Fractionation and Identification of Java Plum Fruit (*Syzygium cumini*) Extract

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ABSTRACT

Java plum (Syzygium cumini) fruit is a tropical, purple fruit that has a potency as a source of natural antioxidant.The objective of this study were to investigate further about the stability of the fruit extract towards pH and UV exposure, to separate the fruit extract by column chromatography filled with silica gel G-60 to its components and to determine as well as to identify which component had highest antioxidant activity. The results showed that the fruit extract was red, orange, yellow, brown, and purple, and blue colors at pH 1-3, 4, 5, 6, 7, and 8 respectively. Among those colors, red had high stability toward low wavelength UV exposure (254 nm) up to 3 hours. The very low degree on slope of the regression line indicated that the fruit extract was particularly stable toward UV light. Separation of the fruit extract by column chromatography filled with silica gel G-60, and followed by gradient elution with EtOAc and MeOH/H2O (1:1) resulted in five fractions including : three were colorless and two were red and pink respectively. The red fraction, however contained anthocyanin and had highest antioxidant activity. The red fraction were then identified by paper chromatography and TLC both as crude (with out hydrolisis) and as acid hydrolyzed extracts. The crude extract used BAW, Bu-HCl, and HCL 1% as developing solvents; whereas hydrolyzed extract used forestal and formic as developing solvents. Anthocyanidin standards were spotted together with the hydrolyzed extract. The identification was based on the Rf values, color of spots visible and under UV light. The results of the hydrolyzed extract showed that there were three spots identified as : pelargonidin, cyanidin, delphinidin; while the non hydrolized extract showed three spots which were identified as : pelargonidin 3-(p-coumaryl-glucoside)-5 glucoside, cyanidin 3-glucoside, and delphinidin 3-rhamnosilglucoside.

Key words : *anthocyanin, polyphenol, pH, UV, fractination.*

INTRODUCTION

Java plum fruit (*Syzygium cumini*) is an Indonesian tropical fruit, has a deep purple color both on the peel and in the pulp. The previous research showed that this pigment was anthocyanin (Lestario et al., 2005b). Anthocyanin is a plant pigment which has either red, orange, blue, and purple color on fruit and flower (Gross, 1987).

Anthocyanin had been only known as pigment that could attract insect that useful for pollination.

However, many recent researches showed that this pigment also had antioxidant activity (Wang et al., 1997). Furthermore, fruits that contain anthocyanin could reduce the risk of degenerative diseases such as coronary heart disease (CHD) and cancer (Amarowicz, et al., 2000).

Most of the researches on fruits that contain anthocyanin were done only on subtropical origin such as grape, blue berry, cranberry, and strawberry, but not those of tropical area origin such as Indonesia. However, in these area there are many kinds of purple, delicious, and exotic fruits, that contained anthocyanins, and one of those is java plum fruit that has a deep purple color on its peel and pulp.

Anthocyanins have also been used as colorants especially on beverages, since many synthetic colorants were recently recognized as toxic and carcinogenic. Color is an important determinant for food, because it influences human preference on a product (Francis, 1999). Anthocyanin had several variation of colors such as red, blue, and orange. These compound are soluble in water, therefore it is easy to be incorporated into aqueous food. However, there are several factors limiting its function such as its low stability.

Fractination by column chromatography separates the pigment extract to its components. Sometimes it must be done with elution gradient (Gross, 1987). Separation by column chromatography filled with silica gel has been done on blue berry with gradient elution of solvent (A) EtOAc and solvent (B) methanol-water (1:1) (Smith et al., 2000).

This research was intended to characterized java plum fruit extract: color stability towards pH and UV light, to separate the fruit extract by column chromatography to its components, and to identify the component that had highest antioxidant activity.

MATERIALS AND METHODS

Sample preparation

Java plum (*Syzygium cumini*) fruits was picked ripe from the plant at Prambanan village, Yogyakarta, and directly brought to the laboratory at the season (October 2004). In the laboratory, the fruits were then washed with tap water. The pulp was separated from the seed, freeze dried, ground and sieved (8 mesh). The freeze dried fruit powder was kept in freezer (-20 °C) until used.

Chemicals

Methanol, hydrochloric acid 37 %, sodium hydroxide, potassium chloride, citric acid, sodium citrate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, silica gel G-60, ethyl acetate, butanol, acetic acid, formic acid, and TLC silica gel were purchased from E. Merck (Darmstadt, Germany). Pelargonidin, Cyanidin and Delphinidin standard were purchased from Extrasynthese (Lyon, French). 1,1-diphenyl-2-picrylhydrazyl (DPPH) from TCI (Tokyo, Japan) and TLC cellulose MN-300 (Polygram®, Doren, Germany) were donated from Prof. Dr. KH Timotius from Satya Wacana Christian University, Salatiga.

Extraction

Approximately 1 g of freeze dried fruit powder was macerated in 60 mL methanol-HCl 1% for overnight in cool room (4 °C), followed by filtration with Whatman no 1, extraction was repeated with 2x20 mL methanol-HCl 1% for 2x1hours so that the waste became colorless. The filtrate was collected and added with methanol until 100 mL. Methanol-HCL 1% was the best solvent for extraction of java plum fruit compared to other solvent (Lestario, et al., 2005a).

Influence of pH and UV exposure

Fruit extract was added to buffer pH 1-8 (1:4 v/v). Fruit extract was diluted in methanol-HCl 1% (1:4), then put under UV light up to 3 hours; every hour the absorbance was measured by spectrophotometer on maximum wavelength, below maximum wavelength, and upper maximum wavelength (535 nm, 534 nm, and 536 nm).

Fractination

Fruit extract (100 mL) was evaporated by rotary evaporator to 5 mL, then 2 mL of it were used for fractination. Fractination was done by column chromatography (1,5 x 30 cm) filled with silica gel 60, eluted with gradien elution of solvent A = EtOAc and solvent B = MeOH-water (1:1). The combination of gradient elution were as follow A = 100 %; A/B = 75/25 %; A/B = 50/50 %; A/B = 25/75 %; B = 100% (40 mL for each of combination solvent). The eluent was put into vials, 10 mL for each vials, so that there were 20 vials. The absorbance of every vials was measured in 280 nm, 310 nm, and 520 nm. The results were then plotted on milimeter paper, then eluent in the vials in the same peak were collected in the same place.

Evaluation of Fractions

Antioxidant activity of every group of fractions were measured with free radical scaveging method with DPPH as free radical (Amarowicz, et al., 2000). The maximum absorbance of every group of fractions were scanned by UV-VIS Spectrophotometer.

Identification: Identification was done with paper chromatography and TLC both for aglycone (hydrolyzed) and for crude extract (non-hydrolyzed). The identification of aglycones used forestal and formic as developing solvents and used pure pelargonidin, cyanidin, and delphinidin as standard of anthocyanidin; whereas the identification of anthocyanins used BAW, Bu-HCL, and HCL 1% as developing solvents and based results of the aglycone identification, color of the spots visible and under UV light, and Rf values of the spots compared to Rf table (Harborne, 1996; Zweig and Whitaker, 1971).

RESULTS AND DISCUSSION

Evaluation of Extract Characteristics

Data on the influence of pH variation towards color stability of fruit extract showed that the fruit extract was red on pH 1-3, orange on pH 4, yellow on pH 5, brownish on pH 6, purple on pH 7; and blue on pH 8 (Table 1). The pH of the fruit extract before mixture with buffer was 1, the color was deep red.

Table 1. Influence of pH Towards Color of Fruit Extract in Methanol-HCl 1%

pH Buffer	Color	Maximum absorbance (nm)
1	Red	307; 522
2	Red	307; 521
3	Red	307; 522
4	Orange	307; 389
5	Yellow	309; 387
6	Brownish	308; 528
7	Purple → bluish	307; 578
8	Blue	309; 613

Maximum absorbance of the extracts on each pH had two peaks : in UV and in visible areas. The maximum absorbance in UV area always located in the same wave length (307-309 nm), whereas in visible area it changes as the change of pH and extract color. It meant that pH had influence on maximum absorbance on visible area, but did not so on UV area.

Influence of UV exposure on color stability of the fruit extract showed that the fruit extract was truly stable towards UV exposure up to three hours. Table 2 showed that the absorbance of the fruit extract was not decrease in every measurement (every hour) during UV exposure. The red color was not change visually. The absorbance measurement of the fruit extract was done in the selected maximum absorbance (λ = 534, 535, dan 536 nm).

Table 2. The effect of UV light (254 nm) towards Color Stability, expressed as % of changing of absorbance at maximum absorbance)

ë (nm)	Absorbance afterUV Exposure				Decreasing/ Increasing of absorbance (%)
	0 min	60 min	120 min	180 min	
534	0,858	0,837	0,848	0,850	0,93 %
535	0,821	0,823	0,834	0,836	1,83 %
536	0,822	0,829	0,836	0,837	1,82 %

Both the decreasing and increasing of absorbance were calculated by the difference of their absorbance values at 180 minutes (end of measurement) and at 0 minutes (beginning of measurement) devided by absorbance values at 0 minutes in percentage. The low percentage of the results (0,93 % - 1,83 %) reflected the high stability of the extract.

Another parameter to measure the stability of fruit extract was with regression line, which used every data of the absorbance (t = 0, 60, 120, and 180 minutes). The formula of the regression line were : $Y = -2{,}2.10^{-5} X + 0{,}85$ (λ = 534 nm) ; $Y = 9{,}3.10^{-5} X + 0{,}82$ (λ =535 nm); dan $Y = 8{,}7.\ 10^{-5} X + 0{,}82$ (λ = 536 nm). All of the formula showed very low A value (slope) which reflected very high stability.

Fractination of Fruit Extract

The result of the fractination of fruit extract could be seen in Figure 1, which showed that the fruit extract could be separated to five groups of fractions.

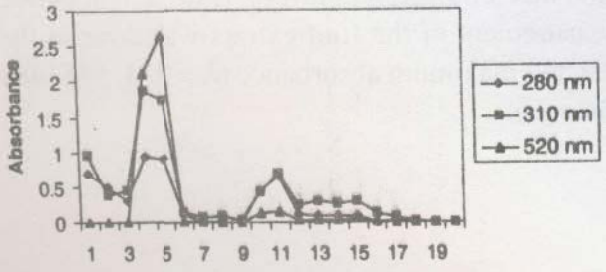

Figure 1. Absorbance of fractions at 280 nm, 310 nm, 520 nm.

Three of the fractions were colorless; while two others were red and pink. All of the fractions contained polyphenols since they had maximum absorbance at UV (around 280 nm). The group number II (red) and number IIIa (pink) contained anthocyanin, based also on their colors and maximum absorbances. Anthocyanin has two maximum absorbance : one on UV area (around 280 nm) and one on visible area (500-550 nm). However, fraction number III b and III c were not anthocyanin because their color were not red and they did not have maximum absorbance at 500-550 nm. Therefore although fraction number III a, b, and c were in the same peak, but they consisted of two different components. Based on their colors and maximum absorbances, the group of fractions number I, III b, III c, IV, and V could be categorized as flavon, biflavonil, flavanon, and flavonol.

Table 3. Fractination of Fruit Extract with Colom Chromatography -Silica Gel 60

No	Color of Extract	ë maximum	Maximum absorbance	Possibility
I.	Colorless	301	0.8518	Flavon/ biflavonil
II.	Red	536; 303	1.2913 1.5253	Anthocyanin
III.a	Pink	533; 297	0.1228 0.6528	Anthocyanin
b		358; 300	0.3645 1.0709	Flavanon/ flavonol
c		362; 297	0.1510 0.3834	Flavanon/ flavonol
IV.	Colorless	362; 298	0.2288 0.4115	Flavanon
V.	Colorless	362; 301	0.2721 0.4366	Flavanon

*) The possibility of the fractions (based on the color and the maximum absorbance as in Harborne, 1996 and Trevor, 1995).

The determination of antioxidant activity with DPPH showed that the red fractions (group of fractions number 2) showed highest antioxidant activity (Table 4). Therefore the identification were continued on fraction number 2 that contained anthocyanin.

Table 4. Antioxidant Activity of the Fractions

No. Fraction	Antioxidant Activity (%) by radical scaveging method with DPPH as free radical
I.	37.08 ± 1.78
II.	54.08 ± 2.42
III.	0.00 ± 0.07
IV.	1.10 ± 0.24
V.	1.13 ± 0.20

Identification of Anthocyanins

Separation of the aglycone of the fruit extract were done on paper chromatography and on TLC cellulose with forestal and formic as developing solvents. The results of the separation with forestal showed three kinds of anthocyanidin : pelargonidin, cyanidin, and delphinidin; separation with formic resulted in two spots: pelargonidin, cyanidin. The Rf values of the spots were also in appropriate with the Rf values of the anthocyanidin standard (Pelargonidin, Cyanidin, and Delphinidin). The Rf of the lowest spot were also in appropriate with Rf values of '*eggplant*' extract which known as source of delphinidin (Table 5). Forestal is known as the most important developing solvent for anthocyanidin, other developing solvents are only for supplement (Harborne, 1996).

Table 5. Rf of Aglycone of the Anthocyanins of Fruit Extract

Developing Solvent	Rf x 100	Color		Possibility
		VIS	UV	
Forestal	70.10	Pink	Dull	Pelargonidin
	55.10	Pink	Dull	Cyanidin
	37.41	Pink	Dull	Delphinidin
Formic	46.06	Pink	Dull	Pelargonidin
	33.33	Pink	Dull	Cyanidin
	22.82	Pink	Dull	Delphinidin

*)Forestal: HOAc-concentrated HCl-H2O (30:3:10 v/v); Formic: HCOOH-concentrated HCl-H2O (5:2:3 v/v).

Separation with BAW and Bu-HCl resulted three spots : Pelargonidin 3-(p-coumaryl-glucoside)-5-glucoside, Cyanidin 3-glucoside, Delphinidin 3-rhamnoglucoside; whereas separation with HCl 1% resulted only two spots : Pelargonidin 3-(p-coumaryl-glucoside)-5-glucoside, and Cyanidin 3-glucoside (Table 6).

Table 6. Rf of Anthocyanins of Fruit Extract

Developing solvent	Rf x 100	Color VIS	UV	Possibility
BAW	43.5	Purple	Flourescent	Pelargonidin 3-(p-coumaryl-glucoside)-5-glucoside
	37.5	Blue	Dull	Cyanidin 3-glucoside
	31	Blue	Dull	Delphinidin 3-rhamno glucoside
Bu-HCl	40.1	Magenta	Flourescent	Pelargonidin 3-(p-coumaryl-glucoside)-5-glucoside
	27.6	Magenta	Dull	Cyanidin 3-glucoside
	15.1	Mauve	Dull	Delphinidin 3-rhamno glucoside
HCl 1%	15.6	Magenta	Flourescent	Pelargonidin 3-(p-coumaryl-glucoside)-5-glucoside
	7.8	Magenta	Dull	Cyanidin 3-glucoside

*)BAW=n-BuOH-HOAc-H2O (4:1:5 v/v, upper layer); Bu-HCl= n-BuOH-2 N HCl (1:1, upper layer); HCl 1%=H2O:12 N HCl (97:3).

In the previous study, separation with BAA resulted in three spots that were thought as pelargonidin-3-(p-coumarylglucoside)-5-glucoside,

pelargonidin-3,5-diglucoside, and cyanidin-3-rhamnosylglucoside-5-glucoside (Lestario, 2003). However, they did not match with the results of hydrolized extract that were identified as three kinds of aglycones (Pelargonidin, Cyanidin, Delphinidin). The un-precise estimation was due to the lack of references and method of identification that only based on the Rf table (Harborne, 1996). In the meantine, we had more complete Rf table which contained about 45 kinds of anthocyanins and aglycones (Zweig and Whitaker, 1971), and also completed the identification process by observed the spots under UV light to see fluresence reflection (to distinguish anthocyanidin 3,5-glucoside or anthocyanidin 3-glucoside).

All of the Rf values of the spots were very closed to the Rf table (Harborne, 1996; Zweig and Whitaker, 1971), and they match each other between results of anthocyanidin and anthocyanins, and between the developing solvents.

Futhérmore, the results of each anthocyanins could be declared very clearly: The red color of the extract was very stable, and it reflected the existence of acylated anthocyanin (Pelargonidin-3-(p-coumaryl-glucoside)-5-glucoside); Cyanidin-3-glucoside was known as the very common anthocyanin that exist in almost every plant; and the Rf of the third spot was very closed with the Rf eggplant extract that was known as source of delphinidin (Harborne, 1996).

CONCLUSIONS

From the results of the research it could be concluded that the red color of the fruit extract was stable on pH 1-3, change to orange on pH 4; yellow on pH 5; brown on pH 6; and purple on pH 7; and blue on pH 8. The red color had high stability on low wavelength UV light (254 nm). The very low slope of the regression line indicated that the fruit extract was very stable. The separation of the crude extract by column chromatography filled with silica gel G-60, resulted in five fractions (two contained anthocyanin and three were not contain anthocyanin). All of them were polyphenols for they had maximum absorbance at UV area. The group of fractions that had red color (group of fractions number 2) and contained anthocyanin, had highest antioxidant activity compared to other fractions. Java plum extract contained three kinds of anthocyanins : acylated anthocyanin (Pelargonidin-3-(p-coumaryl-glucoside)-5-glucoside), mono-glucoside anthocyanin (Cyanidin 3-glucoside), and diglucoside anthocyanin (Delphinidin 3-rhamnoglucoside).

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