Antioxidant Activity of Methanol and Chloroform Extracts of Avocado Seeds (Persea americana Mill.) using the DPPH Method

Ambar Pratiwi*, Adilla Shafa Nafisa
Prodi Biologi, Fakultas Sains dan Teknologi Terapan, Universitas Ahmad Dahlan, Yogyakarta, Indonesia
*Corresponding author: ambar@bio.uad.ac.id

Abstract

One part of the avocado (*Persea americana* Mill.) that is often unused and considered waste is the avocado seed. Avocado seeds have the potential to be made into an extract because they contain antioxidant compounds. The aim of this research was to determine the highest antioxidant activity, total phenolic and total flavonoid content in methanol and chloroform extracts, and to determine the relationship between total phenolic and total flavonoid content on the IC50 values of methanol and chloroform extracts. Antioxidant activity was tested using the DPPH method. Data analysis used the classic assumption test, independent sample t-test, and Pearson correlation test. The results showed that the highest IC50 was found in the chloroform extract at 349.28 ± 137.29 µg/mL DW. The highest phenolic content was found in the methanol extract at 2,419.51 ± 389.54 mgGAE/g DW. The highest total flavonoid content was found in the chloroform extract at 232.13 ± 54.32 mgQE/g DW. Based on the Pearson correlation test, the total flavonoid content had a correlation (0.028<0.05) with the IC50 value of methanol and chloroform extracts of avocado seeds, while the total phenolic content had no correlation (0.749>0.05) with the IC50 value of methanol and chloroform extracts of avocado seeds.

Keywords: *antioxidants, avocado seeds, correlation, DPPH, total flavonoids, total phenolic.*

Abstrak

Salah satu tanaman yang memiliki senyawa antioksidan adalah alpukat (*Persea americana* Mill). Pada umumnya biji alpukat sering tidak terpakai, namun ada yang memanfaatkannya menjadi minyak biji alpukat sebagai tepung untuk ransum ayam ras pedaging. Tujuan dari penelitian ini yaitu untuk menentukan aktivitas antioksidan, kadar fenolik total dan kadar flavonoid total pada ekstrak metanol dan kloroform biji alpukat. Selain itu untuk mengetahui hubungan antara kandungan fenolik total dan flavonoid total terhadap nilai aktivitas antioksidan dari ekstrak metanol dan kloroform biji alpukat. Pada penelitian ini biji alpukat dikeringkan, diserbuk dan dimaserasi menggunakan kloroform dan metanol. Ekstrak yang diperoleh kemudian diuji aktivitas antioksidannya dengan metode DPPH serta dihitung kandungan fenol dan flavonoid total. Analisis data menggunakan uji asumsi klasik dan uji korelasi pearson untuk mengetahui korelasi flavonoid total dan fenolik total terhadap aktivitas antioksidan ekstrak kloroform dan metanol biji alpukat (*Persea americana* Mill.). Hasil penelitian menunjukkan bahwa nilai aktivitas antioksidan (IC50) tertinggi terdapat pada ekstrak kloroform biji alpukat yaitu sebesar 349,28 ± 137,29 µg/mL DW, sedangkan pada ekstrak metanol biji alpukat sebesar 668,82 ± 373,69 µg/mL DW. Kadar fenolik total paling tinggi terdapat pada ekstrak metanol biji alpukat yaitu sebesar 2419,51 ± 389,54 mgGAE/g DW, sedangkan pada ekstrak kloroform biji alpukat yaitu sebesar 529,69 ± 52,07 mgGAE/g DW. Kadar flavonoid total paling tinggi terdapat pada ekstrak kloroform biji alpukat yaitu sebesar 232,13 ± 54,32 mgQE/g DW, sedangkan pada ekstrak metanol biji alpukat sebesar 209,81 ± 44,63 mgQE/g DW. Berdasarkan hasil penelitian kali ini sesuai uji korelasi pearson yang telah dilakukan, kandungan flavonoid total memiliki korelasi atau hubungan (0,028<0,05) dengan aktivitas antioksidan ekstrak metanol dan kloroform biji alpukat (*Persea americana* Mill.).

Kata Kunci: *antioksidant, biji alpukat, korelasi, DPPH, total flavonoid content, total fenolic content.*

INTRODUCTION

Antioxidants act as compounds that donate one electron to free radical compounds, so the free radical compound will be stable (Winarti, 2010). Antioxidants play an important role for humans, among other things, to inhibit the oxidation of free radicals and have a role as a defense mechanism for the body against various diseases associated with free radical attacks (Lindsay & Astley, 2006). Sources of antioxidants can be found in vegetable and fruit plants which are categorized as natural antioxidant compounds (Parwata, 2016).

The avocado plant (Persea americana Mill.), is a plant that contains antioxidant compounds. This plant is considered a fruit that has high economic value and has several benefits for human health. Avocados are usually made into various kinds of food preparations, such as steamed cakes, juices and salads. The flesh of the

fruit is rich in nutrients, and the leaves are commonly used as a herbal treatment for kidney disease and high blood pressure (Antia et al., 2005).

In general, avocado seeds are often not used, but some people use them as flour for broiler chicken rations. Avocado seeds are good enough to be used as an alternative as flour for broiler chicken rations up to a percentage of 5% (Harahap et al., 2019). Most Indonesians consider avocado seeds to be waste that cannot be utilized, even though avocado seeds actually contain antioxidants that can be beneficial for health.

According to Vinha et al., (2013), avocado seeds contain various phytochemical compounds including phenolic 704 mg per 100 g, flavonoids 47.9 mg per 100 g, carotene 0.988 mg/100 g, vitamin C 2.6 mg per 100 g and vitamin E 4.82 mg per 100 g. Phenolic compounds are known as the most important plant components, which are able to fight free radicals. Phenolic compounds can act as reducing agents, hydrogen donors and oxidation reducers (Hsu et al., 2007). Flavonoids are known to be good reducing compounds. Flavonoids can inhibit various oxidation reactions, both enzymatic and non-enzymatic (Robinson, 2002).

Utami (2009) explained that the type of solvent is a factor that influences the extraction results of a sample. The extracting solution used must match the polarity of the targeted compound. According to the principle like dissolves like, a solvent will tend to dissolve compounds that have the same polarity level. Polar solvents will dissolve polar compounds and vice versa (Suryani et al., 2015). Based on research by Hartati et al., (2013), the results showed that the methanol extract from mahogany seeds (Swietenia mahagoni) showed the highest antioxidant activity (60.89%) compared to the 70% methanol extract (60.77%), ethanol pa (58.85%), ethanol 70% (59.87%) and acetone pa (46.54%). Yulistian et al., (2015), stated in their research that 80% acetone extract from cowpea seeds (Vigna unguiculata (L.) Walp) is a solvent capable of extracting phenolic compounds, flavonoids and the highest antioxidant activity with total phenolic and flavonoid levels. The resulting total reached 8,081.4 EAG/g sample and 33,308.3 EK/g sample, while the measured antioxidant activity reached 620.9%.

In this study, antioxidant activity tests were carried out on methanol and chloroform extracts of avocado seeds (Persea americana Mill.). Currently there has been no research on the antioxidant activity of avocado seeds using methanol and chloroform solvents in Indonesia. Therefore, research needs to be carried out to determine the most appropriate type of solvent, from methanol and chloroform, to obtain avocado seed extract with the highest antioxidant activity.

METHOD

6 kg of avocados were obtained from the garden in Kec. South Cilacap. Avocado seeds are taken and washed clean. The sample is sliced into small and thin pieces then drained. Then the avocado seeds that have been sliced into small pieces are dried in the oven at 55oC for 8 hours. Dried avocado seeds are ground using a blender, then sieved with a 60 mesh sieve to obtain avocado seed powder (Prasetyowati and Tera, 2010).

A total of 100 grams of avocado seed powder was macerated using methanol pa and chloroform pa solvents in a ratio of 1:5 for 48 hours, then remacerated with the same solvent for 48 hours. The methanol solvent dregs-free filtrate was obtained and evaporated using a rotary vacuum evaporator at a speed of 100 rpm with a temperature of 40°C then air-dried for 2 weeks to obtain a thick paste-shaped extract, while the chloroform solvent dregs-free filtrate was air-dried for 2 days to obtain the extract thick paste. The avocado seed extract was then covered with aluminum foil then stored in a glass jar and placed in the refrigerator for use in further analysis.

Determination of antioxidant activity using the DPPH method refers to the procedure of Brand-William et al., (1995). 1 mg of avocado seed extract (Persea americana Mill.) was weighed then 1 mL of methanol was added (as a stock solution with a concentration of 1000 ppm for each extract) and 6 series of concentrations were made, namely 0 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. The stock solution of each extract was taken as much as 0.05 mL and

added 1.0 mL of DPPH solution in methanol (20 µg/mL) and 0.45 mL of 50 Mm Tris-HCl Buffer (pH 7.5), then left for 30 minutes in dark room at room temperature. Absorbance was measured with a Uv-Vis spectrophotometer at a wavelength of 517 nm. Methanol is useful as a blank solution and ascorbic acid as a positive control.

Determination of total phenolic content using the Folin-Ciocalteau reagent method refers to the procedure of Luthria et al., (2006). A sample of avocado seed extract (1 mg/ml, dissolved in methanol: water = 80:20 %v/v) was taken as 0.1 mL, mixed with 7.9 mL of distilled water and vortexed for 10-20 seconds. Add 0.5 mL of 50% Folin-Ciocalteau reagent and vortex for 20-30 seconds. Add 1.5 mL of 20% sodium carbonate and vortex for 20-30 seconds. Then the mixture was incubated at room temperature for 2 hours. The absorbance of the mixture was measured at a wavelength of 765 nm using a gallic acid standard curve (0-100 µg/mL). The phenol content can be expressed as mg gallic acid equivalent per gram powder weight (mg GAE/g).

Determination of total flavonoid content using the AlCl3 reagent method refers to the procedure of Pourmorad et al., (2006). A total of 0.5 mL of avocado seed extract sample (1 mg/mL, dissolved in methanol: water = 1:1 %v/v) was added with 1.5 mL of methanol, 0.1 mL of 10% AlCl3, 0.1 mL KCH3COO 1 M and 2.8 mL distilled water. Then it was measured with a spectrophotometer with a wavelength of 415 nm. Methanol was used as a blank solution. Total flavonoid content can be determined using the quercetin standard curve (0-100 µg/mL) and total flavonoids are expressed as mg quercetin equivalent (QE)/g extract.

Data analysis used classical assumption tests (normality test and homogeneity test), independent sample t-test and Pearson correlation analysis to determine the correlation between total phenolic and total flavonoid content on antioxidants (IC50) in methanol and chloroform extract samples of avocado seeds.

RESULTS AND DISCUSSION

Based on the results of the antioxidant activity test using the DPPH method, the IC50 value was obtained from the equation formula y = ax + b and it was found that the chloroform extract of avocado seeds (Persea americana Mill.) had higher antioxidant activity than the methanol extract of avocado seeds (Persea americana Mill.) (Table 1). Based on the results of data analysis using the t-test for Equality of Means, a sig value of 0.592 > 0.05 was obtained, therefore it can be concluded that there is no significant difference in the IC50 value of methanol and chloroform extracts of avocado seeds.

Table 1. IC50 Values of Methanol and Chloroform Extracts of Avocado Seeds.

Extract Type	IC50 value (µg/mL)
Methanol Extract	668.82 ± 373.69
Chloroform Extract	349.28 ± 137.29

The IC50 value obtained through antioxidant activity test results shows that the IC50 value of methanol extract of avocado seeds is 668.82 µg/mL DW and chloroform extract of avocado seeds is 349.28 µg/mL DW. Based on the theory of Tristiantini et al., (2016), these two extracts are grouped as very weak antioxidants, because has an IC50 value above 200 µg/mL. Ascorbic acid has an IC50 value of 57.13 µg/mL DW, so it is classified as a strong antioxidant because it has an IC50 value above 50 µg per mL and below 100 µg per mL. This is different from the results of research conducted by Mustopa (2015), which showed that thick avocado seed extract had antioxidant potential to reduce 50% of DPPH free radicals at a concentration of 44.5793 ppm so it was categorized as very strong. This is because Mustopa's (2015) research used a different type of solvent, namely ethanol solvent. Ethanol is a polar solvent and is very good for use in the extraction of natural materials. Ethanol solvent has the property of penetrating cell wall materials so that it can carry out cell diffusion and attract bioactive compounds more quickly (Harbone, 1987).

Secondary metabolites that act as antioxidants in avocado seeds apart from phenolic compounds and flavonoids include alkaloids, polyphenols, saponins, tannins, steroids and triterpenoids (Abubakar et al.,

2017). In testing antioxidant activity using the DPPH method this time, it was found that the chloroform extract of avocado seeds was higher than the methanol extract of avocado seeds. This is possible because of the non-polar antioxidant compounds contained in avocado seed chloroform extract which are active in inhibiting free radicals such as triterpenoids, catechins, flavonols and steroids (Abubakar et al., 2017).

The IC50 value of the methanol extract of avocado seeds was lower than the chloroform extract of avocado seeds. In the process of making a thick, air-dried extract, methanol extract takes longer than chloroform extract. The methanol extract takes a very long time, namely two weeks, while the chloroform extract only takes two days because it is very volatile. The results of determining the antioxidant activity of avocado seed extract are also very weak because it is influenced by several factors, namely the weak ability of the active compounds in the extract and the length of time the avocado seed extract was stored before quantitative testing was carried out. The thick methanol and chloroform extract of avocado seeds is stored for two weeks, due to the use of laboratory equipment which requires queuing. This is confirmed by research by Khotimah et al., (2018), which stated that the antioxidant activity of Miana leaf extract decreased after being stored for more than two weeks. According to Hihat et al., (2017), low antioxidant activity can be seen through a high IC50 value, whereas high antioxidant activity can be seen through a low IC50 value.

In this study, it was found that the total phenol content in avocado seed extract using methanol solvent was 2419.51 ± 389.54 mgGAE/g DW, while avocado seed extract using chloroform solvent had a total phenol content of 529.69 ± 52.07 mgGAE/ g DW (Table 2). The results of the total phenol calculation show that the methanol extract has a higher phenol content compared to the chloroform extract. Based on the results of data analysis using the t-test for Equality of Means, a sig value of 0.002 <0.05 was obtained, therefore it can be concluded that there is a significant difference in the results of the total phenolic content of methanol and chloroform extracts of avocado seeds.

Table 2. Total Phenol Content of Methanol and Chloroform Extracts of Avocado Seeds.

Extract Type	Total phenol content (mgGAE/g)
Methanol Extract	668.82 ± 373.69
Chloroform Extract	349.28 ± 137.29

Based on a study by Tiwari et al., (2011), the solubility of polyphenol compounds is better in methanol solvent than chloroform. This is also appropriate with research by Dai & Mumper (2010), that solvents such as methanol, ethanol, acetone, ethyl acetate and their fractions are commonly used in the extraction of phenol from natural materials. Methanol is the most efficient and common solvent for the extraction process of low molecular weight polyphenolic compounds. This is because methanol and phenol are polar because they both have hydroxyl groups (-OH), so they can react well. According to Iqbal (2012), the use of solvents with high polarity such as methanol can increase the withdrawal of total phenol compounds from the extract.

In this study, it was found that the total flavonoid content in the chloroform extract of avocado seeds was 232.13 ± 54.32 mgQE/g DW, while in the chloroform extract of avocado seeds it was 209.81 ± 44.63 mgQE/g DW (Table 3). The total flavonoid content of the chloroform and methanol extracts of avocado seeds shows that the total flavonoid content of the chloroform extract is higher than the methanol extract. Based on the results of data analysis using the t-test for Equality of Means, a sig value of 0.549 > 0.05 was obtained, therefore it can be concluded that there is no significant difference in the results of the total flavonoid content of methanol and chloroform extracts of avocado seeds.

Table 3. Total Flavonoid Content of Methanol and Chloroform Extracts of Avocado Seeds.

Extract Type	Total flavonoid content (mgQE/g)
Methanol Extract	209.81 ± 44.63
Chloroform Extract	232.13 ± 54.32

Based on the explanation of Tiwari et al., (2011), chloroform solvent is good for the solubility of flavonoid compounds. The types of flavonoids found in avocado seeds include quercetin, flavonols, catechins and procyanidin (Setyawan and Sukardi, 2021). Quercetin is a polyhydroxy flavonoid which is semi-polar. Flavonols and catechins are non-polar flavonoids (aglycones). Flavonoid aglycones have several polar groups such as hydroxyl groups, but the number is limited compared to the non-polar hydrocarbon groups in their structure. Therefore, the polar nature of flavonoid aglycones is not strong enough to make them soluble in polar solvents such as water, but sufficient to interact with non-polar solvents such as chloroform (Ferreira and Pinho, 2012). Flavonoids as antioxidants are able to reduce free radicals because they contain chelated Mg metal and have a porphyrin framework. The chelated metal produces free radicals which tend to donate electrons to Mg metal, resulting in neutral free radical properties (Damogalad et al., 2013).

Based on the results of data analysis using the Pearson correlation test, it was found that the total flavonoid content in the chloroform and methanol extracts of avocado seeds with the amount of DPPH free radical inhibition had a correlation with a coefficient value of 0.761 (Table 4).

Table 4. Pearson Correlation Test Results.

Parameter relationships	*Pearson correlation*
Total phenols with IC50	0.140
Total flavonoids with IC50	0.761*

**. Correlation is significant at the 0.05 level (2-tailed).*

This shows that the total flavonoid content and the antioxidant activity of avocado seeds have a close relationship. In addition, an increase in flavonoid content will increase the antioxidant activity of an ingredient. The relationship between phenolic content and inhibition of DPPH free radicals has a correlation coefficient of 0.140 (Table 4). This shows that the total phenolic content has no correlation with the antioxidant activity of avocado seeds. Just like Ambarwati and Erni's (2022) study, it is clear that the chemical compounds that have a role as antioxidants in avocado seeds are flavonoids and tannins. The antioxidant activity of flavonoid compounds is able to chelate metal ions. This can reduce the capacity of metals to produce free radicals.

CONCLUSION

Based on the studies that have been carried out, the conclusions obtained are:

1. The highest antioxidant activity was in the chloroform extract of avocado seeds at 349.28 ± 137.29 µg/mL DW.
2. The highest total phenolic content was in the methanol extract of avocado seeds, namely 2,419.51 ± 389.54 mgGAE/g DW, while the highest total flavonoid content was in the chlorophorom extract of avocado seeds, namely 232.13 ± 54.32 mgQE/g DW.
3. Total flavonoids were related to the antioxidant activity value of avocado seed extract (Persea americana Mill.) compared to total phenolics.

SUGGESTION

In accordance with the study that the researcher has carried out, the researcher suggests for future researchers, namely the need to conduct a study on total tannin content using the Folin-Ciocalteau reagent method on avocado seeds and the need for research on fractionation to determine the optimal fraction for the antioxidant activity of avocado seeds (Persea americana). Mill.).

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