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Original Article

Analysis of flavonoid content and antioxidant activity of *Curcuma caesia* roxb grown in different geographical areas

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ABSTRACT

Indonesia is a country with a tropical climate, making it home to a large number of flora and fauna species. One of them is the *Curcuma caesia* Roxb plant. The study aims to compare the effects of growing locations on flavonoid content and antioxidant activity of *Curcuma caesia* Roxb. The plants were taken from different locations in Central Java, namely Kemuning (866 asl) and Kebumen (28 asl). Black turmeric rhizome was extracted by the maceration method using ethanol solvent at room temperature for 3 days. Flavonoid content was identified using thin-layer chromatography, while compound levels were determined using the spectrophotometric method. Data were analyzed using a t-test in SPSS. The results of phytochemical screening of the flavonoid compound group using thin-layer chromatography produced a spot with an Rf value of 0.96. Flavonoid level was 22.1904 mg qe/g in the samples from the Kemuning location and 19.3929 mg qe/g in the Kebumen location. The IC50 value was 63.349 mg/ml in the samples from the Kemuning location and 82.8423 mg/ml in the samples from the Kebumen location. The results of the sample measurements in Kemuning and Kebumen produced soil pH of 6.50 and 6.63; temperature of 23.0 ℃ and 29.0 ℃; air humidity of 80.33% and 76.00%; and light intensity of 10,595 and 27,354 lux. It can be concluded that the growing location affects the flavonoid levels and antioxidant activity of *Curcuma caesia* Roxb.

Keywords: Flavonoid, Antioxidant, Kemuning, Kebumen

Introduction

Indonesia lies along the equator and has a tropical climate, making it home to a large number of flora and fauna species [1]. This diversity makes Indonesia rich in biological resources that can be utilized by the community for several purposes, such as food, cosmetics, medicines, and so on [2]. The *Curcuma caesia* Roxb. plant is one of thousands of flora in Indonesia [3].

Curcuma caesia Roxb. can grow well in moist and sandy soil with a high organic matter content. The lighting intensity preference is half-shade. The ideal temperature for growth is warm temperatures around 20°C–35°C. Black turmeric can grow in subtropical areas and places with sandy clay substrate and soil pH around 4.5–6.5, and it has a growth time of up to 9 months to reach maturity [4]. *Curcuma caesia* Roxb. rhizome contains curcuminoids, essential oils, flavonoids, phenolics, amino acids, proteins, and high alkaloid [5]. One of the contents of *Curcuma caesia* Roxb. functions as an antioxidant that can capture free radicals, anti-inflammatory, and anti-carcinogenic. *Curcuma caesia* Roxb. rhizome contains bioactive compounds of the flavonoid, alkaloid, polyphenol, and tannin groups, which are antioxidants. Antioxidants are compounds that can prevent the negative effects of oxidants in the body, such as damage to vital elements of cells, by donating one electron to oxidative compounds to inhibit their activity [6].

Antioxidants play a role in the free radical defense system caused by external factors, such as temperature, soil pH, UV radiation, air pollution in the environment, and other pollution. Flavonoids are phenolic compounds and one of the most abundant compounds in plant tissue. Flavonoids function as

Access this article online	
Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Rohmani Sh, Astirin OP, Marliyana SD, Handayani N. Analysis of flavonoid content and antioxidant activity of *Curcuma caesia* roxb grown in different geographical areas. J Adv Pharm Educ Res. 2024;14(4):69-75. https://doi.org/10.51847/yy1abuEXOj

antioxidants by donating hydrogen atoms and inhibiting lipid oxidation. One of the factors that influences the production of secondary metabolites is environmental conditions. The phytochemical content in the form of secondary metabolites of a plant differs in each region due to several environmental factors, such as temperature, light, humidity, pH, and the quality of the soil where the plant grows, which will affect the phytochemical content of a plant [7]. Several studies show that the height of the growing place is one of the determinant factors of plant metabolism [8]. The content of secondary metabolites varies due to the different climatic conditions of the growing places, and this content varies from time to time in different locations [9]. The study aims to compare the flavonoid content and antioxidant activity of the rhizome of *Curcuma caesia* Roxb grown in two different places located in Central Java, namely Kemuning and Kebumen, using the ABTS (3-ethyl-benzothiazoline-6-sulfonic acid) method. Kemuning is a highland with cool air, while Kebumen is a lowland with hot temperatures. Most of the natural landscape of Kebumen Regency is latosol soil mixed with limestone soil [10], while the type of soil in the Kemuning Area is yellowish brown andosol and lithosol [11].

Materials and Methods

Equipment and materials

The tools used were a 0.5-gram digital analytical scale (Precisa), a 10-mg digital analytical scale (KERN ABJ), glassware (Pyrex), and a UV-Vis spectrophotometer (Genesys™ 10S).

The materials used include black turmeric plants from Kemuning and Kebumen, distilled water (Merck, Germany), 70% alcohol (Merck, Germany), methanol pro analysis (Merck, Germany), ethanol (Merck, Germany), HCI 37% (Merck, Germany), FeCI3 (Merck, Germany), Quercetin (Sigma Aldrich, USA), ABTS (3-ethyl-benzothiazoline-6-sulfonic acid) (Sigma Aldrich, USA).

Extraction procedures

The rhizome of *Curcuma caesia* Roxb. was washed under running water and then dried. The rhizome was cut into small pieces. The sample was extracted by maceration method using 96% ethanol solvent for 3 days at room temperature in dark conditions. The maceration results were then evaporated using a rotary evaporator.

Qualitative analysis of phytochemical screening of flavonoid compounds

The rhizome extract of *Curcuma caesia* Roxb. was weighed at 0.5 grams and then added with 10 mL of distilled water, heated in a water bath, and filtered. It was then dissolved in 1 mL of ethanol (96%) with the addition of magnesium powder P and dissolved in 10 mL of concentrated hydrochloric acid P. If it turns red-purple, it indicates the presence of flavonoids, and if it turns yellow, it indicates the presence of flavones, chalcones, and aurones [12].

Thin-Layer chromatography testing of flavonoid compounds

This thin-layer chromatography test used the Silica G60 F254 stationary phase. The TLC plate was made with 10 cm long and 3 cm wide. The mobile phase used to identify flavonoids is butanol: acetic acid: water (4:1:5), with the appearance of ammonia stains. If the result is positive, observation under UV light before being evaporated with ammonia will show a dark purple color, and light blue fluorescence, and is not visible. The appearance of stains with ammonia shows yellow, yellow green or brown, light blue, red or orange, yellow-green fluorescence, or bright light blue-green fluorescence, and bright to light blue fluorescence, according to the type of flavonoid [13].

Determination of flavonoid levels

Preparation of standard solution (quercetin)

A stock solution of 1000 ppm quercetin was prepared by weighing 10 mg of quercetin dissolved in 10 ml of solvent in a measuring flask. Furthermore, the concentrations were 20, 30, 40, 50, and 60 ppm as reference concentrations. Each concentration was taken as much as 0.5 ml and then put into a test tube. Each was added with 1 ml of 2% aluminum (III) chloride, 120 mM sodium acetate, and 2.8 ml of distilled water, homogenized, and incubated for 30 minutes. Furthermore, the absorbance of the comparison solution (quercetin) was measured using a UV-VIS spectrophotometer with a wavelength of 435 nm. The solution was measured three times, after obtaining the respective absorbance values, and a calibration curve was made until a linear regression line was obtained [14].

Determination of total flavonoid test

A total of 10 mg of extract was dissolved with 10 ml of solvent in a measuring flask to obtain a concentration of 1000 ppm as a stock solution [15]. A total of 1 ml of stock solution was put into a test tube, added with 1 ml of 2% aluminum (III) chloride, 120 mM sodium acetate, and 2.8 ml of distilled water. It was then homogenized and incubated for 30 minutes. In the next step, the absorbance of the solution was measured using a UV-VIS spectrophotometer at a wavelength of 435 nm. The solution was measured three times to obtain the average absorbance value [14].

Determination of antioxidant activity using ABTS method

Determination of maximum wavelength

A total of 1 mL of ABTS solution was pipetted and put into a 5 mL volumetric flask and filled with distilled water to the

boundary line. Absorption was measured using a UV-Vis spectrophotometer with a wavelength set from 400-800 nm until the maximum wavelength was obtained.

Measurement of the absorbance of blank ABTS solution

ABTS stock pipetted as much as 1 mL was put into a 5 mL measuring tube and then filled with water to the boundary line. Furthermore, the solution was incubated for 15 minutes, and the absorbance was measured by spectrophotometry at a wavelength of 734 nm.

Testing of extract solution

Each extract was pipetted into 0.1 mL and then added with 2 mL of ABTS stock solution. Next, they were incubated for 6 minutes, and the absorbance was measured by spectrophotometry at a wavelength of 734 nm.

Testing of vitamin C solution

30 µl of Vitamin C solution was pipetted and added with 1 mL of ABTS solution and the volume was made up to 5 mL with water. The absorbance was then measured by a spectrophotometer at a wavelength of 734 nm.

Calculation of free radical inhibition percentage

The free-radical scavenging activity (antioxidant activity) of the extract was calculated as the percentage of color reduction (percentage of free radical inhibition) using the equation [16]:

$$Inhibition\ (\%) = \frac{blank\ absorbance - sample\ absorbance}{blank\ absorbance} \times 100\% \quad (1)$$

Calculation of IC_{50} value

The IC_{50} value was calculated from the linear regression curve between the percentage of inhibition and the series of concentrations of the test solution (sample). Sample concentration and inhibition percentage were plotted on the x and y axes respectively to obtain a linear regression equation. The equation used to determine the IC_{50} value was a linear regression equation, with sample concentration as the x-axis and the value 50 as the y-axis [17].

Determination of environmental conditions

Air thermometers were used to measure temperature and humidity parameters by placing them at both growing locations, measured at 07.00 am, 12.00 pm, and 5.00 pm. Intensity was measured using a Luxmeter placed at both locations at 12.00. The height of the location was measured using the Barometer application connected to Google Earth.

Data analysis

The data obtained were analyzed using the SPSS t-test to examine the growing location that influences flavonoid levels and antioxidant activity of *C.caesia* Roxb.

Results and Discussion

Qualitative analysis of flavonoid phytochemical screening of flavonoid compounds

The results obtained from phytochemical screening showed the presence of an orange color when reacting with HCI, which indicates a positive reaction, where the presence of flavonoid compounds is characterized by the formation of an orange color (flavone) [18]. The flavonoid test also showed positive results, which was indicated by a color change when the sample was reacted with a strong base like sodium hydroxide (NaOH), which would form red acetophenone. Based on the test results, it can be concluded that the extract samples from Kemuning and Kebumen contain flavonoid phytochemical compounds [19].

Thin-layer chromatography testing of flavonoid compounds

The results of the phytochemical screening of the flavonoid compound group showed a positive reaction, so TLC was necessary to confirm the screening results. Secondary metabolite compounds of flavonoids in black turmeric extract can be separated using eluent butanol: acetic acid: water with a volume ratio of 4:1:5, which tends to be polar.

The results of the Thin-Layer Chromatography test in visible light produced one spot of 0.16 with a yellow color. At 254 nm UV light, there were three blue fluorescent spots with Rf 0.16, 0.78, and 0.96. At 365 nm UV light, there were 3 spots with Rf 0.16, 0.78, and 0.96 showing light blue fluorescence. After being evaporated with I2 under visible light, it showed a brown color with Rf 0.16, 0.78, and 0.96, while on H2SO4 spraying, there were 4 brown spots with Rf 0.5, 0.65, 0.93, and 0.99. The quercetin marker showed a blue color in UV light at 254 nm and 366 nm with an Rf value of 0.96 and a brown spot on I2 and H2SO4 spraying with an Rf of 0.96. Based on the calculation of the Rf value, which was 0.96, in the quercetin marker or the black turmeric sample, it shows that the flavonoid compounds in the black turmeric extract tend to be distributed in the mobile phase, meaning that the separated compounds tend to be non-polar, thus producing a large Rf value [20]. The correlation between spots and flavonoid structures shows that if blue forensics appears in UV light and green, yellow, or blue-green

fluorescence appears after spraying, then the type of flavonoid is suspected to be the flavone, flavonone, and flavonol groups [13].

Determination of flavonoid levels

Quantitative analysis of total flavonoid compounds using UV-Vis spectrophotometry was carried out to determine the level of total flavonoids contained in the ethanol extract of black turmeric. Flavonoid analysis was carried out using UV-Vis Spectrophotometry since flavonoids contain a conjugated aromatic system, so they show strong absorption bands in the ultraviolet and visible light spectrum regions [21].

This study aims to examine the total flavonoid levels in samples using quercetin as a standard solution with concentrations of 20, 30, 40, 50, and 60 ppm. Quercetin is used as a standard solution as quercetin is a flavonoid of the flavonol group with a keto group at C-4 and has a hydroxyl group at the C-3 or C-5 atom neighboring flavone and flavonol [22]. The calibration curve of quercetin can be seen in **Figure 1**.

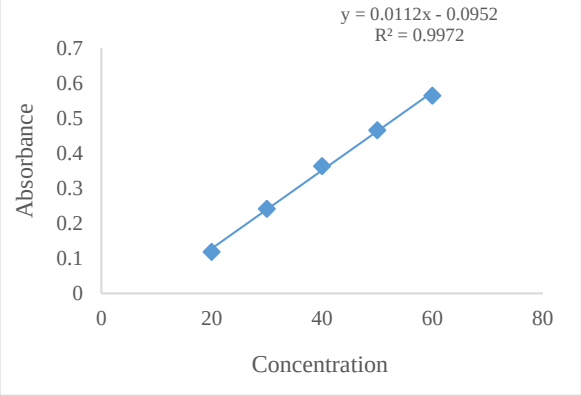

Figure 1. Calibration Curve of Quercetin

Based on the measurement of total flavonoid levels, it can be concluded that the higher the concentration used, the higher the absorbance obtained. The standard quercetin results were plotted between the levels and absorbance, so a linear regression equation was obtained, namely y = 0.0112x - 0.0952 with an R2 value of 0.9972. The quercetin calibration curve equation can be used as a comparison to determine the concentration of total flavonoid compounds in sample extracts. The results of this study showed that the total flavonoid contents of black turmeric ethanol extract from Kemuning and Kebumen were 22.1904 mg qe/g and 19.3929 mg qe/g, respectively.

Antioxidant activity

The IC_{50} value of samples from each region was determined using a linear regression equation based on the correlation curve of sample concentration against the percentage of inhibition. The linear regression equations in **Figure 2**, **Tables 1 and 2** show the relationship between the concentration of the extract and the percentage of inhibition.

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

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC_{50}
Blanko	0	0,622	0	y= 0,496x + 18,579	63, 349 µg/mL
Ekstrak	10	0,471	24,236		
	20	0,443	27,882		
	30	0,414	33,351		
	40	0,387	37,694		
	50	0,347	44,129		

Table 2. Results of Absorbance and % Inhibition of Variation of Curcuma caesia Roxb. Extract from Kebumen

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC_{50}
Blanko	0	0,622	0	y = 0,4011x + 16,772	82,8422 µg/mL
Ekstrak	10	0,495	20,375		
	20	0,464	25,308		
	30	0,441	29,061		
	40	0,420	32,386		
	50	0,392	36,890		

Table 3. Results of Absorbance and % Inhibition of Vitamin C

Sample (% v/v)	Concentration (µg/mL)	Absorbance	% Inhibition	The linear regression equations	IC_{50}
Blanko	0	0,721	0		12,740
Vitamin C	1	0,602	16,505		µg/mL
	2	0,572	20,666	y = 2,653x + 16,2	
	4	0,496	31,207		
	8	0,456	36,708		
	16	0,302	58,159		

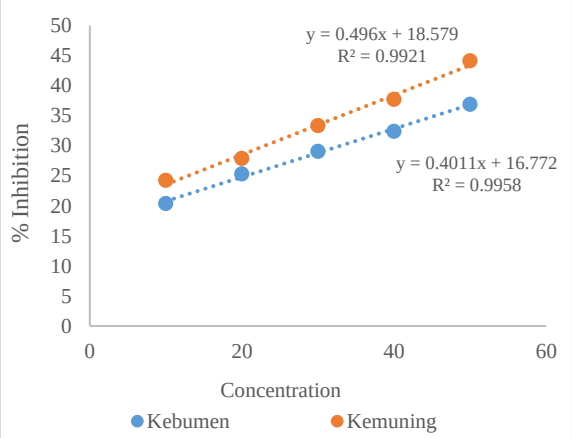

Figure 2. Linear Regression Curve of *Curcuma caesia* Roxb. Extract from Kemuning and Kebumen

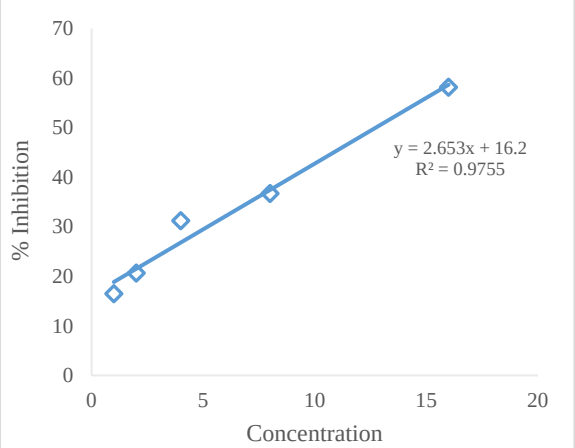

Figure 3. Linear Regression Curve of Vitamin C

In the antioxidant activity test using the linear regression equation, the IC_{50} value for the *C.caesia* Roxb. extract can be determined based on calculations by replacing the y value with 50, so the x or IC_{50} value obtained is an IC_{50} value of 63.349 µg/ml in the Kemuning sample and 82.842 µg / mL in the Kebumen sample. Antioxidant compounds can be classified into 5 groups, including strong antioxidants (IC_{50} <50 µg/mL), active antioxidants (IC_{50} is 50-100 µg/mL), moderate antioxidants (IC_{50} is 101-250 µg/mL), weak antioxidants (IC_{50} is 250-500 µg/mL) and inactive antioxidants IC_{50}> 500 µg/mL [23]. The results of the study showed that the extract of *C.caesia* Roxb. from Kemuning and Kebumen had active antioxidant activity since the IC_{50} value ranged from 50 to 100 µg/mL, while vitamin C used as a standard in this study had very strong antioxidant activity (12,740 µg/mL) since the IC_{50} value was less than 50 µg/mL, can be seen in **Table 3** and **Figure 3**.

Determination of environmental conditions

The results of determination of environmental conditions can be seen in **Table 4**. The two sampling locations, namely Kemuning and Kebumen, have different altitudes. One of the factors that affect the production of secondary metabolites is the environment, where temperature stress in Kebumen has a hotter temperature than in Kemuning. The average level of flavonoids from samples taken from Kemuning was 22.1904 mg qe/g, which was higher than the level of samples taken from Kebumen at low altitude, namely 19.3929 mg qe/g. The results of the antioxidant activity test showed that the antioxidant activity of the sample from Kemuning was higher than the sample from Kebumen. The lower the IC_{50} value, the higher the antioxidant activity. The results showed that a smaller concentration can inhibit 50% of free radicals.

Table 4. Environmental Parameter Values at Sampling Locations

Parameter	Unit	Kemuning	Kebumen
Temperature	°C	23	29
Air humidity	%	80.33	76
Soil pH	-	6,5	6,63
Light Intensity	Lx	10.595	27.354
Location altitude	Mdpl	866	28

Antioxidant activity is also influenced by the total amount of flavonoids and phenolics contained in black turmeric. *C.caesia* Roxb. at low altitudes produces lower total flavonoids due to the correlation between the morphological and anatomical properties of black turmeric with environmental factors, such as high light intensity and temperature, which can inhibit photosynthesis, so flavonoid formation is not optimal. Environmental conditions, such as high light intensity and temperature at low altitudes, can limit photosynthetic efficiency through several mechanisms. Higher light intensity means more packets of light called "photons" hitting the leaf. As the light intensity increases hitting the leaf, the rate of photosynthesis will increase since it receives more light to drive the photosynthesis reaction. However, once the light intensity is high enough, the rate will not increase again since there will be other factors that

limit the rate of photosynthesis. The limiting factor can be the number of chlorophyll molecules that absorb light. Too high light intensity can cause rapid chlorophyll photooxidation, thereby degrading chlorophyll [24].

Increasing light intensity causes more severe photosystem inactivation when leaves are dehydrated. Meanwhile, the greater decrease in photosystem II activity is mainly due to direct photoinhibition by light. The decrease in photosystem I activity is caused by an increased water loss from the leaves under higher light intensity. This phenomenon suggests that light plays different roles in the damage of different sites in the photosystem during leaf dehydration under high light [25]. Meanwhile, high temperatures, up to the maximum limit, may inhibit metabolic reactions in the formation of flavonoids. The reaction rate is greatly influenced by temperature usually, the higher the temperature, the faster the reaction until it reaches a certain threshold. However, the correlation between temperature and biochemical reactions in plants is rarely directly related because of other complex factors [26]. For example, the end products, such as sugar, can accumulate and block further reactions. In some reactions, the availability of nutrients can also be a limiting factor [27].

High light intensity and temperature cause air humidity to decrease, so the respiration process occurs more quickly, causing leaves to tend to lose water. Therefore, regulated photosynthesis is the result of the interaction between drought and excessive light or under double-stress conditions. High temperatures and light intensity can cause dehydration of leaf tissue due to increased evaporation, and high light causes photoinhibition, which can be directly induced by the absorbance of excess light energy. Both of them have the potential to reduce photosynthesis production in plants [28]. This fact shows the phenomenon in this study that although the light intensity is higher at low altitudes, black turmeric at low altitudes produces a smaller total flavonoid due to the inhibition of photosynthesis in producing metabolites. Based on the t-test, the result of the significance value is 0.001 <0.05, so it can be concluded that the growing place affects the difference in flavonoid levels and IC_{50} values.

Conclusion

At the samples from the Kemuning area, the level of flavonoids was 22.1904 mg qe/g, while at the Kebumen location, it was 19.3929 mg qe/g. The samples from the Kemuning location had an IC50 value of 63.349 mg/ml, while the samples from the Kebumen location had an IC50 value of 82.8423 mg/ml. Soil pH values of 6.50 and 6.63, temperature readings of 23.0 and 29.0 degrees Celsius, air humidity values of 80.33% and 76.00%, and light intensity readings of 10,595 and 27,354 lux were obtained from sample measurements conducted in Kemuning and Kebumen. It is clear that Curcuma caesia Roxb's flavonoid content and antioxidant activity are influenced by the plant's growing environment.

Acknowledgments: The author would like to thank the Institute of Research and Community Service, Sebelas Maret University Surakarta for facilitating the authors in obtaining a research grant.

Conflict of interest: None

Financial support: We would like to thank the Ministry of Higher Education, Research, and Technology for providing funding through graduate grants with contract number: 1076.1/UN27.22/PT.01.03/2024

Ethics statement: None

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