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The Effect of Pumpkin Fruit Ripeness (*Cucurbita moschata*. D) on Total Flavonoid Levels and Antioxidant Activity

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Abstract

Pumpkin fruit (*Cucurbita moschata.* D) belongs the Cucurbitaceae family which is a functional vegetable widely distributed in Indonesia because it has nutritional value and health benefits. This study aims to determine the value of total flavonoid content and antioxidant activity in the ethanol extract of unripe, mature and ripened pumpkin. Simplicia powder was extracted by maceration method using 96% solvent. Testing the total flavonoid content with the addition of $AlCl_3$ at a wavelength of 425 nm and the antioxidant activity test was carried out using DPPH (1-1-diphenyl-2-picrylhydrazyl) as a free radical with a maximum wavelength of 515 nm using *microplate reader*. The results of the study concluded that the ethanolic extract of pumpkin flesh had a total flavonoid content of 0,146 mgQE/100g in unripe fruit, 0,221 mgQE/100g in mature pumpkin, 0,191 mgQE/100g in ripened pumpkin. The antioxidant activity of the ethanol extract of unripe, mature, and ripened pumpkin fruit obtained was not active or did not completely reduce free radicals.

Keywords: Pumpkin Fruit (*Cucurbita moschata* D); total flavonoid levels; antioxidant activity.

INTRODUCTION

Pumpkin is an annual plant that has been known to the Indonesian people for a long time and is widely used in traditional food preparations. Pumpkin is considered a functional vegetable that is widespread in Indonesia and is very adaptable to various environmental conditions. The availability of pumpkin in Indonesia is relatively large. However, the high production of pumpkin in Indonesia is not matched by the use of pumpkin (Purwanto *et al*., 2013).

The use of yellow pumpkin are still limited to the household scale, which is processed into cooked vegetable from unripe unripe fruits, or dodol, cakes, compotes, and pastries from ripe fruits (Hamdi *et al*., 2017). Sharma *et al*., (2020) suggested that this plant has a broad spectrum for the treatment of diseases associated with its constituent compounds. The skin, flesh, and seeds of pumpkin have high nutritional value because they contain a lot of total phenols, total carotenoids, flavonoids and a large number of macro and micro nutrients (Hussain *et al*., 2021).

Flavonoids are well-known active substances from plants that act as drugs in the human body. Pumpkin seeds and flesh have higher total flavonoid content due to higher metabolism in plant parts, resulting in more metabolites. Although the total flavonoid content in pumpkin is less than the total phenol content, the total flavonoid content at low concentrations also has strong antioxidant potential (Asif *et al*., 2017).

Gumolung *et al*., (2013) suggested that ethanol extract of pumpkin flesh produced an antidote to free radicals of 62,16%. The ethanolic extract of pumpkin fruit has phenolic compounds, especially flavonoids as antioxidant activity in the moderate category with an IC_{50} (Inhibitory Concentration) value of 175,672 µg/mL and as a comparison is trolox with an IC_{50} value of 33,177 µg/mL (Lukita, 2021). This flavonoid compound can release hydrogen radicals contained in the hydroxyl group (-OH) to attach to DPPH radicals, so that DPPH radicals become stable (Sabarudin *et al*., 2021). In addition, flavonoids have the ability to scavenge free radicals and inhibit lipid oxidation (Zuraida *et al*., 2017). As antioxidants, flavonoids are able to inhibit degenerative and chronic diseases. In addition to antioxidants, flavonoids are said to have hepatoprotector, antithrombotic, anti-inflammatory, and antiviral properties (Dewi *et al*., 2018). The effects of flavonoid compounds are very diverse and very beneficial especially in relation to traditional medicine (Sopan *et al*., 2014).

Previous studies by Mokhtar *et al*., (2021) conducted an analysis of the phenolic content, flavonoids of (*Cucurbita moschata* D) at various stages of ripening (unripe, mature, ripened) and determine antioxidant activity. The content of polyphenolic and flavonoids

compound in riped pumpkin was 97.4 mgGAE/gram and 28.6 mgQE/gram. Ripe pumpkin showed high antioxidant activity against DPPH radicals, which was 0.065 ± 0.010 mol TE/gram.

Based on the description above, it is necessary to conduct research to identify total flavonoid compounds and antioxidant activity of yellow pumpkin fruits during the ripening stage (unripe, mature, ripened). So that this research aims to study the changes in the profile of total flavonoid levels during ripening, and the development of antioxidant activity. Samples had been taken from Tegal Rejo Village which is part of Tegalsari District, Banyuwangi Regency, East Java. The livelihood of the people is 100% in agriculture, one of the cultivated commodities is yellow pumpkin fruit (Fauzi dan Purnomo, 2016). Three fruit samples from each stage of development were selected based on their morphological attributes.

MATERIALS AND METHODS

Material

Unripe, mature, ripened pumpkin fruit obtained from Tegal Rejo Village, Tegalsari District, Banyuwangi Regency, East Java, ethanol solvent 96%, methanol, sodium nitrite (NaNO2) 5%, sodium hydroxide (NaOH) 1 M, Quercetin, DPPH (1,1-diphenyl-2pikrihildrazil), aluminum chloride $AlCl_3$ 10%, and distilled water.

Table 1. Identification of pumpkin fruits (*Cucurbita moschata* D) of different degrees of maturity.

Identification	Description	Picture
Unripe Pumpkin fruit	Light green, the shape of the fruit is flat-rounded. The grooves of the fruit are indistinct, fruit patches are present, the color of the flesh is yellowish-green.	
Mature Pumpkin fruit	Orange green, the shape of the fruit is flat-rounded, the grooves of the fruit are not clear, the color of the flesh is orange.	
Ripened Pumpkin fruit	Brown, flat round fruit shape, fruit spots are present, fruit groove is clear, flesh color is dark orange.	

Procedures

Sample and extract preparation

Unripe, mature, ripened pumpkins were separated from the seeds and skin, then washed with running water and sliced thinly. Then dried using the oven at a temperature of 50º-70º C and put in a blender into powder. Furthermore, 100 grams of simplicia powder were weighed at various levels of fruit maturity and extracted with 500 ml of 96% ethanol for ± 3-5 days and stored at room temperature, after which it was filtered. The result obtained is called the filtrate. The filtrate was then concentrated with rotary evaporatoruntil a thick extract is obtained**.**

Determination of total flavonoid level contents in pumpkin extracts

The method used for total flavonoid contents determination in pumpkin powders was $AlCl_3$ colorimetric assay as described by Dona *et al*., (2020) with slight modification. A sample of 5 mg was dissolved in 5 mL of ethanol, so that the mother liquor with a solution concentration of 1000 ppm was obtained. Then 100 µL was pipetted, and put into a *microwell plate* with three replications. Then added 50 µL $NaNO_2$ 5% and 50 µL $AlCl_3$ 10%, and incubated for 5 minutes in a dark room at room temperature, and added 50 µL NaOH 1 M, then let stand for 30 minutes in a dark place. Then the absorbance was measured using a microplate reader at a wavelength of 425 nm.

Determination of antioxidant activity contents in pumpkin extracts

Determination of antioxidant activity contents in pumpkin extracts was performed through a method by Nasution dan Ardhiyati, 2019). As much of 5 mg of the sample was dissolved in 5 mL of methanol so that the sample concentration was 1000 µg/mL. As much was added 50 µL (plate consists of rows A-H with each

totaling 12 holes). As much as 50 µL was put into each well of rows F, E, D, C, B. Furthermore, samples with a concentration of 1000 µg/mL as much as 50 µL were inserted into rows G and F. Then diluted rows F, E, D, C, B. Row F 50 µL pippeted into row E, row E pipetted 50 µL into row D, row D 50 µL pipetted into row C, row C 50 µL pipetted into row B. Row B 50 µL pipetted then discarded, so that the concentration of the test solution is 32 ppm (µg/mL), 63 ppm (µg/mL), 125 ppm (µg/mL), 250 ppm (µg/mL), 500 ppm (µg/mL), dan 1000 ppm (µg/mL). Next row G, F, E, D, C, B dan A added 80 µL DPPH with a concentration 80 ppm. Then incubated for 30 minutes at room temperature to protect from light. The absorbance of the sample was measured using microplate reader at the maximum wavelength 515 nm. Then the calculation of the value of % inhibition and calculation of IC_{50} were performed.

$$\%\ Inhibition\ = \frac{A\ control\ - A\ sample}{A\ control}\ x\ 100\ \%$$

Description:
$A\ control$: Absorbance of DPPH
$A\ sampel$: Absorbance of the DPPH solution containing the sample

After calculating the % inhibition, the IC_{50} value was calculated from the linear regression equation,

$$y\ =\ ax\ +\ b$$

Description:
x : As sample concentration
y : % antioxidant activity

Data analysis

We analysis of the total flavonoid compounds obtained from the absorbance of the comparison quercetin solution, presented a calibration curve and obtained a linear regression. The average absorbance was fitted in the standard curve equation as the y value, where the x value obtained was the concentration in mgQE/100g. Analysis of antioxidant activity was obtained from the value of % inhibition and calculation of IC_{50}.

RESULTS AND DISCUSSION

Yield of pumpkin fruit ethanol extract

Yield of 96% ethanol extract of unripe pumpkin is 34,2%, ripe pumpkin is 33,1% and mature pumpkin is 19,7% (Table 2). The yield shows the active component that was successfully extracted (Zuraida *et al*., 2017). Yield results can be influenced by several extraction factors, including time, temperature, type of solvent, ratio of material and solvent, particle size and processing of the plant (Chairunnisa *et al*, 2019).

Table 2. Yield of pumpkin fruit ethanol extract.

Sample	Simplicia weight	Extract weight	% Yield
Unripe pumpkin	100 gr	34,2	34,2%
Mature pumpkin	100 gr	19,7	19,7%
Ripened pumpkin	100 gr	33,1	33,1%

Total flavonoid content

This analysis aims to determine the total flavonoid content in the extract obtained from the standard curve equation. The choice of quercetin as a standard solution is due to its compounds that are widely distributed in plants (Hasanah & Novian, 2020). The following absorbance data on the concentration of quercetin produces a standard curve line y= 0,0013x + 0,0783 with an value R^2 = 0,945 (Figure 1). From this equation, the total flavonoid content obtained is 0,146 mgQE/100g extract of unripe pumpkin, 0,221 mgQE/100g extract of mature pumpkin and 0,191 mgQE/100g extract of ripened pumpkin as shown in Table 3.

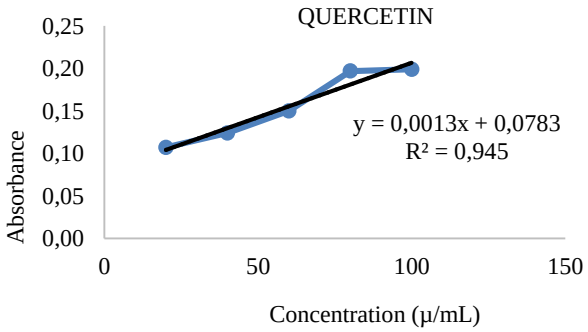

Figure 1. The standard curve of quercetin.

Table 3. Data in the total flavonoid content of the ethanol extract of unripe, mature, ripened pumpkin.

Sample	Absorbance measurement			Average KTF (mgQE/100g)	SD	KTF ± SD (mgQE/100g)
	1	2	3			
Unripe pumpkin	0,111	0,117	0,130	0,146	0,0079	0,146 ± 0,0079
Mature pumpkin	0,164	0,176	0,182	0,221	0,0075	0,221 ± 0,0075
Ripened pumpkin	0,149	0,151	0,153	0,191	0,0016	0,191 ± 0,0016

Antioxidant activity

In the antioxidant activity test, that ripe, mature and unripe pumpkins did not completely reduce the antioxidant activity (Table 4).

Table 4. The results of the measurement if % inhibition and IC_{50} value from ethanol extract of unripe, mature, ripened pumpkin.

Sample	Konsentrasi (µg/mL)	% inhibition	IC_{50} (µg/mL)	Rata-rata IC_{50} ± SD (µg/mL)	IC_{50} (µg/mL)
Raw pumpkin	63	1,1765	1337	1443,8 ± 102,37	Very weak or inactive > 500 (µg/mL) (Waode Rustiah *et al*., 2018)
	125	5,8824			
	250	5,8824			
	500	24,7059			
	1000	35,2941			
	63	1,1765	1454		
	125	7,0588			
	250	9,4118			
	500	15,2941			
	1000	35,2941			
	63	3,5294	1541		
	125	7,0588			
	250	12,9412			
	500	15,2941			
	1000	34,1176			
Mature pumpkin	32	2,198	1637	1751 ± 242,05	Very weak or inactive > 500 (µg/mL) (Waode Rustiah *et al*., 2018)
	63	5,495			
	125	7,692			
	250	10,989			
	500	16,484			
	1000	31,868			
	32	3,297	1587		
	63	5,495			
	125	8,791			
	250	12,088			
	500	17,582			
	1000	32,967			
	32	4,396	2029		
	63	5,495			
	125	7,692			
	250	10,989			
	500	16,484			
	1000	26,374			
Ripened pumpkin	32	2,198	2563	2283,6 ± 319,04	Very weak or inactive > 500 (µg/mL) (Waode Rustiah *et al*., 2018)
	63	4,396			
	125	5,495			
	250	5,495			
	500	12,088			
	1000	20,879			
	32	2,198	2352		
	63	4,396			
	125	6,593			
	250	7,692			
	500	12,088			
	1000	23,077			
	32	4,396	1936		
	63	3,297			
	125	6,593			
	250	6,593			
	500	14,286			
	1000	27,473			

Discussion

Pumpkin has many health benefits, traditionally used to treat skin diseases, measles, jaundice, insomnia, cancer and can help increase endurance due to its antioxidant activity (Sabarudin *et al*., 2021). Different plant parts can have different phytochemical compounds, which can cause different pharmacological effects (Sembiring *et al*., 2018). The extraction method used is maceration.

The maceration method is used because the tools and methods are simple, and it does not use high temperatures which are at risk of damaging the chemical components of materials that are not resistant to high temperatures (Prasetya *et al*., 2020). 96% ethanol is used as a solvent which is polar, so it is good to be used as an extract solvent to extract polyphenol compounds and a solvent that is safe for drugs (Dai & Mumper., 2010).

Analysis of the total flavonoid content test was carried out using the instrument *microplate reader* ELISA (λ) 425 nm, because it is simple, easy to operate, faster, can use many samples at once in measurement and can use smaller volumes such as 200-500µl for *microplate 96 -well* (Berg *et al*., 2016). The measurement of total flavonoid levels using the colorimetric method is based on the formation of a complex reaction between flavonoids and aluminum chloride ($AlCl_3$) (Zuraida *et al*., 2017). Reagent $AlCl_3$ added after $NaNO_2$ will form a stable complex with a C4 keto group and a C3 or C5 hydroxyl group on flavones and flavonols (Syafitri *et al*., 2014), resulting in a shift in wavelength towards the visible which is indicated by the solution producing a more yellow color. The results of the total flavonoid content obtained are in the unripe pumpkin of 0,146 mgQE/100g extract, mature pumpkin of 0,221 mgQE/100g extract, and ripened pumpkin of 0,191 mgQE/100g extract can be seen in Table 2. The values obtained in this study were not much different from those of Hasanah dan Novian (2020) of 0,00288 mg/g. Flavonoids will experience a decrease due to the influence of temperature during the drying process because these compounds are sensitive to light and heat. According to Zainol *et al* (2009) the degradation of flavonoids occurs due to the termination of the molecular chain and the occurrence of an oxidation reaction that causes the oxidation of the hydroxyl group and will form other volatile compounds quickly.

Testing the antioxidant activity of the ethanolic extract of pumpkin fruit by reducing the free radical 1.1-diphenyl-2 pikhrylhydrazil (DPPH) using ELISA *microplate reader* (λ) 515 nm. The results can be seen in Table 4. That unripe, mature, and ripened pumpkins did not completely reduce the antioxidant activity with IC_{50}. The results obtained in unripe pumpkin of 1432,37 µg/mL, mature pumpkin of 1751 µg/mL, ripened pumpkin of 2283,67 µg/mL which could be categorized as very weak or inactive antioxidant activity (Waode Rustiah *et al*., 2018). The difference in activity obtained in each extract is probably due to differences in the content and number of active compounds contained in the extract, so that the antioxidant activity obtained is also different (Purwanto *et al*. 2017). In general, the smaller value IC_{50} obtained means the higher the antioxidant activity (Dona *et al*., 2020). This is possible that in pumpkin which has flavonoid levels in addition to antioxidant activity and also acts as antifungal, diuretic, antihistamine, antihypertensive, insecticide, antiparasitic, anthelmintic, and antiviral (Hasanah dan Novian., 2020).

Another factor that causes weak antioxidant activity is that the compound is still not pure, so it is necessary to do fractionation and purification in the hope that the value will be obtained IC_{50} of specific compounds that have stronger antioxidant activity. The presence of secondary metabolites other than flavonoids may not provide a synergistic response so that the antioxidant activity produced does not completely reduce (inactive) free radicals (Mz *et al*., 2017). In addition, the cause of the ethanol extract of unripe, unripe, and ripe pumpkin fruit does not completely reduce free radicals due to the phytochemical test results which show a small total flavonoid content value. This is supported by research from Gumolung *et al*., (2013) who stated that the part of the pumpkin that contained high free radical scavenging activity specifically was in the skin, stump and seeds compared to the pumpkin flesh. Sopan *et al*., (2014) also suggested that the flesh of pumpkin (*Cucurbita moschata* D) has large phenolic compounds which are very strong chain-breaking antioxidants and it has been reported that phenolic compounds are associated with antioxidant activity. This phenolic compound has many hydroxyl groups including o-hydroxy groups which have very strong antioxidant potential.

According to Supriatna *et al*., (2019), the level of age and maturity of a plant affects the maximum active content of secondary metabolites in the plant. Reinforced by the statement Metusalach (2007) suggests that the growth of a plant is influenced by external and internal factors. External factors such as habitat, season, water temperature, types of food available and other environmental factors, while internal factors, namely age, size and other biological factors determine antioxidant properties. One of the supporting factors of antioxidant activity is the presence of phenolic compounds and flavonoids that can reduce free radicals, as stated by Nuret *et al.* (2019) which states that there is a correlation between phenolic and flavonoid content on antioxidant activity. The content of phenolic compounds and flavonoids in the extract will act as hydrogen donors, reducing agents and unpaired oxidant absorbers. However, another factor that is no less important to the high antioxidant activity is the other bioactive compounds present in the sample, such as tannins and quinones which also have potential as antioxidants whose levels have not been studied in this study. As for the possibility of other compounds in pumpkin fruit that will have potential as antioxidants when extracted with semi-polar and non-polar solvents. There is necessity to aim of taking non-polar fractions in pumpkin which are thought to have potential as antioxidant compounds.

CONCLUSIONS

Pumpkin (*Cucurbita moschata* D) unripe has a total flavonoid content of 0,146 mgQE/100g, mature pumpkin of 0,221 mgQE/100g, ripened pumpkin of 0,191 mgQE/100g. Antioxidant activity in pumpkin fruit (*Cucurbita moschata* D) unripe, mature, and ripened are classified as very weak or inactive because they do not completely scavenge free radicals.

***Conflict of Interests*:** Authors state that there is no conflict of interest in this research output.

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