

HMG-CoA Reductase Inhibitory Activity of *Gnetum gnemon* Seed Extract and Identification of Potential Inhibitors for Lowering Cholesterol Level

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ABSTRACT

Objectives: Melinjo (*Gnetum gnemon*) seeds have been known to have some biological properties. One of them is ant hypercholesterolemia. The present study investigated *in vitro* and *in silico* methods to predict potential antihypercholesterolemic of the Melinjo seed extracts of through HMG-CoA reductase inhibitory activity. **Methods:** Melinjo seed powders were successively extracted by reflux method using five solvents with gradient polarity including: n-hexane, dichloromethane, ethyl acetate and methanol. All extracts were evaluated *in vitro* using HMG-CoA Reductase assay kit, to analyze the inhibitory activity. Molecular docking of the phytochemical content of the seeds were carried out using Auto Dock Vina, and also Ligand Scout to analyses interaction between ligand and receptor. **Results:** Dichloromethane extract demonstrated the highest inhibitory activity against HMG-CoA reductase with IC₅₀ value is 0.40 µg/mL, followed by that of ethyl acetate extract. UPLC-MS analyses showed that dichloromethane extract contained *trans*-resveratrol, piceid, gnetin C, gnetol, isorhapontigenin, ε-viniferin, gneomonol L, and gneomonol M. Molecular docking studies demonstrated that dimer of resveratrol such as gneomonol L, gneomonol

and ε-viniferin have better free binding energy than that of monomer. piceid, gnetin C, gneomonol L, and gneomonol M could be considered as HMG-CoA reductase inhibitor. **Conclusion:** *Gnetum gnemon* seed extract showed strong HMG-CoA reductase activity. Resveratrol dimer promises as a potential lead compound to design/synthesize anti-cholesterol.

Key words: *Gnetum gnemon*, Melinjo, HMG-CoA Reductase, Resveratrol, Stilbene, Molecular Docking.

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INTRODUCTION

Gnetum gnemon belongs to Gnetaceae family is indigenous to Southeast Asia. This plant was known as Melinjo in Indonesia. The leaves and the seeds have been consumed as foodstuff for centuries in Java Island.¹ The seeds flour was reported to be rich in protein, carbohydrates and crude fiber.² The seeds were made to be Emping (local name) by crushing the endosperms that obtained the heated seeds, and the dried drying, and deep-frying.³ Other than as a food ingredient melinjo seeds are also reported to have benefits for health.³

Kato *et al* reported that the seed extracts of Melinjo demonstrated DPPH radical scavenging, pancreatic lipase and α-amylase inhibitory, and anti-immunostimulatory activities.⁴ The administration of the seed extracts rich in resveratrol dimers in mice significantly reduced body weight and anti-metabolic syndrome.⁵ Melinjo seed extract (MSE) demonstrated potent antitumor activity in a colon tumor bearing mice.⁶ A study in mice, showed that MSE could improve several parameters of metabolic syndrome. In a clinical study, administration MSE decreased the serum uric acid levels by inhibiting the reabsorption of uric acid in the renal tubular epithelia as well as by increasing the HDL cholesterol levels by PPAR agonistic activity with no cause of the damage to health.⁷ Ethyl acetate extract of the seed demonstrated strong ACE inhibitory activity, and molecular docking analysis indicated dimer of resveratrol was considered as bioactive compound.⁸

The Gnetaceae family is known to contain stilbenoid group including resveratrol, gneomonols A-L, gneomonosides K, and gnetin E. These

compounds were also successfully isolated the stem and root of *Gnetum gnemon* L.⁹ While, melinjo seeds have been reported containing gnetin L, gnetin C, gneomonosides A, C, D, and resveratrol.⁴ This compound distributed in some species plant including *Veratrum grandiflorum*, *Polygonum cuspidatum*, *Vitis vinifera* and a wide variety of other food sources.^{10,11} Resveratrol has been believed to explain the lower incidence of myocardial incidence.^{12,13} Animal studies have shown that resveratrol could reduce the expression of HMG-CoA reductase mRNA.¹⁴ It has driven considerable interest towards resveratrol in Melinjo molecular activities.

Molecular docking has been use to search and design new potential drug from natural products, such as flavonoid and alkaloid.^{15,16} Based on the biological activity of Melinjo seeds and its stilbene component, this study presented the HMG-CoA inhibitory activity and molecular docking of the stilbene to predict *in silico* HMG-CoA reductase inhibitory activity.

MATERIALS AND METHODS

Melinjo seeds

Fresh Melinjo fruits were used in this study were purchased from a local market Jakarta, Indonesia. Seeds were physically separated from the hard-coat and dried. Dried seeds were then ground to a fine flour (mesh size 30) by using a blender. The powdered seed packed in air-tight polyethylene plastic jar and refrigerated at 4°C, prior to analysis.

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Preparation of extract

Dried melinjo seed powder was successively extracted using gradient solvents: n-hexane, dichloromethane, ethyl acetate, methanol, and distilled water. Crude extract obtained after removing solvent using rotary vacuum evaporator at 50°C (Buchi, Switzerland). While aqueous extract directly freezes dried with freeze dryer (ScanLaf, Denmark) into white powder.

HMG-CoA reductase assay

The HMG-CoA reductase inhibitory activity was performed on the extracts according to Zhou *et al* with slight modification, with pravastatin as the positive control.¹⁷ Briefly, a reaction system *in vitro* consist of NADPH (4 µl) and the substrate (HMG-CoA, 12 µl) was diluted with potassium phosphate buffer (100 mM, pH 7.4) containing KCl (120mM), EDTA (1mM) and 5 m DTT (5mM) until final volume 200 µl. Two µL HMG-CoA reductase (Sigma-Aldrich, Louis, USA) was added to the reaction mixture as initiation time (time 0) and was incubated at 37°C in the presence or absence of samples solution (1 µl).

The absorbance of NADPH was determined spectrophotometrically every 20 sec for up to 10 minutes at 340 nm using a SpectraMax plus 384 microplate reader, equipped with double-beam lamp and a controlled thermostatically cell holder. Softmax Pro® by Molecular Devices, LLC was used for the control the equipment and data evaluation. Therefore, the inhibitory activities were expressed as % inhibition using the following equation:

$$\% \text{ Inhibition} = \frac{\Delta \text{ Positive Blank Absorbance} - \Delta \text{ Sample Absorbance}}{\Delta \text{ Positive Blank Absorbance} - \Delta \text{ Negative Blank Absorbance}} \times 10$$

Resveratrol assay

Resveratrol assay was performed on a Shimadzu HPLC LC-20AT, with UV detector, YMC C₁₈ column (150 x 4.6 mm I.D., 5µm), and temperature column 25°C. Water/acetonitrile (75/25) was adjusted to pH 3.00 as mobile phase with flow rate 1.0 mL/minute.¹⁰ Injection volume was set up at 20 µL, monitored at 306 for 15 minutes.

LC-MS analysis of dichloromethane extract

Liquid chromatographic separations were conducted on a Xevo G2 Acquity UPLC™ with BEH C₁₈ column (50 mm × 2.1 mm, particle size=1.7 µm, Waters Corp, Milford, MA, USA). The column and auto-sampler tray temperature were maintained at 35 and 4 °C, respectively. The mobile phase consisting of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) was used at a flow rate 0.3 ml/min. The gradient elution was from 95% A to 0% A within 6 min and then held for 1 min. From 7 to 7.5 min, the percentage of A was increased to 95% and maintained about 1.5 min, then reduced to the initial condition within 1 min and balanced for 2 mins.

The total running time was 10 min, and the sample injection volume was set at 5 µl. The UPLC system was connected to the mass spectrometer through an ESI interface and was operated in the positive ion detection mode. Nitrogen used for desolvation was 500 L/h, cone gas was set at 16 L/h. The capillary voltage was set at 3 kV, source temperature 110 °C, desolvation temperature 300°C. Data acquisition was processed with Masslynx 4.1 software.

In silico experiments

Dataset and protein-inhibitor complexes

In silico simulations were carried out using platform with core i7 processor. A dataset of stilbene from Melinjo and Pravastatin were obtained from the published literature and used for docking. Structural information was downloaded from <http://www.chemspider.com/Chemical-Structure>. X-ray crystal structures of HMG-CoA reductase in complex with the two

inhibitors were downloaded 3-hidroxy-3-metyl-glutaric acid [MAH] and simvastatin acid from the Protein Data Bank (PDB), complex of catalytic portion of human HMG-CoA reductase (HMGR) (PDB ID: 1DQ8)¹⁸ and HMGR in complex (PDB ID: 1HW9)¹⁹ <http://www.rcsb.org/pdb/>

Molecular docking

AutoDock4.2 was used for molecular docking studies calculation, using a Lamarckian genetic algorithm between flexible ligand and rigid receptor, a population size of 300, a maximum of 250000 generations and 2 500 000 evaluations for 100 GA runs.(20)(20) The root means square deviation tolerance was set to 2.0 Å for the clustering of docking results. Interaction ligand-receptor were processed using pharmacophore feature of LigandScout (Inte:Ligand, Vienna, Austria). Visualization all ligand for hydrophobicity used program UCSF Chimera 1.10.2.

RESULTS

HMG-CoA reductase inhibitory activity

Table 1 showed HMG-CoA reductase inhibitory activity of the extracts. Dichloromethane extract demonstrated the best inhibition activity with 64.78% at 100 ppm, was followed by ethyl acetate extract with inhibition activity 57.86%. Further investigation, the IC₅₀ of dichloromethane extract was 0.40 mg/ml. However, HMG-CoA reductase inhibitory activity of dichloromethane extract is lower than the *trans*-resveratrol.

Resveratrol content

Resveratrol assay was determined by HPLC. Figure 1 shows the chromatogram of resveratrol and dichloromethane extract. Resveratrol content of each extract was presented in Table 2. Resveratrol was found in dichloromethane, methanol and ethyl acetate extracts. Dichloromethane extract contained the highest level of resveratrol, followed by methanolic extract.

LC-MS analysis

LC-MS chromatogram showed that extract contains seven prominent peaks with molecular weight 227, 262.29, 280.296, 395.361, 455.195, 537.331, 617.203, and 779.248 au (Figure 2). Some peaks suggested to originate from the stilbene contained in Melinjo seeds, such as: gnemonoside B (m/z-779.248) and gnetin C (m/z-455.195).

Molecular docking

HMG-CoA Reductase appears as a tetrameric protein complex, structure of enzyme is shown on image below (Fig 3A and 3B). Due to efficiency of time, docking procedure was conducted using dimeric structure of enzyme complex. The validation was carried to assure the accuracy and precision of the used method. HMG-CoA Reductase enzyme complex [PDB ID 1HW9] and [PDB ID 1DQ8] were downloaded from protein data bank and redocked against their original ligand. MAH as the original ligand of 1DQ8 gave free binding energy value -7.19 kcal/mol and RMSD

Table 1: HMG-CoA reductase inhibitory activity of the sample

Sample	Inhibition Activity (%)
n-Hexane extract	22.27
Dichloromethane extract	64.78
Ethyl acetate extract	57.86
Methanolic extract	41.85
Water extract	25.64
<i>Trans</i> -Resveratrol	94.61

*The values are average from the triplicate experiments. Sample concentrations are 100ppm.

Table 2: Resveratrol content in melinjo seed extracts

Extract	Concentration (mg/kg)
n-Hexane extract	-
Ethyl acetate extract	686
Dichloromethane extract	8 190
Methanol extract	3 184
Water extract	-

*The values are average from the triplicate experiments.

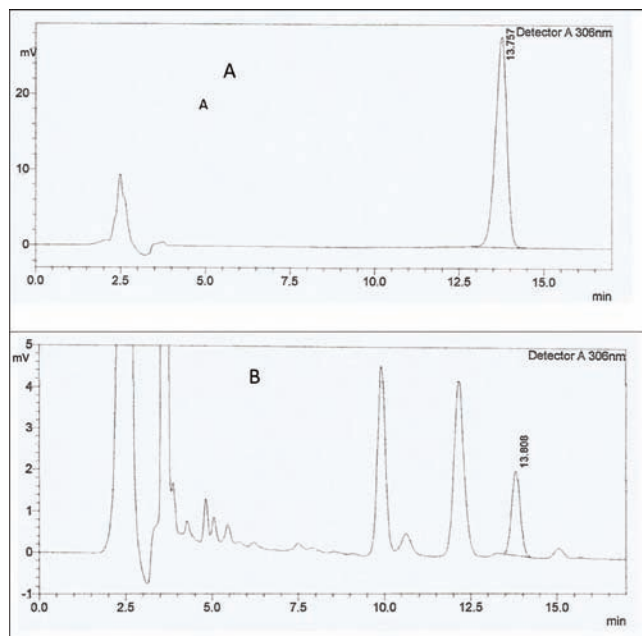


Figure 1: HPLC Chromatogram of resveratrol (A) and (B) dichloromethane extracts

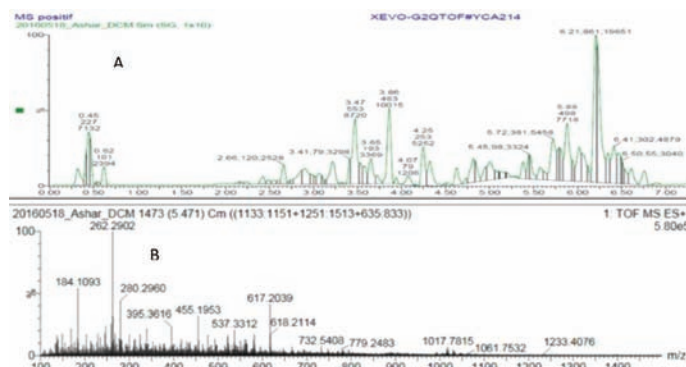


Figure 2: LC-MS/MS chromatogram of dichloromethane extract

value 1.12 Å. HMG-CoA Reductase complex with simvastatin gave free energy binding value as high as -9.76 kcal/mol and RMSD as high as 1.45 Å. Obtained RMSD value of redocked MAH and simvastatin are lower than 2.00 Å. Therefore, the docking method is valid to dock several compounds of dichloromethane melinjo seed extract. Table 3 shows free binding energy of docked stilbenes in Melinjo seed with their ligand.

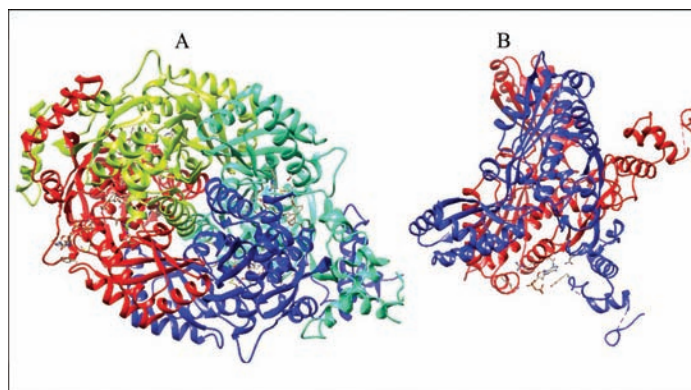


Figure 3: A) Tetrameric and B) dimeric HMG-CoA Reductase enzyme PDB ID 1HW8

DISCUSSION

Gnetaceae family was well known to be rich in stilbenoids and to be used as traditional medicine for treatment of bronchitis, arthritis, and asthma. Stilbenoids oligomers have pharmacological activity, such as: to reduce the level of sugar in the blood, show anti-inflammatory activity and induce apoptosis in colon cancer. Stilbene from *Ampelopsis brevipedunculata* var. *hancei* revealed ACE inhibitory activity *in silico* and *in vitro*. Resveratrol tetramers interact with ACE more favorably than other smaller oligomers. One member of this family is *Gnetum gnemon*. The fruits, leaves, and seeds are used as an ordinary vegetable in Indonesia. The occurrence of various stilbenoids was also reported in the seeds, roots and stems of the plant, however there is no information about the usage of the seeds as traditional plants. In the *in silico* study, dimeric resveratrol from the seeds of the plant demonstrated ACE inhibitory activity.⁸ The seed of Melinjo revealed strong antioxidant and tyrosinase inhibitory activity

Resveratrol as standard was quantified to obtain calibration curve and regression equation. Linearity R-value and regression equation were 0.9998 and $Y=106945X-229881$. Retention time of resveratrol observed under 306-320 nm wavelength was given a maximum peak at 7 minutes (Figure 2).

All samples were evaluated *in vitro* for their capability to inhibit human recombinant HMG-CoA reductase catalytic activity. The IC_{50} value of pravastatin as positive control was determined in preliminary test for the standardization. Calibration curve yielded a linearity with R-value=0.997 and regression equation of $Y=109.8X+45.909$. Obtained IC_{50} value of pravastatin was 87.76 nM, which was in concordance range (44.10 – 70.25 nM) with other study.^{22,23,24}

Sample solutions were observed in the same way of standard. Each peak in chromatogram was identified by comparing the retention time and UV spectra of the samples with that standard. Quantitation was carried out by standard external. Peak's area, then, plotted into regression equation. Resveratrol was present on three samples include ethyl acetate extract, dichloromethane, and methanol extract, with a result of 8.190, 3.184, and 686 mg/kg, respectively (Table 2). Meanwhile, n-hexane and aqueous extract gave a non-significant result on HPLC separation and UV detection. The retention time of samples was similar to resveratrol after 7 minutes. UV spectra also compared with a previous study,¹⁰ which displayed a split peak, shown in Figure 1.

LC-MS chromatogram exhibited that extract contains seven prominent peaks with molecular weight 227, 262.29, 280.296, 395.361, 455.195, 537.331, 617.203, and 779.248 au. In the other hand, there are several small peaks which may have also contributed to enhance the activity.

Cho *et al.* (2008) reported that resveratrol (m/z -229.0625) is responsible for its attenuation against the expression of HMG-CoA reductase mRNA in hamsters.¹⁴ Kato *et al.* (2009) also reported some chemical constituents from Melinjo seed extract such as gnetonoside B (m/z -779.24); gnetin C (m/z -455.14); and resveratrol (m/z -229.0625) are responsible for anti-oxidant, antimicrobial, and other pharmacological properties.⁴ Whereas in present study distinct peaks of molecular weights (m/z -227au) was very similar to resveratrol; (m/z 779.248) was similar to gnetonoside B; and (m/z -455.19) was similar to gnetin C.

The results of *in vitro* test demonstrated that each extract of melinjo seeds have the capability to inhibit activity of HMG-CoA reductase. Dichloromethane and ethyl acetate showed the strongest HMG-CoA reductase inhibitory activity. Dichloromethane extract demonstrated significantly inhibition activity of HMG-CoA reductase and exhibited concentration dependent manner with inhibition activity on the enzyme. Additionally, resveratrol (standard) was tested and the IC_{50} value was determined.

Results of docking were analyzed and the best-docked confirmation was chosen based on their number of conformations in a cluster and the lowest free energy binding. Higher numbers of conformations and the lowest binding energy indicated better affinity of the compound to be docked into HMG-CoA reductase enzyme with PDB ID 1DQ8, 1HW9.

As shown in Table 3, external compounds, piceid, gnetin C, gnetinol L, gnetinol M, viniferin and latifolol docked better and results lower free energy binding than the original ligand MAH in the HMG-CoA reductase enzyme [PDB 1DQ8]. Gnetin C, gnetonoside B, gnetinol M and gnetinol L, docked better than the ligand SIM4 (simvastatin) with PDB 1HW9 and ligand pravastatin as reference drug.

Compounds in Melinjo seed perform contacts with LEU853, LEU857, and LEU862, which these amino acids are the most important residues for hydrophobic interaction. There are several amino acid seed those give similarity with statin drug (Pravastatin) and MAH as the original ligand of HMG-CoA Reductase: LYS691, ASN658, MET657, MET655 with PDB 1DQ8; amino acid LYS691, LEU853, GLU559, LYS735, HIS752, LEU857, ASP690 with PDB 1HW9 (Figure 4, 5 and 6). These results indicate that the interaction of piceid, gnetin C, gnetinol L with HMG-CoA reductase (PDB ID 1DQ8 and 1HW9) residues might lead another way to develop novel HMG-CoA reductase inhibitor. Further study was needed to evaluate anti-hypercholesterolemia activity of the extract and stilbenoid from the seed of this plant through preclinical and clinical studies. In addition, safety study was needed to guarantee the use of the extract.

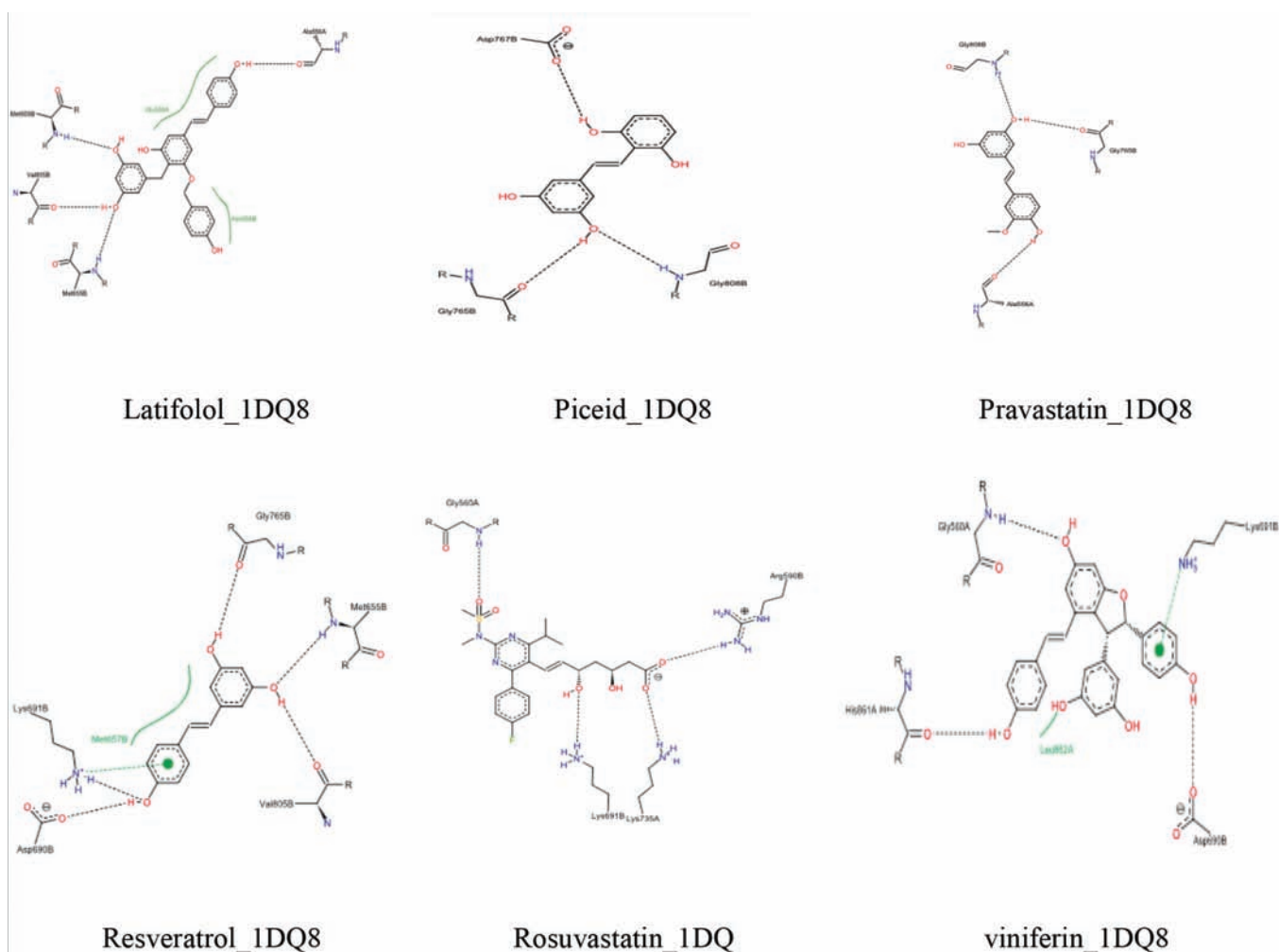


Figure 4: Binding mode of compound compounds with HMG CoA reductase [PDB 1dq8] by docking studies using Autodock4, visualization by PoseView

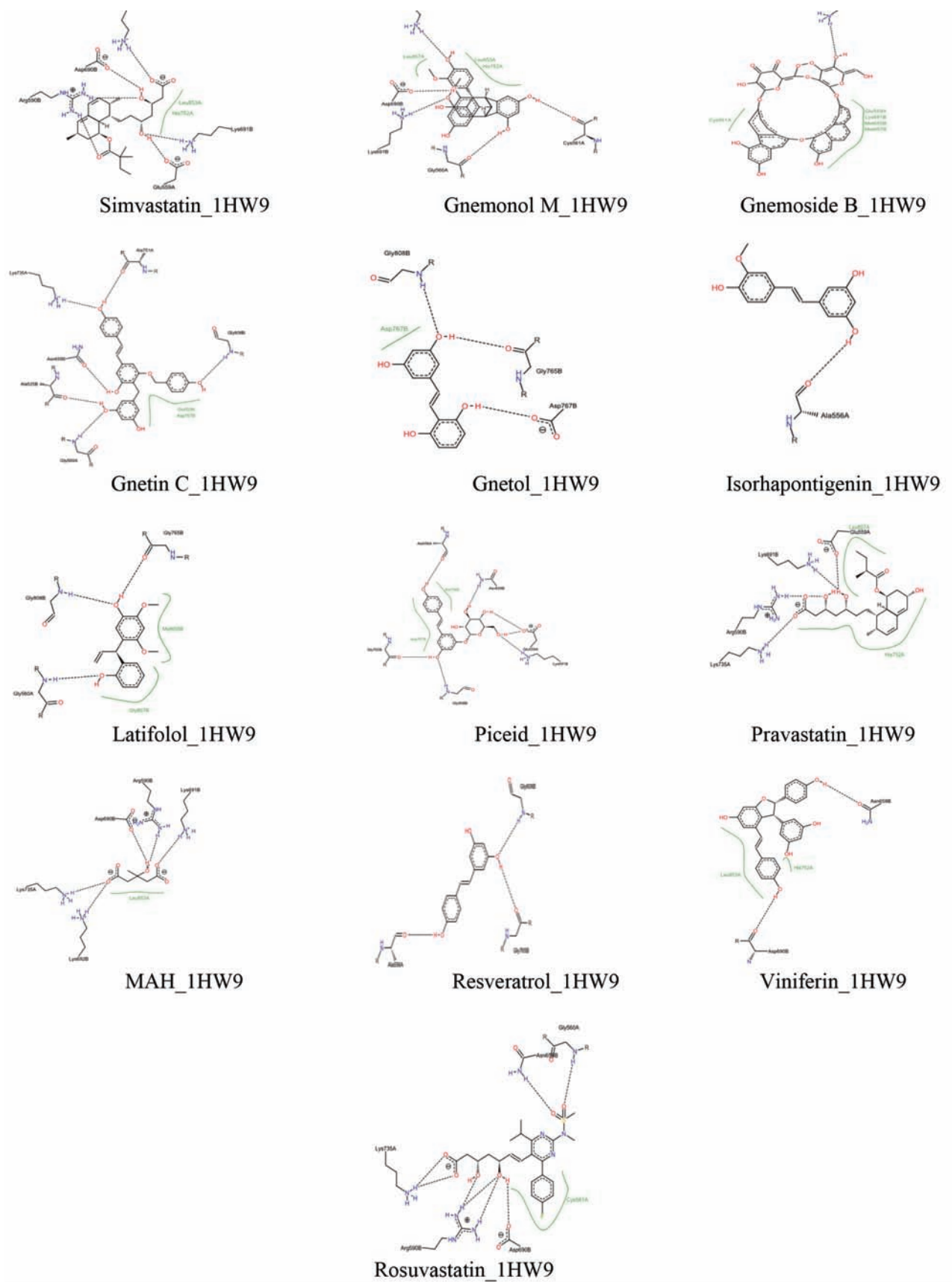


Figure 5: Binding mode of compounds with HMG-CoA reductase [PDB 1hw8] by docking study using Autodock 4, visualization by Pose View

Table 3: Free binding energy (kcal/mol) of MAH, SIM4 (original ligand) resveratrol and isolated compounds from Melinjo seed

Compound	Affinity (kcal/mol) PDB: 1dq8	Affinity (kcal/mol) PDB: 1HW9	Inhibiton constant (1dq8)	Inhibition constant (1HW9)
1 <i>trans</i> -resveratrol	-7.38	-7.35	3.92 μ M	4.11 μ M
2 Piceid	-9.76	-8.69	69.55 nM	428.71 nM
3 Gnetin C	-10.31	-9.33	27.87 nM	144.31 nM
4 Gnetol	-7.74	-5.94	2.11 μ M	44.05 μ M
5 Isorhapontigenin	-7.37	-7.6	3.95 μ M	2.68 μ M
6 ϵ -Viniferin	-9.28	-7.14	156.83 nM	5.87 μ M
7 Gnemonol L	-13.52	-12.2	123.pM	1.15 nM
8 Gnemonol M	-8.64	-7.42	461.04 nM	3.62 μ M
9 Gnemonoside B	-12.3	-10.27	967.72 pM	29.89 nM
10 Latifolol	-7.58	-4.72	2.77 μ M	349.39 μ M
11 Pravastatin	-7.83	-9.28	157.9 nM	1.81 μ M
12 Rosuvastatin	-9.76	-9.26	70.26 nM	163.2 nM
13 MAH	-6.51	-6.55	16.96 μ M	15.77 μ M

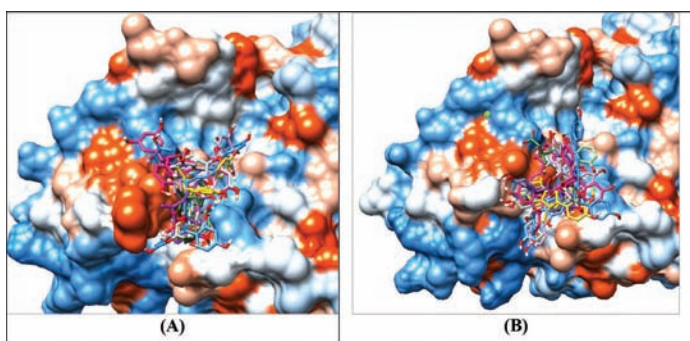


Figure 6: The molecular surface of the binding pocket is coloring and displayed according to hydrophobicity (blue = hydrophilic, orange = hydrophobic), Docking poses of all inhibitors 1-12 obtained for 1DQ8 (A) and 1HW9 (B)

CONCLUSION

Based on the results of the study, it was concluded that melinjo seed dichloromethane extract not only able to hinder the enzymatic activity of HMG-CoA reductase, but also is potentially prohibit with MAH substrate against HMG-CoA Reductase receptor, which might suggest its role in treatment of hypercholesterolemia.

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CONFLICT OF INTEREST

There is no conflict of interest.

ABBREVIATION USED

HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A; **MSE:** Melinjo seed extract; **UPLC-MS:** Ultra-high pressure liquid chromatography-MS-MS.

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