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Effect of various factors on anthocyanins extraction from butterfly pea flower (*Clitoria ternatea* L.)

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Abstract

An antioxidant compound is the main compound that is used to prevent cell damage from free radicals. These unstable molecules can be produced in the environmental condition such as pollution or lifestyle. One of the antioxidant molecules are anthocyanins, which can be found in the butterfly pea flower. This compound could be obtained from the extraction process. However, extraction conditions such as sample/solvent ratio, extraction time, and pH are the main factors in maximizing the yield. In this research, various factors on anthocyanins and phenolic content in butterfly pea extract were studied to get the optimum extraction condition. Extraction of the butterfly pea flower was done using the agitation method with heating and water solvent at 60 °C and various parameters. The sample was a dried butterfly pea flower. Various factors in extraction were: sample/solvent ratio, 1 : 20 and 1 : 50 ($g.mL^{-1}$), extraction time of 90 and 150 min, and pH 1.0 and 7.0. Yield is calculated by comparing the extract weight before and after drying. Total anthocyanins content and total phenolic content are determined spectrophotometrically. Based on the results, the extraction of anthocyanins was affected by the stability of structures at different pH values. The highest total anthocyanins content was 1,206.77 $mg.L^{-1}$ at sample/solvent ratio 1 : 20, 90 min and pH 1.0 conditions. Then, the maximum total phenolic content was 94.04 GAE $mg.mg^{-1}$ sample at the sample/solvent ratio 1 : 50, 90 min and pH 7.0.

Introduction

Free radical has become a threat to health (Pham-Huy *et al.* 2008). This unstable molecule can damage cells causing severe health problems, such as atherosclerosis, arthritis, neurodegenerative disorder, and cancer (Dreher & Junod 1996; Schumacker 2015). According to this health risk, high antioxidant food consumption is needed to ward off the free radical molecules to prevent the risk of severe health problems (Pham-Huy *et al.* 2008). The antioxidant can be obtained from vegetables and fruits with red, orange, yellow, and purple colors. Butterfly pea flower has a purple color and has been known as a rich antioxidant compound. Most people have been using this flower as an herbal food and drink colorant.

Butterfly pea plant (*Clitoria ternatea* L.) is a plant from the Fabaceae family. In Indonesia, this plant is commonly called Telang. The consumption of butterfly pea flower is believed to relieve pain from mouth sprue and reduce insomnia. The high antioxidant content in this flower promotes various pharmacological activities. According to Gollen *et*

al. (2018), butterfly pea flower has several pharmacological activities, such as anti-depressant, anti-anxiety, anti-stress, antioxidant, anti-inflammation, anti-hyperglycaemia, anti-diabetes, analgesic, cytotoxicity, platelet aggregation inhibitory, and hepatoprotective. Various biological activities of this extract are also affected by the content of secondary metabolites. Butterfly pea extract contains various phytochemical compounds, such as alkaloids, tannins, glycosides, resins, steroids, saponins, flavonoids, and phenols (Manjula *et al.* 2013). The content of secondary metabolites underlies the potency of the butterfly pea flower as a valuable medicinal plant.

The main compound that gives antioxidant activity is anthocyanin. Nair *et al.* (2015) identified 12 phenolic metabolites consisting of nine ternatin anthocyanins and three glycosylated quercetins from the butterfly pea flower. The phenolic compound is also contributed to its antioxidant activity. The phenolic compound has a role to perform the reduction of free radical molecules. However, anthocyanins and phenolic content obtained from the butterfly pea flower is closely affected by the extraction conditions.

Other factors of the extraction process are ratio sample/solvent and solvent. Enhancement of yield occurs when the sample/solvent ratio is increased. According to Pham *et al.* (2019), the maximum sample/solvent ratio was 1 : 25 ($g.mL^{-1}$) to obtain a high yield. Nevertheless, other factors also contribute to this result, such as time extraction, temperature, and extraction method. Salacheep *et al.* (2020) obtained high butterfly pea extract efficiency at 45 min, 40 °C, and sample/solvent ratio 1 : 10 ($g.mL^{-1}$) using ultrasound-assisted extraction (UAE). Extraction is also dependent on the solvent choice, which relates to the amount of anthocyanins. Andriani and Murtisiwi (2018) obtained phenolic content 19.43 ± 1.62 GAE (Gallic Acid Equivalents) $mg.g^{-1}$ sample for ethanol extract of butterfly pea flower. For anthocyanins content, Saptarini *et al.* (2015) achieved high anthocyanins at methanol extract for 145.17 ± 0.81 $mg.L^{-1}$. Ethanol 50 % was also known to obtain higher anthocyanins content than water. Methanol and ethanol are suitable solvents for the extraction of anthocyanins. The use of these solvents has proven to produce high content of anthocyanins and phenolic. According to Chemat *et al.* (2012), the manufacturer is currently forced to use a solvent with the absence of risk during the extraction and the safety of solvent traces in the extract. Based on that regulation, water is possible to use as an extraction solvent regarding its polar nature. This polarity can be used to extract proteins, sugars, organic acids, and inorganic substances.

The stability of anthocyanins also affects the extraction process. Based on this reason, a particular pH condition is needed to be considered. Saptarini *et al.* (2015) showed that pH 1.0 gives higher anthocyanins content than pH 2.0. According to Wahyuningsih *et al.* (2017), anthocyanins are more stable in low pH or acid. Anthocyanins also give a different color at different pH conditions. This property underlies the potency of anthocyanins from the butterfly pea flower as an indicator in acid-base titration.

Based on the description above, sample/solvent ratio, pH, and extraction time are factors relating to the obtained extract, specifically for anthocyanins and phenolic compounds. However, the information about the relationship between these factors is still limited. Optimum extraction conditions must be known to obtain high yield or secondary metabolites content.

Therefore, the aim of this study is to assess the effect of different sample/solvent ratio, pH, and extraction time in the extraction of anthocyanins from butterfly pea flower, and to determine the optimal extraction condition of anthocyanins. In this research, we performed the combination of various sample/solvent ratios, time extraction, and pH to extract anthocyanins from butterfly pea flower. Extraction is done using agitation with heating methods and water as a solvent. The optimum conditions of extraction are essential information for designing butterfly pea extraction methods on an industrial scale. The relationship of each factor to the stability of anthocyanins can be observed.

Experimental

Chemicals and equipments

Sodium acetate, hydrochloric acid fuming 37 %, potassium chloride, sodium carbonate anhydrous

were from Merck (Darmstadt, Germany). The gallic acid (>99 %) was from Sigma Aldrich (Saint Louis, USA). The solvents were methanol, ethyl acetate, ethanol (96 %) and aquadest. The Folin-Ciocalteau reagent was from Merck (Darmstadt, Germany). The pH value was monitored using pH meter (Starter 300, OHAUS). Total anthocyanins content and total phenolic content was determined using UV-Vis Spectrophotometer (Genesys 10S, Thermo Scientific).

Extraction of butterfly pea flowers

The dried butterfly pea flower was taken from Kalasan, Sleman, Yogyakarta Special Region. Samples were prepared in powder form. Ten grams of powdered butterfly pea flowers was prepared and added with aquadest, then heated on a magnetic hotplate stirrer (Heidolph, 520 W, 220 V) at 50 °C. The extraction was done in various pH, sample/solvent ratio, and extraction time. Acid condition, pH 1.0, was performed by the addition of 1 M HCl to the sample. Variation of the extraction condition was shown in Table 1.
Then the extracts were filtered with Whatman No. filter paper No. 1. The liquid-liquid extraction method was performed using ethyl acetate. The aqueous extract was evaporated at 50 °C and ethyl-acetate extract at 40 °C with 6,000 rpm for each extract. Furthermore, the butterfly flower's liquid extract was heated using an oven (Mammert, Schwabach, Germany) to remove the remaining solvent. Butterfly pea flower's liquid extract was weighed before and after being heated in the oven to calculate the yield value. This extraction was performed once.

Table 1. Extraction conditions of butterfly pea flower.

Sample Code	Extraction conditions					
	Sample/solvent [g.mL^{-1}]		Extraction time [min]		pH	
	1: 20 (A)	1: 50 (B)	90 (x)	150 (y)	1.0 (1)	Neutral (7)
Ax1	✓		✓		✓	
Ax7	✓		✓			✓
Ay1	✓			✓	✓	
Ay7	✓			✓		✓
Bx1		✓	✓		✓	
Bx7		✓	✓			✓
By1		✓		✓	✓	
By7		✓		✓		✓

Determination of total anthocyanins content

Measurement of the total anthocyanins content was carried out as previously described by Giusti and Wrolstad (2001). Butterfly pea flower extract was diluted using 0.025 M KCl buffer pH 1.0 and 0.4 M CH_3OONa, pH 4.5. Anthocyanins content was measured using UV-Vis spectrophotometer at a wavelength (λ) 510 nm and 700 nm. The blank was an aquadest. Total anthocyanins content was calculated as follows (Eq. 1):

$$\text{Anthocyanins Level} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times L} \quad (1)$$

where $A = (A_{510\ nm} - A_{700\ nm})_{pH\ 1} - (A_{510\ nm} - A_{700\ nm})_{pH\ 4.5}$; MW = molecular weight of cyanidin-3-glucoside (g.mol^{-1}); DF = dilution factor; L = cuvette width (cm); ε = absorptivity concentration of cyanidin-3-glucoside solution (L.mol^{-1}.cm^{-1}).

Preparation of gallic acid calibration curves and determination of total phenolic content (TPC)

Measurement of total phenolic content (TPC) refers to Marjoni *et al.* (2015). Weighed 0.2 g of gallic acid and dissolved in 100 mL of methanol. Then, gallic acid was prepared in various concentrations 200, 266, 400, 534, and 800 µg.mL^{-1}.
From the series of concentrations of the gallic acid solution, 0.2 mL were taken and added with 1 mL of Folin-Ciocalteau reagent, then homogenized and allowed to stand for 8 min in a dark room. Then, 2 mL of sodium carbonate 7.5 % was added, shaken, and left to stand in a dark room for 120 min. Furthermore, the absorption measurement was carried out with a wavelength of 765 nm with aquadest as a blank. The absorbance values of each concentration were used to create a standard curve. This curve was created by plotting the gallic acid concentration as horizontal axis and the absorbance as a vertical axis. The equation from the linear regression was used to measure the phenolics content of the sample in Gallic Acid Equivalents (GAE) mg.mL^{-1}.
In sample preparation for determination of TPC, the butterfly pea flower extract was carried out with a 200x dilution factor. Then, 0.2 mL solution was taken and added with 1 mL of Folin-Ciocalteau reagent, then homogenized and allowed to stand for

8 min in a dark room. 2 mL of Na_2CO_3 7.5 % was added, shaken, and left to stand in a dark room for 120 min. Furthermore, the absorbance was carried out at a wavelength of 765 nm with aquadest as a blank. Total phenolics content was calculated as follows (Eq. 2):

$$TPC = \frac{C \times V}{m} \quad (2)$$

where C = concentration of phenolic compound from linear regression equation (GAE $mg.mL^{-1}$); V = volume of extract used (mL); m = weight of sample used (mg).

Data verification for each assay was carried out by repeating the measurement three times from the same samples in each treatment (n = 3). Then, the standard deviation was calculated, followed by the determination of standard error of mean.

Results and Discussion

Extraction of anthocyanins from butterfly pea flower

Extraction is the primary beginning of isolation, purification, identification, and exploration of active compounds from a specific plant. Several extraction methods are commonly used to isolate anthocyanins, such as maceration, agitation, ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE). The yield of the active compound obtained from the extraction is influenced by several factors, such as solvent, extraction time, size of samples, and sample source. Based on these reasons, the optimization of the extraction condition is needed to increase the obtained yield. Methanol and ethanol are general solvents in many extraction processes regarding their low toxicity and polarity. Apart from those solvents, water can also be used to extract several secondary metabolites regarding the polarity.

Butterfly pea flower is known to contain a high antioxidant compound, anthocyanins. Based on its structures, anthocyanins consist of a hydrophobic hydrocarbon part that is easily dissolved in an organic solvent and a polyphenolic functional group dissolved in a polar solvent. Based on its polarity, water is one of the solvents that can be used to extract anthocyanins. Anthocyanins have different structures in different pH conditions. In neutral pH, anthocyanins produce blueish purple, whereas it gives red extract in an acid condition. According to Syahirah *et al.* (2018), an increase of H^+ ion in an acid condition causes the reduction of the ketone group in the quinone ring producing hydroxyl group. It forms a flavylium cation that gives red color (Fig.1).

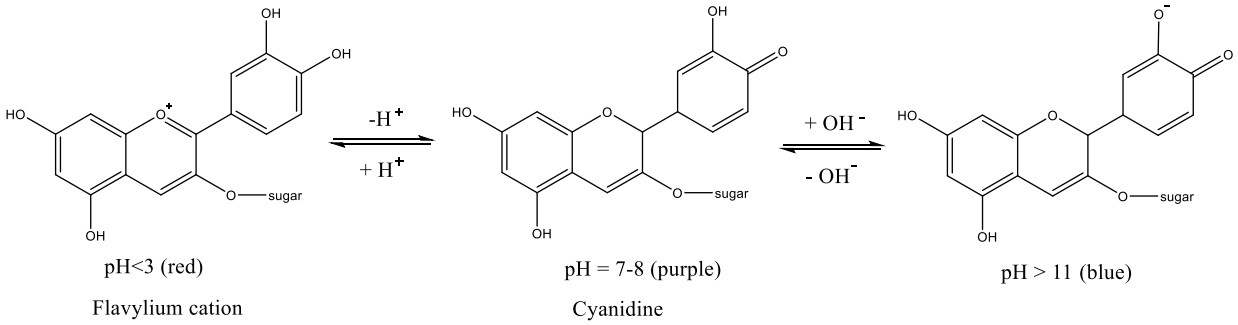

Fig. 1. Changing structures of anthocyanins in different pH condition (Wahyuningsih *et al.* 2017; Syahirah *et al.* 2018).

In this research, extraction of anthocyanins was performed in two different pH conditions, acid for pH 1.0 and neutral for pH 7.0. The extraction was done in a simple method, agitation with heating. The result was shown in Fig. 2. The red extract was obtained for pH 1.0, whereas pH 7.0 was purple. The highest yield was obtained in By1 extract with sample/solvent ratio 1 : 50, 150 min, and pH 1.0. In general, the yield from the acid condition is higher than the neutral condition. This data showed that acid conditions could help the extraction of anthocyanins. Therefore, it increases the yield of extract. Acid condition promotes the breakdown of cell vacuole of the plant, and anthocyanins can be easily obtained. It can also be caused by the ionization of anthocyanins in acid conditions forming flavylium cation. This structure improves the solubility of anthocyanins. However, the

anthocyanins content and phenolic compound must be analysed further. Assay repetition is performed to verify the result of these two assays. The error bar cannot be shown in their graph because of its different scale to the TAC value. However, the standard error mean of each assay is presented in Fig. 4 and Fig. 6. We find out that the data are not significantly different based on their standard deviation. Standard deviation is not presented, but the maximum standard deviation of TAC is 1.30, and for TPC, the maximum standard deviation is 0.66.

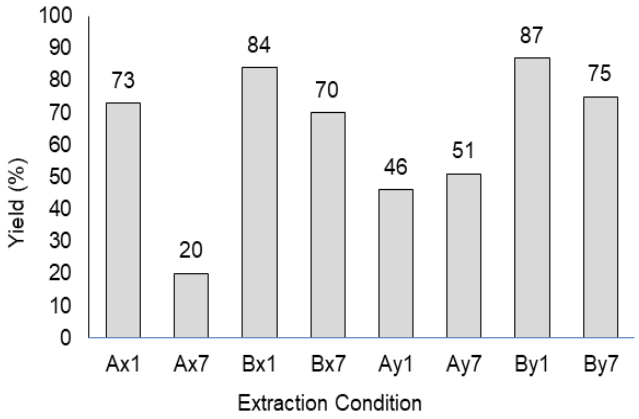

Fig. 2. The yield of butterfly pea flower extract in different sample/solvent ratio, 1 : 20 (A) and 1 : 50 (B), extraction time, 90 min (x) and 150 min (y), and pH condition, acid (1) and neutral (7).

Total anthocyanins content of extract

Total anthocyanins content is used to understand the effect of different extraction conditions to get a high amount of anthocyanins from butterfly pea flower. Monomeric anthocyanins were measured as cyanidin-3-glycoside using the differential pH method. The result is shown in Fig. 3. The highest anthocyanins content is 1,206.77 $mg.L^{-1}$ at Ax1 extract for the following conditions: sample/solvent ratio 1 : 20, extraction time 90 min and pH 1.0.

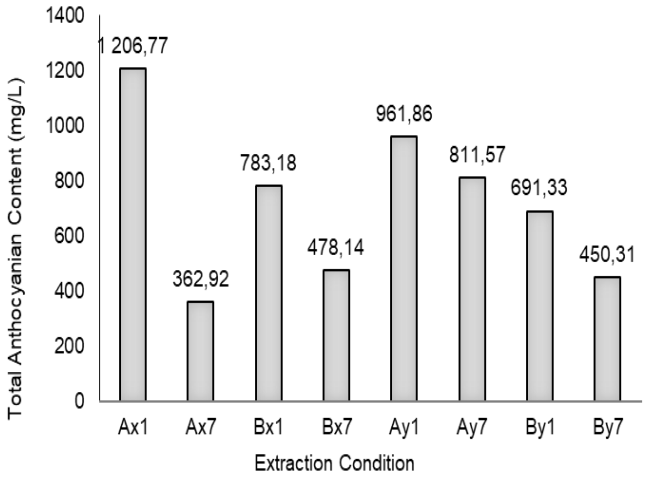

Fig. 3. Total anthocyanins content of butterfly pea flower extract from different sample/solvent ratio, 1 : 20 (A) and 1 : 50 (B), extraction time, 90 min (x) and 150 min (y), and pH 1.0 (1) and 7.0 (7).

Standard error of mean (SEM) is defined as a measure of dispersion of the sample means compared to population means. SEM is calculated by dividing the standard deviation by the square root of sample number. In this study, SEM is used to measure how precise the sample mean is. SEM of total anthocyanins content from each condition is presented in Fig. 4. According to this value, we find out that no significant difference of sample means to population means was significant. The highest of SEM, 1.11, is shown in Ax1, followed by Ax7, Bx7, and By7 which have the same SEM. Then, Bx1, Ay1, Ay7, and By1 give zero SEM. It means that there was no different total anthocyanins content between three replicates. The replication of the assay was performed using same samples from the extraction at same time. Consequently, the difference of mean value between the replicates is low to zero.

Data in Fig. 3 represents high anthocyanins content in acid conditions compared to the neutral condition. This result corresponds to a yield of extract. Low pH increases the obtained anthocyanins content that correlates to the stability of the anthocyanins. Some factors, such as pH, temperature, light, and oxygen, also influence the stability of anthocyanins. In low pH, protonation of cyanidin molecule occurs, producing flavylium cation. The flavylium cation structure contains conjugated double bonds, which can perform delocalization of an electron in double bonds, increasing this molecule's stability. Different from that, an increase in pH causes deprotonation resulting in a negative ion or anion. Inggrid and Iskandar (2016) stated that increasing of the pH caused a shift in the anthocyanins structure's equilibrium to an unstable structure. The

equilibrium of anthocyanins molecules in different pH conditions is shown in Fig. 1. This change is followed by the change of color from purple to blue. This characteristic underlies the potential of anthocyanins to be used as a pH indicator.

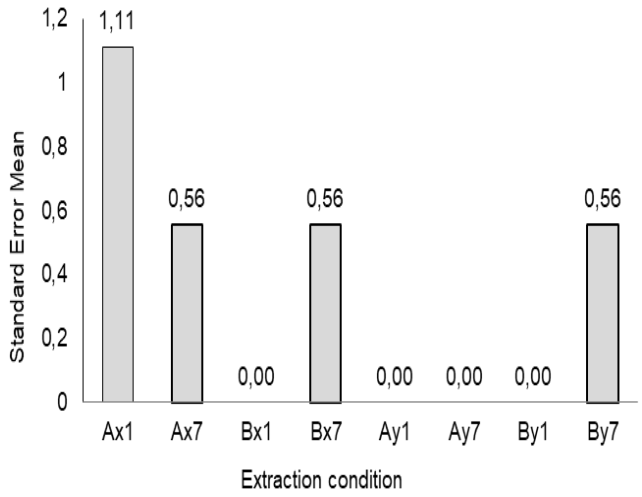

Fig. 4. The standard error of mean of total anthocyanins content from each condition.

High anthocyanins content can also be affected by solubility of molecules, related to the changes of structures in different pH. In acid conditions, anthocyanins are in ion forms. The ionized form has a high solubility in aqueous solution compared to the molecule form. Based on this structure, the highest anthocyanins content is obtained in an acid condition. It also affects the extraction time, which is needed to become shorter. Based on data, an extraction time of 90 min produces higher anthocyanins content than 150 min. A similar result was also observed by Pham *et al*. (2019). Decreasing anthocyanins content occurred during the increase of extraction time. The highest anthocyanins content was produced at 30 min, then decreased at 45 min extraction time. An anticyclonic decomposition of anthocyanins probably occurs at high-temperature exposure in a long extraction time.

Based on the sample/solvent ratio factor, the 1 : 20 ratio produced the highest anthocyanins content. An increase in the amount of solvent was not accompanied by an increase in the extract's anthocyanins. A material or sample has a specific characteristic in the absorption of solvent or water. When its absorption capacity reaches the maximum point, the cell will swell and burst simultaneously. It results in the release of pigment from the cell vacuole. If this condition is not fulfilled, the pigment will be detained inside the cell vacuole, causing low yield (see Fig. 3 on sample Bx1, Bx7, By1, and By7). The amount of solvent, which is more than the maximum amount, causes pigment's captivity inside the cell. In the research of Pham *et al.* (2019), lowering anthocyanins content happened when the solvent amount was increased. The highest anthocyanins content is obtained in the sample/solvent ratio 1 : 25 with 121.58 $mg.L^{-1}$. An increase in extraction time did not have a significant effect on increasing the result. In general, a longer extraction time produces low anthocyanins content. Based on these data, the extraction of secondary metabolites, antioxidant compound in this case, must give attention to the equilibrium parameter in sample/solvent ratio, time, and pH. There is an optimum point in each parameter which has been found to maximize the anthocyanin content. Higher amount of solvent is not necessarily equivalent to the number of obtained anthocyanins.

Total phenolic content of extract

Anthocyanins are secondary metabolites from the flavonoid group. This compound contains a hydroxyl group in its structure, which is positively related to its ability to ward off free radicals. According to that, it is essential to measure the number of phenolic compounds in an extract. The result of phenolic content measurement in this research was shown in Fig. 5. The highest total phenolic content was 94.04 GAE $mg.mg^{-1}$ samples from Bx7 extract with the following conditions: sample/solvent ratio 1 : 50, extraction time 90 min and pH 7.0. According to the data, the total phenolic content was not proportional to yield and total anthocyanins content. Anthocyanin is a derivate single aromatic compound of cyanidin containing functional groups. The number of the hydroxyl groups of each kind of anthocyanins was variated. According to that, the result of total anthocyanins content does not represent total phenolic content. A further assay is needed.

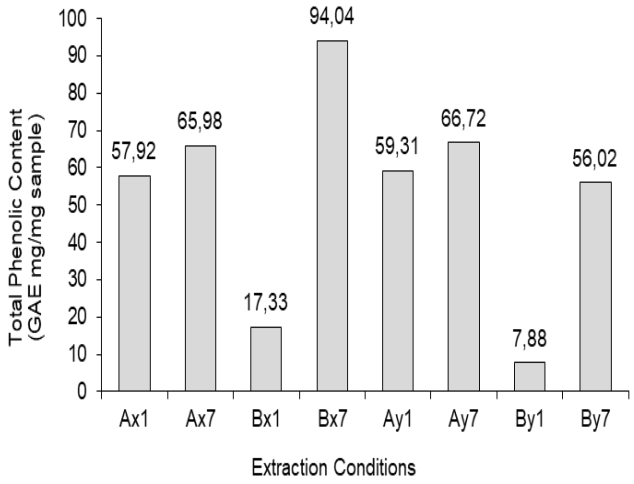

Fig. 5. Total phenolic content of butterfly pea flower extract in different sample/solvent ratio, 1 : 20 (A) and 1 : 50 (B), extraction time, 90 min (x) and 150 min (y), and pH condition, 1.0 (1) and 7.0 (7).

SEM of this assay is presented in Fig. 6. Low value of SEM is low indicates no significant different of total phenolic content mean from three replicates. High SEM values for Ax7 and Ay7 are caused by the difference of measured absorbance that is around 0.01 – 0.02. Therefore, the sensitivity of the instrument affects the final result.

According to the sample/solvent ratio, the result of total phenolic content varies. Total phenolic content at 90 min (x) is higher than 150 min (y). The extraction condition influences this result. In this study, extraction is performed using water and heating. Longer extraction time with heating can promote hydrolysis and oxidation of anthocyanins. It results in decreasing in total phenolic content in the extract.

Based on the medium's acid level, the total phenolic content in pH 1.0 is lower than the neutral condition. The acid condition can help the cell's breakdown, causing pigment release from the vacuole cell. According to the structure of anthocyanins, an acid condition or low pH will increase the hydroxyl group's number by forming flavylium ion. However, the result is different from that theory. Padmaja and Srinivasulu (2016) obtained the highest total phenolic content of butterfly pea extract at pH 7.2. Decreasing phenolic content occurs at high pH from pH 2.0, but increases at pH 7.2. Extraction of the phenolic compound from mulberry (*Morus nigra* L.) also results in the highest total phenolic compound at pH 7.0 (Espada-Bellido *et al*. 2018). According to that, optimum phenolic content obtains at the neutral condition. Anthocyanins are phenolic compounds, but the availability in the plant is only around <10 %. An increase in the total phenolic compound at neutral condition is probably coming from the degradation of other compounds. The product of degradation contributes to the phenolic compound's composition (Espada-Bellido *et al.* 2018).

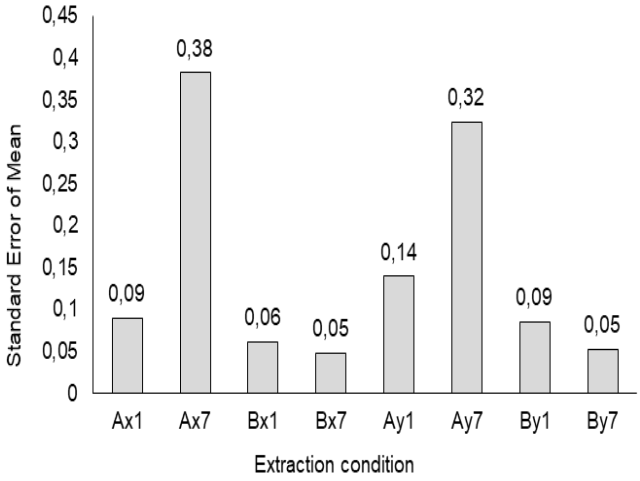

Fig. 6. The standard error of the mean of total phenolic content from each extraction condition.

In this study, we find that the various parameters must be considered to optimize the extraction of anthocyanins and phenolic compounds from plant. These parameters are the sample/solvent ratio, time, and pH. However, the solvents in this process must be considered, in specific for phenolic compounds. Extraction in an acid condition using water as a solvent and heating can promote the hydrolysis of anthocyanins or other secondary metabolites.

Conclusion

Extraction of the butterfly pea flower is a key process to obtain high quantity and quality of product for scale-up production. The process is affected by the ratio sample/solvent, extraction time, and pH. In this study, the optimum extraction condition producing the highest total anthocyanins content is at sample/solvent ratio 1 : 20 ($g.mL^{-1}$), 90 min and pH 1.0. The stability of anthocyanin was reached at acid condition, but is not always proportional to total phenolic content. The highest total phenolic content is produced at the sample/solvent ratio 1 : 50 ($g.mL^{-1}$), 90 min, and neutral condition (pH 7.0). The degradation of other compounds during the extraction contributes to the total phenolic

measurement. Further analysis for antioxidant activity and active compounds is needed and will be performed in the subsequent studies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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