ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Special Issue (October)

Online - 2455-3891
Print - 0974-2441

Research Article

ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY ACTIVITY, TOTAL PHENOLIC AND FLAVONOID CONTENT OF EXTRACT AND FRACTION OF JAM FRUIT LEAVES (*MUNTINGIA CALABURA* L.)

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Received: 20 April 2017, Revised and Accepted: 13 July 2017

ABSTRACT

Objective: Hypertension is one of the most common chronic diseases. Inhibitory activity of angiotensin I-converting enzyme (ACE) is effective on giving hypotensive effect. Jamfruit leaf (*Muntingia calabura* L.) was reported to have an excellent hypotensive effect. This research was aimed to test in the manner of *in vitro* the inhibitory activity of ACE.

Methods: This research used ACE kit-WST, total phenolic content, and total flavonoid from jam fruit leaf ethanol extract, hexane, ethyl acetate, and butanol fraction.

Results: The result showed that Jamfruit leaf extract had ACE inhibitory activity and the most active fraction was ethyl acetate fraction. Inhibitory concentration 50% value of the most active fraction, ethyl acetate fraction was 0.63 µg/mL. Ethyl acetate fraction also provides most flavonoid and phenolic content with a value of 10.91 mg/g extract quercetin equivalent and 74.90 mg/g extract gallic acid equivalent.

Conclusions: Ethyl acetate fraction of jam fruit leaf had most flavonoid, phenolic compound, and ACE inhibitory activity.

Keywords: *Muntingia calabura*, Angiotensin I-converting enzyme inhibition, Antihypertensive, Phenolic, Flavonoid.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10s5.23123

INTRODUCTION

High blood pressure or hypertension is the most important risk factor of death and disability in the world in 2010 [1]. The prevalence of hypertension in Indonesia according to data from the Health Ministry in 2013 reached 25.8% with the highest prevalence in the Bangka Belitung by 30.9% [2]. Thus, there is a challenge in continuing to seek alternative treatment of hypertension which effective and inexpensive.

Indonesia has a variety of plants that grow well and have been used as medicine for generations. One of the most available and has many benefits the jam fruit (*Muntingia calabura* L.). *M. calabura* has been used as traditional medicine for hypertension from time to time. Research in Taiwan found their strong hypotensive effect through activation of nitric oxide signaling pathway of cherry or jam fruit leaf extract [3]. There was no more research on the hypotensive activity by jam fruit leaf extract. More research was necessary included the search for another hypotensive activity pathway. ACE inhibitors are the first-line medicine in the treatment of hypertension because of their effectiveness in decreasing blood pressure. ACE plays a role in the activation of angiotensin I to angiotensin II, which acts as a potent vasoconstrictor and also stop the vasodilatory effects of bradykinin [4]. Clinical analysis and meta-analysis showed a reduction in the morbidity of cardiovascular and death on the use of ACE inhibitors and suggest the use of ACE inhibitors as a treatment of the first choice while ARB working specifically inhibit the receptor that binds to angiotensin II, given to patients who are sensitive to ACE inhibitors [5]. It is also important to classify the active chemical constituents in jam fruit leaf. The search was done through this experiment by comparing the flavonoid, phenolic content, and ACE inhibitory activity of jam fruit fractions.

METHODS

This study was conducted in Phytochemical Laboratory and Quantitative Analysis of Pharmaceutical Chemistry of the Universitas Indonesia, Depok. Work procedures done were material preparations, extractions, fractionations, ACE inhibition percentage measurements, and inhibitory concentration 50% (IC_{50}) test from the extract and also total phenolic and flavonoid content measurements on jam fruit (*M. calabura* L.) fractions.

Material preparation

Plant determination was conducted to confirm that we used the right plant, such as jam fruit (*M. calabura* L.). Plant identification result showed that sample was in Muntingiaceae Family, *M. calabura* L. species.

Extraction

Dry and clean powdered plant was grinded to obtain a smaller size. Extraction was done by maceration. 500 g of powdered leaves were put into maceration container. Ethanol was added to the container (until 3-5 cm above the surface). Extraction was done 2 × 24 hrs; extracts were collected and evaporated using a vacuum rotary evaporator at a temperature of 55°C with a speed of 50 rpm.

Fractionation

Fractionation was done by solvent-solvent fractionation to separate the group of compounds according to their polarity using solvents which do not mix. Fractionation was done to 50.11 g of extract using n-hexane (nonpolar), ethyl acetate (semi polar), and butanol (polar) as solvents. Extracts were put into 600:600 mL polar solvent and water. Fractionations were done to obtain filtrate which is nearly colorless.

ACE Inhibition assay

ACE inhibition assay was performed using ACE kit-WST from Dojindo. Borate buffer pH 8.3 containing 380 mM NaCl was used as a buffer. Absorbance measurements carried out at a wavelength of 450 nm uses filter-based microplate reader. Samples were diluted into 6 concentrations which was 100, 25, 12.5, 6.25, 3.125, and 1.563 µg/mL. Captopril was used as a control standard.

Total phenolic content (TPC)

Determination of TPC of the sample was done using the Folin–Ciocalteu assay and followed the methods of work of Al-Saeedi and Hossain (2015) with some modifications [6]. TPC expressed as the total gallic acid equivalent (GAE). 200 mL and put in a tube, 200 mL of sample was put into the reaction tube. 1.5 mL of Folin–Ciocalteu reagent was added to the tube. Then, the tube was incubated in the dark at room temperature for 5 minutes. After 5 minutes, 1.5 mL of Na_2CO_3 6% was added to the tube and incubated back during the time of incubation in the dark and at room temperature. After incubation measured the solution using a UV-Vis spectrophotometer at a wavelength of optimum.

Total flavonoid (TF)

TF content was determined by the method of Chang *et al*. [7]. A total of 0.5 mL sample was added to 1.5 mL of methanol then followed by the addition of 0.1 mL of$AlCl_3$, 10% 0.1 mL of 1 M sodium acetate, and 2.8 mL of aquadest. After incubation in maximum incubation time, the absorbance was measured at the maximum wavelength. Level of TF was expressed in mg quercetin equivalent (QE)/g extract.

RESULTS AND DISCUSSIONS

ACE inhibitor activity assay

ACE kit-WST was selected for testing fast, accurate, and specific. The test was done using a microplate reader, so it was time saving and requires only small amounts reagents. Most of the conventional methods based on the principle of formation of hippuryl-histidyl- leucine (HHL) by the action of ACE. The product or hippuric acid will be read with a spectrophotometer at a wavelength of 228 nm. ACE activity readings could be disturbed by HHL which were not hydrolyzed which also resulted in strong absorption at a wavelength of 228 nm [8]. ACE is an enzyme that works in a non-specific cut two amino acids from amino acid sequence of the substrate. ACE kit-WST used 3-hidroksibutirilglisil-glisil-glycine (3HB-GGG) as a substrate solution. Previously many researches have been done using substrates glycine-glycine and give good results [9]. Assay using ACE Kit-WST followed the principle of the assay by Lam *et al*. [10]. In principle, as the ACE enzyme dipeptidilkarboksi peptidase works by cutting two peptides from peptide chains on its C terminal. ACE inhibitory activity test is performed by measuring the amount of 3HB obtained from cutting two peptide substrates 3HB-GGG by ACE activity by measuring uptake of WST-1 formazan. Enzymatic reactions occur at a temperature of 37°C. Absorbance measurements carried out at a wavelength of 450 nm uses filter-based microplate reader.

Ethanol extract jam fruit leaves with a concentration of 100.40 µg/mL gave 82.70% of inhibition percentage while the percentage of captopril standard at a concentration of 5×10^{-8} mg/mL was obtained as 34.42%. The inhibition percentage of the ethanol extract of leaves of jam fruit at 100.40 mg/mL was not greater than the inhibition percentage of captopril, but it showed that the extract had activity as an ACE inhibitor. Extract of *M. calabura* leaves gave IC_{50}values of 1.25 µg/mL (Table 1).

Inhibitory activities of ACE by ethanol extract of leaves of jam fruit were included active depended on a category by Elbl and Wagner [11]. For the fractions, IC_{50} of ACE tests were done to fraction which had the most active ACE inhibitory activity. Ethyl acetate gave the smallest value of IC_{50}. Inhibition percentage at 8.33 ppm of the hexane, ethyl acetate, and butanol fractions was 55.21; 81.24; 70.32, consecutively (Table 2).

TPC assay

Folin-Ciocalteu reagent is a redox reagent that will react with the polyphenol compounds. It is believed that the Folin-Ciocalteu reagent contains heteropoly molybdate phosphotungstate. The series of reversible reduction reaction of one or two electrons produces a blue compound which is $PMoW11O40^{4-}$ [12]. Tests performed at the maximum wavelength of 740 nm. Another research on ethyl acetate fraction of jam fruit leaves obtained GAE values of 871.71±8.27 mg/100 g dry weight of crude extract [13]. The results were written in Table 3.

Table 1: Inhibition percentage of ACE by ethanol extract of jam fruit leaf

Concentration (µg/mL)	Inhibition (%)	Regression equation	IC_{50} (µg/mL)
8.37	65.66±2.37	$Y=\frac{100}{1+10^{(0.09583-x*0.3038)}}$	1.25
4.18	58.08±1.67		
2.09	52.66±1.12	R^2=0.9755	
1.05	48.27±0.83		
0.52	44.60±2.34		

ACE: Angiotensin I-converting enzyme, IC_{50}: Inhibitory concentration 50%

Table 2: Inhibition percentage of ACE by ethyl acetate fraction of jam fruit leaf

Concentration (µg/mL)	Inhibition (%)	Regression equation	IC_{50} (µg/mL)
8.33	81.24±0.13	Y=4.0459x+47.469 R^2=0.9894	0.63
4.17	63.32±0.14		
2.08	57.80±1.13		
1.04	52.20±0.56		
0.52	48.11±0.86		

ACE: Angiotensin I-converting enzyme, IC_{50}: Inhibitory concentration 50%

Table 3: TPC content of jam fruit fractions

Sample	TPC content (mg/g extract)
Hexane fraction	33.61±0.62
Ethyl acetate fraction	74.90±1.32
Butanol fraction	41.94±0.83

TPC: Total phenolic content

Table 4: Flavonoid content of jam fruit fractions

Sample	TF (mg/g extract)
Hexane fraction	5.53±0.42
Ethyl acetate fraction	10.91±0.50
Butanol fraction	5.63±0.83

TF: Total flavonoid

TF assay

Measurement of TFs was performed according to the method of Chang *et al*. which used aluminum chloride reagent [7]. The principle of measurement of TFs is complex formation ketone group and a hydroxyl group C ring flavonoids or the hydroxyl group at the ortho position A or B ring flavonoids with aluminum chloride [5]. The complex will provide a bathochromic shift in the absorption of flavonoids that can be tested the levels of TFs. The test result showed the value of TF of jam fruit leaves extract was 47.79 mg QE/g extract. The test was done as well to the fractions, and ethyl acetate gave the highest value of TF.

The study in Taiwan obtained results that water fraction of jam fruit leaf provided significant hypotensive effects compared with control [3]. Researches which were done previously showed that jam fruit leaves contain flavonoids, phenolic compounds, saponins with flavonoids, and steroids as most commonly found in the leaves of jam fruit. ACE inhibitory activity by flavonoids had been extensively tested and resulted that some of the flavonoids effectively inhibit the activity of ACE. Some flavonoids were found to provide ACE inhibitory activity present in jam fruit leaf extract is chalcone, quercetin, and genistein [14,15]. In jam fruit leaf, ethyl acetate which gave the highest value of inhibition activity has the highest value of phenolic content and TF. This may indicates that flavonoid and other phenolic compounds in jam fruit leaves gave ACE inhibitory activity to jam fruit leaves. Further

research can be done to explore further information about active chemical constituents of jam fruit leaf as a hypotensive agent.

CONCLUSION

Ethyl acetate fraction of jam fruit leaf had the highest level of flavonoid, phenolic compound, and ACE inhibitory activity. IC_{50} value of the most active fraction, ethyl acetate fraction was 0.63 µg/mL. The flavonoid and phenolic content of ethyl acetate fraction of jam fruit leaf were 10.91 mg/g extract QE and 74.90 mg/g extract GAE.

ACKNOWLEDGMENT

This study was supported by the Directorate of Research and Community Services, University of Indonesia.

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