Total Anthocyanin Content and Identification of Anthocyanidin From *Plectranthus Scutellarioides* (L.) R. Br Leaves

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Abstract
Plectranthus scutellarioides (L.) R. Br plant is common in Indonesia which has leaves color of purplish red, and it may be due to presence of anthocyanin pigments. This research is aimed to determine the total anthocyanin content and to identify the type of anthocyanidin in Plectranthus scutellarioides leaves extract. The total anthocyanin content was determined by pH-differential method. Identification of anthocyanidin was done by maceration using ethanol acidified by concentrated HCl 0,5% followed by liquid-liquid extraction by n-hexane, ethyl acetate and water.

The purification of anthocyanin from water fraction was done with thin layer chromatography preparative using acetonitrile: water: formic acid (0,7 : 1,3 : 0,1) as eluent, and identification of the isolate was done using Spectrophotometry UV-VIS as well as IR. The result showed that the total anthocyanin content was 0,435 mg/g of wet weight of leaves calculated as cyanidine-3-glucoside. The maximum wavelengths of isolate are 274, 346 and 546 nm. FTIR analysis showed functional group such as -OH, C=C aromatic, C-O and C-H aromatic. Mass spectrometry showed mass compound of 287,0556 with formula $C_{15}H_{11}O_6$. Spectrum analysis showed existence of cyanidin.

Keywords: *Plectranthus scutellarioides* (L.) R. Br, anthocyanin, cyanidin.

Introduction

Most of plants in Indonesia have potential as a drug because of secondary metabolites.[18] One of the plants was reported to have a range of efficacy in the variety of treatment is *Plectranthus scutellarioides* (L.) R. Br. *Plectranthus scutellarioides* plants are believed to be efficacious for treating wounds, inflammation, diarrhea, coughs, hemorrhoids, sore eyes and as a medicine skin disease.

In the North Sulawesi, *Plectranthus scutellarioides* leaves combined with *Piper betle*, honey and egg yolks can be used as a therapy for malaria and enhancing the durability of the body.[10,15]

Secondary metabolites in *Plectranthus scutellarioides* are alkaloids, flavonoids, saponins and tannins and essential oil.[13] Anthocyanins is secondary metabolite derivatives of flavonoid which gave the red, purple, and blue pigments on the plants. Therefore, *Plectranthus scutellarioides* leaves are alleged to contain lot of anthocyanin because of the color of its leaves as purplish red. Anthocyanin have a conjugated double bonds arrangements, that is responsible for the appearance of color, so it is able to absorb light in the visible range. Anthocyanins may also act as antimicrobial, hepatoprotective, anticarcinogenic, antiinflammation and antidiabetic.[4,7]

Characterization of anthocyanin is an important thing to do. Anthocyanin may be have different structures in each plants. This difference depends on the content of the anthocyanidin, the sugar group or the acyl group attached to it.[3] Therefore, research on the total anthocyanin content and identification of anthocyanidin in *Plectranthus scutellarioides* leaves is needed.

Material and Methods

Materials used in this research were: acetonitrile (Merck), alumunium chloride, amyl alcohol, aquadest, cellulose TLC plates (Merck), ethanol 96%, ethyl acetate, filter paper, formic acid (Brataco), hydrochloric acid 96% (Merck), methanol, natrium hydroxide, potassium chloride, sodium chloride, *Plectranthus scutellarioides* leaves from Chideung, Lembang, West Java, Indonesia.

Instruments: The instruments used in this research wer : digital scales (Mettler Toledo), freeze dry, Fourier Transform Infra-Red (FTIR) (Shimadzu, IR Prestige-21), rotatory evaporator (Buchi), UV lamps λ 254 nm and λ 366 nm, UV-Vis spectrophotometry (Specord Analytik Jena, 2000), Mass Spectrometry.

Plant and Materials Collection: *Plectranthus scutellarioides* plants obtained from Cihideung, Lembang were collected in the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematic and Natural Sciences – Padjadjaran University.

Extraction and Fractionation: One kilogram of wet *Plectranthus scutellarioides* leaves that had been cut small were put into a maserator; coated of cotton, ethanol 96% acidified by concentrated HCl 0,5% was added into the maserator until all of the leaves soaked and then left for 24

hours. Repeat maceration up to 3 times. Macerated was evaporated and concentrated by rotatory evaporator at 40^0 C to get a concentrated extract. Fifty gram of concentrated extract was dissolved into 250 mL of water and then inserted into the separating funnel and n-hexane was added as much as the first solvent. Sometimes, the separating funnel was shuffled and then open the valve to remove the air. The separation was repeated until the fraction of n-hexane and water is completely separated. The water fraction is fractionated back using ethyl acetate until got a completely separated ethyl acetate fraction and water fraction. Then the ethyl acetate fraction is concentrated by rotatory evaporator at 40^0 C while the water fraction was concentrated by freeze dry at -80^0 C.

Analysis of Flavonoid Compounds: Put the sample into test tube, add water then heat for 5-10 minutes above the water bath. The filtrate was added a small amount of magnesium powder and 2 N hydrochloric acid solution. Next, into the filtrate was added amyl alcohol, then shook strongly. The presence of yellow to red in amyl alcohol, indicates the presence of flavonoids.

Analysis of Anthocyanin Compound: Put the sample into test tube, 2 M HCl is added, then heated for 5 minutes. Results are positive with the formation of red color. Also add 2 M NaOH a few drops. Positive results appear with the formation of blue green color that fades slowly.

Determination of Total Anthocyanin Content: Determination of total anthocyanin content is calculated as cyanidin 3-glucoside by pH difference method. Anthocyanin extract is dissolved in potassium chloride buffer (0.025 M, pH 1) and sodium acetate buffer (0.4 M, pH 4.5) with a ratio of extract to buffer = 1: 5 (v / v). Each solution measured its absorbance at maximum wavelength and 700 nm after incubation for 15 min at room temperature, the result was put into the formula:

$$A = [(A_{\lambda maks} - A_{700)\ pH = 1}] - [(A_{\lambda mak} - A_{700)\ pH = 4.5}]$$

$$\text{Total Anthocyanin Content} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times b}$$

DF is the dilution factor, b is the thick solution, ε is molar absorptivity and MW is the molecular weight (cyanidin 3-glucoside 449.2 g / mole).[23]

Thin Layer Chromatography: Thin layer chromatography was done for water fraction and ethyl acetate fraction. The chromatographic plate used is a type of cellulose. The mobile phase used is acetonitrile: water: formic acid (0.7: 1.3: 0.1).[1]

Preparative Thin Layer Chromatography: Preparative TLC with sample is water fraction. The mobile phase used is acetonitrile: water: formic acid (0.7: 1.3: 0.1) and the stationery phase is cellulose plate.[9] The color of band was scraped and inserted into vial bottle with methanol solution.

Purity Isolate Test: The isolates obtained were tested for purity by two-dimensional thin layer chromatography using TLC plate with 2 different motion phase systems, then observed and calculated for their Rf values. First eluent used was methanol: water: formic acid (1,6: 0,4: 0,1) and the second eluent used was acetonitrile: water: formic acid (0.7: 1,3: 0,1).

Identification of Isolates

a. UV-Vis spectrophotometry: A blank test with a solvent was used, 0.01% methanol - HCl inserted into the cuvette and reads its absorbance at a wavelength of 230 – 600 nm. Then the isolates were tested with the same treatment as the blanks by adding the isolates. Then the sample was inserted into $AlCl_3$ to see a bathochromic shift.

b. Infra-Red spectroscopy: A small amount of isolate was homogenized with 200 mg of potassium bromide and put into the pressure system at 80 kPa for 5 minutes to form a disk pellet. Previously, the pure potassium bromide plates and the infra-red spectra were measured as blanks. When the pellets were formed, measure the infra-red spectrum at 4000 – 450 $cm^{-1.}$

c. Mass Spectrometry: A small number of isolates were dissolved in methanol and then injected into a spectrophotometer with a 1.0 mL / min flow rate.

Results and Discussion

Plant Determination and Material Collection: The result of determination with the document number 556/HB/11/2016 from Department of Biology, Faculty of Mathematic and Natural Sciences, Padjadjaran University, shows that this plant will be used in accordance with that species *Plectranthus* scutellarioides (L.) R. Br. Then, the leaves were collected as much as ± 1 kilogram and rinsed for dirt removal.

Extraction and Fractionation: The method of extraction used is a maceration method for 3 x 24 hours, this method was used because anthocyanin is a secondary metabolite that is not stable at high temperature. The solvent used is 96% ethanol acidified with concentrated HCl 0.5% of the total solvent. Ethanol 96% is used because the water content in the solvent is not too much so that the extraction will be more viscous. Methanol can extract anthocyanins 20% more effective than ethanol and 73% more effective than water, but the use of methanol in large quantities is avoided because it is not good for health and environment.[8] The pH value greatly influences the color and stability of anthocyanin but the anthocyanin is more stable at acidic pH and will quickly be damaged by warming.[12] Acidic conditions in the solution can be cause a number of vacuoles ruptured, so anthocyanin pigment extracted will be more.[6]

The yield of extract is 8.625% of the total initial weight of 1000 grams. The concentrated extract obtained has a dark red color. The fraction of n-hexane and ethyl acetate obtained was concentrated using a rotatory evaporator at a temperature of 40°C, while the water fraction was concentrated using a freeze dry at a temperature of -80ºC. The yields of n-hexane fraction, ethyl acetate fraction and water fraction were 0,04%, 2.160% and 5.220%, respectively.

Analysis of Flavonoid Compounds: Anthocyanins are derived from flavonoid compounds, so it is important to detect the presence of flavonoid compounds in fresh leaves and extracts first. The test results showed positive sample containing flavonoids with the formation of yellow and red in the amyl alcohol layer, yellow color for fresh leaf samples and red color for extract samples.

Warming for the sample is needed because generally most of the flavonoid group compounds can dissolve in hot water. The presence of yellow to red color in amyl alcohol showed positive samples containing flavonoids, yellow to red color formed due to the reduction by hydrochloric acid and magnesium. [21]

Analysis of Anthocyanin Compounds: Preliminary results of fresh leaves and extracts showed positive results containing anthocyanin. The red color formed in the acidic condition (pH 1-2) is due to the dominant form of the anthocyanin i.e. the cation of flavylium whereas in pH > 3 conditions, the red color slowly becomes blue due to the formation of quinoidal bases.[11]

Determination of Total Anthocyanin Content: Total anthocyanin content obtained was 0,435 mg/g of wet weight leaves (figure 1). The total anthocyanin content is greater when compared with plants of *Coleus scutellarioides* var. Parvifolius of Central Java, 0.1664 mg/g, *Coleus scutellarioides* var. Crispa of Central Java, 0.2937 mg/g dry weight and is smaller when compared with *Coleus scutellarioides* var. Frustescens in China is 0.5047 mg / g dry weight and Coleus scutellarioides from Liman is 0.8209 mg/g wet weight.[5,9]

Total anthocyanin content can be due to different leaf color intensity, different varieties and environmental influences. The higher temperature in the plants area grows, the resulting aglycone is less stable so it can cause less anthocyanin pigment in the plant.[23]

Thin layer chromatography: TLC is best pattern to extract, ethyl acetate fraction and water fraction that is using cellulose plate. Eluents used are acetonitrile: water: formic acid (0.7: 1.3: 0.1). TLC results indicate that the fraction of ethyl acetate contains still a lot of compounds. Then, red color allegedly of anthocyanin was not found in the ethyl acetate fraction of TLC (figure 2).

Preparative Thin Layer Chromatography: Water fraction was done for preparative TLC to separated anthocyanin compounds with other compounds. Selection of water fraction is because anthocyanin fraction is the polar, so anthocyanin will be most in this water fraction.[22] The TLC results become the reference for conducting preparative thin layer chromatography with cellulose plate. Eluents used are acetonitrile: water: formic acid (0.7: 1.3: 0.1). The pink spot at Rf ± 0.3 in visible light is the target for the purification step of the anthocyanin suspected compound.

The pink band with Rf ± 0.3 in preparative TLC was scraped and inserted into a vial bottle containing methanol solution, then left overnight for the polar compound to dissolve into methanol and the cellulose settles at the bottom. Then, isolate was concentrated using rotatory *evaporator* at a temperature of 38ºC. The isolates obtained are ± 1 mg of purple solids.

Purity Isolate Test: The purity test of the isolate was carried out by using two-way TLC using two different types of mobile phase. The first mobile phase used is methanol: water: formic acid (1,6 :0,4: 0.1) and the second mobile phase used is acetonitrile: water: formic acid (0.7: 1,3: 0,1). Two-way KLT results show that the compound obtained is pure compound as evidenced by the appearance of a pink spot at Rf 0.42 with the first mobile phase. Then, after turning 90º to the right and eluting with a second mobile phase, 1 spot pink at Rf 0.07 appears.

Identification of Isolates: Identification of the isolates was first performed by UV-Visible instrument. The results of the identification indicate that the isolates have three absorbance peaks at 274 nm, 346 nm and 548nm wavelengths (figure 3). The existence of a bathochromic shift in isolates or when added to $AlCl_3$ may indicate the presence of ortho-hydroxy group[8] (table 1).

Absorption band at 650 cm^{-1} wave number indicates aromatic CH bonds *(bending)*, the absorption band at 1601 cm^{-1} indicates aromatic C=C bonds. The appearance of these bands indicates that in isolates, they contain an aromatic ring. The band between regions 1604-1576 cm^{-1} indicates the presence of a pyrylium ring group in which this ring is a specific ring possessed by anthocyanin.[20]

Absorption band at wavelength of 1040 cm^{-1} and 1299 cm^{-1} indicates C-O-C bond. The appearance of these bands indicates that in isolates, they contain an aromatic ring. Absorption band at a wavelength of 2343 cm^{-1} indicates -C=O-C bonds.[17] Wavelength absorption band at 2852 cm^{-1} indicates the presence of C-H bonds. Absorption band at 3376 cm^{-1} is of strong intensity and broad band indicates OH group in which the presence of hydrogen bonds helps the interaction intermolecules of anthocyanin.[14] The absence of absorption at 1460

cm^{-1}, indicates the absence of methyl groups on the compound. (figure 4).

The result of identification with mass spectrometry on isolate was found as fragmentation pattern m/z 287,0556; 288,0590 and 289,061. This result indicates that isolate has molecular weight 287,0556 because this molecular weight has the highest intensity among the others. Results from the reading of the mass analysis showed that the molecular weight of 287.0556 with a tolerance of 0.0 MDA has the formula $C_{15}H_{11}O_{6}$. (figure 5). Formula and molecular weight analysis are indicated in accordance with the cyanidin molecular weight compound ($C_6H_{15}O_{6}^{+}$). [2]

The final identification results were confirmed by the addition of 1% Pb-Acetate to the isolate solution. The isolate solution changes color from pink to blue. 1% Pb-acetate can differentiate hydroxyl groups at C-3 'and C-4' positions in ring B. The anthocyanin types of peonidin and malvidin do not have hydroxyl groups. The addition of Pb-Acetate will give rise to purple color. Conversely, if the anthocyanin has a hydroxyl group, then the addition of Pb-acetate will give rise to blue color.[16] Cyanidin is an anthocyanidin having a hydroxyl group located in the C-3 'and C-4' positions in ring B.

Conclusion

1. Plectranthus scutellarioides using ethanol acidified by concentrated HCl 0,5% have total anthocyanin content 0,435 mg/g of wet weight of leaves (anthocyanin calculated as cyanidine-3-glucoside).
2. Anthocyanidin compounds obtained from water fraction using preparative thin layer chromatography with eluent used are acetonitrile: water: formic acid (0,7: 1,3: 0,1) with pink band at Rf 0,3.
3. Spectrophotometry UV-VIS showed maximum wavelengths of isolate are 274 nm, 346 and 546 nm. FTIR analysis showed functional group such as -OH, C=C aromatic, C-O and C-H aromatic. Mass spectrometry showed mass compound around 287,0556 with formula $C_{15}H_{11}O_6$. Spectrum showed existence of cyanidin.

Table 1
Maximum wavelength and batochromic shift of isolate

Sample	Before $AlCl_3$ added	After $AlCl_3$ Added	Batochromic Shift
Isolate	274 nm 346 nm 546 nm	274 nm 396 nm 559 nm	0 nm 50 nm 13 nm

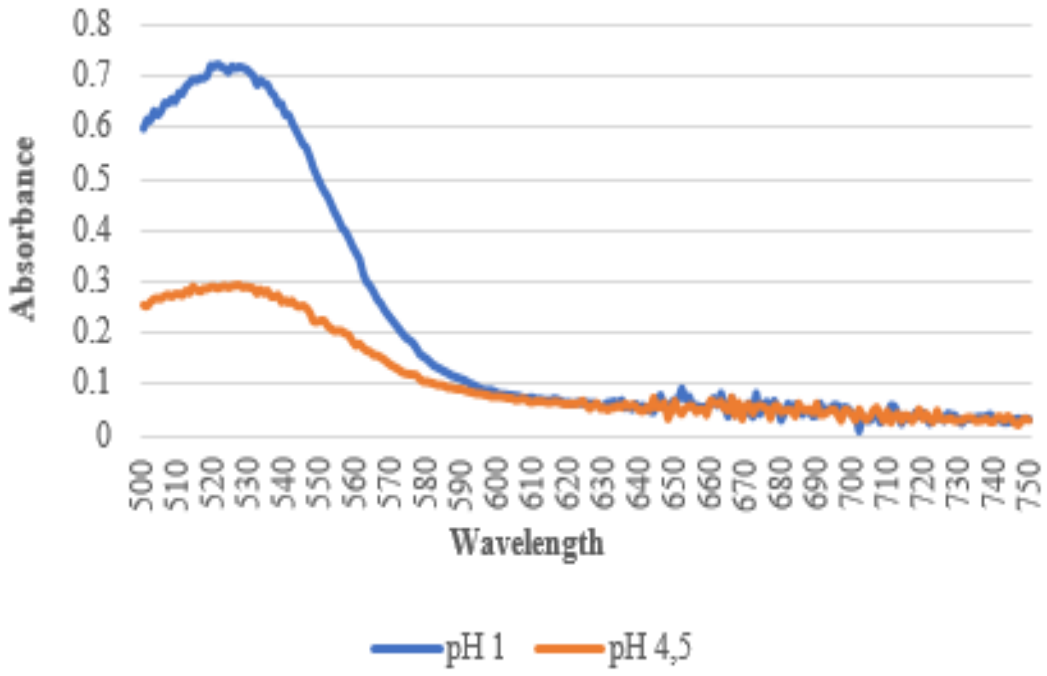


Fig. 1: Spectra of Extract at pH 1 and pH 4.5

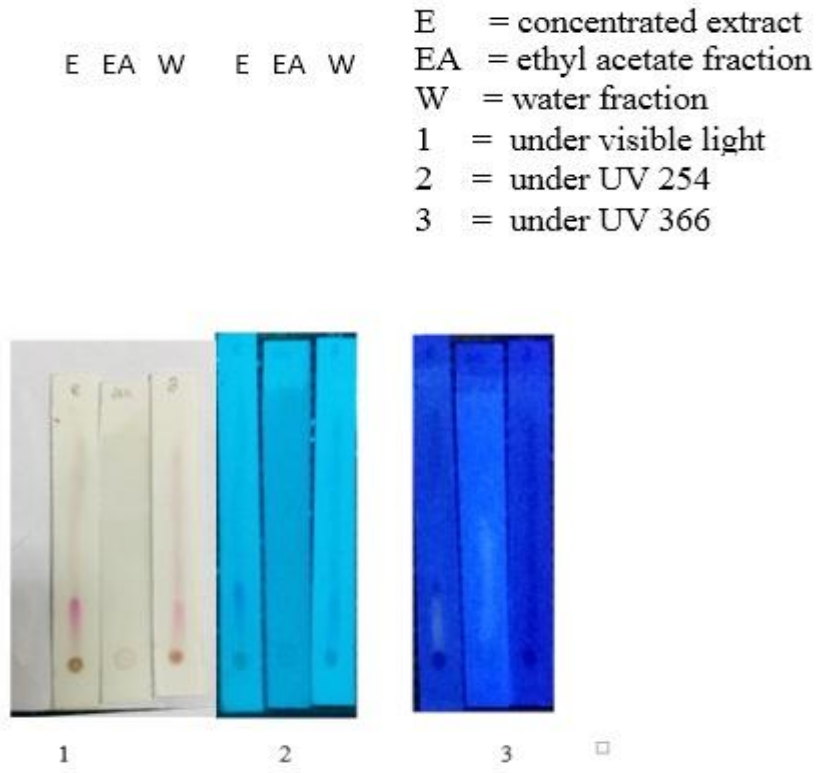


Fig. 2: TLC pattern with acetonitrile: water: formic acid (0.7: 1.3: 0.1)

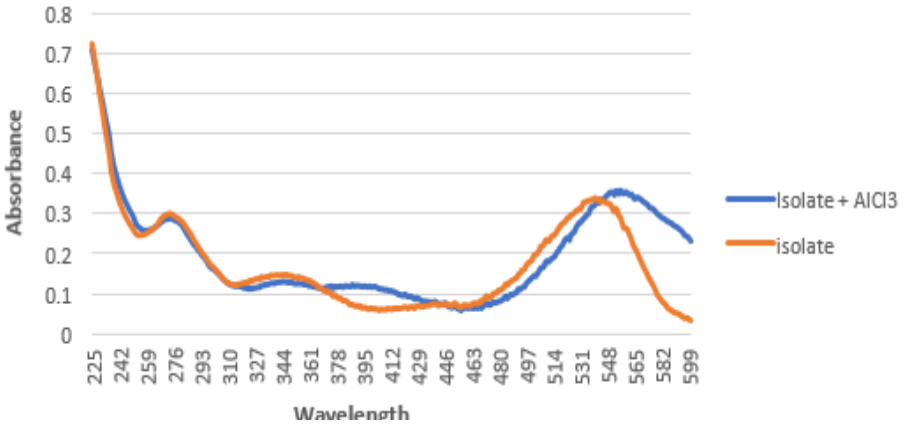


Fig. 3: Absorbance Isolate at Wavelength 225-600 nm

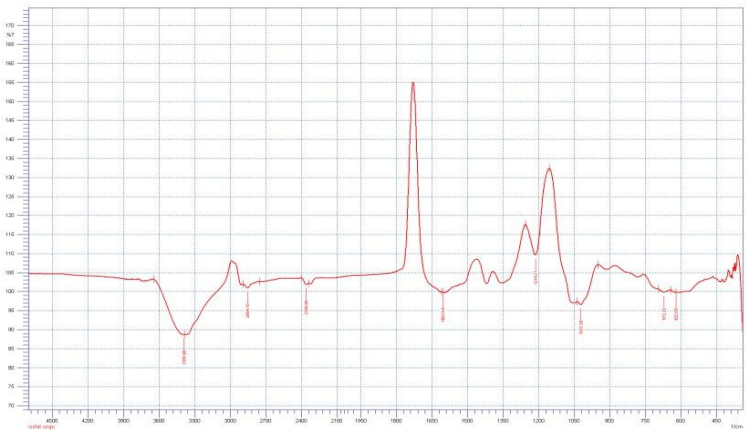
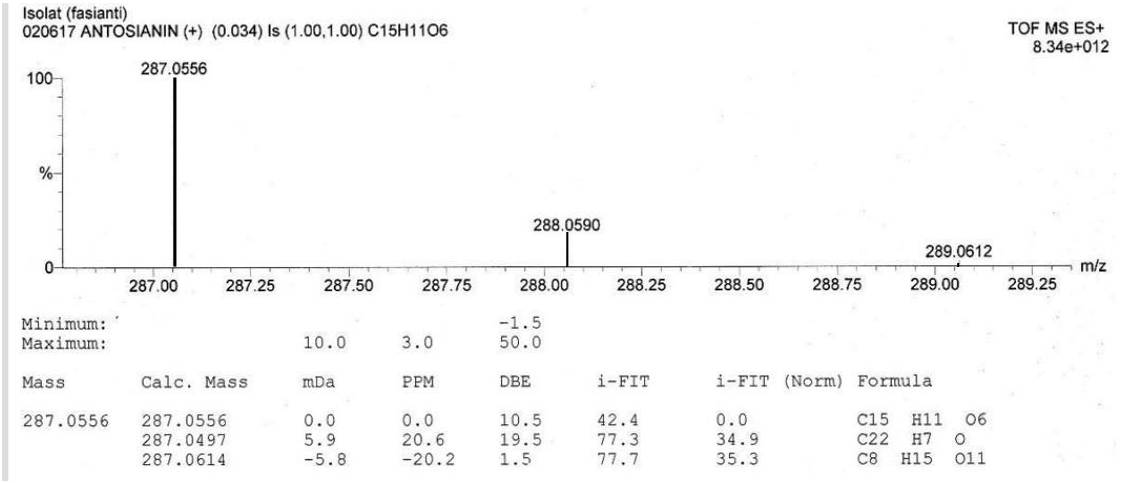


Fig. 4: Infra-Red Spectrum of Isolates



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
Minimum:				-1.5			
Maximum:		10.0	3.0	50.0			
287.0556	287.0556	0.0	0.0	10.5	42.4	0.0	C15 H11 O6
	287.0497	5.9	20.6	19.5	77.3	34.9	C22 H7 O
	287.0614	-5.8	-20.2	1.5	77.7	35.3	C8 H15 O11

Fig. 5: Mass Analysis of Isolate

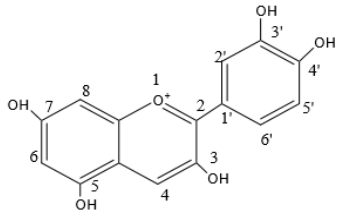


Fig. 6: Cyanidin Structure Cyanidin Structure[18]

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References
1. Anggraini A., Isolasi dan Identifikasi Flavonoid. dari Daun Makuto Dewo (*Phaleria macrocarpa* [Scheff] Boerl.), Available at: http://eprints.ums.ac.id/22364/15/MAKALAH_PUBLIKASI.pdf **(2008)**

2. Anonym, Cyanidin, Available at: https://pubchem.ncbi.nlm.nih.gov/compound/128861#section=Top **(2017)**

3. Brouillard R., Chemical structure of anthocyanins, In Anthocyanins as food colours, Markakis P., ed., Academic Press, London **(1982)**

4. Harborne J. and Metode Fitokimia, Pemantauan Cara Modern Menganalisa Tumbuhan, Terjemahan K. Padmawinata, Edisi II, Penerbit ITB, Bandung **(1987)**

5. Hardiyanti Y., Djaswir D. and Adlis S., Ekstraksi dan Uji Antioksidan dari Daun Miana (*Coleus scutellarioides* L (Benth).) Serta Aplikasi pada Minuman, *J. Kimia Unand*., **2(2)**, 44-49 **(2013)**

6. Hayati E.K., Budi U.S. and Hermawan R., Konsentrasi Total Senyawa Antosianin Ekstrak Kelopak Rosella (*Hibiscus sabdariffa* L.): Pengaruh Temperatur dan pH, *J. Kimia*, **6(2)**, 138-147 **(2012)**

7. Kouakou T.H. et al, Preparative Purification of Delphinidin 3-0 sambubioside from Roselle (*Hibiscus sabdariffa* L.) Petals by Fast Centrifugation Partition Chromatography, *Journal of Advances in Chemistry*, **6**, 994-999 **(2014)**

8. Lestario L.N., Elisabeth R. and Kris H.T., Kandungan Antosianin dan Identifikasi dari Kulit Buah Jenitri (*Elaeocarpus angustifolius* Blume), *Agritech*, **31(2)**, 93-101 **(2011)**

9. Lestario L.N., Hartati S. and Agustine E., Identifikasi Antosianin dan Antosianidin dari Daun Iler (*Coleus scutellarioides*) Var. Crispa dan Var. Parfivolius, Prosiding Seminar Nasional Sains dan Pendidikan Sains IV, **3**, 665-676 **(2009)**

10. Lisdawati V., Mutiatikum D., Alegantina S. and Astuti Y., Karakterisasi Daun Miana *(Plectranthus scutellarioides* (L.) Bth.) dan Buah Sirih (*Piper Betle* L.) Secara Fisik Kimia dari Ramuan Lokal Antimalaria Daerah Sulawesi Utara, *Media Litbang Kesehatan*, **18**, 191-196 **(2008)**

11. Mahmudatissa'adah A., Dedi F., Nuri A. and Feri K., Karakteristik Warna dan Aktivitas Antioksidan Ubi Jalar Ungu, *Jurnal Teknologi dan Industri Pangan*, **25(2)**, 176-184 **(2014)**

12. Markakis P., Anthocyanin as Food Colors, Academic Press, New York **(1982)**

13. Moektiwardoyo M. et al, The Determination of Quercetin in *Plectranthus scutellarioides* (L.) R.Br. Leaves Extract and Its In Silico Study on Histamine H4 Receptor, *Majalah Farmasi Indonesia*, **22**, 191-196 **(2011)**

14. Munawaroh H. et al, The co-pigmentation of anthocyanin isolated from mangosteen pericarp (Garcinia Mangostana L.) as

Natural Dye for Dye Sensitized Solar Cells (DSSC), IOP Conf. Series: Material Science and Engineering, 107 **(2016)**

15. Nugroho Y., Efek Pemberian Kombinasi Buah Sirih (*Piper Betle* L), Daun Miyana (*Plectranthus scutellarioides* (L.) R. Br.), Madu dan Kuning Telur Terhadap Peningkatan Aktivitas dan Kapasitas Fagositosis Sel Makrofag, *Media Litbang Kesehatan*, **22**, 1-5 **(2012)**

16. Nuryanti S., Sabirin M., Chairil A. and Tri J.R., Isolation Anthocyanin from Roselle Petals (*Hibiscus sabdariffa* L) and The Effect of Light on The Stability, *Indo J. Chem*, **12(2)**, 167-171 **(2012)**

17. Nuryanti S., Indarini D.P. and Ratman, Isolation of Anthocyanidin From Wora-Wari Flowers (*Hibiscus rosa sinensis* L.) and Its Application as Indicators of Acid-Base, *Agritech*, **33(3)**, 320 **(2013)**

18. Ogundare A., Antimicrobial effect of *Tithonia diversifolia* and *Jatropha gossypifolia* leaf extracts, *Social Science Research*, **2**, 45-150 **(2007)**

19. Putri Ni K.M., Gunawan I.W.G. and Suarsa I.W., Aktivitas Antioksidan Antosianin dalam Ekstrak Etanol Kulit Buah Naga Super Merah (Hylocereus Costaricensis) dan Analisis Kadar Totalnya, *Jurnal Kimia*, **9(2)**, 243-251 **(2015)**

20. Ribereau-Gayon P., Les Composess Phenoliquesdes Vegetaux Les Anthocyanidin, Dunod, Paris **(1968)**

21. Sangi, Analisis Fitokimia Tumbuhan Ovat di Kabupaten Minahasa Utara, *Chem Prog*., **1(1)**, 47-53 **(2008)**

22. Timberlake C.F. and dan Bridle P., The Anthocyanins, In Horborne J.B., ed., The Flavonoid, Chapman and Hall, London **(1997)**

23. Wrolstad R.E. et al, Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors Texture and Bioactive Food Components, John Wiley and Son, New York, 1-45 **(2005)**.