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Antioxidant Activity Test of Red Spinach Leaf (*Amaranthus Tricolor* L.) Extract and Fraction with Methode (*2,2-DIPHENYL-1 PICRYLHYDRAZYL*)

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Abstract

Red spinach (Amaranthus tricolor L.*) is a local vegetable known to contain bioactive compounds such as flavonoids and phenolics that act as antioxidants. Previous studies have shown the antioxidant potential of crude extracts of this plant, but in-depth studies on antioxidant activity based on solvent fractionation with different polarities are still limited. In fact, fraction separation is important to identify the most potential active compounds in neutralizing free radicals. Therefore, this study aims to evaluate the antioxidant activity of extracts and fractions of red spinach leaves using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Extraction was done through cold maceration with 96% ethanol, followed by fractionation using n-hexane, ethyl acetate, and water. Phytochemical screening showed the presence of alkaloids, flavonoids, quinones, saponins, steroids, and tannins. DPPH test results showed that the ethanol extract had moderate antioxidant activity with an IC50 value of 122.09 µg/mL. The n-hexane and water fractions have similar activity with IC50 of 117.33 µg/mL and 124.36 µg/mL, respectively. The ethyl acetate fraction showed strong antioxidant activity with an IC50 value of 64.77 µg/mL, presumably due to the content of semipolar compounds such as flavonols. The urgency of this study lies in the potential of red spinach as a source of natural antioxidants to prevent degenerative diseases due to oxidative stress. This finding opens up opportunities for the utilization of ethyl acetate fraction from red spinach in the formulation of pharmaceutical products, functional foods, and health supplements based on local natural ingredients.*

Keywords:, Amaranthus tricolor L.*, Antioxidant activity, DPPH, IC50, Red spinach*

Abstrak

Bayam merah (*Amaranthus tricolor* L.) merupakan sayuran lokal yang diketahui mengandung senyawa bioaktif seperti flavonoid dan fenolik yang berperan sebagai antioksidan. Penelitian sebelumnya menunjukkan potensi antioksidan dari ekstrak kasar tanaman ini, namun kajian mendalam mengenai aktivitas antioksidan berdasarkan fraksinasi pelarut dengan kepolaran berbeda masih terbatas. Padahal, pemisahan fraksi penting untuk mengidentifikasi senyawa aktif yang paling potensial dalam menetralkan radikal bebas. Oleh karena itu, penelitian ini bertujuan untuk mengevaluasi aktivitas antioksidan dari ekstrak dan fraksi daun bayam merah menggunakan metode DPPH (2,2-difenil-1-pikrilhidrazil). Ekstraksi dilakukan melalui maserasi dingin dengan etanol 96%, diikuti fraksinasi menggunakan n-heksana, etil asetat, dan air. Skrining fitokimia menunjukkan keberadaan

alkaloid, flavonoid, kuinon, saponin, steroid, dan tanin. Hasil uji DPPH menunjukkan bahwa ekstrak etanol memiliki aktivitas antioksidan sedang dengan nilai IC50 sebesar 122,09 µg/mL. Fraksi n-heksana dan air memiliki aktivitas serupa dengan IC50 masing-masing 117,33 µg/mL dan 124,36 µg/mL. Fraksi etil asetat menunjukkan aktivitas antioksidan kuat dengan nilai IC50 sebesar 64,77 µg/mL, diduga karena kandungan senyawa semipolar seperti flavonol. Urgensi penelitian ini terletak pada potensi bayam merah sebagai sumber antioksidan alami untuk mencegah penyakit degeneratif akibat stres oksidatif. Temuan ini membuka peluang pemanfaatan fraksi etil asetat dari bayam merah dalam formulasi produk farmasi, pangan fungsional, dan suplemen kesehatan berbasis bahan alam lokal.

Kata kunci: *Amaranthus tricolor* L, aktivitas antioksidan, Bayam merah, DPPH, , IC50

INTRODUCTION

Reactive oxygen species (ROS) are highly reactive molecules generated as byproducts of normal cellular metabolism. Excessive ROS can lead to oxidative stress, which is implicated in various chronic diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders. (Snezhkina *et al.,* 2019, Birben *et al.,* 2012).

Antioxidants have gained attention as potential therapeutic agents for various diseases, including cancer and neurodegenerative disorders, due to their ability to neutralize reactive oxygen species (ROS) (Firuzi *et al.,* 2011; Luo *et al*., 2022). Red spinach (*Amaranthus tricolor* L.) is a nutritious leafy vegetable rich in antioxidants and bioactive compounds. Studies have shown that it contains significant amounts of phenolic compounds, flavonoids, and pigments with antioxidant properties (Pulipati *et al*., 2017; Sarker & Oba, 2018).

The antioxidant activity of A. tricolor extracts has been demonstrated through various assays, with methanolic extracts showing the highest potential (Pulipati *et al.,* 2017). Interestingly, salinity stress has been found to enhance the production of bioactive compounds, including leaf pigments, vitamins, polyphenols, and flavonoids, thereby increasing the antioxidant capacity of the plant (Sarker & Oba, 2018). Additionally, A. tricolor exhibits antimicrobial properties, with different compounds showing effectiveness against various microorganisms (Kucab S *et al*., 2023). These findings suggest that A. tricolor is a valuable source of natural antioxidant compounds, with potential applications in the food industry and human health.

Although numerous studies have reported the antioxidant potential of *Amaranthus tricolor* L., most have been limited to crude extract analysis, without further fractionation based on solvent polarity. This represents a critical gap in the literature, as the specific classes of bioactive compounds such as flavonoids, phenolics, and other semi-polar constituents—have not been adequately identified or quantified within individual solvent fractions. In addition, the comparative evaluation of antioxidant activity across fractions with different polarities remains underexplored, thereby limiting a comprehensive understanding of which polarity range contributes most significantly to free radical scavenging activity.

Therefore, this study aims to address that gap by systematically evaluating the antioxidant activity of red spinach leaf extract and its fractions obtained through polarity-based solvent partitioning (n-hexane, ethyl acetate, and water), using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay.

The urgency of this study lies in the global and local need for safe, effective, and natural antioxidants that can help mitigate oxidative stress a major contributor to degenerative diseases such as cardiovascular disorders, cancer, and neurodegenerative conditions. Identifying potent

antioxidant fractions from widely available local plants such as *Amaranthus tricolor* L not only supports preventive health strategies but also fosters the development of value-added products. The discovery of strong antioxidant activity in the ethyl acetate fraction, in particular, opens practical opportunities for its incorporation into pharmaceutical formulations, functional foods, and health supplements, thereby promoting the utilization of indigenous natural resources in health-related industries.

RESEARCH METHODS

Tools

The equipment used in this study included a UV-Vis spectrophotometer (Shimadzu UV-1800, wavelength 517 nm), Thin Layer Chromatography (TLC) apparatus, silica gel TLC plates (Merck, 60 F254), rotary evaporator (Heidolph Laborota 4000), drying oven (Memmert UN30), quartz cuvettes (1 cm path length), incubator (Binder BD115), blender (Philips HR2221), magnifying glass, light microscope (Olympus CX22), analytical balance (Ohaus Pioneer PA214), desiccator, separating funnel (500 mL), beakers (Pyrex, various volumes), measuring cylinders, test tubes, test tube rack, porcelain crucibles, evaporating dishes, tongs, glass vials, Bunsen burner, dropper pipettes, spray bottles, and glass stirring rods.

Material

The plant material used in this study was red spinach (*Amaranthus tricolor* L.) leaves harvested at the optimal physiological age of 30–35 days after planting (DAP) to ensure maximum bioactive compound content. The materials required for this study included fresh red spinach leaves, *n*-hexane, acetone, 96% ethanol, methanol, chloroform ($CHCl_3$), hydrochloric acid (HCl), ammonia, Mayer's reagent, Dragendorff's reagent, magnesium powder (Mg), alcohol, benzene, ether, 2N sodium hydroxide (NaOH), concentrated sulfuric acid (H_2SO_4), anhydrous acetic acid, 1% ferric chloride ($FeCl_3$), DPPH (2,2-diphenyl-1-picrylhydrazyl), vitamin C (ascorbic acid), and distilled water.

Material Preparation

The plant material used in this study was red spinach (*Amaranthus tricolor* L.) leaves obtained from Benteng Village, Campaka District, Purwakarta Regency, West Java Province. The leaves underwent a series of post-harvest treatments, including wet sorting, washing, chopping, drying, and dry sorting. Drying was carried out using an oven at a temperature of 45–50 °C until a constant weight was achieved to preserve the stability of bioactive compounds. Botanical identification was also conducted to confirm the authenticity of the plant material.

Macroscopic Examination

Macroscopic examination was carried out on the dried leaves of red spinach (*Amaranthus tricolor* L.) to evaluate organoleptic characteristics and morphological features. The assessment included observations of shape, color, smell, taste, and leaf dimensions. The leaf shape appeared round with visible signs of dryness, and the color was red-purplish. The aroma was typical of dried spinach leaves, while the taste was bland. Leaf size was measured using a digital caliper, resulting in an average leaf width of 5 cm and height of 6.8 cm. This evaluation was conducted under natural light, and sensory testing was performed cautiously to ensure safety. Macroscopic examination is a standard procedure to confirm the identity and quality of crude plant materials and to detect any contamination or adulteration before further testing (Kementerian Kesehatan RI, 2014; World Health Organization [WHO], 1998).

Testing the Characteristics of Simplisia

The analysis of simplistic characteristics involves a comprehensive set of tests to evaluate its quality and properties. These include macroscopic and microscopic examination, water content determination, total ash content analysis, acid-insoluble ash content measurement, drying shrinkage assessment, as well as testing for water-soluble and ethanol-soluble extractive content. Each of these tests provides critical information about the simplisia's physical and chemical properties, ensuring it meets established quality standards. (Kementerian Kesehatan RI, 2014; World Health Organization [WHO], 1998).

Phytochemical Screening

Phytochemical screening is conducted as a preliminary step to identify the classes of chemical compounds present in the simplisia. This analysis focuses on detecting alkaloids, flavonoids, quinones, saponins, steroids/triterpenoids, and tannins. The results provide insights into the potential bioactive components, forming the foundation for further research into the material's pharmacological properties. (Harborne, 1987; Farnsworth, 1966)

Extract Preparation

The preparation of the extract is carried out using a cold maceration method. The simplisia is soaked in 96% ethanol solvent for three days (3x24 hours) with occasional stirring to enhance the extraction process. This method ensures the efficient extraction of both polar and non-polar compounds from the material (Azwanida, 2015; Harborne, 1987).

Fractionation

Following the preparation of the thick extract, fractionation is performed using the liquid-liquid extraction technique. This process involves sequentially partitioning the extract with solvents of varying polarities: water (polar solvent), n-hexane (non-polar solvent), and ethyl acetate (semi-polar solvent). This step allows for the separation of compounds based on their polarity, facilitating further analysis of specific fractions. (Sasidharan et al., 2011; Wagner & Bladt, 1996).

Measurement of Maximum Wavelength of Blank

To determine the maximum wavelength, a 100 ppm DPPH stock solution is prepared. A 4 mL aliquot of this solution is incubated for 30 minutes and then analyzed using a UV-Vis spectrophotometer across a wavelength range of 400-800 nm. The wavelength at which maximum absorbance occurs is recorded as the maximum wavelength, which is crucial for subsequent antioxidant activity analysis. (Brand-Williams, Cuvelier, & Berset, 1995; Molyneux, 2004).

Antioxidant Test of Vitamin C (Comparator)

As a comparative standard, Vitamin C is used to assess antioxidant activity. A 5 mg sample of Vitamin C is accurately weighed and dissolved in 50 mL of ethanol p.a. in a volumetric flask. This solution serves as a reference for evaluating the antioxidant potential of the test samples. (Blois, 1958; Molyneux, 2004)

Antioxidant Test of Red Spinach Leaf Extract with DPPH Method

The mother liquor of 1000 ppm red spinach leaf extract was diluted into several concentrations namely 50 ppm, 75 ppm, 100 ppm, 125 ppm and 150 ppm into a 5 mL volumetric flask then added 1.4 mL DPPH and added ethanol p.a until the limit mark. The solution was transferred into a vial and then incubated for 30 minutes. The absorbance of the sample was measured using UV-Vis spectrophotometer at the maximum wavelength. (Brand-Williams, Cuvelier, & Berset, 1995; Molyneux, 2004)

Antioxidant Test of Red Spinach Leaf Fractions with DPPH Method
The mother liquor of each water fraction, n-hexane red spinach leaves 1000 ppm was diluted into several concentrations namely 50 ppm, 75 ppm, 100 ppm, 125 ppm, and 150 ppm into a 5 mL volumetric flask then added 1.4 mL DPPH and added ethanol p.a until the limit mark. The solution was transferred into a vial and then incubated for 30 minutes. As for the ethyl acetate fraction, a parent solution of 1000 ppm was made and then diluted into several concentrations, including 10 ppm, 30 ppm, 50 ppm, 70 ppm, and 90 ppm which were entered into a 5 mL volumetric flask and then added 1.4 mL of DPPH and added ethanol p.a to the limit mark. The solution was transferred into a vial and then incubated for 30 minutes. The absorbance of the sample was measured using a UV-Vis spectrophotometer at the maximum wavelength. (Molyneux, 2004; Brand-Williams, Cuvelier, & Berset, 1995).

RESULTS AND DISCUSSION

Plant determination was carried out to determine the type of red spinach (*Amaranthus tricolor L.*). The determination results showed that the samples used were indeed red spinach plants (*Amaranthus tricolor L.*).

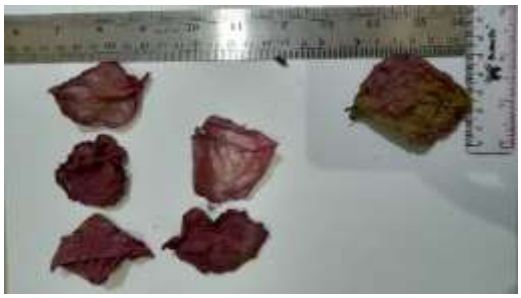

Figure 1: Macroscopic examination of Red Spinach leaves (Amaranthus tricolor L.)

Table 1: Macroscopic Examination Results

Parameter		Results	
Shape		Round dryness	
Color		Red purplish	
Smell		Typical	
Taste		Bland	
Size			
1.	Leaf Width	1.	5 cm
2.	Leaf Height	2.	6.8 cm

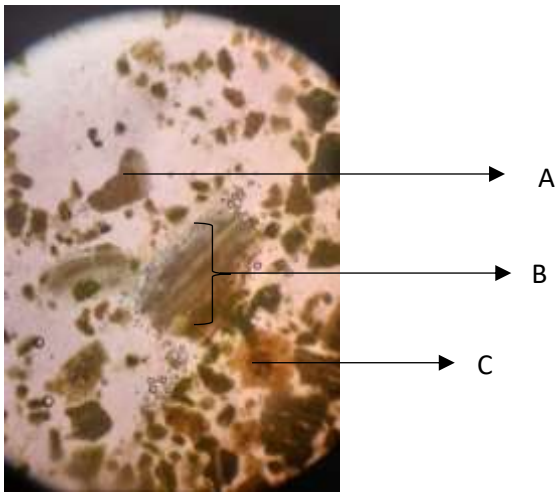

Figure 2: Macroscopic examination of Red Spinach leaves (*Amaranthus tricolor L.*)

Table 2: Microscopic examination results

Parameter	
A	Parenchyma
B	Epidermis
C	Calcium Oxalate Crystals

Microscopic examination of the Red Spinach leaf simplicia (*Amaranthus tricolor L.*) was carried out using a microscope with a magnification of 40x with samples in the form of very fine purplish red powder with identification fragments, namely parenchyma, epidermis, and calcium oxalate crystals.

Table 3: Results of characteristics of Simplicia

Simple Parameter Test	Standards *	Results
Water content	≤ 10.00%	3.32%
Total Ash Content	< 16.6%	12.56%
Acid insoluble ash content	≤ 0.3%	1.82%
Drying shrinkage	< 10.0%	9.93%
Water soluble essence content	< 7.5%	4.66%
Ethanol soluble essence content	< 7.6%	1.29%

*) Indonesian Herbal Pharmacopoeia Standards, 2nd edition, 2017

Based on the results of the examination of simplisia parameters as shown in Table 3, the values for water content, total ash content, drying shrinkage, water-soluble extractive content, and ethanol-soluble extractive content were 3.32%, 12.56%, 9.93%, 4.66%, and 1.29%, respectively. These results comply with the quality standards set by the *Indonesian Herbal Pharmacopoeia*, Edition II, 2017. Meanwhile, for the test of acid-insoluble ash content with a standard ≤ 0.3%, the test result is 1.82%, which means that the value of acid-insoluble ash content that exceeds the normal limit indicates significant contamination in the sample. This test aims to measure the amount of acid-insoluble minerals and foreign substances, such as silica or sand. High levels of acid-insoluble ash can be caused by several factors, including unhygienic production processes, low-quality raw materials, and inadequate storage conditions. The presence of these impurities can reduce product quality. (Pandapotan Marpaung & Septiyani, 2020)

Table 4. Phytochemical Screening Results

Compound	**Standard**	Result
Alkaloid	+	+
Flavonoid	+	+
Kuinon	+	+
Saponin	+	+
Steroid	+	+
Tanin	+	+

Description: (+) = detected
(-) = not detected

Red spinach leaves (*Amaranthus tricolor* L.*)* have been tested for antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The results showed table 4 that extracts and fractions of red spinach leaves had significant free radical scavenging ability. This is closely related to the content of bioactive compounds detected through phytochemical screening, namely alkaloids, flavonoids, quinones, saponins, steroids, and tannins.

Spinach (Spinacia oleracea L.) and related species like red spinach (*Amaranthus tricolor L.*) are recognized as functional foods due to their diverse nutritional composition and bioactive compounds (Roberts & Moreau, 2016; Rahmawati & Retnaningrum, 2022). Phytochemical screening has revealed the presence of various bioactive compounds, including flavonoids,

tannins, alkaloids, and saponins, which contribute to strong antioxidant potential (Pulipati et al., 2017; Rahmawati & Retnaningrum, 2022). These compounds exhibit multiple pharmacological activities, such as antihyperlipidemic, anti-inflammatory, antidiabetic, and antibacterial effects (Rahmawati & Retnaningrum, 2022). The antioxidant properties of spinach extracts make them promising natural alternatives to synthetic antioxidants in food applications (Huda-Faujan *et al.,* 2023). Additionally, spinach-derived phytochemicals can modulate gene expression, curb food intake, and contribute to anti-cancer and anti-obesity properties (Roberts & Moreau, 2016). These findings highlight the potential for utilizing spinach in the pharmaceutical, health, and food industries (Huda-Faujan *et al*., 2023; Rahmawati & Retnaningrum, 2022).

The results obtained are in the form of a calibration curve equation, y = 14.036x - 1.8772 with a correlation coefficient value of R2 = 0.9971. According to the Indonesian Pharmacopoeia VI edition, a good linearity value is ≥ 0.98 or close to 1. (Ministry of Health of the Republic of Indonesia, 2020). Vitamin C has high antioxidant activity, as indicated by its low IC50 value. The IC50 (Inhibition Concentration) value is a concentration value that causes a 50% loss of free radical activity (Santosa & Priya Haresmita, 2015). The IC50 value of vitamin C standard solution is 3.69 µg/mL which can be said that vitamin C has very strong antioxidant activity. This confirms that vitamin C is effective in inhibiting free radicals. The data showed a linear relationship between vitamin C concentration and % inhibition, indicating that the method used was accurate for measuring the antioxidant activity of the standard solution.

The results obtained are in the form of a calibration curve equation, y = 0.4967x - 10.644 with a correlation coefficient value of R2 = 0.9964. The IC50 value of the sample of ethanol extract of red spinach leaves (*Amaranthus tricolor L.*) is 122.09 µg/mL which can be said that the IC50 value is higher than the IC50 value. The ethanol extract of red spinach leaves (Amaranthus tricolor L.) demonstrates moderate to strong antioxidant activity, as evidenced by multiple studies. (Veronica *et al*. 2020) Found that a 0.5% concentration cream formulation of the extract exhibited very strong antioxidant activity. (Yang *et al.* 2023) reported that the ethyl acetate fraction of A. tricolor showed the highest DPPH free radical-scavenging activity. (Rosyidah *et al*. 2021) Observed that a 1.5% concentration of the extract in a face serum formulation displayed very strong antioxidant potential. (Bang *et al.* 2021) noted significant variation in antioxidant activity among different Amaranthus species, with A. tricolor showing both low and high DPPH antioxidant activity depending on the accession. The antioxidant properties of A. tricolor are attributed to its phenolic compounds, including flavonoids, saponins, and tannins (Veronica *et al.,* 2020; Yang *et al*., 2023).

The results obtained from the measurement of antioxidant activity of water and n-hexane fraction samples of red spinach leaves are in the form of linear regression equations, namely y = 0.4977x-11.896 and y = 0.6043x-20.904, respectively, which then obtained IC50 values of 124.36 µg/mL and 117.33 µg/mL, indicating that antioxidant activity in water fraction samples and n-hexane fractions has a moderate level. These results have similarities with ethanol extract samples which both produce moderate levels of antioxidant activity. The results obtained from the ethyl acetate fraction sample y = 0.736x + 2.3258 with an IC50 value of 64.77 µg/mL. The ethyl acetate fraction sample showed a strong level of antioxidant activity.

When viewed from the results of extracts and fractions which are then associated with the level of antioxidant activity, where the ethyl acetate fraction sample produces the least fraction of 0.1447 grams compared to ethanol extract, water fraction, and n-hexane, it can be said that red spinach leaves have semi-polar properties. This can occur because, at the semi-polar level, the

sample has strong antioxidant activity compared to polar and non-polar levels. Flavonoid derivatives, namely flavonols, which are semi-polar, have antioxidant properties that are beneficial to health. This shows that there is a relationship between antioxidant properties and the solubility of a solvent in determining antioxidant activity (Hidayah et al., 2016).

CONCLUSIONS

The results of the antioxidant activity of extracts and fractions of red spinach leaves (Amaranthus tricolor L.) are influenced by the level of polarity of a solvent. The difference can be seen from the test results of antioxidant activity of ethanol extract of 122.09 µg/mL, water fraction of 124.36 µg/mL, n-hexane fraction of 117.33 µg/mL, and ethyl acetate fraction of 64.77 µg/mL. Thus it can be concluded that the ethyl acetate fraction has a strong level of antioxidant activity with an IC50 value of 64.77 µg/mL.

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