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EFFECTIVENESS OF ACIDIFIED ETHANOL EXTRACTION IN EXTRACTING ANTHOCYANIN PIGMENTS OF RED DRAGON FRUIT (*HYLOCEREUS POLYRHIZUS*)

S u m m a r y

Background. Red dragon fruit (*Hylocereus polyrhizus*) contains flavonoid compounds known as anthocyanins. Anthocyanins are polar and can be extracted using polar solvents like ethanol. Citric acid was used to optimize the extraction of anthocyanin compounds from ethanol. This research aimed to determine some parameters of the anthocyanin extract of red dragon fruit extracted using 96 % ethanol and citric acid at different concentrations. Ripe red dragon fruit wasobtained from dragon fruit farmers in Tottong Village, Donri-Donri Sub-District, Soppeng Regency, South Sulawesi, Indonesia.

Results and conclusion. The red dragon fruit was macerated with 96 % ethanol and citric acid at concentrations of 0 %, 5 %, 10 % and 15 %. Total anthocyanin content was analyzed using the differential pH method. A total phenol analysis was performed spectrophotometrically using the *Folin-Ciocalteu* method. The antioxidant activity of anthocyanin extracts from red dragon fruit was determined using the $DPPH^{\bullet}$ method. The data was analyzed using ANOVA and followed with Duncan Multiple Range Test. The results of this study indicate that 96 % ethanol with 15 % citric acid was the best treatment to extract anthocyanin compounds from red dragon fruit. This yielded 37.04 %, total phenol content of 0.82 mg GAE/g, total anthocyanin content of 28.08 mg CyE/100 g, and $DPPH^{\bullet}$ scavenging activity of 56.23 %. In conclusion, the anthocyanin pigments of red dragon fruit were extracted most effectively using 96 % ethanol with 15 % citric acid. It may be used as a natural pigment for the food industry in the future.

Keywords: red dragon fruit, extraction, anthocyanins pigments, citric acid

Introduction

Red dragon fruit (*Hylocereus polyrhizus)* is widely cultivated in some Asian countries, such as Taiwan, China, Vietnam, Thailand, the Philippines, Malaysia and Indonesia [25]. The fruit peel accounts for 30 ÷ 35 % of the whole fruit. It is discarded

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during fruit processing, resulting in waste [11]. Interestingly, the red dragon fruit's flesh and skin can be used as natural dyes instead of synthetic dyes, since they both have red pigments [6].

Red dragon fruit contains anthocyanins, an intense red pigment. Anthocyanins are a group of pigments that can distribute red, purple and blue color [6] to flowers, leaves and fruit of higher plants. Anthocyanins can be a source of natural food coloring and a safe alternative to synthetic dyes [7]. In addition, anthocyanins have several benefits for health, such as antioxidant properties that are associated with the ability to neutralize reactive oxygen species, such as superoxide radicals [20].

Anthocyanins are polar flavonoid compounds that can be extracted using polar solvents [7]. Some polar solvents include ethanol, methanol, distilled water and ethyl acetate. Ethanol is usually used as a solvent for various chemicals intended for human consumption and use, i.e. food flavoring and coloring.

Therefore, acidic conditions are recommended to extract flavonoid compounds [23]. Acidic conditions will denature the vacuole cell wall in plants, thereby extracting the pigment out of the cell [15]. Under acidic conditions, more anthocyanin pigments are present in the form of colored flavylium or oxonium cations, so that the anthocyanin absorbance measured will be even greater [22]. Acidifiers commonly employed in anthocyanin extraction are citric acid, acetic acid, tartaric acid and hydrochloric acid [23]. Citric acid is an organic acid found in most fruit and vegetables. Citric acid is a versatile food additive. In the food industry, it is used to enhance taste and color; it is also utilized to control acidity [11].

This research focuses on extracting natural pigments from red dragon fruit. No research on the effect of citric acid concentration on the anthocyanin pigment extract of red dragon fruit has been conducted yet. The anthocyanin pigments can be used as a natural food colorant. Thus, this research aimed to determine the parameters of the anthocyanin extract of red dragon fruit, extracted using 96 % ethanol and citric acid at different concentrations.

Material and method

Materials

Ripe red dragon fruit was obtained from dragon fruit farmers in Tottong Village, Donri-Donri Sub-District, Soppeng Regency, South Sulawesi, Indonesia. Samples were washed with water, and the exocarpium derivatives contained in the skin of the fruit were removed. The dragon fruit was cut manually using a stainless knife.

Preparation and extraction of anthocyanin

A 100 g portion of red dragon fruit was blended using a blender (Philips, Germany) for 1 min, then 96 % ethanol was added with citric acid ($C_6H_8O_7$; Merck, Darm-

stadt, Germany) at concentrations of 0 %, 5 %, 10 % and 15 %, with a 1:4 (w/v) ratio of ingredients to solvent. The red dragon fruit was extracted at room temperature (25 ÷ 30^{o}C) for 24 h [23] with modification by maceration methods. The extract was filtered with a vacuum filter (Whatman paper No. 42) to separate the pulp from the filtrate. The filtrate obtained was concentrated using a rotary vacuum evaporator at 40 °C until a thick extract was obtained.

Extraction yield measurement

The measurement of the extraction yield was calculated using the anthocyanin yield formula:

$$\text{Extraction yield [\%]} = \frac{Concentrated\ extract\ weight}{Sample\ weight} x100\ \%$$

Total anthocyanin content

Total anthocyanin content was analyzed using the differential pH method. A total of 1 g of red dragon fruit extract was put into two test tubes. The first test tube was filled with 0.025 M potassium chloride (KCl, Merck, Darmstadt, Germany) pH 1.0, until avolume of 10 cm^3 was obtained. The second test tube was filled with 0.4 M sodium acetate (CH_3CO_2Na) buffer solution (Merck, Darmstadt, Germany) pH 4.5, until a volume of 10 cm^3 was reached. The absorbance of the two pH treatments was measured using a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, USA) at wavelengths of 510 and 700 nm after being allowed to stand for 15 min [12]. The absorbance value was calculated applying the formula:

The absorbance (A) = [($A_{510\ nm} - A_{700\ nm}$) pH 1.0 − ($A_{510\ nm} - A_{700\ nm}$) pH 4.5]

The anthocyanin concentration was calculated as cyanidin-3-glucoside (Sigma Aldrich, St. Louis, Missouri, The United States) using the equation:

$$Total\ anthocyanin = \frac{A\ x\ MW\ x\ DF\ x\ 1000}{\varepsilon\ x\ l}$$

where:
A= ($A_{510\ nm} - A_{700\ nm}$) pH 1.0 − ($A_{510\ nm} - A_{700\ nm}$) pH 4.5;
MW = molecular weight (449.2 g/mol for cyanidin-3-glucoside);
DF = dilution factor;
ε = molar extinction coefficient (26,900 L/mol/cm for cyanidin-3-glucoside);
l = pathlength (1 cm).

Total phenolic content

A total phenol analysis was performed spectrophotometrically using the *Folin-Ciocalteu* method, and gallic acid (Merck, Darmstadt, Germany) was used as a reference. A 5 mg sample was dissolved with 2 cm^3 95 % ethanol (Merck, Darmstadt, Germany). Then, 5 mL of distilled water and 0.5 cm^3 of 50 % (v/v) *Folin-Ciocalteau* reagent (Merck, Darmstadt, Germany) were added. The sample was allowed to stand for 5 min, and 5 % Na_2CO_3 (w/v) solution was added until a total volume of 10 cm^3 was reached. The solution was allowed to stand in a dark room for 1 h. Then, the absorbance was measured using a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, USA) at a wavelength of 752 nm [13]. The results are expressed as mg equivalent gallic acid (GAE)/g.

$DPPH^{\bullet}$ radical scavenging activity

The antioxidant activity of anthocyanin extracts from red dragon fruit was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Anthocyanin extract samples were added to 2 cm^3 of 0.1 mM $DPPH^{\bullet}$ (Sigma Aldrich, St. Louis, Missouri, the United States) in a methanolic solution. The mixture was homogenized and incubated at room temperature for 30 min in a dark place. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm [2]. The same treatment was conducted for a blank solution comprising 2 cm^3 $DPPH^{\bullet}$ and 0.1 mM ($DPPH^{\bullet}$ solution containing no test material). Absorbance measurement results show the percentage of antioxidant activity using the formula:

$$\%\ antioxidant\ activity = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{Absorbance\ of\ control} x\ 100$$

Statistical analysis

The experimental design employed a Completely Randomized Design with one factor, namely the concentration of citric acid. The data obtained was analyzed using the SPSS version 25 application, with analysis using ANOVA to determine whether differences in treatment exist at the level of $\alpha = 0.01$, followed by the Duncan Multiple Range Test at the same α level.

Results and discussion

The yield calculation is conducted to decipher how much pigment concentration each treatment produces. Figure 1 depicts the results of the yield analysis. The extraction process using ethanol and 15 % citric acid resulted in the highest yield at 37.04 %. Meanwhile, the lowest yield at 14.15 % used ethanol only. A statistical analysis shows that a significant difference exists among the four treatments ($\alpha = 0.01$).

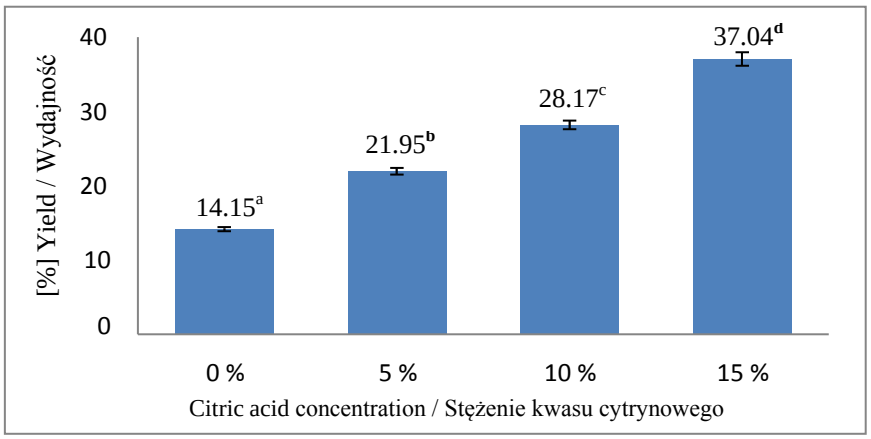

Figure 1. The effect of citric acid concentration on extraction yield
Rycina 1. Wpływ stężenia kwasu cytrynowego na wydajność ekstrakcji
Explanatory notes / Objaśnienia:
Different letters in the graph indicate statistically significant differences ($p \leq 0.01$) / Różne litery na wykresie oznaczają statystycznie istotne różnice ($p \leq 0{,}01$).

An increase in citric acid concentration leads to an increase in extract yield. This is attributed to the citric acid's nature to denature cells. This would cause the vacuole cell wall in plants to lysis, thereby extracting the pigment contained in cells [17]. Increased citric acid concentration increases degraded cell membranes, allowing the pigment component to easily diffuse, thereby producing substantial yield. Hence, an acidic condition is recommended to extract flavonoid components.

Some solvent extraction methods were applied to extract pigment from plant sources, i.e. solvent extraction methods (SEM), ultrasound-assisted extraction (UAE), microwave-assisted extraction, supercritical carbon dioxide extraction and combined extraction methods [21]. Some applied SEM due to its convenient and simple procedure. The solvents are water, acidified water, methanol, ethanol and acidified ethanol [12]. Plant sources contain different anthocyanins. The anthocyanin content of purple heart radish, purple sweet potato, roselle, purple corn and rose flower is 6.125 ± 0.034 mg/g [5], 9.76 ± 0.22 mg/g [14], 11.06 ± mg/g ÷ 15.37 ± 0.41 mg/g [17], 14.04 ± 0.02 mg/g [23], and 8.265 mg/g [9], respectively. Nevertheless, there is no report about pigment extraction from red dragon fruit using acidified ethanol.

Total phenolic content

Figure 2 depicts the total phenol content for the four treatments. The extract with the highest total phenol content at 0.82 mg GAE/g used 15 % citric acid. Meanwhile, the extract with the lowest total phenol content at 0.23 mg GAE/g did not use citric acid (0 %). Furthermore, the statistical analysis shows that all treatments had signifi-

cant differences (α = 0.01). Figure 2 shows that the total phenolic content determined is directly proportional to citric acid concentration. This is due to the citric acid's nature to degrade cell membranes, allowing phenols to easily diffuse out of the cell.

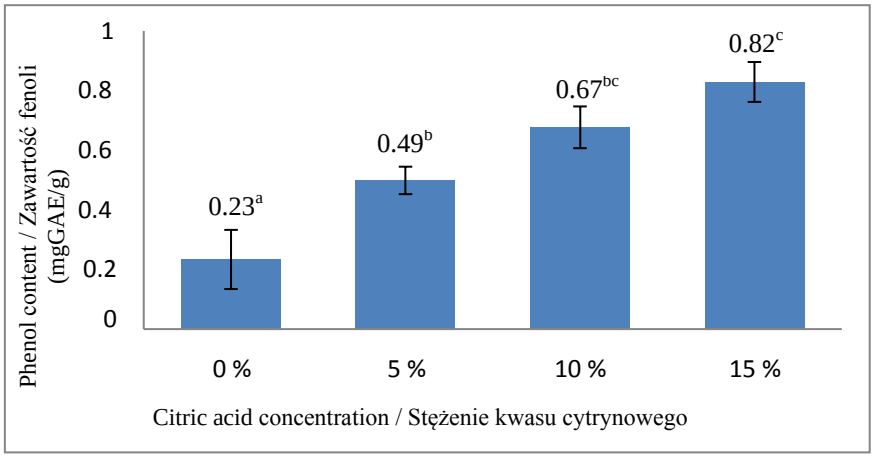

Figure 2. The effect of citric acid concentration on total polyphenol content
Rycina 2. Wpływ stężenia kwasu cytrynowego na całkowitą zawartość polifenoli
Explanatory notes / Objaśnienia:
Different letters in the graph indicate statistically significant differences ($p \leq 0.01$) / Różne litery na wykresie oznaczają statystycznie istotne różnice ($p \leq 0{,}01$).

The research conducted in Butterfly pea (*Clitorea ternatea L.*) flowers using ethanol with 1 % citric acid resulted in the highest phenolic content among other SEM [10]. Phenolic compound extraction was conducted by some researchers using different methods and sources. For instance, using an enzyme-assisted method derived from eggplant [3], UAE from cornelian cherry fruit [8] and hog pulm [1], and ethanol extraction from purple corn flour [16] and cashew leaves [6]. Phenolic acid is the major class of phenolic compounds detected in red dragon fruit samples. Specifically, these are hydroxybenzoic acid, hydroxycinnamic acid, hydroxyphenylacetic acid and hydroxyphenylpropanoic acid derivatives [6]. The phenol compounds are derived from anthocyanins [21], phenolic acids and tannins [11] dissolved during extraction, and are usually analyzed as total phenols.

Total anthocyanins content

Figure 3 shows the total anthocyanins content with different addition of citric acid. The extract with the highest total anthocyanin at 28.08 mg CyE/100g used ethanol and 15 % citric acid. Meanwhile, the extract with the lowest total anthocyanin at 4.15 mg CyE/100 g did not add citric acid (0 %). The statistical analysis shows a significant difference for all treatments (α = 0.01).

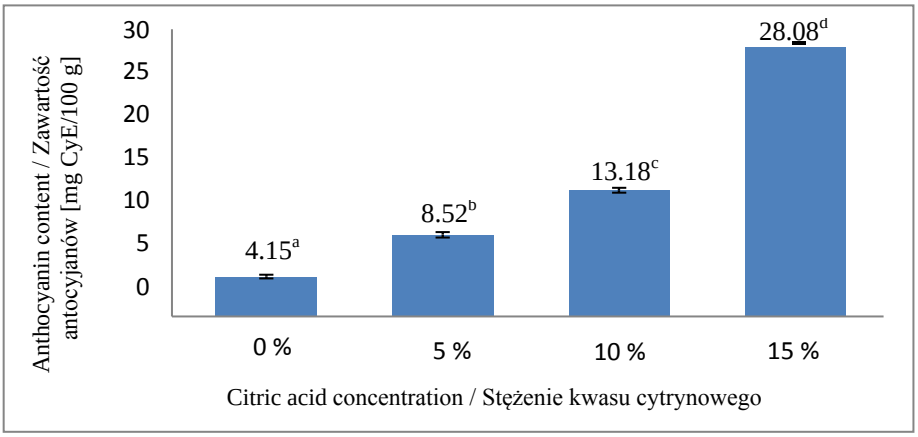

Figure 3. The effect of citric acid concentration on total anthocyanins content
Rycina 3. Wpływ stężenia kwasu cytrynowego na całkowitą zawartość antocyjanów
Explanatory notes / Objaśnienia:
Different letters on the graph indicate statistically significant differences ($p \leq 0.01$) / Różne litery na wykresie oznaczają statystycznie istotne różnice ($p \leq 0{,}01$).

Anthocyanins are water-soluble phenolic glucoside pigments found in fruit cell vacuoles and frequently in the epidermal layers [18]. Using ethanol of different concentrations could significantly affect the extraction yield of anthocyanins [5]. To enhance the yield, it can be acidified using strong organic acid, such as citric acid. Numerous studies demonstrated that the interaction of acidified solvents enhances the release of pigments, such as anthocyanins, from the cell wall membrane, albeit the effect varies depending on the type of acid utilized [10]. Low pH (1.5) was positively correlated with high amounts of anthocyanins, hydroxycinnamic acids and flavonols [4].

DPPH radical scavenging activity

The results of the antioxidant activity test expressed as % antioxidant activity from the four treatments show a significant difference ($\alpha = 0.01$). Figure 4 shows that the extraction treatment that used ethanol and 15 % citric acid had the highest % antioxidant activity at 56.23 %. Meanwhile, the extraction treatment that used ethanol without citric acid (0 %) had the lowest % antioxidant activity at 22.08 %.

Antioxidant activity for the samples from the extraction treatment was high in the extraction treatment using ethanol solvent and citric acid concentration due to antioxidant compounds dissolved during extraction. Adding citric acid would stabilize antioxidant activity. It acts to inhibit the oxidation of other molecules by scavenging the oxidation agent [19]. The antioxidant activity correlates with the total phenol content in

the extract, because phenolic and polyphenolic compounds are the main class of natural antioxidants found in plants that are strong antioxidants.

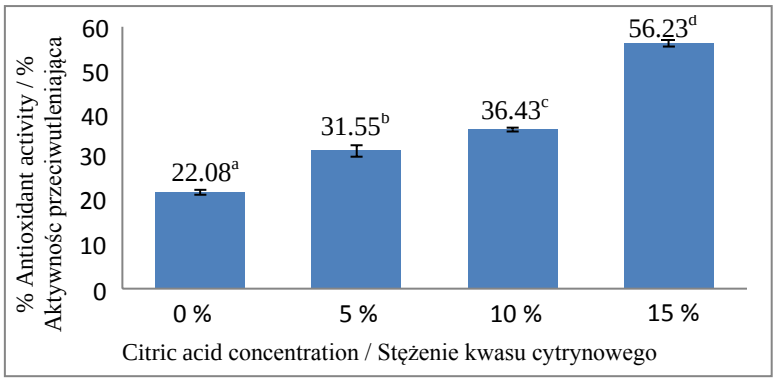

Figure 4. The effect of citric acid concentration on antioxidant activity
Rycina 4. Wpływ stężenia kwasu cytrynowego na aktywność przeciwutleniającą
Explanatory notes / Objaśnienia:
Different letters on the graph indicate statistically significant differences ($p \leq 0.01$) / Różne litery na wykresie oznaczają statystycznie istotne różnice ($p \leq 0{,}01$).

Conclusions

1. Anthocyanin can be extracted from red dragon fruit using maceration method that utilizes a combination of ethanol solvent and citric acid.
2. The highest anthocyanin pigment extract was obtained using 96 % ethanol solvent and 15 % citric acid concentration.
3. The method of preserving anthocyanin from red dragon fruit should be an area of research, since it can be used as a natural food color.

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SKUTECZNOŚĆ EKSTRAKCJI ZAKWASZONYM ETANOLEM BARWNIKÓW ANTOCYJANOWYCH Z CZERWONEGO OWOCU SMOCZEGO (*HYLOCEREUS POLYRHIZUS*)

S t r e s z c z e n i e

Wprowadzenie. Czerwony smoczy owoc (*Hylocereus polyrhizus*) zawiera barwniki flawonoidowe zwane antocyjanami. Antocyjany są polarne i można je ekstrahować przy użyciu polarnych rozpuszczalników, takich jak etanol. Kwas cytrynowy jest stosowany w celu optymalizacji ekstrakcji związków antocyjanowych z etanolu. Celem niniejszych badań było określenie właściwości ekstraktu antocyjanowego z czerwonego smoczego owocu ekstrahowanego przy użyciu 96 % etanolu i kwasu cytrynowego w różnych stężeniach. Dojrzałe czerwone smocze owoce uzyskano od rolników uprawiających smocze owoce w miejscowości Tottong, podokręg Donri-Donri, regencja Soppeng, Sulawesi Południowe, Indonezja.

Wyniki i wnioski. Czerwony smoczy owoc został zmacerowany przy użyciu 96 % etanolu z dodatkiem kwasu cytrynowego w stężeniach wynoszących 0 %, 5 %, 10 % i 15 %. Całkowitą zawartość antocyjanów analizowano przy użyciu metody różnicowego pH. Całkowitą analizę fenoli wykonano spektrofotometrycznie przy użyciu metody Folin-Ciocalteu. Aktywność przeciwutleniającą ekstraktów antocyjanowych z czerwonego smoczego owocu określono metodą DPPH. Dane analizowano przy użyciu analizy wariancji oraz testu wielokrotnego zakresu Duncana. Wyniki przeprowadzonych badań wskazują, że 96 % etanol z 15 % kwasem cytrynowym to najlepszy sposób ekstrakcji związków antocyjanowych z czerwonego smoczego owocu. Wydajność ekstrakcji wynosiła 37,04 %, całkowita zawartość fenoli 0,82 mg GAE/g, całkowita zawartość antocyjanów 28,08 mg CyE/100 g, a aktywność antyoksydacyjna 56,23 %. Podsumowując, najlepszą efektywność ekstrakcji barwników antocyjanowych z czerwonego smoczego owocu uzyskano przy użyciu 96 % etanolu z 15 % kwasem cytrynowym. Może on być stosowany jako naturalny pigment w przemyśle spożywczym w przyszłości.

Słowa kluczowe: czerwony smoczy owoc, ekstrakcja, pigment antocyjanowy, kwas cytrynowy