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Research Article

Antioxidant, Total Phenolic, and Total Flavonoid of 70% Ethanol Extract of Avocado Seeds (*Persea americana* Mill.)

Sri Wahyu Ningsih Munthe, Riskianto Riskianto, Denny Juvi, Jessica Novia*

ABSTRACT

Avocado seeds (*Persea americana* Mill.) are high in phytochemicals and are utilized in herbal medicine. The objective of this study is to analyze the antioxidant activities, total phenolics and flavonoids of the *P. americana* seed extract. *P. americana* seed extraction was obtained through maceration and reflux using a 70% ethanol solvent. The results obtained were compared in terms of yield productivity, with yields of 43.07 (%) and 39.58 (%) respectively. Phytochemical compounds extracted from *P. americana* seeds were tested using the phytochemical screening method, the antioxidant activity assay, the total phenolic analysis, and the total flavonoid analysis. The phytochemical screening showed that *P. americana* seeds contain flavonoids, saponins, phenols, tannins, alkaloids, and quinones. The antioxidant activity of the 70% ethanol extract of *P. americana* seeds obtained by maceration and reflux method was 77.298 g/mL and 98.626 g/mL, respectively, meanwhile the IC_{50} values of vitamin C were 12.883 g/mL. The 70% ethanol extract of *P. americana* seeds obtained by maceration and reflux method had total phenolic content of 276.96 mgGAE/g and 294.96 mgGAE/g, and total flavonoid content of 1.73 mgQE/g and 12.70 mgQE/g respectively. This simply implies that the 70% ethanolic extracts from *P. americana* seeds obtained through maceration and reflux have strong antioxidant activity.

Key words: *Persea americana* Mill., Avocado seeds, Antioxidants, DPPH, Total flavonoids, Total phenolics.

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INTRODUCTION

Medicinal plants have herbal properties and do not cause excess side effects. In general, people process medicinal plants by boiling or brewing them with water.[1] One type of medicinal plant, namely avocado, is known to have various benefits in its fruit,[2] leaves,[3] skin,[4] and seed.[5] Avocado seeds (*Persea americana* Mill.) have an anti-diabetic effect and were historically utilized as traditional medicine by the local people by drying, grinding, then brewing.[6] An example of *P. americana* seeds utilization as traditional medicine is shown in the form of tea bags, which are proven to lower blood sugar levels in rats.[7] Another benefit of *P. americana* seeds is as a remedy for constipation, inflammation, and boost the immune system.[8]

P. americana seeds are also proven for its antibacterial effect against *Pseudomonas* spp., *Staphylococcus aureus, Bacillus cereus*, *Listeria monocytogenes*, and *Escherichia coli.*[9,10] Moreover, *P. americana* seeds also acts as analgesic, anti-inflammatory, antiviral,[11] antiulcer,[12] antiproliferation, free radical-scavenging agents.[5] In addition, *P. americana* seeds also promote amylase enzyme inhibitory effects, therapy for type 2 diabetes mellitus,[13] inhibition of human lung (A549), stomach (BGC823), breast (MCF-7), hepatocellular (HepG2), and colon (HCT116) cancer cell line proliferation.[5,14]

All of those stated above are due to the rich phytochemical compounds of *P. americana* seeds, such as flavonoids, phenolics, carotenoids,[15] sterols, triterpenes, phenolic acids, catechins, linolenic acid, oleic acid, p-coumaric acid, kaempferol, apigenin, vitexin,[13] and triterpenoids.[14]

Flavonoids and phenolics are compounds that play an important role in the body's metabolism, functioning as antioxidants that can form bonds with free radicals. The presence of free radicals in the body can disrupt metabolism and damage cells that trigger diseases. Prevention can be achieved by consuming foods rich in antioxidants, which can bind to free radicals. Based on the benefits and compounds contained in P. *americana* seeds, this research examines the potential antioxidant activity, total phenolics, and total flavonoids of the 70% ethanol extract of P. *americana* seeds using maceration and reflux extraction methods.

MATERIALS AND METHODS

Materials

Spectrophotometer (Thermo Scientific), oven (Memmert), rotary evaporator (Heidolph), soxhlet (Iwaki), desiccator (Iwaki), vortex (Heidoplh), analytical balance (Ohaus), Hot plate (Thermo Scientific), Micropipette (Socorex). Ethanol 70% (Smart Lab), ethanol 96% (Merck), aluminum chloride (Merck), quercetin (Merck), gallic acid (Merck), Folin-Ciocalteu reagent (Merck), ascorbic acid (Merck), sodium acetate (Merck), DPPH radical (Merck), sodium bicarbonate (Merck).

Plant determination

P. americana seeds were determined to derive from the *Persea americana* Mill (No. B-785/V/DI.05.07/3/2022) plant species at the Badan Riset Dan Inovasi Nasional (BRIN) Cibinong, Indonesia.

Extraction

The 70% ethanol extract from *P. americana* seeds was prepared using two methods, maceration (cold methods),[8] dan reflux (hot methods).[16] The maceration method is made by weighing 100 g of

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herb powder, putting it in a container, and soaking it in 1 L of 70% ethanol solvent, every 24 hours a new solvent is replaced three times and followed by evaporation. The reflux method was made by weighing 50 grams of herb powder into a container and heating it in a boiling flask using 0.3 L of 70% ethanol, every 3 hours a new solvent was replaced five times, then evaporated in a rotary evaporator and dried in an oven at temperature 40°C.

Phytochemical screening

Phytochemical screening of *P. americana* seed extract was carried out using a screening method, namely the identification of bioactive compounds through color testing reactions using certain reagents.[17,18] This method aims to determine the presence of flavonoids, saponins, phenols, tannins, alkaloids, steroids, triterpenoids, and quinones in *P. americana* seed herb and extract.

Total phenolic

The total phenolic content of *P. americana* seeds was analyzed by the colorimetric method and measured using a spectrophotometer.[19] Preparation of a standard solution by weighing 5 mg of gallic acid dissolved in 50 mL of 96% ethanol to obtain a concentration of 100 ppm gallic acid. Gallic acid standard curves were obtained by making concentration series of 20, 40, 60, 80, and 100 µg/mL. Total phenolics of *P. americana* seed extract were determined using maceration and reflux methods, with 50 mg of extract dissolved in 50 mL of 96% ethanol to obtain a concentration of 1000 ppm.

Absorbance was measured using a spectrophotometer at a wavelength of 765 nm by measuring 0.1 mL of each series of gallic acid and extract concentrations, then adding 0.5 mL of Folin-Ciocalteu reagent and 0.4 mL of 7.5% sodium carbonate. with as many replications as three times and incubated at room temperature for 30 minutes. The total phenolic content is expressed as mg gallic acid equivalent per gram of extract weight.[20]

Total flavonoid

The total flavonoid content of *P. americana* seeds was analyzed by the colorimetric aluminum chloride method and measured using spectrophotometer.[21] Preparation of a standard solution by weighing 5 mg of quercetin dissolved in 50 mL of 96% ethanol to obtain a concentration of 100 ppm gallic acid. The quercetin standard curve was obtained by making a concentration series of 20, 40, 60, 80, and 100 µg/mL. Analysis of the total flavonoids of *P. americana* seed extract by maceration and reflux methods was obtained by dissolving 50 mg of the extracts in 50 mL of 96% ethanol to obtain a concentration of 1000 ppm extract solution.

Absorbance was measured using a spectrophotometer at a wavelength of 415 nm by measuring 0.33 mL of each series of quercetin and extract concentrations and then adding 0.33 mL of 10% AlCl3 and 0.33 mL of 1 M sodium acetate with three replications and incubated at room temperature for 30 minutes. The total flavonoid content is expressed as mg quercetin equivalent per gram of extract.[22]

Antioxidant activity

Antioxidant activity determined by DPPH assay of *P. americana* seeds maceration and reflux method refers to the procedure[23] with some modifications.[24] Preparation of a standard solution by weighing 5 mg of vitamin C dissolved in 50 mL of 96% ethanol to obtain a concentration of 100 ppm. Formulation of standard vitamin C curves with series concentrations of 2, 4, 6, 8, 10 µg/mL. Maceration and reflux method *P. americana* seed extract 12.5 mg dissolved in 50 mL 96% ethanol to obtain a concentration of 250 ppm each extract and diluted into a series of concentrations of 5, 10, 25, 50, 125 µg/mL.

Absorbance was measured using a spectrophotometer at a wavelength of 517 nm by measuring 0.25 mL of each concentration and extract series and then adding 0.75 mL of DPPH 60 ppm by replication three times and incubated at room temperature for 1 hour. Sample concentration and inhibition percent obtained were substituted on the x and y axes in the regression equation liner to determine the IC_{50} value.[25]

$$\%\ DPPH\ scavenging\ activity\ (IC50) = \frac{Ao - A}{Ao} x100\%$$

A_0: Absorbance blank

A: Absorbance sample

RESULTS AND DISCUSSION

Extraction

P. americana seed powder was extracted using maceration and reflux methods. The 70% ethanol extract of *P. americana* seeds using the maceration method (ME) obtained yields of 43.07% and the 70% ethanol extract of *P. americana* seeds using the reflux method (RE) obtained yields of 39,58%, the data presented in Table 1.

Phytochemical screening

The results of the phytochemical tests in Table 2 show that the herb and *P. americana* seed extract using maceration and reflux extraction contain flavonoids, saponins, phenols, tannins, alkaloids, and quinones. Based on the results of phytochemical testing, it is known that the maceration and reflux methods can extract the content of phytochemical compounds that have antioxidant activity.[26]

Total phenolic

Total phenolic of *P. americana* seeds used the Folin-Ciocalteu reagent and was analyzed with a spectrophotometer. Phenolics are a group of compounds that have an aromatic ring and contain one or two hydroxyl groups.[27] Commonly found phenolic compounds are resveratrol, piceid, tyrosol, hydroxytyrosol, and gallic acid. The compound used as a comparison in this analysis is gallic acid (3,4,5-trihydroxybenzoic acid) which is a stable and simple phenolic compound derived from hydroxybenzoic acid.[28]

The results of measuring the absorbance of gallic acid standard solutions with concentration series of 20, 40, 60, 80, and 100 µg/mL

Table 1: The results of herb extraction from *P. americana* seeds with 70% ethanol extract.

Part of Plant	Weight of Herb (g)	Weight of Extract (g)	Extract Yield (%)
***P. americana* Seeds**			
Maseration	100	43.07	43.07
Reflux	50	19,79	39.58

Table 2: Phytochemical screening of 70% ethanol extract of *P. americana* seeds.

Phytochemical Compounds	ME	RE	HP
Flavonoids	+	+	+
Saponins	+	+	+
Phenol	+	+	+
Tannins	+	+	+
Alkaloids	+	+	+
Steroids	-	-	-
Triterpenoids	-	-	-
Quinone	+	+	+

70% ethanol extract maceration method (ME), 70% ethanol extract reflux method (RE), Herb Powder (HP)

were obtained in the form of a calibration curve with a linear regression equation, y = 0.0058x + 0.0039 with a value of R2 = 0.9989 (Figure 1).

Total phenolic content of the extract was obtained by entering the absorbance value as y in the linear regression equation of the Gallic Acid Equivalent (GAE) calibration curve. The total phenolic levels contained in the extract samples in Table 2 are ME 276.96 ± 5.61 and RE 294.96 ± 11.55. These data show that every gram of avocado seed maceration extract 50 mL with concentration 1000 ppm contains 276.96 mg of gallic acid and every gram of avocado seed reflux extract contains 294.96 mg of gallic acid.

Total flavonoid

Total flavonoid content in *P. americana* seed extract was analyzed using a spectrophotometer with the complex reaction formation method. The reaction between aluminum chloride and the keto group on the C4 atom and the hydroxy group on the C3 or C5 atom in the flavones or flavonols (quercetin) will produce a complex.[21]

The results of standard quercetin absorbance measurements with concentration series of 20, 40, 60, 80, and 100 µg/mL were obtained in the form of linear regression, namely y = 0.0018x + 0.0097 with a value of R2 = 0.9945 (Figure 2). Total flavonoid content in the extract was obtained from the absorbance value as y in the linear regression equation of the quercetin equivalent (QE) calibration curve. The total flavonoid levels contained in the extract samples in Table 4 are ME 1.73 ± 0,00 and RE 12.70 ± 1.28. These data show that every gram of avocado seed maceration extract 50 mL with concentration 1000 ppm contains 1.73 mg of quercetin, and every gram of avocado seed reflux extract contains 12.70 mg of quercetin.

Antioxidant activity

Antioxidant activity of plant extracts or natural materials can be analyzed using the DPPH method. Qualitative antioxidant activity can be observed by reducing the intensity of the purple DPPH light color and is equivalent to reducing the concentration of DPPH. The reaction of DPPH molecules with hydrogen atoms released by molecules in the extract compounds will form diphenyl picryl hydrazine compounds with a reduction of purple to yellow, which is known as the damping reaction.[29]

The antioxidant activity results of the 70% ethanol extract of *P. americana* seeds were presented in Table 5 as a percentage of free radical inhibition of DPPH and compared to the antioxidant activity of vitamin C. The antioxidant activity of an extract (Figure 3 and 4) and vitamin C (Figure 5) can be determined using the IC_{50} parameter, namely the ability of the substrate or sample extract at a certain concentration to absorb free radical activity (DPPH) by 50%.

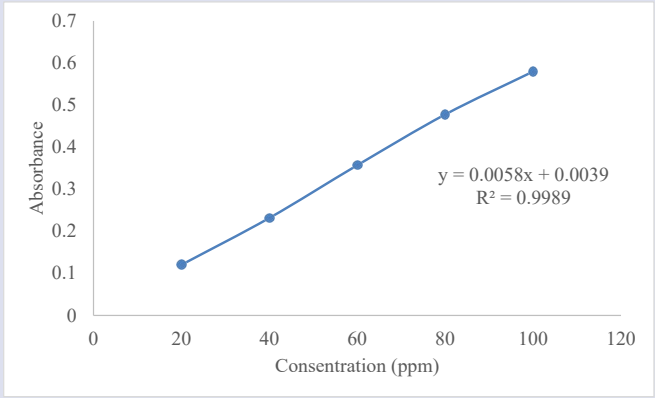

Figure 1: Gallic acid linear regression

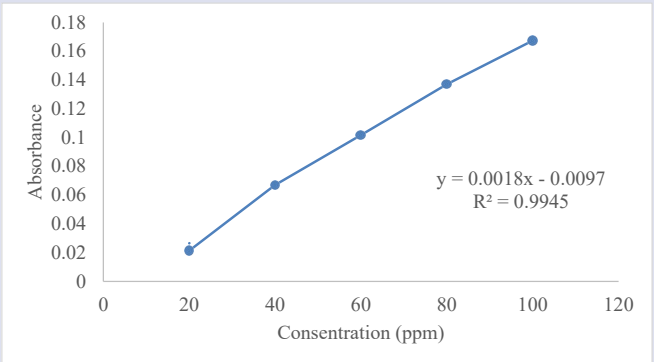

Figure 2: Quercetin linear regression

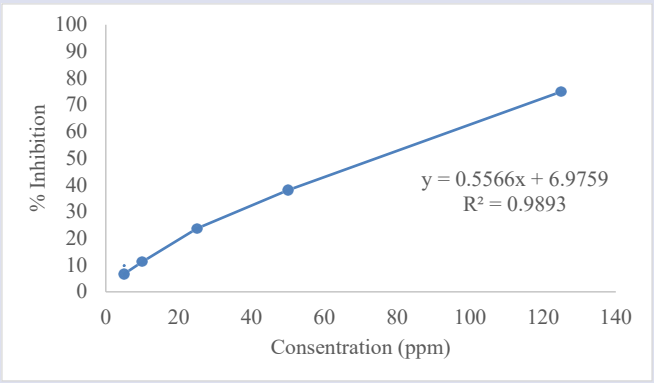

Figure 3: Antioxidant activity of 70% ethanol maseration extract (ME) on DPPH

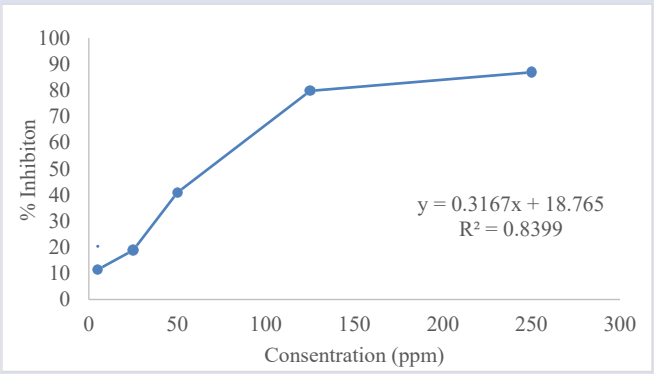

Figure 4: Antioxidant activity of 70% ethanol reflux extract (RE) on DPPH

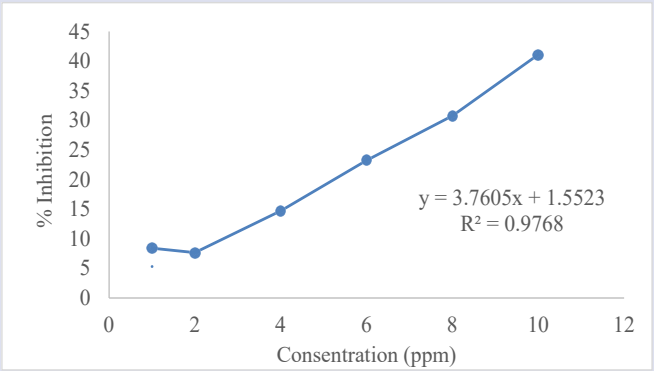

Figure 5: Antioxidant activity of vitamin C on DPPH

Table 3: Total phenolic content 70% ethanol extract of *P. americana* seeds.

Replication	Total Fenolik (mgGAE/G ME)	Total Fenolik (mgGAE/G RE)
1	274.72	286.79
2	272.81	289.90
3	283.35	308.17
X ± SD	276.96 ± 5.61	294.96 ± 11.55

70% ethanol extract maceration method (ME), 70% ethanol extract reflux method (RE)

Table 4: Total flavonoid content 70% ethanol extract of *P. americana* seeds (*P. americana*).

Replication	Flavonoid Total (mgQE/G ME)	Flavonoid Total (mgQE/G RE)
1	1.73	12.70
2	1.73	13.98
3	1.73	11.42
X ± SD	1.73 ± 0,00	12.70 ± 1.28

70% ethanol extract maceration method (ME), 70% ethanol extract reflux method (RE)

Table 5: Antioxidant activity 70% ethanol extract *P. americana* seeds.

Sample	Concentration (µg/mL)	Absorbance	Inhibition (%)	IC_{50} (µg/mL)
ME	5	1.134 ± 0.020	6.667	77.298
	10	1.079 ± 0.030	11.221	
	25	0.927 ± 0.021	23.704	
	50	0.753 ± 0.007	38.025	
	125	0.305 ± 0.028	74.925	
	Control	1.215 ± 0.001		
RE	5	1.076 ± 0.020	11.440	98.626
	25	0.986 ± 0.023	18.859	
	50	0.718 ± 0.023	40.905	
	125	0.245 ± 0.020	79.808	
	250	0.159 ± 0.006	86.914	
	Control	1.215 ± 0.001		
Vit C	1	0.781 ± 0.013	35.659	12.883
	2	0.787 ± 0.023	35.137	
	4	0.727 ± 0.020	40.082	
	6	0.654 ± 0.007	46.071	
	8	0.590 ± 0.012	51.374	
	10	0.502 ± 0.013	58.626	
	Control	1.213 ± 0.001		

70% ethanol extract maceration method (ME), 70% ethanol extract reflux method (RE)

The percentage inhibition value of 70% ethanol extract by maceration and reflux and vitamin C in the concentration series is used as a reference in calculating the IC_{50} of the extracts. High antioxidant activity is inversely proportional to the IC_{50} value of the extracts, meaning that the smaller the IC_{50} value, the extracts have high antioxidant activity. Test results on vitamin C had an IC_{50} of 12.88 µg/mL, 70% ethanol extract of *P. americana* seeds from the maceration method (ME) of 77.29 µg/mL, and 70% ethanol extract of *P. americana* seeds from the reflux method (RE) of 98.62µg/mL.

According to Molyneux (2004),[30] the level of antioxidant strength is divided into four levels, namely very strong (IC_{50} <50 µg/mL), strong (IC_{50}: 50–100 µg/mL), moderate (IC_{50}:101–150 µg/mL), and weak (IC_{50} > 150 µg/mL). Based on these categories, ME and RE have strong antioxidant activity.

These results reinforce the results of previous studies, which stated that *P. americana* seed extract has antioxidant activity.[31,32] The content of secondary metabolites in complex extracts and various reactive oxygen species compounds, so several methods can be used to assess antioxidant activity in plant extracts. This study used the DPPH method to investigate and compare the antioxidant activity of *P. americana* seed extracts.

The antioxidant activity of plant extracts is generally associated with the presence of antioxidants such as phenolic compounds[33] and flavonoid compounds.[34] The results showed that 96% ethanol was extracted from the seeds of *P. americana* obtained from the maceration and reflux extraction methods contain flavonoids and phenolic compounds which have varying abilities to scavenge DPPH free radicals.

Polyphenols are structured from one or more aromatic benzene rings linked by mono- or poly-hydroxyl groups. Furthermore, these polyphenols have the potential to donate electrons or hydrogen, as well as reduce and chelate metals.[35] Previous research has discovered a correlation between the DPPH assay's antioxidant activity and the levels of phenolic compounds, owing to the redox properties of these compounds.[36-38] As a result, phenolic compounds can donate electrons or hydrogen radicals to DPPH free radicals, converting them into neutralized, stable diamagnetic molecules.[33]

Flavonoid group compounds have antioxidant activity because of the hypothetical affinity sequence between flavonoids and amino acid residues, which mostly appears to provide a significant application in theoretical predictions of flavonoid-protein interactions as a high-quality approach to understanding flavonoid biological activity.[39]

Most flavonoid compounds activity as antioxidants. Flavonoids can prevent damage caused by free radicals in various ways and one of them is the capture of free radical molecules directly. Flavonoids are oxidized by radical compounds, producing radicals that are more stable and less reactive. In other words, flavonoids stabilize reactive oxygen species through reactions with free radical compounds. Due to the high reactivity of the hydroxyl groups in flavonoids, the radicals become inactive, as described in the following equation given by Korkina & Afanas'ev (1997).[40]

"Flavonoid (OH) + r(O) + RH"

R represents a free radical and O represents an oxygen free radical. Some flavonoids can actively scavenge superoxide, while others can scavenge peroxynitrites, which are highly reactive oxygen-derived radicals.[41] The differences in the DPPH free radical scavenging capacity of the two *P. americana* seed extracts can be attributed to their phenolic and flavonoid compounds. The antioxidant activity of the 70% ethanol extract of *P. americana* seeds from maceration and reflux methods can be assumed to have the same antioxidant activity. These results depend not only on the content of phenolic compounds and flavonoids but also on the presence of other groups of compounds that have an important role in antioxidant activity[42,43] such as terpenoids and tannin group compounds. Further studies are needed regarding the antioxidant activity of *P. americana* seed extract using other free radical scavenging methods, and an analysis of the total content of other secondary metabolites that have antioxidant activity is needed.

CONCLUSION

P. americana seeds contain flavonoids, saponins, phenols, tannins, alkaloids, and quinones. The antioxidant activity of 70% ethanol extract of *P. americana* seeds obtained by maceration and reflux method had IC_{50} values of 77.298 µg/mL and 98.626 µg/mL. The 70% ethanol extract of *P. americana* seeds obtained by maceration and reflux methods had a total phenolic of 276.96 mgGAE/gand 294,96 mgGAE/g and total flavonoid of 1.73 mgQE/g and 12.70 mgQE/g respectively. Extract ethanol 70% of *P. americana* seeds obtained from the maceration and reflux method has strong antioxidant activity.

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SUMMARY

Avocado seed (*Persea americana* Mill.) contained flavonoids, saponins, phenols, tannins, alkaloids, and quinones. The antioxidant activity showed that the IC_{50} values of the 70% ethanol extract of avocado seeds obtained by maceration and reflux method were 77.298 µg/mL and 98.626 µg/mL, whereas the IC_{50} values of vitamin C were 12.883 µg/mL. The 70% ethanol extract of P. americana seeds obtained by maceration and reflux methods had a total phenolic of 276.96 mgGAE/gand 294,96 mgGAE/g and total flavonoid of 1.73 mgQE/g and 12.70 mgQE/g respectively. Extract ethanol 70% of P. americana seeds obtained from the maceration and reflux method has strong antioxidant activity.

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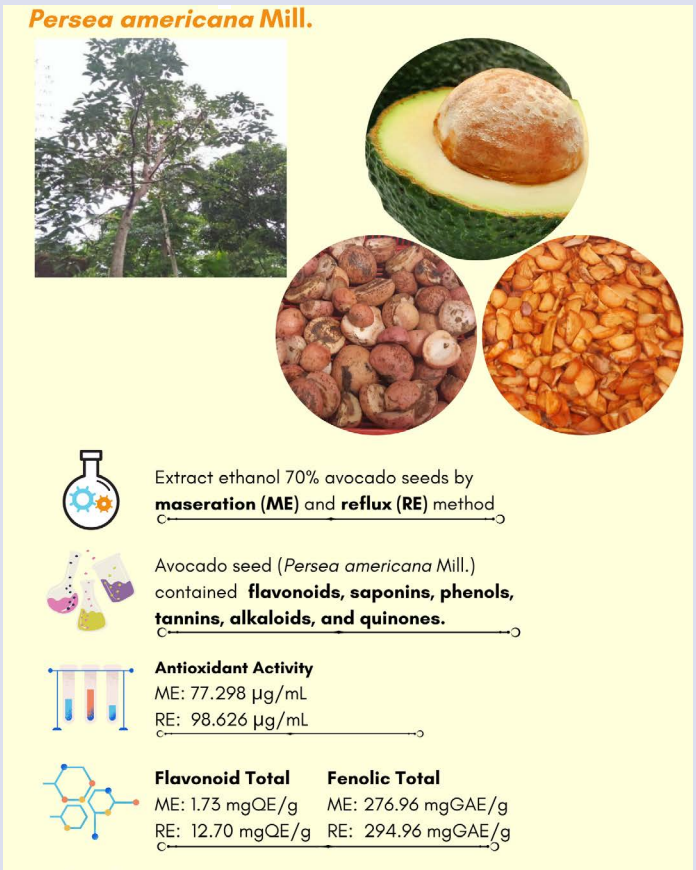
GRAPHICAL ABSTRACT



SUMMARY

Avocado seed (*Persea americana* Mill.) contained flavonoids, saponins, phenols, tannins, alkaloids, and quinones. The antioxidant activity showed that the IC_{50} values of the 70% ethanol extract of avocado seeds obtained by maceration and reflux method were 77.298 µg/mL and 98.626 µg/mL, whereas the IC_{50} values of vitamin C were 12.883 µg/mL. The 70% ethanol extract of P. americana seeds obtained by maceration and reflux methods had a total phenolic of 276.96 mgGAE/gand 294,96 mgGAE/g and total flavonoid of 1.73 mgQE/g and 12.70 mgQE/g respectively. Extract ethanol 70% of P. americana seeds obtained from the maceration and reflux method has strong antioxidant activity.

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