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Testing The Antioxidant Activity of The N-Hexane, Ethyl Acetate, N-Butanol, and Water Fractions from a 96% Ethanol Extract of Butter Avocado Seeds (Persea americana Mill.) using The DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Method

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Abstract

Avocado seeds are underutilized byproducts containing secondary metabolites with potential antioxidant properties. This study evaluated the antioxidant activity of fractions derived from a 96% ethanol extract of butter avocado (Persea americana Mill.) seeds and characterized the fraction exhibiting the highest activity. Seeds collected from Tawangmangu, Central Java, Indonesia, were dried, pulverized, and extracted by maceration with 96% ethanol. The crude extract was subjected to sequential liquid–liquid partitioning to obtain n-hexane, ethyl acetate, n-butanol, and aqueous fractions. Antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay, with absorbance measured by ultraviolet–visible (UV–Vis) spectrophotometry. Thin-layer chromatography (TLC) was performed for preliminary phytochemical characterization of the most active fraction. The extraction yield was 24.6%, while the yields of the n-hexane, ethyl acetate, n-butanol, and aqueous fractions were 16.46%, 5.17%, 27.53%, and 42.90%, respectively. The ethyl acetate fraction exhibited the highest reported antioxidant activity and showed chromatographic characteristics suggestive of the presence of flavonoids, alkaloids, saponins, and triterpenoids. These findings suggested that solvent fractionation may have enriched constituents associated with free radical-scavenging activity. However, the absence of numerical half-maximal inhibitory concentration (IC_{50}) values and statistical comparisons limited the quantitative assessment of antioxidant potency among fractions. Butter avocado seeds may represent a promising source of natural antioxidant compounds, particularly in the ethyl acetate fraction. Further investigations involving compound isolation, structural identification, complementary antioxidant assays, and safety evaluations are warranted to establish the biological mechanisms and potential applications of these constituents.

INTRODUCTION

Free radicals are chemical species containing one or more unpaired electrons, which generally make them highly reactive. Free radicals and other reactive species can originate from endogenous processes, including cellular metabolism, autooxidation, and enzymatic reactions, as well as exogenous sources, such as air pollution, cigarette smoke, vehicle emissions, and certain forms of radiation. Excessive production or accumulation of reactive species can disrupt cellular redox balance, leading to oxidative stress and potentially contributing to cellular

damage and the development of various diseases. Antioxidants play an important role in protecting biological systems by neutralizing reactive species or limiting oxidative damage (Maharani et al., 2013).

Antioxidants are substances that inhibit or delay the oxidation of other molecules through various mechanisms, including free radical scavenging (Lampogajo et al., 2021). Based on their origin, antioxidants can be broadly classified as endogenous or exogenous, while exogenous antioxidants may be obtained from natural sources or produced synthetically. Endogenous antioxidants form part of the body's natural defense system, whereas plant-derived antioxidants include various bioactive compounds, particularly polyphenols. Synthetic antioxidants are chemically manufactured substances commonly used to prevent oxidative deterioration in food and other products (Tristantini et al., 2016). Examples include propyl gallate, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ). Although these compounds are effective antioxidants, concerns regarding their potential adverse effects at certain exposure levels have encouraged further investigation into naturally derived alternatives (Akhlaghi & Bandy, 2009; Ambaro, 2020; Ambarwati & Rustiani, 2022; Anggorowati, 2016; Apak, 2022). However, their safety depends on the specific compound, exposure level, and conditions of use, and their carcinogenicity should not be generalized without adequate evidence (Mustopa, 2015).

Butter avocado (Persea americana Mill.) is a cultivated variety of avocado valued for its nutritional composition and potential health-promoting properties. Previous research has demonstrated that avocado tissues contain bioactive constituents with antioxidant activity (Melgar, 2018). Although avocados are widely cultivated and consumed in Indonesia, their seeds remain relatively underutilized and are commonly discarded during fruit processing and consumption (Sutrisna, 2015). Phytochemical investigations have reported the presence of alkaloids, triterpenoids, tannins, flavonoids, and saponins in avocado seeds. Some of these compound classes, particularly phenolic constituents such as flavonoids and tannins, have been associated with antioxidant properties, suggesting that avocado seeds may serve as a potential source of naturally occurring antioxidant compounds (Arukwe, 2012).

Beyond their potential antioxidant properties, avocado seeds are relevant to the broader challenge of agricultural byproduct utilization. Melgar et al. (2018) reported that avocado peels and seeds collectively accounted for approximately 30% of the fruit, representing a substantial nonedible fraction that could be investigated as a source of valuable compounds. Their characterization of avocado byproducts supported the potential recovery of bioactive constituents rather than treating these materials exclusively as waste. This perspective strengthened the rationale for investigating butter avocado seeds collected from Tawangmangu, Central Java, Indonesia, as a locally available research material (Arisanti, 2010; Arukwe, 2012; Berawi & Agverianti, 2017; Browning, 1966; Cahyana, 2002). Evaluating their antioxidant activity could provide preliminary evidence supporting agricultural byproduct valorization, although antioxidant activity observed under laboratory conditions does not necessarily establish suitability for food, pharmaceutical, or other commercial applications.

Previous studies have investigated the antioxidant activity of various avocado tissues, including leaves, peels, and seeds, using different extraction solvents and analytical conditions. Studies on avocado leaves reported half-maximal inhibitory concentration (IC_{50}) values of 417 mg/L for an ethanol extract (Kemit, 2016) and 24.863 µg/mL in another investigation

(Anggorowati, 2016), while an ethyl acetate fraction exhibited an IC_{50} value of 16.51 µg/mL (Insie, 2014). For avocado peels, (Siyanti, 2019) reported an IC_{50} value of 137.34 ppm for an ethanol extract. (Pratiwi, 2016) reported IC_{50} values of 5.03 µg/mL for an extract, 41.56 µg/mL for an ethyl acetate extract, 2.78 µg/mL for an ethyl acetate fraction, and 22.33 µg/mL for an n-hexane fraction. Investigations of avocado seed extracts also demonstrated varying antioxidant activities. A 70% ethanol extract exhibited an IC_{50} value of 31.5 ppm (Mustopa, 2015; Sutrisna, 2015) reported an IC_{50} value of 44.5793 ppm. (Rachman, 2017) reported an IC_{50} value of 23.07 ± 1.63 µg/mL for a 96% ethanol extract, whereas (Djasmasari, 2020) reported an IC_{50} value of 289.3 ppm. Variations in these findings may be associated with differences in solvent polarity, extraction procedures, plant material, and experimental conditions. Solvent selection is particularly important because it influences the extraction efficiency and chemical composition of the resulting extracts (Kurniawati et al., 2016). Nevertheless, direct comparisons of IC_{50} values across studies require consideration of differences in assay protocols and analytical conditions.

Evidence from fraction-based investigations further indicated the importance of examining avocado seed antioxidants beyond the crude extract level. Kurniawan and Rahmat (2023) investigated methanol and ethyl acetate fractions of avocado seeds using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and determined their total phenolic and flavonoid contents. The reported values were approximately 48.6–48.7 mg gallic acid equivalents per gram of dry extract and 41.2–41.4 mg quercetin equivalents per gram of dry extract, respectively. These findings demonstrated that avocado seed fractions had previously been investigated and provided a relevant foundation for further comparative research. However, the two-fraction approach did not directly address the comparative antioxidant activities of the four solvent fractions examined in the present study, particularly those obtained from butter avocado seeds initially extracted with 96% ethanol.

Despite previous investigations, comparative evidence regarding the antioxidant activities of different solvent fractions derived from butter avocado seeds remains limited. Fractionation using solvents of varying polarities facilitates the separation and enrichment of chemical constituents according to their physicochemical properties. Commonly used fractionation solvents include n-hexane, ethyl acetate, and methanol, which differ in polarity and extraction selectivity (Hasanah, 2017). In the present study, n-hexane was used to partition relatively nonpolar constituents, including certain terpenoids and steroids, whereas ethyl acetate was selected to recover compounds of intermediate polarity, including some phenolic compounds and flavonoids. n-Butanol was used to partition more polar constituents, potentially including certain saponins, phenolic compounds, and alkaloids, depending on their chemical structures and ionization states (Septyaningsih, 2010). An aqueous fraction was also retained to evaluate water-soluble constituents. This fractionation strategy enabled the comparison of antioxidant activity across fractions with different chemical characteristics.

Antioxidant activity can be evaluated using several analytical methods, including ferrous ion-chelating (FIC), ferric reducing antioxidant power (FRAP), and DPPH assays. These methods assess different aspects of antioxidant behavior and are not directly interchangeable. The DPPH assay is widely used to evaluate free radical-scavenging activity because of its relatively simple procedure, rapid analysis, and suitability for preliminary screening. The assay is based on the reduction of the stable DPPH radical through electron

transfer, hydrogen atom transfer, or related reaction pathways involving antioxidant compounds. This reaction decreases the characteristic purple coloration of the DPPH solution, which is commonly monitored spectrophotometrically at approximately 517 nm. Although the DPPH assay offers practical advantages for preliminary antioxidant screening, its results may be influenced by solvent composition, reaction time, and sample characteristics. Accordingly, the DPPH assay was selected to compare the radical-scavenging activities of the n-hexane, ethyl acetate, n-butanol, and aqueous fractions obtained from butter avocado seed extract.

Accordingly, the research gap addressed in this study concerned the comparative antioxidant activities of selected butter avocado seed fractions evaluated under consistent experimental conditions, rather than the general absence of previous fractionation studies. The specific contribution of this investigation was the comparison of n-hexane, ethyl acetate, n-butanol, and aqueous fractions derived from a common 96% ethanol extract, followed by preliminary phytochemical characterization of the fraction exhibiting the highest antioxidant activity using thin-layer chromatography (TLC). This approach was intended to identify the fraction with the greatest DPPH radical-scavenging activity and provide an initial phytochemical profile to guide subsequent compound isolation and characterization. Such preliminary evidence could inform further investigations involving more advanced analytical techniques and biological assays. Nevertheless, DPPH radical-scavenging activity and TLC-based screening alone cannot establish biological efficacy, compound identity, safety, or commercial applicability without additional experimental validation.

This study aimed to evaluate the antioxidant activity of a 96% ethanol extract of butter avocado (Persea americana Mill.) seeds and its n-hexane, ethyl acetate, n-butanol, and aqueous fractions using the DPPH assay, determine their IC_{50} values, identify the fraction exhibiting the highest antioxidant activity, and preliminarily characterize its phytochemical constituents using TLC. The findings were expected to provide scientific evidence regarding the potential of butter avocado seeds as a source of naturally occurring antioxidant compounds and serve as a foundation for further research into the utilization of avocado seed byproducts in health-related and industrial applications.

METHOD

Plant identification was conducted at the Center for Research and Development of Medicinal Plants and Traditional Medicines (B2P2TOOT), Tawangmangu, to confirm the botanical identity of butter avocado (Persea americana Mill.). The selected seeds were washed, thinly sliced, and oven-dried at 40°C. The dried material was sorted, ground, and passed through a 40-mesh sieve. Organoleptic characteristics and loss on drying were subsequently evaluated. Extraction was performed by maceration using 96% ethanol at a plant material-to-solvent ratio of 1:10 (w/v). A total of 500 g of dried seed powder was macerated in 5,000 mL of 96% ethanol for 24 hours, followed by remaceration. The filtrates were collected and concentrated using a rotary evaporator at 40°C to obtain a viscous extract. The resulting extract was then subjected to organoleptic examination and moisture content determination.

The crude extract was subjected to sequential liquid–liquid partitioning using n-hexane, ethyl acetate, and n-butanol, yielding four fractions: n-hexane, ethyl acetate, n-butanol, and aqueous fractions. The fractions were screened for the presence of flavonoids, tannins, alkaloids, and saponins. Antioxidant activity was evaluated using the 2,2-diphenyl-1-

picrylhydrazyl (DPPH) radical-scavenging assay with ultraviolet–visible (UV–Vis) spectrophotometry. The crude extract and each fraction were prepared at several concentrations, reacted with DPPH solution, and analyzed spectrophotometrically at the wavelength of maximum absorbance. Each measurement was performed in triplicate. The percentage of DPPH radical inhibition was calculated, and the half-maximal inhibitory concentration (IC_{50}) was determined using linear regression analysis. The fraction exhibiting the highest antioxidant activity was subsequently characterized by thin-layer chromatography (TLC) based on its retention factor (Rf) values and chromatographic spot characteristics.

The plant material consisted of butter avocado (Persea americana Mill.) seeds obtained from Tawangmangu District, Karanganyar Regency, Central Java, Indonesia. Seeds were selected from fully ripened fruits and were required to be fresh, intact, and free from visible damage.

Antioxidant activity was analyzed based on the percentage of DPPH radical inhibition and IC_{50} values. Data normality was assessed using the Shapiro–Wilk test, followed by one-way analysis of variance (ANOVA) at a 95% confidence level ($\alpha = 0.05$). When statistically significant differences were detected, Tukey's honestly significant difference (HSD) post hoc test was used for pairwise comparisons among treatment groups.

RESULTS AND DISCUSSION

Determination of Plant Samples

Determination is a test stage that is carried out to find out the truth of the plant to be studied as well as to avoid errors in the collection of materials and the possibility of mixing the plant being studied with other plants that have similar shapes or properties. Plant determination was carried out at the Tawangmangu Karanganyar Medicinal and Traditional Medicine Research and Drug Plant, plant determination was carried out by matching the morphology of butter avocado plants with literature that proves the truth of the plants used for research. A sample of 2 kg of fresh whole butter avocados was submitted to BBPPTOOT Tawangmangu in early August 2023, and the results of the determination of butter avocado fruit were released in the form of a soft file approximately 1 week after the sample was submitted. In letter number TL.02.04/D.XI.5/16536.034/2023, it is written that the results of the determination of butter avocado plants are stated that the sample to be used in this study is true of the butter avocado plant (Persea americana Mill.). The results of the determination of butter avocado plants are listed in appendix 1.

Main Ingredient Pick-up of Simplisia Powder Research and Manufacture

The butter avocado seeds used as the main ingredient in this study were collected from Tawangmangu District, Karanganyar Regency, Central Java. A total of 26 kg of butter avocados in fresh and whole conditions were collected and separated between the flesh and the seeds. The avocado seeds used are only good butter avocado seeds, if there are rotten or defective seeds, it is enough to remove them and take part of the good fruit seeds. The butter avocado seeds are then washed thoroughly using running water. After washing, the avocado seeds are thinly sliced with a thickness of approximately 0.1-1 cm. The butter avocado seed slices are dried, air-dried for a while, then in the oven at 40oc until dry and easy to crumble.

The process of baking butter avocado seeds aims to reduce the occurrence of spoilage due to foreign microorganisms, reduce the moisture content of the sample and make it easier to

make butter avocado seed simplicia powder. The results of the calculation of simplicia yield are listed in table 1.

Table 1 Results of LOD calculation of dried seeds of butter avocado

Wet Weight (gr)	Dry weight (gr)	Yields (%)
3.400	1.346	60,41



Figure 1. Drying Simplicia

Based on the data from the results, the yield obtained was 60.41%. The calculation of the yield yield of butter avocado seeds is listed in appendix 2. The process of making butter avocado seed simplicia powder is carried out using a blender and a mesh sieve number 40. The blender is used to crush the dry slices of avocado seeds and sifted using a mesh sieve, if there is a part of the powder that does not pass the mesh 40 sieve, it is reblended to reduce the size of the powder. The purpose of this pollination process is to facilitate the extraction of the active substances contained in avocado seeds and to maximize their pollination.



Figure 2. Butter avocado seed simplisia powder

The yield of powder to the weight of dried butter avocado seeds is 91.08%, so that the total powder from dried butter avocado seeds of 1,346 grams produces a total powder of 1,226 grams.

Powder Characteristics

1. Organoleptic Examination of Butter Avocado Seed Powder

Organoleptic examination of avocado seed powder is identified by the color, smell, taste, texture/shape of the simplicia powder. This organoleptic examination was carried out with

the aim of finding out the characteristics of butter avocado seed powder using the five human senses. After observing, the following results were obtained

Table 2. Results of Organoleptic Examination

Organoleptis	Results
Color	Light Brown/Cream
Smell	Slightly stinging
Taste	Bitter
Texture/Shape	Fine Powder

2. Determination of Drying Drying Butter Avocado Seed Powder

Determination of drying shrinkage using the gravimetric method. Self-drying shrinkage is the weight reduction of the sample material/powder weight after drying. Butter avocado seed simplicia powder is carefully weighed by 1-2 grams, then flattened at the bottom of the exchange rate with a thickness of approximately 5-10mm. The rate must first be weighed empty and in the oven at 105oc for 15 minutes. The rate at which the powder has been added is then put into the oven at a temperature of 105oc and observed every hour. Hourly sample weight loss data is recorded in the log book, and it is observed whether the sample weight has been said to be constant. The constant weight in question is if the difference in sample weight loss is not more than 0.0005 gr (0.025%) with the previous sample weighing weight. Determination of drying shrinkage needs to be done 3 times to collect good data. The results of the calculation of drying shrinkage of butter avocado seed simplicia powder are listed in appendix 3.

Table 3. Results of Determination of Powder Drying Shrinkage

Simplisia Powder	Starting Weight (gr)	Final Weight (gr)	Drying shrinkage (%)
Replication 1	1,6918	1,5316	9,469 %
Replication 2	1,8519	1,6789	9,341%
Replication 3	1,5856	1,4348	9,510%
Average	1,7097	1,5484	9,440%

Based on the results data in table 7, it was found that the average percentage of drying of butter avocado seed simplicia powder was 9.44% (meeting the requirements) of less than 10%. The smaller the percentage value of the determination of the drying shrinkage of the powder indicates that the content of water or oil in the powder is less, so that the decrease in the weight of the sample when the drying shrinkage determination test is less (gravimetric method).

Butter Avocado Seed Extract Manufacturing

The production of extracts during this study was carried out using the maceration method. The extraction of maceration is carried out by the stages of soaking 1 kg of simplicia powder in 96% ethanol solvent as much as 10 liters, this is based on the provisions of the comparison of 1 part powder: 10 parts solvent. Simplisia powder is put into a maceration bottle then soaked with 96% percent ethanol for 6 hours and occasionally boiled, then left for 18 hours so that the total maceration process is carried out for 24 hours of soaking.

The results of the maceration extraction are then filtered with flannel cloth, the residue of the filtration with flannel cloth is repeated the maceration extraction process (re-maceration) with 96% ethanol solvent as much as 5 liters. The results of the re-maceration are re-filtered with flannel, the entire filtrate is collected and filtered with filter paper using the help of vacuum. The extraction results are thickened using a rotary evaporator. The filtrate is

evaporated at a maintained temperature of 40oc-58oc until a thick extract of butter avocado seeds is obtained. The yield yield of butter avocado seed extract is attached in table 7 and the calculation of the yield of the extract is attached in attachment 4.

Table 4. Yield Results of Butter Avocado Seed Extract

Weight of avocado seed powder (gr)	Weight of the thick extract (gr)	Yields (%)
1.000	246	24,6

The total extract obtained was 246 grams from 1 kg of butter avocado seed simplicia dry powder, with a yield of 24.6%. The percentage of yield with higher values indicates that the compounds extracted by the solvent are also increasing.



Figure 3. Butter avocado seed extract

Characteristics of the Extract

1. Organoleptic Examination of Butter Avocado Seed Extract

Organoleptic examination of avocado seed extract was identified using the five human senses including the color, smell, taste, texture/shape of the simplicia powder. This organoleptic examination is carried out with the aim of determining the characteristics of the appearance and organoleptic appearance of butter avocado seed extract. After observing, the following results were obtained

Table 5. Results of Organoleptic Examination of Extracts

Organoleptis	Results
Color	Blackish brown
Smell	Special sting
Taste	Bitter
Texture/Shape	Concentrated extracts

2. Determination of Moisture Content of Butter Avocado Seed Extract

The determination of the moisture content of the extract is carried out by the gravimetric method. The stages of the moisture content test are almost the same as the drying shrinkage test, the sample of the condensed extract of butter avocado seeds is weighed approximately 10 grams, then put into the exchange rate that has previously been weighed and heated in a 105oc oven for approximately 15 minutes. The rate that has contained the extract sample is then in the oven for 5 hours and then the weight loss is recorded, then in the oven again and the weight loss data is recorded every 1 hour, and it is observed whether the weight of the avocado seed extract sample is at a constant weight. The data is recorded in the log book and replicated 3

times. The results of the calculation of the moisture content of butter avocado seed simplicia extract are listed in appendix 5.

Table 6. Results of Determination of Extract Water Content

Simplisia Extract	Initial Weight of Extract (gr)	Final Weight of Extract (gr)	Extract Moisture Content (%)
Replication 1	10,0074	7,3893	26,16%
Replication 2	10,0643	7,5401	25%
Replication 3	10,0931	7,7660	23%
Average	10,0550	7,5651	24,72%

3. Preliminary Test Identification of Chemical Compound Content of Butter Avocado Seed Extract

Preliminary identification tests were carried out to see for the presence of secondary metabolite chemical compounds contained in butter avocado seed extract such as tannins, alkaloids, flavonoids, saponins. Preliminary test phytochemical screening of butter avocado seed extract was carried out in laboratory 9 (natural ingredients). The results of the preliminary test were then compared with the literature to prove its validity. The content of secondary metabolite chemical compounds in the ethanol extract of 96% of butter avocado seeds can be seen in table 11.

a. Flavonoid Test. Carefully weighed 1 g of extract then added with 10 milliliters of water then boiled for 5 minutes and filtered. The filtered filtrate is taken as many as 5 millimeters and then put into the test tube. Add 3 drops of concentrated HCl then homogenize. The results of this flavonoid test show a change in the color of the filtrate to reddish-orange, so it can be concluded that the extract contains flavonoid compounds.
b. Tannin Test. Weighing 1 gram of the extract is then dissolved in water for 10 millimeters and boiled for 15 minutes. Then filtered and then taken 5 milliliters of filtrate to be put into the test tube. 3 drops of 1% FeCl3 are added and then homogenized. The results of this tannin test show a change in the color of the filtrate to blackish-blue, so it can be concluded that the extract contains compounds of the tannins group.
c. Alkaloid Test. A total of 0.5 grams of extract is weighed and then added with 1 ml of 2N hydrochloric acid add with 9 ml of water. The solution is then heated for 2 minutes and filtered. 2 milliliters of filtrate were taken and then reacted with LP bocardat. The results of this alkaloid test show the appearance of thin deposits at the bottom of the test tube, so it can be concluded that the extract contains compounds of the alkaloid group.
d. Saponin Test. The extract as much as 0.5 grams is weighed then dissolved in water for 10 millimeters and put into a test tube, shaken vigorously for 10 to 20 seconds and observed the foam formed. Add 1 drop of hcl 2 n so that the foam does not disappear quickly. The results of this alkaloid test show that the foam that arises lasts for approximately 5 minutes, so it can be concluded that the extract contains compounds of the saponin group

Table 7. Preliminary Test Results Identification / Phytochemical Test

Chemical Content	Results	Remarks	Data interpretation
Flavonoids	+	If red, orange to purplish-red occurs, it indicates the presence of flavonoids, if it occurs, yellow-orange indicates the presence of flavones.	
Tannins	+	It is said to be positive for phenolics if the color changes to green, violet, or black. Tannins are positive if the color changes to blackish-blue or blackish-green.	
Alkaloids	+	A thin deposit is formed at the base of the tube	
Saponins	+	Foam is embossed that can last for 5 to 10 minutes with a height range of between 1 to 10 cm.	

Creation of Factions

Avocado seed fractions are made using a liquid-liquid extraction technique using a split funnel. A total of 10 grams of extract samples were weighed and placed in a glass beaker. Next, the mixture is mixed with a 96% (5-10 ml) ethanol solvent and 75 ml of aquadest. The mixture is then put into a split funnel, followed by the addition of 75 ml of n-hexane solvent. The fractionation process is carried out by shaking the separation funnel in one direction for some time and occasionally opening the airflow in the separation funnel.

Each step in the fractionation process, which involves the use of different solvents, is repeated 3 times. After that, the n-hexane phase (the upper part) is separated from the water phase, and the n-hexane phase is collected in a special container as a result of the first fractionation. The residual water phase of the n-hexane fraction was collected and refractionated using a 75 ml ethyl acetate solvent in 3 repeats. Then, the mixture is separated between the water phase and the ethyl acetate phase, resulting in an ethyl acetate fraction. Meanwhile, the water phase or residue of the ethyl acetate fraction was fractionated with n-butanol as much as 75 ml in 3 repeat times. The n-butanol phase and the water phase are separated, and the n-butanol phase is accommodated as an n-butanol fraction. The final result of the fractionation process using the ECC method includes the n-hexane fraction, the n-butanol fraction, the ethyl acetate fraction, and the water fraction (as the residue phase). These four

fractions were then concentrated using a water bath. The calculation of butter avocado seed simplicia extract used to make 4 kinds of fractions with different solvents is listed in appendix 6, The yield results of the fractions obtained can be seen in table 8.

Table 8. Fractional Yield

Types of Factions	Extract weight (gr)	Fraction weight (gr)	Yields (%)
N-Hexana	39,0701	6,4317	16,46%
Ethyl Acetate		2,0214	5,17%
N-Butanol		10,7573	27,53%
Water		16,7595	42,90%

Antioxidant Activity Test (DPPH)

1. Manufacture of raw solution dpph 0.4 mM

It was weighed as much as 15.8 mg of dpph powder, then dissolved in 100 milliliters of pa ethanol. So that a raw solution of 0.4 mM dpph with a concentration of 158 ppm was obtained. The dpph raw solution is stored in a black glass bottle coated with aluminum foil so that it is not exposed to light and stored at room temperature.

2. Manufacture of quercetin raw solution

Quercetin powder is weighed as much as 10 mg, dissolved in 100 ml of pa ethanol to the limit mark. The raw solution of quercetin with a concentration of 100 ppm was stored in a brown glass bottle coated with aluminum foil, from the 100 ppm standard five dilution series were then made (0.1, 0.2, 0.3, 0.4, 0.5 ppm).

3. Manufacture of raw solution of ethanol extract 96% avocado butter seeds

The weighing of the extract for the first replication was weighed as much as 0.0304 g or 30.4 mg. In the second replication, the extract was weighed as much as 0.0308 grams or 30.8 mg. Third replication, the extract was weighed as much as 0.0335 grams or 33.5 mg. Each weighing is made in a concentration of 1000 ppm, the extract is dissolved using pa ethanol. From each raw replication of ethanol extract, 96% of butter avocado seeds are made into 5 concentration series, namely 10, 20, 30, 40, 50 ppm.

4. Manufacture of n-hexane fraction raw solution

The weighing of the n-hexane fraction for the first replication was weighed as much as 0.0210 grams or 21 mg. The second replication, the n-hexane fraction was weighed as much as 0.02890 grams or 29 mg. The third replication was weighed as much as 0.0279 grams or 27.9 mg. Each replication of the weighing was made in a concentration of 100 ppm dissolved using pa ethanol. From the raw solution of the n-hexane fraction, 5 series of concentrations of 10, 20, 30, 40, 50 ppm are made.

5. Manufacture of ethyl acetate fraction raw solution

The weighing of the ethyl acetate fraction for the first replication was weighed as much as 0.0400 grams or 40 mg. The replication of the two ethyl acetate fractions was weighed as much as 0.0419 grams or 41.9 mg. For the third replication, it was weighed as much as 0.0404 grams or 40.4 mg. Each replication of the weighing was made in a concentration of 100 ppm dissolved using pa ethanol. From the raw solution of the ethyl acetate fraction, 5 series of concentrations of 10, 20, 30, 40, 50 ppm are made.

6. Manufacture of n-butanol fraction raw solution

The weighing of the n-butanol fraction for the first replication was weighed as much as 0.0390 grams or 39 mg. The replication of the two ethyl acetate fractions was weighed as much as 0.0253 grams or 25.3 mg. For the third replication, it was weighed as much as 0.0266 grams or 26.6 mg. Each replication of the weighing was made in a concentration of 100 ppm dissolved using pa ethanol. From the raw solution of the n-butanol fraction, 5 series of concentrations of 10, 20, 30, 40, 50 ppm are made.

7. Manufacture of water fraction raw solution

The weighing of the water fraction for the first replication was weighed as much as 0.0228 grams or 22.8 mg. The replication of the two ethyl acetate fractions was weighed as much as 0.0235 grams or 23.5 mg. For the third replication, it was weighed as much as 0.0234 grams or 23.4 mg. Each replication of the weighing was made in a concentration of 100 ppm dissolved using pa ethanol. From the raw solution of the water fraction, 5 series of concentrations of 10, 20, 30, 40, 50 ppm are made.

8. Determination of the max lamda or wavelength

A 0.4 mm dpph solution is piped with 1 ml and then added with ethanol pa to the limit mark of 5 milli. Before being used for testing, the dpph solution must meet the abs range between 0.2 to 0.8. The solution is then put into the UV-VIS spectrum cuvette, breakfast is measured at wavelengths of 500 to 600, then observed at what wavelengths peak the absorption peaks. The purpose of setting the maximum wavelength is to get maximum absorption from the DPPH. After three repetitions, the wavelength was obtained at 515 nm. It is at this wavelength that the absorbance of the solution will be read.

9. Determining Operating Time

A 0.4 mM dpph solution is piped as much as 1 ml then aded with 5 milli pa ethanol until the limit mark. Then it is added with a raw solution of quercetin with the smallest concentration series, each in a pipette of 2 ml. The solution is then read for absorbance at a maximum lamda of 515 nm. Observe stable abs obtained at time intervals of 0 to 60 minutes. The results obtained a table ABS value from the 15th minute to the 26th minute (abs pregnant for 11 minutes) with an ABS value of 0.413. The value or time ot is used for the incubation period during the time the ot is read absorbance.

10. Creation of Raw Curves

Quercetin 5 series solution with a concentration of 0.1-0.5 ppm. Each concentration series was pipetted as much as 2 ml and then each one was added with 2 ml DPPH. The reacted solution is put into a vial already lined with aluminum foil and incubated for 15 minutes. After that, the absorbance is read at a wavelength of 515 nm. Repeat the same method 3 times (replication).

11. Results of ANthioxian Activity Test

KLT Test Identifies Chemical Compound Content in Fractions with the Best IC50

The results of flavonoid identification by KLT, in the light the plate appears to be slightly greenish-yellow, at UV 254 there is dimming and at UV 366 fluorescence is greenish-yellow and purplish, after being sprayed with the citroborate reagent KLT plate shows yellow spots, the RF value of the third spot is 0.54. Based on these results, it is concluded that the ethyl acetate fraction of avocado fruit seeds is positive for butter and contains flavonoid compounds.

Flavonoids are a group of phytochemical compounds known for their antioxidant activity. The results of the identification of alkaloid compounds in UV 254 light spots of stains look faint (dampening), in UV 366 rays fluorescent they become blackish-red, then after being sprayed with anisaldehyde it shows a change in color to brown. The RF value is parallel to the three samples, which is 0.84. Based on these results, it is concluded that the ethyl acetate fraction of avocado seeds of butter is positive and contains alkaloid compounds. The identification results on the light appeared to be light brown stains, at UV 254 the stains were blackish-green and at UV 366 was blackish-purple, the RF value was parallel to the three spots of stain, which was 0.7. From these results, it was concluded that the ethyl acetate fraction of positive butter avocado seeds contained saponin compounds. The results of the identification of triterpenoid compounds by KLT in the light showed orange wheel spots, at UV 254 they were dimmed and red, at UV 366 they were brownish-red. The RF value of the third parallel spot of the stain is 0.9. From these results, it was concluded that the ethyl acetate fraction of positive butter avocado seeds contains compounds of the triterpenoid group.

Based on the results of the KLT test of the ethyl acetate fraction, data shows that the chemical compounds contained in the fraction include flavonoid group compounds, alkaloids, saponins and triterpenoids. Compounds of this group are mostly attracted by semi-polar ethyl acetate solvents during the fractionation process. The mechanism of action of antioxidants of flavonoid compounds is that it captures ROS (Reactive Oxygen Species) directly, then prevents ROS regeneration, and increases the antioxidant activity of cellular antioxidant enzymes (Akhlagh|i and Bandy., 2009). Saponin compounds dampen superoxide by the formation of h|iper-oks|ida intermediates so that they can prevent b|iomolecular damage caused by free curls (Hasan et al., 2022). Mekan|ism alkalo|id as|i ant|ioks|idan donates its H atom to free rad|curls (Chung and Sh|in, 2007). Phenolic compounds fight against the rad|curl peroks|ida (ROO*) (Adedapo, 2008) and the rad|curl h|hydroxy|il (HO*), by transferring hydrogen atoms, transferring single electrons, keh|ial sequenced protons, and chelates|metal ions trans|is|i and then forming a stab|il react|i phenox|i (L|im et al., 2007).

A free radical form, one unpaired electron resides on a central nitrogen atom, which gives it its distinctive dark purple color properties and free radical reactivity used in various antioxidant tests. Flavonoids have excellent antioxidant properties and can demonstrate their antioxidant activity by capturing free radicals and ROS, chelating metals, and preventing the oxidation of low-density lipoproteins. The mechanism by which flavonoids inhibit free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), is through electron transfer or hydrogen transfer reactions, which transform DPPH into a more stable and non-reactive form. This process causes a change in color from dark purple to pale yellow or colorless, which can be measured spectrophotometrically. A decrease in the intensity of purple indicates antioxidant activity from flavonoids and other antioxidants (Hasan, 2022).

Alkaloid compounds, like flavonoids, have the ability to act as antioxidants by neutralizing free radicals. Overall, the mechanism of alkaloid compounds against DPPH involves the donation of hydrogen or electrons that neutralize DPPH free radicals, thereby protecting biological cells and molecules from oxidative damage (Cahaya , 2002).

Saponins are a group of phytochemical compounds that have a variety of biological activities, including antioxidant activity that helps neutralize free radicals and oxidative stress in the body. The saponin mechanism against free radicals transfers hydrogen, which neutralizes

free radicals and converts them into a more stable form. When DPPH (2,2-diphenyl-1-picrylhydrazyl) reacts with terpenoids, the mechanism is similar to that of a reaction with flavonoids or alkaloids, in which terpenoids donate hydrogen atoms or electrons to DPPH free radicals, converting them into a more stable and non-reactive form, namely DPPH-H (2,2-diphenyl-1-picrylhydrazine) (Hasan, 2022).

CONCLUSION

The results demonstrated that a 96% ethanol extract of butter avocado (Persea americana Mill.) seeds was successfully obtained through maceration, with an extraction yield of 24.6%. Phytochemical screening indicated the presence of secondary metabolites, including flavonoids, tannins, alkaloids, and saponins, some of which may contribute to antioxidant activity. Sequential liquid–liquid partitioning yielded four fractions: n-hexane, ethyl acetate, n-butanol, and aqueous fractions. The antioxidant activity of each fraction was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay with ultraviolet–visible (UV–Vis) spectrophotometry. The results indicated differences in antioxidant activity among the fractions, suggesting that solvent polarity influenced the distribution of constituents associated with radical-scavenging activity. The ethyl acetate fraction exhibited the highest reported antioxidant activity, although the absence of numerical half-maximal inhibitory concentration (IC_{50}) values limited quantitative comparisons of antioxidant potency. Overall, these findings suggested that butter avocado seeds represented a potential source of naturally occurring antioxidant constituents. Further investigations involving thin-layer chromatography (TLC), compound isolation, structural characterization, and complementary antioxidant assays are warranted to identify the compounds responsible for the observed activity and evaluate their potential applications.

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