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Flavonoid Compounds of Buah Merah (*Pandanus conoideus* Lamk) as a Potent Oxidative Stress Modulator in ROS-induced Cancer: *In Silico* Approach

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ABSTRACT

Buah Merah, a typical fruit from Papua, Indonesia which is used empirically in cancer therapy is rich in carotenoids and flavonoids. However, the mechanisms by which Buah Merah ameliorates cancer remained unknown. Natural antioxidant enzymes and pro-oxidant enzymes modulation significantly suppressed ROS production and cancer growth. Therefore, the determination of target enzymes of Buah Merah contents was studied through an *in silico* approach. Carotenoid and flavonoid compounds from Buah Merah were docked to 7 ROS modulating enzymes using Autodock Vina and the interaction stability was studied using the CABS Flex 2.0 server. The crucial amino acids of each enzyme were determined using DockFlin and prediction of acute oral toxicity of each test ligand was studied using ProTox-II. Based on the molecular docking results, quercetin 3'-glucoside is the most potent compound in binding to CAT, GR, GPx, SOD, LOX, and NOX with binding energy values of -11.2, -9.7, -8.6, -10.2, -10.7, and -12.8 kcal/mol, respectively. Meanwhile, taxifolin 3-O-α-arabinopyranose produced the highest binding affinity of -10.0 kcal/mol at the XO. Each test ligand formed stable interactions with ROS modulating enzymes and formed bonds with crucial amino acids resulting in strong adhesion compared to native and reference ligands. The glucoside group of quercetin 3'-glucoside plays an essential role in determining the proper position in the attachment and supports the formation of hydrogen bonds with receptors. With low acute oral toxicity, it can be concluded that quercetin 3'-glucoside from Buah Merah is a potent oxidative stress modulator in cancer prevention and therapy.
Keywords: ROS-induced cancer; Buah Merah; *Pandanus conoideus* Lamk; Quercetin 3'-glucoside; *In silico*

INTRODUCTION

Oxidative stress results from an imbalance between the number of free radicals (ROS) and reactive metabolites (antioxidants) in the body. High levels of ROS can increase the oxidation process in normal cells, causing damage. The most common impacts are premature aging and the onset of chronic diseases such as cancer. It is well known that oxidative stress contributes to tumor initiation and development by inducing genomic instability (Weinberg and Chandel, 2009). As a result, focusing on redox-sensitive pathways and transcription factors holds considerable potential for cancer prevention and treatment (Reuter *et al.*, 2010; Gào and Schöttker, 2017). Lipoxygenase (LOX), NADPH oxidase (NOX), and xanthine oxidase (XO) are enzymes that play a role in modulating the production of ROS. Inhibition of these three enzymes will significantly suppress ROS production and cancer growth (Bishayee and Khuda-Bukhsh, 2013; Landry and Cotter, 2014; Meitzler *et al.*, 2014; Subramanian, Mendez and Becerra, 2016; Gào and Schöttker, 2017; Oh *et al.*, 2019). Likewise, activation of the catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), and superoxide dismutase (SOD) can reduce ROS levels and prevent cell damage (Goh *et al.*, 2011; Bauer, 2012; Glorieux and Calderon, 2018; Wang *et al.*, 2018; Chen *et al.*, 2019; Kennedy *et al.*, 2020).

Natural antioxidants such as flavonoids can act directly through ROS scavenging and metal chelation and indirectly by inhibiting pro-oxidant enzymes and activating antioxidant enzymes (Kopustinskiene *et al.*, 2020). Therefore flavonoids have a robust anticancer activity (Abotaleb *et al.*, 2018; Chirumbolo *et al.*, 2018; Rodríguez-García, Sánchez-Quesada and Gaforio, 2019). Buah Merah (*Pandanus conoideus* Lamk) is an endemic fruit from Central Papua which is often used empirically in cancer therapy (Nuringtyas *et al.*, 2015). Buah Merah is reported to contain a high quantity of carotenoids and flavonoids. The contents of buah merah can be seen in Supplemental Table I.

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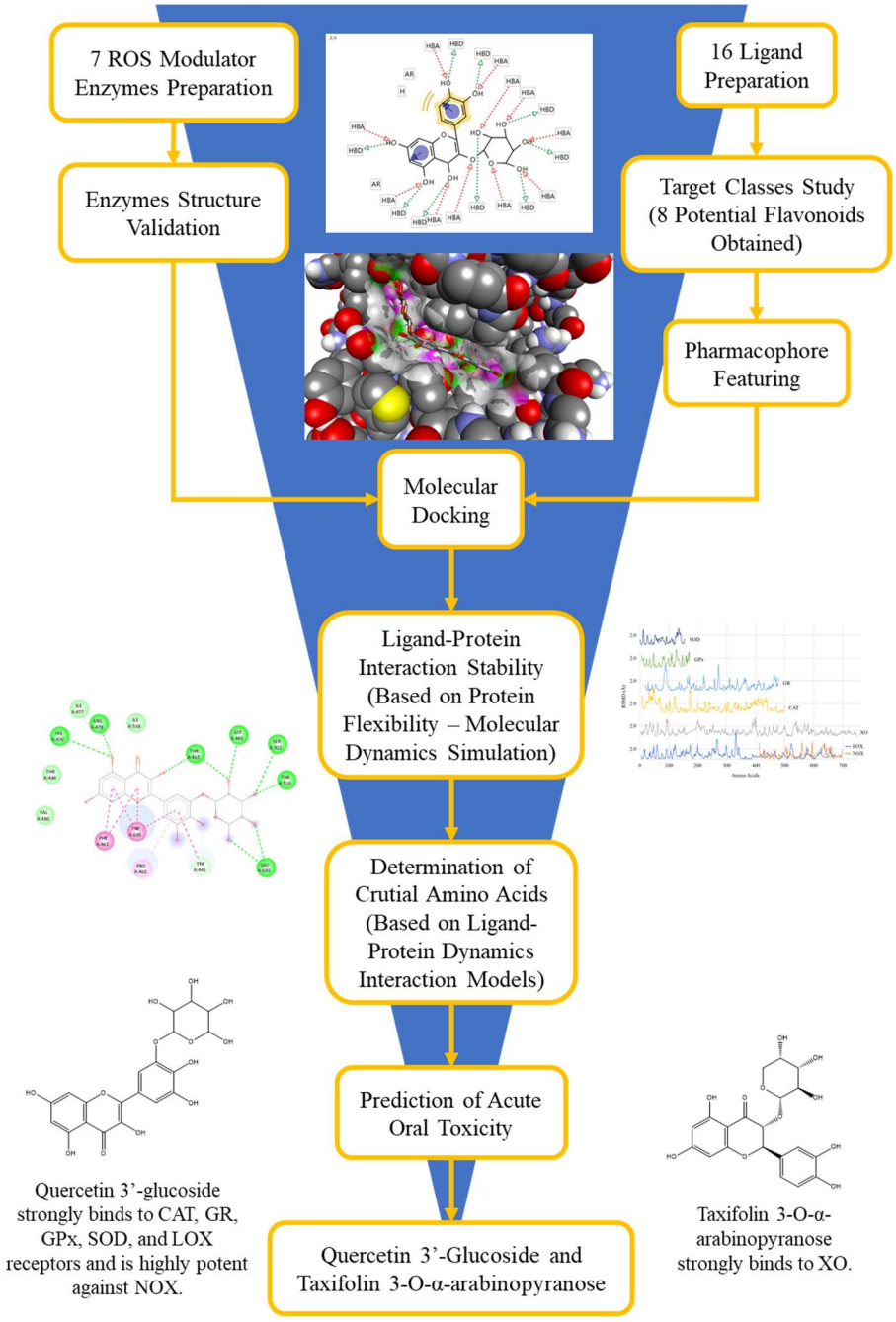

Graphical Abstract

Table I. The ROS enzymes' gridbox parameters

Enzymes	**Active sites Coordinate (Å)**
Lipoxygenase	4,976 x 21,401 x 0,286
NADPH Oxidase	65,738 x 0,875 x 62,590
Xanthin Oxidase	17,175 x -17,782 x 16,527
Catalase	14,877 x 15,793 x 78,537
Glutathione Reductase	82,170 x -5,827 x 36,154
Glutathione Peroxidase	42,474 x 14,099 x -18,061
Superoxide Dismutase	95,611 x 47,146 x 112,872

However, the anticancer mechanism of Buah Merah is still not described. Therefore, this study was conducted to see whether the flavonoids and carotenoids of Buah Merah inhibit LOX, NOX, and XO enzymes and activate or competitively protect CAT, GR, GPx, and SOD receptors from their inhibitors. This study also systematically describes the quantitative relationship between the flavonoid structure of Buah Merah and its activity, the stability of its interaction with ROS modulating enzymes, the formation of bonds with crucial amino acids, and its acute oral toxicity.

METHODOLOGY

Ligand Preparation

The test compounds from Buah Merah consisted of a total of 8 carotenoids (5,6-diepicapsokarpoxanthin, capsorubin, capsanthin 5,6-epoxide, capsanthin 3,6-epoxide, capsanthin, cryptocapsin, β-cryptoxanthin 5,6-epoxide, and cryptoxanthin) and a total of 8 flavonoids (4',6,6',8-tetrahydroxy-3-methoxy-flavon, 3,4',5-trihydroxy-7,3'-dimethoxy flavon, taxifolin 3-O-α-arabinopyranose, quercetin 3-O-glucose, quercetin 3-methyl-ether, quercetin, taxifolin, and quercetin 3'-glucoside). Some ligand structures were downloaded from PubChem, and the rest were drawn with ChemDraw Pro 12.0 (PerkinElmer Informatics, PerkinElmer Inc, USA). Structural errors were checked using the "Check Structure" feature, and then the structures were cleaned using the "Clean Up Structure" feature in ChemDraw. The default geometry of each ligand was removed using the "Clean Geometry" feature in Discovery Studio 2021 Client (DS) (BIOVIA, San Diego, CA, USA). Energy minimization (MM2) was performed using Chem3D. Each ligand was then optimized using AutoDockTools 1.5.6 (ADT) (The Scripps Research Institute, USA) to add Gasteiger charges, set rotatable bonds, and TORSDOF. Furthermore, the ligands are stored in PDBQT file format.

Protein Preparation

The structure of the proteins was obtained from the Protein Data Bank (PDB) website. The code for each protein used is lipoxygenase (3O8Y), NADPH oxidase (5O0X), xanthine oxidase (3BDJ), catalase (1DGF), glutathione reductase (1XAN), glutathione peroxidase (6ELW), and superoxide dismutase (2C9V). Native ligands and proteins were separated with the DS. The protein was optimized with ADT to remove water, regulate the charges (Kollman charges), and add polar hydrogen. The protein was then stored in PDBQT file format. The grid position was arranged based on the active binding site to which the native ligand is attached. The grid dimensions were set to 40 x 40 x 40 magnification with a spacing of 0.375Å. Gridbox parameters can be seen in Table I.

After going through the preparation process, the protein structures were validated with the SAVES V6.0 server (UCLA, England). The PROCHECK feature was used to determine the stereochemical quality of the protein structure (Laskowski *et al.*, 1993). The ERRAT analysis aimed to differentiate between correctly and incorrectly determined regions of protein structures based on characteristic atomic interaction (Colovos and Yeates, 1993). The PROVE analysis aimed to assess the quality of protein crystal structures (Pontius, Richelle and Wodak, 1996).

Target Classes

This test was performed to identify the target classes of the test ligands with SwissTargetPrediction (Swiss Institute of Bioinformatics, Swiss) (Michielin and Zoete, 2019). All test ligands were converted to the SMILES file type. Identification of target classes is only aimed at the *Homo sapiens* protein. This test aimed to evaluate which compounds can interact with enzymes that play a role in oxidative stress.

Pharmacophore Features

Pharmacophore featuring with LigandScout® 4.4.3 (Inte: Ligand GmbH, Austria) was carried out to study the important groups of each test ligand and predict the form of its potential interaction with the receptor. The test ligands were converted to PDB format, then pharmacophore featuring was performed by selecting Pharmacophore > Create Pharmacophore or by pressing Ctrl-F9.

Molecular Docking

Molecular docking was performed with DockFlin software (ETFLIN, Indonesia). This software is a tool for systematically scheduling multi-ligand and multi-protein docking processes by the Autodock Vina. Ligands and proteins were added to the respective list panel, then the docking parameters per protein were loaded in the order in the grid list panel. The docking parameters used were an energy range of 4 and exhaustiveness of 8. The operating system used was Windows 10 Home Single Language 64 bit with AMD Ryzen 5 3500U, Radeon Vega Mobile Gfx 2.10 GHz, and RAM of 8 GB.

Molecular Dynamics Study

The protein's stable structure was studied using the CABS Flex 2.0 server, which is based on coarse-grained simulations of protein motion (Kurcinski *et al.*, 2019). Distance restraints generator mode was SS2 with minimal restraint length of 3.8 Å and maximal restraint length of 8.0 Å. The number of cycles and trajectory frames was set to 50, with a global weight of 1.0 and a temperature of 1.4. The distance restraints generator was set to default values. This test aims to see whether the ligand-protein interaction remains stable during attachment (Arora *et al.*, 2020).

Determination of Crucial Amino Acid

This test was carried out to determine the most active amino acid residues at the binding site of the proteins. The output file of the docking process produces 9 ligand-protein interaction models for each ligand on each protein. All the active amino acid residues bind to the ligand, and their number of occurrences has been scored with DockFlin.

Acute Oral Toxicity Study (LD_{50})

Prediction of acute oral toxicity (LD50) was carried out based on the Globally Harmonized System using ProTox-II (Charite University of Medicine, Germany). The test ligands were converted into SMILES format, then the prediction parameters of the acute oral toxicity model were activated.

RESULTS AND DISCUSSION

Results

Protein Validation

The proteins used were stable and of good quality. It can be seen from the percentage of amino acid residues in quadrant I (most favored regions) and quadrant II (additional allowed regions), which exceeds 90%, while quadrant III (generously allowed regions) and quadrant IV (disallowed regions) have less than 1% amino acid residues (Kleywegt and Jones, 1996; Wlodawer, 2017). All test enzymes have good chi1-chi2 plots. The overall quality factor from the ERRAT analysis also showed a value of more than 90 in all the tested enzymes, while the total buried outlier protein atoms from the PROVE analysis was <2.9% (Kleywegt and Jones, 1996). The validation results showed that all the test enzymes produced values that matched the specifications for all parameters to be suitable for use in the molecular docking process. The validation results can be seen in Table II.

Target Classes

Based on the target classes test, it was found that the flavonoid group compounds have a high probability (10.1 - 100.0%) to interact with oxidative stress modulating enzymes. Taxifolin and its derivative do not have anything in common with the compounds in the SwissTarget database, so the target class prediction is not found. However, because the structure of taxifolin and its derivative is nearly identical to that of quercetin, research on taxifolin and its derivative was continued. The carotenoid compounds did not show sufficient interaction probability (<10%). Thus, further tests were carried out using only flavonoid compounds. Prediction results of target classes can be seen in Table III.

Pharmacophore Features

The test results indicated the presence of pharmacophore features on each test ligand that plays a vital role in biological activity, as shown in Figure 1. Pharmacophore features consist of hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), hydrophobic (H), positive ion (PI), negative ion (NI), and aromatic (AR) (23).

Compound 4',6,6', 8-tetrahydroxy-3-methoxy-flavone has 2 pharmacophore features consisting of 6 HBA and 6 HBD formed from phenol and 2 AR. Compound 3,4',5-trihydroxy-7,3'-dimethoxy-flavone has 6 HBA, 5 HBD, and 2 AR. Taxifolin 3-O-α-arabinopyranose has 11 HBA, 8 HBD, and 2 AR. Quercetin 3-O-glucose has 11 HBA, 9 HBD, and 2 AR. Quercetin 3-methyl-ether has the same pharmacophore features as quercetin, namely 6 HBA, 5 HBD, and 2 AR. Taxifolin has 7 HBA, 6 HBD, and 2 AR, while quercetin 3'-glucoside has 12 HBA, 10 HBD, and 2 AR. With many pharmacophore features of HBA and HBD formed from phenol groups, the compound quercetin 3'-glucoside has been predicted to produce high biological activity.

Molecular Docking

Overall, the test ligands have a significantly greater binding affinity than the native and reference ligands in each test enzyme. Each ligand has a potent effect on a different enzyme (see Table IV).

Antioxidant Enzymes

Several test ligands from the quercetin and taxifolin groups have binding affinity more remarkable than the reference compounds (rutin, astragalin, and trifolin) and native ligands (NADP, XAN, and trehalose). In the CAT enzyme, the highest binding affinity was produced by quercetin

Table II. Proteins validation result.

No	Parameters	Enzymes						
		CAT	**GR**	**GPx**	**SOD**	**LOX**	**NOX**	**XO**
1.	**Ramachandran Plots**	Ok	Ok	Ok	Ok	Ok	Ok	Ok
	Favoured	87.1%	91.7%	96.5%	91.7%	88.8%	92.8%	89.5%]
	Allowed	12.3%	7.8%	3.5%	8.3%	10.7%	7.2%	9.7%
	Generous	0.3%	0.5%	0.0%	0.0%	0.3%	0.0%	0.5%
	Disallowed	0.3%	0.0%	0.0%	0.0%	0.2%	0.0%	0.3%
2.	**Chi1-chi2 Plots**	Pass	Pass	Pass	Pass	Pass	Pass	Pass
3.	**ERRAT overal quality factor**	97.645	97.5391	99.359	92.9329	96.1019	95.9677	96.4215
4.	**PROVE**	0.3%	1.0%	0.8%	1.1%	1.6%	2.4%	0.9%

Table III. Identification of the test ligands target classes

No	Compounds	Probability (%)						
		Antioxidant Enzymes				**Pro-oxidant Enzymes**		
		CAT	**GR**	**GPx**	**SOD**	**LOX**	**NOX**	**XO**
	Flavonoid							
1.	4’,6,6’,8-tetrahydroxy-3-methoxy-flavon	-	-	-	-	10.2	10.2	-
2.	3,4',5-trihydroxy-7,3'-dimethoxy flavon	-	-	-	-	11.3	29.9	-
3.	Taxifolin 3-O-α-arabinopyranose	-	-	-	-	-	-	-
4.	Quercetin 3-O-glucose	-	-	-	-	10.7	87.9	-
5.	Quercetin 3- methyl-ether	-	-	-	-	18.5	71.0	-
6.	Quercetin	-	-	-	-	100	100	-
7.	Taxifolin	-	-	-	-	-	-	-
8.	Quercetin 3’-Glucoside	-	-	-	-	10.1	10.1	-
	Carotenoid							
1.	5,6-diepicapsokarpoxanthin	-	-	-	-	-	-	-
2.	Capsorubin	-	-	-	-	7.5	-	-
3.	Capsanthin 5,6-epoxide	-	-	-	-	7.5	-	-
4.	Capsanthin 3,6-epoxide	-	-	-	-	7.5	-	-
5.	Capsanthin	-	-	-	-	-	-	-
6.	Cryptocapsin	-	-	-	-	8.2	-	-
7.	β-cryptoxanthin 5,6-epoxide	-	-	-	-	8.2	-	-
8.	Cryptoxanthin	-	-	-	-	7.4	-	-

3'-glucoside (-11.2), followed by quercetin 3-O-glucose (-10.3), quercetin (-9.5), and taxifolin (-9.5). This value is significantly higher than the native ligand, namely NADP (-7.9). Quercetin 3'-glucoside forms hydrogen bonds with the amino acids Arg203, Ser201, Gln455, Tyr215, His305, and Lys306. In some models, quercetin 3'-glucoside also tends to form hydrogen bonds with Val450, Phe198, Val302, and His194 in the CAT binding pocket. Another significant difference was found in the GR, GPx, and SOD enzymes wherein quercetin 3'-glucoside produced binding energies of -9.7, -8.6, and -10.2, respectively. However, the reference and native ligands only produced binding energies ranging from -6.3 to -8.0 at GR, GPs, and SOD. Quercetin 3'-glucoside also formed many hydrogen bonds (> 5 bonds) with GR, GPx, and SOD enzymes. The rest is π bonds (see Figure 2).

Pro-oxidant Enzymes

Several test ligand compounds of the quercetin, taxifolin and, flavone groups have binding energy more significant than the reference compounds (rutin, astragalin, and trifolin) and native ligands (oxypurinol, meclofenamate, and zileuton). At the LOX enzyme, the highest binding affinity was produced by quercetin 3'-glucoside (-10.7), followed by trifolin (-9.9) and quercetin 3-O-glucose (-9.7). This value is significantly higher than the native ligands, namely meclofenamate (-7.4) and zileuton (-7.4). All test ligands and reference compounds have a high binding affinity

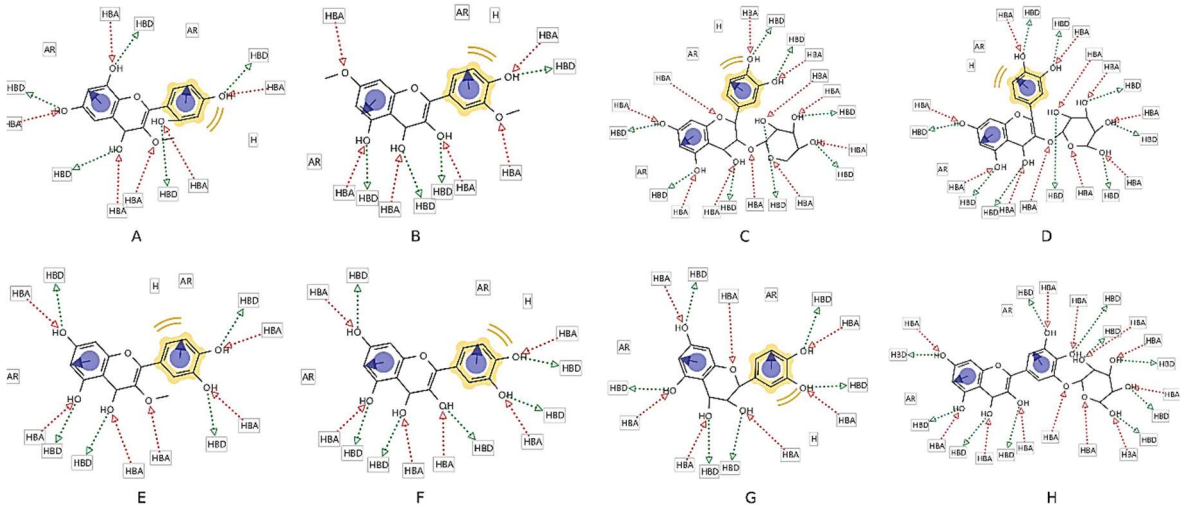

Figure 1. Pharmacophore features of 4',6,6',8-tetrahydroxy-3-methoxy-flavon (A), 3,4',5-trihydroxy-7,3'-dimethoxy-flavon (B), taxifolin 3-O-α-arabinopyranose (C), quercetin 3-O-glucose (D), quercetin 3-methyl-ether (E), quercetin (F), taxifolin (G), and quercetin 3'-glucoside (H)

Table IV. The binding affinity of test ligands and reference ligands to the antioxidant enzymes and pro-oxidant enzymes

No	Compounds	Antioxidant Enzymes				Pro-oxidant Enzymes		
		CAT	GR	GPx	SOD	LOX	NOX	XO
1.	4',6,6',8-tetrahydroxy-3-methoxy-flavon	-8.3	-8.7	-7.2	-7.8	-8.6	-9.9	-6.9
2.	3,4',5-trihydroxy-7,3'-dimethoxy-flavon	-9.1	-7.7	-7.1	-8.8	-8.1	-10.4	-8.5
3.	Taxifolin 3-O-α-arabinopyranose	-9.0	-7.8	-7.5	-7.6	-8.2	-10.0	-10.0
4.	Quercetin 3-O-glucose	-10.3	-8.6	-8.0	-7.8	-9.7	-10.9	-8.3
5.	Quercetin 3-methyl-ether	-9.1	-8.0	-7.1	-8.6	-8.3	-9.6	-7.4
6.	Quercetin	-9.5	-8.8	-7.5	-9.0	-9.1	-10.8	-9.6
7.	Taxifolin	-9.5	-7.8	-7.4	-9.0	-9.3	-9.5	-10.0
8.	Quercetin 3'-Glucoside	-11.2	-9.7	-8.6	-10.2	-10.7	-12.8	-8.8
9.	Native – NADP	-7.9	--	--	--	--	--	--
10.	Native – XAN	--	-7.8	--	--	--	--	--
11.	Oxypurinol	--	--	--	--	--	--	-6.7
12.	Ref - Trehalose	--	--	--	-6.3	--	--	--
13.	Ref - Meclofenamate	--	--	--	--	-7.4	--	--
14.	Ref - Zileuton	--	--	--	--	-7.4	--	--
15.	Ref - Rutin	-8.8	-7.5	-7.2	-7.6	-8.8	-9.6	-7.8
16.	Ref - Astragalin	-8.7	-7.4	-7.7	-7.2	-8.3	-9.2	-8.9
17.	Ref - Trifolin	-9.2	-8.0	-7.7	-7.8	-9.9	-9.0	-7.0

on the NOX enzyme (< -9.0). Quercetin 3'-glucoside is still the highest (-12.8) and can be a potent inhibitor of NOX. On the other hand, taxifolin and its derivatives have a higher binding affinity (-10.0) for the XO. At LOX and NOX enzymes, quercetin 3'-glucoside forms 6 and 8 hydrogen bonds, respectively. Taxifolin and its derivative form 5 hydrogen bonds in the XO enzyme, the rest being pi-cation and pi-alkyl bonds (see Figure 3).

Molecular Dynamics Study

Based on the molecular dynamics simulation, it can be seen that the flexibility of the protein structure does not change the 3D pattern

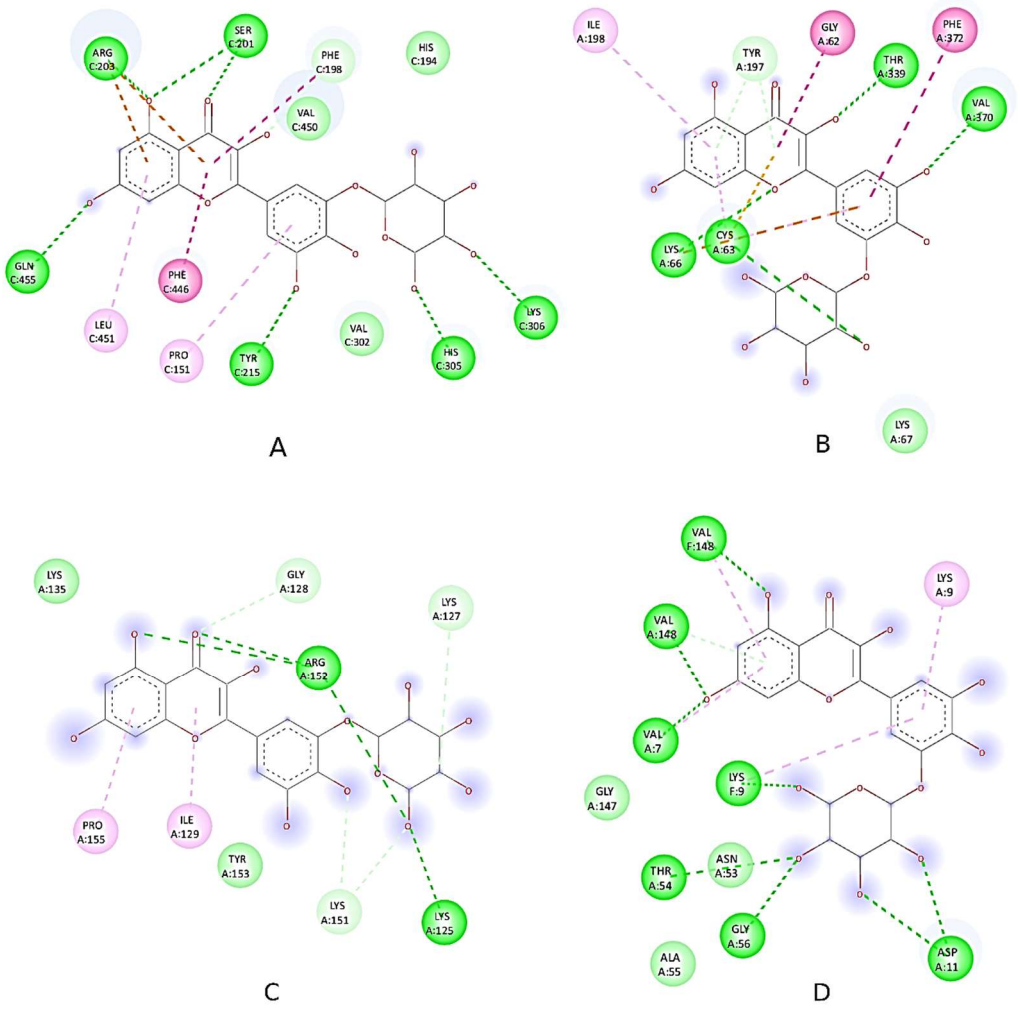

Figure 2. Interaction of quercetin 3'-glucoside with catalase (A), glutathione reductase (B), glutathione peroxidase (C), and superoxide dismutase (D).

of the enzyme significantly (see Figure 4). Each enzyme's binding site retains a similar shape and coordinates indicating that the ligand-binding was not disturbed.

The root mean square fluctuation (RMSF) of the test enzymes was still mostly below 2.0 Å. There is no crucial amFtableino acid in the binding pocket protein with RMSF above 2.0 Å (see Figure 5). RMSF fluctuations more than 2.0 occurred in the CAT amino acid sequences of 21-55, 63-70, 118-123, 171-177, and 390-439; GR amino acids sequences of 83-94, 163-171, 269-274, and 462-471; GPx amino acids sequences of 79-81, 107-112, 124-129, and 156-170; SOD amino acids sequence of 128-139; LOX amino acids sequences of 116-120, 264-270, 329-335, and 521-527; NOX amino acids sequences of 503-505, 558-561, 632-636, and 659-661; and XO amino acids sequences of 33-37, 61-63, 91-97, 197-204, 338-402, 537-541, and 567-570.

Determination of Crucial Amino Acid

The crucial amino acids of each protein were obtained through the occurrences score method (see Table V). CAT has an average bond length ranging from 4,372 – 5,561 Å with a score range of 10.625 – 36.625. GR has an average bond length ranging from 4.393 – 5.356 Å with a score range of 6.25 – 23.5. GPx has an average bond length ranging from 4.265 – 4.99 Å with a score range of 9 – 20.375. SOD has an average bond length ranging from 4.308 – 4.985 Å with a score range of 7 -24.25. LOX has an average bond length ranging from 4.443 – 5.284 Å, with a score range of 7 -25.25. NOX has an average bond length ranging from 4.168 – 5.167 Å with a score range of 9.125 – 53.75. XO has

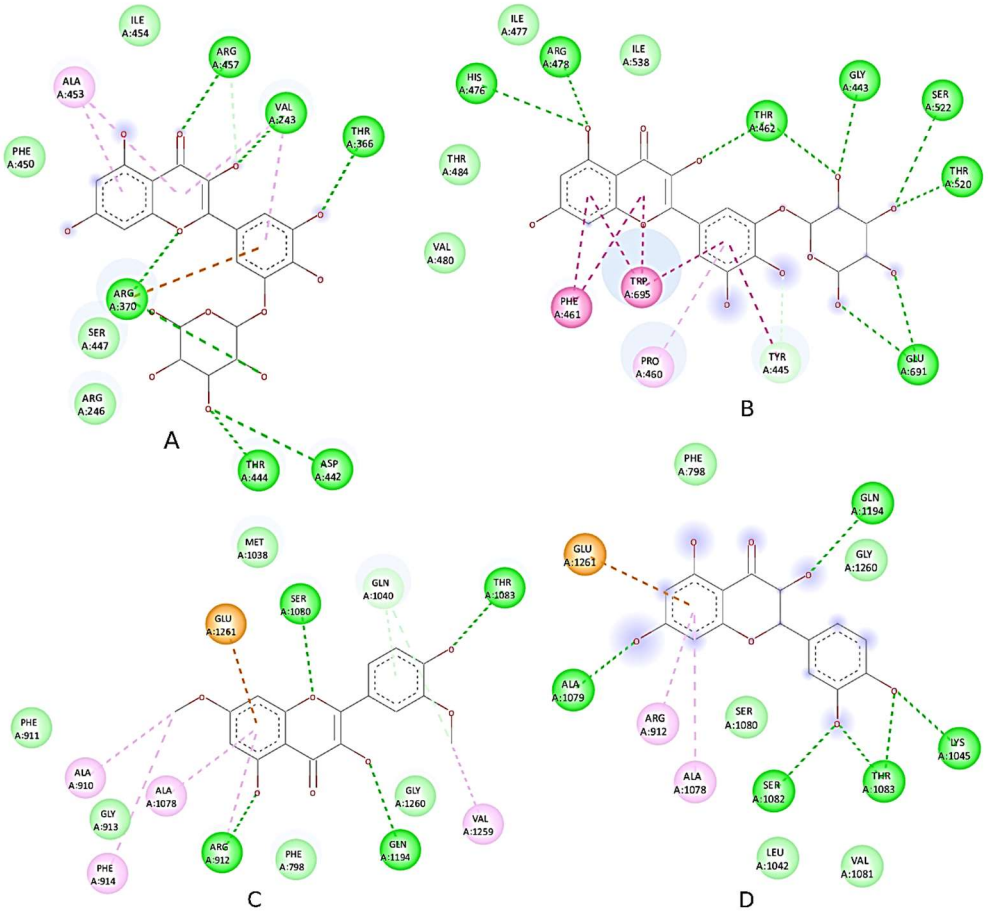

Figure 3. Interaction of quercetin 3'-glucoside with lipoxygenase (A), quercetin 3'-glucoside with NADPH oxidase (B), taxifolin 3-O-α-arabinopyranose with xanthine oxidase (C), and taxifolin with xanthine oxidase (D)

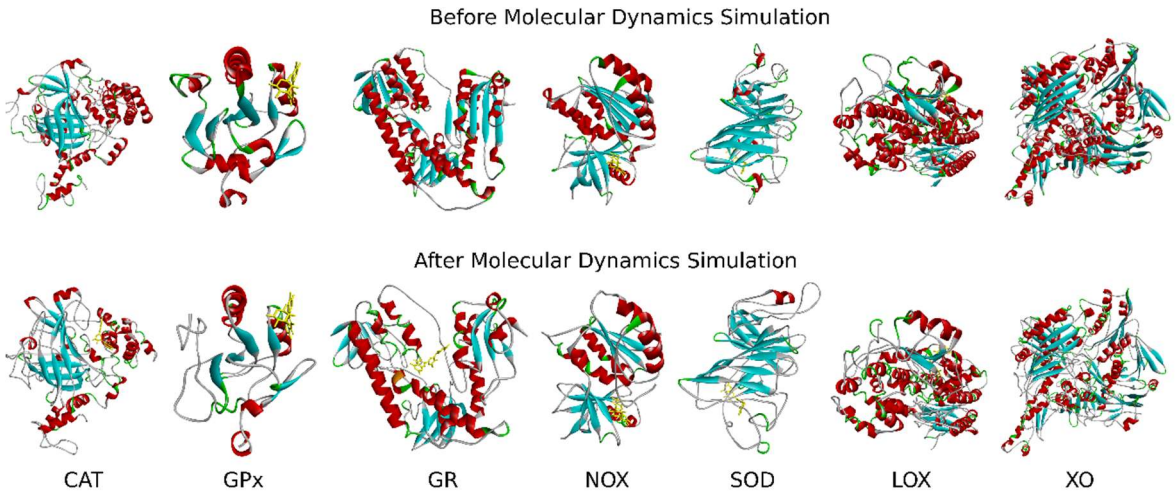

Figure 4. Structure flexibility of the enzymes in molecular dynamics simulation.

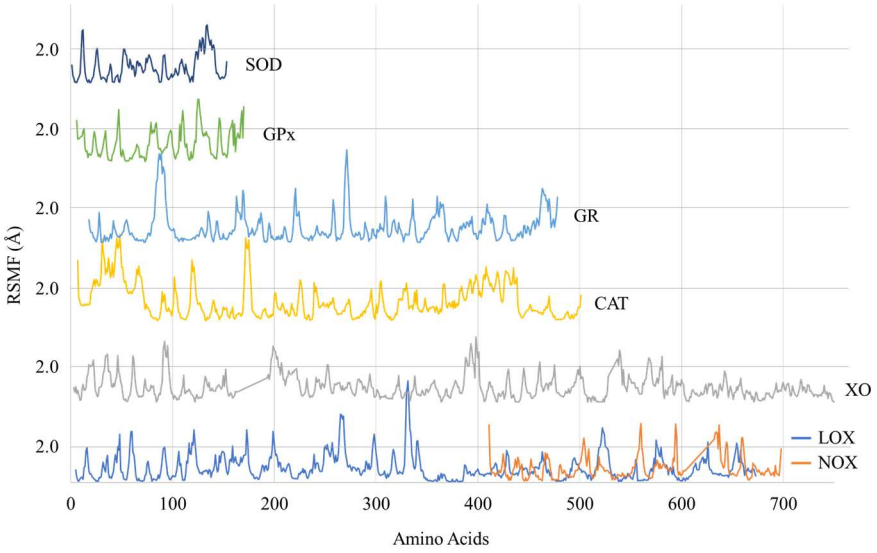

Figure 5. Root mean square fluctuations (RMSF) of protein structures in molecular dynamics simulation responding to quercetin 3'-glucoside binding

Table V. Top 10 crucial amino acids on each protein binding pocket

Protein	Amino Acid	Average Bond Length (Å)	Score
CAT	His305, Phe198, Phe446, Ser201, Arg203, Asp202, Gln442, Ala445, His194, Val450	4.665, 4.421, 4.808, 4.594, 4.763, 4.774, 4.372, 5.037, 4.786, 5.561	36.625, 28.375, 24.375, 20.75, 20.375, 15.125, 13.375, 13, 12.25, 10.625
GR	Val370, Cys63, Asp441, Gly439, Val64, Phe372, Lys66, Thr369, Gln445, His80	4.576, 4.982, 4.912, 4.719, 4.963, 5.356, 4.79, 4.602, 4.393, 4.626	23.5, 16.75, 15.25, 11.5, 9.5, 7.75, 7.75, 6.875, 6.375, 6.25
GPx	Asp101, Met102, Phe100, Lys105, Val98, Arg152, Lys90, Asp23, Gly154, Gly128	4.786, 4.814, 4.99, 4.545, 4.788, 4.393, 4.379, 4.775, 4.265, 4.602	20.375, 19.25, 15.625, 13.875, 13.375, 13, 11.875, 10.125, 10.125, 9
SOD	Val148, Val7, Asn53, Gly10, Lys9, Asp11, Gly147, His63, Asn65, Cys146	4.654, 4.856, 4.895, 4.308, 4.957, 4.814, 4.934, 4.662, 4.985, 4.928	24.25, 20.875, 14.625, 13.125, 12.75, 11.625, 10.25, 8.5, 7.25, 7
LOX	Val243, Ser447, Ala453, Asp442, Arg370, Phe450, Leu244, Asp285, Leu448, Ile365	4.989, 5.061, 4.812, 4.987, 4.443, 4.697, 4.855, 4.632, 4.686, 5.284	25.25, 19.25, 16.25, 13.5, 12.25, 10.5, 9.75, 9.375, 8, 7
NOX	Thr462, Pro460, Arg478, Phe461, His459, His476, Trp695, Thr484, Ile477, Tyr445	4.168, 4.692, 4.672, 4.794, 5.087, 4.851, 4.948, 4.728, 4.722, 5.167	53.75, 31.75, 26.625, 25.625, 15.25, 15, 13.625, 10.5, 9.625, 9.125
XO	Ser876, Arg912, Phe649, Leu648, Lys771, His875, Glu802, Glu879, Phe911, Gly913	4.675, 4.333, 4.791, 5.265, 4.158, 5.319, 5.14, 4.547, 4.524, 4.603	19.75, 9.75, 8.375, 8.25, 7.875, 7.75, 5.875, 5.375, 4.5, 4.375

an average bond length ranging from 4.158 – 5.319 Å with a score range of 4.375 – 19.75.

Acute Oral Toxicity Study (LD_{50})

Based on the results of acute oral toxicity prediction, it is known that flavone compounds and quercetin derivatives are included in class 5 (2000 < LD50 5000) with a low possibility of toxicity. However, taxifolin, taxifolin 3-O-α-arabinopyranose, and quercetin were predicted to be toxic if swallowed (see Table VI).

Discussion

This initial examination was conducted to study how Buah Merah can provide anticancer activity. It is well known that ROS has a significant

Table VI. Prediction of acute oral toxicity (LD_{50})

Compounds	LD_{50} Acute (mg/kg)	Class	Average similarity (%)	Prediction accuracy (%)
4',6,6',8-tetrahydroxy-3-methoxy-flavon	3919	5	74.19	69.26
3,4',5-trihydroxy-7,3'-dimethoxy flavon	5000	5	87.78	70.97
Taxifolin 3-O-α-arabinopyranose	2000	4	75.84	69.26
Quercetin 3-O-glucose	5000	5	84.89	70.97
Quercetin 3- methyl-ether	5000	5	88.31	70.97
Quercetin	159	3	100	100
Taxifolin	2000	4	100	100
Quercetin 3'-Glucoside	5000	5	78.57	69.26

role in the different phases of tumorigenesis (Chirumbolo *et al.*, 2018). By targeting enzymes in redox-sensitive pathways, cancer prevention and therapy will hold considerable prospects (Reuter *et al.*, 2010; Gào and Schöttker, 2017). Therefore, the target enzyme analysis of Buah Merah content was carried out on antioxidant enzymes (CAT. GR, GPx, and SOD) and pro-oxidant enzymes (LOX, NOX, and XO). There are 16 identified metabolites contained in Buah Merah. Eight of them are flavonoid compounds, and the rest are carotenoids. Based on target class studies, only flavonoid compounds have the potential and have a high probability of interacting with oxidative stress modulating enzymes.

The flavonoid compounds used are flavone, quercetin, and taxifolin. Based on the molecular docking results, quercetin compounds produced the highest binding affinity. Therefore we focused on studying the quercetin compounds. There are 4 quercetin compounds used, namely quercetin, quercetin 3-O-glucose, quercetin 3-methyl-ether, and quercetin 3'-glucoside. The presence of 3-O-glucose or 3'-glucoside groups in the core structure of quercetin results in additional hydrogen bonds, either acceptor or donor. Substitution of the hydroxyl group into a methyl-ether group at the C-3 atomic position does not increase the potential for bond formation. The addition or substitution of alkyl groups on quercetin compounds greatly affects its activity. The substitution of the hydroxyl group with a glucoside group on the C-3 atom of quercetin caused an increase in the binding affinity for CAT, GPx, and all pro-oxidant enzymes by 0.93 - 8.42%, but a decrease in binding affinity occurred with GR and SOD enzymes. This complies with the literature wherein the β-glycosidase compound also has a high binding affinity because it has a glucoside group (Materska, 2008). Except for XO, all ROS regulating enzymes show a considerable increase in binding affinity when the glucoside group is substituted for the C-3' atom (10.22 - 18.52%). Methyl ether substitution on the C-3 atom decreased the binding affinity towards all the ROS modulating enzymes (see Table VII). The methyl ether group is reported to be able to block the hydrogen bonding or break hydrogen bonds. Perhaps the hydrogen bonds weren't completely broken, but they were weakened (Muchtaridi, Megantara and Purnomo, 2018).

Based on the results of molecular docking, it can be seen that all the flavonoids of Buah Merah worked synergistically to bind strongly to different ROS modulating enzymes. Judging from the structure and pharmacophore studies, all the test ligands have great potential to form hydrogen bonds either as donors or acceptors. This property makes the test ligands superior to native and reference ligands. In Figure 6, it can also be seen that quercetin 3'-glucoside attaches to the NOX receptor in a fitting position compared to quercetin. The addition of the glycoside group increases the binding energy through the formation of 5 hydrogen bonds. It can be analogized that the glucoside group acts as an anchor in strengthening the attachment of quercetin 3'-glucoside.

In addition to the position of the structure (rotation and size of the molecule) that fits the receptor, quercetin 3'-glucoside also binds to crucial amino acid residues stably. RMSF fluctuations > 2.0 Å did not occur in the amino acid residue in the enzyme's binding pocket and thus the attachment of quercetin 3'-glucoside remained stable. Prediction of acute oral toxicity showed quercetin 3'-glucoside belonged to class 5 (LD50 ≥ 5000 mg/kg BW) which is indicative of low toxicity.

CONCLUSION

Flavonoids from Buah Merah were found to elicit anticancer activity through the modulation of antioxidant and pro-oxidant enzymes. Among the several flavonoids tested, quercetin 3'-glucoside is

the most potent compound in binding to CAT, GR, GPx, SOD, LOX, and NOX receptors with binding energy values of -11.2, -9.7, -8.6, -10.2, -10.7, and -12.8 kcal/mol, respectively. Meanwhile, at the XO receptor, taxifolin 3-O-α-arabinopyranose produced the highest binding affinity of -10.0 kcal/mol. Compared to native and reference ligands, each test ligand formed stable interactions with ROS modulating enzymes and bound critical amino acids, resulting in robust adhesion. The glycone moiety (i.e. glucoside) of quercetin 3'-glucoside played an important role in the determination of proper binding position in the attachment and also supports the formation of hydrogen bonds with the receptors. With low acute oral toxicity, it can be concluded that quercetin 3'-glucoside from Buah Merah is a potent oxidative stress modulator in cancer prevention and therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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