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In Silico Approach of Flavonol in *Hibiscus sabdariffa* as Proteasome Inhibitors Targeting the Ubiquitin-Proteasome Pathway

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Abstract: Multiple myeloma is a blood cancer characterized by the abnormal proliferation of B cells that accumulate in the bone marrow. The proliferation of these cells depends on the role of the proteasome through the ubiquitin-proteasome pathway to degrade proteins that regulate the cell cycle, apoptosis, and stress response. The proteasome is the main target of proteasome inhibitors for the treatment of multiple myeloma. Flavonoid compounds in *Hibiscus sabdariffa* flowers, such as isoquercitrin, quercitrin, quercetagetin, isorhamnetin, and astragalin, have the potential to act as proteasome inhibitors. The insilico study aims to determine the potential of these compounds as proteasome inhibitors based on the results of molecular docking and molecular dynamics simulation. Molecular docking was performed using AutoDock Vina, PyMol, and BIOVIA Discovery Studio. The best compounds from the docking results were then tested for interaction stability through molecular dynamics using YASARA software. Quercetagetin, isoquercitrin, and isohamnetin, based on the results of molecular docking, have the lowest binding affinity of -6.0, -5.7, and -5.5 kcal/mol, respectively. The three compounds bind to the active site of the 20S proteasome, namely Thr1, Ala20, Thr21, and Ala49. The RMSD values in the molecular dynamics of quercetagetin (1.290 Å), isorhamnetin (1.839 Å), and carfilzomib (1.843 Å). The binding affinity of quercetagetin (-422.623 kJ/mol), isorhamnetin (-438.388 kJ/mol), and carfilzomib (664.956 kJ/mol). Molecular docking and molecular dynamics findings indicate that isorhamnetin binds to the amino acid residues Thr1 and Ala49, which are the active sites of the 20S proteasome.

Keywords: flavonol, proteasome inhibitor, molecular docking, molecular dynamic, *H. sabdariffa*

Abstrak: *Multiple myeloma adalah kanker darah yang ditandai dengan proliferasi sel B abnormal yang terakumulasi di sumsum tulang. Proliferasi sel ini bergantung pada peran proteasome melalui Ubiquitin-Proteasome Pathway untuk mendegradasi protein yang mengatur siklus sel, apoptosis, dan respon terhadap stres. Proteasome adalah target utama proteasome inhibitor untuk pengobatan multiple myeloma. Senyawa flavonoid pada bunga H. sabdariffa seperti isoquercitrin, quercitrin, quercetagetin, isorhamnetin, dan astragalin memiliki potensi sebagai inhibitor proteasome. Studi insilico bertujuan untuk megetahui potensi senyawa tersebut sebagai inhibitor proteasome berdasarkan hasil molecular docking dan molecular dynamic. Molecular docking dilakukan dengan menggunakan AutoDock Vina, PyMol, dan BIOVIA Discovery Studio. Senyawa terbaik hasil docking kemudian diuji kestabilan interaksinya melalui molecular dynamic menggunakan software YASARA. Quercetagetin, isoquercitrin, dan isohamnetin berdasarkan hasil molecular docking memiliki energi ikatan terendah sebesar -6,0, -5,7 dan -5,5 kkal/mol. Ketiga senyawa mengikat active site proteasome 20S, yaitu Thr1, Ala20, Thr21, dan Ala49. Nilai RMSD pada molecular dynamic quercetagetin (1,290 Å), isorhamnetin (1,839 Å), dan carfilzomib (1,843 Å). Nilai binding affinity quercetagetin (-422,623 kJ/mol), isorhamnetin (-438,388 kJ/mol), dan carfilzomib (664,956 kJ/mol). Berdasarkan molecular docking dan molecular dynamic, menunjukkan bahwa isorhamnetin mengikat residu asam amino Thr1 dan Ala49, yang merupakan situs aktif proteasom 20S.*

Kata kunci: *flavonol, inhibitor proteasome, molecular docking, molecular dynamic, H. sabdariffa*

INTRODUCTION

Multiple myeloma is a blood cancer characterized by the accumulation of abnormal B cells in the bone marrow and paraprotein in the serum or urine. This disease is still considered incurable due to the low survival rate and quality of life of patients (Wijaya *et al*. 2019). One of the strategies in treating this disease is to inhibit the activity of proteins involved in tumorigenesis and the survival of cancer cells. The proteasome's involvement in the proteolysis process, which is primarily facilitated by the ubiquitin-proteasome pathway, is a critical factor in the

survival of malignant cells in multiple myeloma. In multiple myeloma, cancer cells have a very high rate of protein synthesis, especially in the production of paraproteins, which leads to the accumulation of unfolded proteins. Furthermore, multiple myeloma cells frequently undergo elevated endoplasmic reticulum stress because of their excessive protein production. Consequently, the inhibition of proteasome function in multiple myeloma cells has a substantial impact, as these cells are more susceptible to proteasome function disruption than normal cells (Soave *et al*. 2017).

Proteasome inhibitors, such as bortezomib and carfilzomib, bind to the β5 subunit (chymotrypsin-like) on the 20S proteasome, thereby inducing apoptosis in multiple myeloma (Schenkein 2002; Soave *et al*. 2017; Fricker 2020; Mir *et al*. 2023; Yadav *et al*. 2023). Proteasome inhibition can affect several pathways related to cancer cell development, one of which is the NF-κB pathway. NF-κB is usually found in an inactive state in the cytoplasm due to the presence of IκB, which is an endogenous NF-κB inhibitor protein. Constitutive NF-κB activity in multiple myeloma can lead to increased cancer cell proliferation and resistance to apoptosis. This pathway is initiated upon the proteasomal degradation of polyubiquitinated IκB, leading to the activation of the NF-κB heterodimer transcription factor p50/p65, which then translocates from the cytosol to the nucleus, potentially affecting the transcription of various genes. Therefore, proteasome inhibition can prevent the degradation of IκB, causing the transcription factor p50/p65 to remain in the cytosol and preventing the activation of this pathway (Nunes & Annunziata 2017; Fricker 2020; Jayaweera *et al*. 2021). However, the utilization of these two medications is constrained by the emergence of resistance over time, resulting in illness recurrence. Thus, research on proteasome inhibitors continues, including the exploration of natural compounds.

Flavonoids are natural compounds that have been thoroughly investigated for their potential as proteasome inhibitors, particularly the flavonol variants (Soave *et al*. 2017). *H. sabdariffa* is a plant abundant in flavonols. The flavonol compounds in the flowers of this plant include isoquercitrin, quercitrin, quercetagetin, isorhamnetin, and astragalin (McKay 2009; Alara *et al*. 2020). Isoquercetin has an IC_{50} of 45.7864 µM against T24 cells and quercitrin shows an IC_{50} value of 12.26 µg/mL against PC3 cells (references). Certain chemicals have been investigated for their efficacy against various cancer cells; however, no research has been conducted to assess their potential as proteasome inhibitors. This research may provide a basis for in vitro and in vivo studies and act as a way to mitigate the rising incidence of cancer globally. This in silico study aims to examine the interaction of flavonol compounds from *H. sabdariffa* with the proteasome in multiple myeloma using molecular docking and molecular dynamics methods.

MATERIALS AND METHOD

Preparation for ligand and receptor

The structure of the compounds includes isoquercitrin (CID 5484006), quercitrin (CID 5280459), quercetagetin (CID 5281680), isorhamnetin (CID 5281654), astragalin(CID 5282102), and the 20S proteasome protein. The construction for the three-dimensional structure of the ligands, including the test compounds and carfilzomib (CID 11556711) as the control compound, was conducted by entering the compound names on the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) and converting them into 2D structures using the ChemDraw 12.0 application. Subsequently, within the same application, the structure was converted into 3D and geometric optimization was executed. The optimized file is saved in .pdbqt format (Nusantoro & Fadlan, 2020). The 20S proteasome structure complexed with the native ligand Dihydroeponemycin (PDB ID: 5Lf1) at a resolution of 2.00 Å was obtained from the Protein Data Bank (http://www.rcsb.org/pdb). The protein file was opened using Autodock Tools 1.5.7, separating it from water molecules including ions and removing all chains except for chain K (β5) (Naufa *et al*. 2022).

Pharmacokinetic and toxicity prediction

Pharmacokinetic test was conducted using the SwissADME website (http://www.swissadme.ch/) by entering the SMILES notation of the test compounds and carfilzomib. This test produces molecular weight, logP partition coefficient, quantity of hydrogen bond donors, and quantity of hydrogen bond acceptors. Toxicity prediction using pkCSM *webtools* (http://biosig.unimelb.edu.au/pkcsm/) (Bojarska *et al*. 2020).

Molecular docking

The validation of the molecular docking method was performed by redocking using the AutoDock Tools 1.5.7 and PyMOL software. The grid box is arranged by setting the size of the grid's center coordinates and the grid size. The validation parameter for this method is the Root Mean Square Deviation (RMSD) value, where a good RMSD value is considered defined as ≤ 2Å. The molecular docking process used Autodock tools 1.5.7 software. Isoquercitrin, quercitrin, quercetagetin, isorhamnetin, astragalin, and niraparib that had been optimized were then docked to the target protein PARP-1. The docking process was carried out using Autodock Vina with the same grid box size as the validation step. The docking results produced binding affinity values, and the binding complex was visualized using Discovery Studio software (Naufa *et al*. 2022).

Molecular dynamic

Molecular dynamics simulation using YASARA software. The PDB files of the best protein-ligand complex from the molecular docking results and the protein-carfizomib complex were entered into the YASARA program. Molecular dynamics simulation was conducted at a physiological temperature setting of 310°K or 37°C and a physiological pH of 7.4. The procedure was executed for 15 ns, with a snapshot taken every 100 ps (Istyastono 2020). Studi Guterres & Im (2020) using simulations at 10–50 ns was effective in distinguishing active and decoy ligands in 56 target proteins and 280 compounds from the DUD-E dataset. The data obtained includes RMSD, RMSF, and binding affinity values.

RESULT AND DISSCUSION

Pharmacokinetic and toxicity prediction

Bioavailability is a parameter of the extent and rate at which an orally administered is absorbed into the systemic sirculation. The rate of drug absorption affects the effectiveness of the drug reaching the receptors. This study determined drug bioavailability using swissADME, analyzing parameters according to Lipinski's rule of five. This method assesses the probability of formulating the drug into an oral dosage form through physicochemical analysis (Aini *et al*. 2024). The test involves the analysis of molecular weight, hydrogen bond donors and acceptors, and log P. The test compounds fulfill the criteria by possessing a molecular weight under 500 g/mol, fewer than 5 hydrogen bond donors, fewer than 10 hydrogen bond acceptors, and a Log P of less than 5, with a maximum of one violation (Lipinski *et al*. 1997; Khan *et al*. 2020). Table 1 presents the results of the Lipinski test conducted on the test compounds and carfilzomib. The test results indicate that carfilzomib, quercetagetin, and isorhamnetin comply with Lipinski's rule of five, encompassing molecular weight, LogP, the count of H-bond donors, and the count of H-bond acceptors. Isoquercitrin, quercitrin, and astragalin exhibit multiple parameters that fail to comply with Lipinski's rule, thereby increasing the likelihood of poor absorption or permeation for these compounds.

Drugs are absorbed when they successfully through the cell membrane to reach the receptors. Cell membranes exhibit amphipathic properties, characterized by their dual affinity for both hydrophilic and hydrophobic environments. The capacity of a compound to traverse the cell membrane is influenced by its molecular weight and topological polar surface area (TPSA). High molecular weight and TPSA present challenges for cell membrane penetration, as they necessitate extended durations for drug absorption and distribution. TPSA <140 Å defines permeability in the cell plasma membrane (Bojarska *et al*. 2020; Seelig 2020). Daina (2017) indicates that a LogS value of less than -6 categorizes the solubility level as soluble (INSOLU) (Table 2). Non-ionic organic compounds readily engage in hydrogen bonding with water, resulting in modifications to water's properties, including a reduction in freezing point, an elevation in boiling point, and an increase in the osmotic pressure of the solution. Quercetagetin contains a carbonyl functional group (C=O) and a hydroxyl group (O). Water readily forms bonds with ions or non-ionic polar compounds via -OH, -NH, -SH, and -C=O groups, as well as with unpaired electrons on O and N atoms (Aini *et al*. 2024). Consequently, the solubility of quercetagetin exceeds that of isorhamnetin, despite its greater molecular weight and TPSA value.

The lipophilicity of a chemical is indicated by the clogP value; a higher number signifies more lipophilicity and hence enhanced solubility in lipids. A number exceeding 5 prolongs the compound's presence in the lipid bilayer membrane and facilitates its extensive distribution throughout the body, hence diminishing binding selectivity to the target protein (La Kilo *et al*. 2019; Komarudin *et al*. 2021). Carfilzomib lacks a -OH group, resulting in a greater clogP value relative to comparable drugs. Carfilzomib has significant lipophilicity attributed to the presence of the C=O group and oxygen atoms that affect hydrophobic interactions, while isorhamnetin and quercetagetin possess several -OH groups. Robust hydrogen bonds between the C=O groups are established in the absence of water. Small molecules can penetrate the lipid membrane by shedding their water shell, subsequently dissolving in the hydrocarbons in the middle of the membrane. This causes the molecules to diffuse through to the other side of the membrane and be re-encapsulated by water molecules (Aini *et al*. 2024). The quantity of

Table 1. SwissADME screening results

Compound	BM	cLogP	cLogS	TPSA (Å²)	NORTB	HBA	HBD	%ABS
	≤500 Da	≤5		<140	9	<10	<5	
Carfilzomib	719.91	3.59	Moderate	158.47	24	8	4	54.33
Isoquercitrin	464.38	-0.48	Soluble	210.51	4	12	8	36.37
Quercitrin	448.38	0.22	Soluble	190.28	3	11	7	43.35
Quercetagetin	318.24	0.92	Soluble	151.59	1	8	6	56.70
Isorhamnetin	316.26	1.65	Soluble	120.36	2	7	4	67.48
Astragalin	448.38	-0.09	Soluble	190.28	4	11	7	43.35

Table 2. Bioavailability radar results

Compound	LIPO	FLEX	INSATU	INSOLU ($Å^2$)	POLAR	SIZE (g/mol)
Carfilzomib	4.69	24	0.57	-5.84	158.47	719.91
Isoquercitrin	0.36	4	0.29	-3.04	210.51	464.38
Quercitrin	0.86	3	0.29	-3.33	190.28	448.38
Quercetagetin	1.18	1	0.00	-3.01	151.59	318.24
Isorhamnetin	1.87	2	0.06	-3.36	120.36	316.26
Astragalin	0.72	4	0.29	-3.18	190.28	448.38

hydrogen donors and acceptors influences the absorption of compounds penetrate the cell lipid bilayer; an excessive value necessitates greater energy for absorption, as compounds with numerous hydrogen donors and acceptors are generally more polar (La Kilo *et al*. 2019; Komarudin *et al*. 2021). Hydrogen donors and acceptors enhance drug affinity to the membrane through electrostatic interactions, thereby attracting the drug in the lipid environment and releasing it immediately in the aqueous phase (Aini *et al*. 2024).

The compound's bioavailability is analyzed using the swissADME radar plot. This study aims to evaluate the drug-likeness of a molecule efficiently and comprehensively, particularly in relation to its potential oral bioavailability. The radar exhibits a hexagonal configuration, characterized by six axes extending from the central point. Each axis represents a specific physicochemical parameter for oral bioavailability. The pink area delineates the optimal value range for each parameter, ensuring a favorable probability of oral bioavailability for the molecule. Each axis of the bioavailability radar represents a specific physicochemical parameter, with an optimal value range established through drug data analysis. The parameters consist of lipophilicity (XLOGP3), size (molecular weight), polarity (TPSA), solubility (Log S), saturation (fraction Csp3), and flexibility (number of rotatable bonds) as presented in Table 2. The optimal range for XLOGP3 between -0.7 and +5.0; excessive lipophilicity may result in inadequate water solubility and possible toxicity or accumulation in adipose tissues. The compound exhibits favorable permeability, characterized by a molecular weight of less than 500 g/mol. Larger molecules may exhibit reduced diffusion and absorption efficiency. The optimal range for TPSA is 20 to 140 $Å^2$. Excessive polarity may impede passive absorption across the lipid membrane. A log S value exceeding -4 is typically regarded as favorable for solubility. Saturation indicates the complexity of the molecular structure, with an optimal range of ≥ 0.25. Molecules exhibiting a low Csp3 fraction are typically flat and aromatic, which can affect ADME properties. The optimal flexibility range is ≤ 9; excessive flexibility can lead to unfavorable conformations during interactions with biological targets and can influence membrane permeability.

Based on the graph (Figure 1) and bioavailability radar data, carfilzomib is not optimal on the flexibility axis. The compounds isoquercitrin, quercitrin, and astragalin are not optimal on the polar surface area axis of the compounds. Whereas quercetagenin and isorhamnetin are not optimal on the unsaturation axis. TPSA is influenced by the presence of polar atoms, especially O, N, and H, while unsaturation is influenced by the Csp3 fraction. Boiled-egg Prediction (Brain or Intestinal Estimated) aims to predict Human Intestinal Absorption (HIA) and Blood-Brain Barrier (BBB) based on WLOGP and TPSA values. The white area indicates the possibility of passive absorption by the digestive tract, and the yellow area indicates the possibility of compounds being absorbed by the blood-brain barrier. The analysis results present a graphical output with PGP substrate predictions. PGP plays a role in the transport of chemicals and xenobiotics across cell membranes using ATP as a driving force. PGP is expressed in intestinal epithelial cells, brain capillary endothelial cells, renal tubular epithelial cells, and hepatocytes, which limit drug membrane permeability (Aini *et al*. 2024). PGP is classified into two categories: predicted substrates (PGP+) and non-substrates (PGP-). PGP has implications for the absorption, distribution, metabolism, excretion, and toxicity of drugs, which can clinically alter efficacy or cause various adverse side effects due to drug interactions (Chen *et al*. 2018). The analysis results of boiled-egg prediction (Figure 2) show that the test compound and carfilzomib are in the white area, meaning these compounds can be well absorbed by the digestive system and cannot be absorbed by the blood-brain barrier. Carfilzomib has a blue dot (PGP+) that identifies the substrate as an active efflux, whereas the test compound is marked with a red dot (PGP-) that identifies it as a non-substrate. Non-substrate PGP has the potential to be an anticancer agent, and its overexpression in several tumor cells causes cancer that is resistant to many drugs (Daina *et al*. 2017).

pkCSM AMES refers to the AMES test, which is an in vitro test used to assess the mutagenic potential of a chemical compound using bacteria. A positive result indicates that the drug compound is mutagenic and can act as a carcinogen (Fakhruri & Rahmayanti 2021). The results of the pkCSM AMES prediction

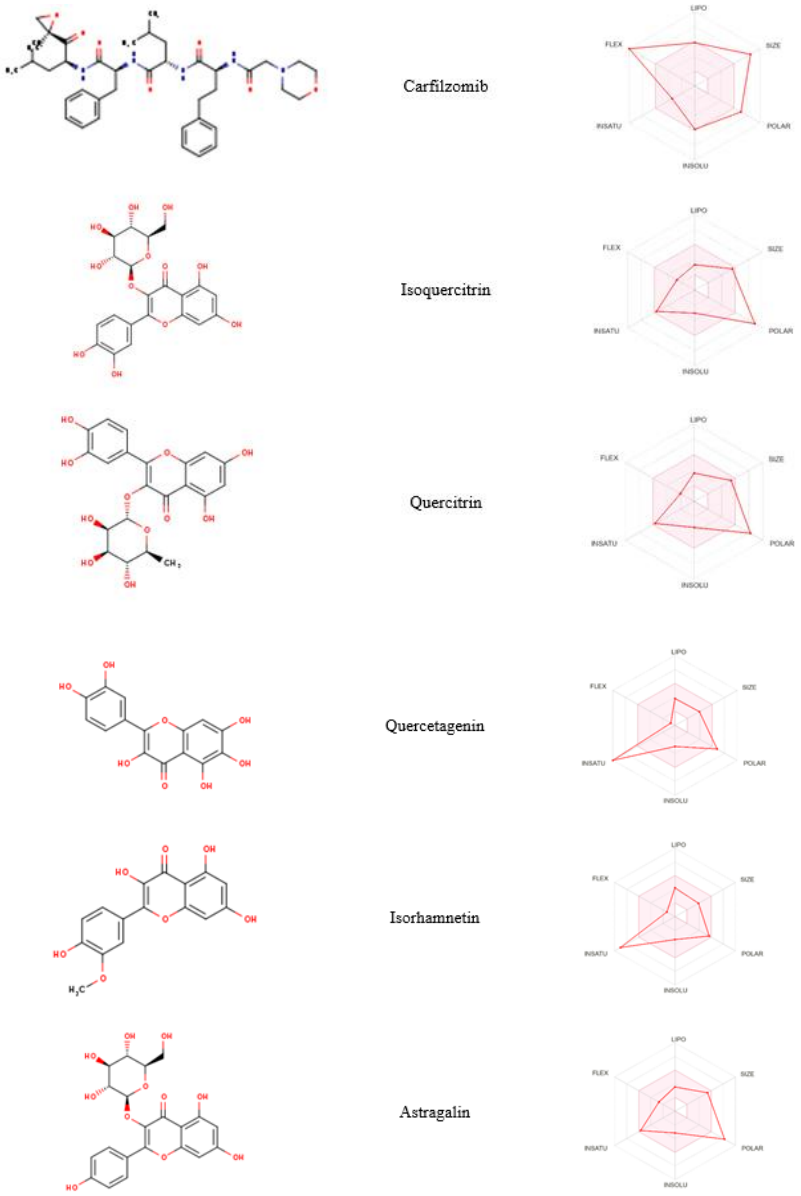

Figure 1. Bioavailability radar chart

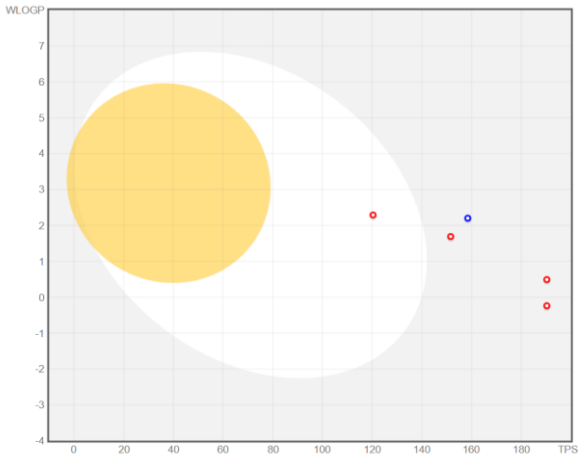

Figure 2. Boiled-egg prediction

(Table 3) show that MTD astragalin has the highest value compared to other compounds. Carfilzomib hERG I and hERG II inhibition, indicating it is inhibitory or toxic. hERG toxicity predicts cardiotoxicity involving the binding of compounds to cardiac potassium channels encoded by the hERG gene (Lee *et al*. 2019). The hERG channel regulates the heartbeat; if the channel is inhibited by a drug compound, it can cause loss of consciousness or increase the risk of sudden death (Kesuma *et al*. 2018). The prediction of the LD50 toxicity test shows that isorhamnetin has the lowest dose value, which is 2.407 mol/kg among other compounds. LD50 determines the dose of a substance that is statistically expected to kill 50% of test animals (Lee *et al*. 2019). A lower LD50 dose result indicates a higher toxicity value. The test chemical is predicted to be non-hepatotoxic, causing no damage to liver organelles or metabolic disruption, whereas carfilzomib yields contrary results. The hepatotoxicity of liver organelles induces alterations in processes and morphology that impact biochemistry. The liver is the body's main detoxification center, transforming toxic compounds and xenobiotics through Phase I and II biotransformation into less harmful and more easily excretable forms. Excessive exposure to these substances can cause various types of liver damage, ranging from inflammation and steatosis to fibrosis, cirrhosis, and even cancer (Lee *et al*. 2019).

Molecular docking

Molecular docking targets the 20S proteasome (PDB: 5LF1) as the target protein with the native ligand dihydroeponemycin. The 20S proteasome protein complex has 14 chains with the β5 subunit located on chain K, which is the main target of proteasome inhibitors (Mir *et al*. 2023). In this study, docking was performed using a flexible-ligand approach where the ligand or test compound is flexible, while the protein is rigid (Nusantoro & Fadlan 2020). The preparation results obtained were in the form of the protein structure and native ligand (Figure 3). This study used a spacing of 1,000 Å with a grid box size of (8 × 14 × 8) Å, which has center coordinates x = 7.441; y = -197.078; and z = 38.499. The best binding mode from the docking validation compared to the native ligand position at the initial condition shows an RMSD value of 1.808 Å. The smaller the RMSD, the better the prediction of the ligand's position, as it converges towards the original position prior to validation. This approach is considered valid as the RMSD is less than 2 Å, permitting the molecular docking of the test molecule to proceed under identical parameters (Nusantoro & Fadlan 2020; Naufa *et al*. 2022; Yuliana *et al*. 2023).

The molecular docking results (Table 4) show that the binding affinity values of the test compounds are lower compared to carfilzomib (-3.9 kcal/mol). The order of binding affinity of the test compounds

Table 3. Toxicity prediction results

Compound	AMES	MTD (log mg/kg/day)	hERG I	hERG II	LD 50 (mol/kg)	Hepatotoxicity
Carfilzomib	No	-0.392	No	Yes	2.553	Yes
Isoquercitrin	No	0.569	No	Yes	2.541	No
Quercitrin	No	0.495	No	No	2.586	No
Quercetagetin	No	0.486	No	No	2.537	No
Isorhamnetin	No	0.576	No	No	2.407	No
Astragalin	No	0.582	No	No	2.546	No

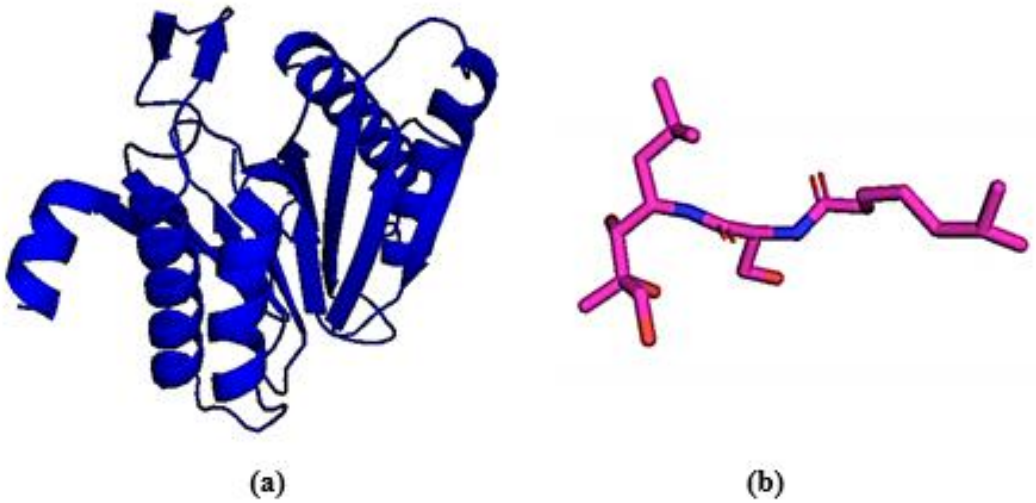

Figure 3. Proteasome (PDB: 5LF1) (a) and native ligand (*dihydroeponemycin)* (b)

Table 4. Results of molecular docking of the 20S proteasome with the compounds quercetagetin, isoquercitrin, isorhamnetin, quercitrin, astragalin, and carfilzomib

Compound	Binding affinity (kkal/mol)	Interactions	Amino acid residues
Quercetagetin	-6.0	Hydrogen	**Thr1**
			Thr21
			Ala49
			Thr21
		Hidrofobic	**Ala20**
			Ala49
			Ala50
Isoquercitrin	-5.7	Hydrogen	**Thr21**
			Gly47
			Thr1
			Thr21
			Gly47
		Hidrofobic	**Ala20**
			Ala22
			Ala27
			Val31
			Ala49
Isorhamnetin	-5.5	Hydrogen	**Thr1**
			Thr1
			Thr21
		Hidrofobic	**Ala20**
			Ala49
Quercitrin	-5.2	Hydrogen	**Thr1**
			Gly23
			Thr21
		Hidrofobic	**Ala20**
			Ala49
			Ala50
Astragalin	-4.8	Hydrogen	**Thr1**
			Arg19
			Thr21
		Hidrofobik	**Ala20**
			Ala22
			Ala49
Carfilzomib	-3.9	Hydrogen	Thr21
			Ala49
			Thr1
			Arg19
		Electrostatic	Lys33
		Hidrofobic	Ala20
			Ala22
			Val31
			Lys33
			Met45
			Gly48
			Ala49
		Unfavorable	Thr1

from lowest to highest is quercetagetin (-6.0 kcal/mol), isoquercitrin (-5.7 kcal/mol), isorhamnetin (-5.5 kcal/mol), quercitrin (-5.2 kcal/mol), and astragalin (-4.8 kcal/mol). The low binding affinity value of the test compounds indicates that these compounds are more stable and more easily interact with the active site of the target protein compared to the positive control. The test compound is predicted to interact with the target protein if it has a binding affinity value equal to or lower than the positive control (Naufa *et al*. 2022).

Based on the docking results, quercetagetin, isoquercitrin, and isorhamnetin have the potential to be candidates for proteasome inhibitors because they have higher affinity compared to carfilzomib. The high affinity is characterized by a low binding affinity value, significantly affecting the stability of the interaction between the ligand and the receptor, as it signifies the minimal energy required by the receptor to interact with the ligand, allowing spontaneous interaction (Naufa *et al*. 2022). In this case, quercetagetin, which has the lowest binding affinity, is likely to demonstrate more stability in complex formation with the 20S proteasome, exhibiting better affinity than the positive control and other test compounds. The visualization results (Figure 4) indicate that these compounds are attached to the active site of the 20S proteasome. The β5 subunit is hydrophobic, with critical amino acid residues identified are Thr1, Ala20, Thr21, and Ala49 (Serrano-Aparicio *et al*. 2021; Yadav *et al*. 2023).

The comparison of the visualization results after docking of the three best test compounds and carfilzomib as a positive control can be seen in Figure 4. Quercetagetin binds to the amino acid residues Thr1, Thr21, and Ala49 through hydrogen bonds. The three hydrogen bonds formed between quercetagetin and Thr1 and Thr21 have a distance of less than 2.3 Å, potentially influencing the compound's affinity, resulting in the lowest binding affinity compared to carfilzomib and other test compounds (Uzzaman *et al*. 2021). These two amino acids play an important role in the catalytic activity of the 20S proteasome (Fernandes *et al*. 2024). Then there are hydrophobic interactions with the amino acid residues Ala20 and Ala49 that can affect CT-L activity (Harshbarger *et al*. 2015). Hydrogen and hydrophobic bonds in ligand interactions with amino acid residues can enhance and stabilize the bonds in the protein-ligand complex.

Isoquercitrin has a higher binding affinity value compared to quercetagetin. However, isoquercitrin is capable of binding to important amino acid residues in the inhibition of the 20S proteasome, namely Thr1, Ala20, Thr21, and Ala49, through hydrogen and hydrophobic bonds. The same occurs with isorhamnetin, which interacts with the same amino acid residues, but the number of hydrogen bonds in the proteasome complex with this compound is fewer compared to isoquercitrin. Hydrogen bonds in the interaction between ligands and target proteins are crucial for attaining a favorable affinity value, which explain why the binding affinity of isoquercitrin is lower than that of isorhamnetin (Pantsar & Poso 2018). Quercitrin and astragalin have higher energies compared to isorhamnetin, namely -5.2 and -4.8 kcal/mol. Although both compounds bind to Thr1 and Thr21 through hydrogen bonds like the other test compounds, the quantity of their bonds is smaller. Furthermore, quercitrin interacts with the adjacent amino acid residue, Thr1, at a distance of 2.695 Å, but astragalin binds to Thr1 at a distance of 3.073 Å.

All flavonol compounds in *H. sabdariffa* have the potential to inhibit the 20S proteasome. This is indicated by the binding affinity value of the test compounds being lower than that of carfilzomib (-3.4 kcal/mol). Nevertheless, carfilzomib can interactions with key amino acid residues by forming seven hydrogen bonds, one electrostatic bond, and ten hydrophobic bonds. Carfilzomib forms hydrogen bonds with Thr1, Thr21, and Ala49, which have bond distances of 2.418 to 3.073 Å. Then, an electrostatic bond is formed with the amino acid residue Lys33. In addition, this positive control also interacts through hydrophobic bonds with the amino acid residues Ala20, Ala22, Val31, Lys33, Met45, Gly48, and Ala49. Hydrophobic bonds are the most prevalent interactions formed in the 20S proteasome and carfilzomib complex. The higher binding affinity value than all the test compounds may be due to the formation of unfavorable donor-donor interactions between carfilzomib and Thr1, which could indicate repulsive forces between the ligand and the protein. This interaction is unfavorable in the complex because it can affect the stability of the ligand-protein complex during docking (Odhar *et al*. 2022). Based on the results of the Lipinski test and molecular docking, it can be predicted that quercetagetin and isorhamnetin are better compared to isoquercitrin, quercitrin, and astragalin. This is evident from the binding affinity values, interactions with important amino acid residues, and the physicochemical properties related to the compound's permeability level. Next, molecular dynamics were performed on both compounds, compared with the carfilzomib control, to ascertain the stability of the ligand-protein complex molecular interactions using the molecular docking results.

Molecular dynamics

Molecular dynamics were conducted under human physiological circumstances at a temperature of 310 K and pH 7.4 (Istyastono 2020). The simulation was conducted for 15 nanoseconds utilizing YASARA software. The results of the molecular dynamics include RMSD, RMSF, binding affinity, and the bonds formed. RMSD is a value that represents the comparison between the conformation of the protein during the simulation process and the initial conformation at each time change. Alterations

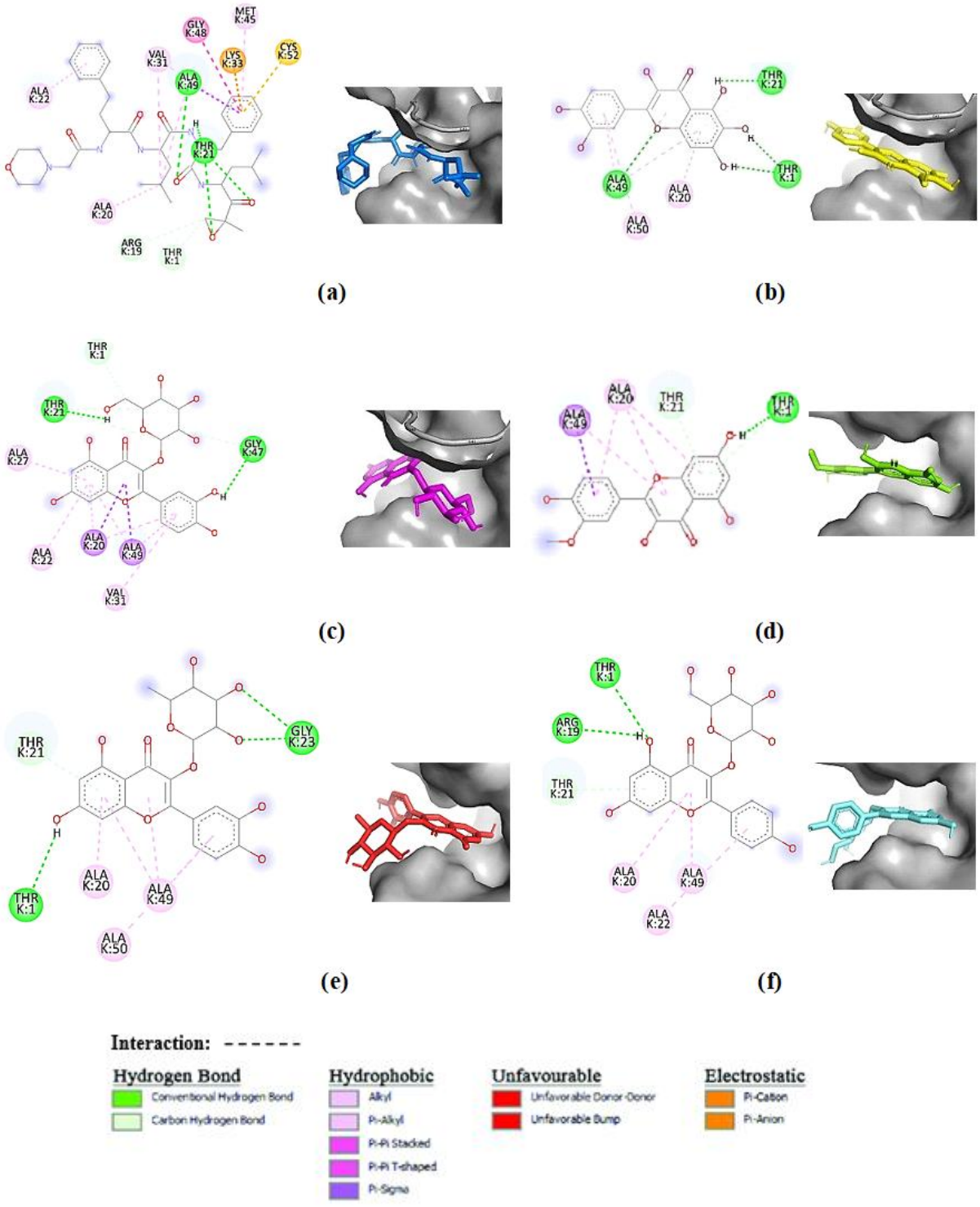

Figure 4. The visualization results of the compounds Carfilzomib (a), Quercetagetin (b), Isoquercitrin (c), Isorhamnetin (d), quercitrin (e), and astragalin (f) against the 20S proteasome using PyMol and BIOVIA discovery studio visualizer.

that diverge significantly from the previous conformation inadequate protein stability (Chairunisa *et al*. 2023). The RMSD values were acquired as presented in Table 5 and Figure 5.

The RMSD graph indicates that carfilzomib, quercetagetin, and isorhamnetin exhibit variable values. The graph shows that the fluctuation of quercetagetin is lower than carfilzomib and ishorhamnetin. Nevertheless, the three compounds are regarded as stable due to their average backbone RMSD values of 1.843, 1.290, and 1.839 Å, respectively. The values are considered stable

Table 5. The average RMSD values of carfilzomib, quercetagetin, and isorhamnetin in molecular dynamics simulations

Compound	RMSD (Å)
Carfilzomib	1.843
Quercetagenin	1.290
Isorhamnetin	1.839

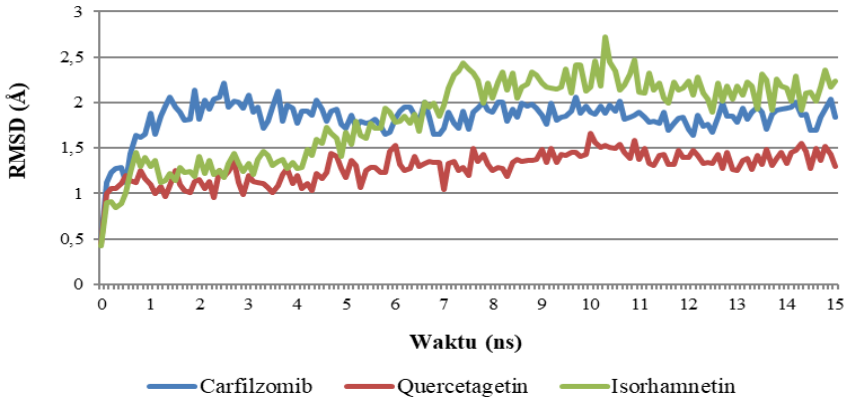

Figure 5. RMSD graph of the compound's carfilzomib, quercetagetin, and isorhamnetin

because they are < 2 Å (Zubair *et al*. 2020). The RMSD value of quercetagetin is lower compared to carfilzomib and isorhamnetin. This suggests that the 20S proteasome complexed with quercetagetin preserves its structure during the simulation, hence enhancing the stability of this ligand-protein interaction.

The second molecular dynamics parameter is RMSF, which can show the fluctuation of amino acid residues in the protein during the simulation, thereby representing its flexibility (Sari *et al.* 2023). The RMSF graph (Figure 6) shows that carfilzomib, quercetagetin, and isorhamnetin experience significant fluctuations. Nevertheless, Table 6 shows that all three can bind to crucial amino acid residues involved in proteasome inhibition, specifically Thr1, Ala20, Thr21, and Ala49. Stability was indicated by the four amino acid residues' RMSF values < 2 Å when they interacted with the test and positive control compounds (Zubair *et al*. 2020).

In comparison to the test compound, carfilzomib shows a lower RMSF value at the Ala20 residue (0.968 Å) and Thr21 (1.034 Å). This indicates that the 20S proteasome bound to carfilzomib experiences low fluctuations at the positions of the amino acid residues Ala20 and Thr21. Meanwhile, quercetagetin has lower RMSF values at residues Thr1 (1.023 Å) and Ala49 (1.168 Å) compared to carfilzomib and isorhamnetin. The low RMSF values at those two residues mean that the 20S proteasome bound to quercetagetin shows low fluctuations at the Thr1 and Ala49 residue positions. Isorhamnetin has a higher RMSF value on the four amino acid residues, so it can be said that the interaction formed between isorhamnetin and the residues is not more stable compared to carfilzomib and quercetagetin. The lower the RMSF value, the more stable the bond between the ligand and the residue (Chairunisa *et al*. 2023).

Molecular dynamics using yasara, the primary method employed to obtain binding affinity value is Molecular mechanics Poisson-Boltzmann Surface Area (MM-PBSA). The method combines energies derivied from molecular mechanics with solvation energy contributions. These solvation energies are calculated using an implicit solvent model and a surface area term. MM-PBSA method is extensively utilized as an effective free energy simulation technique for modelling molecular recognition, particularly in protein-ligand binding interactions (Wang *et al*. 2018). The molecular dynamics parameters other than RMSD and RMSF are the binding affinity values presented in Table 7. The binding affinity value of isorhamnetin (-438.388 kJ/mol) is lower compared to carfilzomib (664.956 kJ/mol) and quercetagetin (-422.623 kJ/mol). The results obtained do not align with the molecular docking results, where a lower binding affinity was found for quercetagetin. This is due to isorhamnetin forming more hydrogen bonds with the 20S proteasome than quercetagetin by the conclusion of the simulation. Meanwhile, carfilzomib has a much higher binding affinity value compared to the test compounds, indicating that the final configuration of the 20S proteasome-carfilzomib complex simulation is unstable (Frimayanti 2020). This also occurred in the studies by Rachmania (2019) and Rendi *et al*. (2021), where the compounds dineolignan, saponin, and alpha-carotene had the highest values compared to other compounds. The elevated binding affinity of

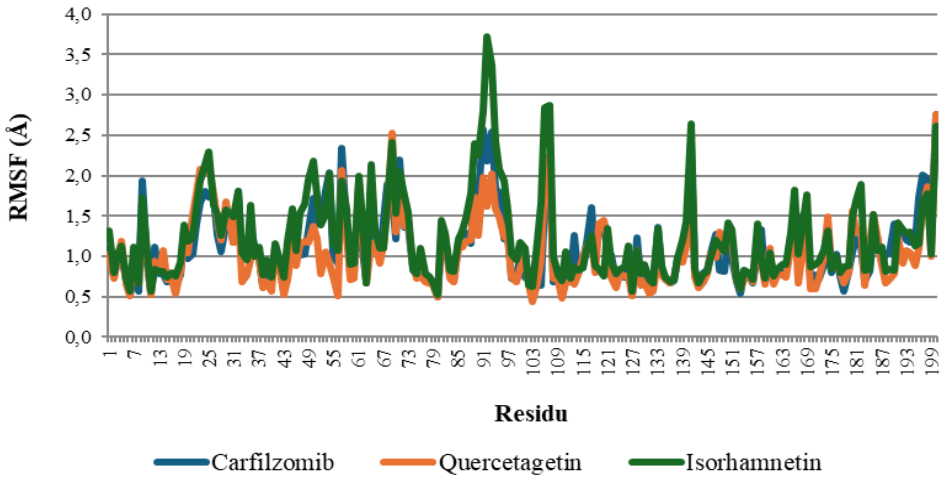

Figure 6. RMSF graph of the compound's carfilzomib, quercetagetin, and isorhamnetin

Table 6. The RMSF values of Carfilzomib, Quercetagetin, and Isorhamnetin against essential amino acid residues

Amino acid residues	RMSF (Å)		
	Carfilzomib	Quercetagenin	Isorhamnetin
Thr1	1.099	1.023	1.322
Ala20	0.968	1.040	1.180
Thr21	1.034	1.515	1.236
Ala49	1.317	1.168	1.954

Table 7. The binding affinity of the compounds carfilzomib, quercetin, and isorhamneton at the end of the molecular dynamics simulation

Compound	Binding affinity (kJ/mol)
Carfilzomib	664.956
Quercetagenin	-422.623
Isorhamnetin	-438.388

the carfilzomib complex arises from its interaction with only two residues via hydrogen and hydrophobic interactions.

Quercetagetin interacts with the four critical amino acid residues to inhibit the 20S proteasome, as demonstrated through molecular docking and molecular dynamics, despite variations in the types of bonds established. In the molecular docking visualization results, hydrogen and hydrophobic bonds were formed with Ala49, whereas at the end of the molecular dynamics simulation (Figure 7), no hydrogen bonds were formed with this residue. Then, in the molecular docking visualization, isorhamnetin formed a hydrogen bond with Thr1 and a hydrophobic bond with Ala20, but the interaction with these residues did not occur at the end of the simulation. Meanwhile, carfilzomib continues to interact with Ala49 through hydrogen bonds and Ala20 hydrophobically. However, carfilzomib does not form interactions with Thr1 and Thr21 through hydrogen bonds, nor does it form hydrophobic bonds with the Ala49 residue. The difference in visualization results at the docking and dynamic stages may be caused by conformational changes, leading to initially interacting amino acid residues no longer interacting during the simulation period.

The results obtained from molecular docking and molecular dynamics vary for both test compounds. The molecular docking studies indicate that the binding affinity of quercetagetin is lower than that of isorhamnetin, whereas molecular dynamics reveal that the binding affinity of isorhamnetin is lower than that of quercetagetin. This phenomenon arises from the semi-flexible docking approach, which treats the ligand as flexible while the protein remains stationary, in contrast to molecular dynamics, where the protein exhibits flexibility and mobility throughout the simulation (Salmaso & Moro 2018). Consequently, the outcomes of the two strategies may vary.

Based on the results of molecular docking, Lipinski's test, and molecular dynamics, it is known that isorhamnetin has the potential to be a proteasome inhibitor candidate. Although quercetagetin showed

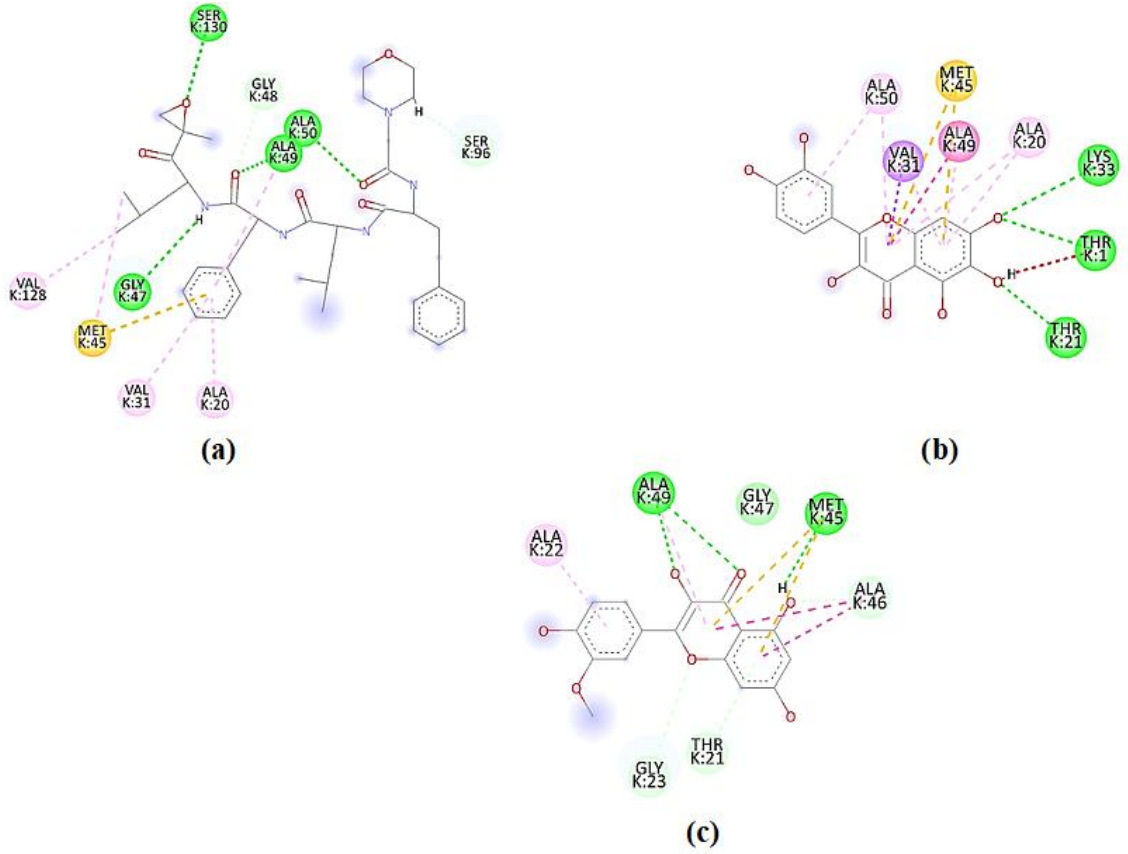

Figure 7. 2D visualization of the final conformation of the carfilzomib complex simulation (a), quercetagetin (b), and isorhamnetin (c)

better results in molecular docking and molecular dynamics compared to isorhamnetin, the Lipinski test results for this compound indicate that quercetagetin has more than 5 hydrogen donors. This affects the compound's ability to penetrate into the cell because the target protein is located inside the cell. A hydrogen donor value of more than 5 reduces the likelihood of the compound being well absorbed because it tends to be more polar and more difficult to pass through the lipid bilayer (Lee *et al*. 2019). In this study, the test compounds, both quercetagetin and isorhamnetin, showed better results compared to the positive control carfilzomib. The test compounds that can bind to the active site are expected to inhibit the activity of the proteasome through the UPP, thereby inhibiting the degradation of specific protein substrates, such as the tumor suppressor p53 and IκB. Inhibition of the proteasome in the UPP can affect the balance or activity of these proteins, thereby inducing apoptosis in cancer cells (Nunes & Annunziata, 2017; Soave *et al*. 2017). Further in vitro studies are needed to revalidate the in-silico test results to precisely determine the potential of isorhamnetin.

CONCLUSION

Isorhamnetin has the potential candidate of proteasome inhibitor compared to other flavonoid compounds in *H. Sabdariffa* flowers, indicated by pharmacokinetic, toxicity, molecular docking, and molecular dynamics data. The pharmacokinetic prediction results indicate that the substamce adheres to Lipinski's rule of five and is suitable for oral administration with well absorption. The boiled egg prediction suggest that it is a non-substrate and is not permeable to the blood-brain barrier. The toxicity prediction results do not suggest toxicity or damage to liver organelles. Molecular docking and molecular dynamics findings indicate that isorhamnetin binds to the amino acid residues Thr1 and Ala49, which are the active sites of the 20S proteasome

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