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Intracellular antioxidant activity of *Muntingia calabura* leaves methanolic extract

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Abstract. *Rahmawati AN, Astirin OP, Pangastuti A. 2018. Intracellular antioxidant activity of* Muntingia calabura *leaves methanolic extract. Nusantara Bioscience 19: 210-214.* Excessive reactive oxygen species (ROS) results in oxidative damage that destroys proteins, lipids, and DNA, resulting in various diseases and accelerating the aging process. Antioxidants are compounds that have the ability to suppress oxidative stress. *Muntingia calabura* has a high phytochemical content, especially the phenolic group that can act as antioxidant. This study aimed to determine the intracellular antioxidant activity of *M. calabura* leaves methanolic extract (MLME) through intracellular ROS levels in 3T3 fibroblast cells under normal condition and oxidative stress due to UVB exposure. Tests were performed using the DPPH (Diphenyl-1-picrylhydrazyl) method and modified NBT (Nitroblue Tetrazolium). Testing of antioxidant activity with DPPH method obtained result that MLME had high antioxidant activity with IC_{50} value of 3.030 µg mL^{-1}, while intracellular antioxidant activity with modified NBT method showed that MLME statistically had significant effect on intracellular ROS level under normal condition ($p < 0.05$) based on dose-dependent manner, but not significant ($p > 0.05$) under oxidative stress condition. Significant effects were only shown at a concentration of 20 µg mL^{-1} against the control of oxidative stress due to UVB exposure.

Keywords: Antioxidant, Intracellular ROS, NBT, *Muntingia calabura*

INTRODUCTION

Reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are products of cellular metabolic activity and serve as second messengers in the cell signaling process when encountered in sufficient quantities. The presence of ROS in high concentrations has a harmful effect on cells because it destroys proteins, lipids, and DNA (Bhattacharryya et al. 2014; Rinnerthaler et al. 2015; Sarangarajan et al. 2017). An increase in the number of ROS occurs in response to UV exposure, cigarette smoke, alcohol consumption, pathogen infection and other external factors that lead to oxidative stress (Bhattacharryya et al. 2014). Oxidative stress that results in oxidative damage is the beginning of many diseases and is included in the main factors that accelerate the aging process (Guerra-Araiza et al. 2013; Pisoschi and Pop 2015; Valko et al. 2016).

Oxidative stress is naturally inhibited by enzymatic antioxidant compounds such as superoxide dismutase, catalase, and glutathione peroxidase, as well as non-enzymatic antioxidant compounds such as glutathione, alpha-tocopherol, ascorbic acid and ubiquinone (Bhattacharryya et al. 2014). However, the high oxidative stress in the cells can cause a disruption of the balance between the amount of ROS with endogenous antioxidants, thus disrupting oxidative homeostasis (Jadoon et al. 2015; Poprac et al. 2017). Exogenous antioxidants help increase oxidative homeostasis through increased endogenous antioxidant activation and direct ROS stabilization (Guerra-Araiza et al. 2013; Nakchat et al. 2014; Jadoon et al. 2015). Exogenous antioxidants can be found as natural antioxidants which are the way out for oxidative stress management because they can affect endogenous antioxidant systems and balance oxidative homeostasis (Jadoon et al. 2015). The search for natural antioxidant sources led to arise of various studies related to phytochemical and antioxidant, including *Muntigia calabura*.

Muntigia calabura or *'kersen'* (in Bahasa Indonesia) is a plant of the Elaeocharpaceae family encountered in almost all tropical regions due to its high adaptability (Lim 2012; Mahmood et al. 2014). *M. calabura* has been widely used in Peru, Colombia, Mexico, Vietnam and the Philippines to reduce ulcers, fever, headaches, to be utilized as tranquilizers. *M. calabura* fruit is often consumed directly, and its leaves can be utilized as a substitute for tea (Mahmood et al. 2014). The high phytochemical content of *M. calabura* leaf extract, especially flavonoids and polyphenols causes the leaf extract of *M. calabura* to have antioxidant activity (Zakaria et al. 2011, 2014a; Balan et al. 2015), antiproliferative (Zakaria et al. 2011), antinociceptive (Zakaria et al. 2014a), anti-inflammatory (Balan et al. 2015), and anticarcinogenic (Nasir et al. 2017). The results of the test with DPPH radical scavenging activity showed that *M. calabura* leaf extract has antioxidant activity above 90% (Zakaria et al. 2011, 2014a; Balan et al. 2015) causing *M. calabura* to potentially as a source of natural antioxidant in the future. However, the effect of *M. calabura* leaf extract on intracellular ROS is unknown. 3T3

fibroblast cells are used as an object because it is easy to culture and produce extracellular matrix (ECM) proteins (Freshney 2010), making it appropriate for research on oxidative damage caused by oxidative stress.

This study aimed to determine the antioxidant activity of *M. calabura* leaves methanolic extract on 3T3 fibroblast cells under normal condition and oxidative stress due to UVB exposure using modified NBT.

MATERIALS AND METHODS

Plant material and preparation of *Muntingia calabura* leaves methanolic extract

Muntingia calabura leaves were collected in October-November 2017 from Wonorejo Village, Polokarto, Central Java, Indonesia. Extraction of *M. calabura* leaves was performed by maceration using MeOH based on Zakaria et al. (2014a, b) and Balan et al. (2015). *M. calabura* mature leaves had been grounded into fine powder after air-drying for about two weeks and as much as 100 g of fine powder was soaked in MeOH at a ratio of 1:20 (w/v) for 72 h. The mixture was filtered with filter paper and evaporated using a rotary evaporator at 40°C to obtain a *M. calabura* leaves methanolic extract (MLME).

Determination of total phenolic content

Determination of total phenolic content (TPC) was performed using Folin-Ciocalteu method based on Singleton et al. (1999) with slight modification. A total of 300 µL of MLME (0.5 mg/1 mL) was mixed with 1.5 mL of Folin-Ciocalteu reagent 10% and then incubated for 1 minute at room temperature. The mixture was added 1.2 mL of Na_2CO_3 and incubated at room temperature for 90 min. Absorbance was measured using a UV-Vis Spectrophotometer at 760 nm. The absorbance measuring results were calibrated to the standard curve obtained from the absorbance of gallic acid with a concentration of 20, 40, 60, 80, and 100 µg mL^{-1}.

Thin layer chromatography

Thin layer chromatography (TLC) was performed by using chloroform: ethyl acetate (40:60) as a mobile phase (eluent) and silica plate as a stationary phase. MLME (1 mg/100 µL) which had been dissolved in methanol: ethyl acetate (60:40) was bottled on a silica plate. The silica plate with MLME was air-dried and then inserted into the eluent. Eluent was allowed to move along the silica plate until it reached a distance of 1 cm before the end of the plate. Quercetin (1 mg/100 µL) was used as a comparison. The TLC results were exposed to ammonia vapor and seen under UV light at 254 nm.

Determination of antioxidant activity

Determination of antioxidant activity was performed using the DPPH (Diphenyl-1-picrylhydrazyl) method based on Zakaria et al. (2014b) and Balan et al. (2015) with slight modification. A total of 50 µL samples with various series concentrations were mixed with 50 µL DPPH 0.1 mM. The concentration series used were 0, 15.625, 31.25, 62.5, 125, 250, and 500 µg mL^{-1}. The mixture was incubated at room temperature for 30 minutes in the dark room. Absorbance was measured using BioRad Microplate Reader at 517 nm. Antioxidant activity was known based on the formula according to Jemli et al. (2016):

$$\% \text{ inhibition} = \left(\frac{A0 - A1}{A0}\right) \times 100\%$$

A_0 is the absorbance of the control, while A_1 is the absorbance of the sample. The percentage of inhibition was used to determine IC_{50} value of antioxidant activity through a regression equation.

3T3 fibroblast cell culture

3T3 fibroblast cell-line was cultured in complete media consisting of DMEM (Gibco®) and FBS (Gibco®). Cells with a density of 7 x 10^3 were distributed into 96 well-plates and then incubated in a CO_2 incubator for 24 h at 37 °C to let cells stick at the bottom of the plate.

Cell viability assay

Cell viability assay was performed using MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium) method in 3T3 fibroblast normal cells. Culture media was discarded and replaced with complete medium that had been mixed with samples in DMSO. Variations in sample concentration used were 3.906, 7.813, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg mL^{-1}. The culture was incubated for 24 h at 37°C in CO_2 incubator. The culture medium was removed and 100 µL of MTT reagent (0.5 mg mL^{-1}) was added. The culture was incubated for 4 h in the CO_2 incubator at 37°C and 100 µL SDS Stopper was added. The culture was incubated at room temperature for overnight under closed conditions of light. The absorbance measuring was performed using Bio-Rad Microplate Reader at 595 nm.

UVB irradiation and treatment

3T3 fibroblast cell-line culture with a density of 5 x 10^3 distributed into 96 well-plates for subsequent incubation in a CO_2 incubator for 24 h at 37°C. Culture media was discarded and replaced with complete medium that had been mixed with samples in DMSO. Variations in sample concentration used were 5, 10, and 20 µg mL^{-1}. The culture was incubated overnight, then replaced with a new medium for further exposure of UVB light at a dose of 50 mJ cm^{-2} using the UVB irradiation system. The culture was incubated at 37°C for 48 h in the CO_2 incubator.

Table 1. The percentage inhibition of DPPH activity by MLME

Concentrations (µg/mL)	% inhibition of DPPH
0	0
15.625	47
31.25	60
62.5	80
125	81
250	82
500	80

Note: The percentage inhibition of DPPH activity showed the potential of the MLME to scavenge DPPH free radical through in vitro assay.

Intracellular ROS assay

Intracellular ROS assay was performed using a modified NBT (Nitroblue Tetrazolium) methods based on Tunc et al. (2008) and Adrian et al. (2013). The culture that had been incubated for 48 h was rinsed with 100 µL PBS, then given 100 µL of NBT in PBS. The culture was incubated at 37 °C for 45 minutes and rinsed with 100 µL PBS twice. The culture was dried and then dissolved with 60 µL KOH 2M and 140 µL DMSO. Samples were allowed to stand for 10 minutes before an absorbance measuring at 450 nm wavelength was performed.

Data analysis

Data analysis was performed using Microsoft Excel 2010. One-way ANOVA and Tukey-Kramer post hoc test were used to evaluate the significance ($p < 0.05$) between samples and controls.

RESULTS AND DISCUSSION

Total phenolic content and thin layer chromatography

The MLME has a high phenolic content which is known based on total phenolic content testing at the extract concentration of 500 µg mL^{-1}. The value of TPC obtained is 1849.716 ± 7.567 mg GAE/100 gram dry weight. Based on Balan et al. (2015), TPC values above 1000 mg GAE/100 gram dry weight are classified into high TPC values.

Identification using TLC method was done to know the type of class of phenolic compounds contained in the MLME. The eluent used was chloroform: ethyl acetate and the solvent used was methanol: ethyl acetate. The use of eluent and solvent for TLC is the result of optimizing some eluents and solvents. The determination of eluent and solvent was also adjusted with quercetin as the comparative solution. The best results were obtained by using the chloroform: ethyl acetate (40:60) as an eluent and methanol: ethyl acetate (60:40) as a solvent. MLME separated into two spots i.e. yellow and green spots when viewed in visible light and two black spots under 254 nm UV light in Figure 1. Clearer results are shown after silica plate was exposed with ammonia vapor and seen under UV light at 254 nm, each plate shows two spots that can be seen in Figure 2. The Rf value of extract and quercetin can be seen in Table 2.

Table 2. Table of Rf value before and after exposure with ammonia vapor

Condition	Rf extract	Rf quercetin
Before exposing	0.859	0.761
After exposing	0.872	0.775

Note**:** Rf value of extract and quercetin was measured before and after exposing with ammonia vapor through calculating the eluent distance from the lower border of the silica plate.

Antioxidant activity and cell viability

Antioxidant activity was measured based on the % inhibition of DPPH that can be seen in Table 1. Based on Table 1, IC_{50} value for antioxidant activity was 3.030 µg mL^{-1}. It showed high antioxidant activity because it is lower than 50 µg mL^{-1} (Hilmi et al. 2014).

The results of the cell viability test of 3T3 fibroblast cells to the MLME done using MTT method can be seen in Figure 3. Based on the data known, the extract did not show the cytotoxicity to 3T3 fibroblast cells. The value of cytotoxicity was expressed in IC_{50} of 782.115 µg mL^{-1}. IC_{50} values above 200 µg mL^{-1} show that the extract is not toxic to cells (Limtrakul et al. 2016). Based on IC_{50} values of antioxidant activity and cytotoxicity, the determination of concentrations of 5, 10, and 20 µg mL^{-1} can be done for subsequent experiments.

Intracellular ROS level

The intracellular ROS level is known through simple assay using modified NBT. The absorbance values obtained indicate intracellular ROS levels based on the interaction between NBT and intracellular ROS (Tunc et al. 2008).

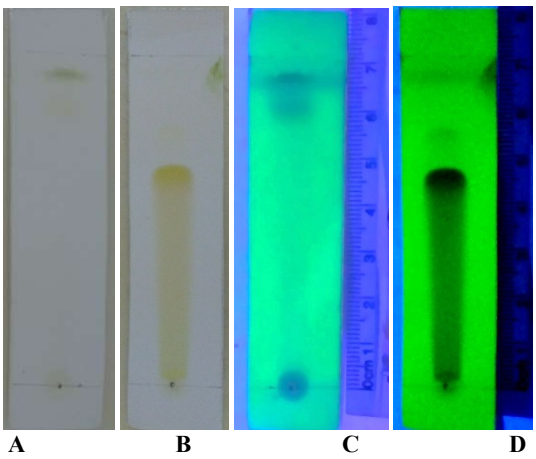

Figure 1. TLC results of MLME (A) and quercetin (B) on visible light. TLC results of MLME (C) and quercetin (D) under UV light at 254 nm before exposed with ammonia vapor

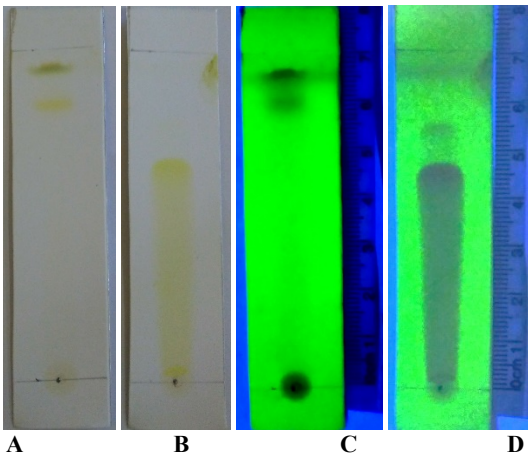

Figure 2. TLC results of MLME (A) and quercetin (B) on visible light. TLC results of MLME (C) and quercetin (D) under UV light at 254 nm after exposed with ammonia vapor

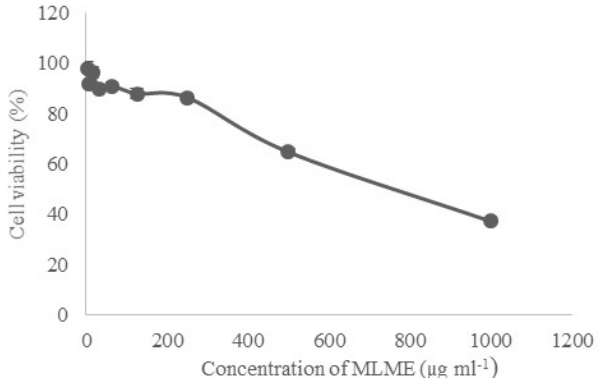

Figure 3. Effect of MLME on the 3T3 fibroblast cells viability. The cells were treated with various concentrations of MLME (3.9 – 1000 µg mL^{-1}) for 24 hours. The cells viability was determined with MTT assay.

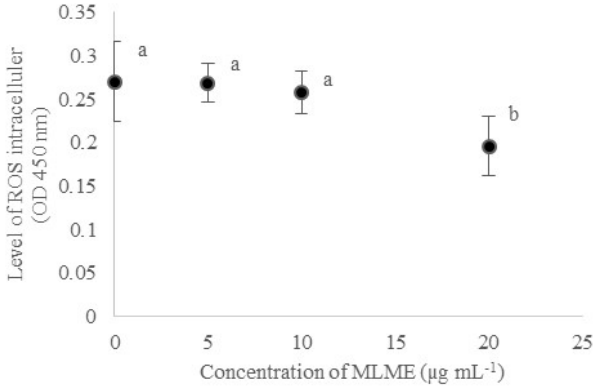

Figure 6. Effect of MLME with different concentrations (0 – 20 µg mL^{-1}) on the intracellular ROS intracellular levels after exposured with UVB at a dose of 50 mJ cm^{-2}. Values with different letter showed significant differences based on one-way ANOVA ($p > 0.05$) followed by Tukey-Kramer post hoc test

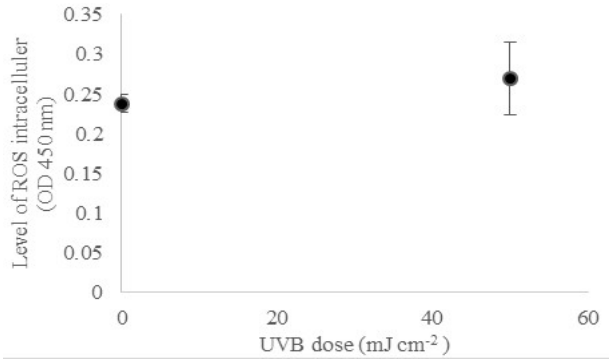

Figure 4. Effect of UVB exposure at a dose of 50 mJ cm^{-2} on the intracellular ROS levels. The two groups were analyzed by using one-way ANOVA followed by Tukey-Kramer post hoc test. Two groups showed difference in mean but not significant ($p > 0.05$).

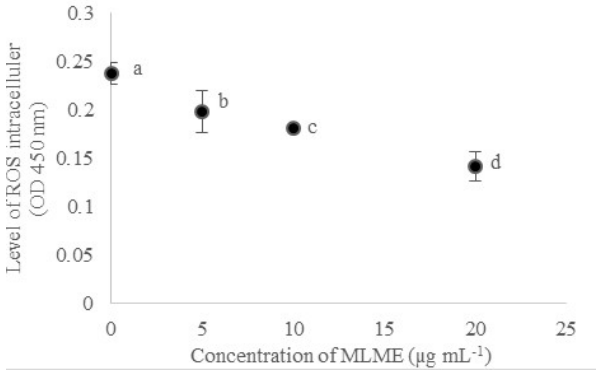

Figure 5. Effect of MLME with different concentrations (0 – 20 µg mL^{-1}) on the intracellular ROS levels under normal condition. Values with different letter showed significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey-Kramer post hoc test.

Giving extract to 3T3 fibroblast culture under normal condition statistically significant to intracellular ROS level ($p < 0.05$) based on dose-dependent manner (Figure 5). The intracellular ROS level of fibroblast cells after administration of an MLME under oxidative stress condition showed a significant effect on the concentration of 20 µg mL^{-1} on the control of oxidative stress without extract. The results can be seen in Figure 6.

Discussion

Oxidative stress in this study was stimulated through UVB exposure at a dose of 50 mJ cm^{-2}. UV light exposure at a dose of 50 mJ cm^{-2} was statistically different in the mean but did not give a significant difference ($p > 0.05$). UV exposure can indirectly affect the production of ROS in greater quantities and ends in the aging process (Rinnerthaler et al. 2015). The results showed that the administration of MLME decreased intracellular ROS level in 3T3 fibroblast cells under normal condition based on dose-dependent manner (Figure 5), while under oxidative stress condition showed significant influence at 20 µg mL^{-1} concentration on control without extract (Figure 6). Decreased ROS levels in 3T3 fibroblast cells under normal conditions and oxidative stress after administration of the MLME correlated polyphenol components measured on the TPC test (Bravo et al. 2017). Flavonoids are a group of phenol compounds identified through the TLC method. MLME had a similar Rf value with the quercetin comparative solution (Table 1) identified that in the MLME there was a compound similar to quercetin which is a flavonoid.

The decrease in intracellular ROS levels in this study is thought to be the effect of phenol compounds, especially flavonoids, which can act as direct scavengers and restore the balance of the endogenous antioxidant system of 3T3 fibroblast cells (Guerra-Araiza et al. 2013). The mechanism of MLME in reducing intracellular ROS still has not been able to be explained through this study, but Nakchat et al. (2014) study showed that the administration of Tamarin

Seed Coat Extract (TSCE) containing phenolic and flavonoid compounds in CCD-1064Sk fibroblast cells reduces oxidative stress through increased GSH activity which is an endogenous antioxidant. The hydroxyl groups present in flavonoid compounds act as electron donors for ROS (Bosch et al. 2015), thus stabilizing ROS and reducing oxidative stress.

The ability of ROS scavenging of MLME was measured through DPPH radical scavenging activity which was expressed as IC_{50} values. IC_{50} values obtained from this study are consistent with the results of previous research (Zakaria et al. 2014a, b; Balan et al. 2015) that MLME has a very high antioxidant activity because it is lower than 50 μg mL^{-1}. Based on Molyneux (2004) the lower the value of IC_{50}, the higher the antioxidant activity. IC_{50} values are also used to express the toxicity of MLME to 3T3 fibroblast cells. IC_{50} values obtained from MTT test of the MLME (Figure 3) showed a value above 200 μg mL^{-1}. Based on Limtrakul et al. (2016) the IC_{50} value above 200 μg mL^{-1} indicates that the extract is safe for subsequent experiments because it has low cytotoxicity.

This research concludes that MLME has an ability in reducing intracellular ROS levels causes it to potentially serve as a natural antioxidant source to combat oxidative stress. This potential allows *M. calabura* to be a potential source of antioxidants for anti-aging cosmetic products. Further research related to anti-aging potential of *M. calabura* needs to be done.

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