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Original Article

Study of Triterpene Saponin Compounds from *Centella asitica* as Renin Inhibitor with Pharmacophore Modeling, Molecular Docking and *In-vitro* Evaluation

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ABSTRACT

Hypertension is a silent killer that causes kidney, heart, and stroke damage if not handled properly. In Indonesia, the prevalence of the population with high blood pressure is 34.11% with women 36.85% higher than men 31.34%, this shows a fairly high value so that special attention is needed on hypertension therapy. It is known that currently there are 6 types of pharmacological therapy for hypertension and one of the newest is the renin inhibitor class (Aliskiren). Indonesia has diverse natural wealth in the form of flora and fauna, with a wealth of more than 30,000 types of medicinal plants with 9500 potential herbal medicines that have not been utilized optimally, with the largest exporter of herbal medicines in the world. *Centella asiatica* plants containing triterpenoid saponins have high renin inhibitor activity, namely the content of Asiaticoside and Madecasoside. The research method was carried out *in silico* using molecular simulation and *in vitro* with fluorometry (328/552 nm) to test the activity of asiaticoside and madecasoside compounds as well as a mixture of asiaticoside and madecasoside in *Centella asiatica* plants. This is supported by the docking outcome. The docking results show that madecososide compounds have a gibbs energy close to the positive control aleskiren (-8.356 kcal/mol) and aleskiren (-9.44 kcal/mol). The experiment results showed that the triterpenoid saponin compound (madecassoside) contained an IC value of 0.71, at a concentration of 5 µg/µl, and absorbance of 1.35 A in the first minute. The strongest renin inhibition was Madecasoside compound with a concentration of 5 µg/µl with an average value of fluorescent adsorption and an average percent inhibition of 135% with the best renin inhibition at Madecasoside 5 ug/ul the first minute with absorbance values 1.19 A. Finally, the *in silico* result corresponded to the *in vitro* experiment. *Centella asiatica* plants have renin inhibitor activity as antihypertensive, especially in secondary metabolites of triterpene saponins with pure madecasoside compounds compared with aliskiren as a renin inhibitor. So that the compound madecasoside has renin inhibitor activity as an antihypertensive.

Key words: Renin inhibitor, *In-vitro*, Asiaticoside, Madecasoside, *Centella asiatica*, Antihypertensive.

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INTRODUCTION

Hypertension is a silent killer, with a prevalence in the Indonesian population of 34.11%, in women 36.85% and in men 31.34%.[1] Hypertension can cause kidney damage, heart, stroke, if not treated properly. Non-pharmacological therapy for hypertension is done by changing a healthier lifestyle, avoiding the stress of a low-salt diet, and exercising regularly from an early age. Pharmacological therapy of hypertension consists of a class of diuretic drugs, Angiotensin converting enzyme (ACE-inhibitors), Angiotensin receptor blockers (ARBs), beta blockers, Calcium channel blockers (CCBs), and renin inhibitors. The renin-angiotensin-aldosterone system (RAAS) has an important role in the development of hypertension. Two drugs that act on SRAA are ACEI and ARB.[2] Both have drawbacks in inhibiting RAAS and side effects. Renin is an important component of RAAS and has specificity for angiotensinogen. Renin inhibitors can block SRAA at the highest levels. Indonesia has diverse natural wealth in the form of flora and fauna.[3] However, there are still many plants that are either utilized or tested scientifically. Renin inhibitors derived from natural ingredients generally come from the class of saponin compounds or polyphenol compounds.[4]

Renin inhibitors are developing as options that can inhibit at the highest levels in RAAS.[5] Renin inhibitors block angiotensin I and angiotensin II, thereby causing no activation at the type 1 angiotensin receptor (AT-1) .[6] Aliskiren was the first renin inhibitor to be administered orally and has progressed to phase III clinical trials. Renin inhibitor drug compounds will reduce plasma renin activity (PRA) and plasma angiotensin I and angiotensin II concentrations proportionally.[7] Plasma immunoreactive renin levels increased rapidly after administration of renin inhibitors.[5] This is due to the loss of the negative feedback of angiotensin II on renin release.[8]

One of Indonesia's native plants that contain saponins and polyphenols is gotu kola (*Centella asiatica*).[9] Since ancient times, gotu kola has been used empirically as a blood pressure-lowering drug, anti-bacterial, skin medicine and medicine for nervous disorders. Pegagan contains a lot of active compounds and the most important compounds are Triterpenoid saponins, namely asiaticoside and madecasoside which have the potential of renin inhibitors as antihypertensives.[10] Due to the very high content of triterpenoid saponins in *Centella asitica*, the researchers wanted to further investigate the effect of asiaticoside, madecasoside on the activity of renin inhibitors as antihypertensives.[11]

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MATERIALS AND METHODS

Proteins (macromolecules)

The crystal structure of Renin form [PDB ID: 3OWN]. We have also selected the protein from host cell, i.e. human cell, which is responsible for renin interaction. Sekuen serta binding site, situs glikosilasi serta struktur sekunde protein chains A by binding with its binding site. 3D structures were obtained from Protein Data Bank (https://www.rcsb.org/), in .pdb format.

Ligand (Centela asiatica) and structures

Preparasi ligand dari senyawa centela asiatica. senyawa triterpen saponin yang terdiri dari *asiatic acid, asiaticoside, madecasoside, dan centelloside.* Kemudian dapat diketahui aktivitas yang paling tinggi terhadap renin inhibitor. The 2D or 3D structures were downloaded from Pubchem (https://pubchem.ncbi.nlm.nih.gov/), save in .sdf format file and structures optimization (checking torsion, and angles) were done using MarvinSketch.

Molecular docking validation

Analisis nilai RMSD (Root Mean Square Difference) dan nilai binding affinity yang telah didapatkan dari hasil docking dalam bentuk format file keluaran (.dlg) antara struktur kristal PDB asli dengan hasil re-docking. dan analisis menggunakan . Grid box for 3OWN develop into three set (40x40x40 Å, 50x50x50 Å and 60x60x60 Å) and optimization grid box in 40x50x60. Run Genetic Algorithm (GA) was set to 100 times. Binding energy (Kcal/mol) and RMSD will evaluate per each docking results.

Molecular docking analysis and visualization

AutoDock software was utilized in all the docking experiments, with the optimized model as the docking target. Ligand and protein optimization were done using Autodocktools 1.5.6. For ligand optimization, the geometry of ligands was cleaned and torsion were set to fewest. For protein optimization, the water was removed, hydrogen polar only were added, hydrogen non polar were merged and Gasteiger charge were added. The docking was performed by using AutoDock4. Run Genetic Algorithm (GA) was set to 10 times. The docking analysis were performed using PyMOL version 2.4.1 for 3D visualization (The PyMOL Molecular Graphics System, Version 2.1 Schro¨ dinger, LLC).

Pharmacopore future compounds

At this point, a 3D pharmacophore model was created with LigandScout 4.09.1 (ligand-based) by following the steps below: The ligand set was prepared by collecting compound files that were known to be active (in.pdb,.mol, or.smi format). The ligand-set conformation is formed. Clustering of active compounds based on chemical features similarities.

In vitro renin inhibitor activity of triterpene saponin

Place 20 L of substrate, 160 L of assay buffer, and 10 L of triterpene saponins (the renin inhibitors with the highest activity after docking) in a well, followed by 10 L of renin substrate solution. The plate was shaken slowly for 10 seconds before being closed and incubated at 37°C for 15 minutes. Fluorescence was used to read the plate at excitation wavelengths of 335-345 nm and emission wavelengths of 485-550 nm.

Calculation

$$\%inhibition = \frac{(average\ SC - average\ compound'sabsorbances)}{average\ SC}\ x\ 100\%$$

SC: solvent control, compound's absorbances: Asiaticoside compound, madecassoside, Cantella asiatica, IC, SC in certain concentration

Measure the fluorescence on the microplate reader in kinetic mode at Ex/Em = 328/552 nm every 3 minutes for at least 30 minutes at 37°C.

RESULTS AND DISCUSSION

Molecular docking validation

Figure 1 depicts the 3D structure of Renin used in this study.

3OWN has active sites at 38 and 226 and uniprot 292. Meanwhile, 3OWN protein renin has glycosylation sites at position 5, uniprot 71, and position 75. Renin was chosen as 3OWN7 (GDP ID) because it has a resolution value of 2.00. Based on molecular docking validation using a re-docking method between 3OWN and its native ligand, the optimum grid box is 40x50x60 with a binding energy value of -8,356 kcal/mol, RMSD value of 1.06, and inhibition constant value of 408.59 nM, with 0.375 spacing for the default setting. The 3OWN protein from the PDB database is an excellent protein for molecular anchoring studies. This protein had a resolution quality of 2.00, no mutations, and a native 3OW ligand bound to the protein's active site. Following the acquisition of the target protein, pharmacophore modelling is used to determine the compatibility of the candidate compounds with the target protein.

Centela asiatica saponic triterpene active compound analysis, as positive controls, the triterpene saponins found in *Centela asiatica* produced madecossic acid, asiaticoside, Asiatic acid, madecasoside, and aliskiren. Characterization of new inhibitor candidates based on Lipinski's "Five" rule (Molecular Weight 500 Daltons, hydrogen bond donor 5, hydrogen bond acceptor 10, logP 5, rotating bonds 10, and Polar Surface Area 140 2) using the Swiss webserver Adme http://www.swissadme.ch/index.php. The analysis results are shown in Table 1. Through molecular anchoring, the structure-based approach is screened. In this study, four triterpene saponins from the *Centela asiatica* herb were used as test ligands: maddecosic acid, Asiatic acid, madecassoside, and asiaticoside, and Aliskiren as a positive control. According to the results of Lipinski's rule of five analyses, only Maddecosic acid and Asiatic acid, as well as Aliskiren, a positive control, complied with Lipinski's rules. The screening results revealed that two compounds (madecossic acid and Asiatic acid) met Lipinski's rule of 5, while two others (asiaticoside and madecasoside) did not because their BM was greater than 500.

Binding energy of molecular docking

Based on the docking results of 4 compounds of *centela asiatica* against the 3OWN receptors (Table 2), two compounds with the best docking results were obtained, namely asiaticososide and Asiatic acid. For Renin inhibitor results, Aliskirene become the best binding energy value against 3 OWN and show the worst binding energy value against Renin.

Ligand Docking and Receptor Testing AutodockTools-1.5.6rc3 was used to configure the grid box parameters. The grid box coordinates were determined using the co-crystal ligand coordinates from the receptor file, as shown in table 2, and the tethering process was carried out using Autodock Vina. Table 2 shows the docking of triterpene saponins with renin. The docking results show that madecososide compounds have a gibbs energy that is close to the positive control aleskirene with a value of -8.356 kcal/mol and aleskirene with a value of -9.44 kcal/mol.

Analysis and Visualisation, Docking Outcomes The docked ligand's conformation (the best pose) is determined by selecting the conformational ligand with the lowest bond energy. The docking results with the best pose are then analysed with the Autodock analyzer, as shown in Figure 2.

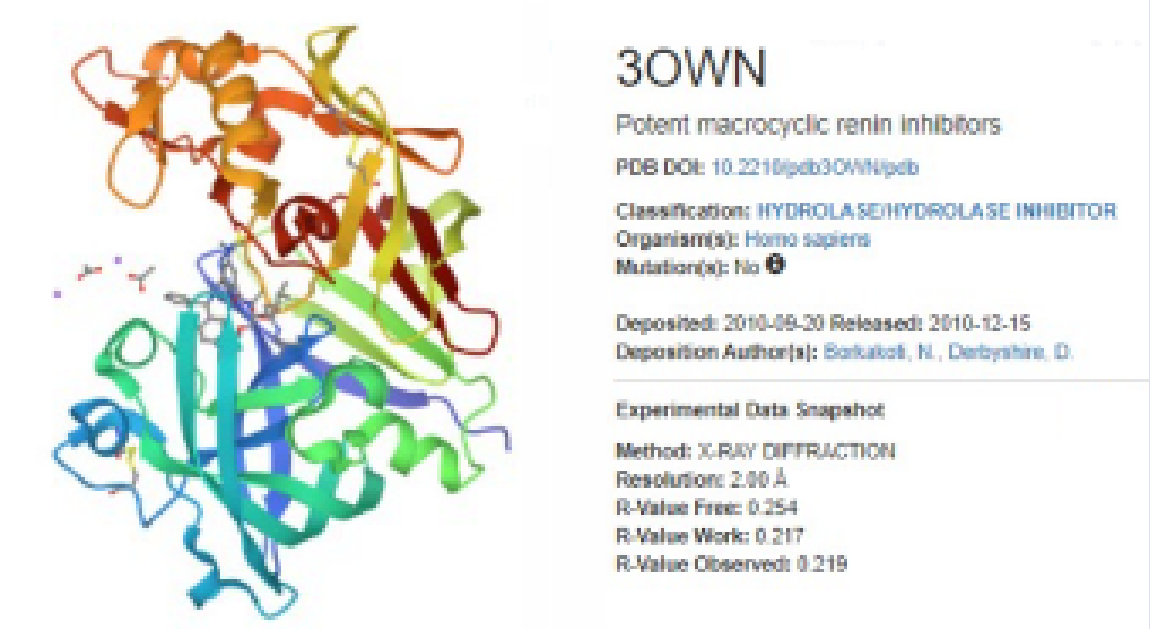

Figure 1: Struktur 3D renin.

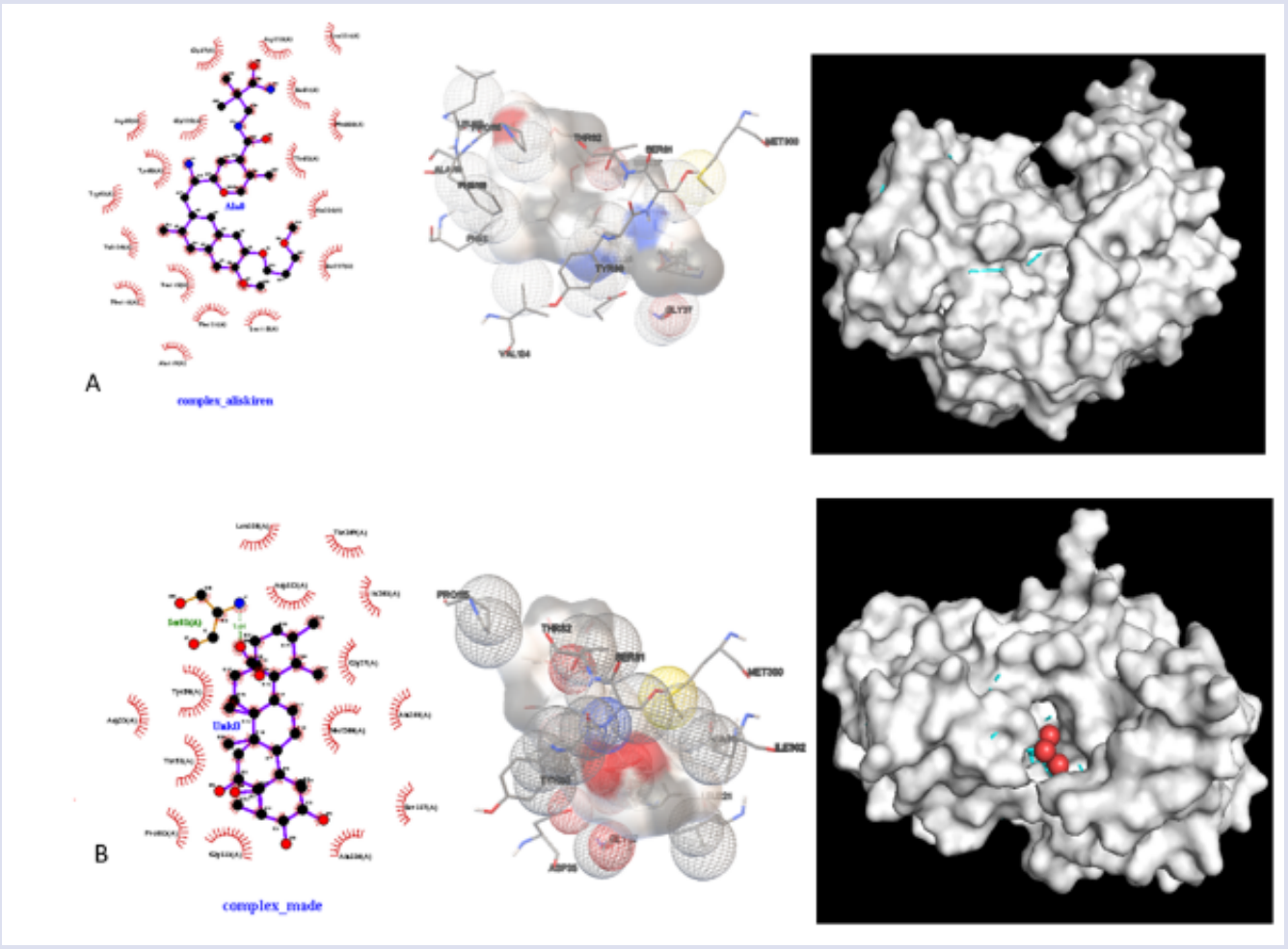

Figure 2: The docking results of the ligand-renin complex are visualised. A. Aliskiren-renin complex with hydrogen bonds and a three-dimensional structure, B. Madecososide-renin complex with hydrogen bonds and a three-dimensional structure.

Table 1: The physicochemical properties of *Centela asiatica's* saponic triterpene active compound were investigated.

	BM	H-bond	H-acc	LogP	TPSA	Lipinski R5
madecossic acid	504.7	6	5	3.56	118.22	yes
asiaticoside	958.51	12	19	2.084	315.21	no
asiatic acid	488.7	4	5	4.45	97.99	yes
aliskiren	551.76	4	7	3.47	146.13	yes
madecasoside	974.51	13	20	1.363	335.44	no

Table 2: Docking result triterpen saponin compound against renin.

	Maddecosic acid	asiaticoside	Asiatic acid	madecasoside	Aliskiren
Gridbox	40x50x60	40x50x60	40x50x60	40x50x60	40x50x60
RMSD	0,9	0,9	1,05	1,06	0,9
Gibbs Energy (kkal/mol)	-7,35	-7,802	-8,162	-8,356	-9,44
H bond	6	12	4	13	4
Hacc	5	19	5	20	7
pKi	6.326	7.118	7.327	8.215	8.521
Hydrogen bond	MET300 ILE302 LEU221 ALA311 ASP35 GLY37	LEU118 ALA119 PHE116 PHE121 VAL124 TYR80 GLY225	MET300 SER81 THR82 PRO115 LEU118 ALA119 TYR80 GLY225	PRO115; THR82 SER61; MET300 ILE302; LEU221 ALA311; ASP35 GLY37; ASP223 TYR80; GLY225 **ALA226**	MET300; SER81; THR82; SER227; PRO115; LEU118; ALA119; PHE116; PHE121; VAL124; TYR80; GLY225; ASP223; GLY37

Table 3: Asiaticoside, Madecasososide, *Cantella asiatica* Fraction, Aliskiren, and control enzyme (renin) IC50 compounds in unit time.

	min1	min2	min3	min4	min5	min6	min7	min8	min9	min10	min11
IC50 Asiaticoside murni	4.058902	3.230943	3.055373	3.912312	2.993218	3.3627	2.915703	2.832791	2.986497	3.211667	3.320066
IC50 Madecasosside murni	2.666347	1.836494	2.043641	1.690761	1.67135	1.571394	1.557578	1.443762	1.473293	2.321469	1.517141
IC50 fraksi Cantella asiatica	0.763479	0.799866	0.834788	0.80982	0.919399	0.898671	0.84515	0.953863	0.899166	0.919743	0.896646
IC50 Aliskiren	1.93639	1.800572	1.828006	1.800278	1.7664	1.792293	1.777268	1.753182	1.758056	1.763014	1.751235
IC50 Enzim control (renin)	8.361515	4.376042	2.070184	1.828141	1.071217	0.912732	1.065564	1.150048	0.945272	0.693881	0.759839

Table 4: Anova significance.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	32.61742	4	8.154355	7.130706	0.000127	2.557179
Within Groups	57.17775	50	1.143555			
Total	89.79517	54				

Amino acid residues, hydrogen bonds, predicted inhibition constants, and bond free energies were among the parameters studied. As shown in Table 2, the molecular docking results show that the madecasoside compound is close to having the same ligand conformation as the binding cavity of aleskiren. These two compounds were then examined in 2 and 3 dimensions for interactions and anchoring at the active site. The validation results show that the RMSD (Root Mean Square Deviation) value 2A at the ligand position is improving as it approaches the original conformation. The lower the re-rank score, the better and more likely it is to have renin inhibitor activity.

Pharmacophore, the pharmacophore feature is used to determine a compound's functional group. Using the Chembl database, the feature search looks for 2D files in the form of smiles. Screening and characterization of bioactive compound functional groups for drug candidates The strategy is ligand-based drug design (LBDD). The LigandScout4 application software was used to search for the active group features of the compound in LBDD. According to the pharmacophore results, the Madecososide compound had 13 H bonding groups and 20 active H acceptor groups, whereas aleskirine had 6 H bonding groups and 5 active H acceptor groups, as shown in Figure 3. The presence of this active group indicates an active future, which in 3D renin has activity as a H acceptor and a H donor, which contributes to Renin binding either hydrogen or covalently.

In vitro study

The effect of the test compound on renin inhibition was investigated *in vitro*. Asiaticoside, Madecasoside, Extract fraction containing (asiaticoside and madecasoside) Compounds tested Comparison, test, and control compounds: Figure 4 shows fluorometric absorbance measurements at excitation/emission wavelengths of 328/552 nm.

The average fluorescence value in the sample represents the percent relative activity or inhibition of each test compound. When each percent inhibition was compared to the percent IC value (71 percent), it was determined that the compound madecasoside M1 5 ug/ul had the highest percent inhibition (135 percent). Based on these findings, the purer the active compound, the better the reaction on renin inhibition.

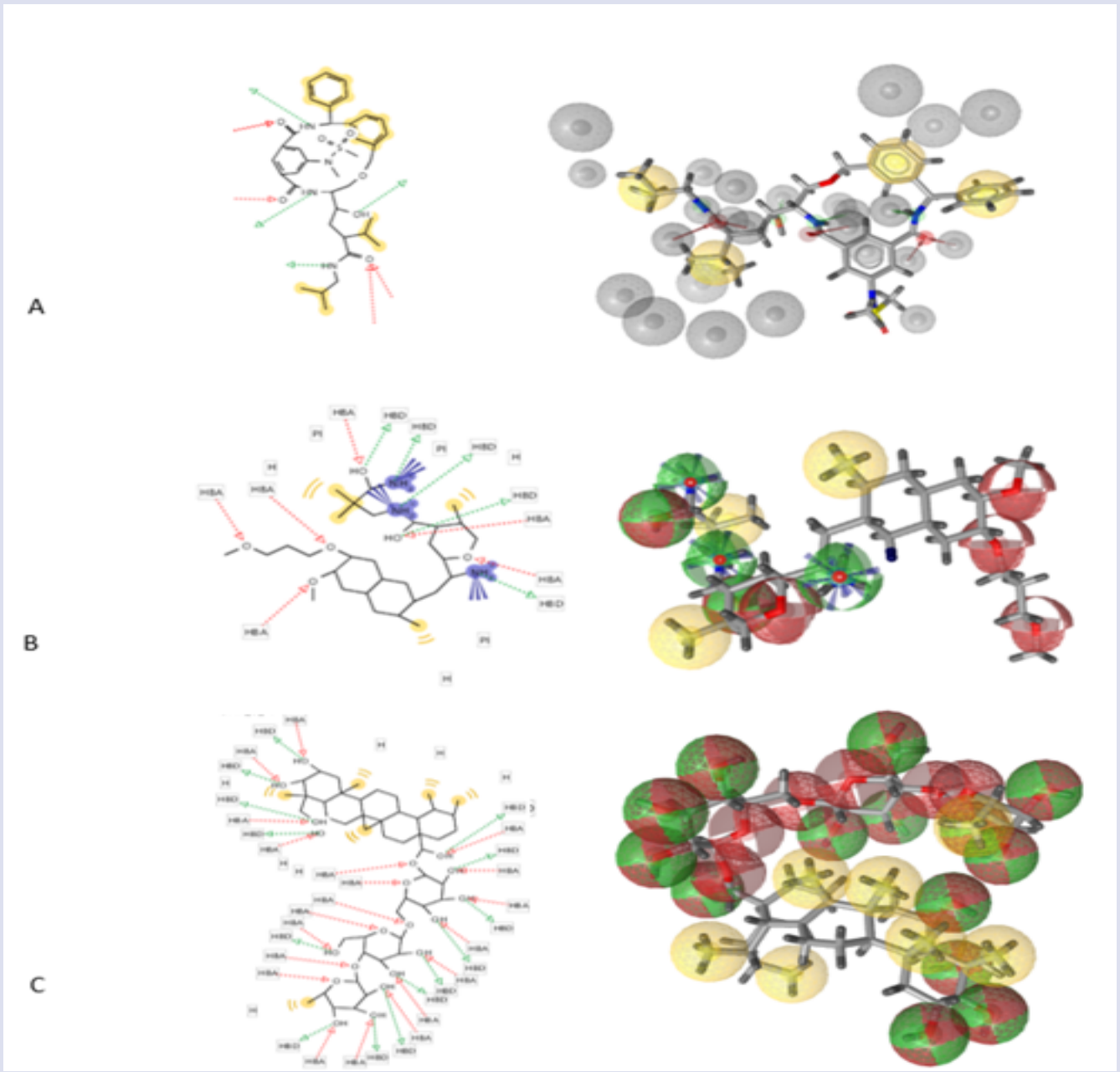

Figure 3: Native compounds in renin, B. renin control inhibitor compounds, and C compounds madecasoside pharmacophore results.

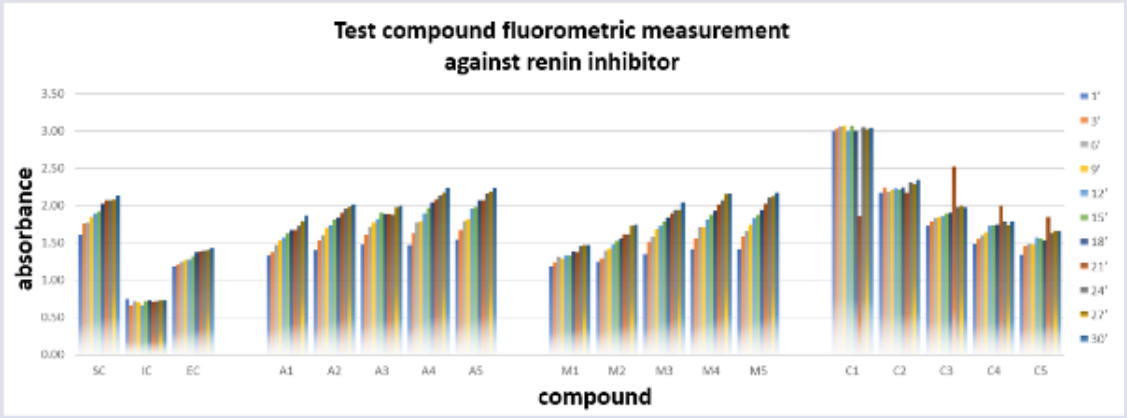

Figure 4: Fluorometric measurements between the test compound and renin inhibitor.

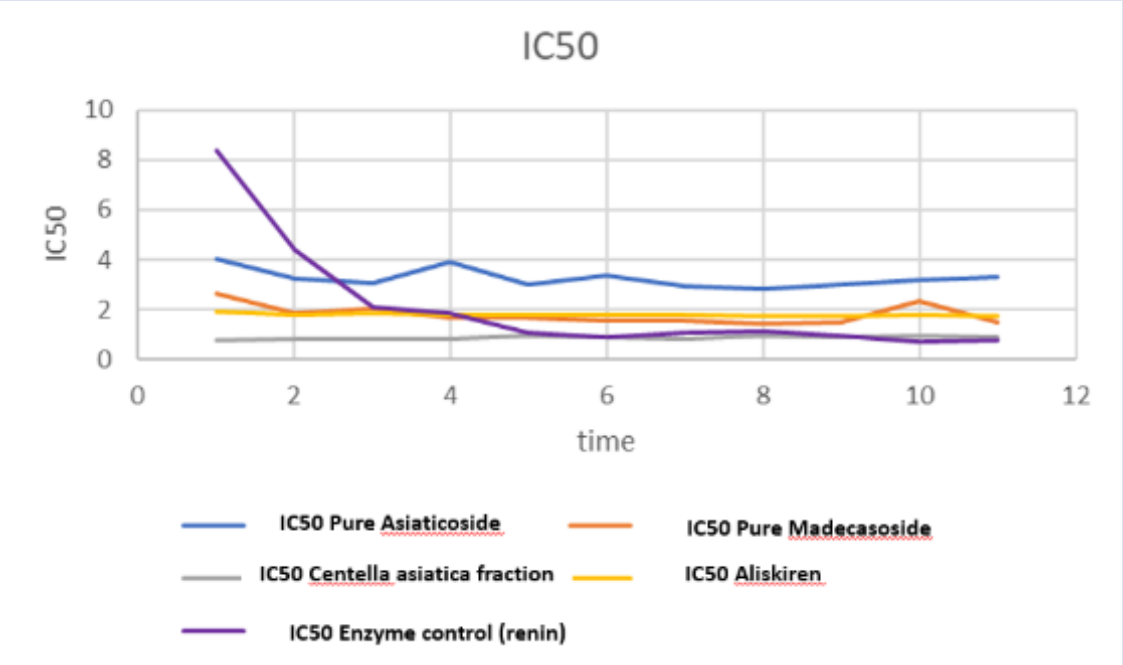

Figure 5: The relationship between the IC50 value and the time interval.

This is demonstrated by comparing the pure compound madecasoside and asiaticoside to the extract fraction madecasoside and asiaticoside. Furthermore, the higher the concentration of the pure compound, the greater the effect on renin inhibition as an antihypertensive. However, not with the mixed fraction. The lower the concentration of asiaticoside and madecasoside extracts, the closer to the percent inhibition value in the control inhibitor, implying that the lower the concentration in the mixed fraction, the more potential it has as an antihypertensive renin inhibitor.

Fluorescence spectroscopy is a type of electromagnetic spectroscopy that analyses a sample's fluorescence. It is also known as fluorometry or spectrofluorometry (test compound). This involves using a light beam, usually ultraviolet light, to move electrons in the molecules of certain compounds, which causes the glow.

Hypertension is a worldwide disease with a high prevalence. We currently know of five classes of antihypertensive drugs. Diuretics, ACE inhibitors, Angiotensin receptor blockers (ARBs), Beta blockers, and CCBs are among them (Chanel Calcium Blockers). Ace-inhibitors and ARBs have the most activity in terms of mechanism of action, namely renin inhibitors. Aliskiren is currently the only renin inhibitor drug on the market. Aliskiren is currently the only renin inhibitor oral drug that is chemically synthetic.

The renin-angiotensin-aldosterone (RAA) system plays a critical role in the pathophysiology of hypertension. Renin is a component that is released by the kidneys when arterial pressure is extremely low. An *in silico* test of the active compound's inhibitory activity against renin was performed in this study. Interleukin-1 protein, PDB code 3OWN, was chosen as the target protein in the docking. Protein selection takes into account several factors, including the presence of native ligands, mutation status, and protein resolution. The protein crystallographic structure in the database to be used in docking should have a resolution less than 2A. This is due to the fact that the lower the resolution, the better the structural quality of the protein, allowing it to conform to its original shape. The test ligand compound's binding is highly dependent on the structure of the target protein's active site. It is hoped that the more precise the molecular docking will be, the better the structural quality of the target protein. In addition to the structure's quality, the presence of ligands at the protein's active site should be noted. With the presence of native ligands in the target protein structure, it is possible to predict which compounds will be used as test ligands in the future. 1 in accordance with *In vitro* studies show that the purer the active compound, the better the response to renin inhibition. This is demonstrated by comparing the pure compound madecasoside and asiaticoside to the extract fraction madecasoside and asiaticoside.

Based on the molecular docking, pharmacopore future and invitro analysis, it was found there are two potential compounds from *Centela asiatica* that showed higher binding affinity score namely madecososide and Asiatic acid. As a result, the considerable effort done in this study to understand the interaction of Renin inhibitor ligands that are considered for hypertension treatment. Sejalan dengan uji *in vitro* bahwa persen inhibisi di bandingkan dengan nilai aliskiren % IC (71%) inhibisi senyawa madecasoside M1 5 ug/ul yaitu 135 % mendekati nuilai aliskiren.

LIST OF ABBREVIATIONS

PDB: Protein Data Bank; RBD: Receptor Binding Domain; RMSD: Root Mean Square Deviation; IC50: Inhibitory Concentration.

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AUTHOR'S CONTRIBUTIONS

Rangki Astiani: Conceptualization, Investigation, Methodology, Software, Validation, Data curation, Resources, Formal analysis, Visualization; Franciscus D. Suyatna: Conceptualization, Investigation, Resources, Formal analysis, Visualization, Supervision; Mohamad Sadikin: Conceptualization, Investigation, Resources, Formal analysis,

Visualization, Supervision; Aprilita Rinayanti: Conceptualization, Methodology, Formal analysis, Data curation; Wawaimuli Arozal, Ani Retno Prijanti, Fadilah, Firdayani, Piter, Guntoro Halim: Formal analysis, Writing - original draft, Writing - review & editing, Visualization.

DECLARATION OF COMPETING INTEREST

The authors declare "No conflicts of interest".

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