Anthocyanin Extraction from *Clidemia hirta* (L.) D. Don Fruit and Its Stability During Storage

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ABSTRACT
There have been concerns regarding the use of artificial food colorant, triggering the food industry to develop natural food colorant with nutraceutical properties, which could be solved by anthocyanins. A series of studies to evaluate the potency of harendong bulu (*Clidemia hirta* (L.) D. Don) fruit as the source of anthocyanin was conducted. First, the selection of time (30, 60, and 90 minutes) and temperature (30°C, 45°C, and 60°C) of aqueous extraction at pH 1. Second, the evaluation of anthocyanin characteristics and stability of the extract at pH 1, 3, 5, 7, and 9. Extraction at 60ºC for 30 minutes gave the best result with 43.81 ± 2.99 mg/L total monomeric anthocyanin and 1.31 ± 0.12 g/L phenolic compound as gallic acid equivalent. *C. hirta* expresses red color at pH 1 and 3. At pH 5, it showed a unique characteristic by expressing deep blue color at pH 5. However, the high browning index and low color stability at the pH have not supported the extract as the potential food colorant. The extract exhibited the best anthocyanin quality and stability at pH 1 by showing bright red color, low browning index, and high color stability (t0.5 = 126.85 d). The best performance of C. hirta at the range of food pH was at pH 3. At the pH, even though the 50% monomeric anthocyanin disappeared in 5.7 days, C. hirta retained its 50% color for 21 days at room temperature. Hence, *C. hirta* showed its promising performance as the red colorant for high acidic food products.

Keywords: *anthocyanin, Clidemia hirta, colorant, stability.*

1. INTRODUCTION

There are two potential benefits of anthocyanins in a food product, first as a coloring agent and second as a functional ingredient. Anthocyanins provide a broad spectrum of colors, such as red, orange, purple and blue. However, most anthocyanins are colorless or easily decolorized at the range of pH of food. Only the exclusive anthocyanins, called poly acylated anthocyanins, show relative high color intensity and stability at the low acidic and neutral conditions [1, 2]. Despite losing its color, anthocyanin remains the health beneficial effect, as an antioxidant, antidiabetic, anti-cancer, antihypertensive, etc [3].

Clidemia hirta (L.) D. Don, commonly called soapbush or *harendong bulu* in Indonesia, is an invasive perennial shrub that is widely found in tropical regions [4]. It has small clustered fruits that have purplish-blue color when ripe (Figure 1), and the taste is sweet. Local people in West Java, Indonesia, commonly eat the berries while walking in the woods. The old and the only study reported that the berry contains acylated delphinidin 3,5-O-diglucoside [5]. There is no specific report to identify the number of acyl groups in the anthocyanins of *C. hirta*.

Figure 1 *Clidemia hirta* berries.

Nevertheless, a study showed that the extract of *C. hirta* almost colorless at pH 4 [6]. This behaviour is an appropriate indicator that the anthocyanin is not polyacylated but monoacylated anthocyanin. Hence, the *C. hirta* berry is not a potential coloring agent for food at pH 4. However, several reports showed that plant extract containing simple anthocyanin performed a relatively high color intensity and stability at pH 3.

With this background, we are interested in studying the color quality of anthocyanin in C. hirta berry extracts and its stability at various pH. This research aimed to evaluate how potent the C. hirta extract is as the source of food colorant, especially at pH 3 [7].

2. MATERIALS AND METHOD

2.1. Materials

The *C. hirta* berries obtained from the plant wildly grown in National Park Halimun-Salak Mountain Bogor, West Java, Indonesia. The fresh berries were collected, steam-blanched for 6 minutes [7], hot air dried at 40ºC 24 hours, powdered and sieved through a 250 µm screen. The powder was stored in a tight dark glass bottle and kept in a freezer (-20ºC) until used. The deionized water obtained from the local market (Amidis®) and the 2,2-diphenyl-2-picrylhydrazyl from Sigma Aldrich, Germany. Buffer solution pH 3, 5, 7, 9. Hydrochloric acid, potassium chloride, sodium carbonate, citric acid, sodium citrate, aluminium chloride, sodium metabisulfite, methanol, potassium acetate, Folin-Ciocalteu reagent, and gallic acid procured from Merck® (Darmstadt, Germany). The reagents were analytical grade.

2.2. Selecting the time and temperature of extraction

The aqueous extraction was conducted using 40 ml of 0.1 N HCl per one gram of berry powder in the dark with continuous shaking. The three different levels of temperature and time were applied (30, 45, 60ºC and 30, 60, 90 minutes, respectively). The filtrate of the suspension collected and centrifuged at 7000 rpm for 5 minutes. The best extraction parameter was chosen based on the total monomeric anthocyanin and total phenolic yielded.

2.3. Anthocyanin quality and stability of the best extract at various pH

The best extraction parameter was chosen based on the total monomeric anthocyanin and total phenolic yielded. The best extraction parameter used to produce *C. hirta* extract that underwent the anthocyanin quality and stability test at pH 1, 3, 5, 7, 9. The quality and stability observed were total monomeric anthocyanins, color intensity, violet index, and browning index.

All samples are bottled in dark vials and stored in a dark container at room temperature. The color intensity and total monomeric anthocyanin observed periodically depend on the pH.

The most stable extract was characterized by the total phenolic content, flavonoid content, and antioxidant activity.

2.4. Total monomeric anthocyanin analysis

The monomeric anthocyanins (TMA) content is measured as delphinidin 3-glucoside by the pH difference method [7]. The method was based on the characteristic of monomeric anthocyanins, which solely exist as red flavylium cation (**AH+**) at pH 1 and converted to colorless carbinol pseudobase (**B**) at pH 4.5. The difference of absorbance at peak wavelength will be proportional to monomeric anthocyanins content.

The analysis was done by placing 0.9 ml of buffer solution pH 1 (potassium chloride - hydrochloric acid) and pH 4.5 (citric acid – sodium citrate) buffer in a test tube and then added with 0.1 ml of the sample. The blank solution is prepared by replacing 0.1 ml of sample with 0.1 ml of distilled water. The pH 1 sample was first scanned by a UV-VIS Spectrophotometer (Genesys 10uv Thermo Electron Corporation, USA) to identify the peak. Then, all samples were measured for absorbance at the peak wavelength and at 700 nm. The absorbance obtained was then used to calculate the monomeric anthocyanins content with the following equations.

$$A = (A_{\lambda max} - A_{700})_{pH\,1}) = (A_{\lambda max} - A_{700})_{pH4.5} \quad \text{.....(1)}$$

$$\text{TMA (mg/L)} = (\text{A x MW x DF x } 1000)/(\varepsilon \text{ x l}) \quad \text{.....(2)}$$

A is the absorbance, MW is molecular weight of delphinidin 3-glucoside (465.2 g.mol^{-1}), DF is a dilution factor, ε is molar absorptivity (29,000 L.mol^{-1}.cm^{-1}), and l is the cuvette width (1 cm).

2.5. Determination color intensity, violet index and browning index

The color intensity (CI) measured to indicate the strength of the color of the extract. The measurement is examined by scanning the extract at the respective pH at the visible region (400 nm to 700 nm) to determine the peak (λmax). The CI calculated by the following equation.

$$CI = (A_{\lambda max} - A_{700})\, X\, DF\,[9] \quad \text{..... (3)}$$

The violet index (VI) determined the strength of violet hue of the extract by dividing the absorbance at 580 nm to the absorbance at 520 nm [10].

$$VI = (A_{580} - A_{700})/(A_{520} - A_{700}) \quad \text{..... (4)}$$

The browning index (BI) is measured to indicate the brown compound in the extract. The brown compound could be contributed either from the degradation of anthocyanins or the polymerization of anthocyanins. The BI calculated the following equation.

$$BI = (A_{420} - A_{700})/(A_{\lambda max} - A_{700})\, X\, DF\,[9] \text{..... (5)}$$

2.6. Analysis of total phenolic content

The total phenolic compound (PC) was determined as gallic acid equivalent (GAE) by the Folin-Ciocalteu modified method [11]. The gallic acid standard was made by diluting a 1000 ppm gallic acid solution to 50, 100, 125, 150, and 200 mg.l gallic acid and then the absorbance was measured at 765 nm.

The analysis was done by placing 0.1 ml of sample in a test tube, added with 0.4 ml sodium carbonate, then mixed with a vortex. Then, 0.5 ml of Folin-C reagent was added to the test tube and mixed with vortex again. The solution was incubated for 60 minutes at room temperature. After incubation, the solution is placed into a cuvette and the absorbance measured at 756 nm.

The PC calculated by the following equation.

$$\text{PC (mg/L GAE)} = (\text{A} \times \text{DF}) / \text{m} \text{..... (6)}$$

A was the absorbance of the sample at 765 nm (A), DF was a dilution factor, and m was the gradient of gallic acid standard curve (A/mg.L^{-1}.)

2.7. Determination of degradation kinetics

The kinetics of the degradation of CI and TMA described by the first-order reaction are as follows.

While the k-values and half-life (t1/2) values for the first-order reactions were calculated with Eq. (7) and Eq. (8) respectively,

$$ln[A] = -kt + ln[A]o \text{ ... ... (7)}$$

$$t_{0.5} = \frac{ln(2)}{k} \text{ ... ... (8)}$$

[A] is the concentration or content at a certain time, k is the kinetic degradation constant (unit of time-1), t is the time (unit of time), $[A]_0$ is the concentration or content at initial time, and t0.5 is the half-live (units of time).

2.8. Statistical analysis

Statistical analyses involved in this experiment was 2 ways Analysis of Variance (ANOVA). The data analyzed with the help of Design-Expert® version 7.0.0. (Stat-Ease Inc.). The post-hoc analysis was Tukey HSD test that conducted using OpenStat®.Statistical Analysis.

All data obtained from the experiments were processed and analyzed with ANOVA (Analysis of Variance) using DesignExpert and OpenStat for Tukey and Post-hoc test. The correlations and the.

3. RESULTS AND DISCUSSION

3.1. Best time and temperature of the extraction

Time and temperature are two parameters that are commonly studied to maximize the anthocyanin extracted from a source [8, 12]. A research of the extraction of anthocyanin from Clitoria ternatea flower revealed that the optimum time and temperature of the extraction are 30 minutes and 60ºC, respectively [8]. The best time and temperature to extract anthocyanins from Bauhinia purpurea flowers are 30 minutes and 45ºC [12]. Meanwhile, the temperature of 60ºC is also stated by the other study as the excellent extraction temperature for anthocyanin from several sources, regardless of the extractant used [13].

A relatively similar result shown by this research. The highest TMA of C. hirta obtained by the extraction at 45ºC and 60ºC, while the highest PC yielded by the extraction at 60ºC (Figure 2). In contrast, there was no significant effect given by the extraction time ($p>0.05$) to the TMA and PC. These results brought us to choose 60ºC and 30 minutes as the selected temperature and time to extract the anthocyanin from C. hirta. The average TMA and PC of *C. hirta* extracted at 60ºC were 43.81 ± 2.99 mg/L and 1314.43 ± 119.97mg GAE/L.

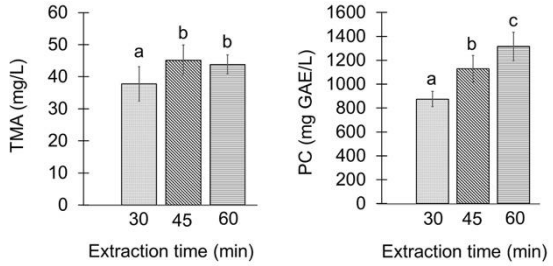
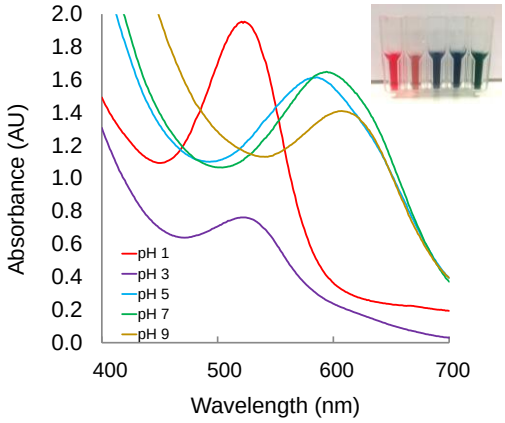


Figure 2 Total monomeric anthocyanin (TMA) (left) and total phenolic content (PC) (right) of *Clidemia hirta* extracted at 30, 45, 60ºC (average of 3 level of time: 30, 60, 90 min ± standard deviation). Averages with the same letter are not significantly different (Tukey HSD, $p>0.05$).

3.2. Anthocyanin quality and at various pH

Through the spectrogram, the color quality of an anthocyanin source extract at various pH can be evaluated. The common color quality parameters of an anthocyanin are λ_{max}, CI, VI, and BI. Several characteristics are interesting to be highlighted from the spectrogram and color quality of *C. hirta* extract (Figure 3).



pH	λ_{max} (nm)	CI	VI	BI
1	523	8.80	0.22	0.61
3	522	3.66	0.42	1.31
5	584	6.10	1.52	1.15
7	594	6.35	1.69	1.26
9	607	5.08	1.16	2.46

Figure 3 Picture, light absorbances and color quality (λmax, color intensity – CI, violet index – VI, and browning index – BI) of Clidemia hirta extract at pH 1, 3, 5, 7, and 9

First, λ_{max} and CI at pH 5. Most simple anthocyanins are colorless or express the lowest color intensity at pH 4 to 5 [2, 12, 14] due to the presence of colorless **B** as the predominant species. The C. *hirta* showed a deep blue color, which indicates that the deprotonation of red $\mathbf{AH^+}$ to purple quinoidal base (**A**) (kinetic reaction) won the competition with the hydration of $\mathbf{AH^+}$ to colorless **B** (thermodynamic reaction) [16]. Hence, based on the intensity of the color, *C. hirta* might be categorized as the potential source of blue colorant for food.

Second, however, the extract showed high adverse BI at pH > 3. All extracts at pH > 3 showed higher absorbance at 420 nm than at λ_{max}. The high BI degrades the quality of red, purple, or blue color of an anthocyanin.

Third, the CI at pH 3 was about 42% of the CI at pH 1. In other words, almost 60% of $\mathbf{AH^+}$ hydrated to **B**. This percentage is lower than the percentage of *Bauhinia purpurea* extract (67%), but higher than the percentage of *Thunbergia erecta* extract (30%) at the same pH [13, 15].

3.3. Anthocyanin stability and at various pH

In general, anthocyanin stability decreases as the increase of the pH. Table 1 shows that the *C. hirta* extract follows the common pattern. Both color and anthocyanin performed the highest stability at pH 1 ($t_{0.5}$ = 126.85 days) and the lowest stability at pH 9 ($t_{0.5}$ = 0.14 days). *C. hirta* extract showed a relatively poor performance both as the colorant and source of monomeric anthocyanin at pH 5 or higher.

Interesting characteristics shown by the extract at pH 1 and pH 3. The color stability of the extract was much higher than the monomeric anthocyanin stability.

At pH 3, the $t_{0.5}$ of the color was 21.19 days, while the $t_{0.5}$ of monomeric anthocyanins was 5.73. The much higher color stability indicated that some monomeric anthocyanin converted to polymeric anthocyanin through a condensation reaction that also occurred in wine during aging [17]. Polymeric anthocyanins are red and are not a pH-dependent pigment [17].

The increase of polymeric anthocyanin in C. hirta extract at pH 1 and 3 during storage was evident in this research (data is not provided). The relatively high color stability and the formation of polymeric anthocyanin during storage at a low pH promotes *C. hirta* as the red colorant for low pH food products such as high acidic fermented beverages.

Table 1 First order degradation kinetics and half-life (t.05) of color intensity and monomeric anthocyanin of C. hirta extract at various pH

pH	Color Intensity			Monomeric Anthocyanin		
	R^2	k, d^{-1}	$t_{0.5}$, d	R^2	k, d^{-1}	$t_{0.5}$, d
1	0.99	0.0055	126.85	0.92	0.0105	66.14
3	0.89	0.0327	21.19	0.91	0.1211	5.73
5	0.78	0.1647	4.21	0.94	0.2246	3.09
7	0.80	0.7022	0.99	0.66	0.4038	1.72
9	0.93	5.0242	0.14	0.98	7.3694	0.09

4. CONCLUSION

The hot aqueous extraction (60º) at pH 1 for 30 minutes could be considered to extract anthocyanin form harendong bulu or *Clidemia hirta* berry. The extraction at a longer time gave no significant effect to the anthocyanin yielded (p>0.05).

Anthocyanin in *C. hirta* extract showed a characteristic that was differ to most simple anthocyanins. It showed a deep blue color at pH 5, but with a relatively high browning index. However, the extract showed low color stability at the pH ($t_{0.5}$ was

lower than five days). Several techniques, such as intermolecular copigmentation with phenolic compounds and matrix development with macromolecules, are suggested to increase the color stability.

The most promising result of the *C. hirta* extract as a potential food colorant is at pH 3. At this pH, *C. hirta* extract retained its 50% color for 21 days at room temperature. As a further evaluation, the study of the application of *C. hirta* extract in several high acidic beverages at various temperatures is recommended. In general, the color of an anthocyanin is much more stable at the lower temperature.

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