JURNAL FARMASIMED (JFM)
E-ISSN: 2655-0814
https://ejournal.medistra.ac.id/index.php/JFM

Multitarget Antihypertensive Potential of Bioactive Compounds from Centella asiatica, Justicia gendarussa, and Imperata cylindrica: An Integrated In Vitro and Molecular Docking Approach

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Abstract

Background: *Hypertension is a leading global health challenge. It has been a major risk factor for cardiovascular disease events that contribute broadly to morbidity and mortality worldwide. With increasing life expectancy and changes in lifestyle and diet, the prevalence of hypertension has increased by up to 40% in certain parts of Asia, particularly in low- and middle-income countries (LMICs). The combination of Centella asiatica (C) leaves, Justicia gandarussa (J) leaves, and Imperata cylindrica (I) roots has been used to treat hypertension.* ***Objective:*** *To identify potential active substances that lower blood pressure, bioactive compounds from CJI were isolated and characterized in vitro, and in silico predictions were generated. Active compounds were identified with LC-HRMS.* ***Methods:*** *We hypothesized that the active compounds of CJI act as multitarget antihypertensives by inhibiting protein targets in the renin-angiotensin-aldosterone system (RAAS), including aspartic proteinase renin (2V0Z), Angiotensin-Converting Enzyme (ACE, 1R4L), angiotensin receptor (AT1R), and mineralocorticoid receptor (MR, 2AA2). LC-HRMS analysis identified 42 active compounds from CJI.* ***Results:*** *Ethanol extract of CJI showed antioxidant activity with an IC50 of 187,866 ppm. Our results indicated that various active substances in CJI could bind to different drug targets. For renin, cylindrin and friedelin, binding affinities of -11 and -10.2 kcal/mol were observed at the S3 binding site, potentially blocking renin activity. For ACE, castillicetin and 1,5-dicaffeoylquinic acid exhibited binding affinities of -10.5 and -10.4 kcal/mol, respectively, and bound to its binding pocket. For AT1R, fernenol and friedelin had binding affinities of around -11 kcal/mol within the binding pocket. Regarding MR, only cylindrin could bind, with an affinity of -8.8 kcal/mol.* ***Conclusion:*** *The CJI combination shows potential activity as an antihypertensive, likely inhibiting multiple drug targets in the RAAS pathway.*

Keywords: *Antihypertension, Bioactive compounds, Herbal combination, Molecular docking, Renin-angiotensin-aldosterone system.*

1. INTRODUCTION

Hypertension is a growing global health issue, particularly in low- and middle-income countries (LMICs), with an estimated 1.28 billion adults aged 30-79 affected [1]. The increasing burden of hypertension in Asia is of great concern. From 1990 to 2019, the prevalence of hypertension increased by up to 40% in certain parts of Asia. With increasing life expectancy and changes in lifestyle and diet, the prevalence of hypertension is steadily rising, particularly in urban areas [2]. Hypertension is a major risk factor for cardiovascular disease events that contribute broadly to morbidity and mortality worldwide [3]. Despite extensive knowledge about ways to both prevent and treat hypertension, its global incidence, prevalence, and (more importantly) cardiovascular complications are not reduced[4]. In Indonesia, cardiovascular diseases remain the leading cause of death, with hypertension contributing substantially to morbidity and healthcare costs. Rural communities, in particular, show a

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Doi : 10.35451/jfm.v9i1.3784
Received : July 24, 2026, Accepted: September 02, 2026, Published: October 30, 2026
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disproportionately higher burden of cardiovascular risk factors, limited access to healthcare, and low awareness of disease prevention [5]

Pathophysiology of hypertension involves the imbalance in the renin-angiotensin-aldosterone system (RAAS), the pivotal pathways regulating blood pressure [6]. This pathway involves the cleavage of angiotensinogen to angiotensin I by renin, and later to angiotensin II by angiotensin-converting enzyme (ACE) [7]. Increased plasma ACE activity was also shown to hasten the progression of atherosclerosis and lead to atherosclerotic cardiovascular disease (ASCVD). Beyond RAAS activation, oxidative stress and chronic low-grade inflammation also contribute significantly to vascular remodeling, endothelial dysfunction, and increased peripheral resistance [8]. An imbalance in reactive oxygen species (ROS) production via oxidative stress is a hallmark of hypertension. Numerous studies have demonstrated a correlation between the overproduction of reactive oxygen species and cardiovascular disease. These free radicals facilitate lipid peroxidation within cell membranes, leading to the generation of lipid peroxides and other free radicals. Free radicals have unpaired, unstable electrons and tend to stabilize themselves by absorbing electrons from nearby molecules. Numerous cell components may sustain harm as a result[9].

Herbal remedies, rooted in tradition, are often studied by isolating single bioactive compounds for drug development, a hallmark of Western medicine [10,11]. In contrast, traditional herbal medicine uses multi-herb extracts, offering a holistic approach with compounds that may act synergistically on multiple targets or enhance each other's absorption [12–14]. However, scientifically validating these combinations remains challenging. One such combination proposed in this study was *C. asiatica*, *J. gendarussa*, and *I. cylindrica*. *C. asiatica* has been widely used as a blood purifier as well as for treating high blood pressure, for memory enhancement, and promoting longevity. It has been recommended for wound healing of various tissue conditions [15,16]. The leaves, roots, and bark of *J. gendarussa* are known to be effective in treating multiple complaints [17,18].

It has been reported that several substances from *I. cylindrica* exhibit a wide range of biological activities, including hemostatic, diuretic, anti-inflammatory, antibacterial, anticancer, and immune system-enhancing effects[19–21]. Despite their individual pharmacological potentials, the antihypertensive synergistic impact of their combination, referred to here as CJI (*Centella asiatica*, *Justicia gendarussa*, *Imperata cylindrica*)—remains unexplored, highlighting a critical gap in understanding their combined efficacy as a holistic treatment for hypertension. In this study, we hypothesize that bioactive compounds from the CJI combination may act synergistically to modulate multiple targets in the RAAS pathway, including renin, ACE, angiotensin receptor (AT1R), and mineralocorticoid receptor (MR). To test this hypothesis, we integrated in vitro antioxidant assays with in silico molecular docking analyses to identify and characterize potential antihypertensive compounds from the CJI extract. This work aims to provide a scientific basis for developing a multi-target herbal formulation with potential therapeutic relevance for hypertension management.

2. MATERIALS AND METHODS

Maceration Kinetics of CJI

Simplicia of *C. asiatica* leaves, *J. gendarussa* roots, and *I. cylindrica* leaves were purchased from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT) at Karanganyar, Central Java province, Indonesia. 16 grams of CJI simplicia (combination 5:5:3) were extracted with 520 mL of 96% ethanol (simplicia-to-solvent ratio of 1:20) using the kinetic maceration method for 24 hours. The extract was then evaporated using an evaporator. The extract was stored in an oven at 40ºC until it became concentrated. The concentrated extract was analyzed using LC-HRMS and evaluated for antioxidant activity using the DPPH method [22]. As shown in Appendix Figure 1, the initial experimental procedure was the extraction of the CJI, and then we continued with the in silico and in vitro evaluations of potential antihypertensive and antioxidant activities of CJI.

Fractionation of CJI Ethanol Extract

The concentrated extract was diluted with distilled water at a 1:3 ratio and stirred until a homogeneous mixture was obtained. The mixture was then transferred into a separatory funnel. An equal volume of n-hexane (1:1) was added, and the funnel was shaken for approximately 10 minutes, followed by phase separation for about 1 hour. This process resulted in two distinct layers: an upper oil phase and a lower aqueous phase. The oil phase was collected and dried in an oven, while the aqueous phase was subsequently treated with ethyl acetate in a 1:1 ratio, following the same shaking and separation procedure described previously. After separation, two layers were formed again. The resulting oil phase from this step was mixed with 96% ethanol at a 1:1 ratio and processed using the same extraction procedure. Finally, the obtained extract was dried in an oven at 40°C until a thick consistency was achieved.

LC-HRMS Analysis

LC-HRMS was conducted at MarkHerb, Bandung, with the condition that mobile Phase B (0.1% formic acid in acetonitrile solvent, water, methanol, acetonitrile in MS grade) was injected at 5% and gradually increased to 90% over 16 minutes. Mobile Phase B was maintained at 90% for 4 minutes and then reduced to 5% for 25 minutes. The column temperature was set at 40°C with an injection volume of 3 µL. The HRMS mode was operated in full MS/dd-MS² acquisition mode under positive ionization using Xcalibur 4.4 software. Screening was performed using nitrogen gas as sheath (32 AU), auxiliary (8 AU), and sweep gases (4 AU). The spray voltage was set at 3.30 kV with a capillary temperature of 320°C and an auxiliary gas heater temperature of 30°C. The m/z range was set from 66.7–1000 m/z with a resolution of 70,000 (Full MS) and 17,500 (dd-MS²).

Collection of CJI Active Compounds

To predict the antihypertensive effects of bioactive compounds in CJI, molecular docking was used. The proteins used as receptors were (PDB IDs: 2AA2, 1R4L, 4ZUD, and 2V0Z). These were downloaded from https://www.rcsb.org as .pdb files [23–26].

Ligand and Protein Preprocessing

Preprocessing of the ligand and protein was conducted using AutoDock Tools by The Scripps Research Institute, followed by molecular docking using AutoDock Vina. The ligands were downloaded as an .sdf file and then converted to a .pdb file using Biovia Discovery Studio. The ligands were prepared by minimizing the energy through Vina's AutoDock software. The preparation of proteins and ligands was done by removing the water using the Biovia Discovery Studio software. The protein was then docked and saved as a .pdb file. The molecular docking method was validated using pose selection, where the natural ligand of each receptor was redocked and examined for its RMSD value, with a value between 1.5 and 2.5 Å being accepted. After docking, the ligand and receptor poses were obtained using PyMOL, and their ligand-receptor amino acid interactions were observed using Biovia Discovery Studio and LigPlot+.

Molecular Docking

Molecular docking was performed using AutoDock Vina implemented in PyRx. Blind docking was employed, with the search space (grid box) configured to encompass the entire macromolecule to allow unbiased identification of potential binding sites. Exhaustiveness was set to 8, and the lowest-energy binding modes were retained. The docking results were then visualized using the PyMOL program. To see the interaction between receptors and ligands, we used the Biovia Discovery Studio program. The binding affinity results were selected based on the most negative value. Negative values indicated the most stable conformation of the ligand with the receptor. The interpretations were based on the binding affinity value, which was more negative than the control, and the number of amino acids bound through the same hydrogen bonds as the control.

Antioxidant Assay

Test solutions were prepared for the Negative Control, Positive Control, and Sample. Each solution (3 mL) was pipetted into a separate test tube. Subsequently, 3 mL of 40 ppm DPPH solution was added to each tube, covered with aluminum foil, and shaken gently until homogeneous. The shaking process was carried out consistently for all test solutions to ensure uniform mixing. The mixtures were then incubated at room temperature (27°C) under

dark conditions to prevent light-induced degradation of DPPH. After incubation, the absorbance of each solution was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm, with 70% ethanol used as the solvent for auto-zero calibration. The absorbance values were recorded carefully. Before each measurement, the cuvette was rinsed with the solution to be measured, and the readings were performed starting from the lowest concentration to the highest to avoid contamination and carry-over effects.

$$\%Inhibition = \frac{A\ blank - A\ sample}{A\ Blank} x\ 100\%$$

3. RESULT

A bioactive compound of CJI identified with LC HRMS has 42 compounds, as shown in Appendix Table 1. Most compounds are 9-Oxo-10(E),12(E)-octadecadienoic acid; α-Eleostearic acid; Asiaticoside; 5-methoxy-2-phenyl-4H-chromen-4-one; and kaempferol. Asiaticoside and madecassoside are known for their pharmacological properties, including neuroprotective, cardioprotective, hepatoprotective, wound healing, anti-inflammatory, antioxidant, anti-allergic, antidepressant, anxiolytic, antifibrotic, antibacterial, antiarthritic, anti-tumor, and immunomodulatory activities [21,27–29].

Based on the data from Table 1, it is shown that the crude ethanol extract from CJI has better antioxidant activity compared to the fraction, with a value of 187,866 ppm. This may result from synergistic interactions among antioxidant compounds in the crude CJI extract, which are lost upon fractionation. When fractionation occurs, compounds are separated based on their polarity, which reduces their antioxidant activity. Active compounds that possess antioxidant activity are flavonoids and terpenoid groups. The active compounds in the terpenoid group in CJI include asiaticoside and madecassoside. Meanwhile, the active compounds of CJI in the flavonoid group are 5-methoxy-2-phenyl-4H-chromen-4-one, kaempferol, and quercetin. Based on *in vitro* tests, CJI has the potential to be used as an antihypertensive through the mechanism of reducing oxidative stress in patients with hypertension.

Table 1. Results of the antioxidant activity measurement of CJI using DPPH method

No	Sample	Concentration (ppm)	Absorbation	Antioxidant Activity (%)	IC_{50} (ppm)
1	Ascorbic acid	2	0.539	8.277	8.040
		4	0.435	26.077	
		6	0.313	46.769	
		20	0.079	86.08	
		30	0.042	92.914	
		40	0.035	93.991	
2	Crude Extract EtOH	100	0.542	6.494	187.866
		150	0.352	39.310	
		200	0.279	51.954	
		250	0.229	60.460	
		300	0.196	66.207	
3	Fraction EtOH	100	0.692	9.39	684.727
		200	0.602	21.14	
		300	0.532	30.262	
		400	0.467	38.777	
		500	0.420	45.022	
4	Fraction EA	100	0.228	59.334	435.063
		150	0.155	72.295	
		200	0.083	85.196	
		250	0.056	89.952	
		300	0.049	91.201	
5	Fraction N-Hexane	150	0.041	6.897	435.063
		250	0.336	29.994	
		350	0.276	41.731	
		450	0.240	49.261	
		500	0.208	56.158	

Molecular docking of active compounds from *C. asiatica*, *J. gendarussa,* and *I. cylindrica* against protein targets

The role of the RAAS in regulating blood pressure has been well documented[7,30]. Renin is an important protein produced by the kidneys, which, with angiotensin-converting enzymes, converts angiotensinogen into angiotensin I and II. Angiotensin II acts on AT1R and AT2R, which causes vasoconstriction and vasodilation, thus directly influencing blood pressure by changes in peripheral vascular resistance. On the other hand, the adrenal gland secretes aldosterone, which plays a role in sodium-potassium balance via MR, thus influencing blood pressure by indirectly altering cardiac output[7,30]. The proteins related to this pathway are often used as classical drug targets for regulating blood pressure and, therefore, can be targets for treating hypertension. However, conventional Western medicine often uses a one-drug-one-target approach. Several studies have highlighted the benefit of targeting multiple drug targets in a pathway using combinatory drug therapy in the treatment of certain diseases, including hypertension[14,31,32]. This study predicted the binding affinity of dominant active compounds from CJI against drug targets in the RAAS.

Renin (PDB ID: 2V0Z) is an aspartic protease that plays a role in the conversion of angiotensinogen into angiotensin II. Renin is a two-chain protein, 36-42 kDa in size, with several designated subsites. The enzymatic activity of renin is determined in subsites S3 and S5, whereas S3 coincidentally is the binding site for angiotensinogen, stabilized by subsites S1 and S1. Aliskerin is known to occupy the S3-S2' subsites and therefore lowers the catalytic activity of renin [26]. Several different compounds from CJI were able to bind to renin (as shown in Table 3) in the subsite that is similar to Aliskerin, namely cylindrin, friedelin, arundoin, 3,5-dicaffeoylquinic acid, and Asiatic acid, whilst other compounds could potentially synergistically destabilize angiotensinogen binding by occupying subsite S1 and S1', such as lupeol and fernenol.

As shown in **Appendix Table 2**, these compounds were able to bind to key amino acids similar to inhibitors, with Cylindrin having interactions with the most amino acids in the binding pocket[24]. However, hydrogen bonds were only found with fernenol, patentiflorin A, 3,5-dicaffeoylquinic acid, Asiatic acid, and germinone A. From these compounds, only 3,5-dicaffeoylquinic acid and Asiatic acid showed similar amino acid interactions (Ser219 and Thr-77) with a renin inhibitor as described in the literature [26]. It can be hypothesized that these two compounds would have the most potential to act as renin inhibitors with a high affinity (-9.9 kcal/mol). Both compounds were found in *C. asiatica*. Thus, it is necessary to define the natural resources that possess renin antagonists as an adjuvant in the treatment of hypertension.

Table 2. **Several ligands from *C. asiatica*, *J. gendarussa* or *I. cylindrica* with high binding affinity against aspartic proteinase renin (2V0Z)**

Rank #	Ligand Name	CID	Plant Source	Binding Affinity (kcal/mol)	Binding Pocket
1	Cylindrin	189045	*I. cylindrica*	-11	S3
2	Friedelin	91472	*I. cylindrica*	-10.2	S3
3	Arundoin	12308619	*I. cylindrica*	-10.1	S3sp
4	Lupeol	259846	*C. asiatica*	-10.1	S1
5	Fernenol	12305178	*I. cylindrica*	-10	S1'
6	Patentiflorin A	11785812	*J. gendarussa*	-10	Other
7	3,5-Dicaffeoylquinic acid	6474310	*C. asiatica*	-9.9	S3sp, S1'
8	Asiatic Acid	119034	*C. asiatica*	-9.9	S3, S3sp
9	Graminone A	101915994	*I. cylindrica*	-9.9	Other

* Key amino acids against aspartic proteinase renin (2V0Z) were based on the literature[24]. Currently, there are no renin inhibitors with 3D structure available. The binding pockets in human renin residues is categorized into (a) S3sp: Gln13, Tyr14, Val30, Val120, Tyr155, Gly217, Ala218, Ser219, Ala303; (b) S3: Thr12, Gln13, Pro111, Phe112, Leu114, Ala115, Phe117, Ser219; (c) S1: Val30, Asp32, Tyr75, Thr77, Phe112, Phe117, Val120, Asp215; (d) S1': Ser76, Leu213, Asp215, Ile291, Pro292, Thr295, Thr298, Ala300, Gly217, and (e) S2': Gly34, Ser35, Leu73, Arg74, Tyr75, Gln128, Ile130.

Based on **Appendix Table 3**, only rutin, castillicetin, and naringin had a similar hydrogen bond with benazepril-HCl (mainly on His345).

Table 3. Several ligands from *C. asiatica*, *J. gendarussa* or *I. cylindrica* with high binding affinity against ACE (1R4L) compared to Benazepril-HCl (Lotensin) as a control

Rank #	Ligand Name	CID	Plant Source	Binding Affinity (kcal/mol)	Interact with Key Amino Acids*	Similar Binding Pocket with Antagonist Control**
	Benazepril-HCl (Lotensin)	5362123	-	-9.4		
1	Rutin	5280805	*C. asiatica*	-10.7	No.	Yes
2	Castillicetin	102394640	*C. asiatica*	-10.5	Yes	Yes
3	Friedelin	91472	*I. cylindrica*	-10.4	No	No
4	1,5-Dicaffeoylquinic acid	122685	*C. asiatica*	-10.2	Yes	Yes
5	Daucosterol	5742590	*I. cylindrica*	-10.2	No	No
6	Alnusenone	10071029	*I. cylindrica*	-10.1	No	No
7	Quadranoside IV	10372074	*C. asiatica*	-10.1	Yes	No
8	Lupeol	259846	*C. asiatica*	-10.0	No	No
9	Naringin	442428	*C. asiatica*	-10.0	No	Yes
10	Patentiflorin A	11785812	*J. gendarrusa*	-9.9	Yes	Yes

* The binding site of ACE2 was at His374 to His378, and His401 to Glu406, with three metal binding ligands at His374, His378, and Glu402. Tyr510, Arg514, Phe504, and Thr347 form the S1 subsite to limit substrate size, therefore playing a role in substrate specificity.

**The binding pocket was based on a molecular docking experiment in this study between the antagonist and angiotensin-converting enzyme. Benazepril-HCl interacts with 1R4L, has a hydrogen bond with His345, Thr371, and Asp367 (carbon hydrogen bond), a pi-bond with Trp271, Phe274, Pro346, Met360, Lys363, and an Unfavorable Acceptor-acceptor with Tyr127. The van der Waals binding pocket consists of Phe504, His505, Leu503, Asp269, Asp368, Thr276, Thr445, Asn149, and Leu144.

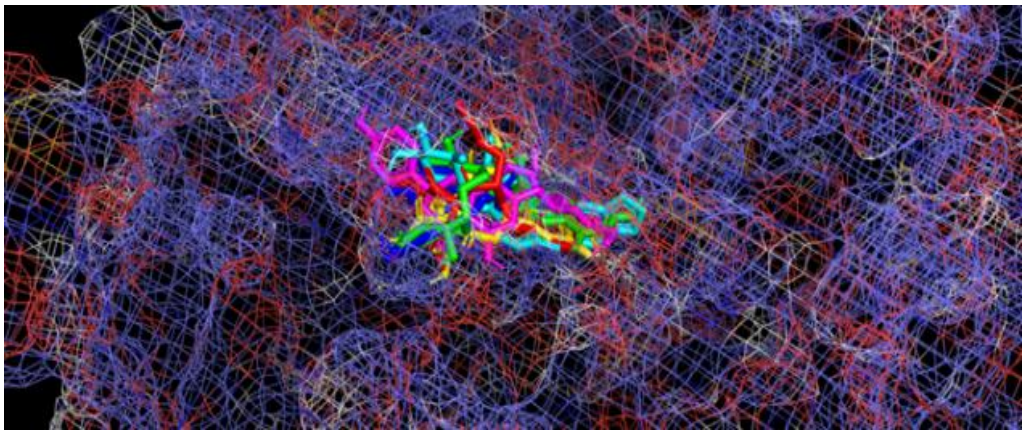


Figure 1. Superposition of ligands from CJI occupying the binding pocket of ACE (1R4L). Shown in this image: Benazepril-HCl (Red), Rutin (Green), Castillicetin (Blue), 1,5-Dicaffeoylquinic acid (Yellow), Naringin (Magenta), and Patentiflorin A (Cyan)

Table 4. Several ligands from *C. asiatica*, *J. gendarussa*, or *I. cylindrica* with high binding affinity against angiotensin receptor AT1R (4ZUD) compared to Olmesartan and Valsartan as inhibitors

Rank #	Ligand Name	CID	Plant Source	Binding Affinity (kcal/mol)	Interact with Key Amino Acids*	Similar Binding Pocket with Antagonist Control**
	Olmesartan (Inverse agonist)	158781	-	-9.0	Yes	-
	Valsartan	60846	-	-8.3	Yes	-
1	Fernenol	12305178	*I. cylindrica*	-11.4	Yes	Yes
2	Friedelin	91472	*I. cylindrica*	-11.4	Yes	Yes
3	Arundoin	12308619	*I. cylindrica*	-11.2	Yes	Yes
4	Simiarenol	12442794	*I. cylindrica*	-11.1	Yes	Yes
5	Alnusenone	10071029	*I. cylindrica*	-10.8	Yes	Yes
6	Cylindrin	189045	*I. cylindrica*	-10.8	Yes	Yes
7	α-Amyrin	73170	*I. cylindrica*	-10.6	Yes	Yes
8	Pomolic Acid	382831	*C. asiatica*	-10.5	Yes	Yes
9	Daucosterol	5742590	*I. cylindrica*	-10.4	Yes	Yes

* Key amino acids in protein AT1R (4ZUD) are as noted in literature 17[39], based on Olmesartan as an inverse agonist. Three residues, Tyr35, Trp84, and Arg167, were found to be critical for the agonist binding site.
**The binding pocket was identified through a molecular docking experiment in this study between Olmesartan and Valsartan with AT1R. Based on the literature, the binding pocket was formed by Phe182, Ala163, Lys199, Ser109, Phe77, Leu112, Tyr292, Leu81, Ile288, Thr88, Tyr92, Tyr87, and Cys180. This was similarly found in the molecular docking results of Olmesartan, with slight differences: Trp84 and Tyr35 formed a Pi-binding interaction, while Arg167 had a van der Waals interaction with Olmesartan. In Olmesartan, an additional hydrogen bond was formed with Asp281, and other Pi binding was formed with Ile288, Pro285, and Ala181, while Valsartan had a slightly different configuration.

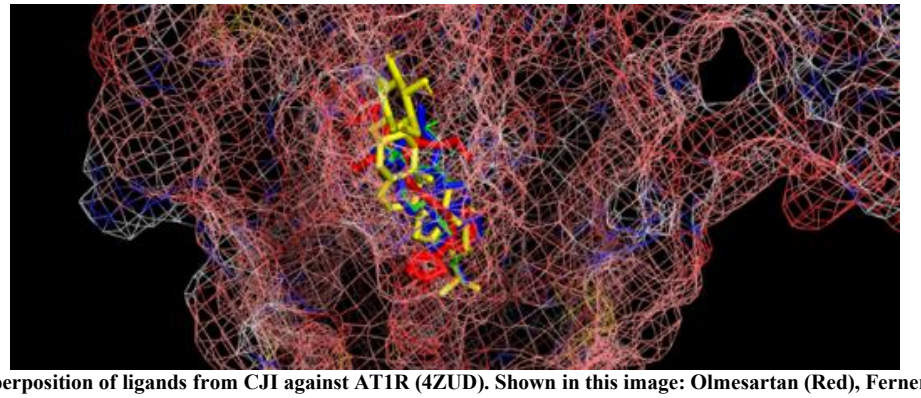


Figure 2. Superposition of ligands from CJI against AT1R (4ZUD). Shown in this image: Olmesartan (Red), Fernenol (Green), Arundoin (Blue), and Daucosterol (Yellow)

Table 5. Several ligands from *C. asiatica*, *I. cylindrica*, or *J. gendarussa* with high binding affinity against the mineralocorticoid receptor (MR, 2AA2) compared to Aldosterone as an agonist, and spironolactone and eplerenone as inhibitors

Rank #	Ligand Name	CID	Plant Source	Binding Affinity (kcal/mol)	Interact with Key Amino Acids*	Similar Binding Pocket with Antagonist Control**
	Aldosterone (activates MR)	5839	-	-6.7	-	-
	Spironolactone	5833	-	-7.3	-	-
	Eplerenone	443872	-	-7.1	-	-
1	4-Hydroxy-5-Methoxyflavone	676305	*I. cylindrica*	-9.3	Yes	No
2	Quercetin	5280343	*C. asiatica*	-9.3	Yes	No
3	5-Hydroxyflavone	68112	*I. cylindrica*	-8.9	Yes	No
4	5-Methoxyflavone	94525	*I. cylindrica*	-8.9	Yes	No
5	Alnusenone	10071029	*I. cylindrica*	-8.9	No	No
6	Flidersiachromone	441964	*I. cylindrica*	-8.9	Yes	No
7	Cylindrin	189045	*I. cylindrica*	-8.8	No	Yes
8	Patuletin	5281678	*C. asiatica*	-8.8	Yes	No
9	Kaempferol	5280863	*C. asiatica or J. gendarrusa*	-8.8	Yes	No

* Key amino acids in protein 2AA2: examines the crystal structure of the protein with aldosterone as noted in literature[23]. The protein is stabilized by Asn770 and Ser767, and activated by hydrogen bonding on Thr945. An extensive hydrogen bond network was also found between aldosterone and MR on Gly776 and Arg817. Unique to Aldosterone is a hydrogen bond to Cys942.
**The binding pocket was based on a molecular docking experiment in this study between the antagonist and MR. Eplerenone has alkyl interactions with Tyr944, Phe941, and Leu984; hydrogen bonds with Glu940; and van der Waals interactions with Asp937 and Glu847. Spironolactone has alkyl interactions with Pro978, Ala976, Val971, and Leu939; hydrogen bond with Arg983, and van der Waals bonds with Tyr980, Ser936, His932, Thr880, Tyr804 and Val935.

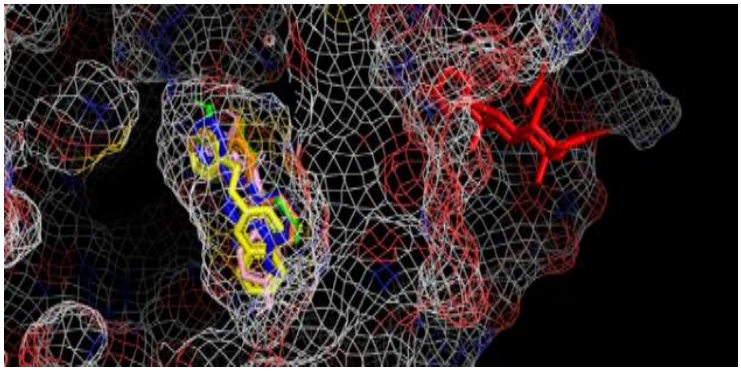


Figure 3. Superposition of ligands in the mineralocorticoid receptor (2aa2) showing a different binding site from the control. Shown in this figure: Spironolactone (Red), 5-Hydroxyflavone (Blue), 5-methoxyflavone (Green), Flidersiachromone (Magenta), Kaempferol (Orange), and Flavone (Cyan)

4. DISCUSSION

A parameter to measure the binding affinity of a ligand with a receptor for determining the stability of a conformation formed between a ligand and a receptor is Gibbs free energy (ΔG binding). The lower the free energy of a reacting molecule, the more stable the molecule formed and the reaction that occurs spontaneously. This is consistent with the law of thermodynamic balance. Therefore, the more negative the free energy of the reacting molecule, the more spontaneous the reaction will be, and the molecule will become more stable or will quickly form a stable configuration [33].

Several active compounds from CJI have been characterized, and the resulting publication served as the basis for selecting the ligands in this study. The compounds in *C. asiatica* are well characterized, and it is known to contain at least 57 major compounds within the isoprenoid (sesquiterpenes, plant sterols, pentacyclic triterpenoids, and saponins) and phenylpropanoid scaffold (eugenol derivatives, caffeoylquinic acids, and flavonoids) [15,16,34]. In this study, 60 compounds from C. asiatica were used in the docking, which include bicyclogermacrene (sesquiterpenes), pentacyclic triterpenoids, derivatives of cinnamic acids, asiaticosides and madecassosides, several isomeric dicaffeoylquinic acids, and ubiquitous flavonoids such as catechins, kaempferols, quercetins, rutins, and naringin.

Compounds in *I. cylindrica* were also well characterized, with 72 chemical constituents isolated and identified, including saponins, flavonoids, phenols, and glycosides [21]. However, not all these compounds had 3D structures available and therefore were not included in this study. From *I. cylindrica*, 49 compounds were used, which include saponins such as arundoin, cylindrin, and friedelin; flavonoids such as methoxy and hydroxyflavones; glycosides such as seguinbosides; phenols such as cylindol and graminones; coumarins such as siderin; and other compounds such as cylindrene. These compounds have various known properties, which function as antioxidants by donating hydrogen atoms or chelating metals, neutralizing free radicals, and preventing cell damage caused by oxidative stress. In addition, recent studies have demonstrated its anti-inflammatory activity in models for conditions like carrageenan-induced edema [35], zymosan-induced paw swelling [36], and dimethylbenzene-induced auricular edema [20].

Unfortunately, phytochemical constituents of *J. gendarussa* have not been well characterized, with few studies focusing on the plant. *J. gendarussa* is commonly a wild plant and is mainly found in Southeast Asia, and until the date of writing, relatively few studies have examined the phytochemical characterization of the plant [37,38]. Studies that have done some phytochemical characterization of J. gendarussa have found it to contain compounds used in this study, such as naringenin and kaempferol (from its leaves) [17,18], alkaloids such as squalene, lupeol, and Patentiflorin A [39]. Traditionally, by itself, this plant was used in the treatment of rheumatism, liver disorders, inflammations, and skin diseases [39]. In this study, only five compounds from J. gendarussa were used.

Molecular docking of active compounds from *C. asiatica*, *J. gendarussa,* and *I. cylindrica* against protein targets

Aside from renin, the conversion of angiotensinogen into angiotensin II relies on angiotensin-converting enzyme (ACE, PDB ID: 1R4L). ACE is a membrane-bound protein that binds to angiotensin and converts it into angiotensin II. Based on electron density maps for native and inhibitor-bound structures, ACE was found to have a binding site from His374 to His378 and from His401 to Glu406. The enzyme has a zinc metallopeptidase domain (residues 19 – 611) in which two catalytic subdomains can be found [23]. As shown in Table 3 and Appendix Table 3, Benazepril-HCl or Lotensin was able to bind to ACE with a binding affinity of -9.4 and had hydrogen bonds at His345, Thr371, and Asp367, which are located within the binding site of ACE. Thus, it can be hypothesized that Benazepril-HCl's mechanism of action is by occupying the binding site of ACE. Benazepril-HCl has been reported to be used in the therapy of hypertension[40,41].

The mechanism of action is currently unclear. Furthermore, the active compounds CJI, which have a strong affinity for the 1R4L receptor protein, were derivatives of flavonoids. One of these compounds was rutin with a Gibbs free energy of -10.7 kcal/mol. Several ligands from CJI were able to bind to ACE with a higher binding affinity and occupy the same space compared to Benazepril-HCl, as also shown in Table 3, namely, rutin, castillicetin, 1,5-Dicaffeoylquinic acid, naringin, and patentiflorin A. ACE inhibitors are widely used in the treatment of hypertension as well as heart failure[40,42] Our study showed that for protein ACE2 (1R4L receptor), all ligands had a stronger binding affinity compared to benazepril-HCl. Most of the ligands worked by occupying an inhibitor binding site in the receptor, although some of these overlap with the proposed amino acids thought to be the binding site of ACE-2 agonists. Notable ligands that may act as an ACE-2 antagonist were rutin, castillicetin, 1,5-dicaffeoylquinic acid, naringin, and patentiflorin. However, most of these had one or two unfavorable bonds.

The binding affinity of rutin is stronger than the control (-9.4 kcal/mol). The interaction of rutin with the ACE receptor protein (1R4L) is shown in green in Figure 1. In this figure, the interaction of all CJI ligands is seen in the same binding site on the ACE receptor protein (1R4L) shown. CJI ligands, namely Benazepril-HCl, Rutin, Castillicin, 1,5-Dcaffeoylquinic acid, naringin, and patentiflorin, are seen overlapping on the active side of the same receptor protein. CJI ligand interactions include hydrogen bonds, phi interactions, and van der Waals interactions. Rutin is a rutinoside, which is quercetin with a hydroxyl group at the C-3 position substituted by a glucose group and rhamnose sugar. Hidroxil group has a hydrogen bond with NH2 and COO- group in amino acid residue (Gly268, Asn149, Asp367, Thr445, His345, Thr276).

Our findings show that the active compounds in CJI that had the strongest affinity for the 4ZUD receptor protein (AT1R) are derivatives of terpenoids, namely friedelin and fernenol, with the Gibbs free energy for both being -11.4 kcal/mol, as shown in Table 4. As shown in Appendix Table 4, our findings showed that all ligands bound to the same binding pocket (also shown in Figure 2); however, ligands from CJI had a higher binding affinity and therefore have more potential for binding compared to controls. Furthermore, the ligands with potential to occupy the same binding pocket as controls, particularly on Tyr35, Trp84, and Arg167, were Fernenol, Arundoin, and Daucosterol. Furthermore, while most ligands were found bound to Tyr35, Trp84, and/or Arg167, only Alnusenone and Quadranoside IV were predicted to have hydrogen bonds with Tyr35 and Arg167, respectively. Generally, angiotensin II type 1 receptor (AT1R) antagonists have been widely used for the treatment of many diseases such as hypertension, heart failure, myocardial infarction, and diabetic nephropathy[43]. Their beneficial effects are related to the inhibition of Angiotensin II by blocking AT1R.

Molecular docking of CJI compounds with the AT1R receptor protein (4ZUD) revealed that ferneol and friedelin exhibited the strongest interactions, with binding affinities of –11.4 kcal/mol (Table 7). Their binding affinities were stronger than those of the control compound, olmesartan. The interaction of ferneol with the AT1R receptor (4ZUD) is illustrated in Figure 2 (green). The figure also demonstrates that all CJI ligands bind at the same active site on the AT1R receptor, suggesting that these compounds may act through a similar mechanism as the control drug.

Based on the binding results of CJI active compounds against MR receptor proteins, the compounds with the most antihypertensive potential are 4-hydroxy-5-methoxyflavone and quercetin, both of which have a Gibbs free energy of -9.3 kcal/mol (as shown in Table 5). The magnitude of the Gibbs free energy from these two compounds is lower compared to the control, spironolactone. Both active compounds are derivatives of flavonoids. Mineralocorticoid receptors are nuclear receptors expressed in various organs (particularly the kidney) that bind to aldosterone and act as transcription factors to regulate sodium balance in the organism[44].

The MR also has a role in the central nervous system that is poorly understood. It is expressed in the hippocampus and is involved in the regulation of different cellular mechanisms (apoptosis, etc.) and physiological processes (regulation of blood pressure and appetite for salt, etc.) [45]. Although tested ligands show some similar interaction (Table 5), based on the visual depiction below, the ligands do not occupy the same binding pocket as aldosterone or spironolactone (as shown in Figure 3). It can be speculated that several of these ligands could activate the MR, thus acting as aldosterone agonists; such as, 4-Hydroxy-5-methoxyflavone, Quercetin, and Patuletin have hydrogen binding against Gln776. Therefore, several ligands that did not have hydrogen binding with Gln776 were potentially competitive inhibitors: 5-Hydroxyflavone, 5-Methoxyflavone, Flidersiachromone, Kaempferol, and Flavone (Appendix Table 5).

This study presents the integration of *in vitro* and molecular docking approaches to discover multi-target antihypertensive activity of bioactive compounds from *C. asiatica*, *J. gendarussa*, and *I. cylindrica*. The use of multiple RAAS-related protein targets also provides a comprehensive view of their potential mechanisms. However, this study has certain limitations that should be acknowledged. To our knowledge, this is the first study to combine *in vitro* methods and molecular docking to discover the multi-target antihypertensive potential of the CJI combination. However, this approach may not fully capture the complexity of physiological systems, including the pharmacokinetic properties and potential toxicity of the identified compounds. However, the *in vitro* activity might actually be due to a different compound in the extract and lead to an incorrect mechanism when the it was docked. In addition, a compound must be present in sufficient concentration to deliver simultaneously affects to the tissue organs. Further research and *in vivo* studies should be conducted to confirm the therapeutic potential and safety of these plant-derived bioactive compounds.

5. CONCLUSIONS

This study demonstrated that the combined extract of CJI possesses potential multi-target antihypertensive activity based on in vitro antioxidant tests and molecular docking through molecular interactions with key proteins in the RAAS pathway, including renin, ACE, AT1R, and MR. LC–HRMS profiling identified several bioactive constituents such as asiaticoside, madecassoside, gendarusin A, and cylindrin that showed strong binding affinities and favorable pharmacokinetic properties, suggesting their contribution to the observed antihypertensive effects. Further biological validation studies are needed to confirm whether active substances of CJI extract interact with living systems to prove they are accurate and clinically relevant.

The integration of in vitro and in silico approaches provides a comprehensive understanding of the pharmacological mechanisms underlying the CJI combination. By targeting multiple components of the RAAS pathway, these compounds may exert synergistic effects in reducing oxidative stress and modulating vascular homeostasis, two key processes in the pathogenesis of hypertension. Collectively, these findings support the potential of CJI as a natural multitarget antihypertensive formulation and justify further in vivo and clinical investigations. Future studies should explore its pharmacokinetic behavior, safety profile, and therapeutic efficacy in hypertensive models to confirm its translational potential. The identification of bioactive compounds in CJI also provides a scientific basis for developing standardized herbal products or drug leads for hypertension management.

ACKNOWLEDGMENTS

The authors would like to thank the Direktorat Riset dan Pengembangan, Kementerian Pendidikan Tinggi, Sains dan Teknologi Republik Indonesia, for the research funding (No. 195/DST/C3/DT.05.00/2026).

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