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Full Length Article

In Vitro Study of Anti-Elastase and Antioxidant Activities on Mabolo *(Diospyros blancoi*) Leaf and Stem Bark Extracts

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Abstract

The mabolo plant (*Diospyros blancoi* A. DC.) has anti-aging potential. This research aimed to investigate the anti-elastase and antioxidant properties of mabolo leaf extract (LE) and stem bark extract (SBE). Using the ultrasound-assisted extraction method, the method used started with the extraction of mabolo plant parts of leaves and stem bark with methanol solvent. Yields and phytochemical screens were determined for each extract and tested for Total Phenolics Content (TPC), Total Flavonoid Content (TFC), anti-elastase and antioxidant [2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-azinobis (3-ethylbenzothiazo-line-6-sulfonic acid) diammonium (ABTS) free radical scavenging methods and ferric reducing power (FRAP) method] activities. According to the findings, LE and SBE have strong anti-elastase and antioxidant activities with 50% inhibitory concentration (IC_{50}) values below 50 μg/mL. LE and SBE had anti-elastase IC_{50} values of 41.06 and 26.52 μg/mL, respectively. The IC_{50} value for DPPH and ABTS in LE were 4.34 and 2.95 μg/mL, whereas in SBE they were 3.73 and 1.96 μg/mL. LE and SBE had FRAP values of 6308.61 and 6835.29 μmol/g, respectively. In conclusion, the findings of this investigation revealed that LE and SBE exhibited strong anti-elastase and antioxidant properties. Furthermore, SBE demonstrated stronger anti-elastase and antioxidant activity than LE. As a result of these findings, the mabolo plant has the potential to be developed as an anti-aging agent.

Keywords: *Diospyros blancoi*; Anti-elastase; Antioxidant; Mabolo; Anti-aging

Introduction

Aging affects all cells, tissues and organs, causing homeostasis to be disrupted and vulnerability to increase, including the skin. Skin aging occurs due to an imbalance between the degradation and production of the dermal matrix in maintaining a healthy skin structure. This imbalance triggers changes in the skin structure that generally occur with age (intrinsic factors). In addition, excessive exposure to ultraviolet radiation by sunlight (extrinsic factors) can also accelerate the skin's aging process (Naylor *et al.* 2011; Shanbhag *et al.* 2015; Sakamoto *et al.* 2017; Nur *et al.* 2022).

In skin aging, both intrinsic and extrinsic factors cause the generation of reactive oxygen species (ROS). Excessive production of ROS results in a cumulative effect of oxidative stress, which might modify the increase in elastase enzyme activity. Elastase enzymes are known to degrade elastin fibers that play an important role in maintaining skin elasticity. This causes changes in the skin structure, which are recognized as indications of skin aging such as wrinkles, sagging, and rough skin texture. Therefore, there is a need for agents with potential as anti-elastase and antioxidant to fight skin aging (anti-aging) (Mukherjee *et al.* 2011; Roy and Sahu 2013; Shanbhag *et al.* 2015).

One plant that could be developed as an anti-aging plant by inhibiting the skin aging process is mabolo (*Diospyros blancoi* A. DC.) which is also known as bisbul or butter fruit (Lim 2012; GBIF 2022; POWO 2023). Studies on mabolo leaf parts have shown to have anti-aging activity (Lee *et al.* 2009). Similarly, antioxidant activity has been reported from the leaves, stem bark, fruit peel and seeds of mabolo (Lee *et al.* 2006; Lim 2012; Khan *et al.* 2016; Setu *et al.* 2017; Saha *et al.* 2018; Arrisujaya *et al.* 2020).

Studies of anti-elastase and antioxidant activities of the same genus (*Diospyros kaki*) have been reported previously (An *et al.* 2005; Kashif *et al.* 2017; Rauf *et al.* 2017). Although the antioxidant activity of mabolo has been reported previously, its anti-elastase activity has never been

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reported. Therefore, further research is needed to explore the anti-elastase (anti-aging) and antioxidant activities of the mabolo plant, especially from its leaves and stem bark.

Materials and Methods

Materials

Preparation and extraction: The samples used were the leaves and stem bark of the mabolo plant (*D. blancoi*) plant, obtained from the collection of the Research Center for Plant Conservation and Botanical Gardens - Indonesian Institute of Sciences (No. B-3990/IPH.3/KS/XI/2019). A total of 25 g of leaf and stem bark simplicia powder were extracted using the ultrasonic-assisted extraction (UAE) method with a QSONICA Sonicator - Q2000 with direct probe type immersed in methanol solvent medium at room temperature, at a frequency of 20 kHz, extraction time of 15 min and sample-solvent ratio = 1:10 (g/mL). After that, the extraction was repeated three times with a different solvent and a sample-solvent ratio of 1:5 (g/mL). To obtain LE and SBE, the extraction results were filtered and evaporated using a rotary evaporator. Furthermore, the yield calculation was carried out by comparing the weight of the extract with the weight of the initial simplicia. TPC, TFC, anti-elastase activity and antioxidant activity were all determined in LE and SBE.

Phytochemical screening: Each LE and SBE mabolo was subjected to phytochemical screening based on procedures listed in the Indonesian Pharmacopeia (4th edition) and Harborne as has been done in previous studies (Radjah *et al.* 2021). Phytochemical compounds identified included saponins, tannins, terpenoids, glycosides, anthraquinones, alkaloids and flavonoids.

Total phenolics content (TPC) assay

TPC testing used Folin Ciocalteau reagent (FCR) with modified method of Radjah *et al.* (2021). A total of 75 μL of methanol extract sample (1000 μg/mL) was combined with 0.5 mL of FCR solution (1:10) into the vial. Then it was shaken for 1 min and let stand for 3 min. Following that, 1 mL of 7.5% (w/v) sodium carbonate solution was added. Next, aquadest was added up to 5 mL, mixed for 1 min and incubated in a dark environment at room temperature for 1 h. Finally, the solution was measured at 671 nm using a Uv-Vis spectrophotometer. The gallic acid standard curve (3-7 μg/mL) was used as reference. The testing was carried out three times. The findings were reported in milligrams of gallic acid equivalent per gram of extract.

Total flavonoid content (TFC) assay

TFC testing utilized modified procedure of Radjah *et al.* (2021). Each extract sample (1000 μg/mL) of LE and SBE was taken as much as 1.0 mL and 3.5 mL, respectively then added 0.1 mL of 10% (w/v) aluminum chloride and allowed to stand for 3 min. Then added 0.1 mL of 1 M sodium acetate and added methanol up to 5 mL. After 1 min of shaking, the mixture was incubated at room temperature for 30 min. A Uv-Vis spectrophotometer was then used to measure absorbance at 441 nm. A blank solution was created by adding a volume of sample and up to 5 mL of methanol. TFC were quantified using a standard curve of quercetin (2–6 g/mL) and results were expressed in milligrams of quercetin equivalents per gram of extract.

Anti-elastase assay

Anti-elastase testing was performed by colorimetric method using a microplate reader based on Sigma-Aldrich protocol modified from previous studies and under with the optimization conditions obtained (Desmiaty and Elya 2021). Samples/positive controls were prepared in various concentrations from the stock solution (1000 μg/mL). The assay was prepared by preincubating 30 μL of sample/positive control solution, 125 μL of Tris-HCl buffer solution pH 8.0 and 15 μL of Porcine Pancreas Elastase (PPE) enzyme solution 0.22 U/mL in a 96-well microplate for 20 min at 25°C. The wells were then filled with 30 μL of 1.3 m*M* N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA) substrate solution. The samples were then incubated at 25°C for 50 min before being quantified at 405 nm with a microplate reader (Promega GloMax® Discover, Madison, USA). Quercetin was used as a positive control. Each sample was tested three times, and the percent inhibition formula was used to calculate the anti-elastase activity:

$$\%\ Inhibition = \frac{A_0 - A_1}{A_0} \times 100\ \%$$

A_0 = Absorbance of blank corrected by blank control
A_1 = Absorbance of sample corrected by sample control.

Based on the percentage inhibition curve of anti-elastase enzyme activity from the concentration series of sample solutions and positive controls, the IC_{50} value was calculated. The log of sample concentration series on the X axis and the percent inhibition on the Y axis was then graphed until a linear regression equation y = a + bx was found. The formula below was used to calculate the IC_{50} value.

$$IC_{50} = \frac{50 - a}{b}$$

Antioxidant assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,20-azinobis (3-ethylbenzothiazo-line-6-sulfonic acid) diammonium (ABTS) free radical scavenging methods, as well as the ferric reducing antioxidant power (FRAP) method, were used to investigate antioxidant activity. Each 20 mg of LE and SBE was dissolved in 20 mL of methanol to obtain a stock solution with a concentration of 1000 μg/mL. Meanwhile, 2.5 mg of positive control (ascorbic acid) was dissolved in 5 mL of methanol to obtain a stock solution with a concentration of 500 μg/mL.

DPPH assay

Antioxidant activity was measured using the DPPH free radical scavenging technique, with minor modifications (Bobo-García *et al.* 2015; Wiliantari *et al.* 2022). Stock solutions of LE, SBE, and positive control were made into concentration series by sampling 10, 15, 20, 25 and 30 μL, respectively. After that, 1 mL of DPPH was added to each mixture, then methanol was added up to 5 mL. The sample was then incubated for 30 min in the dark at room temperature. The sample's absorbance was then measured at 515 nm using a Uv-Vis spectrophotometer. Each technique was carried out three times.

ABTS assay

Antioxidant activity assay used ABTS free radical scavenging method with modification (Tungjai *et al.* 2018; Wiliantari *et al.* 2022). Before usage, an ABTS radical stock solution was produced and stored in the dark for 12-16 h. Then, a certain amount of ABTS radical stock solution was taken and mixed with 95% ethanol to obtain a ratio of 1:15 for use as a test solution. Each stock solution of LE and SBE was diluted to a concentration of 100 μg/mL. Then a concentration series was made by sampling 50, 75, 100, 125 and 150 μL for LE samples, while 25, 40, 55, 70, and 85 μL were sampled for SBE samples. Meanwhile, the positive control concentration series was made by sampling 10, 15, 20, 25 and 30 μL from the stock solution. Next, each mixture was added with 1 mL of ABTS (1:15), then ethanol was added up to 5 mL. Finally, the sample was incubated for 30 min at room temperature in a dark environment. The sample's absorbance was then measured at 752 nm using a Uv-Vis spectrophotometer. Every procedure was carried out three times.

IC_{50} antioxidant

The 50% inhibitory concentration (IC_{50}) of an antioxidant is the concentration of the sample that can reduce the DPPH and ABTS radical by 50% (Arianti *et al.* 2020). The formula for calculating percent inhibition based on the DPPH and ABTS radical scavenging capabilities of each concentration of sample solution was:

$$\%\ \text{Inhibition} = \frac{(A - B)}{\text{A}} \text{x} 100\%$$

Where A = blank absorbance and B = sample absorbance.

After computing the percentage of inhibition for each concentration, the equation y = a + bx is determined by generating a linear regression curve with x equalling the concentration (μg/mL) and y equalling the percentage of inhibition (%). The following formula is used to get the IC_{50} value:

$$IC_{50} = \frac{50 - a}{b}$$

FRAP assay

Antioxidant activity assay by FRAP method followed the existing procedure of Arianti *et al.* (2020) with modification. A total of 25 μL was sampled from the LE and SBE stock solutions, while 50 μL was sampled from the positive control stock solution. Next, 1 mL of FRAP II reagent was added to each mixture, and then distilled water was added up to 5 mL. The sample was then incubated in the dark for 30 min at 37°C. The sample's absorbance was then measured with a Uv-Vis spectrophotometer at 596 nm. The experiment was repeated three times. The calibration curve was made from the plot of concentration (x) in µM vs. absorbance (y) of iron (II) sulfate heptahydrate standard series solution to obtain a linear regression equation y = bx + a. The FRAP value was determined using the following equation:

$$C = \frac{\Delta A596\ \text{nm} - a}{b}$$

$$\text{FRAP value (µmol FeEAC/g)} = \frac{C \text{ x } V \text{ x } Fp}{m}$$

C = Sample concentration (µM)
V = Sample volume (mL)
Fp = Dilution factor
m = Sample weight (mg).

The FRAP value was expressed as µmol/g sample of the proportion of Fe^{3+} reduction to Fe^{2+} by the sample equivalent to $FeSO_4$.

Statistical analysis

The research data were represented as mean ± standard deviation (SD), which was determined by the results of three replications in each test.

Results

Extraction

The yield results of mabolo leaf and stem bark extracts using methanol solvent by UAE method are shown in Fig. 1. LE and SBE respectively obtained extract weights of 4.05 g and 2.67 g from 25 g simplicia. Then, the LE yield was obtained to reach 16.19%, while the SBE yield was 10.66%.

Phytochemical screening

Table 1 indicates the occurrence of various phytochemical compound classes in LE and SBE, including saponins, tannins, terpenoids, glycosides, anthraquinones, alkaloids, and flavonoids.

TPC and TFC assay

Determination of TPC of LE and SBE using gallic acid calibration curve from the equation y = 0.0901x + 0.112 (R^2 = 0.9993). TPC obtained from LE and SBE respectively

Table 1: Phytochemical screening of mabolo methanol extract

Chemical Compound	Result	
	LE	SBE
Saponins	+	+
Tannins	+	+
Terpenes	+	+
Glycosides	+	+
Anthraquinones	+	+
Alkaloids	+	+
Flavonoids	+	+

Table 2: TPC and TFC results of mabolo methanol extract

Sample	TPC (mg GAE/ g extract)	TFC (mg QE/ g extract)
LE	317.07 ± 6.43	1.96 ± 0.07
SBE	339.52 ± 6.14	0.68 ± 0.03

Table 3: Anti-elastase assay results of mabolo methanol extract

Sample	Concentration (µg/mL)	Log concentration (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Quercetin	2.34	0.37	34.79 ± 1.09	5.30
	4.69	0.67	49.91 ± 2.52	
	9.38	0.97	57.54 ± 0.58	
	18.75	1.27	74.07 ± 5.45	
	37.50	1.57	84.77 ± 1.30	
LE	4.69	0.67	14.77 ± 1.02	41.06
	9.38	0.97	24.98 ± 0.74	
	18.75	1.27	36.45 ± 0.65	
	37.50	1.57	47.16 ± 0.33	
	75.00	1.88	61.39 ± 1.62	
SBE	4.69	0.67	21.38 ± 1.02	26.52
	9.38	0.97	31.01 ± 0.96	
	18.75	1.27	41.63 ± 0.75	
	37.50	1.57	57.03 ± 0.39	
	75.00	1.88	69.25 ± 0.42	

Table 4: DPPH assay results of mabolo methanol extract

Sample	Concentration (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Ascorbic acid	1.00	22.93 ± 1.94	2.68
	1.50	30.52 ± 2.59	
	2.00	39.00 ± 0.78	
	2.50	47.78 ± 0.82	
	3.00	54.90 ± 0.45	
LE	2.00	26.36 ± 0.57	4.34
	3.00	35.80 ± 1.18	
	4.00	47.10 ± 0.55	
	5.00	56.36 ± 0.63	
	6.00	67.17 ± 0.82	
SBE	2.00	28.90 ± 0.57	3.73
	3.00	42.02 ± 0.43	
	4.00	54.20 ± 0.36	
	5.00	65.34 ± 0.50	
	6.00	75.17 ± 0.54	

amounted to 317.07 ± 6.43 and 339.52 ± 6.14 mg QE/g extract (Table 2). The calculation of TFC test results was determined using the quercetin standard with the equation y = 0.1354x - 0.0156 (R^2 = 0.9991). The TFC of LE was obtained at 1.96 ± 0.07 mg QE/g extract and SBE at 0.68 ± 0.03 mg QE/g extract. The TPC result of SBE was higher than LE, but the TFC of SBE was lower than LE.

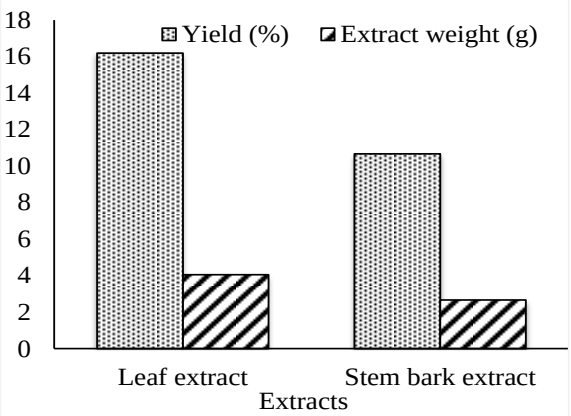

Fig. 1: Yield of mabolo methanol extract

Anti-elastase activity

The regression equations of the anti-elastase activity of LE, SBE and positive control were obtained from the comparison between percent elastase inhibition and log concentration. The regression equations were y = 38.338x - 11.856 (R^2 = 0.9964), y = 40.782x - 8.0555 (R^2 = 0.9913), and y = 41.229x + 20.143 (R^2 = 0.9909), respectively. As a positive control, quercetin showed the highest percent inhibition with an IC_{50} value of 5.30 μg/mL, followed by SBE (IC_{50} = 26.52 μg/mL), then the lowest LE (IC_{50} = 41.06 μg/mL) as shown in Table 3.

Antioxidant activities

The linear regression equations of the percent inhibition of DPPH inhibition to the concentration of LE, SBE and ascorbic acid were obtained including y = 10.219x + 5.6817 (R^2 = 0.9992), y = 11.586x + 6.78 (R^2 = 0.9969) and y = 16.24x + 6.5473 (R^2 = 0.9989), respectively. SBE has a higher DPPH radical scavenging activity with an IC_{50} of 3.73 μg/mL compared to LE of 4.34 μg/mL (Table 4). However, the DPPH radical scavenging activity by LE and SBE obtained in this study was lower than that of ascorbic acid (IC_{50} = 2.68 μg/mL).

The ABTS free radical scavenging test results were presented in Table 5. The linear regression equations of LE, SBE and ascorbic acid obtained were y = 13.407x + 10.484 (R^2 = 0.9982), y = 24.365x + 2.1938 (R^2 = 0.9971) and y = 23.262x + 5.3814 (R^2 = 0.9984), respectively. LE, SBE and ascorbic acid had IC_{50} values of 2.95, 1.96, and 1.92 μg/mL, respectively. SBE had higher ABTS free radical scavenging activity than LE.

The calculation of FRAP test results was obtained from the equation of the calibration curve of iron (II) sulfate heptahydrate (14.39, 21.58, 28.77, 35.97 and 43.16 μM), which was y = 0.0204x + 0.0158 (R^2 = 0.9998). The FRAP values of LE, SBE and ascorbic acid were 6308.61±70.92, 6835.29±161.39 and 11492,16±51,88 μmol/g, respectively as shown in Fig. 2. SBE had a higher

Table 5: ABTS assay results of mabolo methanol extract

Sample	Concentration (µg/mL)	Inhibition (%)	IC_{50} (µg/mL)
Ascorbic acid	1.00	28.07 ± 0.34	1.92
	1.50	40.70 ± 0.30	
	2.00	51.94 ± 0.57	
	2.50	64.49 ± 0.34	
	3.00	74.33 ± 0.46	
LE	1.00	23.65 ± 0.55	2.95
	1.50	30.53 ± 0.85	
	2.00	37.61 ± 0.32	
	2.50	44.57 ± 1.04	
	3.00	50.14 ± 1.02	
SBE	0.80	21.71 ± 0.26	1.96
	1.10	29.63 ± 0.38	
	1.40	35.55 ± 0.39	
	1.70	43.12 ± 0.52	
	2.00	51.51 ± 0.87	

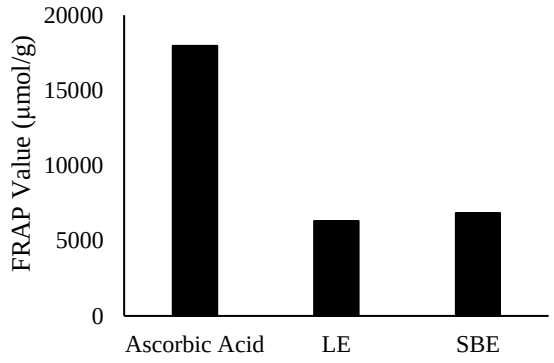

Fig. 2: Ferric reduction power result of mabolo extracts by FRAP assay

FRAP value than LE, indicating that the higher the FRAP value, the greater the potential antioxidant activity of a sample.

Discussion

LE had a greater yield (16.19%) than SBE (10.66%). Another study of *D. villosa* species, extraction using methanol solvent obtained similar results where the yield of leaf extract (10.8%) was higher than that of stem bark extract (7.2%) (Adu *et al.* 2023). However, the extraction results from *D. malabarica* species obtained the higher yield of methanol extract of stem bark (17.8%) than methanol extract of leaves (13.8%) (Zreen *et al.* 2022). This difference in results might be due to the presence of phytochemicals that vary from one species to another. In the phytochemical screening of LE and SBE mabolo, positive results were obtained for the group of phytochemicals. This is in line with other studies that screened mabolo leaf extracts (Demetillo *et al.* 2019). However, screening studies on mabolo bark extracts are still limited.

The anti-elastase and antioxidant activities of the three methods (DPPH, ABTS and FRAP) correlated with the phytochemical content present in LE and SBE. Several studies have suggested that a high TPC and TFC contents were associated with anti-elastase and antioxidant activity (Anggraini *et al.* 2020; Arianti *et al.* 2020). High anti-elastase activity has been documented due to the presence of phenolic compounds (such as catechins, epicatechins, resveratrol and procyanidin B2) and flavonoids (such as quercetin, kaempferol, and myricetin) in the extract or as single bioactive molecules. Furthermore, the presence of phenolic substances (carotenoids, flavonoids and polyphenols) is often responsible for plants' powerful antioxidant action (Hussin *et al.* 2019).

The ability of anti-elastase activity might be due to the phenolic compounds and flavonoids that could inhibit the action of the elastase enzyme. The hydroxyl group of phenolic and flavonoid substances bonds with the carboxyl group of the amino acid serine on the active site of the elastase enzyme, hence maintaining the elastin network (Nur *et al.* 2023). On the other hand, the antioxidant ability through DPPH free radical scavenging method through single electron transfer (SET) or hydrogen atom abstraction (HAT) mechanism, while ABTS free radical scavenging through the HAT mechanism and FRAP reducing power through SET mechanism. The antioxidant's ionization potential was essential in measuring antioxidant activity in the SET mechanism. Meanwhile, the bond dissociation enthalpy of the antioxidant was a crucial measure in measuring antioxidant activity in the HAT mechanism (Liang and Kitts 2014; Aisyah *et al.* 2022).

In this study, both LE and SBE mabolo extracts demonstrated strong anti-elastase and antioxidant activities with IC_{50} values less than 50 μg/mL. A lower IC_{50} value indicates a higher inhibitory activity (Phongpaichit *et al.* 2007; Arianti *et al.* 2020). The findings of this study also suggest that SBE has greater anti-elastase and antioxidant activity than LE. Other investigations have found that the antioxidant activity of an ethanol extract of mabolo stem bark (IC_{50} = 3.10 ± 0.06 μg /mL) is greater than that of an ethanol extract of its leaves (IC_{50} = 3.40 ± 0.08 μg /mL) (Khan *et al.* 2016).

Conclusion

Both LE and SBE have potent antioxidant and anti-elastase properties. Furthermore, SBE exhibits stronger anti-elastase and antioxidant activity than LE. The result revealed that the mabolo plant has the potential to be developed as an anti-aging agent.

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Author Contributions

All authors contributed equally to this work and have given permission to publish it.

Conflict of Interest

The authors state that they have no conflicts of interest.

Data Availability

The corresponding author will offer access to the data contained in this work upon reasonable request.

Ethics Approval

Outside the scope of this paper.

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