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Short Communication

The Potency of Water Clover (*Marsilea crenata* C. Presl.) Leaves as Anticholesterolemic Functional Foods Through *In Silico* Study

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Abstract

Water clover (*Marsilea crenata* C. Presl.) is a widely available plant in Indonesia and often utilized as a traditional food ingredient. This plant is also traditionally believed to contain compounds that can decrease blood cholesterol. This study aimed to determine the compounds in water clover which have the potential to decrease blood cholesterol through inhibition of the HMG-CoA enzyme using *in silico* approach. This research was done in several steps, i.e., extraction using ethyl acetate solvent, identification of chemical compounds using GC-MS, and screening of compounds with potential to be anticholesterolemic agent through *in silico* using PyRx 0.8 (AutoDockVina and Open Babel GUI version 2.4.1), Discovery Studio Visualizer 2021, and PyMOL™ 1.7.4.5 software. Results showed that ethyl acetate extract of water clover contained 26 compounds, 6 of which were potential to be anticholesterolemic agent, i.e., phytol, 1,2-benzenedicarboxylic acid, 2,4-di- tert-butylphenol, diethyl phthalate, 1,2,3,4-tetramethylbenzene, and dipentene. Binding affinity values of those six compounds were lower than the native ligand of the HMG-CoA reductase, although still higher compared to pravastatin. The binding affinity value of pravastatin was -7.13 kcal/mol and the binding affinity value of 3-methyl glutaric acid as a native ligand was -5.33 kcal/mol, meanwhile, the lowest binding affinity value of compounds in water clover was phytol (-6.37 kcal/mol) and the highest was dipentene (-5.40 kcal/mol). Through *in silico* study, there were six compounds from water clover leaf's ethyl acetate extract that could inhibit the HMG-CoA reductase. Therefore, water clover leaf has the potential to become an anticholesterolemic functional food ingredient.

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1. Introduction

Water clover (*M. crenata*) is one of the aquatic plants widely found in rice fields, ponds, lakes, swamps, and rivers (Titisari *et al*., 2016; Nurjanah *et al*., 2012). In Indonesia, water clover is often used as a vegetable source in traditional food called '*pecel*' (Ma'arif *et al*., 2016). Based on its nutritional content, water clover is a source of potassium, calcium, iron, magnesium, and crude protein (Afriastini, 2003). Water clover is also reported to contain reducing sugar, steroid, carbohydrate, flavonoids, saponin, free triterpenoid, alkaloid, polyphenols, amino acid, and oil (Jacoeb *et al*., 2010; Nurjanah *et al*., 2012; Ma'arif *et al*., 2016). These phytochemicals have the potential to be developed into functional foods. Thus, increasing water clover's economic value (Kurniawan *et al*., 2010; Muslim *et al*., 2020).

Functional foods are types of foods containing active compounds that can give health benefits, other than their nutritional content (Yuniastuti, 2014). One of the functional foods that can be developed is an anticholesterolemic functional food. Anticholesterolemic foods could be developed after both *in vitro* and *in vivo* assessments. Several functional food ingredients that have been reported to have anticholesterolemic effects *in vivo* included grass jelly leaf, *melinjo* peel, curry leaf (*Murraya koenigii*), heart leaf (*Anredera cordifolia*), tamarind, moringa leaf (*Moringa olifera*), kersen leaf (*Muntingia calabura*), bay leaf (*Syzygium polyanthum*), and okra fruit. The use of green grass jelly leaf of 9.37 g/kg body weight/day could inhibit the increase of fat cells and infiltration of macrophages in Wistar rats that have been induced with a high-fat diet (Winthoko *et al*., 2020). This extract contains tannin, flavonoid, polyphenol, alkaloids, and saponin (Tibe *et al*., 2018). The *melinjo* peel extract of 54.15 g/kg body weight for 14 days could decrease triglycerides levels in rats that have been induced with high cholesterol diets (Demitri *et al*., 2018). This extract contains alkaloids, flavonoids, steroids, saponin, lycopene, and vitamin C (Wardani *et al*., 2019). Methanol extract and water extract from curry leaf could decrease total cholesterol and LDL level. This extract contains carbazole alkaloids, essential oil, carotenoids-lutein, phenolics, terpenoids, α-tocopherol, minerals, fibers, nicotinic acid, and vitamin C (Choudhury and Sinha, 2015; Phatak *et al*., 2019). Ethanol extract of heartleaf could inhibit hypercholesterolemia in rats that have been induced with high cholesterol diet. This extract contains alkaloids, flavonoids, phenols, tannin, steroid/terpenoid, and saponin (Sukandar *et al*., 2016). Mesocarps of tamarind could give a hypocholesterolemic effect by suppressing triglycerides accumulation and inhibiting cholesterol biosynthesis (Lim *et al*., 2013). Tamarind fruit contains tannin, saponin, sesquiterpenes, alkaloids, and phlobatannins (Silalahi, 2020). Moringa leaf extract (Jain *et al*., 2010; Nwamarah *et al*., 2015; Saleem and Naureen, 2021), kersen leaf (Alimar *et al*., 2022), bay leaf bark (Sutrisna *et al*., 2018), and okra fruit extract (Rahayuningsih *et al*., 2018) were also reported to decrease blood cholesterol of Wistar rats or exhibiting a hypolipidemic effect. Moringa leaf extract contains phenolics, flavonoids, and condensed tannins. Kersen leaf and bay leaf extract contain saponins, flavonoids, and tannins (Rahmayanti *et al*., 2021), meanwhile, okra fruit contains alkaloids, flavonoids, tannins, polyphenols, steroids, and monoterpenoids. Ethanol extract of heart leaf (Anggraini and Ali, 2017), okra fruit (Djamil *et al*., 2020), edible hibiscus leaf (Ilyas *et al*., 2020), croton leaf (Sahara *et al*., 2021), *suji* leaf (Anggraini and Nabillah, 2018), lime peel (Lutfiyati *et al*., 2021), moringa leaf (Reddy *et al*., 2017), and bay leaf (Hartanti *et al*., 2019) could *in vitro* inhibit cholesterol or lipase enzyme or inhibit HMG-CoA reductase enzyme. Phytochemically, okra fruit contains flavonoids, steroids, triterpenoid, saponin, and coumarin, meanwhile, edible hibiscus extract contains condensed tannin, phenolics, flavonoids, and steroids. Lime peel extract contains flavonoids, saponin, polyphenols, eriocitrin, naringin, tangeretin, essential oils, and pectin. Croton leaf extract contains flavonoids, phenolics, triterpenoids, steroids, and alkaloids.

Various phytochemical compounds in different sample extracts could not conclude the phytochemical compounds that are potential to have anticholesterolemic properties. Meanwhile, the potency of phytochemical compounds with an anticholesterolemic effect could be screened using *in silico* method. *In silico* is a computation-based method to analyze certain chemical compounds and their interactions (Bare *et al*., 2019; Makatita *et al*., 2020).

Water clover is traditionally believed to have the ability to decrease blood cholesterol. Ethyl acetate extract of water clover has been reported to have the highest *in vitro* inhibitory activity against the HMG-CoA reductase enzyme (Hardoko *et al*., 2019). HMG-CoA reductase is a key enzyme in the initial synthesis of cholesterol by converting HMG-CoA into mevalonate which is irreversible (Burg and Espenshade, 2011). Limiting cholesterol biosynthesis to reduce cholesterol levels can be done by inhibiting HMG-CoA reductase (Rinto *et al*., 2015). Water clover has also been reported to contain steroids, flavonoids, saponin, free triterpenoid,

alkaloids, and polyphenols (Jacoeb *et al*., 2010; Nurjanah *et al*., 2012; Ma'arif *et al*., 2016). However, the phytochemical compounds that can inhibit HMG-CoA reductase have not been identified. It is required to identify the phytochemical compounds that have the potential to possess anticholesterolemic properties using *in silico* approach, therefore water clover can be further utilized as anticholesterolemic functional food ingredient. The purpose of this study was to identify the types of phytochemical compounds in water clover leaf through *in silico* study that have the potential as the anticholesterolemic agent by inhibiting the HMG-CoA reductase enzyme.

2. Materials and Methods

2.1 Materials

The material used in this research was water clover (*M. crenata* C. Presl.) leaf obtained from rice fields area in Golokan village, Sidayu sub-district, Gresik district, East Java, Indonesia. The material used for extraction was ethyl acetate (Merck). Materials used for analysis using *in silico* method were HMG-CoA reductase (1DQ8) macromolecules obtained from the Protein Data Bank website (https://www.rscb.org/), pravastatin, native ligand (3-methyl glutaric acid) as control and ligand of compounds from water clover obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/).

Equipment used in this research were oven (Heraeus), freezer, and blender (Phillip). Equipment used for extraction were rotary evaporator (Buchi RE111), UV-Vis double beam spectrophotometer, analytical balance, vortex, measuring cylinder, volumetric pipette, Erlenmeyer (Duran), Whatman no. 43 filter paper, and round-bottom flask. Equipment used for the analysis of bioactive compounds was GC-MS (Gas Chromatography-Mass Spectroscopy) (Agilent) and a notebook (Acer Aspire 3 A311-31-C67G, Intel® Celeron® Dual-Core Processor N4020, RAM 4.00 GB, HDD 500 GB, Windows 10 OS).

2.2 Methods

2.2.1 Extraction of water clover and GC-MS analysis

Water clover leaves were washed, strained, cut, and dried using the oven at 50°C for 6 hours. Leaves were then cooled down until room temperature and size-reduced using a dry blender for 2 minutes until it became powder. Water clover leaf powder was added with ethyl acetate solvent with a ratio of 1:20, homogenized, and macerated using a shaker at room temperature for 24 hours. The mixture from maceration was then filtered using Whatman no. 43 filter paper and the filtrate obtained was concentrated using a rotary evaporator at 50°C and 0.3 atm pressure. Ethyl acetate extract of water clover leaf was obtained and analyzed. Previous research found that ethyl acetate extract of water clover leaf produced higher anti-cholesterol activity *in vitro* compared to hexane extract and methanol extract (Hardoko *et al*., 2019).

Analysis of chemical compounds by GC-MS refers to the method of Suharta *et al.* (2021) with modifications. Ethyl acetate extract of water clover was analyzed using GC-MS (7890A-5975C, Agilent technology), with a column length of 60 cm and an inner diameter of 0.25 mm. The initial temperature of the column was 100°C and the final temperature was 190°C with an increase of 15°C per minute at a pressure of 10.4 psi. The mobile phase used was helium gas and the stationary phase was methyl polysiloxane. About 5 µL of the sample was injected with a flow rate of 1 mL/min, using Ultra High Purity (UHP) helium gas as a carrier gas. The GC detector used was Mass Spectrophotometry Ionization (MS).Compounds released from the column were separated by ionization. Temporary identification of volatile compounds was obtained by comparing the mass spectrum of compounds to the data library (NIST02) and (Wiley275).

2.2.2 Inhibition of HMG-CoA reductase by in silico approach

Bioactive compounds that were identified from ethyl acetate extract of water clover were then analyzed using *in silico* approach by molecular docking. *In silico* method was done using software such as PyRx (It contains AutoDockVina and Open Babel GUI version 2.4.1), Discovery Studio Visualizer and PyMOL. Molecular docking of the active compound obtained from the GC-MS results of water clover leaf with the enzyme HMG-CoA reductase (1T02; resolution 2.15Å) as a receptor using PyRx software. The HMG-CoA reductase enzyme receptor was obtained from the https://www.rcsb.org. The ligands used were obtained from the chemical compound of water clover leaf, native ligand, and Pravastatin. The 3D structure of the ligand was obtained from PubChem https://pubchem.ncbi.nlm.nih.go in sdf format. Then it was converted into pdb format using Discovery Studio software. Receptor preparation was carried out by separating the C chain structure from the intact structure, and then saving it in *.pdbqt format. After being stored, the water molecule was removed, and the natural ligand was separated from the enzyme chain C structure. The file was then saved in *.pdbqt format. The molecular docking process was carried out using AutoDockVina program. After docking was

complete, the results of several docking modes along with the value of binding affinity (kcal/mol) were obtained. Next, the docking results were visualized in 2D using Discovery Studio and 3D using PyMOL.

3. Results and Discussion

3.1 Identification of Chemical Compounds from Ethyl Acetate Extract of Water Clover

There were 26 compounds identified using GC-MS (Table 1). There are some similar chemical compounds and some different compounds identified from ethyl acetate extract of water clover compared to its *n*-hexane extract from previous research by Ma'arif *et al.* (2016). Some similar compounds identified were neophytadiene phytol, palmitic acid, and oleic acid.

Table 1. Compounds that were identified in ethyl acetate extract of water clover

No	Retention Time (min)	Chemical Compound
1	6.18	4-Methyl-2-pentanone
2	6.24	sec-Butyl acetate
3	6.32	Isobutyl acetate
4	6.40	Toluene
5	6.69	Tetrachloroethylene
6	7.07	Ethylbenzene
7	7.13	1,4-Dimethylbenzene
8	7.36	Cyclohexanone
9	7.94	2-Ethyltoluene
10	8.26	1,2,3-Trimethylbenzene
11	8.42	Palmitic acid
12	8.57	Dipentene
13	8.63	Eucalyptol
14	8.96	2-Butoxyethyl acetate
15	9.52	1,2,3,4-tetramethylbenzene
16	11.83	8-Heptadecene
17	12.41	Oleic acid
18	12.99	2,4-Di-tert-butylphenol
19	13.48	1-Hexadecene
20	13.69	Diethyl phthalate
21	14.98	1-Octadecene
22	15.34	Neophytadiene
23	15.65	1,2-Epoxynonadecane
24	16.41	Ethyl palmitate
25	17.39	Phytol
26	21.83	1,2-benzenedicarboxylic acid

The difference can be caused by the difference in solvent's polarity, *n*-hexane is non-polar and ethyl acetate is semi-polar. Furthermore, neophytadiene was reported to have antipyretic, analgesic, anti-inflammation, antimicrobial, and antioxidant properties, meanwhile, phytol had anti-inflammation, antimicrobial, antioxidant, and diuretic properties. Palmitic acid was reported to have antioxidant, hypocholesterolemia, anti-androgenic properties, and inhibitor of 5-alpha reductase, whereas oleic acid had anticancer and anti-androgenic properties.

The compounds from ethyl acetate extract of water clover leaf that have the potential to become antihypercholesterolemic agents were isobutyl acetate, palmitic acid, dipentene, oleic acid, 2,4-di-tert-butylphenol, and phytol (Table 1). Ester (especially methyl butanoic and isobutyl acetate) was also found in cashew "Cerrado" fruit juice and was reported for its ability to decrease cholesterol, triglycerides, and LDL level (dos Santos *et al*., 2020). Palmitic acid was reported to delay alcoholic fatty liver disease and decrease blood cholesterol (Yang *et al*., 2018). Oleic acid had antiradical activity and could act as a plasma antioxidant (Ismail *et al*., 2010). Eucalyptol is a monoterpene oxide that shows antioxidant and anti-inflammatory activity on high cholesterol zebrafish and in rainbow trout that were subjected to crowding stress (Borges *et al*., 2018; Mirghaed *et al*., 2018). 2,4-di-tert-butylphenol is a lipophilic phenol that shows antioxidant activity against LDL oxidation; therefore, it has the potential to prevent atherosclerosis and anti-inflammation properties by decreasing TNF-α, interleukin IL-6, and IL-1b expression in rat macrophage cell lines (Al Hageh *et al*., 2020). Phytol is a diterpene that has antimicrobial, antioxidant, and anticancer properties. Neophytadiene is a compound that has analgesic, antipyretic, anti-inflammation, antimicrobial, and antioxidant activity. The presence of stigmasterol, γ-sitosterol, lupeol, phytol, and neophytadiene in high amounts are known to be responsible for decreasing cholesterol, acting as antioxidants and anti-peroxidase on cardiotoxic rats mediated by doxorubicin (Olorundare *et al*., 2020).

3.2 Screening of Compounds in Ethyl Acetate Extract of Water Clover Leaf that Have Anti-cholesterol Potential

The screening was done using *in silico* method through HMG-CoA reductase inhibition. Molecular docking between the HMG-CoA reductase enzyme and the anti-cholesterol drug pravastatin, native ligands, and water clover extract compounds resulted in different binding affinity energy values. The lower the binding affinity value, the more stable the bond between the enzyme and the ligand (Masula *et al*., 2018).

Table 2. Binding affinity values between native ligand, pravastatin, and bioactive compounds in water clover and HMG-CoA reductase

Retention Time (min)	Chemical Compound	Binding Affinity (Kcal/mol)
-	3-methyl glutaric acid (native ligand)	-5.33 ± 0.21
-	Pravastatin (Control)	-7.13 ± 0.78
6.18	4-Methyl-2-pentanone	-4.33 ± 0.32
6.24	sec-Butyl acetate	-4.87 ± 0.32
6.32	Isobutyl acetate	-4.40 ± 0.10
6.40	Toluene	-4.70 ± 0.00
6.69	Tetrachloroethylene	-3.50 ± 0.10
7.07	Ethylbenzene	-4.87 ± 0.06
7.13	1,4-Dimethylbenzene	-5.03 ± 0.06
7.36	Cyclohexanone	-4.83 ± 0.12
7.94	2-Ethyltoluene	-5.23 ± 0.15
8.26	1,2,3-Trimethylbenzene	-5.30 ± 0.10
8.42	Palmitic acid	-5.27 ± 0.06
8.57	Dipentene	-5.40 ± 0.00
8.63	Eucalyptol	-5.17 ± 0.12
8.96	2-Butoxyethyl acetate	-4.53 ± 0.06
9.52	1,2,3,4-tetramethylbenzene	-5.50 ± 0.00
11.83	8-Heptadecene	-5.03 ± 0.57
12.41	Oleic acid	-4.93 ± 0.21
12.99	2,4-Di-tert-butylphenol	-6.13 ± 0.06
13.48	1-Hexadecene	-4.60 ± 0.36
13.69	Diethyl phthalate	-5.87 ± 0.12
14.98	1-Octadecene	-4.33 ± 0.15
15.34	Neophytadiene	-5.37 ± 0.06
15.65	1,2-Epoxynonadecane	-4.63 ± 0.42
16.41	Ethyl palmitate	-4.93 ± 0.25
17.39	Phytol	-6.37 ± 0.12
21.83	1,2-benzenedicarboxylic acid	-6.33 ± 0.15

Compounds that have the potential as anticholesterolemic agents are the ones inhibiting HMG-CoA reductase with lower than or equal binding affinity to native ligand (3-methyl glutaric acid) (Saputri *et al.*, 2016). Compounds in ethyl acetate extract of water clover which had lower binding affinity compared to native ligand were dipentene, 1,2,3,4-tetramethylbenzene, 2,4-di- tert-butylphenol, diethyl phthalate, phytol, and 1,2-benzenedicarboxylic acid. However, the binding affinity values of compounds from water clover were still higher compared to pravastatin (Table 2). Therefore, bioactive compounds from ethyl acetate extract of water clover are not able to replace pravastatin's role in inhibiting HMG-CoA reductase.

The binding affinity value of pravastatin towards HMG-CoA reductase was -7.13 ± 0.78 kcal/mol (Table 2), lower compared to previous research by Alvi *et al.* (2015), who reported that the binding affinity of pravastatin was -5.63 kcal/mol. This could be caused by the difference in the amount of amino acid residue that binds on HMG-CoA reductase receptors. However, this binding affinity value is still higher compared to the gnemono L compound from *melinjo* with a value of -13.52 kcal/mol (Hafidz *et al.*, 2017).

3.3 Visualization of Protein-Ligand Binding

Visualization was done to explain the inhibitory mechanism of ligand on HMG-CoA reductase, related

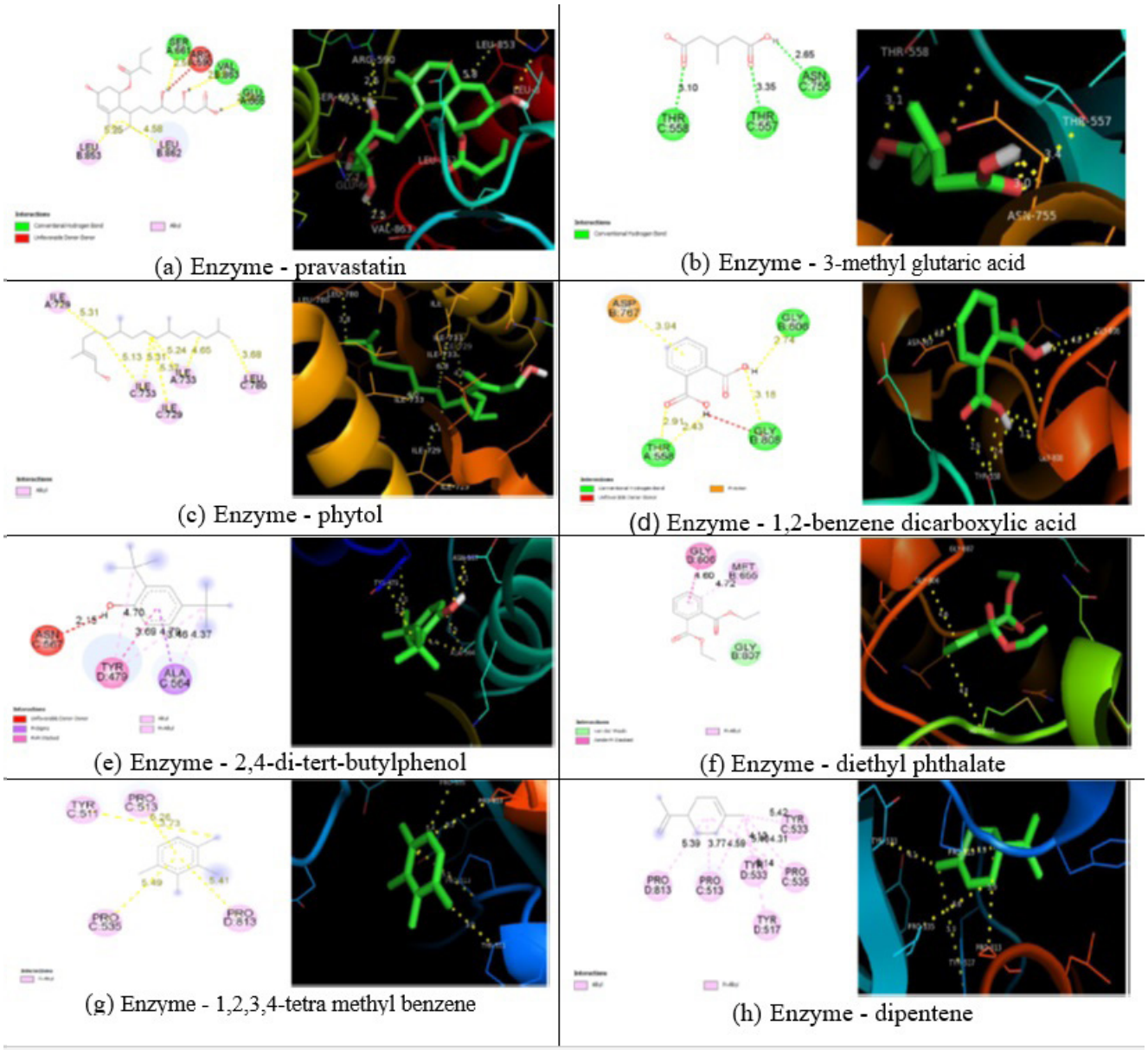

Figure 1. 2D and 3D visualization of the interaction between a) pravastatin; b) 3-methyl glutaric acid; c)phytol; d) 1,2-benzene dicarboxylic acid; e) 2,4-di-tert-butyl phenol; f) diethyl phthalate; g) 1,2,3,4-tetramethyl benzene; h) dipentene and HMG-CoA reductase

to its binding affinity. 2D and 3D visualization will show the type and distance of the bond formed between the amino acids of the receptor and the ligand.

Based on visualization, it can be observed that enzyme-ligand interaction produced different inhibitory mechanisms through binding between amino acid residues and ligands (Figure 1). Native ligand interacts with enzyme on its active site. Amino acid residues of HMG-CoA reductase that interact with pravastatin or phytol are different from amino acid residues that interact with native ligand (3-methyl glutaric acid) or substrate. This indicates that interaction between pravastatin or phytol and HMG-CoA reductase is non-competitive inhibition. In non-competitive inhibition, the inhibitor binds with an enzyme site other than its active site (where the substrate binds), therefore it changes the conformation of the enzyme molecule and subsequently, inactivates the enzyme. Non-competitive inhibitor generally has no similar structure to the substrate (Ouertani *et al*., 2019)

Interaction between amino acid residues of HMG-CoA reductase and 3-methyl glutaric acid (native ligand) or 1,2-benzenedicarboxylic acid ligand shows a similar conventional hydrogen bond on THR558. The similarity in interactions shows similarity in their activities (Nursamsiar *et al*., 2020). Therefore, it can be assumed that 1,2-benzenedicarboxylic acid has competitive inhibitory activity. An inhibitor that has a competitive inhibitory mechanism is a compound that

has a similar structure to the substrate, therefore it will compete with the substrate (native ligand) to bind to the enzyme's active site to decrease or stop the enzyme activity (Eff *et al*., 2016).

Molecule interactions on ligand-receptor include electrostatic interaction, hydrophobic interaction, and hydrogen bond that contribute to the binding energy value (ΔG) of ligand-receptor (Arwansyah and Hasrianti, 2014). The type of interaction and binding distance determine the ligand inhibitory power towards enzyme or binding affinity (Rachmania *et al*., 2015). The hydrogen bond has an important role in determining the binding affinity value obtained from the docking process. This is because hydrogen bond energy is higher compared to hydrophobic binding (Hernandez and Appu, 2006). However, in HMG-CoA reductase-ligand interaction, the lowest binding affinity was obtained from the one that shows both hydrogen bond and alkyl hydrophobic bond, i.e., on pravastatin ligand. If the hydrogen bond alone does not show a lower binding affinity value as in the 3-methyl glutaric acid ligand and also if there is only an alkyl hydrophobic bond as in the phytol ligand. If there are hydrogen bonds and pi-anion electrostatic bonds, it will result in lower binding affinity, as in 1,2-benzenedicarboxylic acid and 3-methyl glutaric acid. Other hydrophobic bonds result in higher binding affinity, in order from high to low, i.e., alkyl – pi-alkyl, pi-alkyl – pi-alkyl, pi-alkyl – amide-pi stacked, pi-alkyl – alkyl – pi-pi stacked – pi-sigma interactions. Although the hydrophobic bond is weaker, it plays role in determining the stability of ligand and target protein. Residue from hydrophobic interactions that is present on the interior side of protein will maintain the stability of the tertiary structure of the protein (Arwansyah and Hasrianti, 2014).

Other factor that influences the binding affinity value is binding distance. The smaller the distance between hydrogen and receptor, the higher the affinity between them (Ruslin *et al*., 2020). This can be observed on pravastatin which has a shorter binding distance compared to other ligand compounds, such as phytol, 1,2-benzenedicarboxylic acid, 2,4-di-tert-butylphenol, diethyl phthalate, 1,2,3,4-tetramethylbenzene, and dipentene. Another factor that also influences the binding affinity value is the type of amino acid residue that is bound to ligands. Pravastatin binds with SER661, VAL863, and GLU665 residues through a hydrogen bond, and binds with LEU853 and LEU862 residues through an alkyl hydrophobic bond (Figure 1a). Meanwhile, 3-methyl glutaric acid (native ligand) binds with THR557, THR558, and ASN755 residues through a conventional hydrogen bond (Figure 1b). Phytol binds with ILE729, ILE733 and LEU780 residues through an alkyl-type hydrophobic bond (Figure 1c). 1,2-benzenedicarboxylic acid binds with THR558, GLY808, and GLY806 residue with a conventional hydrogen bond and binds with ASP767 residue through a pi-anion type of electrostatic bond (Figure 1d). The 2,4-di-tert-butylphenol compound binds with ALA564 residue through pi-sigmatype of hydrophobic bond, binds with TYR479 residue through pi-sigma, pi-pi stacked, and pi- alkyl hydrophobic bonds, and binds with ALA564 residue through alkyl type of hydrophobic interaction (Figure 1e).

A hydrophobic bond is a bond that avoids water environment and has a tendency to group inside the globular protein structure. The residue involved in this interaction is the residue of amino acid that has non-polar properties (Arfi *et al*., 2020). Most compounds from medicine have non-polar parts (alkyl or aryl group) that combine with non-polar receptor sites through hydrophobic interaction. This interaction is very weak but very important in medicine-receptor interaction. The alkyl group can interact with the hydrophobic pocket in the receptor's binding site. The larger and longer the alkyl group, the stronger the binding interaction with the receptor (Pratiwi *et al*., 2016). This inhibitory mechanism is different from diethyl phthalate (Figure 1f). This compound forms an amide-pi stacked type of hydrophobic bond with GLY806 dan GLY807 amino acids and pi-alkyl type on MET655 amino acid. 1,2,3,4-tetramethylbenzene compound binds with TYR511, PRO513, PRO535, and PRO813 residues through a pi-alkyl type of hydrophobic bond, while dipentene binds with PRO513, PRO535, and PRO813 residues through alkyl type hydrophobic bond, and binds with TYR533 and TYR517 residues pi-alkyl type hydrophobic bond (Figure 1g).

4. Conclusion

Ethyl acetate extract of water clover (*Marsilea crenata* C. Persl.) contained 26 compounds, six of which have the potential to become anti-hypercholesterolemic functional food ingredients. Even though their activities were slightly lower than pravastatin, they are still higher than native substrate or ligand. Based on *in silico* approach, compounds that were potential as anti-hypercholesterolemic agents were phytol, 1,2-benzenedicarboxylic acid, 2,4-di-tert-butylphenol, diethyl phthalate, 1,2,3,4-tetramethylbenzene, and dipentene.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author is asfollows, Hardoko; led the research, designed the experiment, and contributed to critical input for the manuscript. Syahrani Nurul Mutmainannah; GC-MS analysis, *in silico* analysis, interpreted the data, and prepared the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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