

The comparison of antioxidant activity test of fractions of *Curcuma caesia* Roxb. originating from Kemuning, Indonesia

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ABSTRACT

Curcuma caesia Roxb. is a rich source of bioactive compounds, including flavonoids, alkaloids, polyphenols, and tannins, which act as antioxidants. This research aims to examine and compare the antioxidant activity of n-hexane, ethyl acetate, and water fractions of *Curcuma caesia* Roxb. rhizome extracts originating from Kemuning, Central Java, Indonesia, using DPPH and ABTS free radical immersion methods. *Curcuma caesia* Roxb. rhizome was extracted using an ethanol solvent, and then fractionated with n-hexane, ethyl acetate, and water. The DPPH and ABTS techniques were then used to assess the fractions' antioxidant activity. The wavelengths used for spectrophotometric measurements were 517 nm and 734 nm. The IC₅₀ value metric expresses antioxidant activity. The results of the antioxidant activity test showed that all concentrations had the potential to capture ABTS and DPPH free radicals, with IC₅₀ values for the n-hexane, ethyl acetate, and water fractions. Based on the two antioxidant testing methods, the highest results were found in the ABTS method. The results of the antioxidant activity test showed that all concentrations could capture ABTS free radicals, with IC₅₀ values for the n-hexane, ethyl acetate, and water fractions of 66.0604 ppm (strong), 40.4678 ppm (very strong), and 39.9784 ppm (very strong), respectively. Meanwhile, the IC₅₀ values in the DPPH method were respectively 52.1287 ppm (strong), 110.3383 ppm (moderate), and 98.15651 ppm (strong). The IC₅₀ value of the positive control, with vitamin C as a comparison, showed very strong antioxidant activity.

Keywords: *Curcuma caesia*, Antioxidant, Fraction, DPPH, ABTS

Introduction

The skin undergoes a degeneration process as it ages. Both internal and external factors of the body are responsible for aging. Internal factors include health, stamina, stress, and hormonal changes [1-3]. All of these are natural processes that

humans cannot avoid but can minimize through proper, regular, and gentle facial care as well as stress reduction. External factors include free radicals and sunlight that can damage skin cells [4]. External factors are also known as photoaging, such as exposure to ultraviolet rays and free radicals. Environmental factors, including cigarette smoke and air pollution, also contribute to skin aging. However, 90% of skin aging results from skin exposure to UV radiation [5]. Antioxidants stabilise free radicals by making up for the electrons they lose, preventing chain reactions and defending the organism against free radical attacks. Antioxidants can act as hydrogen radical donors or free radical acceptors to delay the initiation stage of free radical formation [6].

Indonesia is a tropical country rich in various plants with great potential to be used as natural antioxidants. One of them is the

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rhizome of *Curcuma caesia* Roxb. This plant contains bioactive compounds, including flavonoids, alkaloids, polyphenols, and tannins, which act as antioxidants. Previous studies have revealed some activities of *Curcuma caesia* Roxb., including antifungal, antiasthmatic, bronchodilator, antioxidant, cerebrospinal neuro system (CNS) depressant, anticonvulsant, anthelmintic, antimutagenic, antibacterial, and antiulcer activities [7].

Curcuma caesia Roxb. rhizome consists of various phytochemical contents, such as carbohydrates, proteins, amino acids, steroids, glycosides, flavonoids, alkaloids, and tannins. *Curcuma caesia* Roxb. rhizome also has a higher concentration of phytochemical compounds than other turmeric plants [8].

An antioxidant activity test of an ethanol extract of black turmeric rhizome using DPPH and ABTS methods produced IC₅₀ values of 108.8304 ± 1.24 ppm and 124.8576 ppm, respectively [9]. The ethanol extract of black turmeric had a total flavonoid concentration of 22.1904 mg qe/g. Furthermore, *C. caesia* has 61.823 mgGAE/g of secondary metabolites and phenolic compounds [10].

Fractionation is expected to enhance antioxidant activity compared to that of the extract. Given this context, it is necessary to conduct research on the antioxidant activity of various fractions based on their polarity levels using the DPPH and ABTS methods [11, 12].

Materials and Methods

Equipment

This study employed several tools, including a 0.5-gram digital analytical balance (Precisa), a 10 10-mg digital analytical balance (KERN ABJ), a micropipette (DIAB), a UV-Vis spectrophotometer (Genesys™ 10S), a vortex (Thermo), and a refrigerator (Phillips).

Materials

The materials used in this study included *Curcuma caesia* Roxb. rhizome, distilled water (Merck, Germany), ethyl acetate, phosphate buffer pH 7 (Merck, Germany), 70% alcohol (Merck, Germany), DPPH indicator (Sigma Aldrich, USA), ABTS indicator (Sigma Aldrich, USA), methanol pro analysis (Merck, Germany), blue tip (Nesco), and Ethanol (Merck, Germany) [13-16].

Fractionation

The separation process used liquid-liquid extraction with water, ethyl acetate, and n-hexane solvents. The concentrated ethanol extract of black turmeric (*Curcuma caesia* Roxb.) was dissolved in 100 mL of hot water and then cooled. The obtained filtrate was added with n-hexane in a 1:1 ratio and shaken in a separating funnel. The separating funnel was then opened and set aside. Further, the n-hexane fraction was collected in a container. The water fraction was added with ethyl acetate in a ratio of 1:2. After

the ethyl acetate fraction was separated, the remaining water fraction was used for each solvent in 3 repetitions [17-20].

As a result, the water fraction was dried with a freeze-drying device until the fractions of each solvent were produced, and the n-hexane and ethyl acetate fractions were evaporated [21].

In vitro antioxidant activity testing of black turmeric rhizome extract fractions using the DPPH method

Preparation of 0.4 mM DPPH stock solution

A total of 4 mg of DPPH powder with a molecular weight of 394.32 g/mol was put into a 25 mL volumetric flask, followed by adding methanol of pro analysis to the line mark. The DPPH stock solution was homogenized with a vortex for 20 seconds, kept at a low temperature, and protected from light [22].

Determination of the maximum wavelength of DPPH

A total of 2 mL of a 0.4 mM DPPH stock solution was placed into a 10 mL volumetric flask, and methanol pro analysis was added up to the line mark. The solution was vortexed for 20 seconds and incubated for 30 minutes at 37 °C, and the absorbance was measured at a wavelength of 400-600 nm using a UV-Vis spectrophotometer [23].

Preparation of blank solution

A total of 2 mL of prepared 0.4 mM DPPH was put into a 10 mL volumetric flask, and methanol pro analysis was added to the line mark. The solution was vortexed for 20 seconds and incubated for 30 minutes at 37 °C, and the absorbance was measured at the maximum wavelength obtained [23].

Antioxidant activity test of black turmeric

Black turmeric solution (*Curcuma caesia* Roxb.) was prepared in 5 concentrations (50, 100, 150, 200, and 250 ppm) in a 10 mL volumetric flask. Each solution with different concentrations was added to the line mark with 2 mL of 0.4 mM DPPH and methanol pro analysis. The absorbance was measured at the highest wavelength attained after the solution was vortexed for 20 seconds and incubated for 30 minutes at 37 °C [23].

Antioxidant activity test of pure vitamin C (positive control)

A total of 5 mg of vitamin C was placed into a 50 mL volumetric flask, and methanol of pro analysis was added until the line mark (100 ppm vitamin C stock solution). The stock solution was diluted into 5 concentration series (1, 2, 3, 4, and 5 ppm) in a 10 mL volumetric flask, and 2 mL of 0.4 mM DPPH solution and pro analysis methanol were added to the line mark. All

concentration series were vortexed for 20 seconds and incubated for 30 minutes at 37 °C, and the absorbance was measured at the maximum wavelength obtained [23].

In vitro antioxidant activity testing of black turmeric rhizome extract fraction (curcuma caesia roxb.) using the ABTS method

Preparation of potassium persulfate solution

As much as 3.5 mg of potassium persulfate was dissolved in 5 mL of water [24].

Preparation of ABTS solution

As much as 18 mg of ABTS was dissolved in 5 mL of deionized water [24].

Preparation of ABTS stock solution

A total of 5 mL of ABTS was added with 5 mL of potassium persulfate solution, followed by adding water until the volume reached 25 mL. It was then incubated in a dark room at 22- 24 °C for 12-16 hours [24].

Preparation of vitamin C control solution

As much as 10 mg of pure vitamin C was dissolved in 10 mL and added to the line mark [24].

Antioxidant activity test of the ABTS method

Determination of maximum wavelength

As much as 1 mL of ABTS solution was pipetted into a 5 mL volumetric flask and then filled with distilled water up to the line mark. Absorption was recorded using a UV-Vis spectrophotometer, adjusting the wavelength from 400 to 800 nm until the maximum wavelength was reached [24].

Absorption measurement of blank ABTS solution

ABTS stock was pipetted to a volume of 1 mL and transferred to a 5 mL measuring cylinder, which was then filled with water up to the line mark. The solution was incubated for 15 minutes, and the absorbance was measured using spectrophotometry at a wavelength of 734 nm [24].

Testing of the fraction and extract solution

A volume of up to 0.1 mL of each extract was pipetted, followed by adding 2 mL of ABTS stock solution. The mixture was then incubated for 6 minutes, and the absorbance was measured using spectrophotometry at a wavelength of 734 nm [24].

Testing of vitamin C solution

As much as 30 µl of vitamin C solution was pipetted and added to 1 mL of ABTS solution. The total volume was adjusted with water to 5 mL. Next, the absorbance was measured using a spectrophotometer at a wavelength of 734 nm [24].

Calculation of free radical inhibition percentage

The free radical scavenging activity (antioxidant activity) of the extract and vitamin C was determined by the percentage reduction in color (indicating free radical inhibition) [22].

Calculation of IC₅₀ value

The IC₅₀ value was calculated from the linear regression curve that related the percentage of inhibition to the concentrations of the test solution (sample). A linear regression equation was formulated by plotting the sample concentration on the x-axis and the percentage of inhibition on the y-axis. This equation was then used to determine the IC₅₀ value. The calculation employed the linear regression equation with sample concentration as the x-axis and the value of 50 as the y-axis [25].

Results and Discussion

Antioxidant testing results

Antioxidant testing utilizing the DPPH and ABTS methods is quantified in terms of IC₅₀, which represents the concentration of the test compound required to decrease radicals by 50%. The IC₅₀ value is derived from a curve depicting the percentage inhibition of the test solution. A lower IC₅₀ value indicates superior antioxidant activity [26].

The antioxidant activity tests of the water, n-hexane, and ethyl acetate fractions were conducted using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) method, due to the ability of the *C. caesia* rhizome fraction to reduce or capture DPPH radicals. This DPPH method is commonly employed to evaluate antioxidant activity, as it relies on antioxidants' capacity to inhibit free radicals by donating hydrogen atoms to DPPH. The antioxidant activity measurement using the ABTS method [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] is adaptable at varying pH levels. ABTS demonstrates sensitivity to acidic pH, dissolving effectively in both organic solvents and water, which facilitates the detection of lipophilic and hydrophilic compounds [27].

ABTS testing was performed due to its higher sensitivity compared to DPPH, enabling analysis of antioxidants in food products. There are noted differences in the reaction mechanisms between DPPH and ABTS concerning antioxidant capability. For DPPH, the antioxidant capacity of a compound is assessed based on its ability to donate hydrogen. Conversely, the ABTS method evaluates the antioxidant's ability to stabilize free radicals through proton radical donation [26].

Vitamin C was used as a comparison (positive control) in this antioxidant activity test. Vitamin C is a secondary antioxidant that captures free radicals and inhibits chain reactions. Employing a positive control in this antioxidant activity test helps assess the relative strength of the *C. caesia* rhizome fraction's antioxidant potential compared to vitamin C.

The antioxidant activity results for the n-hexane, ethyl acetate, and water fractions from the *C. caesia* rhizome extract, along with vitamin C, using the DPPH and ABTS methods, can be found in **Tables 1-4**. Linear regression curve of n-hexane, ethyl acetate, and water fractions of *C. caesia* extract in the ABTS and DPPH method can be found in the **Figures 1 and 2** below.

Table 1. Results of Absorbance and % Inhibition of Variation of *Curcuma caesia* Roxb. Extract from Kemuning Using ABTS Method

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC50
Blanko	0	0,621	0		
n-hexane fraction	10	0,589	5,187	$y = 0,809x - 3,495$	66,060 ppm
	20	0,553	11,087		
	30	0,484	22,122		
	40	0,443	28,745		
	50	0,392	36,848		
Ethyl acetate fraction	10	0,611	1,741	$y = 1,584x - 14,105$	40,467 ppm
	20	0,496	20,150		
	30	0,434	30,150		
	40	0,323	48,060		
	50	0,205	66,993		
Water fraction	10	0,451	27,453	$y = 0,742x + 20,332$	39,978 ppm
	20	0,397	36,085		
	30	0,364	41,394		
	40	0,305	50,884		
	50	0,266	57,158		

Table 2. Results of Absorbance and % Inhibition of *Vitamin C* With ABTS Method

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC50
Blanko	0	0,721	0		
Vitamin C	1	0,602	16,504	$y = 2,653x + 16,2$	12,740 ppm
	2	0,572	20,665		
	4	0,496	31,206		
	8	0,4563	36,708		
	16	0,3017	58,159		

Table 3. Results of Absorbance and % Inhibition of Variation of *Curcuma caesia* Roxb. Extract from Kemuning With DPPH Method

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC50
Blanko	0	0,732	0		
n-hexane fraction	10	0,585	19,963	$y = 716x + 12,655$	52,128 ppm
	25	0,502	31,366		
	50	0,384	47,504		
	75	0,253	65,409		
	100	0,107	85,300		
Ethyl acetate fraction	10	0,400	45,355	$y = 0,047x + 44,781$	110,338 ppm
	25	0,394	46,174		
	50	0,390	46,675		
	75	0,378	48,269		
	100	0,368	49,726		
Water fraction	10	0,485	33,743	$y = 0,190x + 31,311$	98,156 ppm
	25	0,466	36,247		
	50	0,443	39,480		
	75	0,396	45,856		
	100	0,360	50,728		

Table 4. Results of Absorbance and % Inhibition of *Vitamin C* With DPPH Method

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC50
Blanko	0	0,732	0		
Vitamin C	1	0,452	38,160	$y = 3,260x + 36,093$	4,265 ppm
	2	0,406	44,489		
	3	0,397	45,765		
	4	0,377	48,497		
	5	0,348	52,459		

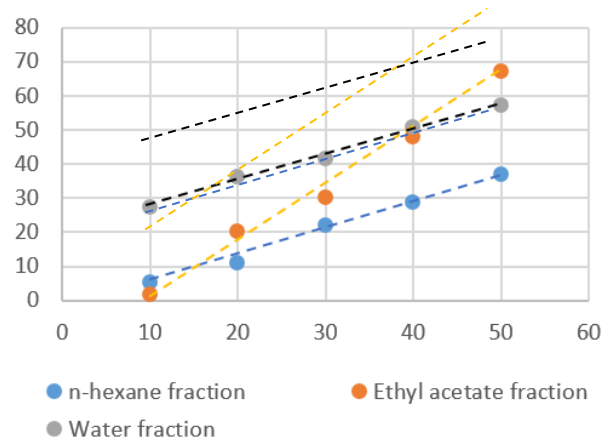


Figure 1. Linear regression curve of n-hexane, ethyl acetate, and water fractions of *C. caesia* extract in the ABTS method

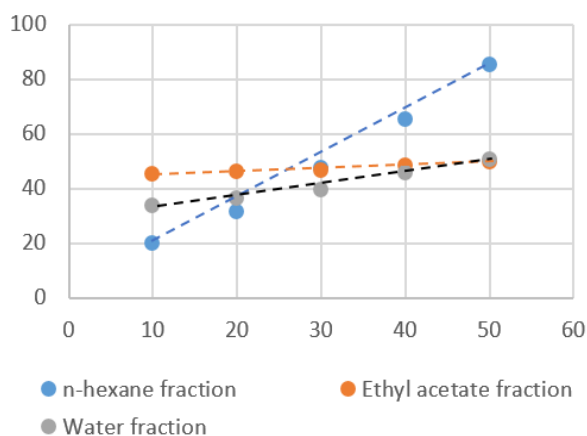


Figure 2. Linear regression curve of n-hexane, ethyl acetate, and water fractions of *C. caesia* extract in the DPPH method

Antioxidants are classified as very strong if the value is < 50 ppm, strong at 50-100 ppm, moderate at 101-150 ppm, and weak if >150 ppm. A sample demonstrates stronger antioxidant activity with a smaller IC_{50} value. Results indicate that the *C. caesia* rhizome fraction exhibits inhibitory activity against antioxidants. The IC_{50} values from the hexane fraction, ethyl acetate fraction, and water fraction of the *C. caesia* rhizome, tested using the ABTS method, were 66.0604 ppm (strong), 40.4678 ppm (very strong), and 39.9784 ppm (very strong), respectively. In the DPPH method, the IC_{50} values were 52.1287 ppm (strong), 110.3383 ppm (moderate), and 98.15651 ppm (strong). Vitamin C, the positive control, exhibits very strong antioxidant activity. The IC_{50} value indicates the concentration of the test compound that can reduce 50% of free radicals; thus, a lower IC_{50} value signifies higher antioxidant activity of the sample [23]. Because antioxidant activity and IC_{50} value are negatively correlated, more antioxidant activity is associated with a lower IC_{50} value [28].

Testing of the *C. caesia* rhizome fractions using the ABTS method revealed that the water fraction had the highest antioxidant activity, with an IC_{50} value of 39.9784 ppm, outperforming the hexane and ethyl acetate fractions. This is influenced by the active compounds extracted in each solvent, where the water fraction contains phenol, flavonoids, saponins, alkaloids, and tannins [29]. The use of polar water solvents effectively extracts these polar compounds. Flavonoid compounds with antioxidant potential, such as kaempferol, along with alkaloids like papaverine and saponins like alpha-hederin, were identified. Saponins, secondary metabolites in glycoside form prevalent in many plants, possess potential antioxidant, antibacterial, and antiviral properties. Alkaloids, commonly found in plants, contain hydrogen in a heterocyclic form and demonstrate pharmacological effects, such as antioxidant, antibacterial, and antiviral activities [29].

The DPPH method results showed that the n-hexane fraction exhibited antioxidant activity with an IC_{50} value of 52.1287 ppm, superior to the water and ethyl acetate fractions. Antioxidant activity can stem not only from polar compounds but also from non-polar ones, including non-polar flavonoids, alkaloids, and

triterpenoids. Non-polar aglycone forms of flavonoid glycosides demonstrate higher antioxidant activity than their polar glycone counterparts. Generally, a higher content of secondary metabolites corresponds to stronger antioxidant activity.

The DPPH and ABTS methods share the same principle: they reduce free radicals, lower redox-active compounds, and employ appropriate standards to measure antioxidant capacity using spectrophotometry. The DPPH method relies on an oxidation-reduction reaction, wherein DPPH, a synthetic free radical, dissolves in polar solvents like ethanol and methanol. DPPH can react via two mechanisms: hydrogen atom donation and electron donation, where antioxidant compounds donate hydrogen atoms or electron pairs to the DPPH radical, decreasing free radical presence in the sample. In contrast, the ABTS method assesses the ability of antioxidant compounds to stabilize free radicals by donating protons, indicated by a color shift from blue-green to colorless. This method measures colour at a certain wavelength using the ABTS radical cation as an indicator. Each approach has unique benefits and drawbacks. The study of hydrophilic chemicals is made more difficult by the DPPH method's restriction to organic solvents, despite its popularity due to its ease of use, rapidity, and sensitivity to low-concentration samples. Conversely, the ABTS method excels compared to DPPH, yielding specific absorbance at visible wavelengths and quicker reactions. Furthermore, ABTS is soluble in both organic solvents and water, enabling detection of both lipophilic and hydrophilic compounds. Nonetheless, the ABTS method cannot accurately represent the body's free radical defense system and is best used for comparison purposes, whereas the DPPH method is preferred by many researchers for its capacity to reflect the biological system's response to free radicals.

Conclusion

Research on the antioxidant activity of n-hexane, ethyl acetate, and water fractions from *C. caesia* rhizomes, conducted using various methods, indicates that the selection of the antioxidant activity testing method can influence the antioxidant activity of a natural substance. Across both testing methods, the ABTS test yields the highest results. The findings demonstrate that all concentrations can capture ABTS free radicals, with IC_{50} values of 66.0604 ppm (strong) for n-hexane, 40.4678 ppm (very strong) for ethyl acetate, and 39.9784 ppm (very strong) for water fractions. In contrast, the DPPH method provided IC_{50} values of 52.1287 ppm (strong) for n-hexane, 110.3383 ppm (medium) for ethyl acetate, and 98.15651 ppm (strong) for water fractions.

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Conflict of interest: None

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