

SYZYGium CUMINI (L.) SKEELS LEAF EXTRACT FRACTIONS AS ARGINASE INHIBITORS AND THE EFFECTS OF TANNINS ON THEIR ACTIVITY

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ABSTRACT

Objective: Arginase inhibition could be a potential therapeutic approach for endothelial dysfunction. *Syzygium cumini* (L.) Skeels leaves contain phenolic acids and flavonoids, which have been predicted to exhibit arginase inhibitory activity. Moreover, these leaves contain tannins, which can form complexes with enzymes and lead to false-positive results during biological testing. Therefore, this study was conducted to evaluate the arginase inhibitory activity of *S. cumini* leaf extract and fractions as well as to elucidate the effects of tannins on this activity.

Methods: *S. cumini* leaves were fractionated using n-hexane, ethyl acetate, and methanol. A colorimetric method was employed to evaluate arginase inhibitory activity. Tannin elimination was performed through the gelatin precipitation method. Total phenolic and flavonoid contents of the fractions were calculated using the Folin-Ciocalteu and aluminum chloride methods, respectively.

Results: Ethyl acetate and methanol fractions showed arginase inhibitory activity with half-maximal inhibitory concentrations (IC₅₀) of 46.96 and 15.35 µg/mL, respectively. The methanol fraction was positive for tannins. After tannin elimination, this fraction exhibited less potent arginase inhibitory activity, with an IC₅₀ value of 53.03 µg/mL. The ethyl acetate fraction showed higher total phenolic and flavonoid contents than the methanol fraction.

Conclusion: Tannins affected the arginase inhibitory activity of the methanol fraction of *S. cumini* leaves; however, the ethyl acetate fraction did not contain tannins and could inhibit arginase activity.

Keywords: Arginase inhibitor, Ethyl acetate fraction, Methanol fraction, *Syzygium cumini*, Tannins.

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INTRODUCTION

The endothelium plays an important role as the central hub for vascular structure, tone, and homeostasis regulation in cardiovascular physiology. Endothelial dysfunction could serve as a predictor of various vascular diseases that lead to pathological conditions. Endothelial dysfunction is characterized by depleted levels of nitric oxide (NO) – a potent vasodilator biomarker [1].

NO synthesis is catalyzed by endothelial NO synthase (eNOS) from the amino acid L-arginine with cofactor tetrahydrobiopterin (BH4) [2]. L-arginine is also utilized by arginase as a substrate to produce L-ornithine and urea. Competition between eNOS and arginase may occur under pathophysiological conditions. Thus, increased expression and/or activity of arginase in endothelial cells can disrupt NO synthesis [3].

When L-arginine levels decrease due to arginase activity, eNOS may become unstable and uncoupled. Hence, decreased NO production can result in the generation of more superoxide radicals. Superoxide can react with NO to form peroxynitrite, which can oxidize BH4 and cause eNOS uncoupling, thus down regulating NO production [2]. Furthermore, reactive nitrogen and oxygen species may oxidize iron in the Hem Group (Fe²⁺ to Fe³⁺) located within soluble guanylyl cyclase (sGC). This oxidation makes sGC unresponsive to NO and vasodilatation is blocked [4]. Therefore, arginase inhibition and oxidation prevention may be considered as potential therapeutic approaches for endothelial dysfunction [1,2].

Studies have shown arginase inhibitory activities of plant substances or extracts. Some examples include *Theobroma cacao* flavonol mixture,

Caesalpinia sappan heartwood ethyl acetate extract, Korean red ginseng root extract, *Scutellaria indica* flavanones, and hydroalcoholic extract from *Melastoma malabathricum* leaves [5-9]. Some natural compounds have also been reported to show arginase inhibitory activity *in vitro*, such as chlorogenic acid, piceatannol, resveratrol, (-)-epicatechin, taxifolin, quercetin, and caffeic acid [10].

Syzygium cumini (L.) Skeels hydroethanolic leaf extract possesses pharmacological activities that impact vascular diseases such as antihypertensive effects, antihyperglycemic activity, and antioxidant properties [11,12]. Various phytoconstituents such as phenolic acids, tannins, flavonoids, and terpenes have already been identified in this extract [13-16]. Phenolic acids and flavonoids of *S. cumini* are hypothesized to exhibit arginase inhibitory activity. However, tannins could serve as inhibitors of enzymatic activity through the formation of complexes with enzymes, resulting in false-positive results during biological testing [17]. Therefore, this study was conducted to evaluate the arginase inhibitory activity of the *S. cumini* leaf fractions and to elucidate the effects of tannins on this activity.

METHODS

Chemicals

Bovine liver arginase, L-arginine, maleic acid, mangan sulfate, gallic acid, resveratrol, aluminum chloride, and iron (III) chloride were purchased from Sigma-Aldrich (Singapore). The Urea Assay Kit was purchased from Abnova (Taiwan). Dimethyl sulfoxide, Folin-Ciocalteu reagent, methanol, ethyl acetate, n-hexane, and other solvents were of analytical grade and obtained from Merck (Germany).

Sample preparation

S. cumini leaves were collected from Bogor Botanical Garden, Indonesia, and positively identified by Indonesia Science Institution. After harvesting, leaves were cleaned and air-dried. Dried leaves were pulverized and extracted using 70% ethanol through maceration at room temperature [11]. The filtrate concentrated using a rotary vacuum evaporator and then vacuum-dried. Dried extract was dispersed in aquadest and fractionated by liquid-liquid partition using n-hexane and ethyl acetate until the solvent became colorless; this was then evaporated under vacuum conditions to obtain the n-hexane and ethyl acetate fractions [18]. Thereafter, the aqueous residue was further evaporated and vacuum-dried. Dried aqueous residue was dissolved in methanol; when the methanol phase became intensely colored, it was separated from the solid phase. Methanol fractionation was repeated until the methanol phase became colorless and the methanol filtrate was collected and evaporated to obtain the methanol fraction.

Tannin elimination

First, the n-hexane, ethyl acetate, and methanol fractions were screened for tannins using a gelatin test and iron (III) chloride. The methanol fraction was positive for tannins and tannin elimination was then performed through the gelatin precipitation method [19]. A gelatin solution was added to the methanol fraction to precipitate tannins. The solution was centrifuged at 100 rpm for 15 min to separate the precipitate. Supernatant was collected to test its arginase inhibitory activity following tannin elimination.

Arginase inhibitory activity assay

Enzyme inhibition activity was determined using arginase enzymatic assay (Sigma-Aldrich, Singapore) and urea determination assay kits (Abnova Corporation, Taiwan), as described elsewhere [9]. The test (10 µL), arginase 1 U/mL (15 µL), and L-arginine 380 mM (20 µL) solutions were mixed in 96-well microplates and incubated at 37°C for 30 min. Test solution was substituted by dimethyl sulfoxide solution in control wells. For blank wells, an arginase solution was added following the first incubation. Then, 100 µL of reagent mixture from the Urea Assay Kit (reagents A: B, 1:1) was added to each well. Absorbance was measured using a microplate reader (Versamax, USA) after a second incubation at 25°C for 60 min. Resveratrol was used as the positive standard, which was tested at a concentration range of 8.76–43.81 µM. Absorbance was measured at 430 nm. Percent inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{(A_c - A_{cb}) - (A_s - A_{sb})}{(A_c - A_{cb})} \times 100$$

where A_c is absorbance of the control, A_{cb} is absorbance of the control blank, A_s is absorbance of the test sample, and A_{sb} is absorbance of the blank of test sample.

Total phenolic content determination

Total phenolic content of the active fractions that inhibited arginase was determined using the Folin-Ciocalteu method [9]. In a 96-well microplate, 100 µL of diluted Folin-Ciocalteu reagent (1:4) was added to 25 µL of either a fraction or standard solution. The mixture was homogenized by shaking for 1 min and left to stand for 4 min. Next, 75 µL of Na_2CO_3 solution (100 g/L) was added to each well followed by homogenization by shaking for 1 min. After incubation for 2 h at room temperature in the dark, absorbance was measured at 750 nm using the microplate reader. Gallic acid was used as a standard to quantify phenolic content; the calibration curve was prepared using gallic acid at a concentration of 25–150 µg/mL. Total phenolic content of the fractions was expressed as gallic acid equivalents (i.e., mg of gallic acid equivalents per g of fraction).

Total flavonoid content determination

Determination of total flavonoid content of the active fractions was performed using the aluminum chloride method, as previously

described with slight modifications [20]. Briefly, 0.5 mL of the fraction or standard solution was sequentially added to 1.5 mL of ethanol, 0.1 mL of AlCl_3 solution (10%), 0.1 mL of sodium acetate solution (1 M), and 2.8 mL distilled water. The mixture was homogenized by vortexing for 1 min. For the sample blank, the AlCl_3 solution was substituted by distilled water of the same quantity. After 30 min of incubation at room temperature, absorbance was measured at 430 nm using a ultraviolet-visible spectrophotometer (Shimadzu 265, Japan). The calibration curve was prepared using quercetin at a concentration of 30–80 µg/mL. Total flavonoid content of the fractions was expressed as quercetin equivalents (i.e., mg of quercetin equivalents per g of fraction).

RESULTS

Arginase inhibitory activity

Resveratrol was used as positive control and showed arginase inhibitory activity with half-maximal inhibitory concentration (IC_{50}) value 17.56 µM. The n-Hexane, ethyl acetate, and methanol fractions of *S. cumini* leaves were evaluated for arginase inhibitory activity. At 100 µg/mL, the n-hexane and ethyl acetate fractions inhibited arginase at 20.90% and 89.74%, respectively. At 30 µg/mL, the methanol fraction inhibited arginase at 89.21%. In this study, the fraction was considered to have *in vitro* arginase inhibitory activity if enzyme activity was inhibited by >50% at concentrations <100 µg/mL. Because the n-hexane fraction did not obtain 50% inhibition, this fraction was not considered to be an active arginase inhibitor and further IC_{50} determination was not conducted. IC_{50} values for the ethyl acetate and methanol fractions are presented in Table 1.

Total phenolic content determination

The total phenolic content of the active fraction was calculated using a gallic acid calibration curve using the equation $y=0.0062x+0.0502$. The total phenolic contents of the ethyl acetate and methanol fractions are presented in Table 2. The ethyl acetate fraction of *S. cumini* leaves showed higher phenolic content than the methanol fraction.

Total flavonoid content determination

The total phenolic content of the active fraction was calculated using a quercetin calibration curve with the equation $y=0.0086x-0.0229$. The total flavonoid content of the ethyl acetate and methanol fractions is presented in Table 2. The ethyl acetate fraction exhibited higher flavonoid content than the methanol fraction.

DISCUSSION

In this study, arginase inhibitory activity was evaluated *in vitro* using an indirect technique through urea measurement as urea is a product of an L-arginine and arginase reaction [3]. Resveratrol was used as a positive control base on previous data by Bordage *et al.* who showed that

Table 1: Arginase inhibitory activity of active fractions derived from *Syzygium cumini* leaves presented as IC_{50}

Sample	Linear regression equation	IC_{50} (µg/mL)
Ethyl acetate fraction	$y=0.702x+17.011$	46.96
Methanol fraction	$y=2.855x+6.163$	15.35
Methanol fraction after tannin elimination	$y=0.641x+16.026$	53.03

IC_{50} : Half-maximal inhibitory concentration

Table 2: Phytoconstituents content *Syzygium cumini* leaf fractions

Sample fraction	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
Ethyl acetate	645.16±12.18	25.22±2.13
Methanol	532.93±7.27	8.08±0.51



Fig. 1: *Syzygium cumini* (L.) Skeels leaves

resveratrol inhibited arginase activity *in vitro*, with a IC_{50} of 18.2 μ M [10]. The IC_{50} value obtained in this study was slightly different; this difference may be caused by different assay methods or conditions employed.

As investigated in previous studies, *S. cumini* leaves (Fig. 1) possess pharmacological activities [11,12]. Arginase inhibitory activity of the fractions of *S. cumini* leaves was evaluated. The methanol fraction exhibited a lower IC_{50} value than the ethyl acetate fraction, indicating higher arginase inhibition potency. However, after screening for tannins through gelatin test and iron (III) chloride, the n-hexane and ethyl acetate fractions contained no tannins, while the methanol fraction tested positive. Tannins within the methanol fraction were eliminated. Following tannin elimination, this fraction exhibited arginase inhibitory activity, but it was weaker than that of the crude methanol fraction. This finding indicates the tannins affect arginase inhibition. Tannins may form precipitates with protein, including enzymes, through binding and aggregation [17].

Some phytoconstituents in *S. cumini* leaves such as quercetin, kaempferol, catechin, gallic acid, caffeic acid, and chlorogenic acid have been reported to show arginase inhibitory activity [10,13,14,21-23]. At 1 mM, gallic acid and catechin exhibited arginase inhibitory activity at 20% and 26%, respectively [23]. Kaempferol, caffeic acid, quercetin, and chlorogenic acid showed arginase inhibition with IC_{50} values of 179.1, 86.7, 31.2, and 10.6 μ M, respectively [10].

The phenolic and flavonoid compounds contained within *S. cumini* leaves might be responsible for their arginase inhibitory activity. The ethyl acetate fraction showed higher phenolic and flavonoid contents than the methanol fraction. The ethyl acetate fraction also showed an IC_{50} value lower than that of the methanol fraction following tannin elimination, indicating a higher potency for arginase inhibition.

CONCLUSION

This study demonstrated that tannins could contribute to arginase inhibitory activity. The ethyl acetate fraction of *S. cumini* leaves did not contain tannins and might be considered as having the most inhibitory potential among all the fractions.

CONFLICTS OF INTEREST

All authors have none to declare.

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