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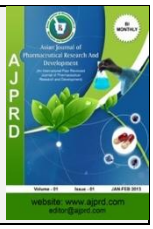

Open Access Research Article

Isolation and identification of flavonoids from avocado leaves (*Persea americana* Mill)

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A B S T R A C T

Leaves of avocado (*Persea americana* Mill) contains bioactive compounds that can act as natural antioxidants. This study was conducted to isolate and identify the flavonoid compounds of avocado leaves. Research stages includes maceration, fractionation, thin-layer chromatography analysis, isolation and purification of compounds also identification of isolates in two-dimensional thin-layer chromatography and by using three different types of solvents. Isolates were identified using ultraviolet-visible spectrophotometry and infrared spectrophotometry. From the results obtained one isolate FA6 showed a single compound. Based on the results by testing the purity of isolates using two-dimensional thin-layer chromatography and by using three different types of solvents, isolates FA6 is a pure compound. Identification by using ray-visible spectrophotometry FA6 isolates showed maximum wavelength at 280.6 nm and 342.0 nm on bands II and on band I. Infrared spectrum ofisolate FA6 showed aromatic OH groups, aliphatic CH, C = O stretching, C = C aromatic, and C-O-C stretch. From the results obtained isolates FA6 is a class of flavonoids group flavones or flavonol (3-OH substitional).

Keywords: *Persea americana*,isolation, flavonoid, UV spectrophotometery, IR-spectrophometry

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INTRODUCTION:

Persea americana Mill or the common people refer to as avocado, avocado, or advocate is a wild plant in the forests, but is widely planted in gardens and yards where the soil layer is loose, fertile and not waterlogged. The results will be satisfactory if planted at an altitude of 200-1,000 m above sea level in tropical and subtropical areas with a lot of rainfall [1].

Avocado leaves are empirically believed to be a diuretic, namely increasing the volume of urine produced during urination to reduce blood pressure and kidney stone problems [2]. Research conducted by Adeyemi et al. in 2002 [3]has reported that avocado leaves can act as anti-inflammatory and analgesic.

The bioactive content contained in avocado leaves can also act as a natural antioxidant [4]. In 2012, a study was conducted on the leaves, seeds and fruit of avocado and it was known that the chemical content was saponins, tannins, flavonoids, cyanogenic glycosides, alkaloids, phenols, and steroids. The amount of this chemical content varies depending on the part of the plant used [5].

Flavonoids are the largest group of phenolic compounds found in nature. These compounds are red, purple and blue as yellow dyes found in plants. Flavonoids have a basic carbon skeleton consisting of 15 carbon atoms, in which two benzene rings (C6) are bonded to a propane chain (C3) to form a C6-C3-C6 arrangement. The rings are marked A, B, and C; carbon atoms are numbered according to a numbering system that uses the usual numbers for the A and C rings, as well as an 'accented' number for the B ring [6]. Flavonoids contain a conjugated aromatic system and therefore exhibit strong absorption bands in the UV and visible spectrum regions. Finally, flavonoids are common in plants, bound to sugars as glycosides and any of the flavonoid aglycones may be present in a single plant in several combinations of glycosides [7]. This article reports

Isolation and identification of flavonoids from avocado leaves, *P. americana*.

Materials and Methods

Tools and Materials:

The chemicals used were 96% ethanol (Brataco), 2N hydrochloric acid, chloroform, Mayer reagent, Dragendroff reagent, Bouchardat reagent, 1% gelatin, magnesium powder, sodium acetate powder, 2N sodium hydroxide solution, boric acid, potassium bromide, potassium hydroxide 5%, ammonia, amyl alcohol, aluminum chloride 5% solution in ethanol, toluene, acetone, ether, methanol (Brataco), n-hexane (Brataco), ethyl acetate (Brataco).

Macerator, UV-spectrophotometer (specord 200-analytic jena).), FT-IR 8400 spectrophotometer (Shimadzu) glassware and commonly used glassware in the Biological Pharmacy Laboratory.

Plant Material:

The plant material used in this study was the leaves of the avocado plant (*Persea americana* Mill) obtained from the Lembang area, Bandung, West Java.

Method:

Determination, Collection and Processing of Research Materials: Avocado leaves were collected in March 2020 obtained from Lembang, Bandung. Materials were determined at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University. Simplicia chopped or finely blended.

Phytochemical Screening: Examination of secondary metabolites of alkaloids, flavonoids, tannins, polyphenolic compounds, monoterpenoids and sesquiterpenoids, triterpenoids and steroids, quinones and saponins.

Extraction: The dried and smooth avocado leaves were weighed and then extracted by maceration for 3 x 24 hours using 96% ethanol as solvent. The macerate was thickened with a rotary evaporator and then evaporated over a water bath to remove any remaining solvent.

Fractionation: A number of viscous extracts were fractionated by liquid extraction method (ECC) using water, n-hexane and ethyl acetate as solvents. The obtained fractions were then concentrated with a rotary evaporator and then evaporated over a water bath so that the weight was constant.

Extract and Fraction Analysis: The mobile phase was optimized for use on the ethanol extract, n-hexane fraction, ethyl acetate fraction and water fraction using thin layer chromatography (TLC) stationary phase with GF254 as adsorbent. The mobile phase used was n-hexane: ethyl acetate (3:7). Extracts and fractions were spotted on a GF254 TLC plate. When the vessel is saturated, the TLC plate is inserted. After the mobile phase removed the boundary marks, the TLC plate was removed and dried. Seen in UV light at 254 nm and 366 nm and sprayed using 5% AlCl3 spotting in ethanol.

Isolation and Purification of Compounds: Using column chromatography and preparative thin layer chromatography methods. Based on the TLC results from the extract and fraction analysis, the fraction suspected to contain flavonoid compounds, namely the ethyl acetate fraction with yellowish-blue fluorescence spots was selected after the addition of 5% AlCl3 spotting in ethanol at 366 nm UV light.

The ethyl acetate fraction was separated by column chromatography using 60 H ciliated gel stationary phase, n-hexane : ethyl acetate (3:7) elution, and using 60 H silica gel as stationary phase. Sub fractions obtained from column chromatography were thin layer chromatography with motion of n-hexane: ethyl acetate (3:7). The sub-fraction suspected to contain flavonoid compounds was further analyzed using preparative thin layer chromatography using the mobile phase n-hexane: ethyl acetate (3:7). The ribbon suspected to contain flavonoid compounds was scraped and dissolved in ethyl acetate solvent in the vial, then allowed to stand for 24 hours.

The purity of the isolates was determined by two-dimensional thin layer chromatography (first mobile phase; n-hexane: ethyl acetate (3:7) and second mobile phase; chloroform: methanol (9:1) and chromatography using three different types of developer (first mobile phase). ; ethyl acetate: n-hexane (3:7), the second mobile phase; toluene: methanol (7,5:2,5) and the third mobile phase; ether: acetone (3:7).

Isolate Identification: Identification of isolates using ultraviolet-visible spectrophotometry and infrared spectrophotometry.

The scrapings that have been soaked in ethyl acetate are removed and allowed to evaporate. Then dissolved in methanol to take as much as 2.5-3 ml and put into a cuvette. A blank test was performed using methanol. The cuvette for comparison is left in the spectrophotometer while the other cuvette is for the sample. For the identification of flavonoids, shear reagent was used with the following steps, after measuring the sample in methanol, three drops of 2N sodium hydroxide were added to the cuvette, mixed and the spectra were designed. After five minutes of mixing, the sodium hydroxide spectrum was measured again, the solution was discarded and the cuvette was washed.

The sample was put into a cuvette and six drops of aluminum chloride reagent was added and the spectrum was measured. Then three drops of hydrochloric acid were added, mixed and the spectrum was re-measured. After measurement, the sample is discarded and the cuvette is washed. The sample was put into a cuvette and sodium acetate was added, the mixture was shaken and the spectrum was measured. Boric acid was added as much as about half of the amount of sodium acetate then compared with data from the literature.

Identification of isolates using infrared spectrophotometry: The results of scrapings that have been soaked in ethyl acetate in the form of a clear solution are transferred to another vial and then allowed to evaporate the solvent so that the isolate is in solid form. The solid isolate was then made into pellets, mixed with KBr, crushed and put into a special printing device. Previously, KBr .

plates were made and the infrared spectrum was measured which was used as a blank. After the sample pellet was formed, the infrared spectrum was measured. The spectrum obtained was then analyzed.

RESULTS AND DISCUSSION

Phytochemical Screening Results: As initial information about what chemical compounds are contained in a plant and to detect plant compounds based on their group. The results of phytochemical screening from avocado leaf simplicia can be seen in Table 1.

Table 1: Phytochemical Screening of Avocado Leaf

No.	Metabolite seconder	Simplisia Avocado leaf
1.	Alkaloids	-
2.	Poliphenolic	+
3.	Tannins	+
4.	Flavonoids	+
5.	Monoterpenoid dan sesquiterpenoid	+
6.	Steroids	+
7.	Triterpenoids	-
8.	Quinone	+
9.	Saponins	+

Notes: +: detected; -: not detected

Phytochemical Screening: Examination of secondary metabolites of alkaloids, flavonoids, tannins, polyphenolic compounds, monoterpenoids and sesquiterpenoids, triterpenoids and steroids, quinones and saponins. The screening was based on existing method [7-9]. Our results quite similar to the work of Rahman et.al [10]. Tanzanian avocado seed was reported containing tannin and flavonoids [11]. Aletan [12] mentioned that the presence of tannins, phenols, flavonoids and steroids in all the three parts of avocado skin, seed and oil, while saponins and alkaloids were detected only in the skin and seed.

Extraction: The dried and smooth avocado leaves were weighed and then extracted by maceration for 3 x 24 hours using 96% ethanol as solvent. The macerate was thickened with a rotary evaporator and then evaporated over a water bath to remove any remaining solvent. The yield of avocado leaf ethanol extract was 11.47%.

Fractionation: A number of viscous extracts were fractionated by liquid extraction method (ECC) using water, n-hexane and ethyl acetate as solvents. The obtained fractions were then concentrated with a rotary evaporator and then evaporated over a water bath so that the weight was constant. The yields of the n-hexane fraction and the ethyl acetate fraction of avocado leaf ethanol extract were 4.089% and 0.582%, respectively.

Extract and Fraction Analysis: The mobile phase was optimized for use on the ethanol extract, n-hexane fraction, ethyl acetate fraction and water fraction using thin layer chromatography (TLC) stationary phase with GF254 as adsorbent. The mobile phase used was n-hexane: ethyl acetate (3:7). Extracts and fractions were spotted on a GF254 TLC plate. When the vessel is saturated, the TLC plate is inserted. After the mobile phase removed the boundary marks, the TLC plate was removed and dried. Seen in UV light at 254 nm and 366 nm and sprayed using 5% $AlCl_3$ spotting in ethanol.

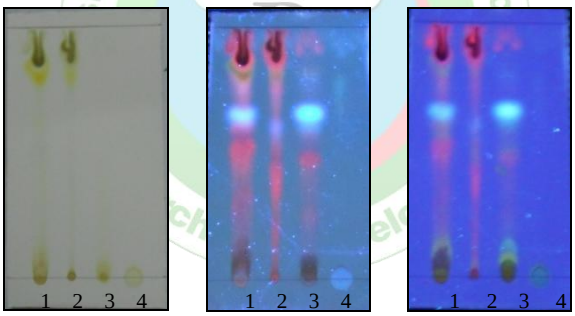
Isolation and Purification of Compounds: Using column chromatography and preparative thin layer chromatography methods. Based on the TLC results from the extract and fraction analysis, the fraction suspected to contain flavonoid compounds, namely the ethyl acetate fraction with yellowish-blue fluorescence spots was selected after the addition of 5% AlCl3 spotting in ethanol at 366 nm UV light.

TLC:

Results of Thin Layer Chromatography of Extracts and Fractions: After optimization of thin layer chromatography (TLC) to find a suitable mobile phase to be used. TLC analysis of ethanol extract, n-hexane fraction, ethyl acetate fraction and water fraction was carried out using silica GF254 stationary phase and n-hexane: ethyl acetate mobile phase (3:7). In the ethyl acetate fraction, the spots with yellowish blue fluorescence were suspected to be flavonoid compounds after being sprayed with 5% $AlCl_3$ spotting in ethanol and viewed under UV light at 366 nm. The results of TLC extracts and fractions can be seen in Table 2 and Figure 1 below:

Table 2: TLC Results of Ethanol Extract, n-hexane Fraction, Ethyl Acetate Fraction and Avocado Leaf Water Fraction

Sampel	R_F	Visible light	UV 366 nm	$AlCl_3$ 5% in ethanol
Ethanol extract	1. 0,87 2. 0,81 3. 0,75 4. 0,66 5. 0,60 6. 0,50	reen yellow - - - -	red yellow faded red faded blue faded yellow red	red yellow faded red Yellowish blue fluorescence yellow green
n-hexane fraction	1. 0,87 2. 0,81 3. 0,75 4. 0,63 5. 0,60 6. 0,38	green yellow - - - -	red yellow faded red faded blue faded yellow red	red yellow faded red blue yellow red
Ethyl acetate fraction	1. 0,87 2. 0,84 3. 0,66 4. 0,60 5. 0,44 6. 0,25	- - - - - -	faded red faded blue Yellowish blue fluorescence yellow red faded red	red blue Yellowish blue fluorescence yellow red yellow
Water fraction	-	-	-	-



Visible light UV 366 nm $AlCl_3$ 5% in ethanol

Figure 1: TLC results of ethanol extract (1), n-hexane fraction (2), ethyl acetate fraction (3) and water fraction (4) using Silica GF254 stationary phase and n-hexane mobile phase: ethyl acetate (3:7)

Column Chromatography Results: In column chromatography, the stationary phase used was silica gel 60 and the mobile phase was n-hexane: ethyl acetate (3:7). The fraction followed by column chromatography was the ethyl acetate fraction because the nature of the flavonoid itself is polar, so it tends to be in the ethyl acetate fraction. Each sub-fraction accommodated as much as 5 ml. The sub-fraction obtained from column chromatography was further analyzed using thin layer chromatography with n-hexane: ethyl acetate (3:7) developer. Based on the research, 36 sub-fractions were obtained, then monitored by TLC.

From the results of the thin layer chromatography, the sub-fractions with the same spot pattern were grouped based on the magnitude of the RF and the color obtained for further purification. From the results of column chromatography, a combination of sub-fractions numbered 14 to 27 (FA) was obtained which was suspected to have the target compound, namely yellowish-blue fluorescence after being sprayed with 5% $AlCl_3$ spray reagent in ethanol at 366 nm UV light.

Results of Preparative Thin-Layer Chromatography: The combined FA sub-fraction was continued with preparative thin-layer chromatography analysis with silica gel 60 PF254 as stationary phase and n-hexane: ethyl acetate (3:7) mobile phase. From these results obtained 7 bands. The results of preparative thin layer chromatography of the FA sub fraction ethyl acetate ethanol extract of avocado leaves can be seen in Table 3 and Figure 2 below:

Table 3: Results of Combined Preparative Thin Layer Chromatography Sub Fraction FA Ethyl Acetate Fraction Ethanol Extract of Avocado Leaf

Band no-	R_F	UV 366 nm
1	0,94	faded blue
2	0,93	red
3	0,92	faded yellow
4	0,7	faded blue
5	0,64	faded red
6	0,6	Yellowish blue fluorescence
7	0,5	yellow

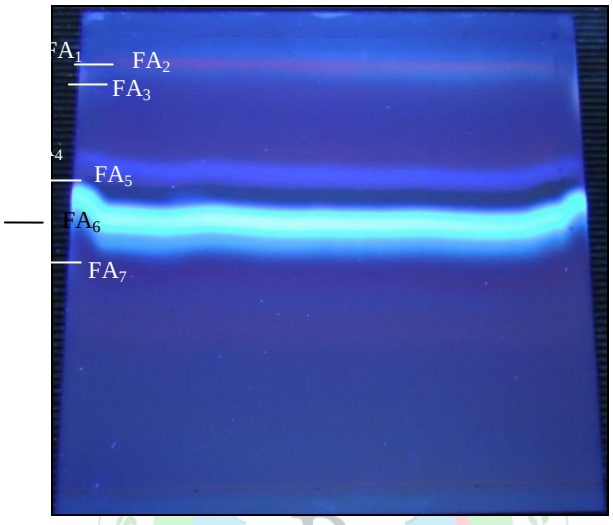


Figure 2: Results of Combined Preparative Thin Layer Chromatography of FA Sub Fraction using Silica gel 60 PF254 Stationary Phase and n-hexane Mobile Phase: Ethyl Acetate (3:7) under UV Light 366 nm

The sixth band (FA6) with RF 0.6 was scraped and put into a vial containing ethyl acetate solvent, then allowed to stand for 24 hours. After settling, the clear solution FA6 was filtered and transferred to another vial. The clear solution FA6 was evaporated over a water bath at a temperature of 50-60°C so that the ethyl acetate solvent evaporated and what remained was the isolate of the target compound (FA6).

Purity Test Results: The isolate purity test was carried out using two-way thin layer chromatography (TLC) and thin layer chromatography using three different types of developer. In the test using the two-way TLC method, two different developers were used, namely the first developer of n-hexane: ethyl acetate (3:7) while the second developer was chloroform: methanol (9:1). Two-way TLC performed on FA6 isolates showed a yellowish-blue fluorescence spot after viewing under 366 nm UV light. While TLC uses three different types of developers, namely the first developer; ethyl acetate : n-hexane (3:7), second developer; toluene : methanol (7.5:2.5) and third developer; ether:acetone (7:3) also produced one significant spot. From the results of the resulting chromatogram, it was concluded that the FA6 isolate was a single compound (see Fig. 3).

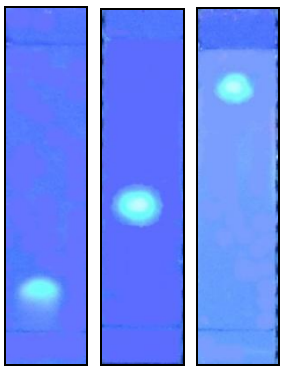


Figure 3: TLC results using Silica GF254 stationary phase and mobile phase; (1) Ethyl Acetate :n-hexane (3:7), (2) Toluene : Methanol (7,5:2.5) and (3) Ether : Acetone (7:3)

Isolate Identification Results: Identification of FA6 isolates was carried out using ultraviolet spectrophotometry and infrared spectrophotometry.

Ultraviolet-Visible Light Spectrophotometry: The FA6 isolate produced two peaks with a maximum wavelength of 280.6 nm band II and 342.0 band I. The appearance of these two peaks was due to the presence of two benzene rings that provided absorption at these wavelengths. This benzene system is derived from rings A and B of flavonoid compounds. Judging from the ultraviolet absorption range, FA6 isolates were in the absorption range of band II 250-280 nm and the absorption range of band I was approximately 310-360 nm, this indicates that FA6 is a flavonoid compound of the flavone group or flavonol (3-OH substituted) [6].The ultraviolet spectrum of FA6 isolate in methanol can be seen in Figure 4 below:

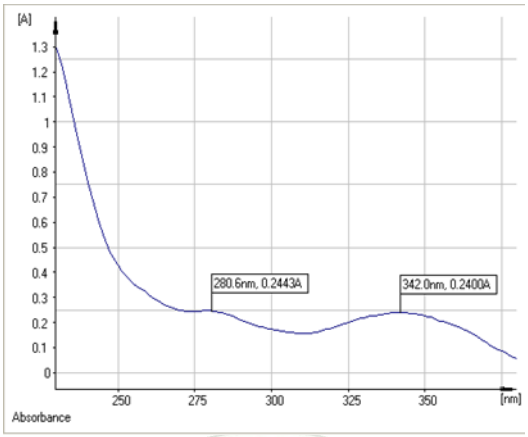


Figure 4: Ultraviolet Spectrum of FA6 Isolate in Methanol

The addition of shear reagent was done to find out more information about the hydroxylation pattern. The shear reagents used were NaOH, AlCl3, AlCl3/HCl, NaOAc, and NaOAc/H3BO3. When compared with the methanol spectrum, the addition of NaOH causes a hypochromic shift in band II of 5 nm and a bathochromic shift of band I of 34 nm. The strength of the I band did not decrease indicating the presence of a 4'-OH group.

After the addition of NaOAc shear reagent, the band II showed a hypochromic shift of 5.3 nm. This happens because with increasing time the strength decreases indicating the presence of a base-sensitive group. It was suspected that the FA6 isolate had a 7-OH group. Then, with the addition of H3BO3, there was a bathochromic shift in band I of 33 nm (relative to the methanol spectrum). This indicated that the FA6 isolate had an ortho dihydroxyl group on ring B.

Infrared Spectrophotometry: The results of infrared spectrophotometry showed absorption at several wave numbers. Infrared spectrum of FA6 isolate showed aliphatic CH strain at 1375.26 cm^{-1} (strong band intensity), 1458.68 cm^{-1} (strong band intensity) and 2859.01 cm^{-1} (strong band intensity), strain C=C aromatic at 806.25 cm^{-1} (strong band intensity) and 1597.07 cm^{-1} (medium band intensity), COC strain at 1272.55 cm^{-1} (strong band intensity), C=O strain at 1729.68 cm^{-1} (strong band intensity) and OH strain at 2925.07 cm^{-1} (strong band intensity) and 3301.66 cm^{-1} (weak intensity). The infrared spectrophotometry results of FA6 isolate can be seen in Table 4 and Figure 5 below:

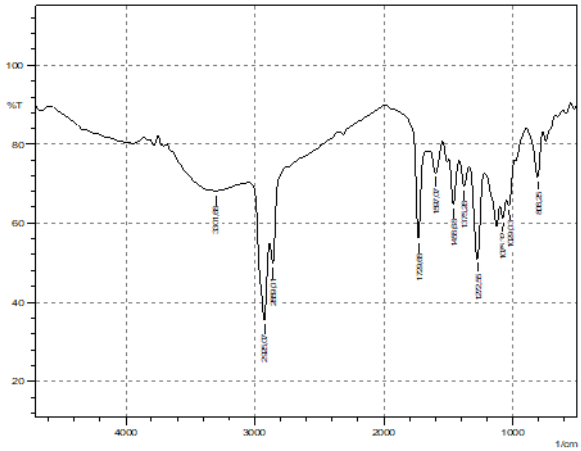


Figure 5: Infrared Spectrum of Isolate FA6

Table 4: Infrared Spectrophotometry of FA6 Isolate

Wave numbers (cm^{-1})	Band shape	Intensity	Sumption
806,25		strong	Aromatic
1272,55	sharp	strong	stretch C-O-C
1375,26	sharp	strong	C-H aliphatic
1458,68	sharp	strong	C-H aliphatic
1597,07	sharp	medium	Aromatic
1729,68	sharp	strong	stretch C=O
2859,01	sharp	strong	C-H aliphatic
2925,07	sharp	strong	stretch O-H
3301,66	sharp width	weak	stretch O-H

Actually, many studies on *P.americana* had been published elsewhere [17-20] but none of them similar to this report.

CONCLUSION

It can be concluded that the isolation process carried out by the maceration method, the separation method using liquid-liquid extraction (ECC), the purification method using column chromatography and layer chromatography. preparative thin. Using an ultraviolet-visible spectrum, isolate FA6 showed a wavelength at 280.6 nm in band II and 342.0 nm in band I and the infrared spectrum reading of isolate FA6 showed the presence of bound OH groups, aliphatic CH, stretch C=O, C. =C aromatic, and stretch COC. Then it can be identified that the ethyl acetate fraction of the ethanolic leaf extract avocado (*Persea americana* Mill), contained flavonoid compounds of the flavone group or flavonol (3-OH substituted).

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CONFLICT OF INTEREST

The authors declare that there was no conflict of interest regarding the publication of this paper.

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