RASĀYAN *J. Chem.*
Vol. 16 | No. 2 |565-572| April - June | 2023
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

PHYSICOCHEMICAL, PHYTOCHEMICAL, AND EVALUATION ANGIOTENSIN-CONVERTING ENZYME INHIBITORY ACTIVITY OF TAMOENJU (*Hibiscus surattensis* L.) LEAVES EXTRACT

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ABSTRACT
Tamoenju (*Hibiscus surattensis* L.) is one of the ethnomedicinal plants used in treating hypertension. One of the potential antihypertensive mechanisms includes angiotensin-converting enzyme ACE inhibitors, considered the first-choice treatment for hypertension. This research presents a physicochemical, phytochemical, and ACE inhibitory activity test of tamoenju leaf extract (TLE). The leaf extract was evaluated to determine the physicochemical and phytochemical profiles using standard methods. The physicochemical evaluation indicated 20.66 ± 3.51% water content, 26.67±5.77% drying shrinkage, 2.0 ± 1.0% total ash value, 0.30 ± 0.13% acid insoluble ash value, 20.00 ± 4.00% water soluble extractive value, 57.00 ± 4.61% alcohol soluble extractive value, and 0.89±0.01 g/ml specific gravity extract. The total plate and yeast mold numbers were $15x10^3$ CFU/g and 92 x 10 CFU/g, respectively. The results of metal contamination showed Pb 1.05 ± 0.15 mg/kg and Cd 0.14 mg/kg. The phytochemical evaluation revealed the presence of tannins (69.02%), flavonoids (27.63%), alkaloids (14.18%), saponins (2.12%), and steroids (1.06%). ACE inhibitory activity was tested and obtained at 38.50±1.41 µg/mL for TLE and 9.44±0.58 µg/mL for captopril. Based on the research results, TLE qualifies as a standardized extract and can be used as a reference for developing herbal medicines. Tamoenju leaves extract is quite high as a natural ACE inhibitor and has the potential as an antihypertensive.
Keywords: Angiotensin Converting Enzyme, Antihypertensive, Physicochemical, Phytochemical, Tamoenju.
RASĀYAN *J. Chem.*, Vol. 16, No.2, 2023

INTRODUCTION

Tamoenju (*Hibiscus surattensis* L.) potherb of Malvaceae is widely available in Africa and Asia, including Indonesia.[1] The plant is harvested from the wild for local use as food, medicine, and a source of materials. Ethnopharmacological studies show this plant's efficacy for treating wounds[2], urethritis, venereal disease, abscesses[2,3], and hypertension.[4] There has been no further research on the antihypertensive activity of tamoenju leaf extract. The pathophysiology of hypertension is primarily determined by the Angiotensin-Converting Enzyme (ACE) activity. Angiotensin Converting Enzyme is a membrane-anchored dipeptidyl peptidase that plays a crucial role in blood pressure homeostasis by hydrolyzing the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II. In addition, ACE also catalyzes the degradation of bradykinin, a nonapeptide vasodilator, to inactive fragments. Inhibition of ACE performance is considered the main therapy for hypertension.[5,6] ACE inhibitors such as captopril, lisinopril, and enalapril show good effectiveness but cause various side effects, such as dry cough, skin rashes, and angioedema.[7,8] Current trends in antihypertensive drug research are looking for potential ACE inhibitors from natural ingredients that provide health benefits without side effects. Previous studies reported that the secondary metabolite content of ethanol extract tamoenju leaves showed the presence of alkaloids, glycosides, tannins, saponins, flavone, flavanone, and steroids.[1,9] These phytochemical components have ACE inhibitory activity in vitro. Therefore, it is exciting to develop ACE inhibitor antihypertensive drugs from plants commonly used by the public for hypertension therapy, especially tamoenju leaves. The extract is an

http://doi.org/10.31788/RJC.2023.1628199

essential material for herbal medicine. Herbal drug formulation requires consistent biological activity, chemical profile, or quality assurance programs that can be achieved by standardizing the extracts. Standardization is a system to ensure medication being marketed as the correct substances in the right amount will induce its therapeutic effect. Maintaining the consistency of biological activity, chemical profile, or quality assurance programs for producing and manufacturing herbal drug preparation is crucial. Furthermore, extract standardization can also increase the economic value of herbal medicine producers.[10,11] This standardization is carried out by specific and non-specific parameters based on generalized standardization parameters of medicinal plant extract issued by the Indonesian Ministry of Health.

EXPERIMENTAL

Material and Methods

The materials used were an extraction set, maceration container, blender (Signora), UV lamp 254 and 366 nm (CAMAG), vacuum rotary evaporator (EYELA N-1 200 B), oven (Oxone), incubator (Eyela), hotplate (Vendille), autoclave (Eyela), petri dish (Pyrex Iwaki), analytical balance (Ohaus), vortex (Labnex VX 200), UV-Vis spectrophotometer (CECIL 7000 series), micropipette (Labnet Biopette), calliper (NANKAI), chamber, shaker water bath (Grant), Laminary Air Flow (Stream Line), spare steel (well), and Accuris SmartReader™ 96 Microplate Reader. The materials used were tamoenju leaves obtained from Alindau village, Donggala district, Central Sulawesi. The ingredients used included distilled water, Water One, 96% ethanol (Bratachem, Indonesia), *n*-hexane (Bratachem, Indonesia), ethyl acetate (Bratachem, Indonesia), *n*-butanol (Merck, Germany), DMSO (Merck, Germany), GF254 TLC plate (Merck, Germany), 10% sulfuric acid (H_2SO_4) (Merck, Germany), 1% $FeCl_3$ (Merck, Germany), anisaldehyde-sulfuric acid reagent, Lieberman Burchard reagent, 0.9% NaCl solution, ACE Kit-WST DOJINDO. Captopril was obtained from Indonesian FDA Laboratory Services (INFALABS).

Sample Extraction

Extraction methods use previously reported methods[12] and calculate the yield of the extract.

Physicochemical Evaluation

The organoleptic test is to show the characteristics early, simply, and objectively by using the sense. The organoleptic parameters of tamoenju leaf extract were described, including the shape, odour, colour, and taste.[13]

Determination of Extract Compound Based on Solvent

Water Soluble Compound Assay

One gram of tamoenju leaf extract was macerated for 24 h using 25 ml of water-chloroform in a plugged Erlenmeyer while shaking for the first 6 hours. Furthermore, allowed to stand for 18 h, then filtered. The filtrate steam is dried in a cup. The residue was heated at 105°C until the weight was constant. Then calculate the percentage of water-soluble compounds to the weight of the initial extract.[14]

Ethanol Soluble Compound Assay

One gram of tamoenju leaf extract was macerated for 24 h with 25 ml of ethanol (96%) into a plugged Erlenmeyer while shaking for the first 6 h and then allowed to stand for 18 h. It was filtered with filter paper quickly, then the filtrate was evaporated to dryness in a cup and the residue was heated at 105°C until it reached a constant weight.[15]

Water Content

A total of one gram of tamoenju leaf extract was weighed in a calibrated container. It was dried at 105°C for ± 5 h and weighed. Continue drying and weighing at 1 h intervals until the difference between 2 consecutive weighing is less than 0.25%.[16]

Density Extract

The density of the extract was determined by dilution of 5% of the extract in ethanol solvent with a pycnometer. Pycnometer was dried and calibrated by assigning weights pycnometer and freshly boiled water to 25°C, then put into the pycnometer at 25°C.[17]

Total Ash Content
The ash content is determined by weighing 3 g of extracted tamoenju leaves, place on asbestos, flattened, and heated until ash. Then the ash was weighed.[17]

Acid Insoluble Ash Content
The obtained solution is filtered using filter paper. The ash obtained dissolved 25 ml chloride acid 10% v/v for 5 min in a porcelain cup and then boiled. The remaining ash on the filter paper is then washed with hot water. Filter paper and residual ash were heated until the weight was fixed.[17]

Determination of Total Yeast Mold
A total of 1 ml of extract from dilutions 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} was pipetted with a sterile pipette, then implanted in PDA medium, then incubated at 25°C for 3 days. Then observed and counted the number of colonies that grew and multiplied them by the dilution factor. It was replicated three times.

Determination of Total Microbes
The total plate number of tamoenju leaves extract using count plate methods. A total of 1 ml of extract from dilutions 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} was pipetted with a sterile pipette, then implanted in NA medium, then incubated at 37°C for 24 h. Then observed and counted the number of colonies that grew and multiplied them by the dilution factor. It was replicated 3 times.

Metal Contamination (Pb and Cd)
Determination of heavy metal (Pb and Cd) content was carried out by atomic absorption spectrophotometer (AAS) method using the destruction method for sample preparation. Destruction begins with weighing 1 gram of the tamoenju leaf extract and adding 10 ml of aqua regia (HCl-HNO_3) 3:1, then heating with a heating mantle until dry or thick and then filtering with Whatman paper no. 41 and put into a 50 ml volumetric flask. Samples were measured using SSA. Read the absorbance of the standard series solution of blanks and extract samples. A calibration curve is created with the Y as absorbance.[18]

Qualitative Phytochemical Analysis
Qualitative phytochemical screening was carried out according to standard procedures with minor modifications.[19]

Alkaloid Test
The sample was basified with 10% ammonia solution chloroform. The chloroform layer formed was then pipetted and filtered. After filtering, the filtrate was added to a 2 N hydrochloric acid solution and shaken vigorously until two layers were formed. The separated top layer is then divided into three parts and treated as follows:

- The first part is used as a blank.
- The second part is dripped with Mayer reagent and then observed. The occurrence of turbidity or white precipitate indicates the presence of alkaloids.
- The third part was dripped with Dragendorff's reagent and then observed. The formation of an orange-brown precipitate indicates the presence of alkaloids.

Flavonoid Test
One gram of tamoenju leaves extract was put into the test tube and was dissolved with 1 ml 96% ethanol, magnesium powder was added, and hydrochloric acid was added. If it is formed orange, red, yellow, or purple it means that it positively contains flavonoids.

Saponin Test
One gram of tamoenju extract was put into a test tube, dissolved in 10 ml of hot water, cooled down then shaken vertically with a little ether for about 10 seconds. With the addition of HCl 2N, the foam did not disappear.

Tannin Test
One gram of tamoenju leaf extract was dissolved in 100 ml of water, heated for 5 min, and then filtered. The filtrate of 5 ml was divided into three and put into a test tube. The first tube is added with a few drops

of 1% iron (III) chloride solution. It may contain tannins if a dark blue or greenish-black color is formed. The second tube is added with a few drops of gelatine solution. The formation of a white precipitate indicates the presence of tannins. The third tube was added with a few drops of Stiasny reagent (a mixture of 30% formaldehyde and 2:1 concentrated HCl) and heated using a water bath. The presence of catechate tannins is indicated by the formation of a pink precipitate. The result of the reaction from the third tube is filtered, the filtrate is added with sodium acetate until it is saturated, then a few drops of 1% iron (III) chloride solution are added. The formation of a blue-black color indicates the presence of the gallic tannin group.

Steroid and Triterpenoid Test

The tamoenju extract was mixed with 3 ml of chloroform or 3 ml of 70% ethanol and 2 ml of concentrated sulfuric acid, and 2 ml of anhydrous acetic acid. The color change from purple to blue or green indicates the presence of steroid compounds, and the formation of a brownish color between the surfaces suggests the presence of triterpenoid compounds.

Quantitative Phytochemical Analysis

A phytochemical quantitative test of the extracts of tamoenju leaves was performed per standard protocols to reveal the existence and level of alkaloids[20], flavonoids[21], saponins[22], tannins[23], and steroids[24] with minor alterations.

ACE Inhibition Assay

ACE inhibitory activity was measured using ACE kit-WST, and captopril was used as a standard[25]. The testing procedures were performed strictly according to kit guidelines from the manufacturer using a 96-well plate without modification (Dojindo Laboratories). The assay used 3-hydroxybutyrate glycine (3HB-GGG) as a substrate for screening the ACE inhibition rate. The absorbance of the resultant of the extract/standard sample was measured using a microplate reader at 450 nm. The ACE inhibitory activity was calculated based on the comparison of absorbance of the extract/standard sample (As), positive control (Ac), and reagent blank (Ab) as in the equation below. Samples were diluted into 6 concentrations which were 12.5; 25; 50; 100, and 200 µg/ml. Captopril was a control standard with 2, 4, 8, 18, 32, and 64 µg/ml. The ACE inhibitory activity was calculated based on the comparison of absorbance of the extract/standard sample (As), positive control (Ac), and reagent blank (Ab) as in the equation below:

$$\text{ACE Inhibitory Activity (\%)} = \frac{(Ac- \quad)}{(Ac-Ab)} \text{ x } 100$$

RESULTS AND DISCUSSION

Sample Extraction

The yield obtained from the maceration process was 11.25%. Yield is the ratio between the amount of extract obtained from the extraction process and the initial weight of the simplicia. The heavier the simplicia used, the more weight the extract and yield produced. The calculation of the yield percentage aims to determine the amount of simplicia required for extraction to obtain the desired amount of extract.

Physicochemical Evaluation

Physicochemical testing of tamoenju leaf extract was carried out to focus on the chemical, physical, and microbiological aspects, namely those that play a direct role in consumer safety. Non-specific parameters are responsible for the quality and safety of a natural material. The results of physicochemical testing can be seen in Table-1. Dissolving the extract with certain solvents, namely water, and alcohol, to determine the number of dissolved compounds gravimetrically. To find out or provide an initial description of compounds containing natural ingredients. The test results showed that tamoenju leaf extract was more soluble in ethanol than in water. This indicates that the number of non-polar and semi-polar compounds that dissolve in ethanol is greater than that of polar compounds that dissolve in water. Determination of drying shrinkage aims to provide an overview of the range of compounds lost in the drying process. Specific gravity is related to the contamination or purity of the extract. Determination of ash content aims to provide an overview of the internal and external mineral content from the beginning to the formation of the extract. The results of testing the water content, drying shrinkage, density extract, total ash, and acid insoluble ash

value of ethanol extract of tamoenju leaves showed that the extract had met the requirements based on the literature.[26] Aspects of microbial contamination aim to determine the presence of microbes whose nature can damage the extract so that efforts can be made to prevent contamination or eliminate contamination by the permitted microbial contamination requirements. The presence of microbial contamination can affect the stability of the extract and is toxic to health. Parameter determination of heavy metals is closely related to the quality and safety of the extract. Examination of metal contamination can guarantee an extract does not contain certain heavy metals such as Cd and Pb.[26, 27] Contamination results from bacteria, mold, and heavy metals (Pb and Cd) were found within the limits and comparable with pharmacopoeial standards.

Table-1: The Result of Physicochemical Evaluation of Tamoenju Leaves Extract

No	Essay	Result
1	Organoleptic	Thick extract, brownish green color, distinctive odor, astringent taste
2	Water soluble compound content (%)	20.00 ± 4.00
3	Ethanol soluble compound content (%)	57.00 ± 4.61
4	Water content (%)	20.66 ± 3.51
5	Density extract (g/ml)	0.89±0.01
6	Drying shrinkage	26.67±5.77
7	Total ash value (%)	2.0 ± 1.0
8	Acid insoluble ash value (%)	0.30 ± 0.13
9	Total plate number (CFU/g)	$15x10^3$
10	Yeast mold number (CFU/g)	92 x 10
11	Pb (mg/kg)	1.05 ± 0.15
12	Cd (mg/kg)	0.14

Phytochemical Analysis

Phytochemical testing is carried out on specific parameters focused on the active compounds responsible for providing pharmacological effects. The test is aimed at identifying qualitatively[28] and quantitatively an active compound that plays a role in a natural ingredient. Based on Table-2, the qualitative phytochemical test tamoenju leaves extract showed the presence of alkaloids, flavonoids, saponins, tannins, and steroids. The results of the quantitative test showed that tamoenju leaf extract contained the highest concentration of tannins, followed by flavonoids, alkaloids, and saponins. Steroids have the smallest amount. The results obtained support the results of previous studies that tamoenju leaf extract contains phenolic and flavonoid compounds.[12] Previous studies have shown that tannin, which are natural polyphenols, and flavonoids, have inhibitory action on the angiotensin-converting enzyme (ACE). So the content of these compounds contributes to the activity as an ACE inhibitor.[29,30]

Table-2: The Result of Phytochemical Analysis of Tamoenju Leaves Extract

No	Assay	Result
1	Qualitative analysis	Alkaloids, flavonoids, saponins, tannins, and steroids
2	Quantitative analysis	
	Tannins	69.02%
	Flavonoids	27.63%
	Alkaloids	14.18%
	Saponins	2.12%
	Steroids	1.06%

ACE Inhibition Assay

Previous ethnobotanical research stated that tamoenju leaves (*H. surattensis* L.) could be used for antihypertensives[4], but there is no scientific evidence to support the results of these studies. Therefore, this study conducted an initial screening to scientifically prove tamoenju leaf extract's antihypertensive activity. Tests were carried out in vitro on its activity as an angiotensin-converting enzyme inhibitor. ACE inhibitors are used as first-line drugs for treatment as antihypertensives.[31] Screening of antihypertensive effects in traditional medicine has been carried out over the years. Several compounds from plants have been

identified to have ACE inhibitory activity, such as flavonoids, tannins, and alkaloids.[32] The results showed that tamoenju leaf extract had a relatively high inhibitory effect on the angiotensin-converting enzyme. This finding is important to support finding natural ACE inhibitors from biological resources. However, the ACE inhibitory activity of captopril as a positive control was more potent than that of the tested extracts. The IC_{50} calculation results are presented in Table-3.

Table-3: IC_{50} Values of Tamoenju Leaves Extract

No	Sample	IC_{50} (µg/mL)
1	Tamoenju leaves extract	38.50±1.41
2	Captopril	9.44±0.58

The ACE inhibitor activity of the tamoenju leaves extract obtained was better than the previous study using the *Hibiscus rosa sinensis* (271 µg/mL) plant which has the same genus.[33] This study shows that tamoenju leaf extract is a potential source of bioactive compounds that can inhibit ACE activity. The presence of kaempferol, morin, and trifolin compounds contributes to the activity as an angiotensin-converting enzyme inhibitor in the tamoenju leaf extract. The activity is related to the chemical structure of the compound, namely the double bond between C2 and C3 in the C-ring; the catechol group in the B-ring (3',4'dihydroxy), and ketone group on C4 carbon on the C-ring.[34,35] These results also scientifically proved the tamoenju plant's traditional use as an antihypertensive drug with its mechanism of action as an ACE inhibitor. However, further research must confirm its pharmacological activity and mechanism *in vivo*.

CONCLUSION

Based on the result of physicochemical and phytochemicals, tamoenju leaf extract has met the specified requirements. The results of the ACE inhibition assay showed that the tamoenju leaf extract could be developed as a natural ACE inhibitory activity.

ACKNOWLEDGMENTS

This work was funded by the Ministry of Education, Culture, Research, and Technology with grant scheme National Competitive Basic Research (PDKN) No 146/E5/PG.02.00.PT/2022.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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