Pharmacogn J. 2019; 11(6):1231-1234
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Original Article

Inhibitory Effects of Sangketan (*Achyranthes aspera* L.) Roots Extract on Arginase Activity and Determination of Its Total Phenolic and Flavonoid Contents

Dieah Siti Rahmawati, Berna Elya*, Arikadia Noviani

ABSTRACT

Background: *Achyranthes aspera*, or commonly called as Sangketan in Indonesian is a wild plant that is used as a traditional medicine. The roots of Sangketan can be used as a wound healer by involving the role of arginine and its metabolites, nitric oxide, that directly affect the wound healing process itself. **Objective:** The aim of this study is to determine the potential of Sangketan roots extract in inhibiting arginase activity. **Methods:** The roots were extracted using multistage ultrasound-assisted extraction method with n-hexane, ethyl acetate and methanol solvent. Each extract from different solvents was tested for the inhibition of arginase activity using a microplate-based colorimetric method, followed by determination of total phenolic concentration and total flavonoid concentration. **Results:** The results of inhibition test of arginase activity by n-hexane, ethyl acetate and methanolic extracts were 9.56; 17.58; and 29.77% sequentially/respectively at concentration of 100 µg/ml; the total phenolic concentration were 3.91; 4.83; dan 11.18 mgGAE/g of sample respectively; and the total flavonoid concentration are 0.29; 0.80; and 0.88 mgQE/g of sample respectively. **Conclusion:** From this research, it can be concluded that Sangketan roots extract had low potency of arginase inhibitory activity.

Key words: *Achyranthes aspera*, Arginase, Inhibitory effect, Sangketan, Total flavonoid content, Total phenolic content.

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History

- Submission Date: 10-12-2018;
- Review completed: 12-04-2019;
- Accepted Date: 24-05-2019.

DOI : 10.5530/pj.2019.11.191

Article Available online

http://www.phcogj.com/v11/i6

INTRODUCTION

Sangketan or *Achyranthes aspera L.* is a plant that is often used in Indonesia. Traditionally, the roots of Sangketan are used as wound healers. Several studies also mentioned that Sangketan can act as diuretic agent, antiobesity, antibacterial, antioxidant, wound healer and immunomodulatory.[1] It contained several chemical compounds like alkaloid, saponin, amino acid, steroid, triterpenoid, also phenolic compound and flavonoid.[2]

Optimal wound healing can be achieved by paying more attention to factors that can affect the continuity of the process. Arginine is one of the important factors because its metabolite—nitric oxide—is needed during the healing process. Nitric oxide is produced by endothelial nitric oxide synthase (eNOS) with L-arginine as substrate on endothelial cells. When the wound occurs, the amount of arginine can decrease dramatically, affecting the amount of NO and the healing process of the wound itself.[3]

Besides being influenced by arginine, the amount of NO can also be affected by the presence of the arginase which will hydrolyze arginine to form ornithine and urea in the urea cycle. However, due to a lot of arginase expressions in cells that are related to the wound healing process, arginase will compete with eNOS to use arginine in the urea cycle.[4] That is why the use of arginase inhibitors can increase the amount of arginine availability in the wound environment.[5] Several studies have found that inhibition activity of arginase enzymes is presence in plant extracts that contain polyphenols such as flavonoids and resveratrol.[6] Research conducted by Bordage *et al.*[6] showed the existence of inhibition activity of arginase from several plant compounds such as resveratrol, quercetin, chlorogenic acid and epicatecin. According to Cray *et al.*[7] polyphenol compounds can act as chaotropic agents—compounds that can disrupt hydrogen bonds between water molecules. These previous researches' results showed that further research to find other effective compounds in inhibiting arginase is needed, especially using plants which can potentially be a source of new active agents.[8]

MATERIALS AND METHODS

Materials

The roots of Sangketan was obtained from Klaten, Central Java, Indonesia. The chemicals and reagents were sources commercially. Arginase, L-arginine, maleic acid and $MnSO_4$ from Sigma Aldrich (Singapore); quercetin and gallic acid from Sigma Aldrich (India); resveratrol from Wako (Japan); urea kit assay from Abnova (Taiwan); n-hexane, ethyl acetate and methanol from Merck (Germany).

Preparation of Sangketan roots extract

Extraction

Dried Sangketan roots were grinded into powder (500 g) which then was extracted using multistage

Cite this article: Rahmawati DS, Elya B, Noviani A. Inhibitory Effects of Sangketan (*Achyranthes aspera* L.) Roots Extract on Arginase Activity and Determination of Its Total Phenolic and Flavonoid Contents. Pharmacog J. 2019;11(6):1231-4.

ultrasound-assisted extraction and evaporated. n-hexane, ethyl acetate and methanol in sequence were used as the solvent of multistage extraction.

Arginase activity inhibition test

The inhibition test was modified from the protocols published by Sigma Aldrich (EC 3.5.3.2). The sample solution was made by mixing 10 µL extract, 15 µL arginase 1 U/mL and 20 µL L-arginine substrate 380 mM. Then the sample solution was preincubated at 37°C for 30 min. Right after the preincubation, 100 µL urea assay reagent was added and then incubated at room temperature for an h. The inhibition activity of arginase was determined on microplate reader (Epoch, USA).[9] Resveratrol was used as positive control of arginase inhibition because resveratrol easily found on plant and it could be on higher rank in terms of inhibitory effect (IC_{50}) amongst other compounds such as epicatechin, quercetin, caffeic acid, kaempferol and quinic acid.[6]

Identification of phenol and flavonoid

Identification of phenol and flavonoid were done/conducted using qualitative method of phytochemical screening and reagents spraying on Thin Layer Chromatography (TLC) plates.

For phenol identification, solution of iron trichloride 3% was added to the sample solutions to see whether the color changed or not. For identification with spray reagents on the TLC plate, standard gallic acid was used as reference, dissolved in ethanol. Silica gel 60 F254 was used as the stationary phase and chloroform-acetone-formic acid (9:3:1) were used as the mobile phase. After the plate eluted, it was sprayed with solution of iron trichloride 3%.

For flavonoid identification, zinc powder, magnesium powder and alumunium chloride spray reagent were used to see the color changes on sample solutions. For identification with spray reagents on the TLC plate, standard quercetin was used as reference, dissolved in ethanol. Silica gel 60 F254 was used as the stationary phase and chloroform-acetone-formic acid (10:2:1) were used as the mobile phase. After the plate eluted, it was sprayed with alumunium chloride 5% and then viewed under 366 nm UV light.

Determination of total phenolic content

The determination of total phenol content was determined on microplate reader.[10] Sample solutions were made in 5000 µg/mL. Measurements started by preparing 20 µL of sample solution mixed with 100 µL of Folin-Ciocalteu reagent (1:4), shaken for a min in 96-well microplate. The mixtures were left for two min. Then 75 µl sodium carbonate solutions (100 g/mL) were added and shaken for a min. After being left for 2 h at room temperature, the mixtures were measured using the microplate reader at 750 nm to get the absorbances. Blank solutions were measured without addition of samples or standard solution in well, which was replaced with methanol P. Gallic acid was used as standard, which were made in concentrations of 25, 50, 75,100, 125 and 150 µg/mL to develop a calibration range.

Determination of total flavonoid flavonoid content

The determination of total flavonoid content was determined on spectrophotometer UV-Vis referred to Pharmacopoeia Herba Indonesia Suplement III. Sample solutions were made in 4000-10,000 µg/mL. Measurements started by preparing 0.5 ml sample solutions on the sample tubes. Then 1.5 mL of ethanol P, 0.1 mL of aluminum chloride 10%, 0.1 mL sodium acetate (100 g/mL) and 2.8 ml of distilled water were added to the sample tubes. The mixtures were shaken and left at room temperature for 30 min. After that, the mixtures were measured using spectrophotometer UV-Vis at 415 nm to get the absorbances. Blank solutions were measured without addition of aluminum chloride 10%, which was replaced with distilled water. Quercetin was used as standard, which were made in concentrations of 10, 20, 30, 40, 50 and 60 µg/mL to develop a calibration range.

RESULTS AND DISCUSSION

Extraction

Extraction is a separation process that separates the active compound from its matrix in plant using a selective solvent. Multistage Ultrasound-Assisted Extraction (UAE) using n-hexane, ethyl acetate and methanol solvents were used based on the differences in polarity. The UAE method is used because this method is assisted by the use of ultrasound which can increase the permeability of the cell wall and form cavities to facilitate the release of bioactive compounds in simplicia.[11] The polar methanol solvent showed the highest yield, followed by n-hexane which is non-polar and ethyl acetate which is semi-polar (Table 1).

Arginase activity inhibition test

Arginase activity inhibition test was determined using a microplate reader with a modified method of the Sigma Aldrich enzyme test protocol, which measured the production of urea—final product of the arginase reaction with L-arginine. Theoretically, the extract is considered to have a good enzyme inhibitory activity if it is able to inhibit enzyme activity more than or equal to 50% at concentration less than or equal to 100 µg/mL. The results of extracts with 100 µg/mL final concentration showed that all three extracts had low potency of arginase inhibitory activity, which didn't reach 50% (Table 2).

Identification of phenol and flavonoid

Identification of phenol on n-hexane, ethyl acetate and methanol extract showed positive results using iron trichloride reagent, which would form a blackish green color after being added. The presence of phenol groups is characterized by the form of dark green or dark blue color due to the formation of complex compounds because of the addition of Fe^{3+}. In addition, identification was also carried out using spray reagents on the TLC plates. The solution of gallic acid as standard and all three extracts were applied as spots on the TLC plate which was then eluted using the mobile phase of chloroform-acetone-formic acid (9:3:1). After the elution process was complete, the plate then sprayed with an iron trichloride. The results shown in the form of visible spots on the positive control of gallic acid and methanol extract with similar Rf.

Identification of flavonoid on n-hexane, ethyl acetate and methanol extract showed different results. The results shown was negative for n-hexane extract and positive for ethyl acetate and methanol extract. It is the results classified as positive if orange or red color presence in the reaction using magnesium powder with hydrochloric acid and zinc powder with hydrochloric acid. The magnesium and zinc with the hydrochloric acid in the test would form a reaction that will reduce

Table 1: The percentage yield of plant extracts in different solvents.

No.	Solvent	Simplicia's Weight (g)	Extract's Weight (g)	Yield (%)
1	n-Hexane	300.07	7.42	2.472
2	Ethyl acetate	300.07	4.67	1.556
3	Methanol	300.07	13.59	4.528

Table 2: The percentage inhibition of Sangketan extracts.

Sample	% Inhibition	SD	CV (%)
n-Hexane extract	9.5617	0.7355	7.6923
Ethyl acetate extract	17.5818	2.8440	12.4243
Methanol extract	29.7765	4.5250	15.1966

the nucleus of benzopirone in the flavonoid structure which led to the color changes into orange or red. It can also be classified as positive when yellow fluorescent was seen when viewed under UV light at 366 nm after spraying alumunium chloride. In addition, quercetin as standard and three extracts on the TLC plate were also applied as spots and then eluted using the mobile phase of chloroform-acetone-formic acid (10:2:1). When the elution process was complete, the plate then sprayed with alumunium chloride. The results showed that there were fluorescent spots on the plates where the solution of quercetin, n-hexane extract, ethyl acetate extract and methanol extract with similar Rf viewed under UV light at 366 nm.

The identification results can be associated with previously performed enzyme test results that phenolic and flavonoid compound may play a role in inhibiting the arginase, but it can't be denied that other compounds may also have a role in it as well (Table 3).

Determination of total phenolic content

The determination of the total phenol content was carried out using Folin-Ciocalteu reagent with a microplate reader. The principle of the reaction that occurs between Folin-Ciocalteu reagents and phenolic compounds in the sample is the oxidation reaction and colorimetric reduction. The phosphomolybdate-phosphotungstate compounds in Folin-Ciocalteu reagent will be reduced by hydroxyl groups in phenolic compounds to form a blue molybdenum-tungsten complex.[12] The addition of Na_2CO_3 solution was aimed to produce an alkaline atmosphere. This alkaline atmosphere was needed because the colorimetric reduction reaction can only occur in an alkaline atmosphere. The high intensity of blue color that formed was equivalent to the amount of phenolic compounds in the sample. The more intense the blue color produced indicates that the higher the content of phenol compounds. The determination started by making the standard gallic acid calibration curve. The linear regression equation that resulted will be used to calculate the total phenol content of the sample. Measurements were made using a microplate reader at a wavelength of 750 nm.[13] The linear regression equation was y = 0.0068x - 0.0069 (r = 0.99985). The average total phenol content were obtained by inserting the absorbance of the extract into the linear regression equation. If the percentage value of enzyme inhibitory activity was associated, then the result of total phenolic content can also be said to have a correlation. The lowest n-hexane extract with inhibition percentage also had the lowest amount of total phenol content, while methanol extract with the highest inhibition percent had the highest amount of total phenol content. It is known in several previous studies that several compounds which able to inhibit the activity of arginase enzymes are generally classified as flavonoids, where flavonoids are derivatives of the phenol group. Therefore, the high phenolic content in a sample can indicate the high content of flavonoids in the sample (Table 4).[12]

Determination of total flavonoid content

The determination of total flavonoid content was carried out using the colorimetric method using UV-Vis spectrophotometry with aluminum chloride and sodium acetate reagents. The calculation results are then stated in milligram quercetin equivalent/g sample. Quercetin was selected as a standard because it is classified as flavonoid in flavonol group which has keto group on C-4 atom and hydroxyl group in C-3 and C-5 atoms. The principle of this determination of total flavonoid content using alumunium chloride reagent is the formation of acid-resistant complexes between aluminum ions with C-4 keto groups and C-3 or C-5 hydroxyl groups in flavonoids resulted in a form of yellow color. The addition of sodium acetate can detect the presence of free 7-hydroxyl groups in flavones and flavonols. This study began with making the standard quercetin calibration curve. The linear regression equation that resulted will be used to calculate the total flavonoid content of the sample. Measurements were made using UV-Vis spectrophotometry at 430 nm, which wavelength shown the maximum absorption. The linear regression equation was y = 0.0076x + 0.1327 (r = 0.99905). The average total flavonoid content were obtained by inserting the absorbance of the extract into the linear regression equation that previously obtained from the quercetin calibration curve. Similar to the results of total phenolic content, when the results of total flavonoid content were associated with the value of enzyme inhibitory activity, it showed a correlation. N-hexane extract with the lowest inhibition percent turned out to have the lowest amount of total flavonoid levels, while methanol extract with the highest inhibition percentage shown the highest amount of total flavonoid levels. It is known in several previous studies that some compounds which able to inhibit the activity of arginase enzymes are generally classified as flavonoids (Table 5).

Table 3: Identifiction of phenol and flavonoid in Sangketan extracts.

Chemical Content	n-Hexane extract	Ethyl acetate extract	Methanol extract
Phenol	+	+	+
Flavonoid	-	+	+

Table 4: Total phenolic content of Sangketan extracts.

Sample	Absorbance	Average Phenol Content (mgGAE/g sampel)
n-Hexane Extract	0,345 0,349 0,337	3,917
Ethyl Acetate Extract	0,482 0,470 0,466	4,830
Methanol Extract	1,039 1,041 1,020	11,189

Table 5: Total flavonoid content of Sangketan extracts.

Sample	Absorbance	Average Flavonoid Content (mgQE/g sampel)
n-Hexane Extract	0,431 0,437 0,438	0,296
Ethyl Acetate Extract	0,596 0,589 0,580	0,804
Methanol Extract	0,513 0,475 0,495	0,884

CONCLUSION

The roots extract of Sangketan had low potency of arginase inhibitory activity.

ACKNOWLEDGEMENT

This research was funded by Doctoral Grant 2018 from Universitas Indonesia.

CONFLICTS OF INTEREST

There is no conflicts of interest.

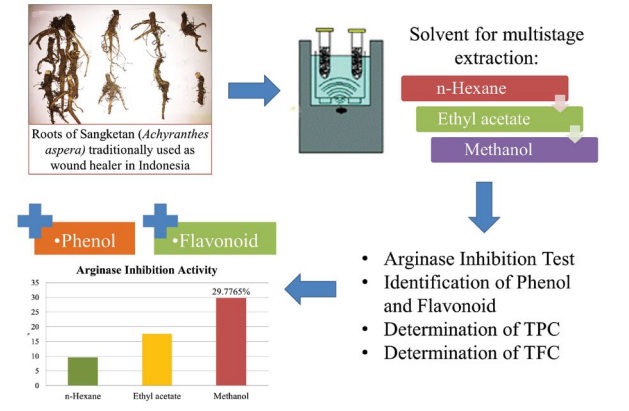
ABBREVIATIONS

eNOS: Endothelial nitric oxide synthase; **NO:** Nitric oxide; **g:** Gram; **µg:** Microgram; **ml:** Milliliter; **µl:** Microliter; **mgGAE/gram:** Milligram gallic acid equivalent per gram of sample; **mgQE/gram:** Milligram quercetin equivalent per gram of sample; **UV-Vis:** Ultraviolet-visible; **$MnSO_4$:** Manganese sulfate; **U:** Unit; **mM:** Millimolar; **h:** Hour; **min:** Minute; **C:** Celcius; **IC_{50}:** Inhibition concentration 50%; **%:** Percentage; **TLC:** thin layer chromatography; **nm:** Nanometer; **UAE:** Ultrasound-assisted extraction; **SD:** Standard deviation; **CV:** Coefficient of variation.

REFERENCES

1. Pandey NK, Sharma HP, Amit P, Paras J. A review on potential magic folk herbal medicinal plant: *Achyranthes aspera* L. International Journal of Medicinal Plants Photon. 2013;105:350-63.
2. Bhosale UA, Yegnanarayan R, Pophale P, Somani R. Effect of aqueous extracts of *Achyranthes aspera* Linn. on experimental animal model for inflammation. Anc Sci Life. 2012;31(4):202-6.
3. Gould A, Naidoo C, Candy GP. Arginine metabolism and wound healing. WHSA. 2008;1(1):48-50.
4. Steppan J, Nyhan D, Berkowitz DE. Development of novel arginase inhibitors for therapy of endothelial dysfunction. Front Immunol. 2013;4(SEP):1-6.
5. Kavalukas SL, Uzgare AR, Bivalacqua TJ, Barbul A. Arginase inhibition promotes wound healing in mice. Surgery. 2012;151(2):287-95.
6. Bordage S, Pham TN, Zedet A, Gugglielmetti AS, Nappey M, Demougeot C, *et al*. Investigation of mammal arginase inhibitory properties of natural ubiquitous polyphenols by using an optimized colorimetric microplate assay. Planta Med. 2017;83(7):647-53.
7. Cray JA, Bhaganna P, Singhal RS, Patil SV, Saha D, Chakraborty R, *et al*. Chaotropic and hydrophobic stress mechanism of antifungal substances. In: Dehne HW, Deising HB, Fraaije B, Gisi U, Hermann D, Mehl A, *et al*. editors. Modern fungicides and antifungal compounds, vol VII. Braunschweig: Deutsche Phytomedizinische Gesellschaft. 2014;73-80.
8. Girard-Thernier C, Pham TN, Demougeot C. The promise of plant-derived substances as inhibitors of arginase. Mini Rev Med Chem. 2015;15(10):798-808.
9. Zalsabela TL, Elya B, Noviani A. Arginase inhibition activity of stem bark extract of *Caesalpinia pulcherrima*. J Young Pharm. 2018;10(2):111-3.
10. Bobo-garcía G, Davidov-pardo G, Arroqui C, Marín-arroyo MR. Intra-laboratory validation of microplate methods for total phenolic content and antioxidant activity on polyphenolic extracts and comparison with conventional spectrophotometric methods. J Sci Food Agric. 2014;95(1):204-9.
11. Medina-Torres N, Ayora-Talavera T, Espinosa-Andrews H, Sánchez-Contreras A, Pacheco N. Ultrasound assisted extraction for the recovery of phenolic compounds from vegetable sources. Agronomy. 2017;7(3):47.
12. Adawiah A, Sukandar D, Muawanah A. Antioxidant Activity and the content of bioactive components NAMNAM juice. Jurnal Kimia Valensi. 2015;1(2):130-6.
13. Indonesian Herbal Pharmacopeia Ed I. 3th Supplement. Jakarta: Ministry of Health Republic of Indonesia. 2013.

GRAPHICAL ABSTRACT



SUMMARY

The results of total phenolic and flavonoid content were associated with the value of enzyme inhibitory activity because it showed a correlation. It is known in several previous studies that some compounds which able to inhibit the activity of arginase enzymes are generally classified as flavonoids, where flavonoids are derivatives of the phenol group. But the extract of *Sangketan* roots still had low potency of arginase inhibitory activity, consecutively from the lowest potential were n-hexane extract (9.56%), ethyl acetate extract (17.58%) and methanol extract (29.77%) with 100 µg/mL final concentration. Phenolic and flavonoid compounds may play a role in inhibiting the arginase, but it can't be denied that other compounds may also have a role in it as well.

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