

Determination of Total Flavonoid And Antioxidant Activity of The Lempuyang Gajah (*Zingiber zerumbet* Sm.) Rhizome Fraction Using Spectrophotometry Method

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ABSTRACT

Oxidative stress induced by free radicals contributes to cellular damage and various degenerative diseases. *Zingiber zerumbet* (lempuyang gajah) rhizomes are rich in flavonoids, which exhibit potential antioxidant and anti-inflammatory properties. This study investigated the total flavonoid content and antioxidant activity of rhizome fractions to identify natural antioxidant sources. Rhizomes were subjected to liquid-liquid extraction, yielding water, ethyl acetate, methanol, and n-hexane fractions. Total flavonoids were quantified via colorimetric assay, and antioxidant activity was assessed using the DPPH method with UV-Vis spectrophotometry. The water fraction provided the highest extract yield (76.00%), followed by ethyl acetate (20.33%), methanol (2.67%), and n-hexane (1.00%). Flavonoid content was highest in the ethyl acetate fraction (29.25 mg QE/g), while methanol, water, and n-hexane fractions contained 5.64, 5.55, and 5.55 mg QE/g, respectively. Antioxidant assays revealed that the ethyl acetate fraction exhibited very strong activity with an IC₅₀ of 17.11 µg/mL, whereas methanol and water fractions showed strong activity with IC₅₀ values of 53.55 µg/mL and 64.44 µg/mL, respectively. The n-hexane fraction demonstrated negligible activity (IC₅₀ = 905.55 µg/mL). In conclusion, the water fraction offers the highest extraction yield, whereas the ethyl acetate fraction possesses the greatest flavonoid content and strongest antioxidant potential, highlighting its promise as a natural antioxidant source.

1. INTRODUCTION

Lempuyang gajah (*Zingiber zerumbet* Sm.) is one of the herbal plants that contains flavonoids, which have the potential to have high levels of antioxidants. Flavonoids are a group of polyphenols known for their ability to neutralize free radicals and reduce oxidative stress, both of which are important factors in the development of various degenerative diseases (Aminah et al., 2017). The free radical capture function of these compounds is made possible by the hydroxyl groups present in their structure, which allows them to prevent cell and tissue damage (Fatmawati & Rohmah, 2022).

The determination of total flavonoids from the lempuyang gajah rhizome fraction was carried out by measuring color formation using the AlCl₃ reagent (Oktaria & Marpaung, 2023). Quercetin is used as a comparator because it is a flavonoid of the flavonol group that has a keto group in C-4 and a

hydroxy group in C-3 or C-5. This process of discoloration is then measured with a spectrophotometer to determine the concentration of flavonoids. The addition of potassium acetate aims to maintain the stability of flavonoid compounds that have a keto group at the C-4 position and a hydroxyl group (–OH) at the C-3 or C-5 position (Ipandi et al., 2016).

Testing of antioxidant activity can be done by several methods, such as FRAV, ABTS, and DPPH. In this study, the method used is DPPH (1,1-Diphenyl-2-picrylhydrazyl) which utilizes DPPH as a free radical synthesis (Aryanti et al., 2021). The principle of the DPPH method is that there is an interaction between antioxidant compounds and free radicals of DPPH through the electron or hydrogen transfer mechanism, which converts DPPH into a stable molecule (Sukandiansyah et al., 2023). The advantages of this method are simple, fast, easy, and high sensitivity analysis of samples with small concentrations. In addition, this method is easier to apply because the radical compounds used are more stable compared to other methods (Scott, 2023).

2. MATERIALS AND METHODS

Research Design: This study employed an exploratory descriptive design. The descriptive component aimed to systematically analyze and present data to facilitate understanding and interpretation, while the exploratory approach was applied to investigate preliminary insights into the total flavonoid content and antioxidant activity of lempuyang gajah rhizome fractions. A quantitative approach was adopted, as the data consisted of measurable outcomes analyzed using statistical methods.

Materials: The materials used included distilled water (DPH), methanol (DPH), n-hexane (DPH), ethyl acetate (DPH), 70% ethanol extract of *Z. zerumbet* rhizomes, quercetin standard (Merck), AlCl_3 reagent (Merck), potassium acetate (Merck), and DPPH (SLI).

Equipment: The instruments utilized comprised a separation funnel (Pyrex), Erlenmeyer flasks (Pyrex), steam cup (local), water bath (Labtech), spatula (local), Cary 60 Agilent UV-Vis spectrophotometer, cuvettes, multichannel micropipette (Pipette), 5 and 10 μL micropipettes (Accumax), incubator (Mettler), oven (Labtech), analytical balance (Sartorius), volumetric flasks (Pyrex), sonicator (WLKS), beakers (Pyrex), spray bottle (local), aluminum foil, and brown vials (local).

Fractionation of 70% Ethanol Extract of Lempuyang Gajah Rhizomes

A 3 gram 70% ethanol extract of lempuyang gajah rhizomes was dissolved in 5 mL of methanol. The extract was then subjected to liquid-liquid fractionation by sequentially adding 200 mL of water and n-hexane, followed by phase separation. The aqueous layer was further partitioned with 200 mL of ethyl acetate, while the n-hexane layer was further extracted with 200 mL of methanol. This procedure yielded four distinct fractions: water, methanol, ethyl acetate, and n-hexane, as illustrated in Figure 1.

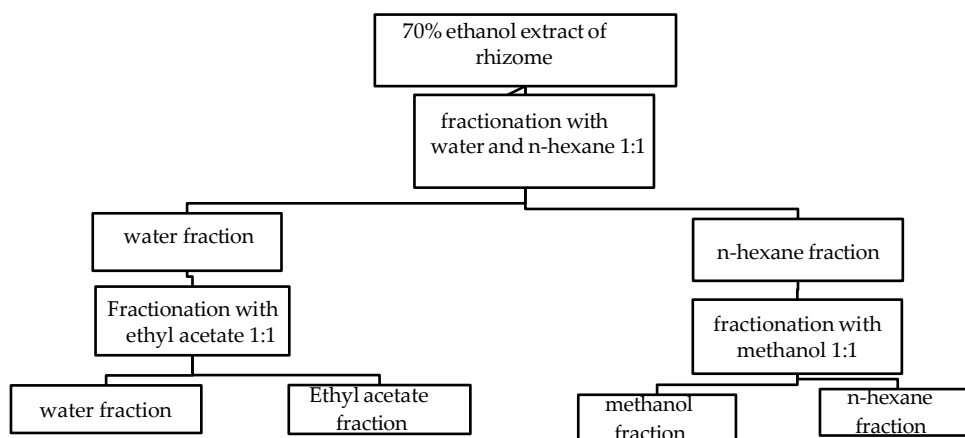


Figure 1. Chart Fractionation of ethanol extract 70% lempuyang gajah rhizome

$$\text{Fraction Yield}(\%) = \frac{\text{Weight of fraction obtained}}{\text{Initial Extract Weight}} \quad (1)$$

Determination of Total Flavonoid Content

Preparation of Test Solutions: Each fraction (10 mg) was dissolved in 10 mL of methanol in a volumetric flask, stirred for 5 minutes, filtered into a 10 mL volumetric flask, and the filter was rinsed with methanol to the calibration mark. *Preparation of Standard Solutions:* Quercetin (10 mg) was dissolved in methanol to prepare a 1,000 ppm stock solution, which was subsequently diluted to obtain standard solutions with concentrations of 25, 50, 60, 75, and 100 µg/mL.

Flavonoid Assay: Aliquots of 0.05 mL from each sample and standard solution were mixed with reagents according to Table 1, including 2% AlCl₃, potassium acetate (CH₃CO₂K), and methanol, and brought to the final volume with distilled water. The mixtures were shaken and incubated at room temperature for 30 minutes. Absorbance was measured at 435 nm using a UV-Vis spectrophotometer.

Table 1. Total flavonoid content and dilution and supplementation solution

No.	Volume Quercetin (mL)	Methanol Volume (mL)	AlCl ₃ volume 2% (mL)	Volume CH ₃ CO ₂ K (mL)	Air Volume (mL)	Quercetin Concentration (µg/mL)
1	0.05	0.15	0.01	0.01	0.28	25
2	0.05	0.15	0.01	0.01	0.28	50
3	0.05	0.15	0.01	0.01	0.28	60
4	0.05	0.15	0.01	0.01	0.28	75
5	0.05	0.15	0.01	0.01	0.28	100

The average absorbance of the samples was substituted into the linear regression equation derived from the quercetin calibration curve to determine the total flavonoid content of each fraction. The flavonoid concentration was expressed as mg quercetin equivalents per gram of extract (mg QE/g) and subsequently converted to % (w/w) following the formula described by (Umiyati, 2021).

$$\text{Total of flavonoid} = \frac{X.V.FP}{g} \quad (2)$$

Information:

X= Concentration (ppm)

V= Sample solution volume (mL)

FP= Dilution factor of the sample solution

g = Sample weight (g)

Determination of Antioxidant Activity

Preparation of Samples: Each fraction (10 mg) was dissolved in methanol and adjusted to a final volume of 10 mL in a volumetric flask, followed by thorough homogenization. A 150 ppm DPPH stock solution was prepared by weighing 15 mg of DPPH, dissolving it in methanol, and diluting to 100 mL in a volumetric flask, followed by homogenization.

Antioxidant Activity Assay: The antioxidant activity of each fraction was evaluated using five concentration levels: 40, 80, 160, 320, and 640 ppm. For each concentration, appropriate volumes of the 1000 ppm sample stock solutions (0.2, 0.4, 0.8, 1.6, and 3.2 mL) were mixed with 1 mL of 150 ppm DPPH solution, and methanol was added to reach a final volume of 5 mL. Blank solutions were prepared by mixing 1 mL of 150 ppm DPPH solution with 4 mL of methanol, as summarized in Table 2.

Table 2. DPPH reagent dilution and addition series

Series	Sample (mL)	Metanol (mL)	DPPH 150 ppm (mL)	Concentration (ppm)
Blank	0	4	1	0
1	0.2	3.8	1	40
2	0.4	3.6	1	80
3	0.8	3.2	1	160
4	1.6	2.4	1	320
5	3.2	0.8	1	640

Incubation and Determination of Antioxidant Activity: The reaction mixtures were incubated at 37 °C for 30 minutes. Following incubation, absorbance was measured at 515 nm using a UV-Vis spectrophotometer. The percentage of inhibition (% inhibition) was calculated according using the equation (3) (Maravirnadita et al., 2019).

$$\% \text{ inhibition} = \frac{\text{DPPH Absorbance} - \text{absorbance of the test sample}}{\text{DPPH Absorbance}} \times 100\% \dots \dots \dots (3)$$

The % inhibition values obtained for each concentration were used to construct a linear regression curve (% inhibition versus sample concentration in ppm) following the equation $y=a+bx$. The IC_{50} value, representing the concentration required to inhibit 50% of DPPH radicals, was determined by substituting $y=50$ into the regression equation and solving for x . This procedure enabled the quantification of the IC_{50} for each sample based on its corresponding linear regression parameters.

3. RESULTS AND DISCUSSIONS

Results of Fractionation of Lempuyang Gajah Rhizome Extract

The 70% ethanol extract of lempuyang gajah rhizomes was fractionated using four solvents of varying polarity—water, methanol, ethyl acetate, and n-hexane—yielding the results summarized in Table 4. The water fraction, with a polarity index of 10.2, produced the highest mass (2.28 g) and yield (76.00%), indicating a predominance of water-soluble metabolites. The ethyl acetate fraction, characterized as semi-polar (polarity index 4.4), yielded 0.61 g (20.33%), followed by the methanol fraction (polarity index 5.1) at 0.08 g (2.67%), and the nonpolar n-hexane fraction (polarity index 0.1) with the lowest yield of 0.03 g (1.00%) (Yana, Adhiksana, & Amborowati, 2023).

These findings suggest that the ethanol extract of *Z. zerumbet* is dominated by polar constituents, consistent with the high solubility of polar phytochemicals in aqueous media. The efficiency of fractionation indicates successful extraction of bioactive compounds, particularly those soluble in polar solvents. The solvent polarity index plays a critical role in determining the extraction efficiency of specific metabolites. Highly polar solvents such as water effectively extract flavonoids and glycosides, while semi-polar solvents like ethyl acetate are capable of dissolving moderately polar compounds. Conversely, nonpolar solvents such as n-hexane preferentially extract lipophilic constituents, resulting in lower yields when applied to polar-rich plant matrices (Sholikhah, Riyanti, & Wahyono, 2023).

Table 4. Fractionation Yield of Elephant Lempuyang Rhizome Extract

No.	Faction Name	Weight of Condensed Fraction(g)	Rendemen (%)
1	Water fraction	2,28	76,00
2	Methanol fraction	0,08	2,67
3	Ethyl acetate fraction	0,61	20,33
4	N-hexane fraction	0,03	1,00
	Total	3,00	100

Total Flavonoid Content

In the determination of total flavonoid content, samples were reacted with AlCl_3 to form a stable complex (Ipandi et al., 2016). The colorless AlCl_3 reagent interacts with flavonoid compounds, producing a yellow complex that remains stable under alkaline conditions but tends to degrade in acidic environments, leading to a bathochromic shift (Sari & Hastuti, 2020). The reaction mechanism is illustrated in Figure 2.

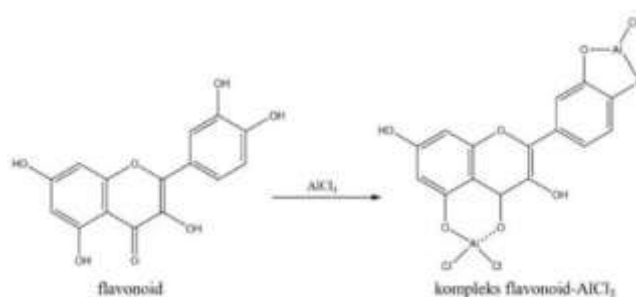


Figure 2. Reaction of the formation of flavonoid complex compounds - AlCl_3

The addition of potassium acetate helps maintain the visible wavelength range, enhancing measurement consistency (Aminah et al., 2017). The reaction mixtures were incubated for 30 minutes prior to measurement to ensure complete complex formation between AlCl_3 and flavonoid compounds, thereby optimizing color intensity.

Theoretically, a compound's maximum absorbance wavelength remains constant regardless of concentration, although absorbance intensity varies proportionally (Ipandi et al., 2016). Therefore, quantitative analysis is performed at the compound's maximum wavelength to ensure high sensitivity and accuracy. For quercetin, the optimal wavelength lies within 400–500 nm (Sari & Hastuti, 2020). In this study, absorbance measurements were conducted at 435 nm, as presented in Figure 3.

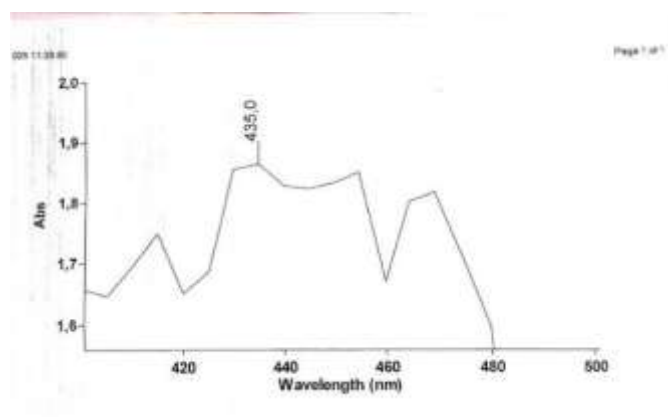


Figure 3. Maximum wavelength curve of standard quercetin

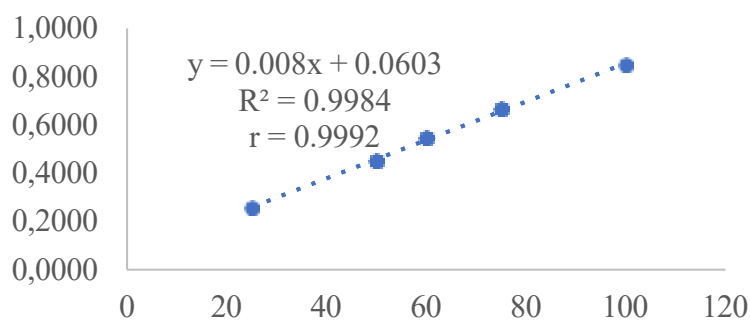


Figure 4. Quercetin Standard Calibration Curve

The absorbance values measured at the maximum wavelength were used to construct a calibration curve for determining total flavonoid content, as illustrated in Figure 4. The calibration curve represents the relationship between concentration and absorbance. When following the Lambert–Beer law, the standard curve forms a straight line with a correlation coefficient (r) approaching ± 1 , indicating a strong linear relationship between concentration and absorbance (Rohman et al., 2024). This linearity implies that an increase in concentration corresponds proportionally to an increase in absorbance.

The total flavonoid content of each lempuyang gajah rhizome fraction was calculated by substituting the sample absorbance value (y) into the linear regression equation ($y = ax + b$) derived from the quercetin standard calibration curve to obtain the sample concentration (x) (Nopianti, 2022). The obtained concentration values were then applied to the formula for determining total flavonoid content. The results of these calculations are summarized in Table 5.

Table 5. Total Flavonoid Level Results

Sample	Absorbansi	concentration	
		(ppm)	concentration (mg QE/g extract)
Water fraction	0,1047	5,55	5,55
Methanol fraction	0,1054	5,64	5,64
Ethyl Acetate Fraction	0,2943	29,25	29,25
N-Hexan Faction	0,1047	5,55	5,55
Total			45,99

Although the water fraction has the highest yield, the flavonoid content is actually lower. This suggests that although many compounds are soluble in the water fraction, not all of them are flavonoids or quercetin, the water fraction may contain a variety of other compounds, such as glucose, that do not have flavonoid levels. The methanol and n-hexane fractions also show a similar pattern, where the weight of the smaller fraction is not always directly proportional to the levels of flavonoids produced. These findings confirm that yield cannot be used as the only indicator to determine the flavonoid content in extracts, and that the selection of the right solvent is essential in the process of extracting bioactive compounds.

The ethyl acetate fraction exhibited the highest flavonoid concentration among all tested fractions. This observation can be attributed to the chemical characteristics of ethyl acetate, which provide superior extraction efficiency for flavonoid compounds that are more soluble in semi-polar solvents. Although the total yield of the ethyl acetate fraction was smaller compared to other fractions, its extraction effectiveness resulted in a significantly higher flavonoid content. This finding indicates that

the intermediate polarity of ethyl acetate facilitates stronger interactions with flavonoid molecules (Manalu et al., 2022).

Conversely, the aqueous fraction, despite yielding the largest extract mass, showed a lower flavonoid concentration. This suggests that although many compounds are soluble in water, not all of them are flavonoids or quercetin derivatives. The aqueous extract may contain various other polar compounds such as sugars or glycosides, which do not contribute to total flavonoid levels. Similarly, the methanol and *n*-hexane fractions followed a comparable trend, indicating that the fraction yield does not necessarily correlate with flavonoid concentration. These results emphasize that solvent selection based on polarity is a critical factor influencing the efficiency of bioactive compound extraction, particularly flavonoids.

Antioxidant Activity Results

The antioxidant activity of the water, methanol, ethyl acetate, and *n*-hexane fractions obtained from the ethanol extract of lempuyang gajah rhizomes was evaluated using the DPPH free radical scavenging assay. This method operates on an electron transfer mechanism, in which antioxidant molecules donate electrons or hydrogen atoms to neutralize the DPPH radical, leading to a measurable decrease in absorbance. The reduction in absorbance corresponds to a color change of the DPPH solution from deep purple to yellow, signifying the formation of the stable DPPH₂ compound (Nurkhasanah et al., 2023). This reaction demonstrates the capacity of antioxidant compounds to stabilize free radicals through electron delocalization within their aromatic rings (Maravirnadita et al., 2019).

The antioxidant potential of each fraction was expressed in terms of the IC₅₀ value, which represents the concentration of the sample required to inhibit 50% of DPPH radicals. A lower IC₅₀ value indicates stronger antioxidant activity, whereas a higher IC₅₀ reflects a weaker radical-scavenging capacity.

Table 6. Antioxidant Activity Results

Sample	IC₅₀ Value	Classification of Antioxidants
Water fraction	64,44	Strong
Methanol fraction	53,55	Strong
Ethyl acetate fraction	17,11	Very Powerful
N-hexane fraction	905,55	Not having

The results revealed that the ethyl acetate fraction exhibited the strongest antioxidant activity among all tested fractions. This finding is consistent with its high flavonoid content, suggesting that the elevated concentration of flavonoids contributes significantly to its superior free radical scavenging capacity. The effectiveness of this fraction can be attributed to the chemical structure of flavonoids, which possess hydroxyl groups capable of donating hydrogen atoms or electrons to stabilize reactive oxygen species. The semipolar nature of ethyl acetate further enhances the extraction of these bioactive compounds, optimizing antioxidant potential.

The water and methanol fractions demonstrated lower antioxidant activity compared to the ethyl acetate fraction, although both were still categorized as strong antioxidants. This trend corresponds with their relatively lower flavonoid concentrations. The aqueous fraction, while yielding a large amount of extract, may contain other polar compounds such as sugars or organic acids that exhibit moderate antioxidant effects but lack the potency of flavonoids. Meanwhile, the methanol fraction showed strong antioxidant activity, consistent with its relatively high flavonoid levels, ranking just below the ethyl acetate fraction in potency.

In contrast, the *n*-hexane fraction exhibited negligible antioxidant activity. Despite containing measurable amounts of flavonoids, the nonpolar nature of *n*-hexane limits its ability to extract polar antioxidant constituents effectively. According to Manalu et al. (2022), *n*-hexane fractions of plant extracts typically display weak or absent antioxidant activity, even when flavonoid levels appear comparable to those of more active fractions, such as water or methanol extracts. The low antioxidant response of the *n*-hexane fraction, with a flavonoid content of 5.55 mg QE/g extract, may also be attributed to the presence of non-reactive flavonoid analogs, such as diketo or dienol derivatives, which lack significant radical scavenging functionality (Figure 5).

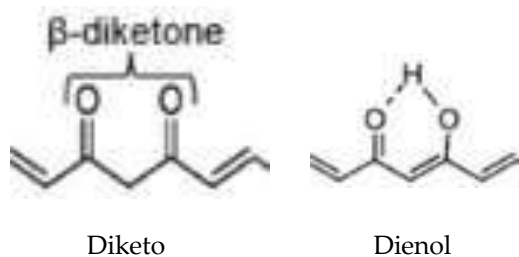


Figure 5. Diketo and Dienol

A notable limitation of this method lies in the use of AlCl_3 as a complexing reagent, which lacks specificity toward flavonoid compounds. This non-selectivity can potentially lead to false positive results, particularly in the determination of total flavonoid content in the *n*-hexane fraction. The reaction mechanism of AlCl_3 with flavonoids does not target the flavonoid core structure directly; instead, it interacts with adjacent diketo or dienol functional groups. These groups are typically located between the hydroxyl group on the A-ring (benzene) and the carbonyl group on the C-ring (pyran) of flavonoid molecules. Consequently, AlCl_3 may also react with other compounds possessing similar structural motifs, thereby compromising analytical specificity. This observation aligns with the findings of Aji et al. (2022), who reported that flavonoid determination using the AlCl_3 colorimetric method can also yield positive reactions with curcumin and its derivatives, which share similar diketo or dienol functional characteristics.

4. CONCLUSION

Based on the findings of this study, the following conclusions can be drawn:

- a. Fractionation outcome: The fractionation of the 70% ethanol extract of lempuyang gajah rhizomes yielded four fractions with varying proportions. The water fraction exhibited the highest yield (76.00%), followed by the ethyl acetate fraction (20.33%), methanol fraction (2.67%), and *n*-hexane fraction (1.00%).
- b. Total flavonoid content: Quantitative analysis of total flavonoid levels revealed that the ethyl acetate fraction contained the highest concentration, reaching 29.25 mg QE/g extract. The methanol and water fractions exhibited moderate flavonoid contents of 5.64 mg QE/g and 5.55 mg QE/g, respectively, while the *n*-hexane fraction displayed a comparable level to the water fraction (5.55 mg QE/g extract).
- c. Antioxidant activity: The antioxidant activity assay demonstrated that the ethyl acetate fraction possessed the strongest radical scavenging potential, with an IC_{50} value of 17.11 $\mu\text{g/mL}$, classified as *very strong*. The methanol and water fractions exhibited *strong* antioxidant activity, with IC_{50} values of 53.55 $\mu\text{g/mL}$ and 64.44 $\mu\text{g/mL}$, respectively. In contrast, the *n*-hexane fraction showed negligible antioxidant activity, indicated by an IC_{50} value of 905.55 $\mu\text{g/mL}$.

Overall, these findings suggest that the ethyl acetate fraction of lempuyang gajah rhizome ethanol extract possesses the highest flavonoid content and strongest antioxidant potential, highlighting its potential as a promising source of natural antioxidants.

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