

On the Hypolipidemic Activity of Elicited Soybeans: Evidences Based on Computational Analysis

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Abstract: Dietary intervention plays a significant role in regulating hyperlipidemia. Besides, elicited soybean has greater bioactive contents with numerous health benefits potential. However, there is no evidence of the hypolipidemic activity of elicited soybean. This study will explore the potential mechanism of hypolipidemic activity of bioactive compounds from elicited soybean through computational modeling. The phytocompounds from elicited soybean were identified by Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS), then screened for potential toxicity and drug-likeness properties. Compounds with low potential toxicity and excellent drug-likeness properties were screened for hypolipidemic activity against 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) and Peroxisome Proliferator Activator Receptor-gamma (PPAR- γ) through molecular docking coupled with molecular dynamics. The result showed that phytocompounds from the isoflavonoid group have an excellent affinity to bind with the HMGCR and PPAR- γ . Daidzein, Genistein, and Glycitein interacted with the catalytic residues of HMGCR to act as potential inhibitors with great affinity and stability. Genistein and Glycitein showed strong affinity and stability during their interaction with the agonistic sites of PPAR- γ . Further, the protein network described that targeting HMGCR inhibitor and PPAR- γ had the advantage in orchestrating cholesterol metabolism homeostasis. In summary, isoflavonoids from elicited soybean may have hypolipidemic activity through HMGCR inhibition and PPAR- γ activation.

Keywords: elicitation; HMGCR; hyperlipidemia; PPAR- γ ; soybeans

■ INTRODUCTION

Dysregulation of blood cholesterol levels remains a concern for global health risks. With the growing population year by year, many metabolic-related diseases become elevated, mainly related to high blood cholesterol levels or hyperlipidemia [1]. Hyperlipidemia could appear due to genetic or epigenetic factors [2-5]. An uncontrolled number of cases becomes a severe threat to global health conditions because it strongly relates to the risk of cardiovascular diseases [6-7]. Numerous efforts have been issued to combat these health issues, including targeting

several target proteins associated with cholesterol metabolism. The most systematic attempt to alleviate blood cholesterol levels is diminishing cholesterol biosynthesis by inhibiting the HMGCR enzyme as a limiting factor for cholesterol metabolism [8-9]. Recent studies also revealed the involvement of the PPAR- γ in physiological cholesterol regulation [10-11]. Targeting this protein has shown to have promising effects in shrinking unregulated blood cholesterol levels.

Typical medical treatment accompanied by dietary interventions has increased patient health condition

based on blood lipid profile [12]. Legumes consumption has a beneficiary effect on improving the lipid profile [13]. However, the exact mechanism underlying this effect remains elusive. Some researchers suggest that the protective effect of legumes appears due to the high protein content in legumes [14-16]. Contrary, several studies also underline that bioactive compounds such as phenolic and flavonoid groups contribute to repair lipid profiles by regulating several pathways related to cholesterol metabolism [17-20]. Nevertheless, those results are still in line with the protective effect of legume consumption in maintaining blood cholesterol at a harmless level.

As widely available legumes, soybean may serve great potential to overcome this condition. Further, several biotechnological approaches have been developed to increase its beneficial effect by augmenting bioactive compounds through the elicitation process [21-23]. Previously, the elicitation using a traditional food fermenter called *Ragi Tape* (RT) has improved soybean's bioactive compound [23]. Those compounds, mainly from isoflavonoids, have been significantly increased, compared to non-elicited soybeans [23]. A meta-analysis from clinical trials reported that soy isoflavones supplementation could repair the patient's blood lipid profiles [24]. *In vitro* and *in vivo* experiments also described the hypolipidemic activity of soy isoflavones that occurred at the transcriptomic scale by modulating PPAR, ATP-Binding Cassette super-family G member 5 (ABCG5), and ABCG8 [18,25]. However, the mechanism at the proteomic level remains inconclusive. Further, no study evaluated the hypolipidemic activity of isoflavones derived from soybean elicitation. Thus, this study will reveal the potential mechanism of the anti-cholesterol effect of several bioactive compounds from elicited soybeans to comprehend the puzzle of that mechanism using computational predictions enriched with biological network analysis.

■ EXPERIMENTAL SECTION

Materials

Soybean (*Glycine max*) var. Anjasmoro was purchased from Research Center for Legumes and Tuber

(BALITKABI), Malang, East Java, Indonesia. RT (NKL) was obtained from a local market in Sawojajar, Malang, Indonesia. All the reagents for compound extraction and chromatography were purchased from Merck.

Instrumentation

A rotary evaporator and freeze dryer were used for the extraction stage. Thermo Scientific Dionex Ultimate 3000 RSLCnano coupled with Thermo Scientific Q Exactive mass spectrometer was employed to do phytochemical chromatography of the elicited soybean extract.

Procedure

Elicitation and extraction

Elicitation and extraction of elicited soybean were performed according to a previously described method with minor modification [23]. The elicitation was begun with surface sterilization of soybean using 70% ethanol for an hour, then washed using the sterilized water for three times. The pre-sterilized soybean was soaked with the sterilized water for 24 h and inoculated with powdered RT (100 g/kg). The inoculated soybean germinated under 60 Watt lightbulb (17.00–09.00) on moisturized cotton in an aseptic milieu for 3 d. The elicited soybean was then harvested and washed with water to proceed with the extraction process. The extraction was performed by grinding the harvested soybean with 80% ethanol and soaking it at 50 °C for an hour. The soaked extract was kept until it reached room temperature and continued to centrifugation (4,000 rpm, 15 min, room temperature). The supernatant was filtered through GF/B grade Whatman paper and used for evaporation and freeze-dried to obtain the final crude extract of the elicited soybean.

Phytochemical screening

The procedure for phytochemical screening was done as previous study [26]. The extract was dissolved in water in advance of chromatography analysis. The chromatography step used the following conditions: 0.1% of formic acid in water (solvent A), 0.1% of formic acid in acetonitrile (solvent B), a Hypersil GOLD aQ 50 1 mm 1.9 m particle size column with a flow rate of

40 $\mu\text{L}/\text{min}$, and a run time of 40 min. The obtained peaks from the chromatography were then analyzed with Compound Discoverer using mzCloud MS/MS Library as the reference database. Compound with best match score greater than 70% then directed into the subsequent analysis.

Three-dimensional structure retrieval of proteins and compounds

Three-dimensional structures and SMILES code of each compound were retrieved from the PubChem database. On the other hand, protein structures were downloaded from the RCSB PDB database with PDB ID 1DQ8 for HMGCR and 1ZEO for PPAR- γ . Simvastatin (PubChem CID: 54454) was used as a control molecule for HMGCR inhibitor, while Rosiglitazone (PubChem CID: 77999) was used as a standard PPAR- γ agonist.

Toxicity estimations and drug-likeness prediction

The compounds from phytochemical screening were first screened using Protox II [27] to determine the possible toxicity according to Lethal Dose 50 (LD_{50}), toxicity class, and potential toxicity properties like Hepatotoxicity, Carcinogenicity, Immunotoxicity, Mutagenicity, and Cytotoxicity. Compounds with toxicity class below class IV and toxicity scores more prominent than 0.7 were not considered for further analysis. Next, the compound that passed toxicity screening was evaluated for drug-likeness properties according to Lipinski's Rule of 5 (LRO5) with Molinspiration web server (<https://www.molinspiration.com/cgi-bin/properties>) as a properties calculator. Finally, the compounds which passed toxicity estimations and complied with LRO5 were directed into a molecular docking study.

Molecular docking

Molecular docking analysis was performed by AutoDock Vina [28] in PyRx 0.9.5 software [29]. Docking was targeted into catalytic sites of HMGCR [30] with grid setting as follows: grid center (x,y,z) = 10.918; 13.570; -3.583 with dimension (x,y,z) = 24.508; 24.695; 32.649. Taken differently, docking for PPAR- γ was directed into agonistic residues [31] with following grid settings: grid center (x,y,z) = 20.752; 8.069; 18.936 with dimension (x,y,z) = 27.533; 31.511; 29.521. Compounds were energy minimized using the Open Babel plugin in PyRx, and

protein structures were prepared by removing the pre-attached ligand and the water molecules with Discovery Studio 2019 prior to the docking process. The complex of compound-ligand with binding affinity lesser than -7.0 kcal/mol then continued for ligand-residues interaction analysis using Discovery Studio 2019. Compound with a larger number of interactions with the catalytic site of HMGCR and targeted residues for agonist interaction with PPAR- γ were directed into molecular dynamics analysis.

Molecular dynamics

Molecular dynamics were performed for 50 ns with AMBER14 forcefield [32] accompanied by the following parameters: pH 7.4; 0.9% NaCl concentration; 0.997 water density; 1 atm pressure; and 310 °K temperature with cubic grid shape. All simulations were performed in YASARA software version 21 [33]. The binding energy calculations were also performed by fast boundary method with YASARA binding energy macros.

Network analysis

The protein-protein network was constructed by Cytoscape 3.9.1 [34-35]. First, the protein network for HMGCR and PPAR- γ were separately built with the STRING protein query [36]. A network for hyperlipidemia-related proteins was also constructed using the STRING PubMed query. All obtained networks were then merged and analyzed for certain proteins directly interacting with HMGCR and/or PPAR- γ . All proteins which directly interacting with both target proteins were then annotated for pathway contribution with DAVID bioinformatics resources [37]. The pathways related to cholesterol metabolism with an adjusted p -value lower than 0.05 (according to the value of Bonferroni adjusted p -value calculations) were analyzed as a possible outcome for proteins target interaction with studied compounds.

RESULTS AND DISCUSSION

Phytochemical Contents, Toxicity Screening, and Drug-Likeness Prediction

Twenty-seven compounds have been identified from the liquid chromatography screening. Most of the

identified compounds were amino acids commonly found in soybeans. Several isoflavonoids were discovered, such as Daidzin, Daidzein, Glycitein, and Genistein (Table 1). Those identified compounds were then screened for possible toxicity according to ProTox II estimations. 2-Amino-4-methylpyrimidine was found to have a possible hepatotoxicity profile, while D-(+)-Maltose and DL-Alanine were out due to the toxic features according to the LD₅₀ value (Table S1). The drug-likeness estimations revealed that three out of twenty-two compounds have at least one violation. Those compounds were DL-Arginine, Hexadecanamide, and Oleoyl Ethanolamide. DL-Arginine had too many hydrogen bond donors, whereas Hexadecanamide and Oleoyl Ethanolamide have issues

with the molecular lipophilicity and flexibility according to the miLogP values and the number of the rotatable bond, respectively (Table S2). Finally, the compound that passed toxicity and drug-likeness screening then continued to the molecular docking process to identify the potential interaction of those compounds with the target proteins.

Molecular Docking

The molecular docking study was applied to screen the interaction of potential compounds with the target proteins. A compound with a lower binding affinity will most likely bind with the target protein at the lowest energy state [38], meaning that the compound will have

Table 1. Identified compounds from elicited soybeans

Compound	Formula	Molecular weight	RT [min]	Area (Max.)	mzCloud best match
2-Amino-4-methylpyrimidine	C ₅ H ₇ N ₃	109.06382	0.887	1311983.309	83.9
2-Hydroxyphenylalanine	C ₉ H ₁₁ NO ₃	164.04673	0.783	8270183.059	90.8
4-Piperidone	C ₅ H ₉ NO	99.0682	1.171	14191771.62	84.2
Choline	C ₅ H ₁₃ NO	103.09947	1.172	410265087.5	97.1
D-(+)-Maltose	C ₁₂ H ₂₂ O ₁₁	364.09657	0.779	1788810.247	84
D-(+)-Proline	C ₅ H ₉ NO ₂	115.06307	0.106	3401508.434	82.5
Daidzein	C ₁₅ H ₁₀ O ₄	254.05696	1.302	2226330.007	86.8
Daidzin	C ₂₁ H ₂₀ O ₉	378.15324	0.746	1171272.058	78
DL-Alanine	C ₃ H ₇ NO ₂	89.04769	0.791	11015320.41	80.5
DL-Arginine	C ₆ H ₁₄ N ₄ O ₂	174.11112	1.05	2087367.731	87.5
DL-Carnitine	C ₇ H ₁₅ NO ₃	161.10452	0.825	2846167.878	82.3
Genistein	C ₁₅ H ₁₀ O ₅	270.05214	1.304	1097771.052	83.5
Glycitein	C ₁₆ H ₁₂ O ₅	284.0674	1.304	1734281.135	88.5
Hexadecanamide	C ₁₆ H ₃₃ NO	255.25517	0.59	41651087.62	94.2
Indole-3-acrylic acid	C ₁₁ H ₉ NO ₂	187.06254	0.8	5417342.154	79.9
L-Aspartic acid	C ₄ H ₇ NO ₄	133.03697	0.779	2205969.197	77.5
L-Glutamic acid	C ₅ H ₉ NO ₄	147.05252	0.126	3115695.536	84.6
L-Histidine	C ₆ H ₉ N ₃ O ₂	155.06902	0.123	4532141.832	97.1
L-Norleucine	C ₆ H ₁₃ NO ₂	131.09418	1.243	26860652.75	86.9
L-Phenylalanine	C ₉ H ₁₁ NO ₂	165.07837	1.228	11348033.16	86.2
L-Pyroglutamic acid	C ₅ H ₇ NO ₃	129.04203	0.777	50208100.2	90.3
Oleoyl ethanolamide	C ₂₀ H ₃₉ NO ₂	307.28615	0.694	4604834.084	81.1
Pipecolic acid	C ₆ H ₁₁ NO ₂	129.07857	1.255	3500976.934	71.2
Proline	C ₅ H ₉ NO ₂	115.06305	0.796	22176141.84	83.5
<i>trans</i> -3-Indoleacrylic acid	C ₁₁ H ₉ NO ₂	187.06238	0.094	603385.7943	86.8
Trigonelline	C ₇ H ₇ NO ₂	137.04709	0.82	25223635.13	94.8
Valine	C ₅ H ₁₁ NO ₂	117.07862	12.323	297719.5179	84.3

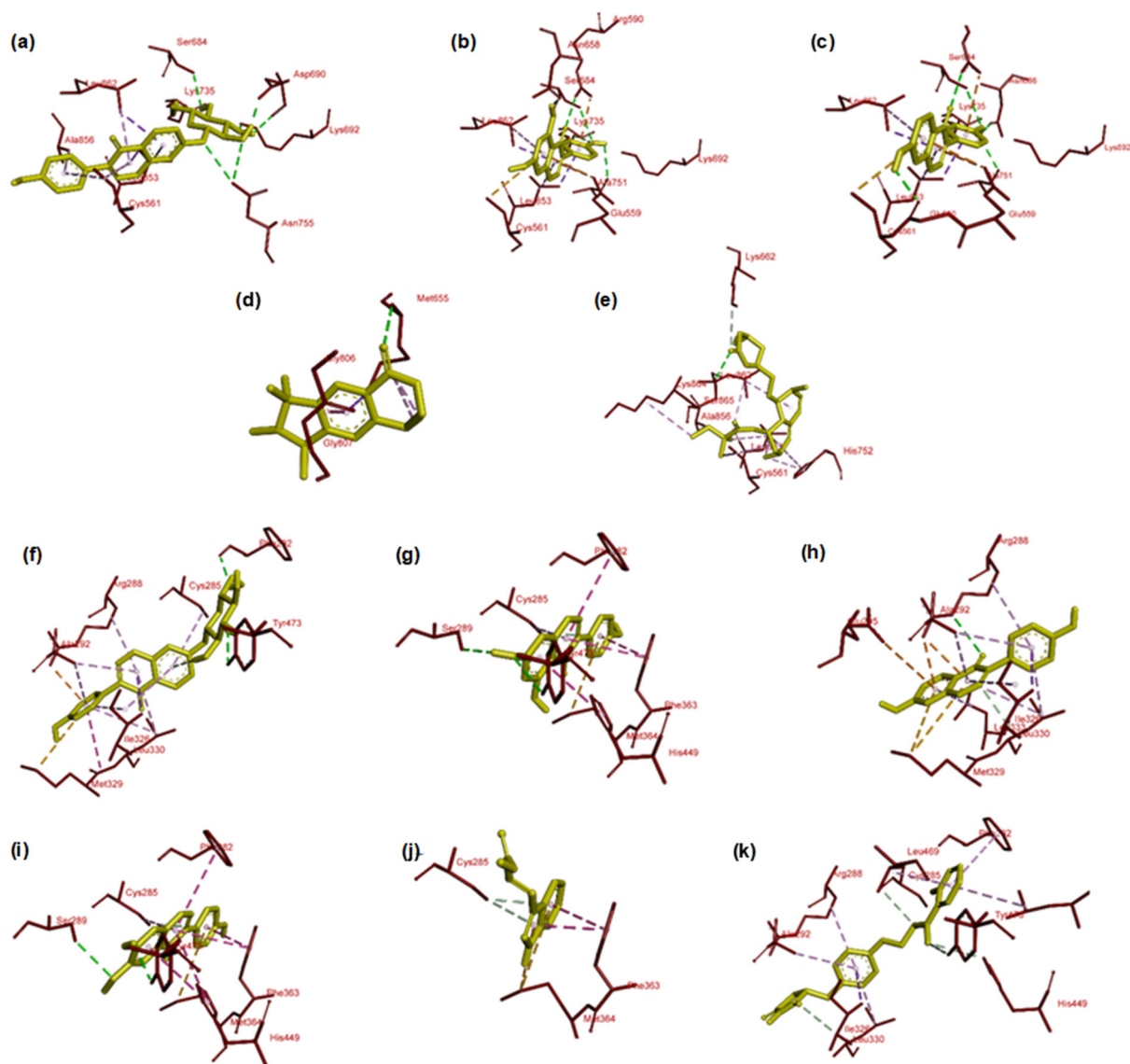


Fig 1. Interacting residues of screened compounds with HMGCR (a-e) and PPAR-γ (f-k). Consecutively, the compounds were Daidzin (a, f); Genistein (b, g); Daidzein (c, h); Glycitein (d, l); Indole-3-acrylic acid (j), Simvastatin (e), and Rosiglitazone (k)

a high probability of acting as an agonist or antagonist. Thus, the binding affinity becomes the first predictor, but not the main one, for predicting the likelihood of a small molecule interacting with the target and performing a biological activity. According to the binding affinity calculations, isoflavonoid compounds performed better than other pre-screened compounds. Daidzin, Daidzein, and Genistein, but not with Glycitein, have lower binding affinity than the known HMGCR inhibitor, i.e., Simvastatin. This result suggests that those three

compounds have a better probability of interacting with the HMGCR as an inhibitor than Simvastatin. Similarly, Daidzin, Genistein, and Glycitein also have lower binding affinity than Rosiglitazone as an agonist compound for PPAR-γ (Table 2). The interacting residues analysis also showed that the isoflavones could bind with the catalytic sites of HMGCR [30] greater in number than Simvastatin (Fig. 1(a-e)). Glu559, Arg590, Ser684, Asp690, Lys692, Lys735, Asn755, and Leu853 were catalytic residues that interacted, mostly with hydrogen

Table 2. The binding affinities of the compound from ESB are lower than -7 kcal/mol

Protein	Binding affinity (kcal/mol)						
	Daidzin	Daidzein	Genistein	Glycitein	Indole-3-acrylic acid	Simvastatin	Rosiglitazone
HMGCR	-9.6	-8.5	-8.6	-8.3	-6.7	-8.4	-
PPAR- γ	-9.4	-8.2	-8.8	-9.0	-7.2	-	-8.8

bond, with the isoflavones (Fig. 1(a-e), Table S3). Therefore, Daidzin, Daidzein, Genistein, and Glycitein were then directed to the molecular dynamic simulations.

During cholesterol metabolism regulation, PPAR- γ plays a role in regulating blood cholesterol levels by organizing oxidized Low-Density Lipoprotein (oxLDL) scavenging [39]. Rosiglitazone is compound with an agonistic effect on PPAR- γ by interacting with several essential residues such as Cys285, Ser289, His323, Ile326, His449, and Tyr473 [31]. Genistein and Glycitein were the compounds that had the most interaction, particularly hydrogen bonds, with those essential residues (Fig. 1(f-k), Table S4). The results agreed with the previous study that soy isoflavones could have an agonistic effect on PPAR- γ expression [25]. Thus, Genistein and Glycitein may have similar action as PPAR- γ agonists as Rosiglitazone. Consequently, those two compounds will go to the

molecular dynamics simulations to understand interaction stability with the PPAR- γ .

Molecular Dynamics Simulations

Selected compounds from the molecular docking have better stability of interaction with the HMGCR than Simvastatin, according to the molecular dynamics results. The Root-Mean-Square Deviation (RMSD) value of the backbone structure revealed that complexes of HMGCR with the isoflavones have better stability than HMGCR-Simvastatin (Fig. 2(a)). Likewise, RMSD of the ligand structure also confirmed that the isoflavones had better structural stability than Simvastatin (Fig. 2(b)). The number of hydrogen bonds among those complexes appeared to format a similar number, with several isoflavones, such as Daidzin and Daidzein having more hydrogen bonds formed during simulations (Fig. 2(c)). The Root Mean Square Fluctuation (RMSF) of each residue

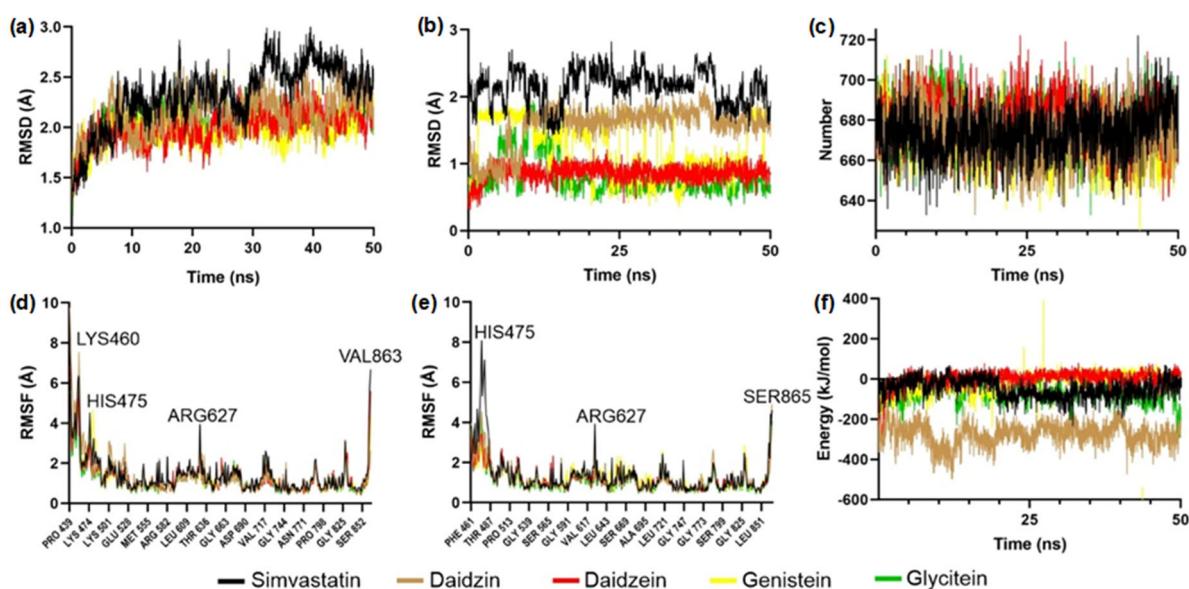


Fig 2. Molecular dynamics simulation on the interaction of HMGCR with selected compounds: RMSD backbone (a), RMSD ligand (b), number of hydrogen bond (c), RMSF of chain A (d), RMSF of chain B (e), and binding energy calculations (f)

in chains A and B of HMGCR was similar to the other parameters, even with significant low fluctuations occurring among analyzed complexes (Fig. 2(d-e)). Nevertheless, binding energy calculations discovered that Daidzein, Genistein, and Glycitein have better stability than Simvastatin (Fig. 2(f)). Those three isoflavonoids were the possible compound to have hypolipidemic activity through modulating *de novo* cholesterol biosynthesis. These results supported previous studies which reported that Daidzein, Genistein, and Glycitein could repair blood lipid profile [40-43] and suggested a new mechanism of hypocholesterolemic activity through the modulation of cholesterol biosynthesis. The inhibition of HMGCR as the rate-limiting factor of cholesterol biosynthesis upregulates LDL Receptor (LDLR) and allows plasma cholesterol uptake to achieve lower blood cholesterol levels [44].

Referring to the molecular dynamics results, Genistein and Glycitein showed better stability than Rosiglitazone. The isoflavones presented better stability according to the RMSD backbone value (Fig. 3(a)) and RMSD of ligand structure (Fig. 3(b)). The number of hydrogen bonds also appeared to increase in Genistein and Glycitein complexed with the PPAR- γ than Rosiglitazone after 25 ns (Fig. 3(c)), suggesting they may have more potent activity as PPAR- γ agonists. Fluctuations are also lower in Genistein- and Glycitein-

PPAR- γ complex than Rosiglitazone-PPAR- γ . Lys263 displayed more significant fluctuations in the Rosiglitazone-PPAR- γ complex (Fig. 3(d)). However, the binding energy calculations demonstrated that only Genistein might have better stability than Glycitein relative to Rosiglitazone as a control (Fig. 3(e)). Still, Genistein and Glycitein showed better performance than Rosiglitazone in modulating PPAR- γ activity. Activating PPAR- γ through agonistic activity modulated apolipoprotein synthesis, resulted in normal plasma cholesterol levels and increasing of atheroprotective effects [45-46]. Therefore, the agonistic activity of Genistein and Glycitein might have beneficial role in achieving average plasma cholesterol levels.

Biological Network

The protein-protein network described the potential implications of targeting HMGCR and PPAR- γ to orchestrate blood lipid levels. HMGCR and PPAR- γ interacted directly with the SREBF1 and SREBF2 as significant transcription factors in cholesterol metabolism [47-48]. Several proteins that have a substantial role in cholesterol metabolism interact with HMGCR and PPAR- γ . Hence, targeting HMGCR and PPAR- γ may serve as a potential mechanism of the hypocholesterolemic activity of isoflavones [49] (Fig. 4(a)). Annotation results from the proteins directly interacting with the target

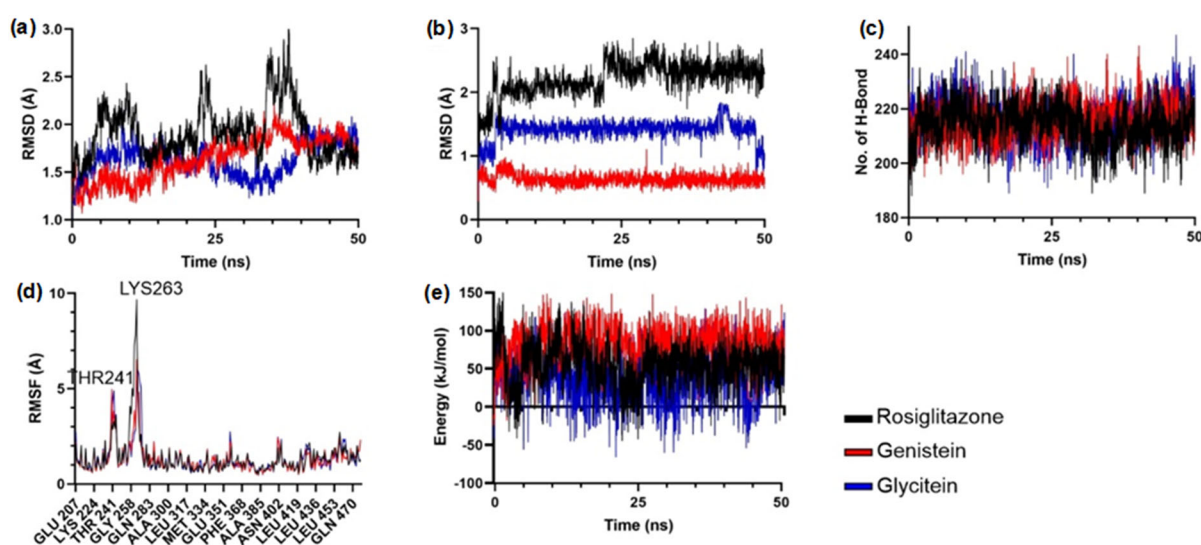


Fig 3. Molecular dynamics simulation on the interaction of PPAR- γ with selected compounds: RMSD backbone (a), RMSD ligand (b), number of hydrogen bonds (c), RMSF of chain A (d), and binding energy calculations (e)

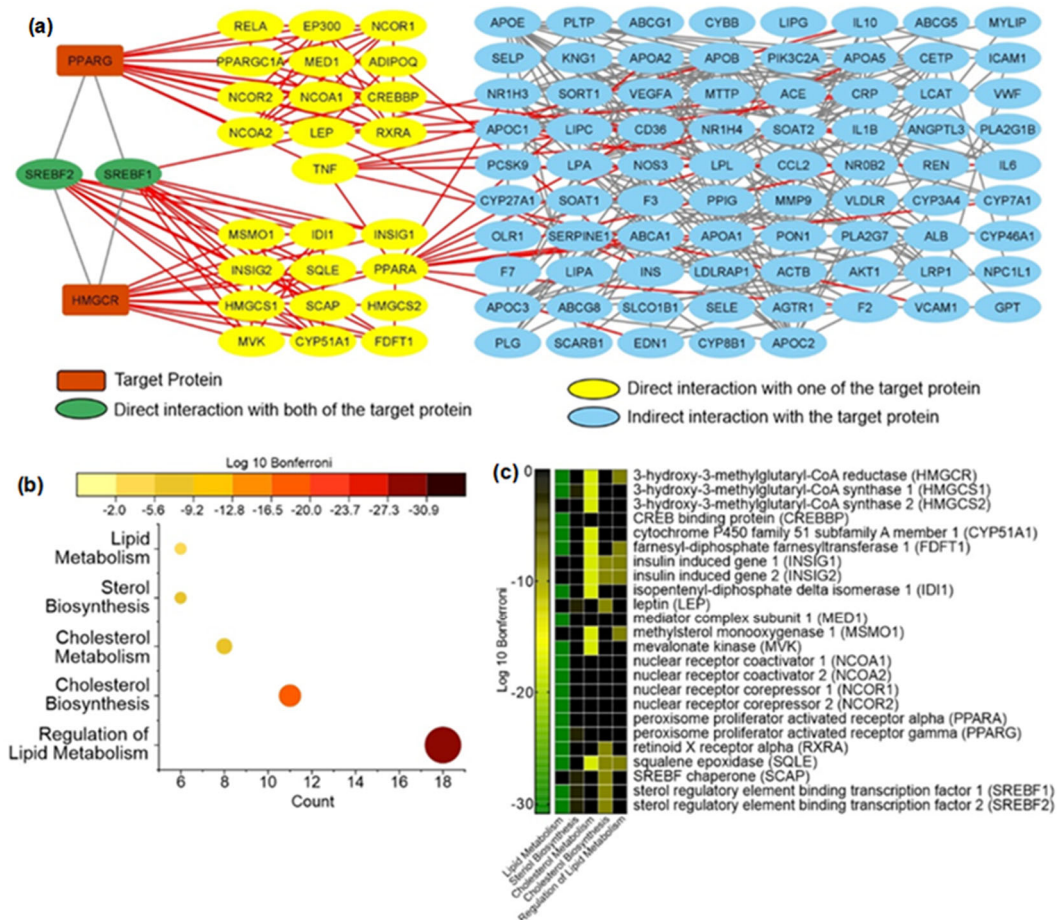


Fig 4. Protein-protein network on the interaction of HMGCR and PPAR- γ (a) and annotation result according to the direct interaction of the proteins with HMGCR and PPAR- γ (b) as well as the list of proteins involved in cholesterol metabolism based on log₁₀ Bonferroni *p*-value (c)

proteins, also titled those involvements in the stage-managing cholesterol metabolism process (Fig. 4(b)). Targeting HMGCR and PPAR- γ will have a direct effect on several cholesterol-limiting factor proteins such as Hydroxymethylglutaryl-CoA Synthase (HMGCS), Cyclic adenosine monophosphate Response Element Binding Protein (CREBBP), Mevalonate Kinase (MVK), Insulin Induced Gene (INSIG), Retinoid X Receptor Alpha (RXRA), and so on (Fig. 4(c)). Essentially, targeting HMGCR and PPAR- γ has a promising impact on mediating the cholesterol-lowering process in people undergoing hyperlipidemia.

CONCLUSION

Several amino acids and isoflavonoids were identified from elicited soybean extract. The screening

based on toxicity and drug-likeness properties showed that most of the identified compounds have low toxicity and a high probability of having properties like a drug. The molecular docking and molecular dynamics analysis revealed that the isoflavonoids from the elicited soybean might become an excellent hypolipidemic agent. Those compounds were Daidzin, Daidzein, Genistein, and Glycitein. The hypolipidemic activity of elicited soybean may occur through regulation of *de novo* cholesterol biosynthesis by inhibiting HMGCR activity and modulating PPAR- γ signaling.

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■ AUTHOR CONTRIBUTIONS

Feri Eko Hermanto conducted the experiment, Warsito conducted the phytochemical analysis, Muhaimin Rifa'i and Nashi Widodo wrote and revised the manuscript and funding acquisition. All authors agreed to the final version of this manuscript.

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