JFSP Vol.8, No.3, December 2022, Page: 294-301, pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

DETERMINATION OF ANTHOCYANIN LEVEL IN KIDNEY BEAN (*Phaseolus vulgaris* L.) TEMPEH AS A HEPATOPROTECTIVE AGENT

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https://doi.org/10.31603/pharmacy.v8i3.3702

Article info:
Submitted : 04-07-2020
Revised : 16-05-2021
Accepted : 11-12-2022

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT

Liver is a part of body that is used as a toxin neutralizer as well as a target for increasing free radicals. In counteracting free radicals, body needs antioxidants in one of the plant secondary metabolites, namely anthocyanins. Anthocyanins are organic compounds from the water-soluble flavonoid family that give red, blue, and violet colors. Fermentation can increase anthocyanin levels in kidney beans. This study aimed to determine the presence of anthocyanins in kidney bean (Phaseolus vulgaris L.) tempeh which can be used as a hepatoprotective agent and to determine the anthocyanin levels in kidney bean (Phaseolus vulgaris L.) tempeh. Qualitative analysis using HCl and NaOH with positive results showed the color change to solid red then to bluish green and then faded. Determination of total anthocyanin levels was carried out using UV-Vis spectrophotometric method with the pH differential principal in anthocyanin showed in mg/100gram sample at a maximum wavelength of 525.5 nm. The result was that the average anthocyanin content was 40.37mg/100gram with a variant coefficient is 0.325%.

Keywords: Anthocyanin; Fermantation; Kidney bean; UV-Vis Spectrophotometry

1. INTRODUCTION

Liver is a part of body that helps neutralize toxins getting into the body and is also a target for free radical accumulation. Some liver disorders in the form of inflammation can be caused by various factors, such as infections, viruses, bacteria, parasites, drugs, alcohol, or autoimmune. Therefore, a hepatoprotective compound that can provide protection against liver damage is required. Hepatoprotective activity is possible due to the presence of anthocyanin compounds which have the ability to counteract free radicals. Free radicals are unstable atoms or molecules containing one or more unpaired electrons, thus, in order to obtain an electron pair these free radicals are very reactive and damage tissues (Pratama & Busman, 2020). Damage caused by free radicals in the body can be overcome by antioxidants (Sayuti & Yenrina, 2015). The body naturally produces endogenous antioxidants that are able to overcome the effects of free radicals. However, when the supply of free radicals increases, it requires a supply of antioxidants from outside. Anthocyanins are organic compounds from the water-soluble flavonoid family that give red, blue, violet colors, which also act as antioxidants. Anthocyanin pigments are potential natural pigments by contributing orange, pink, red, purple and blue colors (Anggriani et al., 2017).

A natural ingredient that potentially contains anthocyanins is kidney bean. In a previous study, kidney beans contained an anthocyanin of 32 mg/100 grams using methanol solvent which was acidified using 1% HCl using UV-Vis spectrophotometry (Sharma, 2015). In general, kidney beans processing techniques in the community are still simple, they are only used as a complement in the meal and processed into porridge. Fermentation technology is an alternative in processing

kidney beans for higher quality food products as it can increase the digestibility and nutritional value contained in the kidney beans (Maryam, 2015).

Tempeh is a popular food product in Indonesia which is obtained from a fermentation process with *Rhizopus sp* (Astawan et al., 2013). *Rhizopus sp* can produce ß-glucosidase enzymes. Related to antioxidant activity, anthocyanin in the aglycone form is stronger than the glycosylated form (anthocyanin) (Arifin & Ibrahim, 2018). Based on the research (Abdullah, 2017) the conversion of anthocyanins to anthocyanidins in black rice bran which was carried out using *Sacharomyces cerevisiae* which is known to contain ß-glucosidase enzyme activity indicated that anthocyanidins have high antioxidant activity. Based on the explanation above, research on the determination of anthocyanin levels in kidney bean tempeh needs to be carried out to obtain scientific data that can be used for clinical purposes, especially for scientific developments regarding liver disease.

2. METHOD

2.1. Tempeh Processing

The kidney beans were obtained from Girimarto, Wonogiri, Central Java, sorted, weighed as much as 500 grams, then boiled using a saucepan with a ratio of water and kidney beans 4:1. The kidney beans were soaked in a basin for 2 x 24 hours, then boiled again with a 4:1 ratio of water and beans. The kidney beans are then shrunk using a blender (Philips brand). The kidney beans were then inoculated using 0.5 gram of tempeh yeast (Raprima). Kidney beans that had been inoculated were then mixed homogeneously and wrapped in banana leaves. The kidney beans are placed at room temperature for 36 hours to ferment.

2.2. Anthocyanin Extraction

Kidney bean tempeh was shrunk then put in the oven at 40°C to be dried, then 100 grams of kidney bean tempeh were blended and weighed. Kidney bean tempeh was macerated with methanol containing 1% HCl, the ratio of sample and solvent used was 1:4 (w/v), for 24 hours at ± 5°C. The filtrate was filtered using Whatman Paper No 1, then macerated again with methanol containing 1% HCl. The same procedure was carried out on the filtrate for the second maceration. The results of the second filtrate was combined with the first filtrate and then concentrated using a water bath at 50°C to obtain a crude extract (Anggriani et al., 2017). The filtrate obtained were weighed to calculate the amount of yield using Eq. 1:

$$Yield = \frac{weight\ of\ concentrated\ extract\ (g)}{\text{weight of extracted sample (g)}} \times 100\% \quad (1)$$

2.3. Anthocyanin Qualitative Test

The content of anthocyanin can be proved in a simple way. The first way was by heating the kidney bean anthocyanin extract with 2M HCL for 2 minutes at 100°C, then observe the color of the extract. If the color does not change, anthocyanins exist. The second way was by adding kidney bean extract with 2M NaOH by drops. If the red color turns blue and then faded slowly, it proves the existence of anthocyanin (Anggriani et al., 2017).

2.4. Determination of λ maximum of extract

Determination of the λ maximum of kidney bean tempeh extract was carried out by UV-Vis spectrophotometry method. 0.5 ml of the macerated extract was dissolved in 5 ml of methanol using a volumetric flask brought to the limit mark, then 1 ml of the 5 ml volumetric flask was dissolved in 10 ml methanol using a 10 ml volumetric flask brought to the limit mark, then the absorbance was measured at a wavelength of 400- 800nm.

2.5. Anthocyanin Quantitative Test

Determination of anthocyanins was conducted using the pH differential method, namely pH 1.0 and pH 4.5. At pH 1.0 anthocyanins were in the form of oxonium compounds and at pH 4.5 they were colorless carbinols. This can be carried out by making an aliquot of anthocyanin

solution whose pH was 1.0 and 4.5 and then the absorbance was measured (F. D. Anggraeni & Sudiyono, 2020). Two sample solutions were prepared from kidney bean tempeh extract which was dissolved at pH 1.0 using KCl buffer and at pH 4.5 using Sodium Acetate buffer. The first sample was dissolved at pH 1.0 and the second sample was dissolved at pH 4.5. Diluted samples were measured at a wavelength of 500-700 nm and 700 nm. The absorbance result from the samples that had been measured (A) was determined by Eq 2:

$$A = (A_{max}–A_{700})_{pH\ 1}–(A_{max}–A_{700})_{pH4,5} \quad (2)$$

The total anthocyanin pigment in kidney bean tempeh was calculated using the Eq 3:

$$Antosianin = \frac{Absorbansi\ x\ MW\ x\ DF\ x\ 1000}{\varepsilon \text{ x L}} \quad (3)$$

Keterangan:

A = Solution Absorbance
ε = Cyanidin-3-glucoside molar absorptivity (26900 L/mol.cm)
L = The width of cuvette is 1 cm
MW = Cyanidin-3-glucoside molecular weight (449.2 g/mol)
DF = Dilution factor
1000 = Conversion from gram to mg

The accuracy of the method for determining the levels of anthocyanins in kidney bean tempeh extract was indicated by the precision parameter. Precision was performed by calculating the coefficient of variation (% CV) in Eq 4:

$$\%CV = \frac{Standard\ Deviation}{Mean} x100\ \% \quad (4)$$

A method is claimed precise if the coefficient of variation (% CV) indicated < 2% (Lestari, 2019).

2.6. Data Analysis

The data analysis technique in this study was that the total anthocyanin content of kidney bean tempeh extract was calculated using the pH differential formula.

3. RESULT AND DISCUSSION

3.1. Tempeh Producing

Kidney beans were sorted to remove dirt or unfit parts for use. The next process was to wash the kidney beans cleanly using running water to remove the dirt and also to separate the damaged kidney beans. Floating kidney beans during washing process means the kidney beans are damaged and discarded. Next, clean kidney beans were boiled in a pot using clean water. Boil the beans for 15 minutes to avoid overheating because anthocyanins are not resistant to heating. Heating process will cause anthocyanins to be damaged (Hambali & Noermansyah, 2015). According to (Nasrullah et al., 2020) heating process that can prevent damage to anthocyanins is heating using high temperatures for a short period of time (High Temperature Shot Time) at 80°C for 1 minute.

After boiling, the kidney beans were removed and cooled. Next, the soaking water was replaced with clean water, then left for 12 hours. This soaking would cause the kidney beans to rise. After that, kidney beans were boiled again to remove odors and other bacteria that could interfere the fermentation process. The odor, bacteria and dirt usually arose when soaking. The boiling process was carried out until the water boils, then left it for about 15 minutes to kill the germs and bacteria.

Fermentation is the most important factor in producing tempeh. This yeast has a role in fermenting the kidney beans to become tempeh. Fermentation was carried out after the kidney

beans were dry and still a bit warm. Kidney beans that are too hot will kill the tempeh yeast, while kidney beans that are too cold will inhibit the growth of yeast or mold. The yeast needed was 0.5 grams by sprinkling the yeast evenly over the kidney beans, then stir until the yeast was evenly mixed. After the fermentation was complete, the kidney beans were wrapped.

Kidney beans were wrapped in banana leaves because packaging using banana leaves will produce a more compact tempeh (Werdiningsih et al., 2018). Inoculation is the process of storing kidney beans that have been given yeast in warm temperatures for 36 hours so that the kidney beans are fermented. 36 hours is chosen because the tempeh that is formed has a compact texture and has the best antioxidant activity (Sidiq et al., 2016).

After the kidney bean tempeh was ready, it was then shrunk and put in the oven with a temperature of 40°C until dried and then was blended. This destruction process effectively damages cell tissue, accelerates the extraction process of anthocyanins, and expands the surface of the material to be extracted. This resulted in a higher dissolution rate of the material to be extracted. Soft tissue can speed up the time needed to dissolve anthocyanins (Mojica et al., 2017).

3.2. Extract Producing

The first step to determine total anthocyanin was extraction to obtain anthocyanin extract. Anthocyanins are found in the part of the cell that is closest to the surface. Anthocyanins contained in these tissues can be obtained by extraction using certain solvents (Mojica et al., 2017). The effectiveness of the extraction process is inseparable from the ability of the extractor to dissolve the extracted components. Polarity is necessary to be considered in the extraction process. The polarity between the extracting materials must be the same as the polarity of the material being extracted. Polar compounds can only dissolve in polar solvents, and non-polar compounds can only be dissolved in non-polar solvents. According to (Inggrid et al., 2018), anthocyanin is a polar component so that the solvent used must also be polar.

The sample used in this extraction was kidney bean tempeh. According to (Amanah, 2019) fermentation can increase anthocyanin levels because the tempeh yeast used for fermentation contains the ß-glucosidase enzyme which can increase anthocyanin levels by converting anthocyanin glycones into aglycones. According to (Mojica et al., 2017), extraction using methanol containing a little acid is the most effective solvent and according to (V. J. Anggraeni et al., 2018) extraction using methanol containing 1% HCl obtains the highest anthocyanin content. This solvent was chosen because of the polarity of anthocyanin and methanol, both of which were polar. A number 1% HCl was added to hydrolyze anthocyanins which were in the form of aglycones so that they could be measured properly at the maximum wavelength.

The process of extracting anthocyanin pigments from kidney bean tempeh was conducted by maceration for 24 hours at ±5°C with periodic stirring. This aims to obtain the maximum anthocyanin. Maceration was carried out at 5°C to prevent anthocyanin degradation. This maceration process produced a red filtrate. This was carried out to optimize the extraction process so that the anthocyanin pigment contained in kidney bean tempeh could be completely extracted. Periodic stirring was performed to increase the effectiveness of the extraction process. The resulting filtrate was then concentrated using a water bath at 50°C. According to (Anggriani et al., 2017) anthocyanins are stable at 50°C. In addition, the use of low temperatures aims to avoid degradation of anthocyanins (Sigurdson et al., 2017).

Yield calculations were performed to determine the ratio of the amount (quantity) of the extract obtained with the amount of the previous material. This yield determination served to discover the levels of secondary metabolites carried by the solvent, however, it cannot determine the type of compound carried by the solvent. This extract was used to determine the anthocyanin levels in kidney bean tempeh. The results indicated that the yield of kidney bean tempeh extract was 48.18% (**Table 1**).

Table 1. The Result of Yield of Kidney Bean Tempeh Extract

Replication	After concentration	Weight of extract (gram)	Yield (%)
Replication 1	Dark red liquid	48.51	48.51
Replication 2	Dark red liquid	48.86	48.86
Replication 3	Dark red liquid	47.18	47.18

3.3. Qualitative Test

The results of the anthocyanin proof test can be seen in **Table 2** which indicated that the kidney bean tempeh extract extracted using methanol containing 1% HCl contained anthocyanins. It was seen when the extract was given the basic compound NaOH, a bluish green color appeared and when the acid compound HCl was added which was heated for 5 minutes, a steady red color appeared. At a high pH, anthocyanins will tend to be bluish and at a low pH, they will be red (**Figure 1**) (Maulida & Guntarti, 2015).

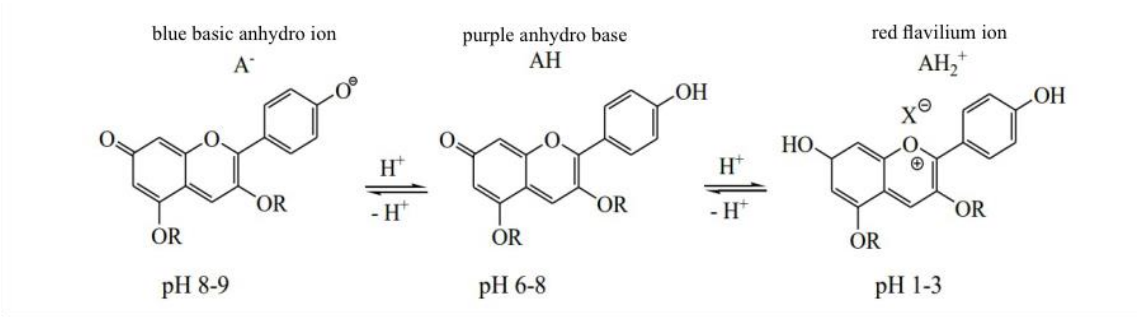

Figure 1. Changes in anthocyanin structure at various pH (Maulida & Guntarti, 2015)

Table 2. The Result of Anthocyanin Qualitative Test

No	Treatment	Anthocyanin Characteristics	Test Result	Information
1	Heated with HCl for 5 minutes at 100°C	Unfaded red color (Maulida & Guntarti, 2015)	Unfaded red color	+
2	Added with NaOH by drops	The red color changes to bluish green and then faded (Maulida & Guntarti, 2015)	Bluish green color	+

3.4. Maximum Wavelength Determination

In this study the determination of the maximum wavelength aims to determine the maximum absorbance of anthocyanins contained in kidney bean tempeh extract. This maximum wavelength determination used the UV-Vis spectrophotometry method at a wavelength of 400-800 nm. According to (V. J. Anggraeni et al., 2018) anthocyanin extraction using methanol solvent produces a wavelength of 515-545 nm where the wavelength is cyanidin-3-glucoside. Based on the results of the maximum wavelength determination, data as seen in **Table 3** was obtained.

Table 3. Maximum Wavelength

No	Wavelength (nm)	Absorbance
1	687.0	0.497
2	678.5	0.494
3	525.5	0.656
4	512.0	0.636

The result of the maximum wavelength determination for kidney bean tempeh extract was at a wavelength of 525.5 nm. This is because this wavelength is included in the wavelength range of anthocyanin cyanidin-3-glucoside and this wavelength has the maximum absorbance.

3.5. Anthocyanin Quantitative Test

Determination of anthocyanin levels was conducted out using the pH differential method, namely pH 1 and pH 4.5. This anthocyanin level determination is at pH of 1.0 as anthocyanins form oxonium compounds (flavinum cations). The more acidic condition approach pH 1, the more

anthocyanins will be in the form of colored oxonium (flavinum cation). At a pH of 4.5, that is in a weak acid, the flavylium cation changes to a more stable form, namely a colorless and chalcone-shaped hemiketal (Kwartiningsih et al., 2016). The difference in absorbance between the two buffers was commensurate with the monomeric anthocyanin pigments. Monomeric anthocyanin underwent a reversible structure with changes in pH; oxonium form was colored at pH 1 and the hemiketal form at pH 4.5. These conditions would be used as a reference for measuring absorbance using a UV-Vis spectrophotometer as it was proportional to the pigment concentration. The following is the structure of anthocyanin color changes due to differences in pH levels, which can be seen in the **Figure 2** below.

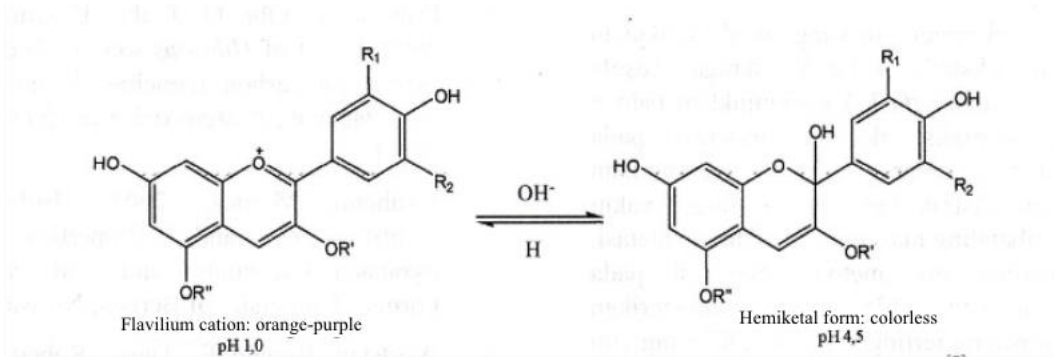

Figure 2. Chemical Structure of Flavylium Cations And Hemiketal (Pratiwi & Priyani, 2019)
Note: R=H or glycoside substituents

Samples were measured at maximum wavelength and at a wavelength of 700 nm. A wavelength of 700 nm was to correct precipitates that still existed in the sample. If the sample is completely clear, the absorbance at 700 nm is 0. In determining total anthocyanins, the dilution factor must be determined first by dissolving the sample with a buffer solution of pH 1 so that an absorbance of less than 1.2 was obtained. The dilution factor had been determined to be 100 times at the maximum wavelength of kidney bean tempeh extract of 525.5 nm. In this study the wavelength measured at 700 nm did not give an absorbance of 0, which was due to the existence of small particles in the sample. 0.5 ml of the sample pipetted was put into a 5 ml volumetric flask and then pH 1 was added and brought to the mark. Samples from a 5 ml volumetric flask were pipetted as much as 1 ml into a 10 ml volumetric flask and pH 1 was added and brought to the mark, then left for 15 minutes so that the sample dissolved at pH 1 formed flavylium cations entirely and at pH 4.5 formed hemiketal and the absorbance was measured using the maximum wavelength and at wavelength of 700 nm, as well as the treatment of a pH 4.5 solution. The absorbance results were put into the formula and the mean and %CV of yielded levels of each replication were then measured.

Table 4. Total Anthocyanin Levels in Kidney Bean Tempeh Extract

Replication	Triplo	Anthocyanin Level mg/100 gram	Mean SD	%CV
Replication 1	1	40.60	40.67	0.284
	2	40.60		
	3	40.80		
Replication 2	1	40.79	40.68	0.258
	2	40.69		
	3	40.58		
Replication 3	1	39.57	39.77	0.435
	2	39.87		
	3	39.87		
The average total anthocyanin content is 40.37 mg/100 gram of extract with a % CV of 0.325%				

Total anthocyanin levels were stated in mg/100 grams. The results in the **Table 4** above indicated an average total anthocyanin content of 40.37 mg/100 grams of extract. Compared to

research of (Sharma, 2015), the anthocyanin in kidney beans is 32 mg/100 grams indicating that the anthocyanin content is higher by fermentation. Based on a study, the high anthocyanin content has the ability of antioxidant compounds that potentially to be a hepatoprotective agent as has been tested in animals (Suriani et al., 2019) and (Hardiningtyas et al., 2014). The coefficient of variation was determined to figure out the proximity of the results of one analysis to others analysis of a series measurements obtained from continuous sampling of homogeneous samples. The %CV result of the anthocyanin level determination of kidney bean tempeh extract was 0.325% <2.0%. A good CV value is less than 2.0%. It indicated that the data was obtained with a good level of work accuracy.

4. CONCLUSION

Based on the result of the research, it can be concluded that the results of anthocyanin qualitative analysis in kidney bean tempeh are positive for containing anthocyanins. Based on the results of anthocyanin level determination, it is obtained an average level of 40.37 mg/100 grams with an average % CV of 0.325%. Based on the results of this study, it is necessary to isolate anthocyanin compounds so that they can be formulated and used as alternative medication derived from natural ingredients.

5. CONFLICT OF INTEREST

The author states that there is no conflict of interest in conducting this research.

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