Virtual Prediction of Phenolic and Glucosinolate Compounds with Keap1 Protein as Anti-aging by Stimulating Nrf2

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Abstract

Aging is caused by an imbalance between antioxidants and ROS. Nuclear Factor Erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates antioxidant genes. Under normal conditions, Nrf2 will bind keap1 and cause degradation of Nrf2. Nrf2 activation can be stimulated by secondary metabolites, such as glucosinolate (glucoraphanin and sulforaphane) and phenolic (kaempferol and quercetin) groups found in broccoli (*Brassica oleracea*). The purposes of this study were to analyze the interaction of the four compounds with Keap1 through molecular docking, to identify interactions that inhibit Keap1, and also to know the bioactivity scores, drug-likeness, and bioactivity prediction of each compound. The Nrf2-Keap1 protein (ID: 2FLU) structure was retrieved from the protein database, whereas the quercetin (CID: 5280343); kaempferol (CID: 5280863), sulforaphane (CID: 5350), and glucoraphanin (CID: 656556) were obtained from the PubChem Database. Molecular docking was done with HEX 8.0. The docking results were visualized with Discovery Studio 2020. Drug-likeness and bioactivity scores of the compounds were identified using mollinspiration. Prediction of bioactivity was carried out with PASS Online. The results showed that the binding energy of quercetin with Keap1 was -268.72 kcal.mol^{-1}, and glucoraphanin with Keap1 was -318.01 kcal.mol^{-1}. We found that quercetin from the phenolic group and glucoraphanin from the glucosinolate group had a strong interaction with Keap1, indicated by the number of interactions occurred and the smaller energy needed. Hence both compounds could inhibit the interaction of Keap1-Nrf2. Consequently, Nrf2 could transcribe antioxidant genes. The interaction between Keap1 and quercetin may play a role related to ROS reduction activities, such as enhancing HMOXI expression. This study indicates that quercetin has more potential in drug development as peroxidase inhibitors.

Keyword: Aging, bioinformatic, glucoraphanin, keap1, quercetin

INTRODUCTION

Aging is a biological and multifactorial phenomenon characterized by a decrease in the physiological function of cells and tissues. One cause of aging is oxidative stress [1]. Oxidative stress is caused by an imbalance between antioxidants and free radicals in the body, such as Reactive Oxygen Species (ROS). ROS levels will increase along with age and can cause cell damage, as well as disrupt the stability and function of molecules, such as proteins, carbohydrates, and fats [2]. Therefore, the body is more susceptible to aging diseases, such as cardiovascular, neurodegenerative, cancer, and diabetes [1].

Nuclear Factor Erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates antioxidant enzymes. Nrf2 levels will diminish with age [3]. Under normal conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1) to initiate interactions with Cullin3 (Cul3) and Rbx 1. As a result, the ubiquitin ligase3 complex is formed. Nrf2 in the ubiquitin ligase3 complex is the target of proteasome degradation. Hence, Nrf2 can not transcribe antioxidant enzymes [4].

Nrf2 activation can be stimulated by bioactive compounds or secondary metabolites plants [4]. Some secondary metabolites of plants, such as glucosinolate and phenolic compounds, are known to have antioxidant activity [5], anticancer, antibacterial, and anti-inflammation [6]. Broccoli (*Brassica oleracea* L.) is a cruciferous edible green plant with high levels of glucosinolate (33%) and phenolic compounds (28%). The glucosinolate compounds found in broccoli are glucoraphanin and sulforaphane, while the most common phenolic compounds are kaempferol and quercetin [7]. Therefore, this study was conducted to find out the inhibition of Keap1-Nrf2 interaction by phenolic compounds (quercetin and kaempferol) and glucosinolate (sulforaphane and glucoraphanin) through *in silico* analysis. We would like to determine which compounds work better to stimulate the Nrf2 pathway. This study also supports existing *in vivo* and *in vitro* research about the stimulation of Nrf2 with flavonoid and glucosinolate compounds.

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MATERIAL AND METHOD

The 3D structure of the compounds was obtained from PubChem (https://pubchem.ncbi.mlm.nih.gov/). The compounds used included the flavonoid group, namely quercetin (CID: 5280343) and kaempferol (CID: 5280863), and the glucosinolate group, namely sulforaphane (CID: 5350) and glucoraphanin (CID: 656556). We obtained the structure of the Nrf2-Keap1 protein complex (ID: 2FLU) from rcsb.pdb.org. Ligands were prepared to minimize energy using Open Babel in PyRx 0.8 software. Water molecules removal was done using Discovery Studio 2020 [8].

Drug-likeness and Bioactivity Prediction

Drug-likeness is an evaluation of drug ability based on several physical-chemical and biological properties of each compound. The *drug-likeness* test was carried out using *Lipinski's Rule of Five*. The rules state that *druglike* molecules must have molecular weight (MW) <500, logp ≤ 5, the number of hydrogen bond acceptors ≤10, the number of hydrogen bond donors ≤5 and should not violate more than one rule [9].

The bioactivity score of the compound was obtained from https://www.molinspiration.com/. These compounds are expected to bind biological targets, such as GPCR ligands, Protease Inhibitors, Enzyme Inhibitors, and kinase inhibitors [9]. The prediction of drug-likeness and bioactivity scores of each compound were performed with https://www.molinspiration.com/ [9].

Biological Activity Prediction Using PASS Online

Biological activities were displayed using the PASS Online server. The biological activity spectrum of each compound showed specific toxicity and pharmacological effects. The compound's activity spectrum was calculated through two parameters, namely Pa (probable activity) and Pi (probable inactivity). Pa and Pi values range from 0-1 and Pa + Pi <1 [10].

Molecular Docking and Visualization

Molecular docking was done using HEX 8.0. The docking results were visualized using Discovery Studio 2020 [8].

RESULT AND DISCUSSION

Evaluation of Drug-likeness

Evaluation of the drug-likeness from four bioactive compounds was based on Lipinski's five rules. These five rules consist of Log P (partition coefficient), molecular weight, the number of hydrogen donors and receptor bonds, and the polar surface area. Based on the physicochemical characterization of the tabulated compounds (Table 1), the four bioactive compounds had molecular weight below 500, which indicated that they are easily transported, diffused, and absorbed compared with heavy molecules [11]. The four bioactive compounds had a log P value below 5, which indicated that they had a high permeability to cell [11].

Sulforaphane, quercetin, and kaempferol possessed bioactive scores in acceptable ranges, such as the number of hydrogen bond acceptors below 10 and the number of hydrogen bond donors below 5. Meanwhile, glucoraphanin had 11 hydrogen bond acceptors, which did not meet the requirements of Lipinski Rules, leading to the reduction in the ability of a molecule to permeate a membrane bilayer [12]. In general, an orally active drug has no more than one violation of the Lipinski Rules. In this study, all four bioactive compounds can be considered as a drug in humans, since the overall score of the compounds is in acceptable ranges.

Bioactivity Score

Biological targets for drugs, such as enzymes, ion channels, and receptors, are targets for drug activity. Bioactivity scores of drugs are calculated based on different parameters such as binding to G protein-coupled receptor ligands (GPCR), nuclear receptor ligands, ion channels, kinase inhibition, protease inhibition, and enzyme inhibition activity [9]. All of these parameters indicated the probability of a molecule to be active, leading to the maximum production of the physiological action [10]. A compound with a bioactivity score of more than 0.00 is most likely to possess considerable biological activities. A compound is moderately active if it has a bioactivity score between -5.0 and 0.0, and inactive if it is less than -5.0 [11].

The bioactivity scores of the four bioactive compounds are tabulated in Table 2. Quercetin and kaempferol had high bioactivity scores as kinase inhibitors, nuclear receptor ligands, and enzyme inhibitors. Sulforaphane had a high bioactivity score on the inhibitor of the enzyme, while glucoraphanin had a high bioactivity score on the enzyme inhibitor, GPCR ligands, protease inhibitors, and nuclear receptor ligands.

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Table 1. Physicochemical characteristics of four bioactive compounds

Compound	Molecular Weight (MW)	Donor atom H	Receptor atom H	Log p	Lipinski's Violation
Quercetin	302.24	5	7	1.68	0
Kaempferol	286.64	4	6	2.17	0
Sulforaphane	177.29	0	2	1.15	0
Glucoraphanin	436.51	4	11	-4.94	1

Table 2. Bioactivity scores of four bioactive compounds

Compound	GPCR Ligand	Kinase Inhibitor	Nuclear Receptor Ligand	Protease Inhibitor	Enzyme Inhibitor
Quercetin	-0.06	0.28	0.36	-0.25	0.28
Kaempferol	-0.10	0.21	0.32	-0.27	0.26
Sulforaphane	-0.35	-1.98	-0.84	-0.72	0.44
Glucoraphanin	0.39	-0.34	0.10	0.21	0.62

Bioactivity Prediction

The bioactivity prediction of all compounds is shown in Table 3. Only activities with Pa > Pi is considered as possible for a particular compound. If Pa > 0.7, the probability of experimental pharmacological action is high, and if 0.5 < Pa < 0.7, probability of experimental pharmacological action is less [9]. Using PASS Online server, selected bioactive constituents were analyzed to evaluate the possible biological activity. Based on the PASS prediction result, sulforaphane was found to have antioxidant activity from the Glutathione S-Transferase Substrate (GST) pathway and CYP2E1 inhibitor. Glutathione S-Transferase Substrate (GST) is a phase II detoxification enzyme that results from the expression of cryoprotective genes induced by Nrf2. GST plays a role in the secondary detoxification of Reactive Oxygen Species (ROS), so DNA damage can be avoided, and cells stay alive [9-10].

Sulforaphane also had inhibitory activity against CYP2E1. CYP2E1 (Cytochrome P450) is a CYP isoform that has a role in the production of ROS. The presence of CYP2E1 in the brain can trigger lipid peroxidation and apoptosis, thereby increase blood permeability to the brain, and can cause brain cell damage. CYP2E1 inhibitor activity in this compound can certainly delay aging by preventing the occurrence of chronic diseases associated with aging, such as neurodegenerative diseases [13].

PASS prediction result showed that the flavonoid compounds group (kaempferol and quercetin) had the activity as HMOXI expression enhancer and peroxidase inhibitor. HMOXI expression enhancer is an antioxidant enzyme secreted to respond to oxidative stress. Activation of this enzyme is regulated by the transcription factor Nrf2. Therefore, if Nrf2 stimulation is increased coupled with the presence of kaempferol and quercetin compounds, which have HMOXI expression enhancer activity, the expression of antioxidant enzymes can elevate, leading to the reduction of the ROS accumulation in the body [14]. One of the peroxidase inhibitors is through the enzyme Glutathione peroxidase 2, which plays a role in catalyzing the reduction reaction of hydrogen peroxide. Glutathione peroxidase is regulated by Nrf-2, which is a transcription factor for the gene product, antioxidant [15]. All these activities from three compounds were related to Nrf2 induction.

Table 3. Bioactivity prediction of four bioactive compounds

Compound	Activity	Pa value	Pi value
Sulforaphane	Glutathione S-Transferase Substrate (GST)	0.936	0.002
	CYP2E1 inhibitor	0.703	0.004
Kaempferol	HMOXI expression enhancer	0.945	0.002
	Peroxidase inhibitor	0.956	0.001
Quercetin	HMOXI expression enhancer	0.957	0.002
	Peroxidase inhibitor	0.962	0.001

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Docking Analysis

The Kelch-Neh2 complex structure was obtained from Protein Data Bank with ID 2FLU. In the Kelch-Neh2 complex, the Nrf2 protein is in the P chain, and the Keap1 protein is in the X chain [16]. The Neh2 domain in Nrf2 with the ETGE and DLG regions is the Kelch-binding region of Keap1 with strong bonds [17]. The Kelch-domain consists of DGR (Double glycine region) and CTR (C¬-terminal region). This domain is the binding site of Neh2 in Nrf2. The Kelch-domain starts with amino acid residues 327-611 (Fig. 1). Based on previous studies, the essential active site is found in serin and arginine residues in the Kelch-domain [17-18].

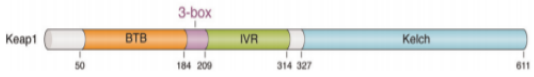

Figure 1. Domain architecture of Keap1[23]

Quercetin is a flavonoid compound that has antioxidant activity. Five hydrogen bonds occurred between quercetin and amino acid residues Val 465, Val 369, Val 463, Ile416, and Ala607 in the Keap1-quercetin complex. Four hydrophobic interactions were also established between quercetin and amino acid residues Ala607, Ala366, and Cys368 (Fig.2). The energy affinity from the docking results between Keap1 and quercetin was -268.72 kcal.mol^{-1} (Table 4). The interaction of Keap1 and quercetin showed that the ligand did not bind to the active side. However, the bonds that occurred between them were still in the Kelch domain, which contributed to the binding of Nrf2. Therefore, quercetin can stimulate Nrf2 expression. In another previous study, quercetin increased the expression of Nrf by *in vivo* [20]. Another *in vitro* study demonstrated that quercetin could activate Nrf2 [21]. The enhancement of Nrf2 expression is important because it is required for binding to ARE (Antioxidant Responses Element), which mediates the transcription of Nrf2-regulated genes with the products of antioxidant and detoxifying enzymes [3].

The conformation with the lowest energy is the conformation with the strongest binding. Binding energy itself is the sum of the intermolecular forces upon the receptor-ligand complex [22].

Table 4. Comparison of energy values and bond types of 4 compounds

	Ligand	Energy (kcal.mol^{-1})	Name	Distance (Å)	Category
Glucosinolate	Glucoraphanin	-318.01	X:ARG326:HH21 - :LIG1:O	2.83488	Hydrogen Bond
			X:VAL420:HN - :LIG1:O	1.7772	Hydrogen Bond
			X:VAL467:HN - :LIG1:O	3.07319	Hydrogen Bond
			:LIG1:H - X:VAL561:O	2.91043	Hydrogen Bond
			X:GLY419:CA - :LIG1:O	2.82909	Hydrogen Bond
			:LIG1:H - X:THR560:OG1	2.51532	Hydrogen Bond
			:LIG1:H - X:VAL561:O	2.84092	Hydrogen Bond
			X:CYS368 - :LIG1	4.85342	Hydrophobic
			X:ALA607 - :LIG1	4.51101	Hydrophobic
	Sulforaphane	-179.66	X:VAL369:HN - :LIG1:O	2.32618	Hydrogen Bond
			X:GLY419:HN - :LIG1:S	2.55596	Hydrogen Bond
			:LIG1:S - X:TYR426:O	3.41717	Hydrogen Bond
			:LIG1:H - X:VAL418:O	2.56679	Hydrogen Bond
Phenolic	Kaempferol	-251.54	X:VAL467:HN - :LIG1:O	2.66797	Hydrogen Bond
			:LIG1:H - :LIG1:O	2.21303	Hydrogen Bond
			:LIG1:H - X:VAL420:O	2.72538	Hydrogen Bond
			:LIG1:H - X:VAL604:O	2.88415	Hydrogen Bond
			X:ALA366:CA - :LIG1:O	2.86789	Hydrogen Bond
			:LIG1 - X:ALA366	3.9726	Hydrophobic
	Quercetin	-268.72	X:VAL465:HN - :LIG1:O	2.3514	Hydrogen Bond
			:LIG1:H - :LIG1:O	2.2166	Hydrogen Bond
			:LIG1:H - X:VAL369:O	2.45654	Hydrogen Bond
			:LIG1:H - X:ILE416:O	2.89961	Hydrogen Bond
			:LIG1:H - X:VAL463:O	2.8104	Hydrogen Bond
			X:ALA607:CA - :LIG1:O	3.21145	Hydrogen Bond
			:LIG1 - X:ALA607	4.66263	Hydrophobic
			:LIG1 - X:CYS368	4.90849	Hydrophobic
			:LIG1 - X:ALA607	4.03925	Hydrophobic
			:LIG1 - X:ALA366	4.97704	Hydrophobic

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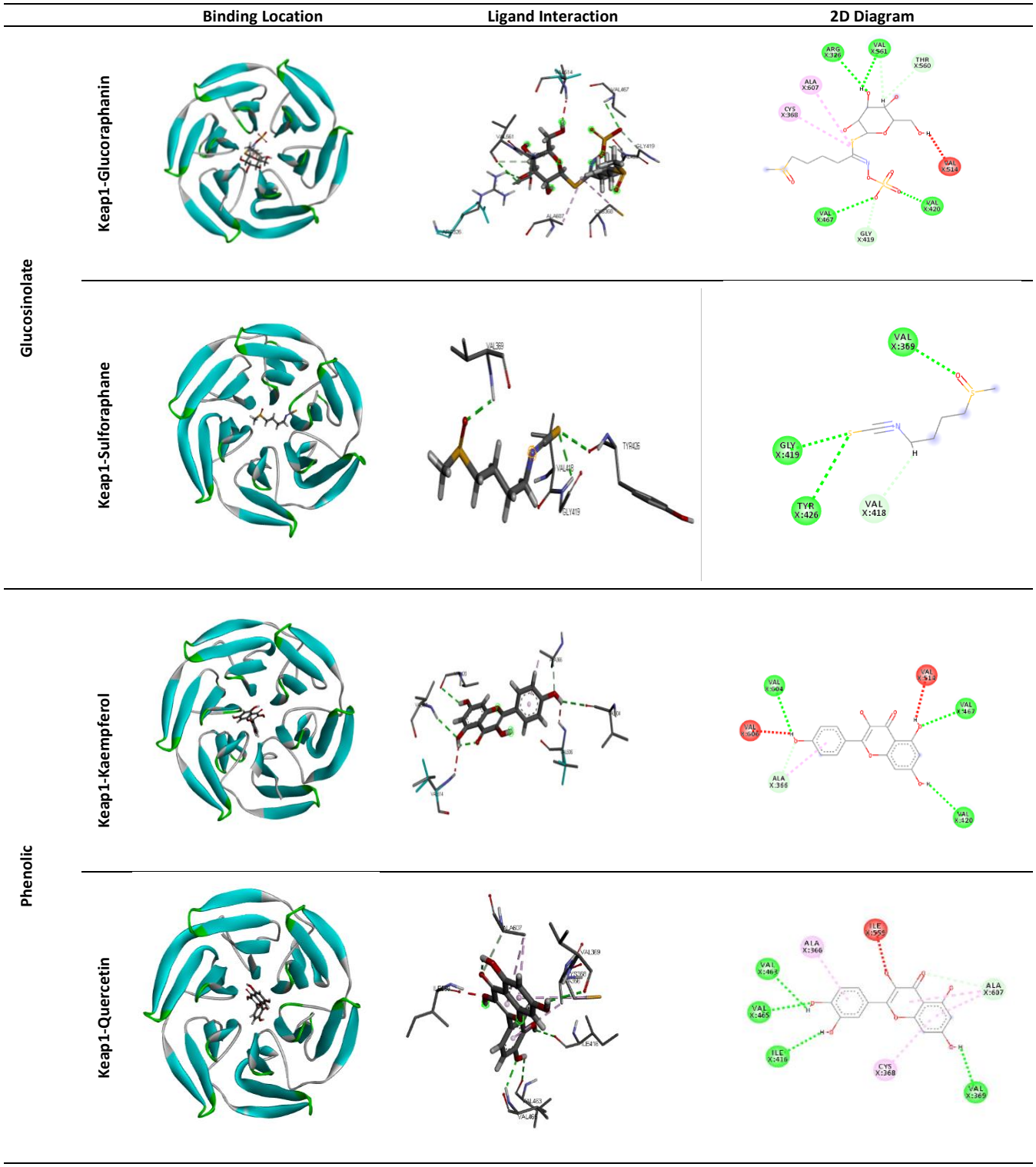

Figure 2. The 3D and 2D structures of molecular docking between Keap1 and glucosinolate group (glucoraphanin and sulforaphane) and or phenolic compounds (kaempferol and quercetin). The binding location of each complex is shown in the left column. The ligand-binding interactions between the ligand and amino acids of Keap1 are shown in the middle column. The 2D interaction between Keap1 and each ligand is shown in the right column.

In previous studies, sulforaphane was able to activate Nrf2 by changing the conformation of Keap1. This conformation involves modifying nucleophilic groups in proteins, including cysteine thiols. Conformation changes of Keap1 may stimulate Nrf2 to transcribe antioxidant genes in the nucleus [16]. The docking results between Keap1 and sulforaphane showed that the ligand did not bind to the active site of the Kelch-domain. There were four interactions of hydrogen bonds in sulforaphane with residues of Gly418, Val419, Val369, and Tyr426 in the Kelch domain (Fig. 2). The interaction between Keap1 and sulforaphane compounds had an energy of -179.66 kcal.mol^{-1} (Table 4).

The most abundant glucosinolate in broccoli is a derivative of methionine 4-methyl-sulfinyl butyl glucosinolate (glucoraphanin), which produces isothiocyanate sulforaphane [24]. This compound can induce Nrf2, a transcription factor that regulates the expression of phase 2 detoxification and antioxidant genes [25]. The docking results between Keap1 and glucoraphanin showed that the ligand did not bind to the active site of the Kelch domain. Seven hydrogen bonds occurred between glucoraphanin with amino acid residues Arg326, Gly419, Val420, Val467, Thr560, Val561. Two hydrophobic interactions were established between glucoraphanin with Cys368 and Ala607 of Keap1 (Fig. 2). The hydrogen bonds help to optimize the hydrophobic interactions and may increase the binding affinity complex [26]. These hydrophobic interactions also help in stabilizing the biochemical environment of the target [27]. The energy affinity from the docking results between Keap1 and glucoraphanin was -318.01 kcal.mol^{-1} (Table 4). This number of energy states the intermolecular forces upon the receptor-ligand complex [22].

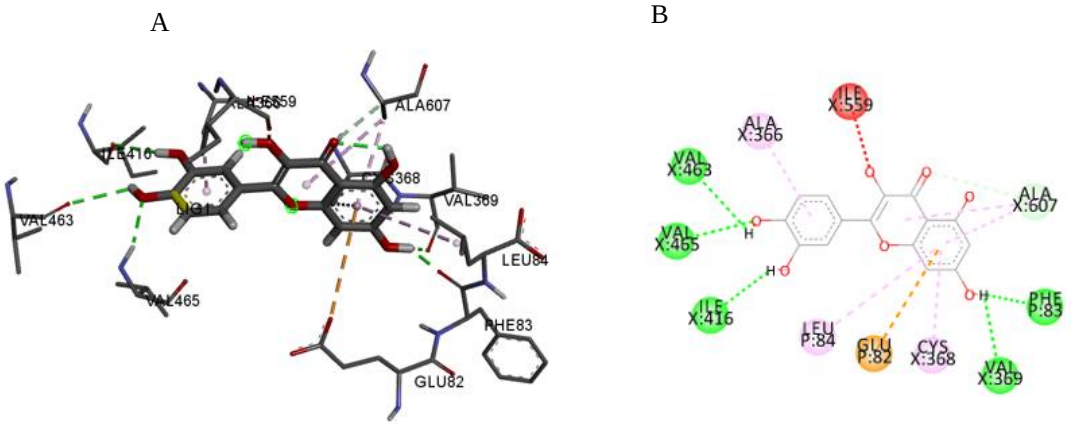

Figure 3. Docking result of Keap1-quercetin complex to Nrf2 A. Ligand-binding Interaction, B. 2D Interaction

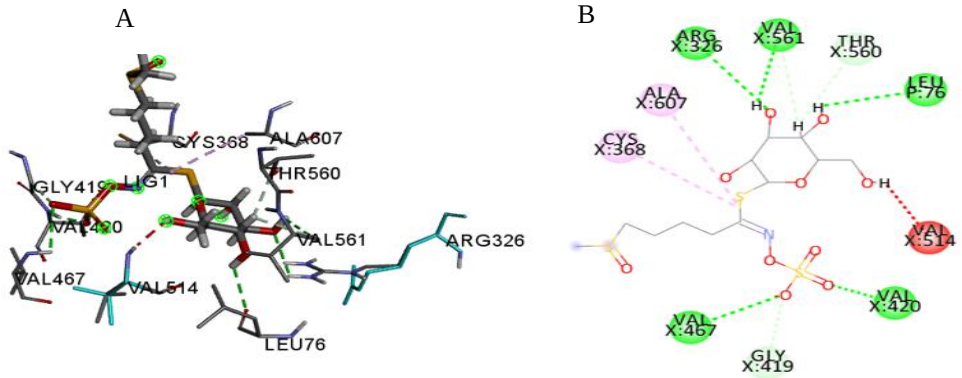

Figure 4. Docking result of Keap1-glucoraphanin complex to Nrf2. A. Ligand-binding Interaction, B. 2D Interaction

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Quercetin and glucoraphanin bound to the Kelch domain, which functioned as the Nrf2 binding region. Both compounds had strong interaction and could easily bind to Keap1, proven by more amount of interactions and smaller binding energy than other compounds [28]. The strong interaction indicates a stronger impact on Nrf2 to prevent being sequestered by Keap1. Therefore, degradation of Nrf2 by proteasome can be prevented. Consequently, Nrf2 will be free, then it can enter the nucleus to form heterodimers with small MAF proteins [4]. The heterodimer complex is recognized by ARE, leading to the induction of antioxidant gene expression [15]. It shows that quercetin and glucoraphanin can change the stability of the Nrf2-Keap1 interaction.

The combination of Keap1 and quercetin in the form of ligand complex could disturb the Nrf2 binding on the Kelch domain of Keap1 (Fig.3). As a result, the Nrf2 binding site to Keap1 changed into Val 463, Val 465, ILE 416, Val 369, and ALA 607 which were different from the residue amino acid in keap1-Nrf2. The same thing also happens in a result docking of complex keap1-glucoraphanin to Nrf2 (Fig. 4). This complex disturbs Nrf2 binding on the Kelch domain of keap, so that, binding site to Keap 1 changed into Val326, Val561, Ala607, Cys368, Val467, and Val420, which were distinct from residue amino acid in Keap1-Nrf2. Keap1-Nrf2 has a binding site in serin and arginine residue amino acid.

Hydrogen bonds are known to increase the binding affinity of ligand interactions. Besides, hydrogen bonds strongly contribute to the transportation, adsorption, distribution, and metabolism of a molecule [29]. The combination of hydrophobic bonds with hydrogen bonds can optimize binding affinity. In addition, the presence of hydrophobic bonds can increase the biological activity of the drug [22].

CONCLUSION

Quercetin and glucoraphanin are the strongest compounds to activate Nrf2 by inhibiting Keap1, followed by kaempferol and sulforaphane. Both of these compounds had low binding energy, more hydrogen bonds, and high hydrophobic interactions. Quercetin has a higher potential in drug development than glucoraphanin based on its biological activity as a peroxidase inhibitor.

ABBREVIATION

Nrf-2	Nuclear Factor Erythroid 2-related factor
Keap1	Kelch ECH-associating protein1
GST	Glutathione S-Transferase Substrate
Pa	probable activity
Pi	probable inactivity
DGR	Double glycine region
CTR	C¬-terminal region
Val	Valin
Ile	Isolysin
Ala	Alanine
Cyst	Cysteine
Arg	Arginine
Gly	Glycine
Tyr	Tyrosine
ARE	Antioxidant Responses Element

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