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Original Article

Renin inhibition activity, phenolic and flavonoid contents of *Centella asiatica* (L.) Urb.

[Inhibición de renina, contenidos de fenoles y flavonoides de *Centella asiatica* (L.) Urb.]

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Abstract

Context: *Centella asiatica* (L.) Urb. and its components may have antihypertensive effects through renin inhibition.

Aims: To assess the *in vitro* renin inhibitory activity of extracts with various solvents from the *C. asiatica* leaves and asiaticoside, asiatic acid, madecacosside, and madecaside acid. Additionally, the study evaluates each extract's total phenolic and flavonoid contents.

Methods: Renin inhibitor activity *in vitro* was assessed using the renin inhibitor screening assay kit. Total phenolics and total flavonoid contents were spectrophotometrically measured using Folin-Ciocalteu and aluminum chloride, respectively.

Results: The extracts of ethanol, hexane, ethyl acetate, and butanol have renin inhibitory activity with IC_{50} = 1.329, 23.73, 53.76, and 61.38 µg/mL, respectively. Meanwhile, asiaticoside, asiatic acid, madecacosside, and madecaside acid showed IC_{50} = 2.09, 63.65, 48.2, and 38.12 µg/mL, respectively. Aliskiren, as a reference, exhibited an $IC_{50} = 3.56 \times 10^{-3}$ µg/mL. The total phenol levels for ethanol, hexane, ethyl acetate, and butanol extracts were 61.03 ± 3.44, 45.75 ± 0.77, 49.60 ± 2.33, and 76.78 ± 3.44 mgGAE/g, respectively. The total flavonoid contents were 51.95 ± 0.74, 70.01 ± 0.50, 50.46 ± 0.53, and 72.95 ± 1.28 mg QE/g, respectively.

Conclusions: The ethanol extract showed the lowest IC_{50} value, indicating better renin inhibitory potency. Asiaticoside may inhibit renin because its IC_{50} is lower than the other three. Since butanol extract displayed the highest total phenol and flavonoid contents, it could inhibit renin due to its antioxidant qualities.

Keywords: *Centella asiatica*; renin inhibitor; total phenol; total flavonoid.

Resumen

Contexto: *Centella asiatica* (L.) Urb. y sus componentes pueden tener efectos antihipertensivos a través de la inhibición de la renina.

Objetivos: Evaluar la actividad inhibidora de la renina *in vitro* de extractos con varios solventes de las hojas de *C. asiatica* y asiaticósido, ácido asiático, madecacósido y ácido madecásido. Además, el estudio evalúa el contenido total de fenoles y flavonoides de cada extracto.

Métodos: La actividad inhibidora de la renina *in vitro* se evaluó utilizando el kit de ensayo de detección de inhibidores de renina. Los contenidos totales de fenoles y flavonoides se midieron espectrofotométricamente utilizando Folin-Ciocalteu y cloruro de aluminio, respectivamente.

Resultados: Los extractos de etanol, hexano, acetato de etilo y butanol tienen actividad inhibidora de la renina con CI_{50} = 1,329, 23,73, 53,76 y 61,38 µg/mL, respectivamente. Mientras tanto, el asiaticósido, el ácido asiático, el madecacósido y el ácido madecásido mostraron una CI_{50} de 2,09, 63,65, 48,2 y 38,12 µg/ml, respectivamente. El aliskiren, como referencia, exhibió una CI_{50} de $3,56 \times 10^{-3}$ µg/mL. Los niveles totales de fenoles para los extractos de etanol, hexano, acetato de etilo y butanol fueron 61,03 ± 3,44, 45,75 ± 0,77, 49,60 ± 2,33 y 76,78 ± 3,44 mgGAE/g, respectivamente. Los contenidos totales de flavonoides fueron 51,95 ± 0,74, 70,01 ± 0,50, 50,46 ± 0,53 y 72,95 ± 1,28 mg QE/g, respectivamente.

Conclusiones: El extracto de etanol mostró el valor CI_{50} más bajo, lo que indica una mejor potencia inhibidora de la renina. El asiaticósido puede inhibir la renina porque su CI_{50} es menor que los otros tres. Dado que el extracto de butanol mostró los contenidos totales más altos de fenoles y flavonoides, podría inhibir la renina debido a sus cualidades antioxidantes.

Palabras Clave: *Centella asiatica*; inhibidor de renina; fenol total; flavonoide total.

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Abbreviations: ACE: angiotensin-converting enzyme; ACEi: angiotensin-converting enzyme inhibitors; ARB: angiotensin receptor blockers; BP: blood pressure; GAE: gallic acid equivalent; MGGA: monoglucuronyl glycyrrhetic acid; RAS: renin-angiotensin system; RAAS: renin-angiotensin-aldosterone system; TFC: total flavonoid compounds; QE: quercetin equivalent.

INTRODUCTION

The renin-angiotensin system (RAS) is essential for regulating blood pressure in the human body. Renin, acting as a crucial enzyme with rate-limiting properties, can break down the N-terminus of angiotensinogen, producing angiotensin I. Subsequently, angiotensin I undergo hydrolysis to form angiotensin II, a potent vasoconstrictor facilitated by the angiotensin-converting enzyme (ACE) catalytic activity. This enzymatic process ultimately leads to an elevation in blood pressure. Therefore, the inhibition of the RAS has become a crucial therapeutic approach for managing hypertension and its associated end-organ damage (Fountain and Lappin, 2021).

Renin inhibitors, angiotensin-converting enzyme inhibitors (ACEi), and angiotensin receptor blockers (ARBs) are the most commonly used antihypertensive drugs acting on the renin-angiotensin-aldosterone system (RAAS). Renin inhibitors have advantages compared to ACE inhibitors and ARBs. Renin inhibitors have relatively safer side effects than ACE inhibitors and ARBs. A drug belonging to the renin inhibitor class is aliskiren. Aliskiren is the only synthetic drug in the renin inhibitor class. In comparison to a placebo, aliskiren demonstrated a dose-dependent reduction in blood pressure, similar to the effects observed with ACEi inhibitors and ARB (Musini et al., 2017). Aliskiren reduces BP in hypertensive patients in a dose-dependent manner and controls blood pressure for 24 hours, up to a dose of about 300 mg once a day. At these levels, aliskiren exhibits tolerability similar to that of a placebo. It has antihypertensive potency comparable to angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, and diuretics. Prolonged suppression of plasma renin activity and lasting BP decrease are reported following rapid removal. When paired with diuretics, the BP reduction is entirely additive. Aliskiren induces significant extra BP decrease when combined with an angiotensin receptor blocker, indicating complementary pharmacology and more comprehensive renin-angiotensin system inhibition. The clinical trial findings will ultimately decide the role of renin inhibition and aliskiren in treating hypertension and related cardiovascular illnesses (Gradman and Kad, 2008). Among the agents that block the RAS, direct renin inhibitors have demonstrated remarkable effectiveness in controlling hypertension and pharmacological tolerance comparable to other suppressors of the renin-angiotensin system. Aliskiren, the sole direct renin inhibitor currently accessible, proves effective in managing blood pressure either as a standalone treatment or when combined with other antihypertensive medications, without regard to the patient's age, ethnicity, or gender. Furthermore, its efficacy extends to individuals with metabolic syndrome, obesity, and diabetes (Juncos, 2013). Currently, the only renin inhibitor available on the market is aliskiren. Therefore, searching for new drugs with antihypertensive effects with a mechanism of action as a renin inhibitor is necessary.

Polyphenols and saponins are standard classifications for renin inhibitors derived from natural sources. The native Indonesian pegagan [*Centella asiatica* (L.) Urb.] plant, family *Apiaceae*, contains saponins and polyphenols (Astiani et al., 2023). Asiaticoside, madasiatic acid, madecassoside, madecassic acid, and asiatic acid are among *C. asiatica*'s active ingredients (Vinolina and Sigalingging, 2022). An *in-silico* study showed that the five test ligands showed affinity energy values ranging from -8.7 kcal/mol to -10.4 kcal/mol. On the other hand, the comparative ligand, aliskiren, has an affinity energy value of -9.0 kcal/mol. The affinity energy values for madecasoside, asiaticoside, and asiatic acid are -10.4 kcal/mol, -9.6 kcal/mol, and -9.2 kcal/mol, respectively (Eff et al., 2023). Pharmacokinetic examination indicated favorable properties for asiatic acid, madecassic acid, madecassoside, and asiaticoside (Eff et al., 2023). Through *in-silico* molecular docking and pharmacokinetics exploration, it is suggested that the fourth of active substances from *C. asiatica* stands out as the most promising candidate for further *in-vitro* research as a renin inhibitor (Eff et al., 2023). Flavonoids are a massive family of polyphenolic compounds in humans. They are an anti-inflammation agent for neurological disorders, cancer, urolithiasis, venous insufficiency, and hypertension. Meanwhile, saponins function as anti-thrombotic, anti-inflammatory, anti-cancer, anti-diabetic, antihypertensive, and treating reproductive disorders (Xu et al, 2021).

C. asiatica has been used in traditional medicine systems for centuries to treat various ailments, including hypertension. This traditional use suggests the plant may contain bioactive compounds with potential therapeutic benefits (Bansal et al., 2024). *C. asiatica* is a source of natural renin inhibitors, specifically focusing on its active compounds: asiaticoside, asiatic acid, madecassoside, and madecassic acid (Sun et al., 2020). The ethnomedical use of *C. asiatica* for hypertension aligns well with its potential renin inhibitory activity (Bunaim et al., 2021). Renin inhibition is a

well-established mechanism for lowering blood pressure. By blocking the renin-angiotensin-aldosterone system (RAAS), renin inhibitors can reduce blood pressure and alleviate hypertension-related symptoms (Fountain and Lappin, 2021). Hypertension is often associated with inflammation and oxidative stress, and some of the compounds in *C. asiatica* have been shown to possess anti-inflammatory and antioxidant properties (Razali et al., 2019). These properties may contribute to its potential renin inhibitory activity and, consequently, its ability to lower blood pressure (Razali et al., 2019). By investigating the renin inhibitory activity of *C. asiatica* extracts and their bioactive compounds, this research can provide valuable insights into the potential of natural products in managing hypertension and other cardiovascular disorders.

In-vitro assays are a common practice in scientific research to perform before engaging in *in-vivo* studies, as they offer valuable insights into the behavior of biological systems. This sequential approach aids researchers in designing more efficient and effective *in-vivo* studies. In-vitro assays serve various purposes, such as screening potential drugs or treatments and elucidating fundamental biological mechanisms (Xu et al., 2021). They are crucial in identifying the specific target molecule or receptor to which a drug binds and determining the required drug concentration for a particular biological response (Tabana et al., 2023). By conducting *in vitro* assays prior to *in vivo* studies, researchers enhance their understanding of biological processes, pinpoint potential drug targets, and assess the efficacy and toxicity of treatments (Madorran et al., 2020). This strategy allows for a comprehensive screening of potential interventions before progressing to more complex and resource-intensive *in-vivo* investigations (Swinney and Lee, 2020). This study aims to assess the renin inhibitory activities of extracts with various solvents from the *C. asiatica* leaves and asiaticoside, asiatic acid, madecacosside, and madecaside acid using *in-vitro* method and to evaluate the total phenolic and flavonoid contents from each extract.

MATERIAL AND METHODS

Chemicals

Asiaticoside, asiatic acid, madecacosside, and madecaside acid were purchased from MarkHerb Indonesia, Aliskren (Sigma, Germany), ethanol (Brataco, Indonesia), ethanol (Brataco, Indonesia), ethanol pro analysis (Merck, Germany), hexane (Brataco, Indonesia), ethyl acetate (Brataco, Indonesia), butanole (Brataco, Indonesia), distilled water (Brataco, Indonesia) and renin inhibitor screening assay kit (Cayman Chemical, USA)

Plant material

Centella asiatica (L.) Urb. leaves obtained from cultivated plants from the Research Institute for Spices and Medicinal Plants (Balitro), Bogor, Indonesia (6.5767° S, 106.7858° E) and have been identified at Herbarium Bogoriensis, Center for Biological Research, Indonesian Institute of Sciences with the number identified B-1406/IV/DI.05.07/5/2022. The voucher specimen was deposited in the Herbarium of Pharmacognosy Phytochemistry Laboratory, Department of Pharmacy, Universitas Esa Unggul.

Extraction

The *C. asiatica* leaves were air-dried at room temperature for 15 days and reduced to a coarse powder. The powdered plant material was extracted using ethanol, ethyl acetate, hexane, and butanol for 72 hours at room temperature. The extraction process was carried out in triplicate. The solvent was removed using a rotary evaporator. The residue was dissolved in the appropriate solvent (ethanol was used to evaluate renin inhibitor activity, and water was used to determine total phenolic and flavonoid content). Phytochemical screenings detected the presence of both primary and secondary metabolites in each extract, including the presence of alkaloids, flavonoids, tannins, saponins, and steroids (Abubakar and Haque, 2020).

Renin inhibitor assay

The evaluation of renin inhibitor activities in *C. asiatica* leaf extracts and asiaticoside, asiatic acid, madecacosside, and madecaside acid through an *in vitro* assay using the renin inhibitor screening assay kit. The sample was initially diluted in ethanol (1000 µg/mL, pH 8.0) to create a stock solution. From this stock solution, test solutions with concentrations of 50, 100, 200, 400 µg/mL, and 800 µg/mL were prepared through dilution. Prior to the reaction, the test solution was prewarmed to 37°C. The assay involved three sets of preparations: Background wells received 20 µL substrate, 160 µL assay buffer, and 10 µL ethanol. Control wells were provided with 20 µL substrate, 150 µL assay buffer, and 10 µL of the sample. Inhibitor (sample) wells were equipped with 20 µL substrate, 150 µL assay buffer, and 10 µL of the sample. The reaction was initiated by adding 10 µL renin to the control and sample wells. The microplate was then shaken for 10 seconds to ensure proper mixing and subsequently incubated at 37°C for 15 minutes. Fluorescence intensity (FI) was recorded using a fluorometric microplate reader (Spectra MAX Gemini,

USA) at excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage of renin inhibition was calculated according to the formula [1].

$$\%\ Inhibition = \left[\frac{\text{FI (control)} - \text{FI (sample)}}{\text{FI (control)}}\right] x\ 100 \quad [1]$$

Where FI: fluorescence intensity on a fluorescence plate reader.

The analyses were performed in triplicate. The determination of the IC_{50} value involved plotting the percentage of inhibition or the percentage of initial activity. The IC_{50} value was identified as the concentration of samples in mg/mL needed to achieve a 50% reduction in renin activity (Gao et al., 2022).

Determination of total phenolic contents

Total phenolic content was determined in *C. asiatica* leaf extract using the Folin-Ciocalteu method, utilizing gallic acid as the standard solution. Various concentrations of standard gallic acid solution (ranging from 45-115 µg/mL) were prepared, and to each flask, 0.5 mL of Folin-Ciocalteau reagent was added. Subsequently, 2 mL of 1% Na_2CO_3 solution was introduced to the mixture, and the volume was adjusted to 5 mL with distilled water. The solution was then incubated for 60 minutes. The absorbance was measured at 740 nm using a UV-Vis spectrophotometer (Tekan infinite 200 M pro®). Total phenol content was calculated as % gallic acid equivalent (GAE) (Özay and Mammadov, 2019)

Determination of total flavonoid content

The total flavonoid content in each extract was investigated using the aluminum chloride colorimetry method. Concisely, approximately 1.5 mL of each extract solution was withdrawn from the 1000 µg/mL main solution. 0.1 mL of 10% $AlCl_3$ was added, followed by 0.1 mL of 1 M CH_3COONa. The volume was then adjusted to 5 mL using ethanol. The solution was incubated for 30 minutes in the dark at room temperature. Subsequently, the absorbance was measured at 425 nm using a UV-Vis spectrophotometer (Tekan infinite 200 M pro®). The content of the total flavonoid compounds (TFC) in the extract was expressed in mg quercetin equivalent (QE)/g of dried extract (Özay and Mammadov, 2019).

Statistical analysis

The data were expressed as a mean ± standard deviation from three observations. The IC_{50} values of *C. asiatica* leaves extract, asiaticoside, asiatic acid, madecacosside, madecaside acid, and total phenolic and flavonoid were analyzed statistically using a one-way ANOVA and multiple comparisons test to determine differences between treatment groups. Data analysis was carried out using SPSS software version 26. The p-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The extraction results using various solvents, namely ethanol, ethyl acetate, hexane, and butanol using maceration, produced yield values of 16.69%, 8.8%, 4.684%, and 9.112%, respectively. The results of examining the chemical content of the extract are presented in Table 1.

The results of the *in-vitro* test for renin inhibitors can be seen in Table 2.

The results of total phenolic and flavonoid content are presented in Table 3.

The C. *asiatica plant* has been widely used by the general public as an alternative therapy, one of which is as an antihypertensive. *C. asiatica* contains pentacyclic triterpenoids such as asiaticoside, brahmoside, and madecassic acid, as well as other substances such as centellose, centelloside, and madecassoside (Gohil et al., 2010). Triterpenes, such as asiaticoside, asiatic acid, madecassoside, and madecassic acid, play a key role in its pharmacological activity (Bansal et al., 2024). The ethanol extract of the *C. asiatica* leaves has antihypertensive effects at doses of 500 mg/kg and 1000 mg/kg in mice induced with L-NAME. After 8 weeks, the use of *C. asiatica* extract and captopril had a significant impact in preventing the increase in systolic blood pressure, lowering serum nitric oxide levels, and increasing cardiac and aortic MDA content, cardiac ACE activity, and serum brain natriuretic peptide levels ($p<0.05$) (Bunaim et al., 2021).

Table 1. Chemical constituent of extract.

Extract	Chemical constituent
Ethanol	Steroids, triterpenoids, tannins, and saponins
Ethyl acetate	Steroids, flavonoids, triterpenoids, and tannins
Hexane	Steroids, triterpenoids, and saponins
Butanol	Alkaloids, flavonoids, steroids, triterpenoids, and tannins

Table 2. *In-vitro* test results for renin inhibitors.

No.	Sample	IC_{50} (µg/mL)
1	Aliskiren	0.0033 ± 0.0002
2	Ethanol extract	1.35 ± 0.08
3	Ethyl acetate extract	53.67 ± 0.19*
4	Hexane extract	23.68 ± 0.23*
5	Butanol extract	61.46 ± 0.11*
6	Asiaticoside	2.09 ± 0.01*
7	Asiatic acid	61.99 ± 1.91*
8	Madecacosside	48.14 ± 0.17*
9	Madecassic acid	38.19 ± 0.07*

Data represent mean ± SD (n = 3); *P<0.05 significant difference as compared to aliskiren.

Table 3. Total phenolic and flavonoid contents.

Extract	Phenolic content (mgGAE/g)	Flavonoids content (mgQE/g)
Ethanol	61.03 ± 3.44*	51.95 ± 0.74*
Ethyl acetate	49.60 ± 2.33*	50.46 ± 0.53*
Hexane	45.75 ± 0.77*	70.01 ± 0.50*
Butanol	76.78 ± 3.44	72.95 ± 1.28

Data represent mean ± SD (n = 3). *P<0.05 significance difference as compared to butanol extract.

Extraction is the initial stage of separating desired natural products from raw materials (Zhang et al., 2018). The extraction process begins with creating simplicia, including wet sorting, washing, drying, cutting, and sorting. Wet sorting removes foreign objects from plants. Washing is done with flowing water to clean the plants of microbes and dust; the length varies depending on the plant. Chopping speeds up the drying process and makes milling and packing easier. Chopping should not be too thin to prevent the loss of active ingredients in the plants. Simplicia drying must meet quality requirements to preserve the simplicia from fungi; the action of the enzyme did not harm the active substance, and its chemical content was not damaged (Abubakar and Haque, 2020). The extraction process is carried out using simplicia in powder form so that the extraction process can run optimally. The extraction process is influenced by particle size; the smaller the particle size, the greater the surface area and the easier it is for the solvent to diffuse and break down the cell walls of *C. asiatica* leaves (Zhang et al., 2018).

The extraction technique is accomplished using maceration. The maceration method is a cold extraction technique that offers numerous benefits. For instance, it does not necessitate the plant part to be extracted as a fine powder, no specialized expertise is needed, solvent loss due to evaporation is minimized, and heating does not damage the compounds present in simplicia (Zhang et al., 2018). Multiple cycles of straightforward soaking and maceration are performed until the solvent becomes transparent. The purpose of re-maceration is to extract the most active compounds possible, as the extraction will be greater the longer the solvent is in contact with the substance (Abubakar and Haque, 2020).

Solvent extraction for soluble plant components is a safe extraction method. It yields comparable outcomes to other frequently employed techniques. The solvents utilized for extraction are ethanol, ethyl acetate, hexane, and butanol. The metabolite content of plant extracts is heavily impacted by the technique and polarity of the solvent used during extraction. Variations in solvent polarity affect the amount of extract produced, the phytochemical content, and the activity test (Abubakar and Haque, 2020).

Ethanol is a commonly utilized solvent due to its safety for infused edibles and compatibility with various types of containers. Ethanol offers reliable outcomes and is readily recovered. Ethanol is a protic organic solvent with a polarity index 5.2 and a dielectric constant of 24.55. Ethyl acetate is a suitable solvent for extraction because it can quickly evaporate, is not hygroscopic, and has low toxicity. Ethyl acetate is

semi-polar with a dielectric constant value of 6, so it can attract both polar and non-polar compounds. N butanol is a polar solvent with a dielectric constant of 12.5. The dielectric constant value is in line with a solvent's polarity level. The more polar a solvent, the higher the dielectric constant value. n-hexane is a non-polar solvent with an alkane hydrocarbon chain with the chemical formula C_6H_{14}, and the dielectric constant is 1.89. Hexane is the result of oil refining. Composition and fractions influenced by oil sources. Generally, around 50% of the chain weight isomers and boils at 60–70°C. All hexane isomers are often used as an organic solvent and are inert because they are non-polar. n-Hexane can attract compounds in *C. asiatica* leaves that are non-polar, namely triterpenoid (Plaskova and Mlcek, 2023).

In the first step of phytochemical screening, steroids, triterpenoids, tannins, flavonoids, and saponins were found in the ethanol, ethyl acetate, hexane, and butanol extracts. Phytochemical screening is a valuable method for identifying the components of plant extracts and determining their relative abundance. Additionally, it aids in searching for bioactive compounds that can produce valuable pharmaceuticals (Pant et al., 2017). Other investigations have supported the previous findings by confirming the presence of alkaloids, phenolics, flavonoids, saponins, tannins, triterpenoids, steroids, and glycosides in *C. asiatica* (Anam et al., 2023; Pokhrel and Neupane, 2021). The primary active ingredient found in *C. asiatica* is saponin, also known as triterpenoids. These include asiaticosides, a polymer class where the trisaccharide group connects with madasiatic acid, madecassoside, asiatic aglycone, brahmoside, and brahminoside (Sun et al., 2020). The efficacy of centelloside and its derivatives in treating venous hypertension has been established. *C. asiatica* also comprises various constituents such as plant sterols, flavonoids, essential acids (specifically beta-caryophyllene, trans-beta-pharnesen, and germachrene D), phytosterols (campesterol, sitosterol, and stigmasterol), flavonoids (specifically quercetin and kaempferol derivatives), an alkaloid (hydrocotin), a bitter component (vallerin), and fatty acids (linoleic, linolenic, oleic, palmitic, and stearic acid) (Gohil et al., 2010).

Flavonoids are a class of compounded polyphenols characterized by the presence of C6-C3-C6 rings (referred to as rings A and B) connected by ring C. Flavonoids are ubiquitously present in the extracts of several green plants. The results of phytochemical testing have indicated the presence of flavonoids in ethyl acetate and butanol extracts. The presence of the yellow tint in all the extracts is apparent (Abubakar and Haque, 2020). Flavonoids possess bioactive compounds that exhibit anti-inflammatory, anti-cancer, neuroprotective, and antioxidant properties (Safe et al., 2021). Flavonoids can potentially prevent cell damage due to oxidative stress by donating hydrogen ions to neutralize the toxic effects of free radicals. Flavonoids inhibit the activity of the ACE and cause relaxation of the blood vessel endothelium so that more blood will flow to the heart and cause a decrease in blood pressure (Maaliki et al., 2019).

Saponins have a relatively high molecular weight. Saponin tests were carried out on the plant materials using a qualitative procedure with foam tests and color tests. A relatively stable form of foam can characterize saponins. Based on the result, it was found that ethanol and hexane extracts contained saponin. The saponin structure is non-polar, which causes saponins like soap or detergent (Ashour et al., 2019). Some saponins are known to be capable of deactivating viruses and have antimicrobial and anti-inflammatory effects (Ashour et al., 2019). The saponin group has the potential to act as a diuretic by reducing plasma volume by removing water and electrolytes, especially sodium, which ultimately causes a decrease in cardiac output (Takahashi et al., 2010).

Steroids encompass a collection of biologically significant secondary metabolites synthesized by various organisms, including plants, animals, and microbes. These chemicals usually have a tetracyclic 1,2-cyclopentenoperhydrophenanthrene (5α- or 5β-gonane) carbon framework. The carbon framework has methyl substituents at positions C-10 and C-13 and an alkyl substituent (side-chain) at position C-17 (Dembitsky, 2023). Steroids are widely present in plants and significantly impact the pharmacological effects of these medications. Plant steroids have interesting medical and pharmacological properties. They can fight tumors, weaken the immune system, protect the liver, kill bacteria and parasites, damage cells, and relax the heart (Dembitsky, 2023). Phytochemical screening results showed that the four extracts of *C. asiatica* contained steroid compounds.

The results of the phytochemical screening showed that alkaloid content was found in the butanol extract. Alkaloids are a class of chemical molecules characterized by their low molecular weight and nitrogen content that exhibit pharmacological properties in humans and animals (Bhambhani et al., 2021). A wide range of alkaloids can be found in many plant components, such as seeds, fruit, stems, roots, leaves, and other organs (Bhambhani et al., 2021). To determine if an extract contains alkaloids, add 1 mL of chloroform and 5 mL of 10% ammonia. Ten drops of 2 N sulfuric acid are added, and then Mayer's reagent is added until a crimson precipitate is formed (Abubakar and Haque, 2020). Alkaloids play a role in reducing blood pressure. The mechanism of action of alka-

loids is analogous to hypertension medications belonging to the β-blocker category. Specifically, alkaloids exert their effects by diminishing the intensity of myocardial contractions and reducing heart rate, resulting in a decrease in cardiac output and peripheral retention (Bhatia et al., 2019).

Table 2 shows that IC_{50} for aliskiren is 0.0033 ± 0.17 µg/mL and has no significant difference as compared to ethanol extract ($p>0.05$). Ethanol is a polar solvent that can extract various compounds, including polar compounds such as flavonoids, phenolics, and terpenoids. These compounds often have biological activity similar to the active compounds in synthetic drugs. Non-polar solvents like hexane extract non-polar compounds, while semi-polar solvents such as ethyl acetate and butanol excel in extracting compounds of intermediate polarity. The composition of compounds extracted by these solvents can vary significantly from that of ethanol extracts (Abubakar and Haque, 2020). *C. asiatica* ethanol extract has the smallest IC_{50} value compared to ethyl acetate, hexane, and butanol extracts. Meanwhile, asiaticoside has the lowest IC_{50} value in pure compounds compared to asiatic acid, madecacoside, and madecaside acid. The study conducted by Chen et al. (2013) demonstrated that patients who received aliskiren treatment achieved superior blood pressure control compared to those treated with ACE inhibitors and receptor blockers. Aliskiren may, therefore, be considered a more efficacious antihypertensive medication as a pharmacological renin inhibitor with an IC_{50} value of 0.6 nM for controlling blood pressure. *C. asiatica* is commonly found in tropical regions, such as India and Southeast Asia (Sun et al., 2020). This herb has been utilized to treat diverse ailments, including fever, wound healing, gastric discomfort, leprosy, bladder hypofunction, and urinary tract infections (Gohil et al., 2010). *C. asiatica* includes biologically active triterpenoid chemicals, including asiatic acid, madecassic acid, asiaticoside, and madecassoside. In addition, the *C. asiatica* plant has successfully extracted various chemicals, including flavonoids, brahmoside, brahminoside, isothankuniside glycosides, and thankuniside (Sun et al., 2020). Our *in-silico* study showed that the active compound *C. asiatica* has the potential as a renin inhibitor based on energy affinity value, and pharmacokinetic analysis revealed that asiatic acid, madecassic acid, madasiatic acid, and asiatic acid have good pharmacokinetic properties (Eff et al., 2023). Soya-saponin was the first renin inhibitor isolated from soybeans. Studies conducted by Takahashi et al. (2010) showed that soya-saponin I and II, glycyrrhizin, monoglucuronyl glycyrrhetic acid (MGGA), chikusetsusaponin IV, and *Kochia scoparia* fruit saponins (momordins) were effective in inhibiting renin activity, while sapogenol (soyasapogenol B and glycyrrhetic acid), saikosaponin b2 and c, and ginsenoside Rb1 had no activity. The research also emphasized the pivotal role of the 3-O-β-d-glucopyranosiduronic moiety in saponins for renin inhibition.

In a rat model with hypertension, the chloroform fraction of *C. asiatica* contains a high concentration of triterpenoids and flavonoids. This extract has been found to effectively reduce abrupt spikes in blood pressure with a single administration. Asiatic acid, a prominent triterpenoid molecule found in *C. asiatica,* has been shown to inhibit the renin-angiotensin-aldosterone system (RAAS) activation in a mouse model of metabolic syndrome. In vitro studies demonstrate the capacity of *C asiatica* to suppress renin and ACE activity (Bunaim et al., 2021). Renin is a crucial enzyme in the renin-angiotensin system. It is responsible for releasing angiotensin I from angiotensinogen, a plasma precursor (Fountain and Lappin, 2021). However, there has been limited research on identifying plant-derived renin inhibitors. Recently, a study was conducted on soybean saponin as a potential source of renin inhibitors. The study discovered several compounds like soya saponin I and II, glycyrrhizin, monoglucoronyl glycyrrhizic acid, chikusetsusaponin IV, and *Kochia scoparia* fruit saponins that have inhibitory effects on renin activity. The study also found that 3-O-B-D glucuronide saponin is necessary to suppress renin (Takahashi et al., 2010).

C. asiatica possesses significant antioxidant capabilities due to its rich presence of triterpenoids and flavonoids. *C. asiatica*'s active chemicals exert a therapeutic impact on conditions associated with oxidative stress, such as hypertension and heart failure (Gohil et al., 2010; Kunjumon et al., 2022). The total phenolic and flavonoid content of each extract is shown in Table 3. The butanol extract has the highest phenolic (76.78 ± 3.44 mgGAE/g) and flavonoid (72.95 ± 1.28 mgQE/g) content compared to hexane, ethyl acetate, and ethanol ($p<0.05$). However, this result is inversely proportional to the activity of butanol extract in inhibiting renin. Butanol extract has the most enormous IC_{50} value (61.46 ± 0.09 µg/mL) compared to ethanol extract, ethyl acetate extract, and hexane extract. The high total phenolic content of *C. asiatica*, primarily due to the presence of flavonoids such as quercetin, kaempferol, catechins, rutin, apigenin, and naringin, is of particular interest. These flavonoids, with their direct impact on blood pressure reduction, have been shown to have a hypotensive effect in hypertensive rodents induced by L-NAME. Furthermore, they enhance the relaxation of cardiovascular smooth muscle, demonstrating an antihypertensive effect (Bunaim et al., 2021).

Polyphenolic chemicals, commonly found in plants, fruits, and vegetables, play a crucial role in cardiovascular protection and improving endothelial function. These chemicals are derivatives and/or isomers of flavones, isoflavones, flavonols, catechins, and phenolic acids. They have been proven to be biologically active as both free radical scavengers and antioxidants. Their antioxidant activity is primarily attributed to redox properties that act as reducing agents, hydrogen donors, and singlet oxygen quenchers, making them a fascinating area of study (Zhang et al., 2022). Flavonoids, with their antioxidant and antiradical actions, are instrumental in benefiting the cardiovascular system through various molecular mechanisms. Both experimental and clinical studies have shown that these bioactive compounds can modify oxidative weakness and lower cardiovascular risk (Godos et al., 2019). They function as signaling molecules, interacting with cellular receptors or proteins, thereby influencing regulatory pathways that can trigger physiological responses or alter gene expression. Of particular interest, flavonoids have a direct impact on the function and regulation of endothelial cells, which in turn affects blood flow. They have the unique ability to inhibit endothelial NAD(P)H oxidase, a key player in the control of nitric oxide (NO) (Hügel et al., 2016). In the vascular endothelium, through inhibiting superoxide production. Some types of phenolic compounds or their metabolites help endothelial function by acting as antioxidants and releasing NO, which widens blood vessels and stops the renin-angiotensin-aldosterone system and angiotensin II receptor activity (Clark et al., 2015).

In recent years, studies have witnessed a surge in the identification of natural renin inhibitors in foodstuffs, a significant development in the field of food science and health. These include saponin from soybeans, oleic acid, and linoleic acid from rice, all of which hold potential for the development of antihypertensive functional foods and plants. Additionally, pea protein hydrolysate contains dipeptides; however, no plant-derived renin inhibitor compounds are currently available. Developing potential antihypertensive functional foods and plants provides another significant method for screening renin inhibitors. Polyphenolic compounds can potentially contribute to renin inhibition, a finding that the renin-inhibitory activities of saponins and polyphenolic extracts of *C. asiatica* may corroborate (Tavassoli et al., 2018).

CONCLUSION

The findings support the potential of *Centela asiatica* as a source of natural renin inhibitors. Among the various solvent extracts (ethanol, ethyl acetate, hexane, and butanol), the ethanol extract stood out with its remarkable and potent renin inhibitory activity. Asiaticoside, a triterpenoid compound isolated from *C. asiatica*, demonstrated the strongest renin inhibitory activity compared to asiatic acid, madecacoside, and madecassic acid. The total phenolic and flavonoid content varied across the extracts but did not directly correlate with renin inhibitory activity. These results, which are in line with previous studies suggesting the potential antihypertensive properties of *C. asiatica*, underscore the need for further research to unravel the mechanisms that underlie the renin-inhibitory effects of *C. asiatica* and its isolated compounds. This study opens doors for future investigations into the development of natural renin inhibitors derived from *C. asiatica* for potential applications in managing hypertension.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Eff ARY	Eden Y	Rahayu ST	Radji M
Concepts or ideas	x			x
Design	x		x	
Definition of intellectual content	x			x
Literature search	x	x	x	x
Experimental studies		x	x	
Data acquisition		x		
Data analysis	x	x		
Statistical analysis	x			
Manuscript preparation	x	x	x	x
Manuscript editing	x	x		x
Manuscript review	x	x	x	x

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