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Original Article

Antioxidant Activity of Acid Hydrolyzed Fraction and Ethanol Extract of *Musa balbisiana* BBB and *Hylocereus polyrhizus*

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Abstract

Background: Plants contain secondary metabolites, including flavonoids, which are bound to glycosides or not in the form of free molecules. The hydrolysis treatment in this study was expected to increase antioxidant activity by converting bound forms into free molecules.

Methods: To evaluate the antioxidant activity, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test was used. The obtained half-maximal inhibitory concentration (IC_{50}) value indicates antioxidant activity. This study aimed to determine the effect of acid hydrolysis treatment on the hexane-insoluble fraction of Kepok banana heart (*Musa balbisiana* BBB) and water fraction of red dragon fruit skin (*Hylocereus polyrhizus*) on increasing DPPH radical scavenging activity. Acid hydrolysis was carried out by adding 2 N HCl and then measuring antioxidant activity through the DPPH radical scavenging test using a UV-Vis spectrophotometer. To determine the compounds in the hexane and water-insoluble fractions that act as antioxidants, qualitative analysis was used and total phenolic and flavonoid contents were determined.

Results: The hexane-insoluble fraction of Kepok banana heart hydrolyzed for 3 hours (IC50 25.00 µg/mL, total phenolics 38.40 µg/mL, and total flavonoids 15.90 µg/mL) and the water fraction of red dragon fruit peel hydrolyzed for 1 hour (IC50 7.74 µg/mL, total phenolics 62.50 µg/mL, and total flavonoids 29.70 µg/mL) showed the highest antioxidant activity.

Conclusion: The highest antioxidant activity of Kepok banana heart was observed in the hexane-insoluble fraction hydrolyzed for 3 hours, while the highest antioxidant activity of the red dragon fruit was observed in the water fraction hydrolyzed for 1 hour. Therefore, acid hydrolysis of the hexane-insoluble fraction of Kepok banana heart for 3 hours and hydrolysis of water fraction of red dragon fruit skin for 1 hour could increase antioxidant activity.

Keywords: Kepok banana heart (*Musa balbisiana* BBB), Red dragon fruit skin (*Hylocereus polyrhizus*), DPPH, Acid hydrolysis, Radical scavenging

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Introduction

Antioxidants play an important role in self-defense mechanisms against cardiovascular disease, cancer, arthritis, asthma, and diabetes. Natural antioxidants can minimize oxidative stress, but synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) can be harmful to health, particularly when consumed in large quantities. Furthermore, the search for effective, safe, and novel natural antioxidant compounds has been increasing in recent years. Herbs and spices are natural sources of antioxidants. The use of fresh herbs in food may contribute to daily antioxidant needs. Phenolic compounds are among the primary antioxidants found in herbs and spices .[1]

Kepok banana heart (*Musa balbisiana* BBB) contains various bioactive compounds rich in antioxidants including phenolic compounds, flavonoids, alkaloids, tannins, and saponins that have the potential to act as free radical scavengers. Research on this plant has shown DPPH radical scavenging activity with an IC50 value of 439.12 µg/mL.[2] The red dragon fruit (*Hylocereus polyrhizus*) seed also has emerged as a noteworthy source of antioxidants with free radical scavenging properties. Ethanol extract of *H. polyrhizus* seeds was able to scavenge 76.76% of free radicals at 1000 µg/mL. Ethanol extract, chloroform extract, and hexane extract had relatively the same level of inhibitory effects on lipid oxidation (95.60%,

98.90%, and 88.46%, respectively).[3]

In plants, flavonoid glycosides are generally less effective than their aglycone forms.[4] Ethyl acetate is considered a semi-polar solvent that is able to attract compounds with semi-polar characteristics such as flavonoid aglycones.[5] Breaking the bonds with glycosides by hydrolysis is a way to obtain free flavonoids. For example, hydrolysis of the water fraction of mengkudu leaves, brotowali stems, and talok fruit is known to increase DPPH radical scavenging activity because hydrolysis is able to free flavonoid aglycones from their glycoside forms.[6] Hydrolysis can be carried out in acidic or basic conditions by producing O-flavonoid glycosides with different cleavage sites. Based on previous studies, the yield of acid-hydrolyzed GBR (Germinated Brown Rice) extract is greater than that of base-hydrolyzed GBR.[7]

From previous research conducted on banana heart and red dragon fruit skin, no treatment with hydrolyzed fractions has ever been carried out. Therefore, this study was conducted to determine the extent of the increase in DPPH radical scavenging activity from the insoluble phase of acid-hydrolyzed hexane of kepok banana blossom and the acid-hydrolyzed water fraction of red dragon fruit peel. Acid hydrolysis treatment on the hexane-insoluble fraction of Kepok banana blossom and the water fraction of red dragon fruit peel can free flavonoid aglycones from their glycoside forms, so that it can increase antioxidant activity. The radical scavenging activity of the hydrolyzed fraction of Kepok banana blossom and red dragon fruit peel was determined based on the IC50 value obtained using the DPPH radical scavenging test.

Materials and Methods

Kepok Banana (Musa balbisiana BBB), red dragon fruit (Hylocereus polyrhizus) peels, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich), ethanol 96% (technical), n-hexane (technical), ethyl acetate (technical), methanol p.a. (Merck, Germany), ethanol p.a. (Merck, Germany), distilled water, chloroform p.a., formic acid p.a., silica plate 60 F254 (Merck, Germany), quercetin (Sigma-Aldrich), 2N HCl (Merck, Germany), filter paper, aluminum foil, AlCl3 spot detector, $FeCl_3$ spot detector, DPPH and ammonia spot detectors, and sitroborate were used in this study.

Plant Identification, Extraction, and Fractionation

Plant identification was conducted in the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy Universitas Gadjah Mada. Extraction was conducted by maceration of 1000 g of dry powder of banana blossom with 70% ethanol (1:7) and 400 g of red dragon fruit skin with 2.5 L of 96% ethanol (technical). The filtrate was evaporated until a thick extract was obtained.

The extract was fractionated using the solid-liquid method. The extract of banana blossom and red dragon fruit peel was fractionated with 600 mL of n-hexane by stirring. The clear filtrate was evaporated and stored as the hexane fraction. Meanwhile, the hexane-insoluble phase was then fractionated using 600 mL of ethyl acetate. The clear filtrate was evaporated to produce the ethyl acetate fraction. The insoluble ethyl acetate phase was stored as the water fraction.

Preparation of Hydrolyzed Fraction

The hydrolyzed fraction was prepared by dissolving the solid extract in 50 mL of 96% ethanol and 50 mL of 2 N HCl. Then, it was refluxed for 10 minutes, 1 hour, and 3 hours. The mixture was then partitioned with 40 mL of ethyl acetate. There were two phases, namely the ethyl acetate soluble phase at the top and the ethyl acetate insoluble solid phase. The ethyl acetate insoluble solid phase was partitioned again with ethyl acetate until the phase at the top became clear. The ethyl acetate soluble phase was filtered with anhydrous sodium sulfate and then evaporated to obtain a thick solution which was the hydrolyzed fraction.

Determination of Total Phenolic and Total Flavonoids

Total phenolics were determined by spectrophotometric method using Folin-Ciocalteu reagent. Gallic acid was used as the comparator. However, total flavonoids were determined by spectrophotometric method using AlCl3 reagent. Quercetin was used as the reference standard for comparison.

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) test was conducted to determine the compounds contained in each extract and fraction. In this TLC test, the stationary phase was silica gel 60 F254 and the mobile phase was chloroform-ethyl acetate-methanol with a ratio of 5:1:1v/v for Kepok banana blossom and chloroform-ethyl acetate-methanol with a ratio of 4:2:1 v/v for red dragon fruit skin. The test sample was used at a concentration of 5 mg/mL. The spots that appeared were then observed under visible light, as well as UV light at 254 and 366 nm. To detect the presence of flavonoids, ammonia vapor (NH3), AlCl3, FeCl3, and DPPH spray reagents were used.

Antioxidant Test

The radical scavenging activity test was carried out by reacting the test solution with DPPH solution. The test solution was added to 1 mL of 0.4 mM DPPH solution and methanol p.a. in a volume of 5 mL. Ethanolic extract and fractions were made at concentrations of 10, 15, 20, 25, 30, and 35 µg/mL, while quercetin was prepared at 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 µg/mL. The time obtained from determining the operating time was used for the incubation of the test solution before reading its absorbance. The highest absorbance wavelength of DPPH was produced in blank methanol p.a. Measurement of DPPH radical scavenging activity for each series of test compound concentrations was replicated 2 times. Afterwards, the color control absorbance reading was

also carried out. Previously, negative control absorbance readings were carried out using a mixture of 1 mL of 0.4 mM DPPH solution and 4 mL of methanol p.a.

Results and Discussion

Extraction and Fractionation

A filtrate in the form of a thick extract weighing 17.20 g (yield 1.72%) was produced from the powder of Kepok banana heart, and a filtrate in the form of a thick extract weighing 48.80 g (yield 12.20%) was produced from the red dragon fruit peel.

The results of the fractionation of the Kepok banana flower, namely the n-hexane fraction, were obtained as much as 0.70 g (yield 4.07%), the ethyl acetate fraction as much as 3.60 g (yield 20.93%), the water fraction as much as 12.20 g (yield 70.93%), the hexane-insoluble fraction hydrolyzed for 10 minutes as much as 0.42 g (yield 14.00%), the hexane-insoluble fraction hydrolyzed for 1 hour as much as 0.80 g (yield 26.67%), and the hexane-insoluble fraction hydrolyzed for 3 hours as much as 0.43 g (yield 14.33%). Meanwhile, the results of the fractionation of red dragon fruit skin, namely the n-hexane fraction, were obtained as much as 0.00 g (yield 0.00%), the ethyl acetate fraction as much as 1.86 g (yield 7.46%), the water fraction as much as 31.66 g (yield 126.94%), the 10-minute hydrolyzed water fraction as much as 2.1 g (yield 26.99%), the 1-hour hydrolyzed water fraction as much as 2.22 g (yield 28.53%), and the 3-hour hydrolyzed water fraction as much as 2.17 g (yield 27.89%).

Total Phenolic and Total Flavonoid Contents

Phenolic compounds have antioxidant activity with a role as free radical inhibitors, metal chelators, lipoxygenase inhibitors, and free radical scavengers. However, flavonoid compounds have water-soluble properties and are included in the phenolic compound group because they can experience color changes when base or ammonia is added, both in chromatograms and in solutions. Flavonoids contain a conjugated aromatic system and show strong absorption bands in the UV-Vis region.[8]

Based on the data presented in Tables 1 and 2, the ethanol extract of Kepok banana heart had the highest total phenolic content, while the 1-hour hydrolyzed water fraction of the red dragon fruit skin had the highest total phenolic content. The results of the total flavonoid test showed that the 3-hour hydrolyzed hexane-insoluble fraction of the Kepok banana heart had the highest total flavonoid content, while the 1-hour hydrolyzed water fraction of the red dragon fruit skin had the highest total flavonoid content.

Thin Layer Chromatography

The compound content test was carried out using the thin layer chromatography method. A UV light of 254 nm was used to observe other compounds that were not visible under the visible light and could produce several previously invisible chromatogram spots. The appearance of dark colors was due to flavonoid compounds that absorb UV light at a wavelength of 254 nm.[9] The occurrence of this blackout indicates that the compounds in the chromatogram are able to absorb UV light at 254 nm. Flavonoids can also be identified as purple, pink, and orange spots when observed under UV light at 366 nm.[10] Meanwhile, flavones, flavanone glycosides, flavonols, and isoflavones can be identified as light blue spots when observed under UV light at 366 nm.[9]

Flavonoid content can be detected with the addition of AlCl3, FeCl3, and ammonia reagents. The appearance of yellowish green fluorescence under UV light at 366 nm indicates the presence of flavonoids. AlCl3 can produce yellow green fluorescent spots due to its reaction with the 5-hydroxy-flavonoid group.[9] In the chromatogram of the banana heart, all the spots that appear are yellow, except for one spot in the 3-hour hydrolysis fraction. In the red dragon fruit skin, yellow fluorescence was observed in quercetin, which was used as the reference standard. However, in the ethanol extract, ethyl acetate fraction, water fraction, 10-minute hydrolyzed water fraction, 1-hour hydrolyzed water fraction, and 3-hour hydrolyzed water fraction, what appears is purple fluorescence.

The purpose of spraying FeCl3 is to detect simple phenol compounds which are marked by the appearance of strong green, red purple, blue, or black spots.[11] The spots that appear on the heart of the kepok banana show spots that appear only on the quercetin, which shows a black color. Meanwhile, the results of spraying FeCl3 on the ethanol extract, ethyl acetate fraction, water fraction, 10-minute hydrolyzed water fraction, 1-hour hydrolyzed water fraction, and 3-hour hydrolyzed water fraction of red dragon fruit skin did not show any spots. Therefore, simple phenol compounds were not detected. The appearance of a faint black color is only on quercetin which is a comparator and should indeed indicate the presence of simple phenols. Observations were also

Table 1. IC50 Values, Total Phenolics, and Total Flavonoids in Kepok Banana

Sample	IC_{50} (µg/mL)	Total phenolics (µg/mL)	Total flavonoids (µg/mL)
Ethanol extract	103.00	54.10	0.363
Ethyl acetate fraction	51.00	24.10	8.61
Water fraction	200.00	2196	0.00
Hexane-insoluble fraction hydrolyzed for 10 minutes	48.00	24.80	9.50
Hexane-insoluble fraction hydrolyzed for 1 hour	27.00	29.74	14.40
Hexane-insoluble fraction hydrolyzed for 3 hours	25.00	38.40	15.90

Table 2. IC50 Values, Total Phenolics, and Total Flavonoids in Red Dragon FruitSkin

Sample	IC_{50} (µg/mL)	Total phenolics (µg/Ml)	Total flavonoid (µg/mL)
Ethanol extract	64.00	16.20	5.90
Ethyl acetate fraction	50.00	8.60	8.60
Water fraction	68.00	13.90	4.10
Hexane-insoluble fraction hydrolyzed for 10 minutes	14.50	40.80	28.50
Hexane-insoluble fraction hydrolyzed for 1 hour	7.74	62.50	29.70
Hexane-insoluble fraction hydrolyzed for 3 hours	52.00	21.70	8.30

made on the chromatogram after being evaporated with ammonia. Evaporation with ammonia aims to increase detection sensitivity by producing color changes that can be used as a reference for a type of flavonoid. Flavonoid compounds in an extract after being given ammonia vapor show yellow spots in visible light observations, then will be blue under UV light at 366 nm.[12] Higher color intensity is directly proportional to its antioxidant activity.

The spots that appeared on the chromatogram of banana heart after exposure to ammonia vapor became clearer and more numerous, both below and above the Rf of quercetin. Most of the spots were fluorescent yellow, but the 3-hour hydrolysis fraction showed a fluorescent blue color. Meanwhile, the detection of UV light at 366 nm on the red dragon fruit skin shows the appearance of yellow fluorescent spots in the 10-minute hydrolyzed water fraction and the 1-hour hydrolyzed water fraction. The results of the TLC test after being sprayed with ammonia using visible light on the banana heart and the red dragon fruit skin are shown in Figure 1 and Figure 2, respectively.

Testing for antioxidant compounds can be performed by spraying DPPH solution on the TLC plate. Radical scavenging compounds will produce a yellow color when sprayed with DPPH solution.[14] This is shown in the chromatogram of the banana heart, all the spots that appear are yellow, while in the comparison, quercetin itself is brown. Meanwhile, on the red dragon fruit skin, no yellow spots were seen in any of the tests carried out, namely in the ethanol extract, ethyl acetate fraction, water fraction, 10-minute hydrolyzed water fraction, 1-hour hydrolyzed water fraction, and 3-hour hydrolyzed water fraction.

Flavonoid aglycones have superior antioxidant activity than their glycoside forms due to steric inhibition by their glycoside groups. This steric hindrance can cause the presence of large groups that prevent the active antioxidant group (flavonoid aglycone) from reacting with oxidants. Therefore, acid hydrolysis in the aqueous fraction can free flavonoid aglycones from their bonds with glycosides in plants.

Antioxidant Activity

The test was conducted on each extract and fraction with certain concentration variations. The higher the concentration, the higher the radical scavenging activity. The color of the solution which was originally purple changed to yellow after the test sample was added.

The DPPH test was used to measure the antioxidant properties of a compound based on its ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. In the DPPH test, a reaction occurs between the hydrogen atom released by the test material and the DPPH radical molecule to form a 1,1-diphenyl-2-picrylhydrazine compound with a yellow color.[13]

The median effective concentration (EC_{50}) or inhibition concentration (IC_{50}) parameter states the ability of a compound to scavenge radicals. The IC_{50} value is the concentration value of the antioxidant compound that is able to reduce 50% of the initial concentration of DPPH radicals. The DPPH test was chosen because it is simple and fast, and the instruments and materials are easy to obtain.[14]

In banana blossom and red dragon fruit skin, all fractions were measured for their activity at 6 concentrations with the same treatment, at a wavelength of 516.2 nm, and an operating time of 30 minutes. Quercetin and HCl control were used for comparison. In this study, color control was also used, namely the absorbance value of the compound without the addition of DPPH solution. The concentration of color control was adjusted to the concentration of each test sample. The absorbance of color control and HCL control in this study was 0.00.

The radical scavenging values by the fraction concentration series were entered into a linear regression equation to determine the IC50 values. The smaller the IC50 value, the greater the DPPH radical scavenging activity of the compound. The radical scavenging activity of the banana blossom extract and red dragon fruit peel was lower than that of the quercetin, which was 0.50 µg/mL. This is because pure quercetin was used as the reference standard,; however, the extract and fraction contain various compounds that may or may not exhibit radical scavenging activity. The IC50 values of each banana blossom fraction and red dragon fruit peel are shown in Tables 1 and 2.

Quantitative analysis such as the DPPH test needs to be tested for linearity and precision. Linearity measures the significant correlation between the two measured quantities, the concentration and DPPH radical scavenging activity in this case. An equation is considered to have good linearity if the linearity value is close to 1

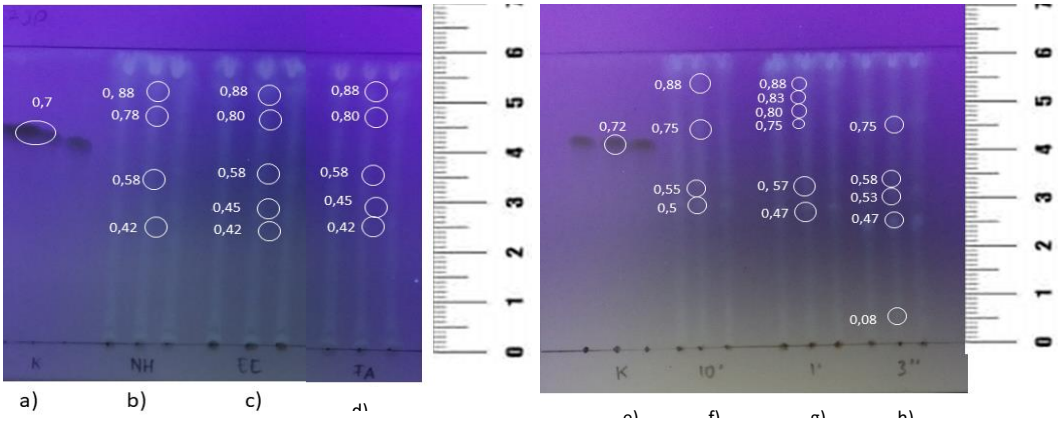

Figure 1. Chromatogram of ethanol extract of banana blossom and its fractions with silica gel 60 F254 as the stationary phase and chloroform-ethyl acetate-methanol as the mobile phase (5:1:1v/v) after being sprayed with NH3 reagent observed under UV light at 366 nm
Description: a) quercetin, b) ethanol extract, c) hexane fraction, d) hexane-insoluble fraction, e) quercetin, f) hexane-insoluble fraction hydrolyzed for 10 minutes, g) hexane-insoluble fraction hydrolyzed for 1 hour, h) hexane-insoluble fraction hydrolyzed for 3 hours

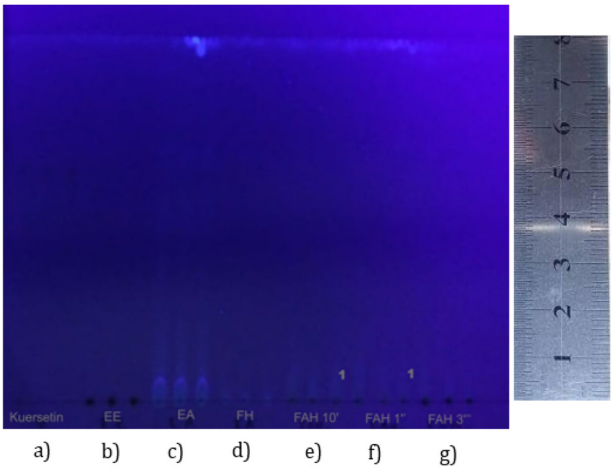

Figure 2. Chromatogram of ethanol extract of red dragon fruit skin and its fractions with silica gel 60 F254 as the stationary phase and chloroform: ethyl acetate: methanol (4:2:1 v/v) as the mobile phase after being sprayed with NH3 reagent observed under UV light at 366 nm
Description: a) quercetin; b) ethanolic extract; c) ethyl acetate fraction; d) water fraction; e) 10-minute hydrolyzed water fraction; f) 1-hour hydrolyzed water fraction; g) 3-hour hydrolyzed water fraction

or -1. In this study, the linearity value of all fractions was higher than 0.9, indicating good linearity.

Antioxidant activity before hydrolysis is related to its total phenolics. If the total phenolic value is high, the antioxidant activity measured before hydrolysis will be higher. However, the antioxidant activity after hydrolysis is related to its total flavonoids. If the total flavonoid value is high, the antioxidant activity measured after hydrolysis will be higher (the IC50 value is smaller).

Conclusion

The highest antioxidant activity of banana blossom was observed in the hexane-insoluble fraction hydrolyzed for 3 hours (IC50 25.00 µg/mL, total phenolics 38.40 µg/mL, and total flavonoids 15.90 µg/mL) and the highest antioxidant activity of red dragon fruit peel was observed in the water fraction hydrolyzed for 1 hour (IC50 7.74 µg/mL, total phenolics 62.50 µg/mL, and total flavonoids 29.70 µg/mL). Therefore, acid hydrolysis of the hexane-insoluble fraction of ethanol extract of banana blossom for 3 hours and acid hydrolysis of the water fraction of red dragon fruit peel for 1 hour can increase antioxidant activity.

Ethics statement

Not applicable.

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Conflict of interests declaration

The authors declare that they have no conflict of interest.

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Data availability statement

All data generated or analyzed from this research are included in this published.

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Consent for publication

Not applicable.

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