Original Article

In vitro anti-aging activity of *Muntingia calabura* L. fruit extract and its fractions

[Actividad anti-envejecimiento *in vitro* del extracto del fruto de *Muntingia calabura* L. y sus fracciones]

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Abstract

Context: Premature aging usually occurred due to free radicals reducing the skins' physiological functions. *Muntingia calabura*, a plant containing rich antioxidants, has the potential to overcome this problem.

Aims: To evaluate the antioxidant capacity of *M. calabura* in inhibiting the premature aging process, to be potentially developed into an antiaging active ingredient.

Methods: The samples were extracted using ethanol 96%, and processed into n-hexane, ethyl acetate, and ethanol fractions, respectively. Total phenolic content was determined, followed by the evaluation of antioxidant capacity through DPPH, FRAP, and ABTS assay. Further, anti-elastase was conducted using human neutrophil elastase as a skin degradation enzyme, followed by an anti-collagenase test. Finally, normal cell proliferation was also evaluated via the MTT method measuring cell viability on HDFa cells.

Results: As the results, ethanol extract, ethyl acetate fraction, and ethanol fraction showed a strong antioxidant effect, having great capacity reducing DPPH, ABTS radicals, and also iron reduction, in contrast to n-hexane fraction that exhibited only weak activity. The antioxidant trend capacities were found directly correlated to total phenolic contents. Furthermore, the ethyl acetate fraction was found to have optimum activity in inhibiting elastase and collagenase enzymes, showing a similar impact on cell viability.

Conclusions: The ethyl acetate fraction from *M. calabura* exhibits the prospect for further development to support its effectiveness as an active ingredient in antiaging cosmetics.

Keywords: antiaging; antioxidant; cell viability; cosmetic; *Muntingia calabura* L.

Resumen

Contexto: El envejecimiento prematuro generalmente se produce debido a que los radicales libres reducen las funciones fisiológicas de la piel. *Muntingia calabura*, una planta que contiene ricos antioxidantes tiene el potencial de superar este problema.

Objetivos: Evaluar la capacidad antioxidante de *M. calabura* para inhibir el proceso de envejecimiento prematuro, para convertirse potencialmente en un ingrediente activo antienvejecimiento.

Métodos: Las muestras se extrajeron con etanol al 96% y se procesaron en fracciones de n-hexano, acetato de etilo y etanol, respectivamente. Se determinó el contenido fenólico total, seguido de la evaluación de la capacidad antioxidante mediante el ensayo DPPH, FRAP y ABTS. Además, la evaluación anti-elastasa se llevó a cabo utilizando elastasa de neutrófilos humanos como enzima de degradación de la piel, seguida de una prueba anti-colagenasa. Finalmente, también se evaluó la proliferación celular normal mediante el método MTT que mide la viabilidad celular en células HDFa.

Resultados: El extracto de etanol, las fracciones de acetato de etilo y de etanol mostraron un fuerte efecto antioxidante, teniendo gran capacidad reductora de radicales DPPH, ABTS y también reducción de hierro, en contraste con la fracción n-hexano que exhibió solo actividad débil. Las capacidades de tendencia antioxidante se correlacionaron directamente con el contenido total de fenoles. Además, se encontró que la fracción de acetato de etilo tiene una actividad óptima para inhibir las enzimas elastasa y colagenasa, mostrando un impacto similar en la viabilidad celular.

Conclusiones: La fracción de acetato de etilo de *M. calabura* presenta la posibilidad de un mayor desarrollo para respaldar su eficacia como ingrediente activo en cosméticos antienvejecimiento.

Palabras Clave: clave: anti-envejecimiento; antioxidante; cosmético; *Muntingia calabura* L.; viabilidad celular.

INTRODUCTION

Aging skin is the first thing an individual can see when interacting with others, so it will greatly affect the quality of life (Xie et al., 2015). In some people, aging will occur according to age, while in certain people, aging will occur earlier and is known as premature aging. This is due to a combination of intrinsic aging, which is influenced by genetic factors related to chronological age, and extrinsic aging that occurs due to the influence of environmental factors such as exposure to ultraviolet (UV) rays, smoking, chemicals, and earth's gravity. A factor in the extrinsic aging process is UV radiation (Lee et al., 2012; Hwang et al., 2017).

UV rays are not the basic cause of skin aging, but about 80% of facial skin aging is caused by UV rays, known as photoaging. Excessive UV exposure will result in the formation of reactive oxygen species (ROS). Increased ROS will cause damage to lipids, proteins, and cell's DNA, triggering the skin aging process (Poljšak et al., 2012). The ROS formed can inhibit the transforming growth factor-β, prevent collagen formation, and increases nuclear activator protein (AP-1) transcription. The increase in transcription from AP-1 then causes an increase in the expression of matrix metalloproteinase, which is a collagen-degrading enzyme (Farage et al., 2008; Helfrich et al., 2008; Pandel et al., 2013). Collagen is a fibrous protein providing strength and flexibility to important tissues and bones. In the field of cosmetics, collagen acts as an active substance that can provide many benefits for the skin, such as preventing wrinkles, increasing skin moisture, protecting skin from free radicals, and maintaining skin elasticity (Thaman and Draelos, 2005).

In addition, the formation of ROS can also increase the formation of the enzyme elastase (human neutrophil elastase), which is an enzyme that degrades elastin. Elastin is an insoluble elastic fiber protein, and together with collagen, affects the mechanical properties of connective tissue, which is responsible for maintaining skin elasticity and resilience. During the aging process, skin elasticity decreases due to the presence of the elastase enzyme, deteriorating elastin so that the skin becomes loose (Steinbrecher et al., 2008; Garg et al., 2017).

In internal aging, there are three combinations of skin aging processes, including a decreased proliferation of skin cells, decreased synthesis of the skin's extracellular matrix, and increased activity of enzymes that degrade collagen in the dermis. One of the skin cells, namely fibroblasts, will experience a decrease in population with age. The decrease in fibroblast cell population causes a decrease in collagen biosynthesis in the dermis layer (Jenkins, 2002; Hwang et al., 2011). Fibroblasts are cells that are widely obtained in connective tissue, especially in the skin. Fibroblast cells are actively involved in the formation of fibers, especially collagen fibers and extracellular amorphous matrix. Fibroblasts are also involved in the normal growth, wound healing process, and physiological activity of every tissue and organ in the body (Freshney, 2011).

Based on this, efforts are made to prevent premature aging from occurring, namely by using antioxidant compounds such as polyphenols. Polyphenols contain OH groups attached to a benzene ring. This phenolic group provides a direct antioxidant effect, reduces metal ions, modulates protein phosphorylation, and can inhibit lipid peroxidation (Pouillot et al., 2011; Halliwell and Gutteridge, 2015).

Kersen (*Muntingia calabura* L., *Muntingiaceae*) is one of the plants containing rich antioxidants that have been scientifically studied, specifically its fruit. The results of the phytochemical analysis of this fruit contain saponins, flavonoids, tannins, and steroids. In addition, *M. calabura* fruit is known to be enriched with phenolic compounds related to its antioxidant capacity, including gallocatechin, epigallocatechin, catechins, flavanols, naringenin, quercetin, gallic acid, vanillic acid, chlorogenic acid, caffeic acid, ρ-coumaric acid, ferulic acid, Ɛ-hydroxycinnamic acid and myricetin (Mastuki et al., 2019). The results of research that has been conducted with fresh ripe *M. calabura* fruit showed an IC_{50} value of 41.10 μg/mL

classified as a very strong antioxidant category. In addition, other studies also stated that the *M. calabura* methanol extract of this fruit provided strong antioxidant capacity in inhibiting DPPH radicals with an IC_{50} value of 90 ± 0.04 µg/mL (Preethi et al., 2010). Based on this background, a study was conducted to see the activity of *M. calabura* fruit as an antiaging candidate.

MATERIAL AND METHODS

Plant material

Muntingia calabura L. fruits were collected from Biringkanaya regency, Makassar city, South Sulawesi, Indonesia (GPS coordinate -5°.11′6.439″N, 119°.51′94.99″E). The species was identified by Dr. A. Mu'nisa, and the voucher specimen was kept at the herbarium of Botanical Laboratory, Department of Biology, Mathematics and Science Faculty, and State Makassar University, Indonesia.

Chemical and reagents

Chloroform, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrochloric acid, acetic acid, n-hexane, ethyl acetate, quercetin, distilled water, hydrochloric acid, iron (III) chloride, 2,4,6-tripyridyl-s-triazine (TPTZ), acetic acid, 2,2'-azino-bis-(3-ethylbenzothiazoline sulfonate) (ABTS) were supplied by Sigma. Ethanol 70%, n-hexane, ethyl acetate, acetone, ethanol, distilled water, human neutrophil elastase kit consisting of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NaCl, Tween 20, dimethyl sulfoxide (DMSO), elastatinal, neutrophil elastase enzyme, substrate (MeOSuc-Ala-Ala-Pro-Val-pNA), vitamin C from Merck (Germany). The MMP-1 collagenase kit obtained from Enzo Life Science consisted of collagenase enzyme (MMP-1). N-isobutyl-N-(4-methoxyphenylsulfo-nyl) glycylhydroxamic acid (NNGH) 1.3 µM, substrate (thiopeptide, Ac-PLG-[2-mercapto-4-methyl-pentatonoyl]-LG-OC25H, 100 µM) from Enzo Life Science. Adult human dermal fibroblast (HDFa) cells obtained from Life Technologies Corporation. Iron (III) chloride, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), glutamine, hydrogen peroxide (H_2O_2), filter paper, trypsin-EDTA solution, phosphate-buffered saline (PBS), penicillin, reagent 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyltetrazolinum bromide (MTT), streptomycin were purchased from Gibco, Life Technology Corporation, New York, USA.

Preparation of extract and fractions of *M. calabura*

The plant material was dried at a temperature of 40°C (in an oven, TEW IL80-EN, Agrowindo company, Makassar city, Indonesia), and 500 g were extracted by maceration with 96% ethanol solvent as much as 3.2 L for 3 times 24 hours in a closed vessel (protected from light) and manually shaken occasionally. Then the filtered was evaporated with a rotary vacuum evaporator (rotary evaporator R-100, Vacum V-100, Buchi, Germany) at 50°C to get viscous extract (ethanol extract). The ethanol extract obtained was then fractionated using a variety of solvents with different polarities.

For the fractionation process, ten grams of the extract were dissolved with 50 mL 70% ethanol and added with 50 mL n-hexane. The mixture was shaken and allowed to stand until completely separated. Fractionation was carried out repeatedly using n-hexane until the solution was clear. Furthermore, the residue was fractionated using ethyl acetate solvent in the same procedure as for the preparation of the n-hexane fraction. The liquid fractions of n-hexane, ethyl acetate, and ethanol (residue) were evaporated to obtain the dry fraction. The ethanol extract (EE), n-hexane fraction (HF), ethyl acetate fraction (EAF), and ethanol fraction (EF) obtained were then tested for their activity. The extract and fractions were dissolved in ethanol p.a. for antioxidant and total phenolic evaluation. Whereas anti-elastase, anti-collagenase, and normal cell proliferation assay, the extract and fractions were dissolved in DMSO.

Antioxidant evaluation

Antioxidant capacity testing was carried out using three different tests, including 1,2-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS).

DPPH assay

A DPPH (0.4 mM) solution was prepared briefly to obtain a solution absorbance of 0.529 ± 0.02. A series of sample concentrations of 6, 12, 18, 24, and 30 μg/mL for ethanol extract; 200, 300, 400, 500, and 600 μg/mL for n-hexane fraction; 4, 8, 12, 16, and 20 μg/mL for ethyl acetate fraction, and 12, 18, 24, and 30 μg/mL for ethanol fraction as well as 2, 4, 6, 8, and 10 μg/mL for quercetin as reference were made (Nur et al., 2019; Yahaya et al, 2020). Several volumes of solution from each sample at a specified concentration were added to 1 mL of DPPH solution. Then, the volume of the mixture was adjusted to 5 mL with ethanol p.a. The mixture was incubated for 30 minutes, and the absorption was measured on a UV-Vis spectrophotometer (Shimadzu UV-1900, Shimadzu Corporation, Kyoto, Japan) at 515 nm. The inhibitory effect was calculated based on the percentage of the color of the DPPH solution that faded to yellowish by the control (DPPH solution only). The effect of extract and fractions to scavenge the DPPH radical was calculated with equation [1].

$$DPPH\ radical\ scavenge\ (\%) = [(A_0 - A_1)/A_0) \times 100] \qquad [1]$$

Where A_0 was the absorbance of the negative control (DPPH solution only) and A_1 was the absorbance of the sample. The IC_{50} value was represented based on the sample concentration necessary to reduce the DPPH to 50%, a value obtained from the linear regression graph.

FRAP assay

The antioxidant capacity in the reducing iron was carried out by colorimetric method using a UV-Vis spectrophotometer (Nur et al., 2019). A ferric chloride (0.02 M) solution in distilled water and a TPTZ solution (0.01 M in 0.04 M hydrochloric acid), and an acetic buffer pH 3.6 were prepared. Each sample solution was pipetted with 500 μL and FRAP reagent was added with a composition of the acetic buffer:TPTZ:FeCl₃ (with a ratio of 10: 1: 1). In addition, the volume of the mixture was added to 5 mL with distilled water and incubated for 30 minutes at room temperature. The absorption of the mixture was measured using UV-Vis spectrophotometer (Shimadzu UV-1900, Shimadzu Corporation, Kyoto, Japan) at a wavelength of 593 nm. The absorbance data were calculated against the serial concentration of the quercetin standard curve solution and recorded as % (w/w) quercetin equivalent antioxidant capacity (%w/w QEAC).

ABTS assay

The antioxidant capacity test using the ABTS method was carried out by spectrophotometry (Paşayeva et al., 2020; Yahaya et al., 2020). The ABTS•+ radicals were prepared by reacting a solution of ABTS (5.4 mM in 10 mL of distilled water) and a solution of potassium persulfate (5.6 mM in 10 mL of distilled water). The mixture was kept in the dark place for 12 hours at room temperature. After the incubation period, the mixture was diluted with ethanol to 50 mL. One mL of the ABTS•+ solution then diluted to 5 mL and obtained an absorbance of 0.746 ± 0.06 using UV-Vis Spectrophotometer (Shimadzu UV-1900, Shimadzu Corporation, Kyoto, Japan) at 752 nm. A volume (1 mL) of each sample solution at a serial concentration of ethanol extract (4–20 μg/mL), n-hexane fraction (100–500 μg/mL), ethyl acetate fraction (1–5 μg/mL) and ethanol fraction (4–20 μg/mL) as well as quercetin (1–5 μg/mL) as reference were added to 1 mL of ABTS•+ solution. The mixture was adjusted to a volume of 5 mL with ethanol p.a. and incubated for 30 minutes in a dark place at room temperature. The inhibitory effect was calculated based on the percentage of the color of the ABTS•+ solution that faded by the control sample (ABTS•+ solution only). The effect of extract and fractions to scavenge the ABTS•+ were calculated with equation [2].

$$ABTS^{•+}\ scavenge\ (\%) = [(A_0 - A_1)/A_0) \times 100] \qquad [2]$$

Where A_0 was the absorbance of the negative control (ABTS•+ solution only) and A_1 was the absorbance of the sample. The IC_{50} value was represented based on the sample concentration necessary to reduce the ABTS•+ to 50%, a value obtained from the linear regression graph.

Total phenolic content

The total phenolic determination was performed using the Folin-Ciocalteu method with slight modifications (Singleton et al., 1999). An aliquot of 100 μL of each sample extract solution in ethanol and added with 500 μL of Folin-Ciocalteu

reagent. Then the mixture was added with 2 mL of 7.5% sodium carbonate solution and let stand for 3 minutes. Furthermore, the volume of the mixture was adjusted to 5 mL with distilled water. Incubated for 30 minutes, and the absorbance was measured with a UV-Vis spectrophotometer (Shimadzu UV-1900, Shimadzu Corporation, Kyoto, Japan) at 778 nm. Gallic acid concentration series was used as the standard curve. Total phenolic was calculated using the standard curve equation for gallic acid, and the total phenolic content of each sample was equivalent to the% (w/w) of gallic acid (%w/w GA).

Anti-elastase and anti-collagenase evaluation

Determination of the inhibitory activity of the elastase enzyme by using human neutrophil elastase was carried out as a skin degradation enzyme that causes skin aging when reacting with the substrate (MeOSuc-Ala-Ala-Pro-Val-pNA). The elastase test was performed following the Product Manual of the Drug Discovery Kit, Enzo Life Science (Manual, Neutrophil Elastase Colorimetric, BML-AK497-0001). For the elastase inhibition test, 20 μL of each sample extract and fractions were diluted with 65 μL of buffer solution (10 mM HEPES, 50 mM NaCl, and 0.05% Tween 20 in DMSO) in 96-well microplates. Elastatinal (100 μM) was used as an inhibitory control, 95 μL of the buffer as a blank, and 85 μL of the buffer as a negative control. Neutrophil elastase enzyme (2.2 μU/μL) as much as 10 μL was added to the sample solution, negative control solution, and control inhibitor (blank with no added enzymes). It was incubated for 10 minutes at 37°C (Memmert BM400 incubator, Memmert GmbH+Co.KG, Southern Germany) and then 5 μL of the substrate (MeOSuc-Ala-Ala-Pro-Val-pNA, 100 μM) was added in each well, and the absorption was measured by a microplate reader (Grating microplate reader SH-1000Lab, Corona Electric Co., Ltd, Tokyo, Japan) at 405 nm, observed for 10 minutes at 1-minute intervals. Activation of the elastase enzyme with the substrate will produce a yellow color.

For the collagenase inhibition test (Manual, MMP-1 Colorimetric Drug Discovery Kit, BML-

AK404-0001), a total of 20 μL of each sample extract and fractions solution were added with 50 μL of buffer solution (50 mM HEPES, 10 mM CaCl$_2$, 0.05% Brij-35, and 1 mM DTNB in DMSO) into 96 well microplates (Sterile microplate 96 well KI171-0001 with a flat bottom, polystyrene, Enzo Life Science, USA). N-isobutyl-N-(4-methoxyphenyl-sulfonyl) glycylhydroxamic acid (NNGH 1.3 μM) was used as a control inhibitor for use as a comparison. A total of 90 μL of buffer solution was used as a blank, and 70 μL of buffer solution was used as a negative control. A volume of 20 μL of MMP-1 enzyme (153 mU/μL) was added to the well containing the sample, control inhibitor, and negative control (blank without added enzymes). Incubated for 30 minutes at 37°C, then each well was added with 10 μL of the substrate (thiopeptide, Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC$_2$H$_5$; 100 μM), and the absorption was measured by a microplate reader (Grating microplate reader SH-1000Lab, Corona Electric Co., Ltd, Tokyo, Japan) at 410 nM. Observations were made for 10 minutes with 1-minute intervals. The percentage of the remaining activity of the elastase and collagenase enzymes was calculated based on the equation in the product manual list (https://www.enzolifesciences.com). Enzyme inhibitory activity was evaluated based on the IC$_{50}$ value obtained from the plot between the concentrations of the sample solution against the enzyme activity (%).

Evaluation of normal cell proliferation

Normal cell proliferation was evaluated by the MTT method (González et al., 2017). This is a method that can be used to measure the percentage of cell viability based on the mitochondrial activity of the fibroblast cells (Bernas and Dobrucki, 2002). In this study, adult human dermal fibroblasts (HDFa), derived from normal adult skin cells that have a cellular response to aging on the skin, were used. HDFa cells obtained from Life Technologies Corporation (GIBCO, California, USA) were cultured on complete DMEM media. Cells that were confluent about >80% were considered harvested cells. For the proliferation test with the MTT assay, it was used with a concentration of

2×10^4 cells/wells. Cell suspension as much as 100 μL (2×10^4 cells/well), distributed into 96-well plates, then incubated 24 hours so that the cells adhere to the wells, and various series levels (62.5–1000 μg/mL) of each sample and quercetin as positive control were added in the culture medium. All samples tested were individually dissolved in DMSO prior to addition into the culture media. The wells were incubated for 24 hours in a 5% CO_2 incubator (Thermoscience 8000Dh series, Waltham, Massachusetts, USA) at 37°C. After the incubation period, the medium was discarded, and the cells were washed with 100 μL of PBS. A total of 100 μL H_2O_2 (1000 μM) was added to each well, then re-incubated for 2 hours in a 5% CO_2 incubator at 37°C. After that, the medium was discarded, and the cells were washed with PBS 100 μL and then added to each well 100 μL MTT reagent (5 mg/mL in PBS) and re-incubated for 4 hours in a 5% CO_2 incubator, at 37°C. Living cells will react with MTT to form purple formazan salts. The MTT reaction was stopped with a stopper reagent (SDS 10% in 0.01 N HCl), then incubated overnight at room temperature and in a dark place. On the following day, the absorption was read with a microplate reader at 595 nm. The results of the viability study of HDFa cells were represented by calculating the IC_{90} value. The IC_{90} value is intended to determine the population of HDFa cells that live after exposure to H_2O_2. The high IC_{90} value indicates that the high concentration of the sample solution can still maintain the cell population up to 90%.

Statistical analysis

Statistical analysis was performed by SPSS 23.0 version (IBM, New York, USA). Research data are represented as mean ± standard deviation (SD) determined from the results of three replications in each test. Comparisons were made using two-sample t-test analysis. The results differed significantly when the p-value was less than 0.05 ($p<0.05$).

RESULTS AND DISCUSSION

Antioxidant capacity testing of extracts and fractions from *M. calabura* was carried out using three methods with different mechanisms, namely DPPH and ABTS radical reduction and iron reduction (FRAP). The three tests were carried out to determine the antioxidant capacity based on the mechanism and the predicted chemical content in a sample. According to Huang et al. (2010), based on the reaction mechanism, the antioxidant capacity test can be classified into two mechanisms, namely (1) a mechanism based on the hydrogen atom transfer (HAT) reaction, and (2) a mechanism based on the single electron transfer (ET) reaction, where those three aforementioned tests are representing these two, HAT and ET mechanism. The antioxidant capacity of a sample was expressed by the IC_{50} value. The IC_{50} value obtained from the linear regression equation was expressed in the relationship between the concentration of the test sample and the percentage of antioxidant activity. The smaller the IC_{50} value of the sample solution, the stronger the antioxidant activity. On the contrary, the greater the IC_{50} value indicates that, the lower the antioxidant activity. The results of testing for antioxidant capacity can be seen in Table 1.

Testing with DPPH is based on reducing radical compounds that are purple to yellow due to the transfer of hydrogen atoms by antioxidant compounds so that DPPH radicals become stable (Floegel et al., 2011; Wootton-Beard and Ryan, 2011). The data in Table 1 indicate that EAF exhibited high antioxidant capacity when compared to other extract samples. The order of antioxidant capacity in the test sample from the highest to the lowest was EAF and followed by EF, EE, and HF, respectively. Blois (1958) stated that antioxidant compounds are categorized as very strong if the IC_{50} value is less than 50 μg/mL, strong antioxidants have an IC_{50} value between 50-100 μg/mL, moderate antioxidants have an IC_{50} value between 100-150 μg/mL and weak antioxidant more than

150 µg/mL. Based on the level of antioxidant power, the EAF, EF and EE from *M. calabura* are very strong category. When compared with positive control, the IC_{50} value of EAF was not significantly different from quercetin (p>0.05).

Similar results were obtained in testing for antioxidants in reducing ABTS radicals. From the test results of *M. calabura* extract and fractions in reducing ABTS radicals, it can be seen that EE, EAF, and EF have very strong antioxidant capacity because they have IC_{50} values <50 µg/mL. The greatest ABTS radical scavenging capacity was found in the EAF sample. The amount of antioxidant capacity of the *M. calabura* sample is thought to be due to the high content of chemical compounds such as flavonoids and phenolics (Pereira et al., 2018).

The antioxidant capacity of the extracts and fractions of *M. calabura* was also used for testing to determine the antioxidant capacity based on the iron ion reduction method. The ability of the sample solution can be seen through the Fenton reaction mechanism by chelating metal ions such as Fe^{2+} ions, which are responsible for converting hydrogen peroxide into hydroxyl radicals that occur on the skin so that testing with the iron ion

reduction method (FRAP) can be used (Abdul Karim et al., 2014). According to Hodzic et al. (2009), testing with the FRAP method was used to determine simple and very fast antioxidant activity and a very reproducible reaction and its linear relationship to the molar concentration of antioxidants. The higher the FRAP value of the sample, the greater the antioxidant capacity because the FRAP value is based on the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), which antioxidant compounds are reducing agents. Antioxidant compounds are able to donate an electron or hydrogen atom during reduction (Apak et al., 2007; Rabeta and Nur Faraniza, 2013).

The reducing power of *M. calabura* extracts and fractions were expressed in quercetin equivalent antioxidant capacity (QEAC) that is antioxidant capacity expressed as mM Fe^{3+} reduced to Fe^{2+} equivalent to quercetin in 1 gram of sample. The sample data of QEAC value were obtained by calculating the absorbance of the sample against the linear regression equation for the quercetin standard curve. Based on the data, Table 1 shows that EAF provides the highest QEAC value compared to others, so this indicates that QEAC has antioxidant power in reducing iron.

Table 1. Antioxidant capacity of extract and fractions of *M. calabura*.

Sample	IC_{50} value (µg/mL)		FRAP assay (mM QEAC/g)
	DPPH assay	ABTS assay	
EE	20.61 ± 0.98[a]	11.17 ± 0.27[a]	1.89 ± 0.06
HF	573.74 ± 1.52[a]	282.97 ± 1.12[a]	0.71 ± 0.03
EAF	7.57 ± 0.76[b]	2.83 ± 0.53[b]	16.89 ± 0.07
EF	14.51 ± 1.02[a]	4.66 ± 0.21[a]	1.82 ± 0.12
PC	4.60 ± 0.59	1.66 ± 0.09	-

The data are expressed as mean ± SD with triplicate (n = 3). [a]p<0.05 statistically significant differences compared with positive control. [b]p>0.05 not significantly different compared with positive control. Quercetin was used as a positive control (PC). The IC_{50} was determined by plotting the percentage of inhibition against the concentration of each sample solution. In the FRAP assay, the antioxidant activities of samples were determined by quercetin equivalent antioxidant capacity (QEAC). QEAC of the sample was obtained by calculating the absorbance of the sample against the concentration series (6.62, 13.24, 19.86, 26.48, 33.1, and 39.72 µM) of the quercetin standard solution and recorded as the equivalent of µmol/g of the sample obtained from the results of the calibration curve with the equation y = 0.0151x + 0.1389 with correlation coefficient r^2=0.998. EE: ethanol extract; HF: n-hexane fraction; EAF: ethyl acetate fraction; EF: ethanol fraction.
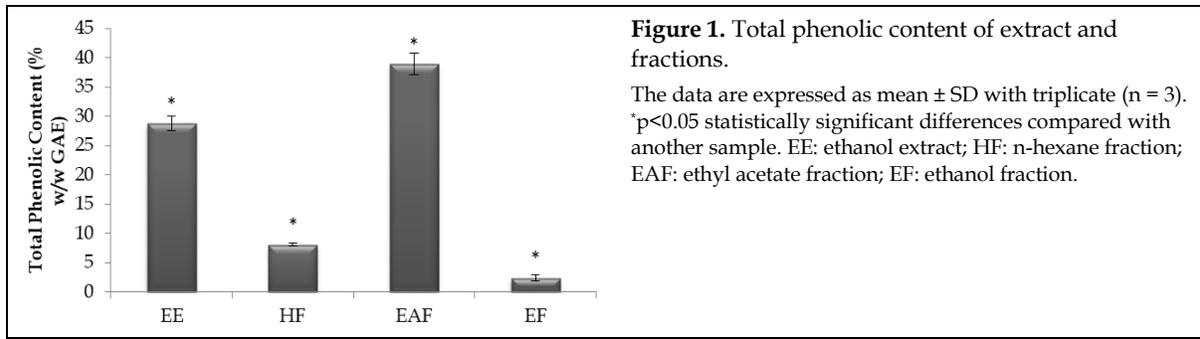
Figure 1. Total phenolic content of extract and fractions.

The data are expressed as mean ± SD with triplicate (n = 3). *p<0.05 statistically significant differences compared with another sample. EE: ethanol extract; HF: n-hexane fraction; EAF: ethyl acetate fraction; EF: ethanol fraction.
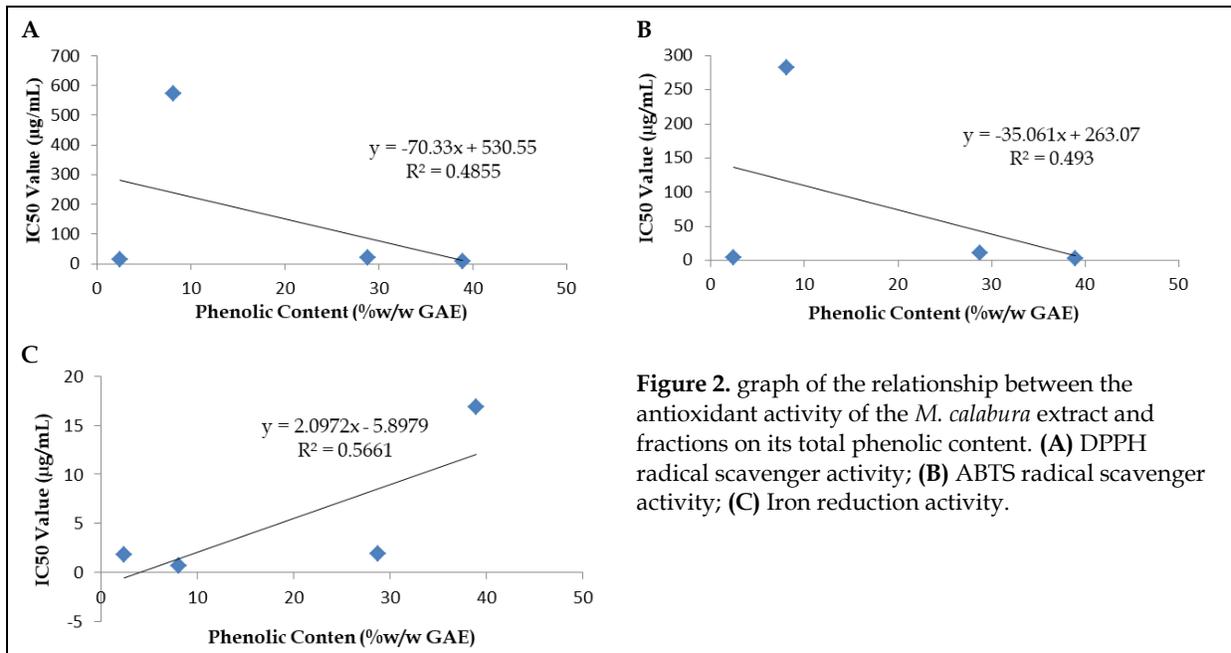

Figure 2. graph of the relationship between the antioxidant activity of the *M. calabura* extract and fractions on its total phenolic content. **(A)** DPPH radical scavenger activity; **(B)** ABTS radical scavenger activity; **(C)** Iron reduction activity.

Pereira et al. (2018) informed that *M. calabura* contains polyphenol compounds such as gallocatechin, epigallocatechin, catechin, flavanols, naringenin, quercetin, gallic acid. The reaction with phenolic compounds will donate electrons (H^+) to the TPTZ-Fe^{3+} complex reduced to TPTZ-Fe^{2+}. The resulting reaction is marked by a color change from colorless to Pers's Prussian Blue color. Antioxidant compounds that have donated an electron will become a radical compound, but they are more stable because of their ability to resonate (Gordon, 1990).

Total phenolic levels were calculated based on the linear regression equation of the standard curve for gallic acid (concentrations 2, 4, 6, 8, and 10 μg/mL). The correlation between gallic acid concentration and its absorbance were expressed in the equation y = 0.1017x + 0.0336 with a correlation coefficient value (r = 0.9998). From the results of these calculations, the highest phenolic content was found in the ethyl acetate fraction of 38.94 ± 1.88 % (w/w GAE).

The difference in antioxidant activity in an extract or fractions is due to differences in the content of compounds such as polyphenols. The presence of large amounts of polyphenol compounds can have an effect on antioxidant capacity (Fig. 1). The large content of antioxidant compounds such as polyphenol in the extract or fractions causes high free radical scavenging activity (Cox et al., 2003). The correlation between the phenolic content of each extract and its antioxidant capacity can be seen in Fig. 2.

Based on Fig. 2, the relationship between total phenolic levels and IC_{50} values in reducing DPPH, ABTS and iron reduction from extracts and fractions were obtained by the equation y = -70.33x + 530.55 (R^2 = 0.4855), y = -35.061x + 263.07 (R^2 = 0.493), and y = 2.0972x-5.8979 (R^2 = 0.5661), respectively. These results indicate that the correlation coefficient is 0.4855 implied that 48.55% of DPPH radical scavenging capacity is the result of the contribution of phenolic compounds. Similar results also occurred in the relationship between the total phenolic content and the IC_{50} value of antioxidant capacity in ABTS radical scavenger and iron reduction from each sample. The correlation coefficients were 0.493 and 0.5661, suggesting that 49.3% and 56.61% of antioxidant capacity were influenced by the presence of phenolic compounds, respectively. From these results, it can be stated that the antioxidant capacity of *M. calabura* extracts and fractions is not only influenced by phenolic compounds but may be influenced by other compounds such as alkaloids, vitamins, carotenoids, and lignans (Mistriyani et al., 2018).

The antioxidant capacity possessed by *M. calabura* has the potential to be developed as an antiaging. Therefore, our research also carried out the development of the activity of *M. calabura* extracts and fractions as antiaging.

Determination of antiaging activity is based on the inhibitory activity of skin-degrading enzymes that are anti-elastase and anti-collagenase enzymes. The data of anti-elastase and collagenase of extract and fractions of *M. calabura* can be seen in Tables 2 and 3.

Therefore, the compounds contained in the extract and fractions of *M. calabura* are expected to be able to inhibit the activity of the elastase enzyme by looking at the visualization of the color formed after incubation and the kinetics of the enzyme activity every minute. In this study, elastatinal, as a control inhibitor, was also used as a standard to determine the suitability of the measurement and analysis method used. In addition, it was done by measuring the blank (without enzymes and inhibitors) and negative control (without inhibitors). Blanks aim to avoid the occurrence of false-positive results at the time of measurement, while negative control was to see the activation of the enzyme that observed kinetic every minute on the micro-plate reader at a wavelength of 405 nm (Abdul Karim et al., 2014).

The data in Table 2 shows that the EE, EAF, and EF gave almost the same inhibitory activity with IC_{50} values of 37.08, 33.53, and 32.06 µg/mL, respectively.

Table 2. Inhibition activities of extract and fractions of *M. calabura* against HNE.

Sample	% Inhibition at concentration (µg/mL)			IC_{50} value (µg/mL)
	100	250	500	
EE	61.54 ± 2.81[ab]	73.08 ± 1.92[b]	81.54 ± 1.88[ab]	37.08
HF	27.69 ± 0.98[ab]	32.31 ± 1.80[ab]	33.85 ± 2.90[ab]	>500
EAF	65.39 ± 0.72[ab]	68.47 ± 1.62[ab]	85.39 ± 0.59[ab]	33.53
EF	60.50 ± 1.16[ab]	65.08 ± 1.72[ab]	79.24 ± 1.18[a]	32.06
Elastatinal	71.54 ± 1.05[b]			-
PC	77.69 ± 1.92[a]			-

Inhibitor control of elastatinal and positive control of quercetin with a concentration of 100 µM was used. The data are expressed as mean ± SD with triplicate (n=3). [a]p<0.05 statistically significant differences compared within the elastatinal concentration. [b]p<0.05 statistically significant differences compared within quercetin concentration. Almost all concentrations of each sample solution gave percent inhibition results that present statistically significant differences with respect to the control. HNE: human neutrophil elastase; EE: ethanol extract; HF: n-hexane fraction; EAF: ethyl acetate fraction; EF: ethanol fraction; PC: positive control.

Table 3. Inhibition activities of extract and fractions of *M. calabura* against MMP-1.

Sample	% Inhibition at concentration (µg/mL)			IC$_{50}$ value (µg/mL)
	50	100	200	
EE	34.91 ± 1.09[ab]	42.34 ± 1.72[ab]	49.11 ± 1.82[ab]	203.47
HF	16.99 ± 1.92[ab]	25.51 ± 1.26[ab]	32.85 ± 2.01[ab]	363.16
EAF	38.46 ± 0.97[ab]	51.48 ± 1.06[ab]	55.66 ± 1.69[ab]	98.40
EF	31.95 ± 1.28[ab]	44.97 ± 1.11[ab]	49.10 ± 1.91[ab]	169.34
NNGH	83.43 ± 1.47[b]			-
PC		62.71 ± 1.79[a]		-

Inhibitor control of NNGH with a concentration of 1.3 µM and positive control of quercetin with a concentration of 100 µM were used. The data are expressed as mean ± SD with triplicate (n=3). [a]$p<0.05$ statistically significant differences compared within NNGH concentration. [b]$p<0.05$ statistically significant differences compared within quercetin concentration. Almost all have results that differ significantly from their respective sample concentrations ($p<0.05$). EE: ethanol extract; HF: n-hexane fraction; EAF: ethyl acetate fraction; EF: ethanol fraction; NNGH: N-isobutyl-N-(4-methoxyphenylsulfonyl) glycylhydroxamic acid as inhibitor control; PC: positive control.

The data in Table 2 shows that low concentrations of 100 µg/mL can inhibit the activity of the elastase enzyme above 50%, while the HF fraction provides low activity in inhibiting the action of the elastase. The activity of the EE, EAF, and EF showed similar inhibition to the control inhibitor and positive control at a concentration of 100 µM. Quercetin was used as a positive control. This is one of the flavonoid derivatives having the activity in inhibiting elastase in modulating the degradation of fibrous elastin found in the dermal matrix (Sahasrabudhe and Deodhar, 2010).

Moreover, polyphenols in a sample will interact with the amino acids of the enzyme such as serine, histamine, and aspartate residues through hydrogen bonds, causing a decrease in catalytic activity and denaturation of the enzyme (Brás et al., 2010; Mardhiyah et al., 2020). The presence of flavonoid polyphenols in the extract and fractions of the *M. calabura* allows for a role in providing anti-elastase activity.

Determination of anti-collagenase activity was carried out by the same method as anti-elastase, but the enzymes used are matrix metalloproteinases-1 (MMP-1) or collagenase, which these enzymes can reduce the aggregate of collagen and elastin molecules (Ghimeray et al., 2015). MMP-1 was used in this study because the enzyme recognizes the substrate through a hemopexin-like domain mechanism and is able to degrade fibrillar

collagen tissue. The degradation causes wrinkle or aging of the skin. Therefore, it is hoped that the compounds contained in the extract and fractions of *M. calabura* fruit can inhibit the work of the enzyme to degrade skin collagen tissue (Pittayapruek et al., 2016).

The data in Table 3 states that the EAF has a strong activity in inhibiting the activity of the collagenase with an IC$_{50}$ value of 98.4 µg/mL. The data in Table 3 also indicate that the EAF at the highest concentration (200 µg/mL) displayed inhibitory activity similar to that of the positive control but not for NNGH. The presence of similar results from the EAF with positive control allows the contribution of flavonoid group polyphenol compounds that can inhibit the activity of collagenase from preventing the wrinkle in the skin layer (Landau, 2007).

In this study, antiaging activity testing was also carried out by involving the presence of fibroblast cells, which aims to determine the molecular phenomenon involved in the aging process. Testing of cell viability of *M. calabura* extract and fractions intend to describe the number of living cells. The data in Table 4 shows cell viability of extract and fractions of *M. calabura* against HDFa cells.

In this study, HDFa, derived from normal adult skin cells that have a cellular response to aging on the skin, were used. Analysis of cell viability was carried out by treating the presence of H$_2$O$_2$ expo-

Table 4. Cell viability (%) of extract and fractions of *M. calabura* against HDFa.

Sample	% Cell viability at concentration (µg/mL)					EC_{90} (µg/mL)
	62.5	125	250	500	1000	
Control	100					
H_2O_2	44.77[a]					
EE	160.92 ± 5.98[ab]	166.54 ± 4.84[ab]	160.43 ± 0.52[ab]	122.70 ± 4.78[ab]	39.53 ± 8.49[a]	673.76
HF	4.65 ± 1.92[ab]	5.01 ± 0.70[ab]	4.13 ± 1.24[ab]	6.91 ± 2.17[ab]	10.42 ± 1.05[ab]	>1000
EAF	136.07 ± 6.46[ab]	144.08 ± 4.57[ab]	159.50 ± 1.38[ab]	117.11 ± 9.69[ab]	55.02 ± 2.52[ab]	715.70
EF	164.15 ± 1.48[ab]	169.35 ± 3.36[ab]	165.71 ± 1.75[ab]	127.83 ± 9.23[ab]	46.03 ± 11.1	717.39
PC	100.36 ± 0.70	112.38 ± 2.79	138.91 ± 4.03	167.8 ± 5.07	198.61 ± 2.7	133.01

The positive control was quercetin with serial concentrations. The data are expressed as mean ± SD (n=3). [a]$p<0.05$ statistically significant differences compared within quercetin concentration. [b]$p<0.05$ statistically significant differences compared within H_2O_2 (1000 µM). HDFa: adult human dermal fibroblast cell; EE: ethanol extract; HF: n-hexane fraction; EAF: ethyl acetate fraction; EF: ethanol fraction PC: positive control.

sure. Cell viability with H_2O_2 exposure aims to determine the ability of the sample to maintain or increase the viability of cells that have been treated with H_2O_2. This occurs because H_2O_2 can cause oxidative damage and will manifest in the premature aging of human skin (Makrantonaki and Zouboulis, 2007; Giampieri et al., 2014; Halliwell and Gutteridge, 2015). Based on the results, Table 4 shows that 1000 µM H_2O_2 concentration is the optimum concentration could reduce the cell viability to below 50%. Therefore, H_2O_2 with a concentration of 1000 µM was used as an effective concentration for exposure to post-treatment HDFa cells with sample extracts and fractions. Similar results were also obtained in another research (Giampieri et al., 2014) in which showed that H_2O_2 at concentrations of 500-1000 µM was able to reduce the percentage of cell viability compared to controls.

The data in Table 4 shows a trend of increasing cell viability that occurs in all samples along with increasing concentrations, although there is a decrease in the highest concentration of 1000 µg/mL. This is contrary to the results in the positive control, where the higher the concentration, the greater the cell viability that occurred. This is possible because many compounds in the extract or fractions in high concentrations could have a toxic effect on cells. The data in Table 4 shows that the EAF presented the highest EC_{90} value, followed by

the EF and EE. This shows that the highest concentration of the sample solution can maintain the cell population up to 90%.

Sample of EAF, EF, and EE almost have a cytoprotective effect on cells at concentrations below 500 µg/mL despite H_2O_2 exposure. In fact, these results show that there is a proliferation of cells after being treated with samples, in comparison with negative control (H_2O_2 exposure without the sample solution treatment) and HF having decreased the population below 50%.

CONCLUSIONS

This study provides an overview of scientific data for the development of *M. calabura* fruit as antiaging. From the results of this study, it can be concluded that the ethyl acetate fraction of *M. calabura* exhibits the best antioxidant, anti-elastase, anti-collagenase, and cell viability activities so that it shows the potential to be developed as an active ingredient for an antiaging candidate.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Nur S	Angelina AA	Aswad M	Yulianti R	Burhan A	Nursamsiar N
Concepts or ideas	x		x	x	x	x
Design	x				x	
Definition of intellectual content	x		x	x		x
Literature search	x		x	x	x	x
Experimental studies	x	x		x		x
Data acquisition	x		x			x
Data analysis	x	x	x	x	x	x
Statistical analysis		x			x	
Manuscript preparation	x	x	x	x	x	x
Manuscript editing	x		x	x	x	x
Manuscript review	x	x	x	x	x	x