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BLACK FRUIT PLANT (*Haplolobus monticola*) ETHANOLIC EXTRACT AS A GOOD SOURCE OF CHEMICAL COMPOUND CONTENT WITH THE POTENTIAL AS A SAFE ANTIOXIDANT AND ANTIBACTERIAL AGENT

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Abstract

Black fruit is a native Papuan plant that faces extinction if it is not propagated. The availability of scientific information on the potential use of black fruit can help proliferate this plant. This study investigates the potential of black fruit plants in the leaves and flesh of the fruit using extraction, characterization, and extract activity testing. Extraction was carried out by maceration using a 96% ethanol solvent. Characterization was carried out by FTIR, UV-Vis spectrophotometers, and phytochemical screening. The antioxidant activity test was carried out by the DPPH method, the antibacterial test was carried out by the well diffusion method, and the toxicity test was carried out by the BSLT (Brine Shrimp Lethality Test) method. The results showed that the ethanol extract of black fruit flesh separated into two layers when stored in the refrigerator, while the leaf extract did not. Leaf extract is richer in phytochemical content compared to fruit flesh. Antioxidant and antibacterial activities were higher in leaf extracts than in fruit pulp extracts. Toxicity test results showed that the ethanol extract of black fruit leaves has the potential to be used as a food additive for antioxidant and antibacterial properties. The potential benefits of the ethanol extract of black fruit leaves and flesh can encourage cultivation and post-harvest management of this plant.

INTRODUCTION

The food system is a key sector in national and global development because it has a reciprocal relationship with other essential fields, namely the economy, socio-culture, health, and the environment. For example, ecosystem degradation [1], climate change [2], as well as depletion of natural resources and increased greenhouse effect [3] can cause and are caused by changes in food systems. Currently, the main problems of the food system are food safety, security, sustainability, and nutrition [4]. The extinction of food-producing plants is one of the food issues related to food sustainability that needs attention. Species extinction can be caused by a lack of utilization information, thereby reducing interest in breeding certain species. Therefore, research related to the potential benefits of threatened plants needs to be carried out.

One of the endemic food plants that is threatened with extinction and needs attention is the black fruit plant. The black fruit plant (Haplolobus monticola) has the local name Pi Airawi (Wandamen) and is a medium-sized tree, 24-30 m high, main stem diameter of ± 50 cm, and single compound leaves. The fruit is oval, 2.5 cm long, 1.2-1.5 cm in diameter. Young fruit is green, ripe fruit is black. Single seed, 1.8-2.2 cm long, 0.7-1 cm in diameter. Black fruit is reported to contain protein, fat, and vitamin C which are generally higher than other types of fruit that are often consumed by the public. Black fruit fat content is 14.73 g and vitamin C content is 277.45 mg [5].

Research on black fruit plants is still relatively new and not much has been done. Initially, research was conducted in the forestry sector to study the ethnobotany of black fruit plants and efforts to conserve them using this plant cultivation approach [5]. Ecological studies of black fruit plant culture have been carried out for

adaptive management [6]. Studies on the effect of the external environment on the content of secondary metabolites in the leaves [7] and black fruit [8] have also been carried out. In that study, secondary metabolites were obtained from black fruit leaf extract, namely alkaloids, flavonoids, and tannins. Research on the utilization of tannin extract from black fruit leaves as a natural corrosion inhibitor has been carried out [9]. Exploratory research on the larvicidal ability of Haplolobus monticola leaf extract is quite high which is thought to be due to the content of flavonoids, alkaloids, and tannins [10], but no phytochemical analysis has been carried out in this regard. The antibacterial ability of the ethyl acetate extract of black fruit seeds against Aeromonas hydrophila bacteria that causes freshwater fish disease is quite high [11]; [12]. Black fruit plant methanol extract contains high levels of saponins and tannins. The results of the antibacterial activity test on E. coli bacteria showed strong activity with an inhibition zone diameter of 11 mm and B. subtilis bacteria showed moderate activity with an inhibition zone with a diameter of 8 mm [8].

These studies have tested the antibacterial and antioxidant parts of the Haplolobus monticola plant, but research on black fruit plants which are used in unripe fruit (a), ripe fruit (b), leaf fruit (c), ripe fruit flesh (d) with the direction of product engineering has never been carried out, especially using ethanol extract. In the study of extracting antibacterial substances from jalapeño peppers, it was found that methanol and ethanol were solvents that produced extracts with the highest antibacterial activity compared to other solvents [13]. The reason for selecting ethanol as the extraction solvent in this study was due to its lower toxicity and better performance than methanol in extracting antibacterial compounds [14].

Figure 1. Black fruit: unripe fruit (a), ripe fruit (b), leaf fruit (c), and ripe fruit flesh (d).

EXPERIMENT

Material

The materials used in this study were leaves and black fruit flesh, DPPH, phytochemical screening reagents (99% purity Merck, Germany), and 96% ethanol. Media for bacterial culture, namely NB (Nutrient Broth) liquid media and NA (Nutrient Agar) solid media, distilled water, cotton, tissue, spirits, Whatman filter paper (Filter Paper) No. 42, alcohol, aluminum foil, disc paper.

Instrumentation

The equipment used in this research was FTIR IR-Prestige 21 from Shimadzu, UV-Vis spectrophotometer Uv-1800 from Shimadzu, used in chemical characterization. Equipment for toxicity and antibacterial tests, namely water baths, hot stirrer plates, stirrers, metler analytical balances, vortexes, incubators, laminar flow, refrigerators, glassware, and others.

Procedure

Extraction, Characterization, and Phytochemical Tests

Water was used to clean samples of black foliage and fruits. Samples of black leaves and fruit were used as much as possible 1000 g and 115.80 g. The black flesh is separated from the skin, dried, and blended. Extraction was carried out by maceration using 96% ethanol solvent to produce an extract of leaves and fruit respectively 70.48 g and 41.40 g. Furthermore, the extract samples were analyzed with FTIR and UV-visible spectrophotometers. Phytochemical screening was saponins, polyphenols, triterpenoids, and steroids. Extract phytochemical testing was carried out according to the guidelines specified [15]; [16] as follows:

Polyphenol test

A total of 0.10 g of extract was added to 5 mL of distilled water and boiled for 5 minutes. Then filtered to obtain the filtrate. The filtrate was then added with 5 drops of 1% $FeCl_3$ and the color change was observed. A change in color from green to blue to black indicates the presence of polyphenolic compounds.

Flavonoid test

A total of 0.10 g of the extract was mixed with 5 mL of ethanol then shaken, heated, and shaken again. The mixture was then filtered and the filtrate was taken. The filtrate was then added with 0.20 g of Mg powder and 3 drops of HCl. The formation of a red color in the ethanol layer indicates the presence of flavonoid compounds [17].

Alkaloid test

A total of 1 g of extract was added with 5 mL of chloroform and 3 drops of 10% ethanol and then shaken. The chloroform fraction was then dissolved in 1 mL H_2SO_4 2N and then shaken until it became homogeneous. After that, 1 drop of Meyer's reagent (Kl+$HgCl_2$) was added. The presence of alkaloids was indicated by the formation of a white precipitate by Meyer's reagent, a red precipitate by Dragendorf's reagent, and a brown precipitate by Wagner's reagent.

Tannin test

1 mL of the test solution is reacted with 10% iron (III) chloride solution, if a dark blue or greenish-black color occurs, it indicates the presence of tannin.

Saponin test

Condensed extract of black fruit seeds of as much as 1 g is added to warm water, shaken with ethanol for 10 seconds, and then left for 10 seconds. Formation of 1–10 cm high foam that is stable for not less than 10 minutes indicates the presence of saponins. On the addition of 1 drop of 2 N HCl, the foam did not disappear.

Triterpenoids and steroids

Examination of triterpenoids and steroids was carried out by the Liebermann-Burchard reaction. 2 mL of the test solution was evaporated in a porcelain cup. The residue is dissolved with 0.5 mL of chloroform, then 0.5 mL of anhydrous acetic acid is added. 2 mL of concentrated sulfuric acid was then added through the tube wall. The formation of a brownish or violet ring at the boundary of the solution indicates the presence of triterpenoids, whereas a greenish-blue ring appears indicating the presence of steroids [18].

Antioxidant Test

The antioxidant test was carried out according to the DPPH method [19]. The mother extract solution was made 100 ppm by dissolving 10 mg of the extract in 100 mL of methanol PA, which was then diluted to 5 ppm, 6 ppm, 7 ppm, 8 ppm, and 9 ppm. A 50 ppm DPPH stock solution was created by dissolving 5 mg of DPPH solid in 100 mL of methanol PA. The control solution was prepared by mixing 2 mL of methanol PA and 1 mL of 50 ppm DPPH solution. The test sample was prepared by mixing 2 mL of extract solution with a certain concentration with 2 mL of DPPH solution. The test solution was incubated in the dark at room temperature for 30 minutes and then the absorbance was measured using a Uv-Vis spectrophotometer at a wavelength of 517 nm. The same test was carried out on a standard solution of β-carotene and a standard solution of vitamin C. The value of antioxidant activity (Radical Scavenging Activity /RSA) was calculated using equation 1.

RSA (%)=1-A_sampel/A_kontrol x 100% (1)

Antibacterial Test

The antibacterial test was carried out where bacteria are grown in the laboratory Microbiology PSPG Gadjah Mada University using the well method [20]. The nutrient agar media was sterilized at 121 ° C for 15 minutes and then cooled to a temperature of ± 40 ° C. A total of ± 107 test bacteria were added to the nutrient agar medium and shaken until homogeneous then poured into a sterile petri dish as much as 20 mL. The mixture is allowed to stand until the nutrient medium solidifies. Media that has been perforated using an iron punch. 30 μL of the test sample was put into the well. The petri dish containing the sample was cooled in the coolroom for 1 hour and then incubated at 37°C for 24 hours. After incubation was completed, the inhibition zone was measured.

Toxicity Test

The toxicity test was carried out using the Brine Shrimp Lethality Test (BSLT) method [21]. As much as 1 g of A. salina Leach larvae eggs were taken, then immersed in 2000 mL of artificial seawater, given a 40-60 watt incandescent lamp, and aerated for 48 hours. Artificial seawater was prepared by dissolving 40 g of salt in water up to a volume of 2000 mL, after which it was filtered. A 2000 ppm stock extract solution was prepared by dissolving 0.2 g of the extract in artificial seawater

in 100 mL, then diluted to 1000, 100, 10, and 1 ppm. 6 mL of the test solution with concentrations of 1000, 100, 10, and 1 ppm was pipetted, and put into a glass, then 10 shrimp larvae that were 2 days old were added. Observation of larval mortality was carried out for 24 hours with an interval of 1 hour, after which the LC50 from the larval mortality data [21].

Data Analysis

Statistical tests were carried out on toxicity calculations using probit analysis using Excel.

RESULT AND DISCUSSION

The solvent ethanol of 2000 mL was used to extract black leaves and fruit to produce a viscous liquid. The top layer remains in a liquid state when cooled, while the bottom layer is solid.

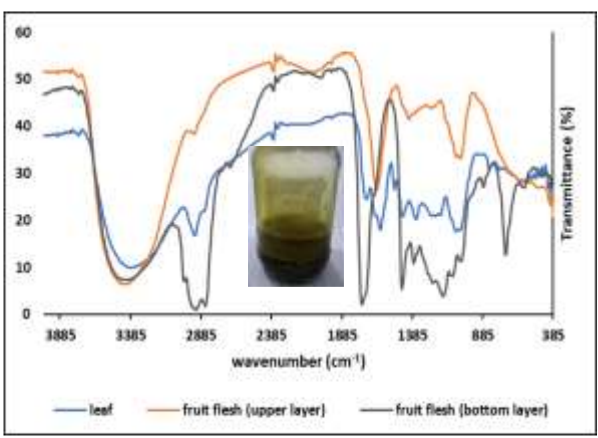

Figure 2. FTIR spectra of ethanol extract of leaves and fruit pulp (liquid and solid); inset is 2 layers of black pulp ethanol extract when cooled.

The FTIR absorptions of both the ethanol extract of the leaves, the liquid and solid layers of the ethanol extract of the black fruit flesh (**Figure 2**) were significantly different, both in terms of wave number and their yield characteristics. This can how the bond strength and functional groups that are different from the three samples. There is a difference in the absorption of the CH bonds from the ethanol extract of black fruit flesh at a wave number around 3000 cm^{-1}, namely that absorption of the top layer appears at a smaller wave number than the solid layer. This shows that the bond between C and H is stronger in the upper layer which is probably due to the presence of a double bond. The presence of a double bond at wave number 1643 cm^{-1} [22] makes the top layer in a more liquid phase than the bottom layer when cooled in the refrigerator. This was confirmed by the stretching vibration C=O in the three samples, namely at 1743 cm^{-1} (bottom layer), 1651 cm^{-1} (top layer), and 1620 cm^{-1} (leaves). Absorption of 1743 cm^{-1} can come from Unconjugated alkyl aldehydes and alkyl esters, absorption of 1651 cm^{-1} shows Hydroxy unsaturated ketones, aldehydes, [23], and absorption of 1620 cm^{-1} can show amide groups [24]. The broad absorption bands of the OH groups in the range indicate the presence of carboxylic acid or alcohol structures, both phenolic and aliphatic [25]. The OH stretching vibrations on leaves and fruit are different. Leaves absorb at 3402 cm^{-1}, while fruits absorb at 3410 cm^{-1}. Absorption in the fingerprint region of the three samples was significantly different indicating a different combination of compound content in the three extracts.

Figure 3 shows the findings of extract characterization with UV-visible spectrophotometry. The highest wavelength appears in the UV area, which is less than 350 nm. The viewable area has very modest absorption, revealing only a few colorful chemicals. Polyphenols have a maximum wavelength of 280-330 nm; flavonoids and quinones are generated by oxidizing polyphenols at 390-420 nm; and chlorophylls at 600-660 nm [26]. Even though the absorbance in the 600-660 nm region was very low, the three samples showed the presence of absorption in the three locations; at a wavelength of 660, the leaf absorbance was 0.014, the top layer was 0.006, and the bottom layer was 0.005. This indicates a small amount of chlorophyll content in the three extracts which is possible due to the nature of ethanol which can extract chlorophyll [27].

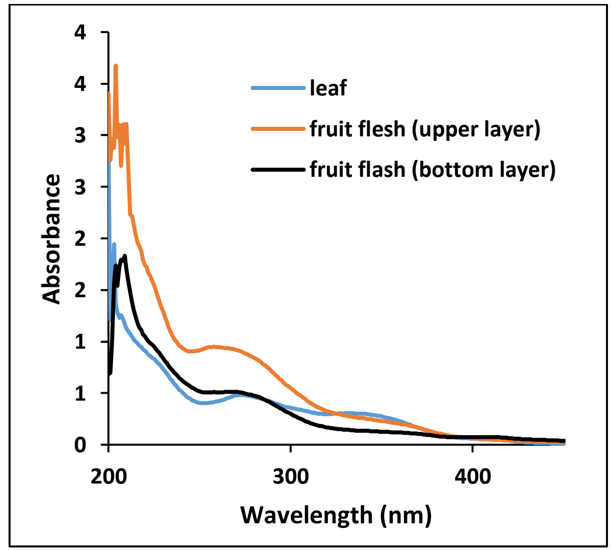

Figure 3. UV-Visible spectra of ethanol extract of black fruit leaves and flesh, (a) blue: leaf; (b) orange: upper layer; (c) black: bottom layer.

Phytochemical Test Results

The results of the phytochemical tests of the ethanol extract of black fruit leaves and flesh are presented in **Table 1**.

Phytochemical analysis confirmed the FTIR and UV-visible results. **Table 1** shows that the ethanol extract of black fruit leaves is carries more secondary metabolites. Leaf extracts contain alkaloids, flavonoids, tannins, saponins, polyphenols, and steroids. The top layer extract contains alkaloids, flavonoids, polyphenols, and triterpenoids. In the table, there are no flavonoids and polyphenols in the bottom layer, whereas in the UV-Vis results, there are flavonoids and polyphenols. This is because the two chemical's quantities are so low that they cannot be seen visually in the phytochemical analysis.

Table 1. Results of the phytochemical test of the ethanol extract of black fruit leaves and flesh of fruit.

Compound Class	Plant part		
	Leaf	Flesh of fruit	
		Upper layer	Bottom layer
Alkaloid (Test using Meyer's Reagen)	+++	-	-
Alkaloid (Test using Wagner Reagen	+++	-	-
Alkaloid (Test using Dragendrof Reagen	+++	++	++
Flavonoids	+	++	-
Tannins	+++	+++	+++
Saponins	+++	-	+
Polyphenols	+++	+++	-
Triterpenoids	-	+++	-
Steroids	+++	-	+++

Antioxidant Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method was used to measure the antioxidant activity of the samples. This approach assessed the sample's capacity to generate electron pairs and scavenge DPPH stable free radicals. Because of its ease of use, high accuracy, and precision, this analytical approach is frequently used to measure antioxidant activity [28]. The three samples in this analysis fall into the category of very strong antioxidant activity (**Figure 4**) because, according to [19], an IC_{50} value of less than 50 ppm is considered to have very strong antioxidant activity, so the three samples in this study have a very strong antioxidant activity category. The antioxidant activity of the leaves was higher with an IC_{50} value of 3.71 ppm the antioxidant activity of the leaves was greater than that of the fruit extract; the values at the top and bottom were almost identical, at 17.84 and 18.99 ppm respectively.

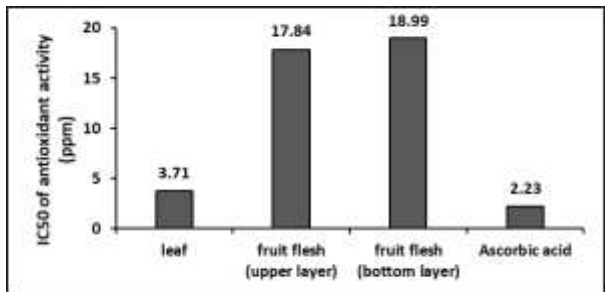

Figure 4. The antioxidant activity of ethanol extract of the leaf, fruit flesh, and ascorbic acid (positive control).

Table 2 shows that the antibacterial ability of the ethanol extract of black fruit leaves is higher than the ethanol extract of black fruit flesh, both in the form of solids and liquids. This is indicated by the size of the inhibition zone. The three samples in the study had lower antibacterial activity than the positive control, namely the antibiotic chloramphenicol. The antibiotic chloramphenicol is a broad-spectrum type of antibiotic, ie antibiotics that can inhibit or kill gram-positive and negative bacteria [29]. According to [30], the classification of bacterial growth inhibitory responses is as follows: inhibition zone diameter <10 mm is defined as No. obstacle, zone inhibition 10-15 mm weak grid, inhibition zone 16-19 mm medium grid an inhibition zone >20 mm is categorized as strong. In this study, the chloramphenicol inhibition zone for E. coli (gram negative) and S. aureus (gram positive) bacteria was almost the same. The ethanol extract of the top leaves and meat had antibacterial activity against E.coli and S.aureus, while the bottom layer only had antibacterial activity against E. coli bacteria. All three samples were more active against E. coli bacteria than S. aureus. E. coli bacteria are gram-negative bacteria that have a cell wall.

Phytochemical tests show that there are secondary metabolite compounds in black fruit leaf extract in the form of tannin, alkaloid, flavonoid, and steroid compounds. Measurement of the inhibitory power of bacterial growth used the well method, and variations in extract concentration were 1.25x102 µg/mL, 2.5x102 µg/mL, 5x102 µg/mL, and 103 µg/mL Antibacterial activity was determined by measuring the diameter of the clear zone for each observation period of 1 day, 2 days, and 3 days. The results of the inhibitory power measurements showed that the diameter of the clear area for all tested bacteria increased with increasing

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extract concentration and observation time. This shows that the 70% acetone extract of black fruit leaves has a broad spectrum and is bactericidal. The inhibitory activity was greater for S. thypi bacteria with the diameter of the clear area at an extract concentration of 103 µg/mL on the third day of 14.1 mm [31].

Table 2. Antibacterial test results of ethanol extract of black fruit leaves and flesh.

Sample	Inhibition zone diameter(mm)			
	E coli bacteria		Staphylococcus aureus bacteria	
Leaf ethanol extract		24		16
Top layer black pulp ethanol extract		20		17
Ethanol extract of the black flesh of the bottom layer		16		0
Positive Control (Chloramphenicol)		30		31
Negative Control (96% ethanol)		0		0

Toxicity Test Results

Toxicity testing aims to assess the safety or danger posed by materials, such as food products, drugs, and cosmetics [32]. One of the inexpensive toxicity tests, but which can provide sufficient information on the toxicity of a sample is the BSLT test. Apart from being a general in vivo test method for assessing potential toxicity, BSLT can also provide early predictions of antitumor activity, as well as detection of the presence of fungal toxins, heavy metals, and pesticides [33]. BSLT is associated with initial tests on cancer cells because the growth of Artemia Salina larvae is very fast which resembles the growth rate of cancer cells [34]. The following are the results of toxicity tests for the leaf extract and the top layer of black fruit ethanol extract. Because the solid coating sample cannot be dissolved in brine with the help of ethanol or DMSO, the results of this sample have not yet been obtained for its toxicity test.

Table 3 shows that the toxicity of the ethanol extract of black fruit leaves is almost the same as the toxicity of the ethanol extract of black fruit flesh, namely 109.71 and 108.64 ppm, respectively. These results provide an initial estimate of the toxicity of the ethanol extract of black fruit leaves and pulp. The IC_{50} value of this study sample was included in the toxic category (less than 1000 ppm) but not very toxic (less than 30 ppm) [35] . Both leaves and fruit were tested under the same conditions and yielded nearly the same values. It is proposed that black fruit leaves are safe to apply as a food additive, for example as a food preservative. Based on ethnobotany studies, black fruit has long been eaten directly or mixed in food, namely sago by the Wondama ethnic community [36] . If the black fruit which has an IC_{50} toxicity value of 108.64 ppm can be eaten directly or mixed in food, then the ethanol extract of black fruit leaves which has an IC_{50} toxicity value of 109.71 ppm is safe to use as a food additive which is applied to food at higher concentrations. lower than the black fruit itself. The use of ethanol as an extraction solvent, apart from being safer, also produces extracts that have lower toxicity than non-polar solvents. In another study, Thymus vulgaris leaf extract using non-polar solvents (chloroform and petroleum ether) was more toxic than polar extracts (hydro alcohol) in the BSLT test [37].

Table 3. Test results for antioxidant activity and BSLT toxicity test.

No.	Sample	Toxicity as IC_{50} (ppm)
1	Leaf ethanol extract	109.71
2	Ethanol extract of the upper fruit flesh	108.64
3	Lower fruit pulp ethanol extract	n.t

CONCLUSION

The ethanol extract of the black pulp separates into two layers when cooled. The phytochemical content of the ethanol extract of black fruit leaves is higher than that of the fruit flesh extract. Ethanol extract from black fruit leaves has higher antioxidant and antibacterial activity than black fruit flesh. The results of toxicity tests on black fruit leaf extract provide important information on the safety of using this extract as a food additive, such as a natural preservative as well as an antioxidant and antibacterial agent in food, medicine and cosmetic products. The safety test of using black fruit ethanol extract can be further confirmed using other methods to ensure safety. Important and first information in research can encourage efforts to cultivate, manage and preserve black fruit plants.

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