Antioxidant and Anti-aging Activity of Parijoto Fruit (*Medinilla Speciosa*, Reinw.ex Bl.) Ethanolic Extract In Vitro

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Abstract. The Human skin can experience physiological changes with age, resulting in skin aging. There are several types of factors that cause skin aging, such as intrinsic and extrinsic factors. Parijoto fruit (*Medinilla speciosa*, Reinw.ex Bl) contains polyphenolic compounds that can improve the balance of ROS (Reactive Oxygen Species) with a mechanism as a free radical inhibitor. Avoid symbols and abbreviations in the abstract. This study aims to determine the biological activity of Medinilla speciosa, Reinw.ex Bl fruit as an anti-aging active ingredient in vitro. The parijoto fruit powder was macerated using 70% ethanol followed by phytochemicals screening. Antioxidant activity was tested using DPPH method (quercetin as a positive control). The in vitro anti-aging activity was tested using MTT Assay on Human Dermal Fibroblast adult (HDFa), meanwhile the elastase enzyme inhibition test was conducted using Human Neutrophil Elastase (HNE). The results showed that parijoto fruits extract contained saponins, tannins, and flavonoids. The activity of parijoto fruits extract with an IC_{50} value of 122.19 g/mL was categorized as a medium antioxidant. Parijoto fruit extract was proven to inhibit elastase enzyme activity with a percentage of inhibition at 50 µg/mL, 100 µg/mL, and 200 µg/mL concentrations is 80%, 80%, and 60%, respectively. The HDFa cell viability test showed that ethanol extract of parijoto fruit could affect maintained HDFa cell viability against exposure to H_2O_2 at a concentration of 31.25;250;500;1000 µg/mL. Based on the results, the ethanolic extract of parijoto fruit possesses anti-aging activity.

Keywords: Anti-aging, Antioxidant, Elastase enzyme, Fibroblast cell, Parijoto (*Medinilla speciosa*).

1 Introduction

Skin is one of the largest organs in the human body and consists of tissue that has a specific function as a protector and covering that keeps other body organs together. Like other organ systems, human skin can undergo physiological changes as we age, resulting in skin aging [1]. The skin aging process consists of factors, namely intrinsic

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and extrinsic. Intrinsic aging is likely caused by genetic control of the individual that occurs as a natural consequence of physiological aging. Intrinsic factors are a combination of three processes, including a decrease in the viability of skin cells, a reduction in the synthesis of the skin's extracellular matrix, and an increase in enzyme activity. On the other hand, conditions such as exposure to UV rays, especially UVB, chemical poisons, and cigarette smoke are causes of extrinsic aging (photoaging) [2]; [3].

The activity of the elastase enzyme can cause wrinkles on the skin and loss of skin elasticity which are signs of aging [4]. Increased Reactive Oxygen Species (ROS) will result in collagenase and elastase activity, which play a role in skin aging [5]. Elastin is the main element of elastic connective tissue, which plays a role in maintaining skin elasticity caused by sunlight, especially UV B, which can cause photoaging.

The use of natural ingredients, especially medicinal plants, tends to increase now. Medicinal plants processed as traditional medicines since the community have been widely used in ancient times. However, there is still little research regarding the use of medicinal plants as antiaging. With the increasing effects of skin damage caused by factors that trigger aging, it is necessary to carry out preventive strategies and develop therapies. One of the developments carried out is by utilizing natural extracts. The mechanism by which natural ingredient extracts protect the skin can occur in several ways by reducing ROS's reactivity. Much research has been carried out on active compounds derived from plants, and several plants are known to have collagenase and anti-elastase activity. The anti-collagenase and anti-elastase activities are caused by various phenolic compounds, such as flavonoids originating from these plants [6].

In a previous study, the IC50 value of parijoto fruit antioxidant activity was 48.24 µg/ml [7]. In another study, it was 44.75 µg/ml [8]. Parijoto fruit (Medinilla speciosa) contains secondary metabolites such as saponins, glycosides, flavonoids and tannins and has antioxidant activity [7]. This is the basis for carrying out this research on parijoto fruit (Medinilla speciosa, Reinw.ex.Bl.) through the mechanism of in vitro inhibition of the elastase enzyme and HDFa cell proliferation as well as to determine antioxidant activity through an action mechanism based on DPPH radical scavenging.

2 Materials and Method

2.1 Instrumens & Material

Instrumens. The instrumens used in this research were Analytical scale (Sartorius®), Alat-alat Gelas (Pyrex®), Lamiran Air Flow Cabinet (Labconco®), Alumunium Foil (Diamond®), Hemositometer (Nebauer®), Sentrifuge (Sorvall®), Spektrofotometer UV-Vis (Shimadzu®), Elisa Reader (Bio-Rad Benchmark®) dan Corona Electic Type SH-1000), Cuvet (Disposible®), Pipet Mikro (Socorex®), Rotary Evaporator (IKA RV10), Vortex (Shimadzu®), Sinar Lampu UV254 dan UV 366, Oven (Memmert®),

Mikroskop Inverted (Zeiss®), Waterbath (Memmert®), Chamber (CAMAG®), Inkubator (Heraeus®), Plate 96 Well (Pyrex®), Pipa Kapiler (Brand®), Plat KLT Silica Gel F254S (Merck®).

Material. The main ingredient used in this research was parijoto fruit (Medinilla speciosa, Reinw.ex Bl.), obtained from Kudus Regency, Central Java, were determined with No. 195/Lab.Bio/B/VII/2020. Meanwhile, supporting materials include Ethanol 70% (General Labora/Technical Grade), Medium Kultur *Dulbecco's Modiefied Eagle Medium* (DMEM), *Fetal Bovine Serum* (FBS), *Dimetylsulfoxide* (DMSO), Hidrogen Peroksida (H_2O_2), SDS 10%, HCl p.a (Merck®), Enzim Porcine Pancreas Elastase (Sigma®), Metanol p.a.

2.2 Material Preparation

The parijoto fruit (Medinilla speciosa, Reinw.ex Bl.) that was obtained was then wet sorted and macerated with a ratio of Simplicia powder to 10% ethanol (1:10). This maceration process is carried out for 2-3 days. The maceration is carried out at room temperature and protected from direct sunlight with several stirrings to optimise the results. The macerate formed is evaporated using a rotary evaporator and water bath at 60°C until a thick extract is obtained [9].

2.3 Phytochemical Screening

Saponin Test. 1 drop of 2 N HCl was added to 1 gram of extract, which had been dissolved in hot water. Samples contain saponin compounds if foam forms and lasts five minutes or more [10]

Alkaloid Test. Fifty grams of the extract was dissolved in several ml of HCl. Then, the extract solution was tested by adding two drops of Dragendorff's reagent. A positive reaction will be indicated by the formation of an orange precipitate in the Dragendorff reagent and the figure of a red-black color in the Wager reagent [11].

Tannin Test. The tannin test was carried out by dissolving 0.1 grams of extract in 10 ml of distilled water and filtering it. Then 5 ml of 1% iron (III) chloride (FeCl3) was added to the filtrate. A positive reaction is indicated by the formation of a dark blue or black color [12].

Steroid Test. A total of 2 ml of ethanol extract was added to 2 ml of n-hexane and then shaken. The n-hexane layer was added with Liebermann-Burchard reagent. A positive reaction is indicated by a color change to greenish blue [13].

Flavonoid Test with 10%NaOH. A total of 1 ml of parijoto fruit ethanol extract was put into a test tube, and then two drops of 10% sodium hydroxide (NaOH) were put into the test tube and shaken. A positive solution contains flavonoids if it experiences an extreme color change to yellow, red, or brown [14].

2.4 Antioxidant Activity Test using the DPPH Method

Testing antioxidant activity begins by preparing 2,2 diphenyl-2-picryhydrazyl (DPPH) with slight modifications [16]. The DPPH testing step was carried out by preparing a 0.4mM DPPH solution and making a series of sample concentrations of 1;3;5;7, and 9 µg/ml. In this test, it was made with a concentration series of 10 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml. The activity test was carried out by taking 2 ml each of ethanolic extract of parijoto fruit (EEPF) and quercetin solution of each level. Then, 2 ml of 0.4 mM DPPH and 6 ml of methanol were added, homogenized using a vortex and left for operating time in a closed room. The absorbance of the sample is read at the maximum DPPH wavelength, and then the IC_{50} value is calculated [17]. Absorbance was measured at a wavelength of 514.5 nm after incubation for 30 minutes at room temperature. The inhibitory effect was calculated based on the percentage of color fading of the DPPH solution to yellowish by the sample compared to the control (only DPPH solution). The IC_{50} value is represented based on the sample concentration required to reduce 50 DPPH with the value obtained from the linear regression graph.

2.5 Elastase Enzyme Inhibition Test

This test was carried out by following the product manual of the Neutrophil Elastase Inhibitor Screening Kit procedure based on the Sigma Aldirch procedure manual. In the elastase inhibition test, 25 µL of sample solution was diluted with 48 µL buffer solution (10 mM HEPES, 50 mM NaCl and 0.05% Tween 20 in DMO) in a 96-well micropipette. Elastatinal (25µL) was used as an inhibitor control, 50µL buffer solution as a blank, and 25µL enzyme solution as an enzyme control. Neutrophil elastase enzyme (2.2µL) was added to the sample solution, 2µL enzyme control solution and control inhibitor (blanks did not add enzyme). It was incubated for 10 minutes at 37°C, and then 5µL of the substrate (MeOSuc-Ala-Ala-Pro-Val-pNA, 100µM) was added to each well. The absorbance was measured at a wavelength of 405nm and observed for 10 minutes at each 1-minute interval [18].

2.6 Fibroblast Cell Viability Test

Parijoto fruit extract solution with concentration series 31; 25; 62.5; 125; 250; 500; Fibroblast Cell Viability Test µg/ml and 1000 µM H2O2. HDFa cells 2 x 104 cells/100 µl well, distributed into 96-well plates, incubated for 24 hours. Added 100 µl of sample was incubated for 24 hours in a 5% CO2 incubator, temperature 370C. Then, the medium was discarded, and the cells were washed with 100µl PBS. Add 100 µl of H_2O_2 and incubate for 2 hours. Added 100 µl of 5 mg/ml MTT reagent in PBS and incubated for 4 hours. The reaction was stopped with a stopper reagent (10% SDS in 0.01 N HCl),

and incubated for 24 hours at room temperature covered with aluminum foil. Absorbance was read at a wavelength of 595 nm. Tests were also carried out without H_2O_2 similarly [19].

3 Results and Discussion

3.1 Phytochemical Screening

The phytochemical examination is a qualitative test to determine what is contained in the ethanolic extract of parijoto fruit (EEPF). This study tested several compounds: alkaloids, tannins, saponins and steroids. EEPF is thought to contain secondary metabolite compounds saponin and tannin, which can be seen changing color when treated in Table.1

Table 1. Phytochemical Screening

Compounds	Result
Alkaloid	Negative (-)
Tannin	Positive (+)
Saponin	Positive (+)
Steroid	Negative (-)
Flavonoid	Positive (+)

3.2 Antioxidant Activity Test using the DPPH Method

The antioxidant capacity of EEPF and quercetin is determined by the activity of DPPH in counteracting free radicals. This method is based on the formation of non-radical DPPH H due to the addition of hydrogen from antioxidants, which is characterized by the reduction of the DPPH alcohol solution. Antioxidant molecules counteract DPPH free radicals, represented by a purple change from the DPPH sample to colorless. The (IC_{50}) value can be seen in (Table 3.) As shown in Table 2, (EEPF) has an IC_{50} value of 122.19 µg/ml and quercetin as an equation has a value of 6.49 µg/ml. The smaller the IC_{50}, the stronger the antioxidant power. The IC_{50} value of quercetin is higher than the IC_{50} value (EEPF). This shows that the antioxidant of quercetin is stronger than the antioxidant of the ethanol extract of parijoto fruit [16].

Table 2. IC_{50} of Antioxidant

Sample	Linear Regression Equations	R^2	IC_{50} (µg/mL)	Statement
Quercetin	y = 4.1136x + 23.291	$R^2 = 0.9123$	6.49	Very Strong
Ethanolic extract of parijoto fruit	y = 0.3293x + 9.7624	$R^2 = 0.9962$	122.19	Medium

3.3 Elastase Enzyme Inhibition Test

The measurement of the inhibitory activity of the Human Neutrophil Elastase enzyme was used as a skin-degrading enzyme. This is the main cause of skin aging when it reacts with the substrate (MeO-Suc-Ala-Ala-Pro-Val-MCA). The test used three series of EEBP concentrations, namely 50, 100 and 200 µg/mL. Inhibitory activity was observed by measuring absorbance every minute within 10 minutes with a 405 nm Elisa reader. The results are plotted against time to obtain a slope value, as shown in Figure 1. Antiaging activity is expressed as a percentage of enzyme inhibition compared to the control. Absorbance data is plotted against time every minute. The results of % enzyme inhibition in EEBF and control can be seen in Table 3.

Table 3. % Inhibition of control elastase enzymes and EEBF with 3 different concentrations

Treatment	% Enzime Inhibition	Slope	Deviation Standard
Enzim Control	90 %	0.0005	0.002
50 µg/mL	80%	0.0001	0.000
100 µg/mL	80%	0.0001	0.001
200 µg/mL	60%	0.0002	0.000

Based on the graph of % Inhibition of the enzyme produced, it can be seen that at concentrations of 50 µg/mL and 100 µg/mL, it is 80%, then at a high concentration of 200 µg/mL, it drops to 60%. These results show that the more controlled the EEBF sample results are, the greater the possibility of the presence of elastin-inhibiting enzyme activity. The same thing happened in the sample solution; EEBF can inhibit the action of the elastase enzyme, which also obtains kinetics. Based on these results, it shows that EEBF works well. It is said to be affected because it is based on the profile of the compounds in EEBF whose anti-elastase activity is similar at each concentration, but at high concentrations, the inhibition is reduced. This activity results from the content of secondary metabolites such as flavanol-type flavonoids, including myricetin, quercetin and kaempferol, which act as inhibitors of elastase depending on van der Waals (vdW) interactions and hydrogen bonds between the enzyme and the inhibitor, making it a phenol with a large number of potential interactions, including ring aromatics for van der Waals (vdW) interactions and hydroxyl groups for hydrogen bonds, are more likely to inhibit elastase better. In these structural properties, the galloyl group, the degree of polymerization in procyanidin, and the structural hydroxyl ion have an essential role in the inhibitory activity of phenol against elastase [20]. However, the presence of glycosidic moieties in the phenolic structure may interfere with the inhibitory interaction with the enzyme due to steric hindrance. This suggests flavonoid aglycones may relate more to the extract's elastase inhibitory capacity than the respective glycoside derivatives [20]; [21].

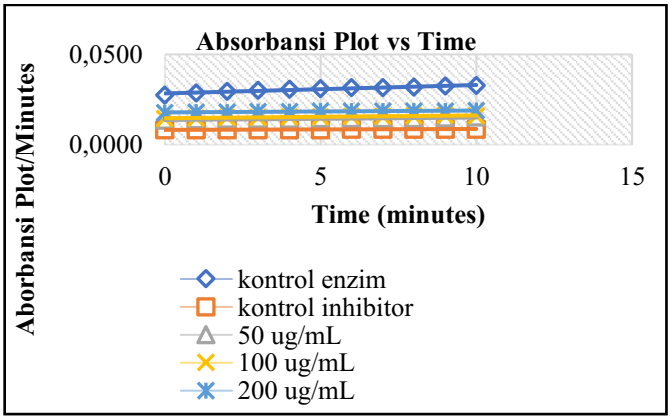

Fig. 1. Kinetic profiles of enzyme inhibition in controls and samples

3.4 Activity Test on HDFa Cell Viability

The HDFa EEBF cell viability test was carried out in vitro to describe the number of viable cells using the MTT {3-(4,5-dimethylthiazol-2-yl) 2,5diphenyltetrazolium bromide} method using Elisa with a wavelength of 595 nm. This test was carried out with two treatments, namely with exposure to H_2O_2 and without exposure to H_2O_2. Cell viability without exposure is intended to see the effect of the sample in accelerating cell viability so that it can be used as an anti-inflammatory for aging. Within this time, exposure to H_2O_2 is to determine the ability to maintain or increase the viability of samples from cells treated with H_2O_2. This happens because H_2O_2 is singlet oxygen, which induces oxidative damage and premature aging in human skin, whereas H_2O_2 is a source of oxidative stress due to an increase in cytotoxic ROS, and UVA rays cause most ROS formation. UV exposure represents an energetic photon that passes through the layers of the skin, and color-carrying molecules or photoreceptors are absorbed, thereby causing biological effects [22]; [23]; [24].

Table 4. Cell viability with H_2O_2 exposure

Sample Concentration (µg/mL)	**Absorbance**	**Cell Viability %**	**SD**
H_2O_2 Control	0.458	61.42	2.27
1000	0.415	89.44	5.83
500	0.333	69.04	7.58
250	0.329	68.09	3.79
125	0.324	66.64	2.39
62.5	0.307	62.40	1.66
31.25	0.325	66.95	2.26
Average	Cell Control	0.458	0.00
Absorbance	Media Control	0.055	0.00

Table 5. Cell viability without H_2O_2 exposure

Sample Concentration (µg/mL)	Absorbance	Cell Viability %	SD
Cell Control	0.246	100	0.00
1000	0.168	60.16	3.79
500	0.122	37.78	1.15
250	0.185	68.54	5.89
125	0.245	97.81	0.86
62.5	0.242	96.61	3.43
31.25	0.234	92.75	8.16
Average	Cell Control	0.246	0.00
Absorbance	Media Control	0.045	0.00

Table 5 shows the viability of HDFa cells from ethanol extract of parijoto fruit without exposure to H_2O_2 compared to control cells. Based on this graph, it can be seen that the treatment group (without exposure to H_2O_2) was at a concentration of 125; 250; 500 and 1000 µg/mL and had significant differences compared to control cells. Gimpieri et al. (2014) reported that H_2O_2 at a concentration of 500 µM to 1000 µM could reduce the percentage of cell viability compared to the witness. Therefore, in this study, optimize the color range from 100 µM to 1000 µM to obtain the optimal concentration that can reduce the viability of cells exposed for 2 hours [24]. Based on the research results, an H_2O_2 concentration of 1000 µM is the optimal concentration, which can reduce cell viability to less than 50%. So H_2O_2 with an attention of 1000 µM was used as an effective concentration for exposure to HDFa cells post-treatment with parijoto fruit extract samples (Medinilla speciosa, Reinw.ex Bl.).

Based on the viability test result, each sample concentration gave different results. The antioxidant effect gives a protective effect on the cells. HDFa can block peroxide radicals that are harmful to cells. So, it can be concluded that the ethanol extract of parijoto fruit has anti-aging activity by protecting FGF_2 levels in human skin and can maintain various functions that support skin rejuvenation. Proteoglycans through mechanisms that maintain FGF_2 levels in the skin by mimicking the protective effects of heparan sulfate. By preventing spontaneous degradation of FGF_2, parijoto fruit extract maintains the bioavailability of this growth factor to its target cells, skin fibroblasts.

In the HDFa viability test, cell morphology was also observed using an inverted microscope. In HDFa cells, the cell shape changes from elongated to wrinkled and becomes irregular Fig. 2 [a]. HDFa cell's morphology changes slightly from elliptical to circular, reducing their size Fig. 2[b].

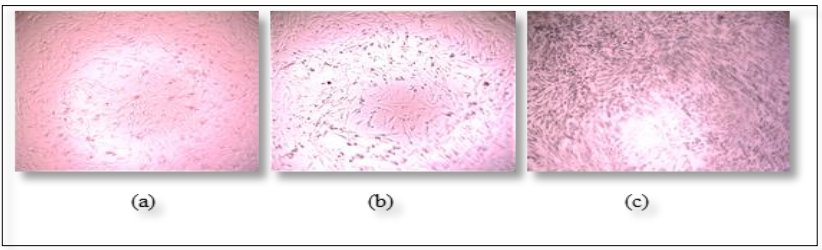

Fig. 2. Changes in the morphology of HDFa cells during treatment with EEBP [a] before treatment [b] immediately after treatment [c] after treatment and incubation and addition of MTT reagent

Living cells ( ); Dead cells ( )

4 Conclusion

The results obtained in the research can be concluded that parijoto fruit contains alkaloids, saponins and flavonoids. Based on the IC_{50} value, parijoto fruit has medium-category antioxidant activity. However, parijoto fruit has good activity in the elastase enzyme inhibition test. Based on these data, the parijoto fruit extract sample has the potential as an active antiaging ingredient.

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