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Quantitative Analysis of Bioactive Compounds in Extract and Fraction of Star Fruit (*Averrhoa carambola* L.) Leaves using High Performance Liquid Chromatography

Analisis Kuantitatif Senyawa Bioaktif dalam Ekstrak dan Fraksi Daun Belimbing Manis (*Averrhoa carambola* L.) dengan Metode Kromatografi Cair Kinerja Tinggi

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Abstract

Star fruit (*Averrhoa carambola* L.) is potential as raw material for medicine, native in tropic areas, including Indonesia. According to other study report, star fruit leaves containing flavonoids apigenin and quercetin as potential anti-inflammatory and anticancer agents. The raw material for the drug in Indonesia mostly obtained through imports from other countries. In order to support the independence of traditional medicine raw materials, it is important to standardize the quality of traditional medicine raw materials, in this case is star fruit leaves by High Performance Liquid Chromatography (HPLC) method. The sample used is star fruit leaves extract obtained from maceration process using ethanol 70%; water fraction, ethyl acetate and hexane fractions obtained from fractionation process of the ethanolic extract. Physical parameters analyzed in sample include appearance, color, odor, taste, extract yield, water content, loss of drying, total ash content, residual solvent. Chemical parameters analyzed include apigenin and quercetin contents. The results shows that star fruit leaves used in this study meet the standards of Indonesian Herbal Pharmacopoeia with highest apigenin (6.37%) and quercetin (4.49%) content are in ethyl acetate fraction.
Keywords: Apigenin; Quercetin; *Averrhoa carambola* L; HPLC

Abstrak

Belimbing manis (Averrhoa carambola L.) merupakan tumbuhan yang potensial sebagai bahan baku obat, tumbuhan asli di daerah tropis termasuk Indonesia. Berdasarkan laporan pada penelitian-penelitian sebelumnya, daun belimbing manis mengandung senyawa flavonoid apigenin and quercetin yang potensial sebagai anti-inflamasi dan antikanker. Bahan baku obat selama ini banyak diperoleh melalui impor dari negara lain. Dalam rangka mendukung program kemandirian bahan baku obat, maka penting untuk dilakukan standarisasi kualitas bahan baku obat produksi dalam negeri dalam hal ini adalah daun belimbing manis, salah satunya dengan analisa menggunakan metode Kromatografi Cair Kinerja Tinggi (KCKT). Sampel yang digunakan adalah ekstrak daun belimbing manis dari proses maserasi menggunakan etanol 70% serta fraksi air, etil asetat dan heksana dari proses fraksinasi ekstrak etanol. Parameter fisik yang dianalisa pada sampel meliputi wujud, warna, bau, rasa, jumlah rendemen ekstrak, kadar air, susut pengeringan, kadar abu total, residu pelarut. Parameter kimia yang dianalisa meliputi kadar apigenin dan quercetin. Hasil analisa menunjukkan bahwa bahan baku daun belimbing manis dalam penelitian ini memenuhi standar Farmakope Herbal Indonesia dengan kandungan apigenin (6.37%) dan quercetin (4.49%) tertinggi berada dalam fraksi etil asetat.
Kata kunci: Apigenin; Kuersetin; Averrhoa carambola L; KCKT

INTRODUCTION

Averrhoa carambola L., also known as star fruit is a plant that found in tropic areas, including Indonesia. Mostly people take advantage of the fruit to be consumed, but the leaves have a lot benefit. Star fruit leaves useful as an anti-inflammatory, appetite enhancer, laxative urine (diuretic), antidiarrheal, and used traditionally as an external medicine to reduce fever and treat eczema.[1,2]

Star fruit leaves contain major flavonoid compound such as apigenin and quercetin. Other compound identified in star fruit leaves are amarin, rutin, saponin and tannin. According to Cabrini *et.al* report, apigenin compounds in star fruit leaves extracts and fractions can inhibit the formation of edema (redness) on ear skin of mice and effectively inhibit the migration of leukocytes to inflammatory.[3,4] Quercetin-3-O-rhamnoside compound isolated from star fruit extract has high antioxidant activity (5.19 µg/mL) and potential as anticancer.[5]

During this time, mostly raw materials of drugs in Indonesia is obtained from another country by import process. In order to support government programs, especially for independence of traditional medicine raw materials, it is important to standardize the raw materials quality. An option to determine the quality of raw materials is by conduct a quantitative analysis to asses bioactive compounds of the traditional medicine.

Quantitative analysis as determination of the active compounds using high performance liquid chromatography (HPLC) is better than using a spectrophotometer or densitometer Thin Layer Chromatography (TLC). HPLC has advantage in more sensitive, specific and requires less sample. According to the benefits of star fruit leaves extracts and fractions as potential raw material for traditional medicine, therefore, needs a study to determine the levels of apigenin and quercetin as bioactive compounds in star fruit leaves extracts and fractions as part of efforts to guarantee product quality.

METHODS

This study was an experimental laboratory, conducted in Pharmacy laboratory, Center for Biomedical and Basic Technology of Health, National Institute of Health Research and Development-Ministry of Health Republic of Indonesia in 2015.

Extraction and fractionation

The leaves of *Averrhoa carambola L*. was obtained from Kelapa Dua, Depok District, West Java. It was authenticated by Center for Plant Conservation Botanic Gardens, Indonesian Institute of Sciences, number 628/IPH.3/KS/XI/2015.

Simplisia powder of star fruit leaves was extracted using ethanol 70% by maceration method, with ratio of powder : solvent (1:10). The maceration process begin with soaking simplisia during the first 6 hours while stirring occasionally and then allowed to stand for 18 hours. Maserat evaporation with a rotary vacuum evaporator is conduct to reduce the solvent until reach the sticky extract. The obtained extract yield is calculated and characterized organoleptic, moisture content, drying shrinkage, total ash content and residual solvent.[6]

Fractionation process of extract to purify the compounds is carried out using n-hexane, ethyl acetate and water. The extract was grinded into powder, suspended in n-hexane and homogenized using sonicator for 10 minutes. The suspension was filtered using filter paper. The filtrat evaporated using rotary evaporator until viscous extract is gained., and then dried using vacuum oven at 40-50 °C until a fixed weight is obtained. The residue then dissolved in ethyl acetate and homogenized using sonicator for 10 minutes. After that, the solution was partitioned by adding distilled water, then shaken in a separator funnel and allowed to stand for 30-60 minutes until two layers were formed (layer of ethyl acetate on top and a layer of distilled water at the bottom). Both layer formed is then separated. Ethyl acetate and water layer is evaporated using rotary evaporator until viscous extract is gained. Furthermore, viscous extract is evaporated in

a fume hood, and then dried using vacuum oven at 40-50 °C until a fixed weight is obtained.[7,8]

Sample preparation

5 mg of each *Averrhoa carambola L*. leaves extract and fraction was accurately weighed, dissolving with methanol, and then transferred into 5 mL volumetric flask, sonicated for ten minutes and filtered through syringe filter 0,45 µL. Filtrate was further used as sample solution for the assay experiment.

Preparation of apigenin and quercetin standard

The standard solution of apigenin and quercetin were prepared by dissolving 5.0 mg of each apigenin and quercetin analytical standard (Sigma-Aldrich) using methanol into 5.0 mL volumetric flask until volume of solution reach 5 mL, followed by sonicating for ten minutes. Prepared a series solution by aliquots 50 µL, 100 µL, 200 µL, 300 µL, 400 µL and 500 µL of each standard solution into 5.0 mL volumetric flask and diluted with methanol to obtain a concentration range of 10-100 ppm respectively.

Quantitative analysis of apigenin in extract and fraction using high performance liquid chromatography (HPLC)

Apigenin standard calibration curve was done by plotting six concentrations, i.e. 10; 20; 40; 60; 80 and 100 ppm. Standards solution and samples (extract and fraction) were analyzed using HPLC (Waters), with 4.6 × 150 mm Sun Fire C18 column size, 1.0 mL/min flow rate, 20 µL injection volume and Photodiode Array (PDA) detector at 340 nm. The mobile phase used isocratic system, comprising of acetonitrile and distilled water (45:55, v/v).[9]

Quantitative analysis of quercetin in extract and fraction using high performance liquid chromatography (hplc)

Quercetin standard calibration curve was done by plotting six concentrations, i.e. 10; 20; 40; 60; 80 and 100 ppm. Standards solution and samples (extract and fraction) were analyzed using HPLC (Waters), with 4.6 × 150 mm Sun Fire C18 column size, 1.0 mL/min flow rate, 20 µL injection volume and Photodiode Array (PDA) detector at 273 nm. The mobile phase used isocratic system, comprising of acetonitrile:water:acetic acid (10:90:0.2, v/v).[10]

RESULT AND DISCUSSION

Extraction process used 2000 g of star fruit leaves simplisia powder. The obtained crude extract respectively 388.42 g. The yield of obtained extract is 19.42%. The characterization results showed in Table 1 proved that the extract met all requirement parameters in The Indonesian Herbal Pharmacopoeia.[6]

Table 1. Characteristics of *Averrhoa carambola* L leaves extract

Characteristics	Results	Requirements
Appearance	Solid, powder	Solid, powder
Color	Green	Green
Odor	Specific star fruit	Specific star fruit
Taste	Slightly astringent	Slightly astringent
Yield of extract	23.5 %	≥ 50 %
Water content	0.84 %	≤ 10 %
Loss on drying	1.26 %	≤ 14 %
Total ash content	0.42 %	≤ 0.5 %
Residual solvent	0 %	≤ 0.5 %

HPLC spectrum of apigenin compounds in the extract, ethyl acetate fraction and water fraction showed in Figure 1 has a retention time 4.055 minutes with a mobile phase acetonitrile:water (45:55, v/v) is similar with retention time of apigenin standard. Hexane fraction did not showed any apigenin spectrum.

HPLC spectrum of quercetin only appears in extract and ethyl acetate fraction (showed in Figure 2) has a retention time 6.116 minutes with a mobile phase acetonitrile: water:acetic acid (10:90:0.2, v/v)

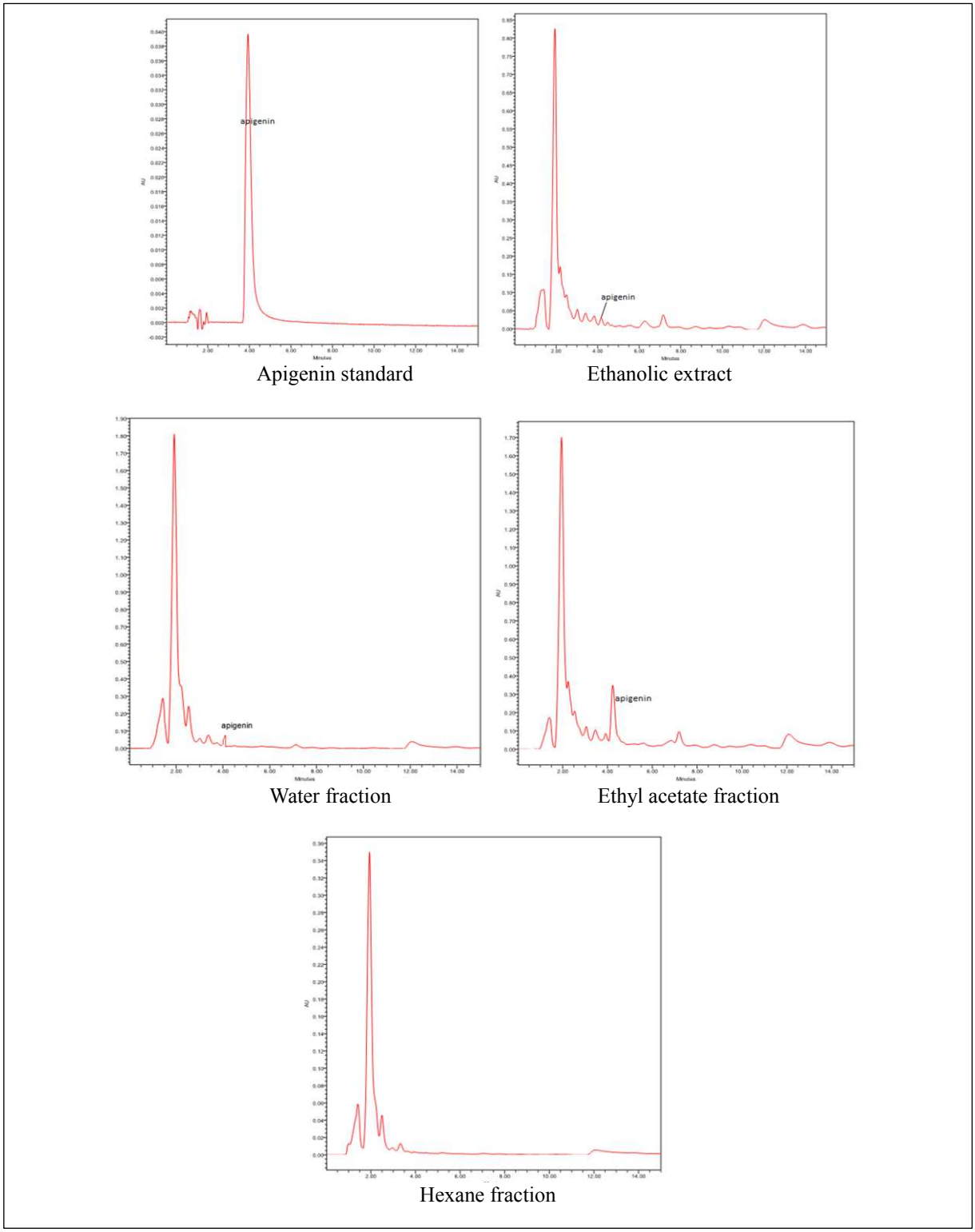

Figure 1. Spectrum chromatogram of apigenin in standard, extract and fraction of *Averrhoa carambola* L. leaves

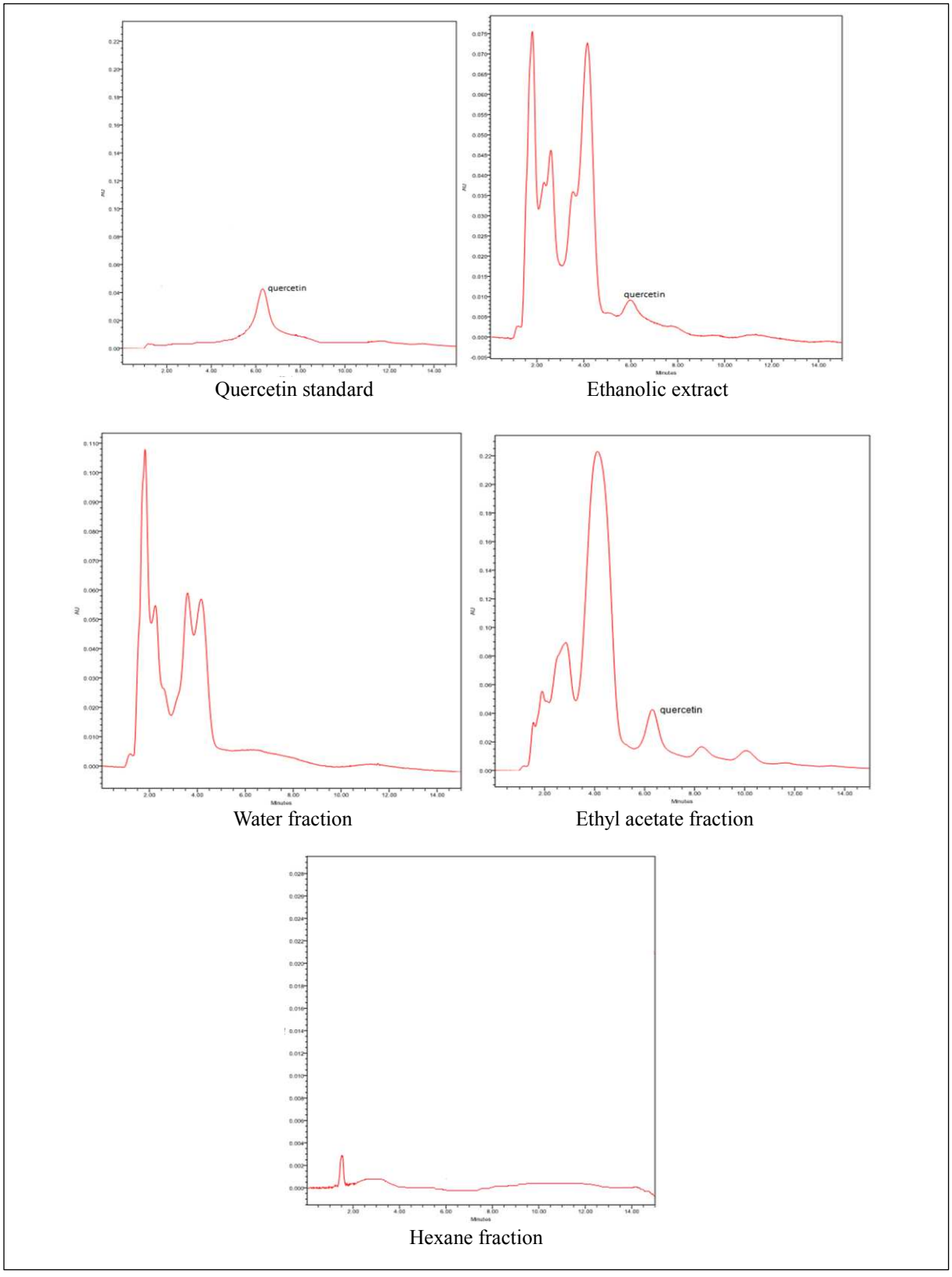

Figure 2. Spectrum chromatogram of quercetin in standard, extract and fraction of *Averrhoa carambola* L. leaves

Table 2. Analysis quantitative of apigenin and quercetin in *Averrhoa carambola* L. extract and fraction

Sample	Concentration of apigenin (%)	Concentration of quercetin (%)
Ethanolic extract	0.48	0.83
Water fraction	0.02	0.00
Ethyl acetate fraction	6.37	4.49
Hexane fraction	0.00	0.00

is similar with retention time of quercetin standard. While in water and hexane fraction HPLC spectra did not showed any quercetin.

The results of apigenin and quercetin assay are shown in Table 2. The highest content of apigenin 6.37% was found in ethyl acetate fraction. While in hexane fraction did not contain apigenin compound, which is proved by zero apigenin levels and in HPLC spectrum did not showed any apigenin spectrum. The highest quercetin level 4.49% was found in ethyl acetate fraction. While in water and hexane fraction did not contain any quercetin compound, it is proved by zero quercetin level and in HPLC spectra of that two fractions did not showed any quercetin.

Characterization of the extract were conducted to determine the physical quality of the obtained extract. Determination of water content in the extract was aimed to measure water level in star fruit leaves extract. The obtained extract has water level 0.84%, which means meets the Herbal Pharmacopoeia requirements (< 10%).[6] The less water content in material ingredients can reduce the risk of microbial growth, fungus or damage caused by insects.[11] Loss of drying examination aims to determine how many loss of components (water and volatile compounds) when done heating at 105^{o}C.

Loss of drying examination results of star fruit leaves extract 1.26%. This loss of drying level is greater than the water content level, which means in addition to the water component there is also a loss of volatile component such as butyl acetate, ethyl decanoate and hexadecanoic acid. Determination of total ash level aims to determine the amount of remaining material after incineration (at 700 ^{o}C). The obtained total ash content 0.42% in accordance with the requirements in Herbal Pharmacopoeia which is less than 0.5%. The small ash content indicates that the remaining material is less. The remaining materials include physiological ash from plant tissue itself and non-physiological ash which is a residue of other materials that adhere to the surface of plants such as sand and soil, so the smaller the ash content means the smaller the impurity in obtained fraction.[11,12]

To calculate of apigenin and quercetin levels in the star fruit leaves extracts and fractions firstly created the calibration curve equation of apigenin and quercetin standard. Calibration curve equation is the relationship between the x-axis and y-axis. A series concentration expressed in the x-axis, while the obtained peak area of chromatograms from the measurement result is expressed as the y-axis. In this method, the construction of apigenin and quercetin standard calibration curve is done by connecting the six-point concentration at 10; 20; 40; 60; 80 and 100 ppm. From the results of measurements and calculations using HPLC, apigenin calibration curve equation is y = 55829x + 127791 with a correlation coefficient (r) 0.9992, while for quercetin calibration curve equation is y = 46018x + 4751 with a correlation coefficient (r) 0.9995. The obtained correlation coefficient are close to 1, states increasingly linear relationship between concentration and peak area of chromatogram. According to Association of Official Analytical Chemists (AOAC) these values meet the specified requirements, ie 0.9990. High correlation coefficient values showed a linear relation-

ship between the measured signal detector and the amount of active compound.[13,14]

In apigenin compound examination, the generated spectrum chromatograms showed that apigenin is detected at wavelength 340 nm with retention time 4.055 minutes (Figure 2), whereas the spectrum chromatogram of quercetin compound is detected at wavelength 273 nm with retention time 6.116 minutes (Figure 3). The content of apigenin and quercetin in star fruit leaves match with a research conducted by Cabrini et.al.[4] The highest apigenin and quercetin levels were found in ethyl acetate fraction 6.37% and 4.49%, while the content of apigenin and quercetin in ethanol extract 0.48% and 0.83% (Table 2). Apigenin and quercetin levels in ethyl acetate fractions and ethanol extract of star fruit leaves in this study were greater than in the star fruit flesh extracts. The research conducted by Khanam et al., the highest content in the ethanol extract of star fruit flesh is luteolin 11.40%, while apigenin and quercetin content were only 0.36% and 0.37%.[2] This good correlation confirms the data presented by Moresco et.al, which apigenin-6-C-β-L-fucopyranoside and apigenin-6-C-(2"-O-α-Lrhamnopyranosyl)-β-L-fucopyranoside isolated from the ethyl acetate fraction.[15] According to these results showed that there are differences of apigenin and quercetin level in star fruit leaves and flesh. The utilization of extract/fractions of star fruit leaves or flesh depends on the medication therapy to be administered. Apigenin is a highly potential flavonoid as anti-inflammatory and has a vasodilatory effect. Quercetin is a flavonoid that is proven as antioxidant and anticancer.[16,17,18]

CONCLUSION

Quantitative analysis of *Averrhoa carambola* L. leaves using HPLC showed it as a good source of flavonoid, especially ethyl acetate fraction which is containing the highest number of apigenin and quercetin.

Validation methods of apigenin and quercetin analytical assay in star fruit leaves is necessary to conduct for further.

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