

Research Article

ANTIOXIDANT ACTIVITY OF ETHYL ACETATE EXTRACT OF RED *Psidium guajava* L. LEAVES GROWN IN MANOKO, LEMBANG - INDONESIA

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Received: 27-10-2011

Revised: 23-02-2012

Accepted: 28-02-2012

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ABSTRACT

Psidium guajava L. (Myrtaceae) is a well known plant in Malaysia and Indonesia. Its leaves extract was found to possess antidiarrhea, antimicrobial, hepatoprotective and antioxidant activities. Objective of this research is to isolate an antioxidant substance from Red *Psidium guajava* L. leaves. The crude leaves was extracted using Soxhlet apparatus by gradual polarity of three different solvents, n-hexane, ethyl acetate and methanol. Antioxidant activity of each extract was tested by using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method. Total phenol, total flavonoid and total tannin content of the extracts were also measured. Ethyl acetate extract was fractionated using vacuum liquid chromatography for fractionation. Purification was performed using TLC preparative. Isolate then characterized using specific spray reagent, UV-Vis spectrophotometry and infrared spectrophotometry. Crude drug of *Psidium guajava* contained flavonoid, tannin, quinone, saponin and steroid/ triterpenoid. Antioxidant activity of ethyl acetate extract is 65.63% with total phenol 4.25%, total flavonoid 0.53% and total tannin 1.16%. Antioxidant compound *N* was isolated from ethyl acetate extract. Antioxidant compound *N* was supposed to be aglycone flavone that has OH at C -4', C-5 and C-7.

Key words: *Psidium guajava* L. , antioxidant activity, ethyl acetate extract

INTRODUCTION

Uncontrolled generation of free radicals together with reduced level of antioxidative vitamins and enzymes is considered to be the main contributor to oxidative stress. Free radicals attack membrane lipids, protein and DNA is believed to be involved in many health disorders (Ikegami, 2001). Antioxidants are known to have beneficial effects on the prevention or progression of diseases related to oxidative stress on account of their high antioxidant activity. An antioxidant is a molecule capable of inhibiting the oxidation of other molecules.

Guava (*Psidium guajava* L.), also known locally as jambubatu, is grown commercially and grown in many home gardens in Malaysia and Indonesia. *Psidium guajava* L. is a tropical tree or shrub. It is native to central America from Mexico to northern south America. It has been introduced to most tropical and sub-tropical locations around the world for its

edible fruit. *Psidium guajava* L. (Myrtaceae) is one of such plants in folk medicine that has been used for the management of various disease conditions and is believed to active. Various parts of the plant has been used in traditional medicine to manage a lots of disease conditions (Metwally, *et al.*, 2011).

The pharmacological actions and the medicinal uses of aqueous extracts of guava leaves in folk medicine include gastrointestinal disturbances such as diarrhea it acts also as antibacterial agent. The leaf extract was found to possess antimicrobial, hepatoprotective and antioxidant activities (Metwally, *et al.*, 2011).

Red guava leaves is alternative of antioxidant resource that can be developed. Objective of this research is to isolate antioxidant compound from red guava leaves extract employing DPPH radical scavenging assay. The isolated compound can be used as the marker of red guava leaves.

METHODOLOGY

Materials

Red guava leaves (*Psidium guajava* L.), ammonia, chloroform, magnesium powder, amyl alcohol, hydrochloric acid, sodium hydroxide, Dragendorff reagent, Steasny reagent, Mayer reagent, Liebermann-Burchard reagent, sodium acetate, ferum (III) chloride, methanol, ethyl acetate, n-hexane, silica gel, aluminium (III) chloride, sulphuric acid, DPPH reagent, Folin-Ciocalteu reagent, aluminium chloride.

Sample Preparation

Red guava leaves (*Psidium guajava* L.) was collected from Pusat Pertanian Manoko and determined in Herbarium Bandungense, School of Biology and Engineering, Institut Teknologi Bandung. The leaves have been dried in the oven under 40-42°C for few days. The dried leaves was grinded coarsely until a crude leaves powder was obtained.

Characterization of Crude Drug

Characterization of crude drug powder including macroscopic and microscopic, water soluble and ethanol soluble extractable matter, loss on drying, water content and total ash content.

Phytochemical Screening

Phytochemical screening was conducted to detect the presence of alkaloids, steroid/triterpenoid, flavonoids, tannins, quinones and saponins.

Extraction and Extract Monitoring

Extraction was performed using Soxhlet method. Crude leaves powder was extracted with three different solvent with increasing polarity. Solvents that were used are n-hexane for non polar solvent, ethyl acetate for semipolar solvent and methanol for polar solvent. Liquid extracts were concentrated using rotavapor. Extracts were monitored by thin layer chromatography using suitable mobile phase for each extract and observed under UV light at λ 254 nm and λ 366 nm. The chromatogram was sprayed with H₂SO₄ 10% in methanol and DPPH 0.2% in methanol. Based on the observation, if the extracts gives yellow color spots with purple background after spraying with DPPH 0.2% in methanol, it

shows the presence of antioxidant.

Total Phenolic, Total Flavonoid and Total Tannin Content

As supporting data in selecting extract that will be fractionated and assayed later with fractionation steps, total phenol, flavonoid and tannin content were also measured .

Determination of Total Phenolic Content

The total phenolic content of extracts were determined with Folin-Ciocalteu reagent using gallic acid as standard. Concentration of standard used were 50 μ g/mL, 100 μ g/mL, 150 μ g/mL, 200 μ g/mL and 250 μ g/mL. Absorbance was measured using UV-Visible spectrophotometry at λ 765 nm. Total phenol content of extracts were determined based on calculation from linear regression equation of calibration curve of gallic acid as standard (Pourmorad, *et al.*, 2006).

Determination of Total Flavonoid Content

Aluminium chloride colorimetric method was used for the determination of total flavonoids. Concentration of standards used were 25 μ g/mL, 50 μ g/mL, 75 μ g/mL, 100 μ g/mL, 125 μ g/mL. Absorbance was measured using UV-Visible spectrophotometry at wavelength of λ 415 nm. Total flavonoid content of extract was determined based on calculation from linear regression equation of calibration curve of quercetin as standard (Ordon *et al.*, 2006).

Determination of Total Tannin Content

Total tannin content was determined using method as stated in Quality Control Methods for Medicinal Plant Material.

Antioxidant activity of extracts

Antioxidant activity of each extract was tested using scavenging DPPH method by reacting each extract with solution of DPPH 50 μ g/mL in methanol (1:1) for 30 minutes. Absorbance was measured at λ 516 nm. Antioxidant activity of each extract were determined based on the reduction of DPPH absorbance by calculating percentage of antioxidant activity (Bedawey, 2007).

Fractionation and Monitoring of Fractions

The extract was chosen then fractionated by vacuum liquid chromatography using silica gel 60 H as the stationary phase and 21 combinations of n-hexane-ethyl acetate-methanol. Eluted gradiently as mobile phase as mobile phase. Fractions collected were monitored using suitable mobile phase. Fractions which showed positive antioxidant activity effect then combined and monitored again. Fractions combination which gave a good separation was chosen for second fractionation using preparative thin layer chromatography.

Purification and Purity Test

Purification was performed by preparative thin layer chromatography using silica gel GF₂₅₄ and suitable mobile phase. Bands that showed positive antioxidant with DPPH reagent were scraped off and monitored. For purity test, single development TLC with three different mobile phase was performed.

Characterization of Isolate

Isolated antioxidant compound was characterized using specific spray reagent, uv-visible spectrophotometry and 2 dimensional paper chromatography.

RESULTS AND DISCUSSION

The red *Psidium guajava* L. leaves was collected from Pusat Pertanian Manoko, Lembang, Indonesia at the end of January 2012. The identification of plant was performed in Herbarium Bandungense, School of Biology and Engineering, Institut Teknologi Bandung.

The characterization of crude drug showed that total ash content is 7.65 %, water soluble extractable matter is 12.50 %, ethanol soluble extractable matter is 9.60 %, loss on drying is 7.50 % and water content is 5.51 %.

Phytochemical screening was done as preliminary steps to determine the presence of chemical compounds in the crude leaves. The results showed that the crude leaves contains flavonoids, tannins, quinones, saponins and steroid/triterpenoid.

The crude leaves was extracted by Soxhlet apparatus using increasing polarity solvents. Solvents used were n-hexane, ethyl acetate and methanol. Three different solvents were chosen to compare the antioxidant activity in different polarities of solvents. The liquid extracts were concentrated using rotavapor. Density of n-hexane, ethyl acetate and methanol extract are 0.66g/mL, 0.88g/mL and 0.72g/mL respectively.

All the three concentrated extracts were monitored using thin layer chromatography (TLC) with stationary phase of silica gel GF₂₅₄ and using n-hexane-ethyl acetate (8:2) for n-hexane extract, ethyl acetate-toluene (8:2) for ethyl acetate extract and ethyl acetate-formic acid-acetic acid water (100:11:11:26) for methanolic extract suitable mobile phase for each extract. TLC plates then observed under UV light at λ 254 nm and λ 366 nm. The TLC plate was sprayed with H₂SO₄ 10% in methanol and DPPH 0.2% in methanol. Based on the observation, if the extracts gives yellow color spots with purple background after spraying with DPPH 0.2% in methanol then it shows the presence of antioxidant.

Antioxidant activity of these three extract was tested based on the reduction of DPPH absorbance at λ 517 nm. Methanol extract has the highest antioxidant activity followed by ethyl acetate and n-hexane extracts.

Tabel I. Data of DPPH scavenging activity, total phenol, total flavonoid, and total tannin contents of *Psidium guajava* L Extract

Result(%)	<i>n</i> -hexane	ethyl acetate	Ethanol
DPPH Scavenging Activity	48.08	65.63	77.06
total phenolic		4.25	16.43
total flavonoid	0.10	0.53	0.88
total tannin		1.16	2.74

Table II. Characterization of isolate N

Reagents	$\Delta\lambda$ max (nm)		$\Delta\lambda$ max (nm)		Interpretation
	Band I	Band II	Band I	Band II	
MeOH	311	253			Flavone
NaOMe	358	271	+47	+18	4'-OH
NaOAc	312	260	+1	+7	7-OH
NaOAc/H ₃ BO ₃	312	260	+1	+7	-
AlCl ₃	367	260	0	0	-
AlCl ₃ /HCl	367	260	56	+7	5-OH

As supporting data in selecting extract that will be continued with fractionation steps, total phenol, flavonoid and tannin contents were also measured. Phytochemical screening was performed on each extract to confirm the presence of phenolic, flavonoids and tannins which usually gives antioxidant activity. The results showed that *n*-hexane extract contains least compounds which are flavonoid and steroid/triterpenoid compared to that of ethyl acetate and methanol extracts. Methanol extract contains almost all the compound tested but alkaloid while ethyl acetate extract contains flavonoid, tannin and steroid/triterpenoid.

Based on the phytochemical screening results, extracts that gives positive result for phenol test was taken to determine the total phenolic content and likewise for flavonoid and tannin.

Total phenolic content was performed using gallic acid as standard with the concentration of 50, 100, 150, 200 and 250 µg/mL. Absorbance was measured using UV-Visible spectrophotometry at λ 765 nm. Equation of calibration curve obtained was $y = 0.0047x + 0.0128$ with $R = 0.9963$. Total phenol content of extract was expressed as mg/g equivalent to gallic acid.

Total flavonoid content determination was performed using quercetin as standard with the concentration of 25, 50, 75, 100 and 125 µg/mL. Absorbance was measured using UV-Visible spectrophotometry at wavelength of λ 415 nm. Equation of calibration curve obtained was $y = 0.016x + 0.0054$ with $R = 0.9987$. Total flavonoid content of extract was expressed as mg/g equivalent to quercetin.

Total tannin content identification was not performed for *n*-hexane extract because tannin compound was not detected during phytochemical screening. Based on the results, methanol extract contains highest amount of total phenolic, flavonoid and tannin, followed by ethyl acetate and *n*-hexane extracts.

Based on determination carried out, even though methanol extract has the highest antioxidant activity, total phenolic, flavonoid and tannin content, the separation of ethanol extract with various mobile phase has not resulting a good result and will cause difficulty to carry out isolation. Therefore ethyl acetate extract was chosen for fractionation. Ethyl acetate extract chromatogram showed better separation pattern compared to that of methanol extract.

Vacuum liquid chromatography was used to fractionate ethyl acetate extract using silica gel 60H as the stationary phase and eluted gradually combinations of *n*-hexane-ethyl acetate-methanol as mobile phase. Fractions collected were monitored using mobile phase ethyl acetate-toluene (9:1).

Fraction 8, 9,10,11,12,13 and 14 showed the presence of antioxidant by giving yellow color spots with purple background. Since fraction 8 and 9 gave the same antioxidant spots, both of these fraction were combined and monitored again. In the other hand fraction 10, 11, 12, 13 and 14 were combined and monitored again.

Combination of F8-F9 showed one antioxidant spot, while F10-F14 showed three antioxidant spots. F8-F9 showed better separation compared with combination of fraction F10-F14. Combination of fraction F8-F9 was purified using preparative thin layer chromatography.

After spraying with DPPH solution, band X showed positive antioxidant activity with yellow color. The scrapped band was extracted in methanol and filtered to remove the silica. The filtrate was concentrated. Purity test by single development thin layer chromatography using three different solvent system were chloroform-ethyl acetate (6:4), *n*-hexane-ethyl acetate (3:7), chloroform-methanol (4:6), showed one spot. Followed by this, band X is called as isolate N.

The isolate N was sprayed with citriboric acid, aluminium (III) chloride and ferrum (III) chloride. It gives positive reaction with 3 spray reagents indicated the presence of phenolic compound and flavonoid group.

Characterization of the isolate N was performed using UV-Vis spectrophotometry giving maximum absorbances at λ 253 nm and λ 311 nm in methanol which showed UV-Vis spectrum of flavonoid group. Band I at λ 311 nm showed that the isolate N belongs to flavone group. Isolate N under UV λ 366 nm gave deep purple color, this indicated that the isolate N has OH at C5. The result of 2 dimensional paper chromatography showed

that position of isolate N at the bottom left, it was indicated that isolate N was aglycone flavone group.

Characterization of isolate N was also carried out by spectrophotometry UV-visible using shift reagent.

Based on UV-Vis spectrum using shift reagents, it emitted a deep purple color under UV λ 366 nm, which indicated that isolate N was supposed to be a aglycone flavone that has OH at C-4', C-5 and C-7.

CONCLUSION

Antioxidant compound N was isolated from ethyl acetate extract. Antioxidant compound N was supposed to be a flavone aglycone that has OH at C-4', C-5 and C-7.

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