

In vitro antioxidant activity test and determination of phenolic and flavonoid content of *Moringa oleifera* pulp and seeds

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Abstract

Moringa oleifera is a plant that is empirically widely used as a medicine to treat hyperglycemia, anti-inflammatory, antidiabetic, antimicrobial, anticancer, antioxidant, gastric ulcer, skin disease, fever, fatigue, hysteria, thrush, bladder, and bronchitis. *Moringa oleifera* contains secondary metabolites such as flavonoids, tannins, terpenoids, alkaloids, and phenolics. This study aimed to investigate the antioxidant activity and levels of phenolic and flavonoids in the extracts and fractions of moringa pulp and seeds. The pulp and seeds of *Moringa* were extracted using the maceration method and fractionated using the liquid-liquid extraction method. Antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Phenolic content was measured using the Folin-Ciocalteu method. Meanwhile, the flavonoid content was determined using the $AlCl_3$ colorimetric method with UV-Vis spectrophotometry. The ethyl acetate fraction of moringa pulp and seeds had the strongest antioxidant activity with IC_{50} values of 5.97 $\mu g/mL$ and 5.08 $\mu g/mL$, respectively. Ethyl acetate fraction also has high phenolic and flavonoid content in the pulp and seeds of moringa with a phenolic content of 43.956 mg GAE/g sample for pulp and 9.429 mg GAE/g sample for moringa seeds. The flavonoid content of 94.257 mg QE/g sample for pulp and 93.719 mg QE/g sample, with the highest correlation between phenolic compounds and flavonoids on antioxidant activity, was obtained in moringa pulp with the highest correlation ($R^2 = 0.6514$) for phenolic and $R^2 = 0.7531$) for flavonoids. In conclusion, the ethyl acetate fraction of moringa pulp and seeds can be further developed as an antioxidant and functional food.

1. Introduction

Natural compounds, such as vitamins and phenolic compounds, are of concern to researchers as natural antioxidants are known to function as chemopreventive agents in fighting diseases. Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxy radical (OH) and hydrogen peroxide (H_2O_2) can cause damage to macromolecules, such as DNA, proteins, lipids, and small macrocellular. Free radicals have a role in the pathology of diseases such as cancer, atherosclerosis, ageing, cancer, hepatic disorders, neurodegenerative diseases, cardiovascular disease, kidney disease, and diabetes (Ekin *et al.*, 2017; Rohman *et al.*, 2020).

Antioxidants are molecules that can interact and inhibit the initiation or propagation of oxidation chain reactions which can cause damage to the cells of organisms or molecules (Sirivibulkovit *et al.*, 2018; Kokila *et al.*, 2020). Antioxidants can stop the chain reaction by stabilizing free radicals and blocking

oxidation with others by oxidizing themselves. Therefore, polyphenol compounds and flavonoids are often reducing factors (Kobus-Cisowska *et al.*, 2020). Antioxidants are known to play an important role in preventing diseases caused by oxidative stress such as cancer, heart disease, diabetes, stroke, rheumatoid arthritis, Alzheimer's disease, ageing, and cataracts (Lukitaningsih *et al.*, 2020). Antioxidants consist of synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ), propyl gallate, and natural antioxidants derived from plants (Rohman *et al.*, 2020; Kokila *et al.*, 2020). Thus, natural antioxidants sourced from natural ingredients are extensively explored as alternative sources of antioxidants (Lukitaningsih *et al.*, 2020).

Polyphenol compounds found in plants are the main secondary metabolites characterized by one or more hydroxyl groups attached to one or more aromatic rings. Several thousand polyphenol molecules found in higher

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plants have been identified, including edible plants. Polyphenols found in plants are divided into two major groups, namely flavonoids and non-flavonoids. Flavonoids are divided into flavanols, flavonols, anthocyanidins, flavones, flavanones, and chalcones. Non-flavonoids include stilbene, phenolic acids, saponins, and tannins (Lukitaningsih *et al.*, 2020; Stagos, 2020)

Most of the antioxidant compounds that come from plant sources have a wide variety and chemical properties. Its antioxidant properties are based on its ability to trap free radicals. *Moringa oleifera* (Moringaceae; Indonesian name: Kelor) is a vegetable plant native to tropical and subtropical regions and is commonly cultivated throughout Indonesia as a vegetable ingredient. All parts of this plant are reported to have various biological activities such as reducing hyperglycemia, anti-inflammatory, anti-diabetic, antimicrobial, anticancer, and antioxidant (Fitriana *et al.*, 2016). In addition, this plant is also known to have various important properties as medicine such as antiasthmatic, hepatoprotective, spasmolytic, bradycardia, hypotension, cholesterol-lowering, and hypoglycemic effects (Vyas *et al.*, 2015). *M. oleifera* has long been known in Ayurvedic medicine for the prevention and treatment of several diseases, such as gastric ulcers, skin diseases, fever, fatigue, hysteria, thrush, bladder, and bronchitis (Gimenis *et al.*, 2018; Lin *et al.*, 2018).

The strength of antioxidant activity *M. oleifera* plants is caused by the high content of phenolic and flavonoids found in plants which are the main compounds responsible for antioxidants (Sulastris *et al.*, 2018). The objectives of this study were to determine the total phenolic and flavonoid levels contained in the extract and fraction of moringa pulp and seeds and antioxidant activity *in vitro* using the DPPH method.

2. Materials and methods

2.1 Material

Moringa pulp and seeds are obtained from Palahidu Village, Binongko District, Wakatobi Regency, Southeast of Sulawesi Province, Indonesia. Methanol, ethyl acetate, n-hexane, DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (Sigma-Aldrich®), Folin-Ciocalteu reagent (Sigma-Aldrich®), aluminium chloride, gallic acid (Sigma-Aldrich®), and quercetin (Sigma-Aldrich®)

2.2 Extraction

A weight of 350 g of pulp and moringa seed pulp

was extracted using the maceration method, a cumulative three rounds for 24 hrs with stirring every 4 hrs. Then, every 24 hrs, it is filtered. The filtrate obtained was concentrated by means of a rotary evaporator at a temperature of 50°C to obtain the methanol extract. Later, the fractionation process using the liquid-liquid extraction method was carried out based on the polarity of the solvent. Approximately 20 g of methanol extract was dissolved in warm water, then fractionated with n-hexane solvent to obtain the n-hexane fraction. The residual water was fractionated again using ethyl acetate as a solvent to obtain ethyl acetate and water fractions. The n-hexane fraction, ethyl acetate fraction, and water fraction were concentrated using a rotary evaporator. The fractionation process is shown in Figure 1.

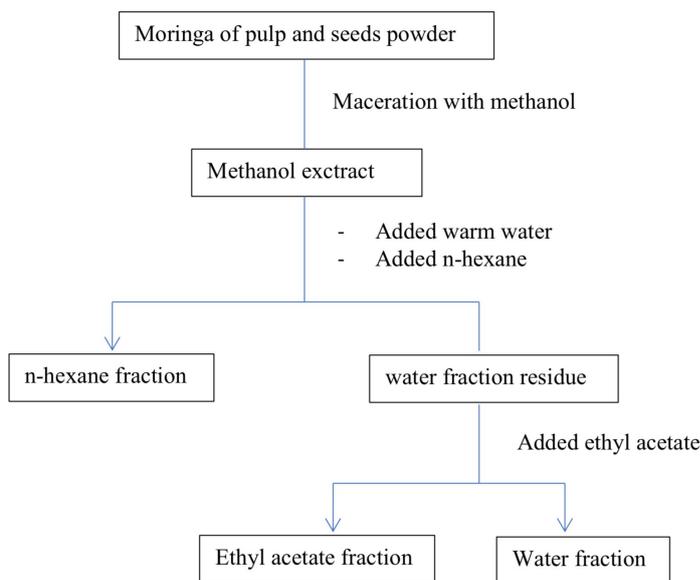


Figure 1. Schematic of extraction and fractionation of Moringa pulp and seeds

2.3 Phytochemical screening

Phytochemical screening is carried out by following the method used by Gul *et al.* (2017), Ngibad (2019) and Yamin *et al.* (2020)

2.4 Determination of total phenolic content

Determination of total phenolic content of moringa pulp and seeds was carried out using the Folin Ciocalteu reagent method as performed by John *et al.* (2014) with slight modification. Briefly, 1 mL of each extract and fraction is placed into a test tube 0.4 mL of Folin Ciocalteu reagent was added and left for 5-8 mins. The solution was added to 4 mL of 7% NO_2CO_3 and shaken until homogeneous. Then 10 mL of aquadest was added. It was then left for 2 hrs at room temperature. The absorbance was measured using UV-Vis spectrophotometry at 750 nm. Total phenolic content is expressed as mg of gallic acid equivalent (GAE)/g sample.

2.5 Determination of total phenolic content

Determination of the total flavonoid content was carried out using the colourimetric method by Vyas *et al.* (2015) and Yamin *et al.* (2020) Briefly, 1 mL of each sample was added to 3 mL of methanol p.a., and later 0.2 mL of 10% AlCl₃ and 0.2 mL of potassium acetate 1 M was added into 10 mL aquabidestilata. It was left for 30 mins at room temperature and the absorbance was obtained by UV-Vis spectrophotometry at 432 nm.

2.6 Determination of antioxidant activity using the DPPH method

Measurement of antioxidant activity using the DPPH radical scavenging was carried out following the method of Arina and Rohman (2013) and Yamin *et al.* (2020). A 10 µL of each test solution was placed into a test tube, where 2 mL of methanol and 2 mL of DPPH 0.6 mM solution were added. Then it was incubated in a dark room for 30 mins. The absorbance was measured at 517 nm. The inhibition ability of antioxidants was calculated based on the equation:

$$\% \text{ inhibition} = \frac{A_b - A_s}{A_b} \times 100\%$$

Where A_b = Absorbance blank and A_s = Absorbance of sample

The percentage inhibition was plotted with the concentration (µg/mL) to obtain the linear regression equation $y = bx + a$. The IC₅₀ value is obtained by replacing y with 50 and calculating the value for x. IC₅₀ is defined as the sample concentration required to inhibit 50% of the DPPH radical (Shahidi and Zhong, 2015)

2.7 Data analysis

All data were calculated using the MS Excel program using mean values.

3. Results and discussion

The extraction process was carried out through the maceration method. This is because maceration is a simple method using simple equipment, that does not require special skills, and is energy efficient. It is used to extract certain substances that are very insoluble in solvents and is ideal for use on substances that require

prolonged contact with the solvent. Also, this method is cheap (Rasul, 2018).

The results of phytochemical screening showed that moringa pulp and seeds contained metabolites of alkaloids, flavonoids, tannins, terpenoids, and saponins. As shown in Table 1, this is in line with the research conducted by Fowoyo and Oladoja (2015).

3.1 Determination of antioxidant activity using the DPPH method

DPPH has been widely used to measure the antioxidant ability of free radicals. The DPPH test is a stable, sensitive, fast and low-cost antioxidant method, whereby the hydrogen binding by the DPPH changes the radical solution that decolourizes from purple to yellow (Indradi *et al.*, 2017; Fatiha and Abdelkader, 2019; Hussain *et al.*, 2019; Chay *et al.*, 2020). The DPPH test is based on the ability of 2, 2-diphenyl-1-picrylhydrazyl free radicals to react with hydrogen donors. DPPH radicals exhibit an intense absorption spectrum in the ultraviolet region. DPPH reactions with antioxidants or reducing compounds produce DPPH-H (diphenyl hydrazine) (Lewoyehu and Amare, 2019).

Table 2 shows the strength of the antioxidant activity. The antioxidant activity that occurs was due to the performance of secondary metabolites contained in plant samples such as alkaloids, saponins, phenols, tannins, terpenoids, and flavonoids. These secondary metabolites play a role in scavenging DPPH radicals by reducing DPPH to 1,2-diphenyl-1-picrihidrazyne (DPPH -H). The change is seen when the DPPH solution changes from purple to yellow. This colour change indicates the potential for free radicals to capture antioxidant agents (Cane *et al.*, 2020).

Table 2. Category of strong antioxidant (Cane *et al.*, 2020)

Strength Category	
	<100 µg/mL (strong)
Activity antioxidant	101-150 µg/mL (moderate)
	151-200 µg/mL (weaker)

Table 3 shows the antioxidant activity of the extract and fraction of the pulp and seeds of Moringa with vitamin C as a comparison. Based on Table 3, the extract

Table 1. Results of the phytochemical screening of Moringa pulp and seeds

Test	Sample							
	Extract		Hexane Fraction		Ethyl Acetate Fraction		Water Fraction	
	Pulp	seed	pulp	seed	pulp	seed	pulp	seed
Alkaloid	+	+	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+	+	+
Tannin	+	+	+	+	+	+	+	+
Terpenoid	+	+	+	+	+	+	+	+
Saponin	+	+	+	+	+	+	+	+

and fraction of moringa flesh and seeds have relatively strong antioxidant activity. This is due to the IC₅₀ value <100 µg/mL. The data in Table 3, shows that the ethyl acetate fraction has a very strong antioxidant activity compared to the extract or fraction. Where the IC₅₀ values of the ethyl acetate fraction of moringa pulp and seeds were recorded at 5.97 µg/mL and 5.08 µg/mL, respectively.

Table 3. Antioxidant activity of Moringa pulp and seeds

Sample	Extract and fraction	IC ₅₀ (µg/mL)
Moringa pulp	Methanol extract	6.23
	Ethyl acetate fraction	5.97
	n-hexane fraction	7.06
	water fraction	7.42
Moringa seeds	Methanol extract	5.09
	Ethyl acetate fraction	5.08
	n-hexane fraction	5.79
	water fraction	8.82
Vitamin C		4.53

Similar results were found in which ethyl acetate fraction is stronger in antioxidant activity compared to other fractions from the ethyl acetate fraction of Ragu (*Dracontomelon dao*) bark (Yamin et al. 2020), ethyl acetate fraction of Rambutan (*Nephelium lappaceum* L.) peel (Mistriyani et al., 2018), and ethyl acetate fraction of *Oroxylum indicum* Linn bark (Trang et al., 2018). Thus, ethyl acetate fraction can be isolated to determine which compounds are responsible for antioxidants.

3.2 Determination of total phenolic and flavonoid content

Flavonoids and phenolic compounds are the most abundant secondary metabolites found in plants and have various biological activities. Scientifically, phenolic compounds have been shown to be compounds that have the most efficient natural antioxidant activity in stabilizing free radicals, and play an important role in the prevention of cardiovascular disease, ageing, and scavenging oxygen free radicals. (Phuriyakorn et al., 2019; Ndanusa et al., 2020). Flavonoid compounds are a group of natural metabolites and have a broad spectrum of chemical and biological properties. Besides being responsible as an antioxidant, flavonoid compounds are

also effective as anti-carcinogenic, anti-diabetic, and anti-arteriosclerosis (Mohd et al., 2020; Yasmin et al., 2020).

Table 4. Phenolic and flavonoid content of Moringa pulp

Sample	Content	
	Phenolic (mg GAE/g sample)	Flavonoid (mg QE/g sample)
Methanol extract	11.524	62.557
n-hexane fraction	4.4760	60.421
Ethyl acetate fraction	43.957	94.257
Water fraction	3.381	41.058

Table 5. Phenolic and flavonoid content of Moringa seeds

Sample	Content	
	Phenolic (mg GAE/g sample)	Flavonoid (mg QE/g sample)
Methanol extract	4.809	79.509
n-hexane fraction	3.524	73.901
Ethyl acetate fraction	9.429	93.719
Water fraction	1.143	64.708

Table 4 and Table 5 show the phenolic and flavonoid content of the extract and the fraction of moringa pulp and seeds. These data indicate that the ethyl acetate fraction has the highest phenolic and flavonoid levels compared to the extract and fraction of n-hexane and water fraction, both found in the pulp and seeds of moringa, with a phenolic content value of 43.957 mg GAE/g sample for pulp and 9.429 mg GAE/g sample for moringa seeds. The flavonoid content of 94.257 mg QE/g sample for pulp and 93.719 mg QE/g sample for moringa seeds.

The data showed that ethyl acetate fraction had higher DPPH free radical scavenging activity compared to methanol extract, n-hexane fraction, chloroform fraction, and water fraction. This is due to the higher phenolic and flavonoid concentrations in the ethyl acetate fraction. An increase in the concentration of phenol and flavonoid compounds in the sample will increase the value of radical scavenging activity (Tandoro et al., 2020).

When the correlation analysis was carried out, it showed that phytochemical compounds such as flavonoids and phenolic compounds contributed to the

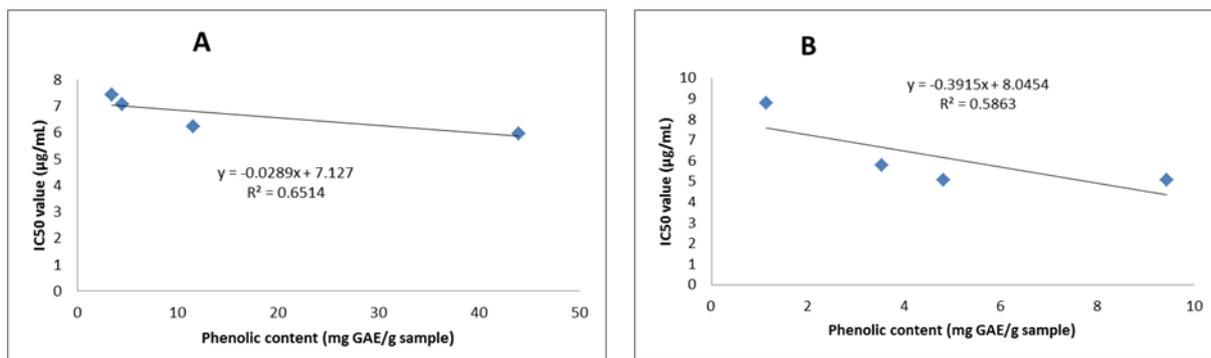


Figure 2. Correlation antioxidant activity and phenolic content of Moringa (A) pulp and (B) seeds

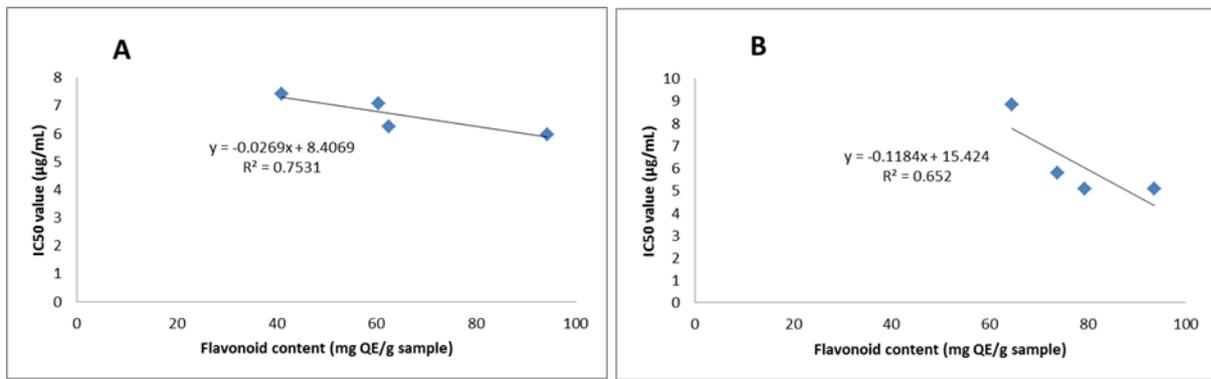


Figure 3. Correlation antioxidant activity and flavonoid content of Moringa (A) pulp and (B) seeds

antioxidant activity of the extracts and fractions of moringa pulp and seeds as shown in Figure 2 and Figure 3. Based on the results of the correlation analysis, it showed that the highest correlation between phenolic compounds and flavonoids on antioxidant activity was obtained in the flesh of moringa with the highest correlation ($R^2 = 0.6514$) for phenolic and $R^2 = 0.7531$ for flavonoids.

4. Conclusion

Extracts and fractions of moringa pulp and seeds have the strongest antioxidant activity. Ethyl acetate fraction had the strongest antioxidant activity and has high phenolic and flavonoid content in the pulp and seeds of moringa. The ethyl acetate fraction of moringa pulp and seeds can be further developed as an antioxidant and functional food.

Conflict of interest

The authors declare no conflict of interest.

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