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# Determination of Flavonoid Content from Ethanol Extract Srikaya Leaf (*Annona Squamosa* L.) Using High Performance Liquid Chromatography (HPLC) Method

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**ABSTRACT**

Research on the determination of flavonoid levels from the ethanol extract of sugar apple (*Annona squamosa* L.) leaves using high performance liquid chromatography (HPLC) has been carried out. The srikaya leaf simplicia was dried and mashed and extracted by maceration with 70% ethanol solvent and then evaporated with a rotary evaporator to obtain a thick extract. Qualitative test of flavonoids from ethanol extract of srikaya leaves using a color test with Mg and HCl powder reagents produced a red-orange color and using the thin layer chromatograph method (TLC) obtained an Rf value of 0.7. The determination of the maximum absorption wavelength of quercetin was 371 nm. Optimization of the mobile phase used methanol:aquadest (65:35).

**Keyword :**Quercetin; Srikaya Leaf; Flavonoids; HPLC

## PRELIMINARY

Indonesia is famous for its natural wealth which has various types of plants that are efficacious as medicine. Traditional medicine has been known and used for generations by Indonesian people. The use of traditional medicine is generally preferred to maintain health, although its use is also intended as a treatment for a disease (Suharmiati et al., 2003).

Sugar apple plant (Annona squamosa L.) is used as a medicinal plant (Erlina et al., 2018). In general, all parts of the srikaya plant can be used for health. The results of the study stated that the pharmacological effects of srikaya leaves include antibacterial, antioxidant, anti-cholesterol, antipyretic, and anthelmintic. The groups of compounds that are thought to have pharmacological effects on srikaya leaves are alkaloids, terpenoids, flavonoids, phenolics, sitosterol, phytosterols, and tannins (Elora et al., 2022).

The content of secondary metabolites in srikaya is glycosides, alkaloids, saponins, flavonoids, tannins, phenolic compounds and phytosterols (Werdiningsih & Zahro, 2020). Like other secondary metabolites, flavonoids have various activities, including having antiviral, anticancer, anti-inflammatory, antioxidant, antihepatotic and antidiabetic effects.Erlina*et*al., 2018).

Research conducted by Mubarokah et al., (2005) showed that srikaya leaf infusion contains flavonoids which have an antioxidant activity of 53.23%. Research conducted by Kusmardiyani et al., 2012 showed that the phytochemical screening of two srikaya leaf simplicia from different places of growth showed that they contained flavonoids, tannins and terpenoids. Simplicia was extracted by refluxing with different solubility increases.

In a study conducted by Werdiningsih & Zahro (2020), the flavonoid content of sikaya leaf extract was determined using the maceration method containing a flavonoid compound of 0.00317 µg QE/mg using the UV-Vis Spectrophotometry method. In a study conducted by Ayantini et al., (2020) fermented srikaya leaf extract contained flavonoid compounds with the highest content of flavonoid compounds at 72 hours of 9.227 ± 0.243 mg QE/g using the UV-Vis Spectrophotometry method.

Determination of flavonoid content can be done by several methods, including: Spectrophotometry and High Performance Liquid Chromatography(HPLC).High-performance liquid chromatography is the latest development of classic column liquid chromatography, more sensitive and sensitive detectors and technological advances in high-pressure pumps that make HPLC a method with a fast and efficient substance separation system (Johnson, 1991). High performance liquid chromatography has several advantages, including relatively short analysis time, small sample volumes used, can analyze organic and inorganic compounds, and reusable columns (Ardianingsih, 2009).

## METHOD

**Tools and Materials**

The tools used in this study were a set of High Performance Liquid Chromatography ((HPLC)) (Shimadzu®), ODS column 4.6 x 250 mm (ShimPack®), UV lamp, TLC plate silica gel 60 F254 (Merck® ), rotary evaporator (Heidolph®), analytical balance (Precisa®), glassware (Pyrex®) and other laboratory tools that support research. Plant materials used in this study were sugar apple leaves, ethanol (C2H5OH) 70% (Merck®), aquadest (H2O) (PT.Bratachem), methanol (CH3OH) (Merck®), ethanol (C2H5OH) 95% (Merck® ), Mg powder (Fision plc®), concentrated hydrochloric acid (HCl) (PT. Smart Lab), n-butanol ($C_4H_{10}O$) (PT. Smart Lab), acetic acid ($CH_3COOH$)(Merck®), quercetin (Sigma®).

**Work procedures**

The srikaya leaves used came from Aia Pacah Village, Koto Tangah District, Padang City, West Sumatra as much as 2 Kg. The matoa plant was identified in the Andalas University Herbarium (ANDA), Biology Department, FMIPA Andalas University, Padang, West Sumatra. The processing of dry simplicia goes through the stages of raw material collection, wet sorting, washing, chopping, drying, dry sorting, and storage (Ministry of Health of the Republic of Indonesia, 1985).

**Preparation of ethanol extract of srikaya leaves**

The ethanol extract of srikaya leaves was prepared by maceration method. Maceration was carried out by soaking 100 grams of simplicia in 1000 mL of 70% ethanol in a vessel, then stirring. The vessel was covered using aluminum foil and left for 1 day while occasionally stirring. After 1 day, the extract was filtered using a funnel to produce the first filtrate and dregs of srikaya leaf extract. The remaining dregs were added with 1000 mL of 70% ethanol solution and covered with aluminum foil, then left for 1 day while occasionally stirring. After 1 day, the extract was filtered using a funnel to produce the second and third filtrate and dregs of srikaya leaf extract. The first, second and third filtrates were mixed together, then concentrated using a rotary evaporator to obtain a thick extract of srikaya leaves.

***Qualitative analysisflavonoid compounds***

**Color reaction**

The test was carried out by means of 0.5 mg sample of ethanol extract of srikaya leaves, then dissolved with 2 mL of ethanol. Then add 0.1 gram of Mg metal and 3 drops of concentrated HCl. If an orange to red solution is formed, then it is positive for flavonoids (Erlina et al., 2018).

**Chromatogram pattern (TLC)**

In the separation with analytical TLC used silica plate GF254which has been activated by heating in an oven at 100°C for 10 minutes, then spot the extraction results and reference standard quercetin at a distance of 1.5 cm from the bottom edge of the plate with a capillary tube then dried and eluted with the mobile phase n-butanol : acetic acid : aqua (BAA) (4:1:5). After the movement of the developer solution reaches the boundary line, the elution is stopped. The stain formed was examined with a UV lamp at a wavelength of 254 nm and then the Rf value was measured(Hanani, 2014).

***Quantitative analysis of flavonoid compounds***

**Preparation of Quercetin Master Solution and Standard Raw Series**

Weigh 10 mg of quercetin carefully then put it into a 100 mL volumetric flask, then dissolve it with 96% ethanol to obtain a concentration of 100 µg/mL.A quercetin series solution was prepared withconcentrations of 10, 20, 30, 40 and 50 µg/mL. Pipette 1, 2, 3, 4, and 5 mL of quercetin standard standard mother liquor (100 µg/mL) into a 10 mL volumetric flask, then add to the mark with 96% ethanol (Rahmawati et al., 2019 ).

**Determination of the maximum absorption wavelength of quercetin**

Serial solution with a concentration of 50 µg/mLdiuMaximum absorption curve using a UV spectrophotometer at a maximum wavelength of 200-400 nm and obtained the wavelength of quercetin (Asmorowati & Novena, 2019).

**Mobile Phase Optimization**

The mobile phase was optimized by means of a series concentration solution of 50 µg/mL in the amount of 20 µL injected into a high performance liquid chromatography system (HPLC) with a certain flow rate and wavelength using methanol: aquadest as the mobile phase. Optimization was carried out 3 times with different ratios of mobile phase, namely with a ratio of 75:25, 80:20 and 65:35. The optimum mobile phase was obtained with a ratio of methanol:aquadest (65 : 35) v/v (Rahmawati et al., 2019).

**Determination of levels of flavonoids**

Samples of ethanol extract of srikaya leaves (Annona squamosa L.) were weighed 10 mg and then put into a 100 mL flask, added with 96% ethanol then ethanol extract of srikaya leaves was sonicated for 15 minutes and filtered to obtain a concentration of 100 µg/mL. The ethanol extract of srikaya leaves was then analyzed by High Performance Liquid Chromatography (HPLC) by injecting 20 µL of the sample into HPLC for analysis using the mobile phase of methanol: aquadest (65:35), measuring three repetitions and calculating the flavonoid content using linear regression equation (Rahmawati et al., 2019; Wahid, 2020).

## RESULTS AND DISCUSSION

The plant samples used in this study were dried sugar apple (Annona squamosa L.) leaves. The extraction method used is the maceration method. The choice of this extraction method is because it is a simple, easy method, and without going through a heating process, so that the possibility of damage to the components of chemical compounds can be minimized, maceration is also used both for small scale and industrial scale. The

extraction process was carried out by immersing the simplicia powder in 70% ethanol solvent at room temperature in a tightly closed container. The filter liquid will penetrate the cell wall and enter the cell cavity containing the active substance, the active substance will dissolve and because there is a difference in concentration between the active substance solution and what is outside the cell, the concentrated solution is forced out.

The solvent used in this study was 70% ethanol to extract flavonoid compounds. The choice of solvent is due to the fact that flavonoid compounds are generally in the form of polar glycosides, so they must be dissolved in polar solvents. 70% ethanol has the ability to extract compounds over a wide polarity range from polar to nonpolar compounds, is not toxic compared to other organic solvents, is easier to evaporate with water, is not easy for microbes to grow and is relatively inexpensive (Saifuddin et al., 2011).

The yield of ethanol extract of srikaya leaves obtained was10.2122grams with a percent reduction of10.2122%.The yield of the extract is calculated by comparing the weight of the viscous extract obtained to the amount of simplicia powder used in the extraction process. The yield uses units of percent (%), the higher the yield value produced indicates the number of bioactive components contained in it (Maryam et al., 2020).

Preliminary tests on the extract were carried out to provide an overview of the class of compounds contained in the ethanol extract of srikaya leaves. The addition of Mg and HCl powders to the identification of flavonoid compounds aims to reduce the benzopiron nuclei present in the flavonoid structure resulting in an orange or red color change. The addition of HCl resulted in an oxidation-reduction reaction between Mg metal as a reducing agent and flavonoid compounds. The results obtained from the chemical content test showed that the ethanol extract of srikaya leaves contained flavonoids. The reactions that occur between flavonoid compounds and Mg and HCl metals can be seen in Figure 1.

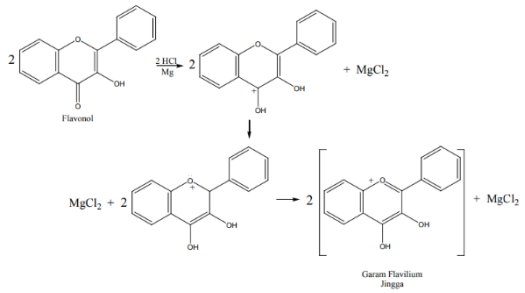


**Image 1**. Flavonoid reaction with metal Mg and HCl (Ergina et al., 2014)

Preliminary tests on the extract were carried out to provide an overview of the class of compounds contained in the ethanol extract of matoa leaves. The group of compounds to be identified is the class of flavonoids which was carried out by adding HCl and Mg powder.

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Figure 2. Test results for the ethanol extract of srikaya leaves.

The TLC test aims to provide an initial description of the composition of the chemical constituents based on the chromatogram pattern (Ministry of Health of the Republic of Indonesia, 2000). Analysis using TLC is a separation of chemical components based on the adsorption and partition principles determined by the stationary phase (adsorbent) and the mobile phase (eluent). This test is carried out by first oriented the eluent with different polarity levels to obtain a solvent that is able to provide good separation and good dye spots. A good eluent is an eluent that can separate compounds in large quantities marked by the appearance of spots. The spots that form are tailless and the distance between one spot and the other is clear. The eluent used in the TLC was n-butanol : acetic acid : water (BAA) with a ratio of 4:1:5. The presence of spots on the extract with qualitatively different Rf values indicates the presence of several compounds contained in the extract. A good Rf value ranges from 0.2 - 0.8. When the TLC plate is detected in a 254 nm UV lamp, the TLC plate fluoresces and the spots will be dark in color. The appearance of the spots on the 254 nm

UV lamp is due to the interaction between the UV lamp and the chromophore groups bound by the ausochrome in the spots (Hanani, 2016). The results

of the spots seen in a UV lamp of 254 nm showed that the sample (ethanol extract of srikaya leaves) had an Rf value of 0.7 and an Rf value of the comparator (quercetin) 0.7. When the TLC plate is detected in a 254 nm UV lamp, the TLC plate fluoresces and the spots will be dark in color. The appearance of the spots on the 254 nm UV lamp is due to the interaction between the UV lamp and the chromophore groups bound by the ausochrome in the spots (Hanani, 2016). The results of the spots seen in a UV lamp of 254 nm showed that the sample (ethanol extract of srikaya leaves) had an Rf value of 0.7 and an Rf value of the comparator (quercetin) 0.7. When the TLC plate is detected in a 254 nm UV lamp, the TLC plate fluoresces and the spots will be dark in color. The appearance of the spots on the 254 nm UV lamp is due to the interaction between the UV lamp and the chromophore groups bound by the ausochrome in the spots (Hanani, 2016). The results of the spots seen in a UV lamp of 254 nm showed that the sample (ethanol extract of srikaya leaves) had an Rf value of 0.7 and an Rf value of the comparator (quercetin) 0.7.

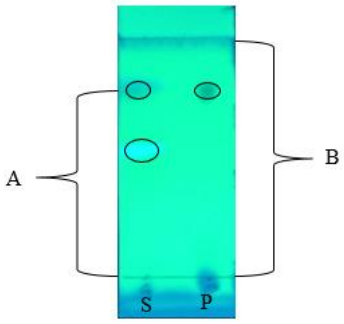


Figure 3. Chromatogram pattern of ethanol extract of srikaya leaves and quercetin.

Measurement of flavonoid levels in ethanol extract of srikaya leaves with a wavelength of 371 nm. Flavonoids were calculated as quercetin using the linear regression equation of the previously measured quercetin calibration curve. Quantitative analysis of the ethanol extract of srikaya leaves was carried out to determine the maximum wavelength of the quercetin standard using a spectrophotometer. The quercetin test solution with a concentration of 50 µg/mL is put into a cuvette that has been rinsed with a blank solution and then put into the spectrophotometer, where the clear part must point to the radiation path that passes it so that the radiation can be transmitted perfectly. The spectrophotometer is running at a wavelength of 200-400 nm. From the results of the absorbance reading, the maximum wavelength is 371 nm with an absorbance of 0.794.

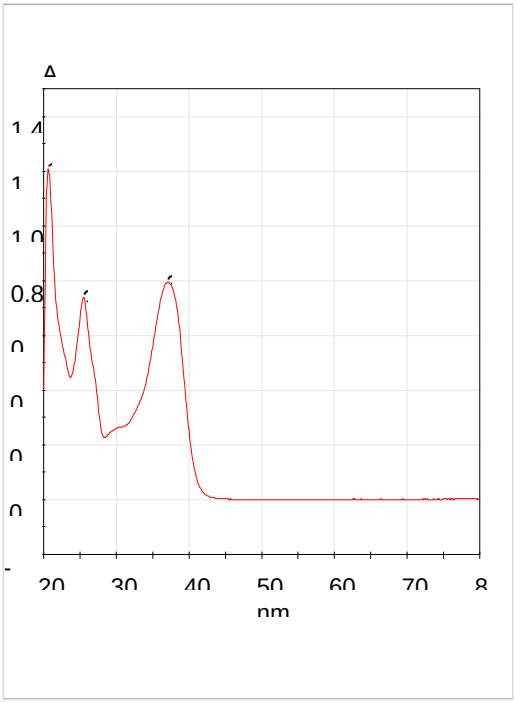


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**Figure 4**. Quercetin Maximum Wavelength

The mobile phase was optimized by injecting a 50 µg/mL quercetin solution into the HPLC injector with an injection volume of 20 µL. Set the measurement with a wavelength of 371 nm and a flow rate of 1 mL/minute, the mobile phase will be pushed by a pump past the injector where the

sample is injected until it reaches the column, separation will occur in the column which will be read by the detector and then data will be obtained in the form of a chromatogram showing the time retention and area under the curve (AUC) of quercetin. The experiment was carried out three repetitions with the mobile phase methanol:aquabidest based on the difference in comparison between the two, namely 75:25, 65:35, and 80:20 and the optimum conditions were obtained at a ratio of 63:35 with AUC 5129722 and retention time of 5,607 minutes.

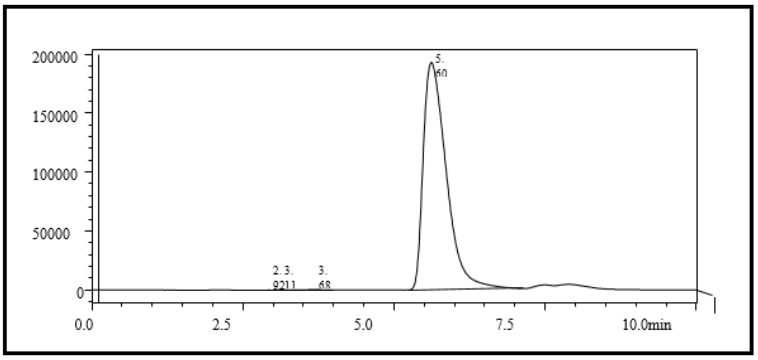


**Figure 5**. Mobile Phase Optimization (65:35)

The column used in this study was an ODS type of 4.6 x 250 mm which has an octadecyl silica (ODS) group which is capable of separating compounds with low to high polarity. The mobile phase used was methanol: aquabidest with a polar ratio of 65:35. The combination of methanol – aquadest is a universal solvent that can elute polar compounds.

The calibration curve was used with concentrations of 10, 20, 30, 40 and 50 µg/mL using the mobile phase of Menol: Aquadest with a ratio of 65:35, the injection volume was 20 µL, the maximum wavelength was 371 nm and the flow rate was 1 mL/minute, the results were in the first iteration, AUC 956906, 1961597, 2979145, 3971163 and 5119876 were obtained with the linear regression equation, namely y = 103355.06x-102914.4. The results obtained from standard curve measurements, namely the higher the concentration, the higher the AUC value, this shows a straight relationship between AUC and retention time.

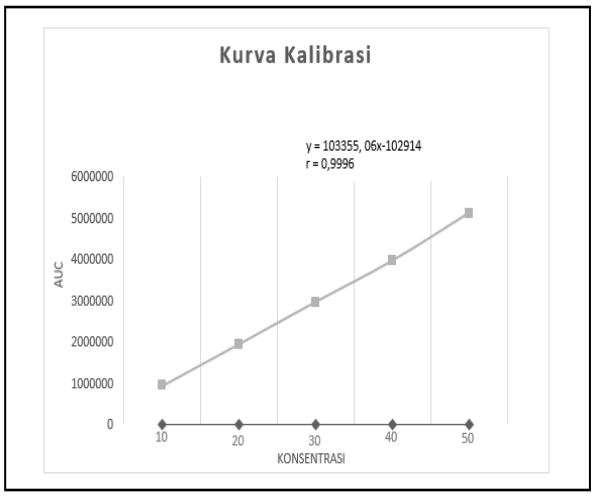


**Figure 6**. Calibration Curve

Determination of the levels of flavonoid compounds in the ethanol extract of srikaya leaves with three repetitions of the experiment will obtain data in the form of a chromatogram. In the first repetition, AUC 1776407 was obtained, in the second repetition, AUC 1742674 was obtained, and in the third repetition, AUC 1763174 was obtained, then the data obtained was processed using a linear regression equation which has a correlation coefficient (r) value of 0.9996 which is closest to 1. The r value is close to 1 indicates that the regression equation is linear, so it can be said that AUC and retention time have a very strong correlation. So that after processing, the levels of flavonoids from the ethanol extract of srikaya leaves with an average percentage of 18.095%, a standard deviation of 0.1644 and a relative standard deviation (RSD) of 9.

## CONCLUSION

From the results of the research that has been done, it can be concluded that the ethanol extract of srikaya leaves (Annona squamosa L.) positively contains flavonoid compounds. Flavonoid levels in the ethanol extract of srikaya leaves using the HPLC method obtained an average percentage value of 18.095%, a standard deviation of 0.1644 and a relative standard deviation (RSD) of 9.085%.

**SUGGESTION**

It is suggested to further researchers to use other methods and use different solvents from this study.

**BIBLIOGRAPHY**


Ardianingsih, R. (2009). The Use of High Performance Liquid Chromatography (HPLC) in the Ion Detection Analysis Process. EIGHT. Aerospace News 10 :101-104.

Aryantini, D., Fita Sari & Citra RW (2020). Total Phenolic and Flavonoid Content in Fermented Srikaya (Annona Squamosa L.) Leaf Extract. Pharmascience, 7(2)

Asmorowati, H & Novena Yety Lindawati. (2019). Determination of total flavonoid content of avocado (Persea americana Mill.) by spectrophotometric method. Pharmacy Scientific Journal 15(2) August-December 2019, 51-63 ISSN: 1693-8666

Backer, CA and B.v. D. Brink. (1963).*Flora of Java Vol. I.*NVP Noordhoff Groningen The Netherlands.

Dillah, A. (2015). Identification of Solid Interactions in the Quercetin-Nicotinamide Binary System. (Thesis). Padang : Faculty of Pharmacy, Andalas University.

Effendy. (2004). High Performance Liquid Chromatography in the Pharmaceutical Field. North Sumatra: FMIPA USU.

Elora, AC, Siti Hazar, Sri Peni Fitrianingsih. (2022). Literary Study of the Pharmacological Effects of Srikaya Leaves (Annona squamosa L.).*Bandung Conference Series: Pharmacy*, Vol. 2 No. 2 (2022), pp: 307-313. ISSN: 2828-2116

Erlina, D., Rosa Juwita H & Meylisa N. (2018). Comparison of Antibacterial Activity of Red & Green Srikaya Leaves Against S.aureus Bacteria. Proceedings of the 1st National Seminar on Science, Technology and Analysis

Ewing, GW (1985). Instrumental of Chemical Analysis Fifth Edition. Singapore: McGraw-Hill.

Ferdinand, A & FS Rizki. (2021). Isolation and Identification of Flavonoid Compounds from the Ethanol Extract of Forest Pandan, a New Type of Freycinetia Sessiliflora. Indonesian Pharmacist Journal, 4(1) May 2021 (1-6) P- ISSN 2621-3184 ; E-ISSN 2621-4032

Gandjar, IG & Rohman, A. (2007). Analytical Pharmaceutical Chemistry. Yogyakarta : Student Library.

Gritter, RJ, et al., (1991). Introduction to Chromatography Edition II. Translator: Kosasih Padmawinata. Bandung: ITB Press.

Hanani, E. (2014). Phytochemical Analysis. Jakarta : EGC Medical Book.

Harmita. (2004). Instructions for Implementing Method Validation and Calculation Methods. Pharmaceutical Science Magazine, 1(3), 117-135.

Husnia, F. & Aqnes Budiarti. (2021). Development of Quercetin Analysis Method in Ethanol Extract of Leunca Fruit (Solanum nigrum L.) Using High Performance Liquid Chromatography. Pharmaceutical MediaISSN 0216-2083ISSN 2622-0962 (17 : 2)

Johnson, EL and Stevenson R., (1991). Basics of Liquid Chromatography. ITBBandung, Bandung.

Kusmardiyani, S. Ferlin Wandasari, Komar Ruslan Wirasutisna. (2012). Phytochemical Study of Srikaya Leaves (Annona squamosa L.) Derived from Two Growing Locations. Acta Pharmaceutical Indonesia, *Vol. XXXVII, No. 1, 2012*

Latifa, C., et al., (2007). Solubility of Flavanoids in Organic Solvents. Journal of Chemical and Engineering Data, 52(5), 1552-1556.

Maulana, M. (2018). Thin Layer Chromatography Profile of Bidara Arab Leaf Extract Based on Solvent Variations. (Thesis). Malang: Faculty of Science & Technology UIN Maulana Malik Ibrahim.

Markham, KR. (1988).*How to Identify Flavonoids*. ITB Press. Bandung.

Marko, V., Damjana, R., & Radovan, K. (2015). Effects of Flavonoids from Food and Dietary Supplements on Glial and Glioblastoma Multiforme Cells. Journal Molecules. ISSN 1420-3049.

Maryam, F., Burhanuddin, T., & Deby, PT (2020). Measurement of Specific and Non-Specific Parameters of Matoa Leaf Ethanol Extract (Allophyllus

cobbe (L.) Raeusch.) Journal of Mandala Pharmacon Indonesia, 6(1), 1-12.

Mubarokah, IZ, et al.,.(2005). Antioxidant Activity Test of Srikaya Leaf Infusion (Annona squamosa L). Proceedings of National Semester Research, Education and Application of MIPA, FMIPA-UNY, Yogyakarta 8 February 200

Raaman, N. (2006).*Phytochemical Techniques*. New India Publishing Agency, New Delhi. Pages: 19 – 20

Rahmawati et al., (2019). Optimization of Separation Conditions for Flavonoid Compounds from the Polar Fraction of Erythrina poeppigiana Using Preparative High Performance Liquid Chromatography (HPLC). Chemica et Natura Acta, 7(1), 27-36.

Robinson, T., 1995, High Plant Organic Compound Content, Sixth Edition, ITB Publisher: Bandung.

Saifuddin, A., Rahayu, V., & Teruna, HY (2011). Standardization of Natural Medicinal Ingredients. Yogyakarta: Graha Science.

Salmia. (2016). Analysis of Total Flavonoid Content of Bangkok Kedondong Stem Bark Extract (Spondias dulcis) with UV-Vis Spectrophotometry Method. (Thesis). Makasar : Alauddin State Islamic University Makassar.

Setiawati, W., et al., (2008). Plants Botanical Pesticides and Methods of Production for the Control of Plant Destruction Organisms (OPT). Prima Tani. Vegetable Crops Research Institute.

Smith, J, A et al., (2011). Cocrystals of Quercetin with Improved Solubility and Oral Bioavailability. Molecular Pharmaceutics, (8), 1867-1876.

Suharmiati MH. (2003). The Efficacy and Benefits of Dutch Teak, the Slimming and Cholesterol Laxative. Jakarta: AgroMedia Pustaka.

Van Steenis, CGGJ, (2003).*Flora*, pp. 233-236, PT Pradya Paramita, Jakarta.

Vonny. (2019). The Effect of Srikaya Leaf Extract (Annona squamosa L.) on the Growth Inhibition of E.Coli Bacteria. Medical School. University of Northern Sumatra. (thesis)

Wahid, RA, (2020).Qualitative and Quantitative Analysis of Flavonoids Extract of White Pomegranate Peel (Punica granatum L.) Using High Performance Liquid Chromatography Method. Indonesian Journal of Pharmacy and Natural Products,*Volume 03, Number 02 , September, 2020 p-ISSN : 2656-3215 e-ISSN : 2615-6903*

Werdiningsih, W & Assolychatu, Zahro. (2020). Determination of Flavonoid and Phenol Levels from Srikaya Leaves (Annona squamosa L.) and Antioxidant Activity. Wiyata Journal, Vol. 7 No. 2 of 2020