

Research Article

Arginase inhibitory and antioxidant activities in *Syzygium cumini* (L.) Skeels leaves extracts collected from three different locations of java

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KEYWORDS:

Antioxidant; Arginase inhibitor;
Gallic acid concentration; Phenolic
content; *Syzygium cumini* leaves

ABSTRACT

Arginase inhibition and antioxidant activities may improve endothelial dysfunction. Previous studies demonstrated that *Syzygium cumini* leaves had pharmacological effects in diseases related to endothelial dysfunction, but there was no source linking the effect with arginase inhibitory activity. The aim of this study was to investigate arginase inhibitory and antioxidant activity of *S. cumini* leaves extract from different sources of simplisia, also determine its total phenolic content and gallic acid concentration. The leaves of *S. cumini* collected from Tangerang, Bogor and Sukoharjo city in Java, Indonesia. Arginase inhibition assay was done *in vitro* using colorimetric determination of urea which generated from L-arginine and arginase reaction. Antioxidant activity was evaluated using DPPH and FRAP methods. Total phenolic content of extract determined using Folin-Ciocalteu method. Gallic acid concentration was estimates using HPTLC. At a concentration of 50 µg/mL, all extracts exhibited arginase inhibition above 80%. However, the most active extract was sample collected from Bogor > Tangerang > Sukoharjo. Antioxidant activity of extract also came in that order. Determination of total phenolic content and gallic acid concentration of extracts revealed that *S. cumini* leaves collected from Bogor had the highest phenolic content (476.18 mgGAE/g) and gallic acid concentration (4.4 mg/g of extract). *S. cumini* leaves from different sources possessed different activity value on arginase inhibition, but not significant statistically. The leaves extract from Bogor, with the highest phenolic content and gallic acid concentration, showed the highest arginase inhibitory and antioxidant activity.

1. INTRODUCTION

Endothelial dysfunction is a pathological condition, which can be defined as an imbalance between endothelium vasodilation and vasoconstriction factors. Endothelial dysfunction can affect development of several diseases, as occurs in diabetic vascular complications and other cardiovascular diseases¹. A decrease bioavailability of nitric oxide (NO), disruption of NO signaling and increase production of reactive oxygen species in endothelium are major contributors in endothelial dysfunction².

In the endothelium, endothelial nitric oxide synthase (eNOS) synthesizes NO from amino acid L-arginine. Arginase, an enzyme that converts L-arginine into L-ornithine and urea, might compete with eNOS for their substrate, L-arginine, and thus regulates NO production. Previous studies showed that arginase upregulation is related to pathophysiology of some diseases such as ageing, cardiovascular, diabetes mellitus, pulmonary, and obesity. Therefore, arginase inhibitors considered as a potential therapy for various diseases³. Adding antioxidants may contribute beneficial effect in the therapy since oxidative stress plays a critical role in endothelial function⁴. Previous studies reported that some plant extract rich in polyphenols such as cocoa bean⁵, *Caesalpinia sappan*⁶, *Scutellaria indica*⁷, and plant-derived polyphenols such as chlorogenic acid, epicatechin, quercetin⁸ exhibited arginase inhibitory effect.

Syzygium cumini (L.) Skeels was known as folklore medicinal plant used for the treatment of diabetes by traditional practitioners in some countries, such as in India, Srilanka, Tibet⁹, Brazil¹⁰, and Indonesia¹¹. Various phytochemicals such as phenolic acids, flavonoids, terpenes, and tannins have been reported in *S. cumini* leaves¹²⁻¹⁵. Chagas *et al.*¹⁵ summarized the activity of *S. cumini* against cardiometabolic diseases such as antihyperglycemic, antihyperlipidemic, cardioprotective, anti-inflammatory and antioxidant activities based on experimental studies. Hydroethanolic extract of *S. cumini* leaves reported having a high total of polyphenol content, antioxidant activity, improves insulin sensitivity¹⁶ and antihypertensive effect¹⁷.

Despite some studies provided the pharmacological effect of *S. cumini* in diseases related to endothelial dysfunction, no source has examined whether the effect is via arginase inhibitory pathway. Phytochemical content and composition of an extract, which determine the activity, also affected by geographical and environmental parameters¹⁸. Therefore the aim of this study was to investigate arginase inhibitory and antioxidant activities of *S. cumini* leaves extract from three different locations in Java with different geographical condition, also determine its phenolic content.

2. MATERIALS AND METHODS

2.1. Sample preparations

2.1.1. Plant materials

S. cumini leaves collected from three

different locations in Java island: Tangerang (Banten province), Sukoharjo (Central Java province) and Bogor Botanical Garden (West Java province). The leaves were harvested in December 2017. The collected leaves were mature leaves, indicated by dark green colour, with a length of 10-13 cm. Plants were identified by Indonesia Science Institution, with reference number B-188/IPH.3/KS/I/2018 for sample from Bogor.

2.1.2. Extraction

The leaves of *S. cumini* were collected and cleaned, then dried at room temperature. Dried leaves powder (50 g) of each sample was extracted at room temperature using 500 mL of 70% ethanol as the solvent in first 24 hours, and continued with 250 mL of 70% ethanol in the second and third days. The solution evaporated using a rotary vacuum evaporator and then dried in vacuum oven to obtain dried extracts.

2.2. Arginase inhibition assay

Arginase inhibitory activity was determined using arginase enzymatic assay protocol (Sigma Aldrich, Singapore) and urea assay kit (Abnova Corporation, Taiwan) as reported by previous authors¹⁹ with slight modification. Using 96-well microplate, 10 μ L of each extract solution at the same concentration was mixed with 15 μ L of arginase solution (1 U/mL) and 20 μ L L-arginine (Sigma, Singapore) 380 mM. The mixtures were incubated at 37°C for 30 minutes. For control wells, the extract was replaced by dimethyl sulfoxide (Merck, Germany) and deionized water. The reaction was terminated with the addition of 100 μ L urea assay kit (reagent A : reagent B = 1 : 1) into each well. Arginase inhibition activity was determined after incubation at 25°C for 60 minutes by measuring the quantity of urea. Absorbance was recorded using Versamax Microplate Reader (USA) at wavelength 430 nm.

Percentage of inhibition was calculated using the formula:

$$\% \text{ Inhibition} = \frac{([A_1 - A_2] - [A_3 - A_4]) \times 100}{[A_1 - A_2]}$$

A_1 is the absorbance of the control, A_2 is the absorbance of the control blank, A_3 is the absorbance of the sample, A_4 is the absorbance of the sample blank.

Calibration curve was used to obtain IC_{50} value, which represented concentration of the inhibitor needed to inhibit arginase. Resveratrol was used as a positive standard at range of concentration 8.76-43.81 μ M.

2.3. Antioxidant assays

2.3.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free radical scavenging activity of each extract was evaluated using DPPH assay adopted from previous study²⁰ with slight modification. The reaction mixture consists of 20 μ L of diluted extracts at the same concentration and 180 μ L of DPPH diluted in methanol (150 μ mol/L). The mixture was shaken for 60 seconds and then incubated for 30 minutes in a dark place at room temperature. Absorbance was recorded at 517 nm using microplate reader. Control wells were prepared by mixing 20 μ L methanol and 180 μ L DPPH solution. The scavenging activity (%S) was calculated as follows:

$$\% S = ([A_c - A_s] / A_c) \times 100$$

A_c is the absorbance of the control, A_s is the absorbance of the test extracts. Quercetin was used as a positive standard. Calibration curve of the sample was applied to determine IC_{50} value on DPPH scavenging activity.

2.3.2. Ferric reducing antioxidant power (FRAP) assay

Reducing potential of all extract was examined using microplate-based FRAP assay according to previous study²¹ with slight modification. The FRAP solution consists of 1-volume of TPTZ (40 mM in 40 mM HCl), 1-volume of ferric chloride (20 mM in water), and 10-volume of acetate buffer (300 mM, pH 3.6). In the 96-well microplate, 20 μ L sample solutions were added with 280 μ L FRAP solution and then the mixtures were shaken. After incubated at 37°C in the dark place for 30 minutes, the absorbance was recorded at 593 nm using microplate reader. Quercetin was used as a positive standard. A calibration curve was prepared using various concentration of ammonium ferrous (II) sulfate (2.5-80 μ mol/L). The FRAP value calculated from the calibration curve was expressed as mmol Fe^{2+} equivalents per g of dried extracts.

2.4. Determination of total phenolic content

Folin-Ciocalteu method was applied for the determination of total phenolic content in extracts, as previously described²². Briefly, 25 μ L of sample solution was added to 100 μ L of 25% Folin-Ciocalteu reagent and homogenized by shaking for 60 seconds in 96-well microplate. After 4 minutes, 75 μ L of sodium carbonate solution (100 g/L) was added into each well and then homogenized by shaking for 60 seconds. The reaction mixture was kept protected from light for 2 hours, and afterward, the absorbance was read at wavelength 765 nm using a microplate reader. The sample solution was substituted by methanol as a blank. A calibration curve for quantifying phenolic contents was prepared using gallic acid dilutions (25-150 mg/L). Total phenolic content expressed as gallic acid equivalents (mg of Gallic Acid Equivalents (GAE) per g of dried extract), all samples were determined in triplicate.

2.5. Gallic acid estimation using HPTLC

Active fractions solution of *S. cumini* leaves and gallic acid as standard was spotted on pre-coated silica gel GF254 (Merck, Germany) and eluted using a solvent system of toluene-ethyl acetate-formic acid (5:4:1 v/v) in a CAMAG glass chamber (20 x 10 cm)²³. Chromatogram was detected using Camag Wincat 3 TLC Scanner (Camag, Switzerland) at wavelength 280 nm. Gallic acid amounts in the extracts were calculated using calibration curve. The standard calibration curve was prepared using gallic acid dilutions at range of concentration 25-400 μ g/mL.

2.6. Statistical analysis

All data values represented the means of three replicates measurement. Data were subjected to analyze using one way analysis of variance by SPSS 16.0 software, followed by least significant difference test for multiple comparison between groups. The mean difference considered as significant at the 0.05 level. Correlation between variables was analyzed using Pearson correlation test. Correlation was known statistically significant at the 0.01 level (2-tailed).

3. RESULTS

3.1. Arginase inhibitory activity

Resveratrol, as positive standard, exerted arginase inhibition with IC_{50} 17.56 μ M. The inhibitory activity of hydroethanolic extracts of *S. cumini* leaves from Tangerang (ScT), Sukoharjo (ScS) and Bogor (ScB) against bovine liver arginase were evaluated. Arginase inhibitory activity of all

extracts at a concentration of 50 μ g/mL is presented in Figure 1. All *S. cumini* leaves extracts showed inhibition above 80% and the most active extract was the sample collected from Bogor (88.87%), followed by Tangerang and Sukoharjo, respectively. Concentration of extract that inhibited 50% arginase activity presented in Table 1. *S. cumini* leaves extracts from Bogor showed lowest IC_{50} value.

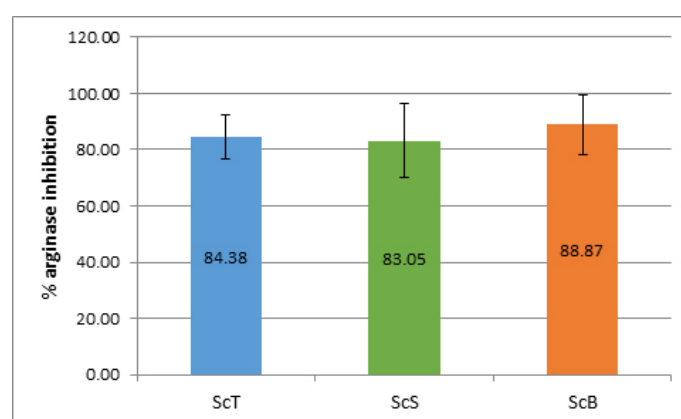


Figure 1. Percentage of arginase inhibition from *S. cumini* leaves extracts from three different locations. Percent inhibition was performed by mean of triplicate measurements

Table 1. Antioxidant activity of *S. cumini* leaves extracts collected from three different locations

Sample	DPPH (%S)	FRAP (mmol Fe(II) eq/g)
ScT	31.38 \pm 1.12	6.22 \pm 0.04
ScS	28.83 \pm 2.53	4.01 \pm 0.15
ScB	33.05 \pm 0.45	7.27 \pm 0.30
Quercetin	87.91 \pm 1.11	15.84 \pm 0.54

All analyses are the mean of triplicate measurement \pm SD.

3.2. Antioxidant activities

The antioxidant activities of *S. cumini* leaves extracts from three different locations of Java were evaluated using DPPH and FRAP assays. DPPH scavenging activity and FRAP value of three extracts are presented in Table 2.

At a concentration of 10 μ g/mL, extracts exerted DPPH scavenging activity expressed as percent scavenging and then observed for IC_{50} value. The lowest IC_{50} value indicated highest

potential as radical scavenger. The highest DPPH scavenging activity was the sample from Bogor (IC_{50} 14.84 μ g/mL). Using FRAP assay, crude extracts also exhibited ferric reducing potential. The lowest FRAP value was the sample from Sukoharjo (4.01) and the highest was the sample from Bogor (7.27). However, the extracts' antioxidant activities still weaker than quercetin itself which exerted DPPH IC_{50} value 5.30 μ g/mL and had FRAP value 15.84 mmol Fe(II) eq/g.

Table 2. Antioxidant activity of *S. cumini* leaves extracts collected from three different locations

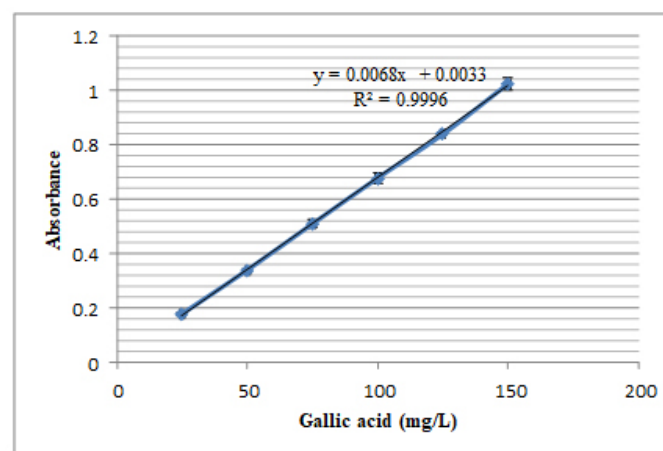
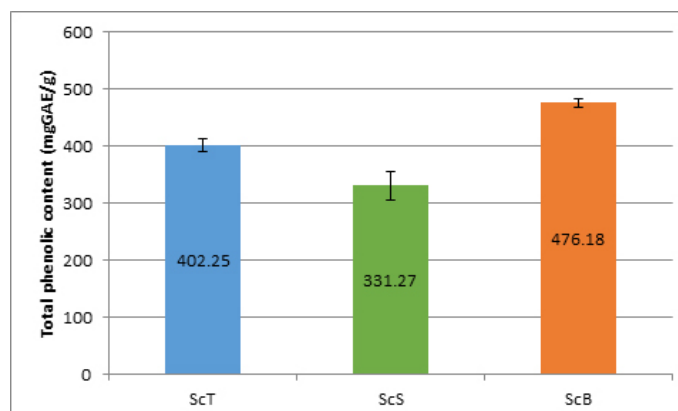
Sample	DPPH		FRAP
	(%S)*	IC ₅₀ (µg/mL)	(mmol Fe(II) eq/g)
ScT	31.38 ± 1.12	17.67	6.22 ± 0.04
ScS	28.83 ± 2.53	19.98	4.01 ± 0.15
ScB	33.05 ± 0.45	14.84	7.27 ± 0.30
Quercetin	87.91 ± 1.11	5.30	15.84 ± 0.54

* % S was done using sample at a concentration of 10 µg/mL.
All analyses are the mean of triplicate measurement ± SD.

3.3. Total phenolic content

The total phenolic contents of three extracts was reported as gallic acid equivalents. The standard calibration curve is presented in Figure 2 with equation $Y=0.0068X+0.0033$. Figure 3 showed the

total phenolic content of three extracts. According to the result, *S. cumini* leaves extracts from Bogor possessed the highest phenolic content (476.18 mgGAE/g), followed by *S. cumini* leaves extracts from Tangerang and Sukoharjo.

**Figure 2.** Calibration curve of gallic acid for determination of total phenolic content**Figure 3.** Total phenolic content of *S. cumini* leaves extracts from three different locations. Result was presented by mean of triplicate measurements.

3.4. Gallic acid estimation using HPTLC

Previous study reported that *S. cumini* leaves contain gallic acid. Thus, each extract was analyzed using HPTLC. Chromatographic profile of three extracts is presented in Figure 4. The standard

calibration curve equation was $y = 41.005x + 563.44$ ($r = 0.989$). Concentration of gallic acid in the extracts presented in Figure 5. The leaves of *S. cumini* collected from Bogor showed highest concentration of gallic acid.

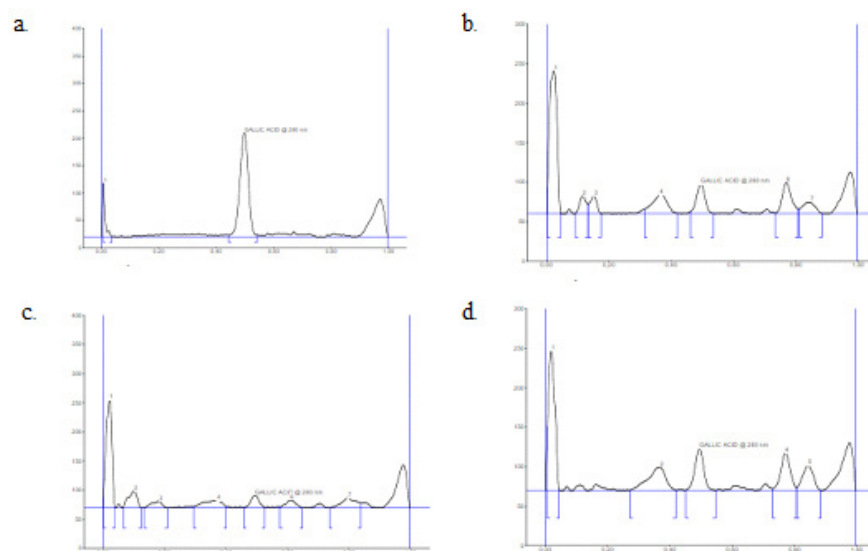


Figure 4. Chromatographic profile of (a) gallic acid; (b) ScT; (c) eScS; and (d) ScB

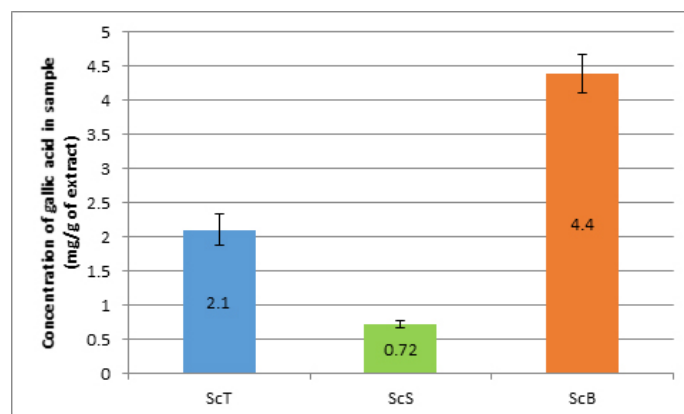


Figure 5. Concentration of gallic acid in *S. cumini* leaves extracts from three different locations. Result was presented by mean of triplicate measurements.

3.5. Statistical analysis

Statistically, there was no significant difference in arginase inhibition and DPPH activity between the leaves extract collected from 3 locations (P -values were 0.795 and 0.050, respectively). The mean difference of FRAP activity, total phenolic content and concentration of gallic acid were significant (P -value < 0.01) between source of

extracts. There was no significant correlation observed between arginase inhibitory activity of extract with antioxidant activity, total phenolic content and concentration of gallic acid (sig. > 0.01).

4. DISCUSSION

Syzygium cumini (L.) Skeels in Indonesia known as jamblang, duwet, juwet, jambu kalang,

or jambulan²⁴. *S. cumini* leaves were collected from three different locations of Java. First sample collected from Tangerang, Banten Province, located in west-north of Java, altitude above sea level \pm 35 m with average rainfall per year 200 mm. Second sample collected from Sukoharjo, located in central Java, altitude above sea level \pm 102 m with average rainfall per year 2790 mm. Third sample collected from Bogor Botanical Garden, located in west Java, altitude above sea level \pm 290 m with average rainfall per year 3500 mm. Samples were extracted using 70% ethanol at room temperature to obtain a high content of phenolic compounds¹⁶ and avoid any degradation of active compounds during extraction which can be caused by heat.

Arginase inhibitory activity was performed *in vitro*. Arginase will hydrolyzed L-arginine into L-ornithine and urea³, thus urea determination utilized to measure enzyme activity. Commonly used method for determination of urea at low concentration is a colorimetric reaction of urea, o-phthalaldehyde, and primaquine²⁵. In this study, we used urea assay kit (Abnova, Taiwan). Resveratrol was used as positive standard based on the previous study⁸ which had tested arginase inhibitory activity of resveratrol and yielded IC_{50} at concentration 18.2 μ M. In this study, IC_{50} of resveratrol was 17.56 μ M. The difference on result value might be due to assay method and conditions that were different from the previous study.

At a concentration of 50 μ g/mL, all extracts possessed arginase inhibition above 80%. Highest activity was shown by the sample from Bogor (88.87%), followed by the sample from Tangerang and Sukoharjo. But this difference was not statistically significant. As described by previous studies, the leaves contains some flavonoids and phenolic acids such as catechin, kaempferol, quercetin, rutin, myricetin, myricitrin, gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and ellagic acid^{12,13,26,27}. Polyphenol compounds contained in *S. cumini* leaves predicted as responsible compounds for its arginase inhibitory activity.

Polyphenols play an important role in enzyme activity because it can alter the active conformation of enzymes through destabilization of the bonds between hydrogen bonds and water molecule²⁸. Extract of *Moringa oleifera* Lam. leaves, which contained gallic acid, catechin,

chlorogenic acid, ellagic acid, epicatechin, rutin, quercitrin, isoquercitrin, quercetin and kaempferol, showed arginase inhibition from rat penis homogenates (IC_{50} 159.59 μ g/mL)²⁹. Other study using isolated rat liver found that at a concentration of 1 mM, catechin and gallic acid inhibited arginase by 26 and 20%, respectively³⁰. Another *in vitro* colorimetric study evaluated the potential of some polyphenols as arginase inhibitor. Chlorogenic acid, quercetin, caffeic acid and kaempferol showed inhibitory activity with IC_{50} 10.6, 31.2, 86.7, 179.1 μ M, in that order. Caffeoyl (3,4-dihydroxycinnamoyl) group might be the key to enzyme inhibitory activity⁸.

In this study, all extracts of *S. cumini* leaves showed antioxidant activity using two methods, DPPH and FRAP assay. Quercetin, a well-known natural antioxidant, was used as a positive standard. The extract using the leaves from Bogor showed highest activity for both assays but weaker than quercetin. Mean difference between groups was not statistically significant for DPPH assay, but statistically significant for FRAP assay. Phenolic compounds might contribute to the antioxidant activity of the extracts. *S. cumini* leaves from different sources might vary in the number of phenolic compounds, thus differs in antioxidant activity.

In line with arginase inhibitory activity, the highest antioxidant activity also exhibited by *S. cumini* leaves collected from Bogor. The result of the total phenolic content and gallic acid concentration of *S. cumini* leaves extract collected from Bogor also showed highest phenolic content and gallic acid concentration compared to sample from Tangerang and Sukoharjo. In this study, it can be considered that arginase inhibitory tends to align with the antioxidant activity of this plant and the high phenolic content also gallic acid concentration. Nevertheless, the correlation could not be observed statistically. The variation of secondary metabolites content and pharmacology activity in the leaves extract collected from different geographical location¹⁸ might due to differences in plant nutrition and environment.

5. CONCLUSIONS

S. cumini leaves possessed arginase inhibition and antioxidant activity which might be beneficial for therapy of diseases related to

endothelial dysfunction. This study revealed that different source of *S. cumini* leaves exhibited different activity value. Sample collected from Bogor exhibited the highest activity, also had the highest phenolic content and gallic acid concentration. Further studies are needed to determine active phytoconstituents from *S. cumini* leaves and to confirm the activity *in vivo*.

6. ACKNOWLEDGEMENTS

This study was supported by University of Indonesia through “Hibah TADOK 2018” grant for research funding and Center for Plant Conservation, Bogor Botanical Garden, Indonesia Science Institution for sample collection. Ari Ariefah Hidayati would thanks to Ministry of Health of the Republic of Indonesia for the scholarship.

Conflict of interest

The authors declare to have no conflict of interest.

Funding

None to declare

Ethical approval

None to declare

Article info:

Received December 5, 2018

Received in revised form March 1, 2019

Accepted March 29, 2019

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