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Original Article

Optimization of Microwave-Assisted Extraction of Active Compounds, Antioxidant Activity and Angiotensin Converting Enzyme (ACE) Inhibitory Activity from *Peperomia pellucida* (L.) Kunth

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ABSTRACT

Introduction: to obtain the optimum extraction conditions of total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (AA) and angiotensin converting enzyme (ACE) inhibitory activity from *Peperomia pellucida* (L.) Kunth. **Method:** TPC, TFC, AA and ACE inhibitory activity was measured by Folin-Ciocalteu method, $AlCl_3$ method, DPPH and FRAP methods, and ACE kit-WST. MAE efficiency parameters used were ethanol concentration, a sample to solvent ratio, extraction time, and microwave power. **Results:** The optimum conditions of MAE for TPC (49.78 mg GAE/g extract) and TFC (37.18 mg QE/g extract) were 80% ethanol, sample-solvent ratio of 1:12, extraction time of 2 min, MAE power of 30% and 80% ethanol, ratio of 1:12, time of 2 min, and 70% power respectively. The optimum AA was obtained at 65% ethanol, ratio of 1:10, time of 3 min, 50% power (DPPH method), and 65% ethanol, the ratio of 1:12, time of 1 min, and 70% power (FRAP method) respectively. The optimum ACE inhibitory activity (54.73% at a concentration of 100 µg/mL) were obtained at 80% ethanol, ratio of 1:12, time of 2 min, and 70% power. **Conclusion:** The analysis of Pearson correlation indicated that there was no correlation between TPC with FRAP method; TPC with ACE inhibitory activity and TFC with DPPH method.

Key words: ACE, Antioxidant Activity, Flavonoid Compounds, Microwave-Assisted Extraction, *Peperomia pellucida* (L.) Kunth, Phenolic Compounds.

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INTRODUCTION

Peperomia pellucida (L.) Kunth is medical herb of Piperaceae that grows in damp places and is widely available in countries America and Asia including Indonesia.[1] The ethanolic extract of *P. pellucida* contains alkaloids, saponins, tannins, flavonoids, terpenoids, and cardiac glycosides.[2] From the previous research, phenolic compounds such as flavonoids and tannins reported having antioxidant activity and the ACE inhibitory caused by phenolic groups. The optimum content to obtain of phenolic compounds, flavonoid compounds activity as ACE inhibitors and antioxidants activity of herbal extracts necessary appropriate extraction method. Extraction with conventional methods produce inferior products and needs a lot of solvents, energy and time.[3] Modern methods such as microwave-assisted extraction (MAE) becomes with benefits, among others results higher extraction, extraction time is short and a little solvent consumption.[3] There are factors that the efficiency MAE extraction with the irradiation time, the volume and concentration of the solvent, and microwave power.[4] The optimizing of MAE need four factors. Response surface methodology (RSM) is a mathematical techniques and statistics in this case can investigate the relationship between the variables of extraction and the results, as well as to optimize the experimental variable. Compared factorial design, experimental design with RSM more efficient in reducing the number of experiments carried out and the time required for optimizing the extraction conditions.[5] In this study used RSM to obtain extraction conditions optimum of phenolic compounds, flavonoid compounds, ACE inhibitory activity, and antioxidants activity. In this study is expected to obtain optimum conditions MAE of total phenolic content, the flavonoid content, ACE inhibitory activity and antioxidant using RSM.

MATERIALS AND METHODS

Materials

Plants materials

Peperomia pellucida (L.) Kunth obtained from *Balai Penelitian Tanaman Obat dan Aromatika* (BALITRO), Bogor. The herbs were stored in drying cabinets and sorted then powdered by a grinding machine to obtain a dry powder.

Materials and reagents

Gallic acid, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sodium carbonate and 2,4,6-Tri(2-pyridil)-s-triazine (TPTZ) were purchased from Sigma-Aldrich. Folin-Ciocalteu, dimethyl sulfoxide, sodium acetate, iron (III) chloride, hydrochloric acid, glacial acetic acid, methanol and ethanol (pro analysis) were purchased from Merck. ACE kit-WST

was purchased from Dojindo, Japan. Captopril was obtained from Kimia Farma, Indonesia. Distilled ethanol comes from Brataco.

Methods

Extraction and experimental design

The herbs powder (20g) extraction with MAE method was performed using ethanol-water in a 1000 mL round bottom flask. The extraction was adjusted based on parameters such as concentrations of solvent extraction, extraction time, sample ratio of the solvent, and MAE power. The extraction parameters were optimized using response surface methodology (Design Expert® software version 10.0.2.0) with Box-Behnken experimental design.[3] All parameters were independent variables which the value ranges were selected based on preliminary test results. Three levels of each variable coded as -1, 0, and +1. Then extract was filtered and evaporated using rotary vacuum evaporator.

Determination of total phenolic content (TPC)

Determination of TPC was performed by spectrophotometric method used the Folin-Ciocalteu reagent according to Shah *et al* (2016) with slight modification, and the results were expressed in mg gallic acid equivalents per g of the extract (mg GAE/g extract).[6] An aliquot (0.5 mL) of diluted sample was mixed with 2.5 mL of 10% v/v Folin-Ciocalteu reagent, then was vortex and incubated for 2 min at 25°C. After that, 2.0 mL of 7.5% w/v $Na_2CO_3$was added. The solution was vortex and incubated at 25°C for 60 min. The absorbance of the mixture was measured at 765 nm against the blank solution using a UV-Vis spectrophotometer. The blank solution contains a mixture of reagents and solvents without the sample. Total phenolic levels are determined using a standard calibration curve of gallic acid.[6,7]

Determination of total flavonoid content (TFC)

Total flavonoid content was measured using 10% $AlCl_3$ with some modifications.[9] The extract (25 mg) was diluted in 25 mL of methanol, 0.5 mL aliquot of the diluted sample was mixed with 1.5 mL methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M sodium acetate, and 2.8 mL distilled water. The mixture was vortex and incubated for 30 minutes at 37°C. The absorbance of the mixture was measured at 415 nm with a UV–VIS spectrophotometer. Quercetin was used as a reference standard, and the results were expressed as mg of quercetin equivalents (QE) per g of the extract (mg QE/g extract).

Determination of radical scavenging activity by the DPPH method

Radical scavenging activity was determined by using DPPH method.[10,11] Dry extract was dissolved in methanol. The diluted sample (1 mL) was reacted with 1mL DPPH (150 ppm) and 2mL of methanol. The mixture was vortex and incubated in the dark for 30 minutes at 37°C. The absorbance was measured with spectrophotometer UV/Vis at 515 nm. Quercetin was used as a reference standard.

Determination of antioxidant activity (AA) by the FRAP method

Ferric Reducing Antioxidant Power (FRAP) assay was performed according to the Benzie and Strain (1999) method with some modifications.[12] Brieftly, an aliquot of extracted sample (100 L) was added with 3.0 mL of FRAP reagent in test tubes, then homogenized. Both samples and blank were incubated in water bath for 30 min at 37°C. Then, the sample absorbance was measured against blank FRAP using spectrophotometer UV/Vis at 595 nm. Series of stock solution at 24, 30, 66, 78, 120, 150 µM were prepared using ethanol of Gallic acid as standard calibration curve. AA of the sample was expressed as µmol of gallic acid equivalent per g of extract (µmol GAE/g extract).[13]

ACE inhibition assay

ACE inhibitory activity was measured using ACE kit-WST (Dojindo Laboratories, Japan) and captopril was used as a standard. The dried extract was dissolved in ddH_2O to obtain a concentration of 100 g/mL. The testing procedures were performed strictly according to kit guidelines from the manufacturer using a 96-well plate without modification (Dojindo Laboratories, 2013). The assay used 3-hydroxybutyrate glycine (3HB-GGG) as a substrate for the screening of the ACE inhibition rate. The absorbance of resultant of the extract/standard sample was measured using micro plate reader (Thermo Scientific, USA) 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.

ACE Inhibitory Activity (%) = [(Ac – As)/(Ac – Ab)]×100

Thin layer chromatography (TLC) method

TLC profile of the extracts were determined by TLC densitometry using silica plate (Merck, Germany) as stationary phase and dichloromethane: methanol (90:10) mixture as mobile phase. The plate which has been eluted then we analyzed using densitometer (Camag, Swiss) at 254 nm and 366 nm.

RESULTS

Selection of extraction parameters

The parameter of extraction conditions of MAE was determined through the study of literature. According to there were several factors that affect the efficiency of MAE, including the type of solvent, the sample-solvent ratio, extraction time and microwave power. The polar solvents such as ethanol-water was suitable to be used for extracting of phenolic compounds which were polar because generally these compounds bind to sugar compounds as glycosides. Independent variables and levels as shown in Table 1. Based on those variables and levels, there are a total 25 run experiments.

Total phenolic content

Graph of TPC of each sample is shown in Figure 1. Based on the graph, it appears that the run 20 gives the average level of the highest, namely 49.78 mg GAE/g extract, with solvent extraction conditions such as 80% ethanol, the sample-solvent ratio of 1:12, extraction time of 2 min, and 30% microwave power. TPC of *P. pellucida* extract obtained in this study (49.78 ± 1.15 mg GAE/g extract) was quite high when compared with the methanol extract at reflux method (35.79 ± 1.10 mg GAE/g extract). Meanwhile, when compared with maceration method (25.09 ± 0.53 mg GAE/g extract), the MAE method also greater yield in TPC. Run 20 was the optimum conditions MAE of total phenolic compounds in the herbs. This indicated that the MAE method was an efficient method to obtain the content of phenolic compounds in the plants.

Thin layer chromatography profile

Figure 2 showed TLC profile of the extract. The results of densitometry at a wavelength of 366 nm shows a sample run 23 and quercetin which has the biggest spot at R_f of 0.28 (1.56%) and 0.5 (7.77%), respectively. The presence of flavonoid in *P. pellucida* were clarified by TLC profile after stained by $AlCl_3$ solution.

Determination of the total flavonoid content (TFC)

Figure 3 showed effect of MAE conditions on total flavonoid content. MAE optimum conditions for the extraction of total flavonoid content is run 23. Run 23 consists of 80% ethanol solvent, the sample-solvent ratio of 1:12, 2 minutes, and power 70.

Free radical scavenging of DPPH

Free radical DPPH scavenging activity of each extract after MAE extraction was presented in Figure 4. Run 1 is the optimum extraction conditions with reduction of the free radical DPPH value of 28.55%. The extraction conditions in run 1 in the form of ethanol 65%, a sample-solvent ratio of 1:10, extraction time of 3 minutes, and 50% microwave power while run 8 with ethanol-water 50%, the ratio of sample to solvent 1:12, time 2 minutes and 30% power.

Correlation between TFC and radical scavenging activity

The results showed no significant relationship (p> 0.05) between the levels of flavonoids to antioxidant activity DPPH method, with a correlation coefficient r = 0.41. Correlation analysis between TFC and free radical DPPH scavenging activity was presented in Figure 5.

Determination of antioxidant activity by FRAP method

In this study, EC_{50} of Gallic acid that indicates the concentration needed to increase the uptake of 50% was 5.29 M. From the graph of antioxidant capacity FRAP methods obtained from each run (Figure 6), it appears that the second run is the optimum extraction conditions with antioxidant capacity values 130.33 ± 5.19 mol GAE/g extract. The extraction conditions in run 2 in the form of 65% ethanol, a sample-solvent ratio of 1:12, extraction time of 1 min, and 70% microwave power.

Correlation between TPC with antioxidant capacity of FRAP method

The analysis showed that no significant relationship (p> 0.05) between TPC and antioxidant capacity of FRAP method (r = 0.359) (Figure 7), which means that the relationship positive, weak and not significant.

ACE inhibition assay

ACE inhibition activity assay was conducted on a sample extract with a concentration of 100 ppm, and captopril solution was used as a standard comparison with IC_{50} values of 1.13×10^{-12} ppm. Run 23 provides the mean percent ACE inhibition above 50%, which is 54.73% (Figure 8). Conditions such as solvent extraction run 23 to 80% ethanol, the sample-solvent ratio of 1:12, extraction time of 2 minutes, and 70% microwave power.

Correlation between TPC with ACE inhibitory activity

The correlation between TPC with ACE inhibitory activity of ethanol-water extract of *P. pellucida* was analyzed using Pearson correlation. In this research, the relationship was not significant (p> 0.05) between the total phenolic content of the percent inhibition of ACE ethanol-water extract of herbs errand (r = 0.322), which means that the relationship included in the category of weak (Figure 9).

When compared to the optimum MAE phenolic compounds, the difference MAE conditions for obtaining optimum ACE inhibitory activity lies only in the micro-wave power, which is 70%. It is possible that the active compound as an ACE inhibitor is not just a phenolic compound, and at 70% power and extraction time of 2 minutes, the compound is not degraded.

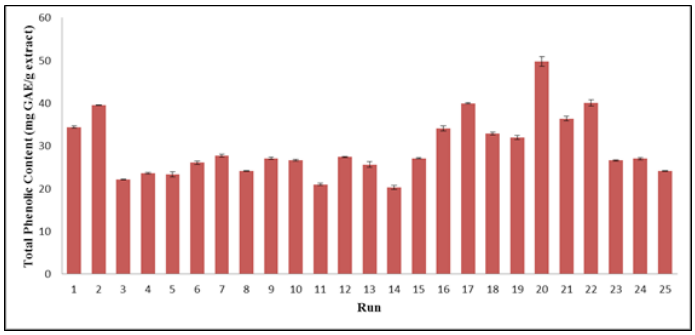

Figure 1: Graph of TPC from *Peperomia pellucida* (L.) Kunth extract (n = 3).

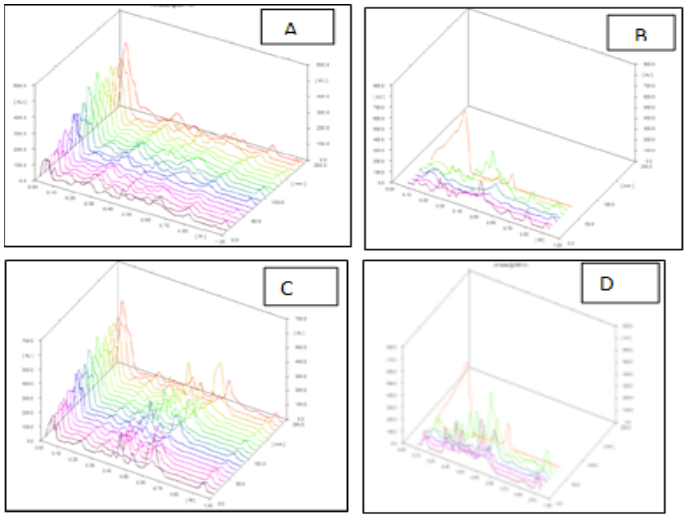

Figure 2: Thin layer chromatography profile of *P. pellucida* extract using densitometry (A) run 1-19 (B) run 20-kuersetin 254 nm wavelength (C)run 1-19 and (D) run 20-25 wavelength 366 nm

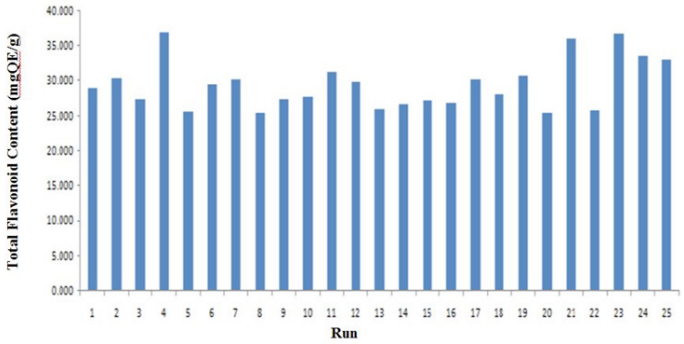

Figure 3: Total flavonoid content of *P. pellucida* (L.) Kunth extract of each MAE condition (Values are the mean of triplicate measurements).

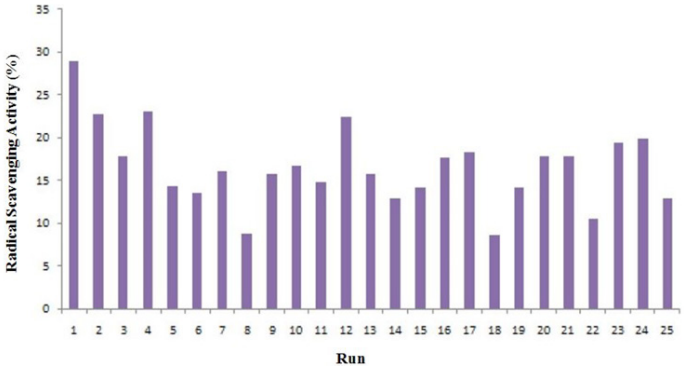

Figure 4: : Effect of MAE condition of *P. pellucida* extraction on DPPH free radical scavenging activity (Values are the mean of triplicate measurements)..

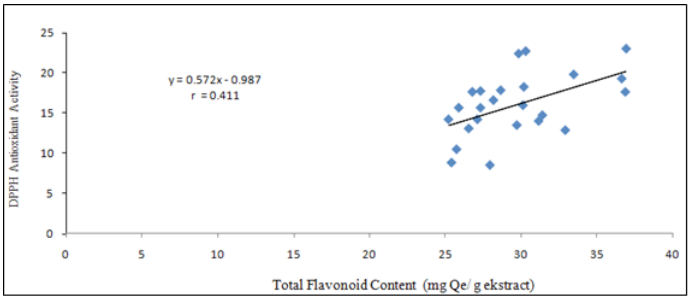

Figure 5: Curve correlation between total flavonoid content and the DPPH free radical scavenging activity.

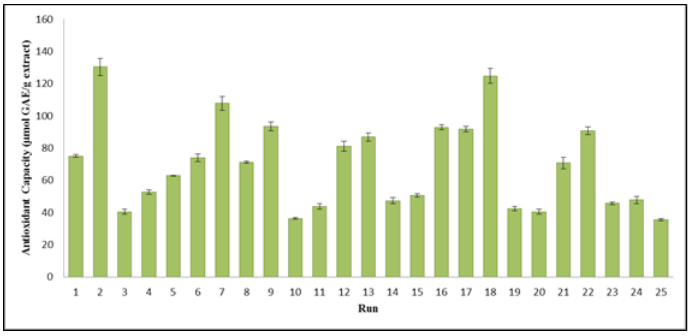

Figure 6: Antioxidant capacity value of FRAP method of *P. pellucida* extracts (Values are the mean of triplicate measurements).

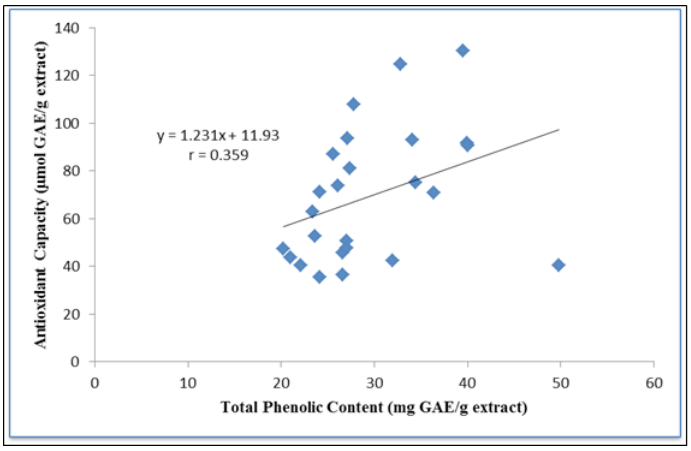

Figure 7: The relationship curve between TPC and antioxidant activity of FRAP method of *P. pellucida* extract.

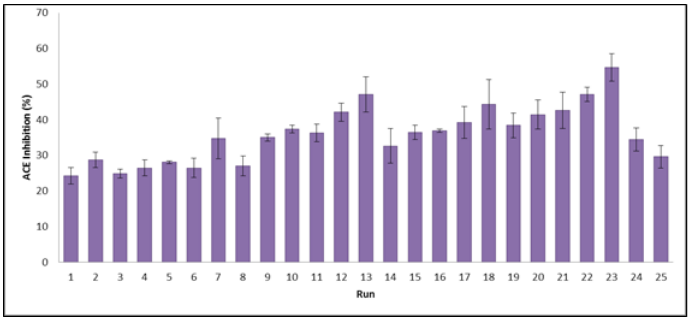

Figure 8: ACE inhibition percentage value of each *P. pellucida* extract (sample concentration= 100 ppm; values are the mean of triplicate measurements).

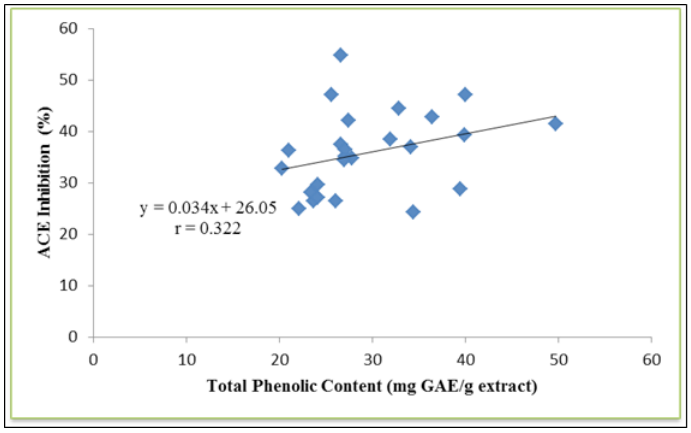

Figure 9: Relationship curve between TPC with ACE inhibition percentage of *P. pellucida* extract.

Table 1: Independent variables and their level

No.	Factors	Levels		
		-1	0	+1
1.	Ethanol concentration	50%	65%	80%
2.	Sample to solvent ratio	1:10	1:12	1:14
3.	Extraction time	1 min	2 min	3 min
4.	Microwave power	30%	50%	70%

DISCUSSIONS

Peperomia pellucida is edible plant and traditionally used medicinal plant in Indonesia. The aerial part of this plat was used to decrease uric acid levels and to treat renal problems. Scientifically, this plant has been proven to demonstrate some biological activities, such as anti-inflammation, analgesic, and fracture healing.[14, 15] In the previous study, methanolic extract of this plant showed strong ACE inhibitory activity.[16] This plant reported to contained flavonoid and phenolic compounds.[16,17]

MAE is advanced extraction technique which effective for extraction phytoconstituent.[18] This technique has been successful extracted flavonoid and phenolic from plants.[19, 20] In this study ethanol-water mixture was selected as an extraction solvent because the mixture has a high dielectric constant and dissipation factor to absorb microwave energy effectively and was able to increase the penetration of the solvent into the sample matrix, and consequently, heating efficiency was increased.[21] Research shows that the presence of a small amount of water in the solvent extraction increased the diffusion of water into the cell matrix, delivers better heating, and facilitates high-speed mass transfer compound into the solvent.[7] Extraction time was selected based on heating efficiency with microwave and to avoid the risk of thermal degradation and oxidation of desired phenolic compounds.[22]

Polyphenols contribute to defend body on the complications involved with free radicals. Variations in the structure and level of phenolic compounds affect their antioxidant activity. Mechanism free radical scavenging DPPH method based electron donation from phenolic compound.[11] Antioxidant activity using FRAP assay is based on a complex reduction of 2,4,6-tripiridil-s-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3.6H_2O$) are almost colorless to slightly brownish-yellow into blue ferro complex after reduction.[12] In this study the gallic acid used as a reference standard because it is a phenolic acid compounds that have strong antioxidant activity. The results of this study indicated that non-phenolic compounds also play a role in providing antioxidant activity in ethanol-water extract of *P. pellucida*. According to Ou, Huang, Woodill,

Flanagan, & Deemer (2002), the above condition is difficult to find and unrealistic. It is caused by several factors, namely because of the potential value reduction of Fe (III)/Fe (II) was 0.77 V, so that any compound including non-phenolic compounds with a potential value reduction below 0.77 V can reduce Fe (III) to Fe (II) and provides uptake value on FRAP methods, and keep in mind is that not all antioxidants can reduce Fe (III) at a time corresponding to the time of incubation.[23]

In this study, there is no correlation between total phenolic content with antioxidant activity. According to Grassmann (2005), is not only the phenolic compounds, but also vitamin C, and vitamin E in fruit and vegetables also have antioxidant activity.[22] Pigment such as chlorophyll, or nitrogen compounds can also act as antioxidants. Therefore, further research is needed to elucidate anti-oxidative compound in ethanol-water extract of *P. pellucida*.

Polyphenol and flavonoid in plant revealed potential ACE inhibitory activity.[17, 24] ACE inhibitory activity assay in the study was conducted using ACE-WST kit based on the enzymatic reaction that was initiated by ACE and aminoacylase in a mixture containing 3HB-GGG substrate and the sample as ACE inhibitors. During incubation, the substrate 3HB-GGG enzymatically was broken into 3HB-G and GG, then 3HB and G. 3HB formed was then observed through the concentration of formazan formed to produce a yellow color, absorbance was measured at 450 nm using a micro plate reader after 10 min at the reaction 25°C.[25] The results of this study indicated that the active compounds which have ACE inhibitory activity in ethanol-water extract of *P. pellucida* not only from phenolic compounds, because ACE has three active sides, where further study is needed on the compounds that can inhibit the activity of ACE. The first part is a carboxylate binding functionality such as guanidinium group of arginine. The second active side is a pocket which accommodates a hydrophobic side chains of C-terminal amino acid residues, and the third is a Zinc ion. In addition, the possibility of activity of ACE inhibitors on ethanol-water extract of *P. pellucida* more influenced by the content of flavonoids. The planar structure of the flavonoids important in inhibiting a metallopeptidase such as ACE.[23] Study of structure-activity relationships of the flavonoid and inhibitory activity of ACE showed that the combination of sub-structures in the framework of flavonoids which enhances the activity of ACE inhibitor consists of the following elements: catechol group in the B-ring, a double bond between C2 and C3 on the C-ring, ketone group in C4 on the C-ring.[25] Susmita & De (2013) are also reported that the content of phenolic total on 19 kinds of fruit did not have a significant relationship with the percent inhibition of ACE, with a correlation value row r = 0.0034, and r = 0.134.[26]

CONCLUSIONS

The optimum conditions of microwave assisted-extraction for TPC were 80% ethanol, sample-solvent ratio of 1:12, extraction time of 2 min, and microwave power of 30%, while TFC were 80% ethanol, ratio of 1:12, time of 2 min, and microwave power of 70%. The result of study indicated that there was no significant correlation between TPC and ACE inhibitory activity and antioxidant activity. Also, there were no significant correlation between TFC and scavenging activity

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CONFLICT OF INTEREST

None.

ABBREVIATIONS USED

AA: Antioxidant activity; **ACE:** Angiotensin converting enzyme; **DPPH:** 1,1-diphenyl-2-picrcylhydrazil; **MAE:** Microwave assisted extraction; **TFC:** Total flavonoid content; **TLC:** Thin layer chromatography; **TPC:** Total phenolic content.

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