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Research Paper

Molecular Docking of Flavonoids from Extract of Roselle (*Hibiscus sabdariffa* L.) Calyx on PBP2a as the Basis for Antibacterial Activity Against Methicillin Resistant *Staphylococcus aureus*

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Abstract
The increasing bacterial resistances to antibiotics are serious threat to world health. In Indonesia, there are resistant bacteria such as Methicillin Resistant *Staphylococcus aureus* (MRSA). In order to overcome the problem, the compounds contained in the *Hibiscus sabdariffa* L. are potential to be developed as new antibacterial against MRSA. To confirm the antibacterial activity, the extract of roselle calyx was tested against MRSA. The twelve compounds contained in the extract was docked into binding site of PBP2a using Autodock 4.2.6. The results showed MIC 2.5% of roselle extract. Two flavonoid compounds comply the Lipinski's rules and the docking results showed all compounds had higher binding affinity than reference ligand ceftobiprole. The quantitative structure-physicochemical property relationship (QSPR) found that steric property (CMR) and energy (E_{total}) of ligand contributed to the binding affinity against PBP2a. It concluded kaempferol-rutinoside was the most potential compound from *H. sabdariffa* that could be selected as lead compound to be develop as antibacterial agents.

Keywords
Flavonoids, *Hibiscus sabdariffa*, Antibacterial, PBP2a, Molecular Docking, QSPR

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1. INTRODUCTION

The increasing number of bacterial resistances to antibiotics is a serious threat to health in the world. Resistance has an impact on the field of treatment, and makes successful empirical therapy more difficult to achieve (Wu et al., 2023). Methicillin-resistant *S. aureus* (MRSA) is one of the most common antibiotic-resistant pathogenic bacteria and is currently endemic in hospitals throughout the world (Larsen et al., 2022), including in Indonesia (Susanti et al., 2020; Fitranda et al., 2023). Finding new antibiotics that can inhibit MRSA bacteria is very important in the field of new drug development. β-lactam antibiotics are a group of antibiotics that are widely used for the treatment of infectious diseases caused by Gram-positive and Gram-negative pathogenic bacteria, but these bacteria have become resistant to most β-lactam antibiotics. Resistance to β-lactams is mainly caused by bacteria producing β-lactamase enzymes that can hydrolyze the β-lactam ring in the structure of penicillin antibiotics so that the drug becomes inactive (Bush and Bradford, 2016).

Methicillin is a penicillin-derived β-lactam group antibiotic that is resistant to β-lactamase hydrolysis, but there are strains of *Staphylococcus aureus* that are resistant to methicillin (Thebti et al., 2023). Resistance to methicillin is caused by changes in the structure of Penicillin-Binding Protein (PBP) to PBP2a which cannot be bound by methicillin, thus taking over the normal function of *Staphylococcus* PBP in cell wall synthesis (Carretto et al., 2018). Ceftobiprole is a β-lactam antibiotic that has been shown to be effective for the treatment of infections caused by MRSA bacteria by inhibiting several transpeptidases of PBP, including PBP2a in MRSA (Morosini, 2019; Giacobbe et al., 2019). PBP2a are essential enzymes involved in the synthesis of cell walls in resistant bacteria. They are the primary targets of β-lactam antibiotics such as penicillins, cephalosporins, and carbapenems. Understanding the interaction between PBPs and antibiotics can provide insights into antibiotic resistance mechanisms and aid in the development of new antibacterial agents. Therefore, the compound ceftobiprole was used as a comparison ligand in antibacterial testing against PBP2a of *S. aureus*.

In order to overcome resistance to antibiotics, research to find compounds derived from plants and potential as antibac-

terials was carried out, one of the plant are *Hibiscus sabdariffa* L. or known as roselle (Alhadrami et al., 2020). The pharmacological activity of various parts of the rosela plant has been reported, one of which is roselle flower calyx as an antibacterial (Hassan et al., 2016). Water extract of roselle flower calyx can inhibit the growth of *S. aureus* and *K. pneumoniae* bacteria with a minimum inhibitory concentration (MIC) value of 0.30 + 0.2 mg/mL (Isnaeni et al., 2020) and shows a 50% inhibitory concentration (IC_{50}) value of 178.62 + 3.63 μg/mL (Jung et al., 2013). Research conducted by Lee et al. (2018) showed that the water extract of roselle calyx inhibited the growth of MRSA with MIC of 112-144 μg/mL.

The aqueous extract of roselle flowers contains several flavonoid compounds and phenolic acids (Da-Costa-Rocha et al., 2014) which are known to inhibit the growth of *S. aureus* bacteria, including: quercetin-3-glycoside and kaempferol-3-glycoside with MIC 0.065 mg/mL (Lade et al., 2022), mirisetin with MIC 0.005 mg/mL (Rashed et al., 2014; Salinas-Moreno et al., 2023) and chlorogenic acid with MIC 40 μg/mL (Abdel-Shafi et al., 2019). In addition, quercetin and protocatechuic acid (5 mg/mL) can inhibit the growth of MRSA strains with MIC of 62.5 mg/mL and 24-44 μg/mL (Lee et al., 2018; Woźnicka et al., 2013).

Flavonoid compounds contain two benzene rings connected by a three-carbon chain that forms a closed heterocyclic pyran ring containing oxygen. The phenolic groups in flavonoid compounds can interact with target molecules in bacterial cells (Kumar and Pandey, 2013; Lobiuc et al., 2023). The flavonoid ring structure consists of a combination of rings A and B connected to ring C, on each ring there is a hydroxy group (OH). OH groups at the 2',4' position (ring C) and at the 5,7 position (rings A-B) in the flavonoid structure play a role in anti-MRSA activity (Kalli et al., 2021).

The aqueous extract of roselle flowers also contains other flavonoid compounds, including: delfinidin, delfinidin-3-glucoside, cyanidin, cyanidin-3-glucoside, quercetin, quercetin-3-glucoside, quercetin-3-rutinoside, mirisetin, mirisetin-3-arabinoside, kaempferol-3-glucoside, and kaempferol-3-rutinoside as well as other phenolic acid compounds, namely protocatechuic acid, chlorogenic acid and gallic acid. These compounds have differences in the number and position of hydroxy groups in the flavonoid ring and the type of sugar bound to position 3 of the flavonoid ring structure (Da-Costa-Rocha et al., 2014).

Although the aqueous extract of roselle flowers showed antibacterial activity against MRSA no one has reported the specific compounds in the aqueous extract of roselle flowers that produced the antibacterial effect. However, several flavonoids including mirisetin, kaempferol glycosides and quercetin and its glycosides showed antibacterial activity against MRSA bacteria. To evaluate which of the compounds in the extract of roselle' calyx have antibacterial activity against MRSA, an in silico test was conducted after the in vitro test of the extract. The antibacterial potency of the compounds contained in the extract against MRSA was tested by molecular docking into the PBP2a from *S. aureus* (PDB: 1MWT). 1MWT is the PDB code for the crystal structure of PBP2a from MRSA that was selected because it contained ligand ceftobiprole, an active inhibitor of PBP2a, and can serve as a reference compound. The parameter used to determine the activity of the compound against the target molecule is free energy (ΔG^0) as a measure of ligand-receptor affinity. The lower the free energy, the higher the affinity of the compounds to the receptor.

2. EXPERIMENTAL SECTION

2.1 Materials

Materials for the antibacterial activity test include ethanol-water extract (dry) of rosela flowers, obtained from one of the pharmaceutical industries. Vancomycin, methanol p.a. (Merck), nutrient agar (Oxoid), NaCl p.a. (Merck), DPPH p.a., distilled water, MRSA 113749 obtained in the form of isolate stock from Institute of Tropical Disease Universitas Airlangga.

The compounds used in molecular docking are flavonoid-derived compounds contained in the water extract of roselle flowers where the structure is shown in Table 1. The docking target protein is PBP2a (PDB: 1MWT) downloaded from Protein Data Bank (www.rcsb.org). The chemical compound structure of flavonoid and test compound can be seen in Figure 1 and Figure 2.

Sofware ChemOffice 19.1, *AutoDockTools* 1.5.7, *Discovery Studio Visualizer*, and computer tools HP-66L8BTFK with *11th Gen Intel (R) Core (TM) i3-1115G4 @3.00 GHz* processor, *2901 Mhz, 2 Core(s), 4 Logical Processor* and *operating system Windows 11 Home 64-bit.*

2.2 Method

2.2.1 Preparation of Nutrient Agar Media

Agar solution was prepared from 28 g nutrient agar powder in 1L distilled water while heating. The media was then filled into several test tubes with part of the volume ±15 mL for the base layer, part of the volume ±10 mL for the seed layer, and agar slant for bacterial rejuvenation. The media was sterilized by autoclaving at 121°C for 15 minutes (Bridson, 2006).

2.2.2 Preparation of MRSA Inoculum

MRSA was taken from isolate stock and cultured in NA medium for 24 hours at 37°C. The culture results were made into suspension with sterile 0.9% NaCl solution. The transmittance of the suspension was measured using a UV-Vis spectrophotometer at a wavelength of 580 nm using a blank 0.9% NaCl solution (diluted if necessary) until a transmittance of 25% was obtained.

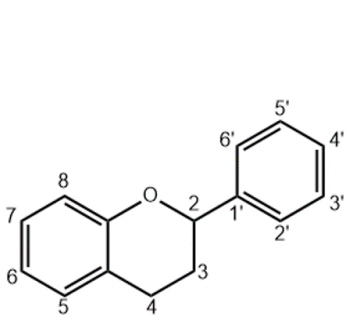
2.2.3 Preparation of Test Solution

A 10% solution of 1 gram roselle flower extract was prepared and diluted in distilled water, then serial dilutions were made to obtain test solutions with concentrations of 5%; 2.5%; 1.25%; and 6.25%.

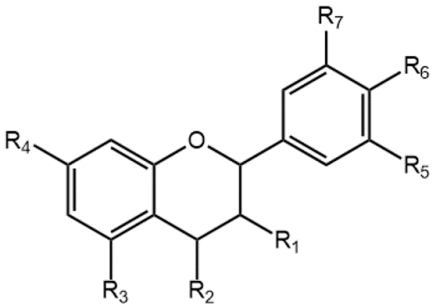
2.2.4 MIC Determination

NA media as much as ± 15 mL (45 - 50°C) was poured into a petri dish aseptically and allowed to stand until solid (base

Table 1. Structure of Flavonoid Compounds Contained in Rosella Flower Extracts

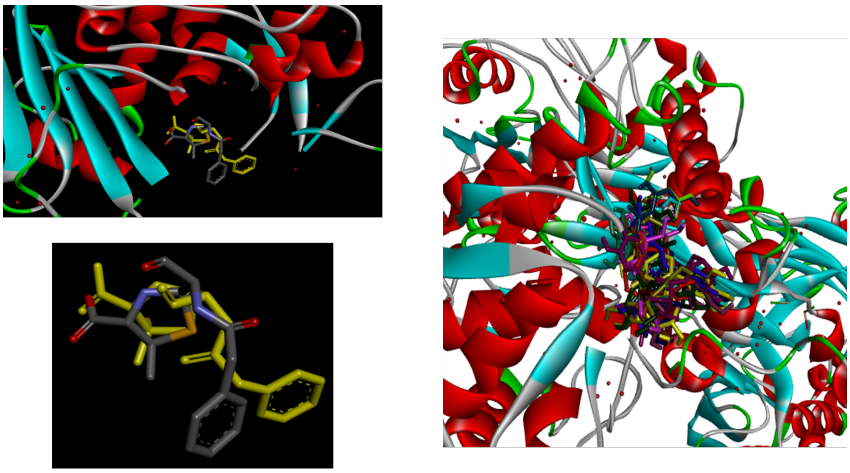


Flavonoid scaffold



Test Compound

Compound	R1	R2	R3	R4	R7	R6	R5
Dephinidine	-OH	-	-OH	-OH	-OH	-OH	-OH
Dephinidine-3-glucoside	-glucoside	-	-OH	-OH	-OH	-OH	-OH
Cyanidin	-OH	-	-OH	-OH	-	-OH	-OH
Cyanidin-3-glocoside	-glucoside	-	-OH	-OH	-	-OH	-OH
Quercetin	-OH	=O	-OH	-OH	-	-OH	-OH
Quercetin-3-glucoside	-glucoside	=O	-OH	-OH	-	-OH	-OH
Quercetin-3-rutinoside	-rutinoside	=O	-OH	-OH	-	-OH	-OH
Myricetin	-OH	=O	-OH	-OH	-OH	-OH	-OH
Myricetin-3-arabinoside	-arabinoside	=O	-OH	-OH	-OH	-OH	-OH
Kaempferol-3-glucoside	-glucoside	=O	-OH	-OH	-	-OH	-
Kaempferol-3-rutinoside	-rutinoside	=O	-OH	-OH	-	-OH	-



a b

Figure 1. 3D-Visualization of the Original Ligand, Penicillin G, Showing Overlap of the β-lactam Ring Before and After Docking (a); Conformational Pose of Test Ligands Post-Docking at the PBP2a Binding Site (b)

layer). Then, 8 μL of MRSA inoculum was taken and included in ± 10 mL of nutrient agar media (45 - 50°C), shaken with a vortex then poured into the base layer and allowed to solidify (seed layer). Holes were made on the test media using a sterile hole puncher. Into each hole was inserted a sample of the test solution and reference drug (vancomycin) as a positive control. Then the media was incubated at 37°C for ±24 hours. The diameter of the inhibition zone formed was observed using a caliper. The smallest concentration that can still inhibit the growth of test bacteria is designated as MIC.

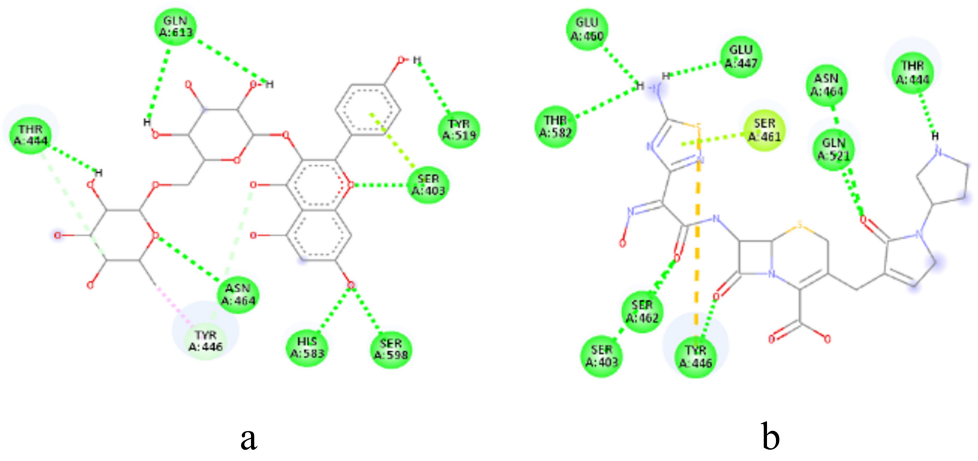


Figure 2. 2D Interaction of Top Score Ligand with Amino Acids in PBP2a (a) Compared with Cephobiprole (b). = Hydrogen Bond; =Pi-Sulfur; = Pi-Lone Pair

2.2.5 Protein Preparation for Docking Study

The macromolecular structure of PBP2a protein (PDB. 1MWT) was downloaded from protein data bank, then processed with AutoDockTools 1.5.7 program. Water molecules and ligand of origin were removed from the protein molecule, then polar hydrogen was added and Kollman Charges were applied. The finished protein was saved in *.pdbqt format.

2.2.6 Ligand Preparation

The two-dimensional structures of the compounds as test ligands were drawn with the ChemDraw 19.1 program, then converted into three-dimensional structures with Chem3D 19.1. The most stable conformation was sought by minimizing the energy using MMFF94, then the structure was saved as *.mol2. PBP2a protein structure (PDB. 1MWT) in PDB format was opened with AutoDockTools 1.5.7 application. Water molecules and all proteins were removed, except for the native ligand, then a hydrogen atom was added to the native ligand molecule and given a Gasteiger charge. The structure of the native ligand was saved in *.pdbqt format.

2.2.7 Docking Procedure

Before docking the test ligand molecule, the docking procedure was validated by re-docking the original ligand to the protein. The docking result in Autodock is the free energy of ligand-protein complex (ΔG). If ΔG has RMSD (Root Mean Square Deviation) value < 2 Å then the method is valid and can be used for docking. Docking of test ligands was performed with the same procedure as the validated ligand of origin.

The autodock4 and autogrid4 programs were prepared in a working folder. The docking process begins by entering the prepared protein and ligand (*.pdbqt) in the Autodock workspace, then the Grid box is set to cover the binding site and test ligand, then the Grid parameters are saved in *.gpf format file. After that, the docking parameters are set with Genetic Algorithm parameters, then stored in the form of Lamarckian GA with *.dpf format. Furthermore, the docking simulation is performed using the Run AutoDock command. The docking result is contained in *.dlg file. Visualization of docking results was done with Discovery Studio Visualizer program. In this study, the molecular docking was carried out in triplicates and the ΔG presented is the average value. The ΔG of 11 compounds have been tested using ANOVA and significant differences were obtained.

3. RESULTS AND DISCUSSIONS

3.1 Antibacterial Activity

Antibacterial activity evaluation of roselle calyx extract and vancomycin against MRSA can be seen in the Table 2. The results showed that there was a zone of inhibition form extract at the concentrations of 10%; 5%; 2.5% and the MIC value of roselle extract was 2.5%. According to literature, to inhibit the bacterial growth of MRSA, roselle aqueous extract was used at a MIC 3% (Isnaeni et al., 2020).

Table 2. Antibacterial Activity of Roselle Flower Extract

Extract and Standard Concentration	Inhibition Zone (mm) Replication I	II	III	Average
10 %	16.03	16.23	16.40	16.22 ± 0.18
5 %	14.20	15.57	15.10	14.95 ± 0.70
2.5 %	10.10	10.10	10.03	10.08 ± 0.04
1.25 %	-	-	-	-
0.625 %	-	-	-	-
Vancomycin 50 ppm	16.73	16.50	15.63	16.29 ± 0.47

Table 3. Physicochemical Properties of Flavonoid Compounds Contained in Rosella Flower Extracts

Compound	MW	LogP	HBA	HBD	CMR	pKa	tPSA	LogS	E_{total}
Delphinidine ($C_{15}H_{11}O_7^+$)	303.25	2.61	6	6	7.47	0.28	132.68	-0.53	41.13
Dephinidine-3-glucoside ($C_{21}H_{21}O_{12}^+$)	465.10	0.09	11	9	10.84	-0.90	211.83	-0.13	146.00
Cyanidin ($C_{15}H_{11}O_6^+$)	287.25	2.91	5	5	7.32	0.29	112.45	-0.62	40.12
Cyanidin-3-glocoside ($C_{21}H_{21}O_{11}^+$)	449.39	0.38	10	8	10.69	-0.90	191.60	-0.23	146.01
Quercetin ($C_{15}H_{10}O_7$)	302.24	0.35	7	5	7.44	6.87	127.45	-3.15	37.49
Quercetin-3-glucoside ($C_{21}H_{20}O_{12}$)	464.10	-1.39	12	8	10.81	6.64	206.60	-2.48	160.46
Quercetin-3-rutinoside ($C_{27}H_{30}O_{16}$)	610.52	-2.28	16	10	14.03	6.63	265.52	-2.33	237.74
Myricetin ($C_{15}H_{10}O_8$)	318.24	-0.04	8	6	7.59	6.86	147.68	-2.47	35.03
Myricetin-3-arabinoside ($C_{20}H_{18}O_{12}$)	450.35	-1.24	12	8	10.35	6.64	206.60	-1.82	133.83
Kaempferol-3-glucoside ($C_{21}H_{20}O_{11}$)	448.38	-1.00	11	7	10.66	6.65	186.37	-2.89	147.23
Kaempferol-3-rutinoside ($C_{27}H_{30}O_{15}$)	594.52	-1.89	15	9	13.88	6.64	245.29	-2.73	240.39

Table 4. Docking Results of Flavonoid Compounds in Rosella Flowers on PBP2a

Compound	ΔG (kcal/mol)	Σ H-bond	Interacting of Amino Acid
Dephinidine	-6,23	5	Ser461; Gln521; Ser403; Thr600; Gly640
Dephinidine-3-glucoside	-6,54	7	Gln613; Gln521; Glu602; Ser462; Ser403; Ser598; His583
Cyanidin	-6,38	4	Glu602; Thr600; Lys597; Ser598
Cyanidin-3-glocoside	-6,68	4	Thr600; Tyr519; Asn464; Gln521
Quercetin	-6,44	3	Asn464; Ser461; Gly640
Quercetin-3-glucoside	-6,95	8	Gln613; Glu602; Gln521; Asn464; Ser403; Ser462; His583; Ser598
Quercetin-3-rutinoside	-6,24	7	Gln613; Glu602; Gly520; Tyr446; Ser462; Ser461; Asn464
Myricetin	-6,32	6	Ser461; Gln521; Ser403; Thr600; Gly640; Asn464
Myricetin-3-arabinoside	-6,74	7	Gln613; Thr444; Gln521; Glu602; Asn464; Ser462; Ser598
Kaempferol-3-glucoside	-6,81	6	Gln613; Glu602; Gln521; Asn464; Ser403; His583
Kaempferol-3-rutinoside	-7,33	7	Thr444; Gln613; Tyr519; Ser403; Ser598; His583; Asn464
Ceftobiprole (Reference ligand)	-8,74	9	Thr444; Asn464; Gln521; Glu 447; Glu460; Thr582; Ser462; Ser403; His583; Tyr446

Based on the classification of the zone of inhibition of bacterial growth, the MIC value of 2.5% which provides a 10 mm zone of inhibition is classified as potentially strong (Kowalska-Krochmal and Dudek-Wicher, 2021). The proven antibacterial activity of roselle calyx extract against MRSA, it will then be investigated what compounds in it are potential as antibacterials. The physicochemical properties of 11 bioactive compounds contained in roselle calyx extract are shown in Table 3.

According to Lipinski's rule of five, requires that compounds will be easily absorbed and have high permeability if the molecular weight is less than 500 g/mol, the partition coefficient (log P) is less than 5, the number of hydrogen bond acceptors is less than 10 and the number of hydrogen bond donors is less than 5 (Lipinski et al., 2012). Based on the data in Table 3, the test compounds that fulfill Lipinski's five rules include quercetin, cyanidin, protocatechuic acid and gallic acid.

3.2 Docking Study

The docking result in Autodock is free energy of ligand-protein complex (ΔG). The docking procedure was validated by re-docking the original ligand to the protein. Docking of ori-

gin ligand Penicillin G on PBP2a (PDB: 1MWT) resulted in average ΔG with RMSD (Root Mean Square Deviation) of 1.96±0.03 Å from triplicates. If RMSD value < 2 Å then the procedure was valid and can be used for docking simulation (Castro-Alvarez et al., 2017). Visualization of the overlay of the position of the ligand of origin before and after redocking and the 3D interaction of the test ligand in the PBP2a binding site can be seen in Figure 1.

The free energies of ligand-protein complexes at the PBP2a binding site are shown in Table 4. All the test ligands have higher ΔG° values than the comparator ligand ceftobiprole, which indicates that the test ligands have lower affinity. The ΔG° values of glycoside-derived compounds were lower than those of aglycone compounds, whereas quercetin and myricetin and their glycosides had lower ΔG° than delphinidin and cyanidin and their glycosides. The interaction of compounds that have the highest affinity to PBP2a is shown in Figure 2.

Based on the data of ΔG° and the hydrogen bonds formed in the interaction with PBP2a, the affinity of the test ligand is lower than that of the reference ligand because the formation of hydrogen bonds by the test ligand is less. Based on the analysis of the relationship between physicochemical properties and free energy of PBP2a ligand-protein complexes, the following relationship model was obtained:

$$\Delta G^0 = -0.192\text{CMR} - 4.674 \quad (p = 0.000; F = 44.009; R = 0.886) \tag{1}$$

$$\Delta G^0 = -0.421\text{CMR} + 0.009\text{E}_{total} - 3.472 \quad (p = 0.000; F = 36.006; R = 0.931) \tag{2}$$

$$\Delta G^0 = -0.503\text{CMR} + 0.008\text{E}_{total} + 0.157\text{HBD} - 3.718 \quad (p = 0.000; F = 25.686; R = 0.941) \tag{3}$$

Based on the equation obtained, it appears that ligand molecular size (CMR) and ligand molecular energy before docking have a contribution in the interaction between ligand and PBP2a. The larger the ligand molecular size will increase the stability of the ligand-protein complex, as indicated by the lower ΔG° value of kaempferol-rutinoside compound. It is likely that a large enough molecular size is needed to better fit the space in the protein binding site.

4. CONCLUSIONS

Based on the research conducted, kaempferol-rutinoside was the most potential compound from *H. sabdariffa* that could be selected as lead compound to be develop as antibacterial agents against MRSA. Flavonoid compounds with larger molecular size and higher total energy would increase their interaction with PBP2a.

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