behnken design does not include the embedded factorial or fractional factorial points, which are located at the midpoint edges of the variables space as well as at the centre.

Linear or multiple regressions are carried out at the last stage to study the interactions between variables. The polynomial equation which is a combination of the first and second order with analysis of variance (ANOVA), provides the statistical measurement.

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \sum_{j=1}^{4} \beta_{ij} X_i X_j + \sum_{j=1}^{4} \beta_{ii} X_{i^2}. \quad (2.1)$$

The above equation provides an idea about the quadratic polynomial function where Y represents the predicted response quantified by an extraction recovery, where X_i is the independent variable, β_0 is the model constant, β_i is the linear co-efficient, β_{ii} is the quadratic co-efficient, and β_{ij} is the cross product of the co-efficient.

Investigations were carried out in groups of three and the findings were recorded as mean. The observations were all analysed using Design-Expert software. Optimization of percentage best molecular interaction study was analysed using Box-Behnken design [16].

3. Results and Discussion

3.1 Analysis of molecular interaction

The docking results of all the biofilm forming proteins (Table 1), namely 3TIP, 3ZYB, 5KED, 5FCE, 5D6H and 6YF6 (Fig. 1) with the quercetin were analysed.

The two-dimensional structures of the interactions between proteins and quercetin shows the various types of amino acid residues in the proteins that interact with the ligands through H-bonds. From these results, it can be concluded that the amino acids that interact with the ligands are mainly lysine, glycine, tyrosine, serine and threonine.

Binding energy is released during the molecular interaction of quercetin with the target biofilm and QS protein molecules.

This binding results in the decrease of the total energy of the complex. The release of binding energy facilitates any necessary conformational changes for the ligand to transition from its minimum energy state to its bound conformation with the protein. Hence, the larger in magnitude the negative value on the ligand - protein binding, the more energy released. The packing energy, binding energy and the Van der Waals energy values were obtained (Table 2) and subjected to further analysis and comparison. From all the observations, it is well conveyed that biofilm forming proteins have a higher binding energy with quercetin. This was especially true for protein 5KED (*Klebsiella pneumonia* MrkH). So, it can be predicted that quercetin has sufficient binding capacity and stability when binding with biofilm forming proteins.

On the basis of similarity of biofilm forming bacterial genes, a phylogenetic relationship can be traced among the members of ESKAPE pathogens. On the basis of nucleotide homology and phylogenetic analysis, the phylogenetic tree of bacterial strains was performed using the Neighbour-joining method (Fig. 2). The tree depicts the similarity between these pathogens as well as the presence of genes for pathogenicity and biofilm formation.

3.2 RSM analysis

In this study, RSM was used to optimize three operational variables namely, torsion angle, glide score and categoric factor for biofilm forming. The maximum docked energy between the biofilm forming protein and quercetin was taken into account. The results were obtained from the Box Behnken Design experiments (Fig. 3). A 95% confidence interval was used for ANOVA calculations. The Y data demonstrated that physical characteristics had a considerable effect on docked energy. The docking energy between protein and quercetin is represented by this 2nd order polynomial model for a three-factor system.

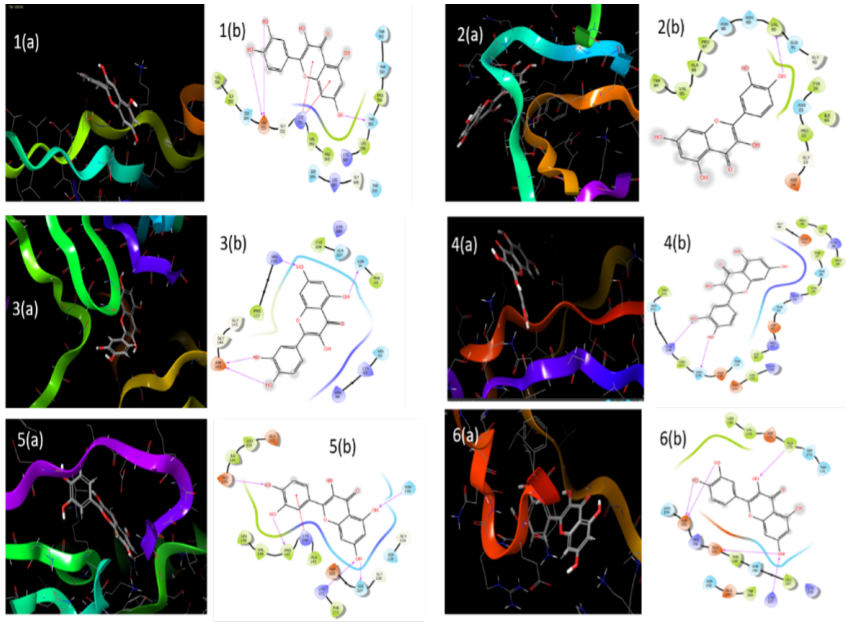

Fig. 1. Docking interactions between quercetin and biofilm forming proteins (3TIP (1a,b), 3ZYB(2a,b), 5KED(3a,b), 5FCE(4a,b), 5D6H(5a,b) and 6YF6(6a,b)) of *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecium, Acinetobacter baumannii,* and *Enterobacter cloacae*, respectively.

Table 1. Details of biofilm forming proteins.

3TIP	Crystal structure of *Staphylococcus* aureus SasG E-G52 module
3ZYB	Crystal structure of PA-IL lectin complexed with GALAG0 AT 2.3 A resolution (*Pseudomonas aeruginosa*)
5KED	Structure of the 2.65 Angstrom P2(1) crystal of *K. pneumonia* MrkH
5FCE	The crystal structure of the ligand binding region of Serine-glutamate repeat protein A (SgrA) of *Enterococcus faecium*
5D6H	Crystal structure of CsuC-CsuA/B chaperone-major subunit pre-assembly complex from Csu biofilm-mediating pili of *Acinetobacter baumannii*
6YF6	EclA C-terminal domain; sugar-binding protein *Enterobacter cloacae* subsp. cloacae ATCC 13047

Table 2. Molecular interactions between quercetin and the biofilm forming proteins of ESKAPE pathogens.

Molecularinteractions between	Glide g score (kcal/mol)	Binding energy (kcal/mol)	Packing energy (kcal/mol)	Van der Waals energy (Kcal/mol)	Interacting Amino acids with Quercetin
Staphylococcus aureus 3TIP vs quercetin	-4.607	-31.73	0	-20.5756	ASP553, LYS551, THR595
Pseudomonas aeruginosa 3ZYB vs quercetin	-4.290	-39.59	-0.80402	-26.2804	VAL90, GLN91, TYR20
Klebsiella pneumoniae 5KED vs quercetin	-6.838	-50.45	-4.53313	-29.6397	ASN34, ARG115, ASP143
Enterococcus faecium 5FCE vs quercetin	-3.912	-26.41	-4.43E-05	-20.2307	ASN136, LYS138
Acinetobacter baumannii 5D6H vs quercetin	-6.259	-49.92	-1.85E-11	-31.8603	ASN139, SER227, LYS171, LYS146, PRO147, GLU235
Enterobacter cloacae 6YF6 vs quercetin	-5.346	-40.25	-0.62251	-24.8469	ALA172, LYS277, GLU151, ASP153

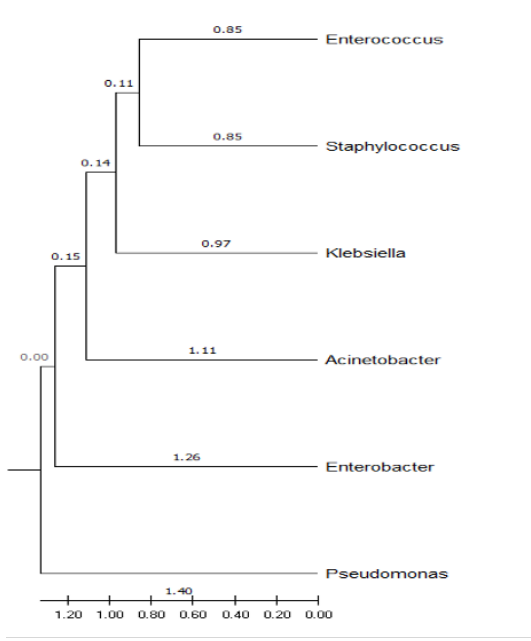

Fig. 2. The similarity of biofilm forming bacterial genes of ESKAPE pathogens were identified through 16s rRNA sequencing, showing the consensus DNA sequence and Clustal analysis.

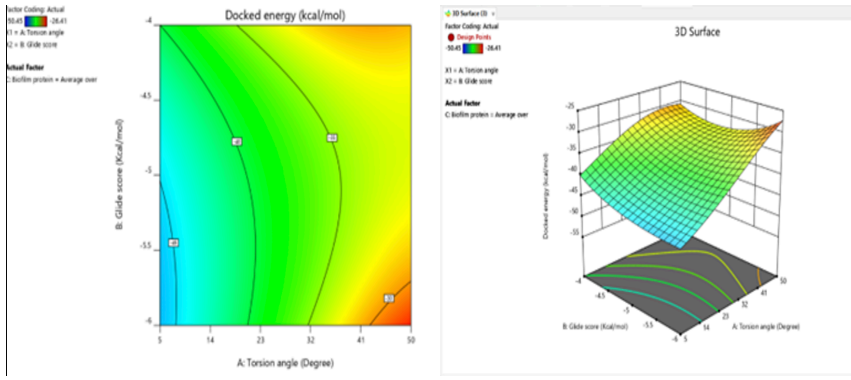

Fig. 3. The 2D and 3D configuration showing the effects of torsion angle, glide score and individual biofilm protein on the docked energy.

3.3 Three-factor Box-Behnken experimental design

3.3.1 Response 1: Docked energy

Transformation: Base log 10 with constant K 52 Quadratic model (Table 3), Numeric parameters [A = Torsion angle $(5, 25, 50)$, B = Glide score $(-4, -5, -6)$] Categoric parameter, C = Biofilm forming proteins (3TIP,3ZYB,5KED,5FCE,5D6H,6YF6)

The model's F-value of 8.98 indicates that it is significant. Such a large F-value might have arisen due to noise (only 0.05% of the time). Model terms with p-values of less than 0.0500 are significant. A, C, AB, and AC are crucial model terms in this scenario. Values larger than 0.1000 imply that the model terms are not signif-

Table 3. Quadratic model and its relationship with model parameters.

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	2.26	20	0.1131	8.98	0.0005	significant
A-Torsion angle	0.7849	1	0.7849	62.34	<0.0001	
B-Glide score	0.0319	1	0.0319	2.54	0.1423	
C-Biofilm protein	0.47	5	0.094	7.47	0.0037	
AB	0.0824	1	0.0824	6.54	0.0285	
AC	0.4182	5	0.0836	6.64	0.0056	
BC	0.0838	5	0.0168	1.33	0.3266	
A^2	0.0261	1	0.0261	2.08	0.1803	
B^2	0.0248	1	0.0248	1.97	0.1904	
Residual	0.1259	10	0.0126			
Lack of Fit	0.1175	8	0.0147	1.47	0.0426	significant
Pure Error	0.0085	2	0.0042			
Cor Total	2.39	30				

Factor coding is Coded. Sum of squares is Type II Classical

Table 4. Fit Statistics of the RSM model.

Std. Dev.	Mean	C.V. %	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
0.1122	1.15	9.76	0.9473	0.8418	-41.7527	13.0483

Table 5. Regression coefficients in terms of coded factors (Sum contrasts).

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	1.14	1	0.0414	1.05	1.23	
A-Torsion angle	0.214	1	0.0276	0.1526	0.2754	1.21
B-Glide score	0.0567	1	0.0286	-0.0069	0.1203	1.3
C[1]	0.1278	1	0.045	0.0275	0.228	
C[2]	0.0225	1	0.0495	-0.0878	0.1329	
C[3]	-0.244	1	0.0562	-0.3692	-0.1188	
C[4]	0.1572	1	0.0511	0.0434	0.271	
C[5]	-0.1506	1	0.0523	-0.2671	-0.0342	
AB	-0.0898	1	0.0351	-0.168	-0.0116	1.64
AC[1]	-0.0569	1	0.0631	-0.1976	0.0838	
AC[2]	-0.1871	1	0.0591	-0.3189	-0.0553	
AC[3]	0.187	1	0.0535	0.0679	0.3062	
AC[4]	0.0272	1	0.0792	-0.1494	0.2037	
AC[5]	0.1715	1	0.0726	0.0097	0.3333	
BC[1]	-0.0302	1	0.064	-0.1729	0.1125	
BC[2]	-0.0357	1	0.0661	-0.183	0.1115	
BC[3]	0.1154	1	0.054	-0.0048	0.2357	
BC[4]	-0.0671	1	0.0688	-0.2204	0.0863	
BC[5]	0.0626	1	0.0716	-0.0971	0.2222	
A^2	-0.0896	1	0.0622	-0.2281	0.049	2.16
B^2	0.0815	1	0.058	-0.0478	0.2108	1.9

icant. Model reduction may improve the model if there are many inconsequential model terms (except those required to support hierarchy). A negative predicted R^2

Table 6. Response of the maximum binding energy between the biofilm forming proteins and quercetin according to the process parameters.

Run	Torsion angle	Glide score	Biofilm proteins	Response
1	25	-6	5FCE	-31.62
2	50	-6	6YF6	-30.12
3	5	-6	5D6H	-49.92
4	26.6667	-5	3TIP	-30.67
5	26.6667	-5	5FCE	-27.31
6	5	-4	5FCE	-39.21
7	50	-5	3TIP	-31.73
8	5	-5	3TIP	-42.36
9	5	-4	6YF6	-35.06
10	50	-4	5D6H	-30.62
11	26.6667	-5	3ZYB	-37.21
12	25	-6	3TIP	-32.41
13	50	-6	3ZYB	-33.65
14	50	-4	3TIP	-29.76
15	25	-4	3TIP	-28.51
16	26.6667	-5	5D6H	-40.66
17	5	-4	5KED	-45.94
18	50	-4	5KED	-30.29
19	5	-6	5KED	-50.45
20	25	-5	6YF6	-40.25
21	50	-4	3ZYB	-39.59
22	50	-6	5KED	-34.61
23	5	-6	3ZYB	-42.36
24	50	-4	5FCE	-26.41
25	50	-4	6YF6	-29.16
26	5	-5	3ZYB	-40.17
27	5	-6	6YF6	-36.12
28	26.6667	-5	5D6H	-41.39
29	50	-6	5D6H	-30.16
30	26.6667	-5	6YF6	-38.91
31	26.6667	-5	5FCE	-33.56

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implies that the overall mean may be a better predictor of the yielded response than the current model. In some cases, a higher order model may also make better predictions. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The obtained ratio of 13.048 (Table 4) indicates an adequate signal. This model can be used to navigate the design space. The F-value, for lack of fit, of 1.47 indicates that the lack of fit is substantial in comparison to the pure error. A significant lack of fit F-value owing to noise has a 24.26% chance of occurring. A non-significant lack of fit is desirable because the model is required to fit.

All other factors constant, the coefficient estimate provides the expected change in response per unit change in factor value. In an orthogonal design, the intercept is the overall average response of all runs (Table 5). The coefficients are modifications based on the factor settings that are made around that average. VIFs are 1 when the factors are orthogonal; VIFs more than 1 imply multi-collinearity; the higher the VIF, the

more severe the factor correlation. VIFs of less than 10 are generally acceptable.

RSM helps to validate the docking efficiency between the biofilm forming proteins of ESKAPE pathogens and quercetin. Each docking parameter has a significant role in the binding. The torsion angle determines (Table 6) the rotation of the ligand and movement of its functional groups. During docking, for providing stability, conformational changes occur for the ligand, which is mainly regulated by the torsion angles. The glide score approximates a thorough systematic search of the docked ligand's structural, orientational, and positional space. A crude positioning and scoring phase that drastically constricts the search area is followed by torsionally elastic optimization of energy on an OPLS-AA nonbonded possible grid for a few hundred remaining alternative poses in this search. Thus, RSM is used to specify the changes of both the parameters on the binding efficiency. It was observed that with a more negative glide score and minimum torsion angle, quercetin provides the most acceptable docked pose with the highest binding energy.

4. Conclusion

As depicted by the molecular docking analysis, it is clear that quercetin, a natural flavonoid, can effectively bind with biofilm forming proteins of ESKAPE pathogens. Hence, either commercially available quercetin or quercetin-containing extracts may be applied to treat biofilm-mediated diseases caused by ESKAPE pathogens. This may be successfully used as an alternative therapeutic strategy for the diseases caused by these pathogens, with the important added benefit of not inducing antibiotic resistance.

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