Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(12):1276-1282

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Simple characterization of anthocyanin from *Ficus padana* Burm.f

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ABSTRACT

Ficus Padana Burm.f is potential source of anthocyanin based on phytochemical test result.This study focuses on the analysis and simple characterisation of anthocyanins from Ficus Padana Burm.f fruits. The fresh Ficus Padana Burm.f fruits were extracted with the acidified ethanol with citric acid at room temperature for 12 hours in the darkenvironment.The pigment was identified with UV-Vis Spectroscopy, HPLC-DAD, and LC-MS. The pigment are pelargonidin 3-(6"-p-coumarylglucoside)-5-(4"'-Malonylglucoside) and pelargonidin 3-(6''-Malonylglucoside). The second one is a major compound that taking up to 91% of the total anthocyanin content in Ficus padana Burm.f. The results confirmed that Ficus Padana Burm.f contained acylated anthocyanin pigments.

Key words: *Ficus padana* Burm.f, anthocyanin, characterization.

INTRODUCTION

Ficus Padana Burm.f is a species of the genus *Ficus*, moreceae family and spread across Java and Sumatra. This plant has another name Hemberang (Sunda), Dedek, Kebeg (Java)[1].This plant is a tree, to 15 m high and, in the western part of Java is growing at an altitude of 300 -1300 m above sea level, on the slopes of the ravine and in secondary forests[2].

Ficus padana Burm.f fruits containing anthocyanins allegedly because of the color of the seeds and pulp of the fruit is colored red. The possibility of anthocyanin compounds in fruit *Ficus Padana* Burm.f also reinforced by the positive results of the flavonoid phytochemical test of the fruit *Ficus Padana* Burm.f. Based on these data the expected fruits of *Ficus Padana* Burm.f is potential source of anthocyanins.

Figure 1 Pictures of *Ficus padana* Burm.f

Anthocyanins are responsible for most of the red, blue and purple colors of fruits, vegetables, flowers and other plant tissues or products[3-6]. The isolation and identification of anthocyanins are difficult as a result of their ability to undergo structural transformations and complex reactionary. Moreover, they are difficult to be analyzed independently from other flavonoids because they have similar reaction characteristics[7].

In this study we investigate the major compounds of anthocyanin in the fruit of *Ficus padana* Burm.f based on the properties of anthocyanin. The structure of the anthocyanins were identify with UV-VIS spectrum, the effect of pH stability, stability against strong acid hydrolysis and mass spectra analysis by comparingwith the spectral data of published data[8].

EXPERIMENTAL SECTION

Plant samples

The fruit samples were picked at Andalas University botanical garden in West Sumatera, and transported to the laboratory immediately.

The fruits sample were identify at Research Centre of Biology, Indonesian Institute of Sciences with identification number 1168/IPH.1.02/If.8/VI/2013

Chemicals

HPLC-grade water, methanol, ethanol and acetonitrile, citricAcid, hydrocholric acid, formic acid were obtained from Merck , Germany. All other chemicals used in this study were analytical grade.

Instrumentation

Shimadzu spectrophotometer UV1800, Shimadzu HPLC with DAD detector (prominence UFLC) . An Agilent LC-MSD 6100 series, equipped with a DAD and ESI-MS detector were used for mass spectra analysis.

Procedure

1. Extraction of anthocyanins

Acidified of ethanol pH 1,5 were prepared by mixed of ethanol with citric acid 35 %(3:7). 200 ml acidified ethanol was added into 1000 ml Erlenmeyer flask containing100 g fruit. Anthocyanins were extracted at room temperatur for 12 hours in dark environment, this procedure was repeated three times to collect the extract solution. The extraction was concentrated under vacum at room temperatur using a rotary evaporator until left 1/3. About 10 ml of extracted solution was passed through a 0.45 μm millipore filter for analysis.

2. Characterization of anthocyanin with the spectrophotometer

Characterization by spectrophotometer measurements performed with a maximum wavelength of anthocyanin in UV-VIS spectrum. Maximum wavelength measurements performed using a double beam spectrophotometer Shimadzu UV-1800 with an area measuring wavelengths between 200-800 nm.

3. Characterization of anthocyanin by measurement of the stability of anthocyanin extract
Measurement of the color intensity of anthocyanin extracts performed on the anthocyanin solution dissolved in five different solution pH conditions (1, 3, 5, 7,and 9).Each solution was measured with a maximum wavelength double beam spectrophotometer Shimadzu UV-1800 with an area measuring wavelengths between 200-800 nm

4. Acid hydrolysis of anthocyanins.
5 ml of 2N HCl was added to 1 ml of the extract of anthocyanin in a screw-cap test tube. The pigments were hydrolysed at 100°C for 1,5 hours.Then the solution was immediately cooled in room temperature.About 1 ml of hydrolysate solution was passed through a 0.45 μm millipore filter for analysis

5. HPLC-DAD- analysis
Shimp-pack ODS column was used. The solvents were (A) aqueous 2% formic acid, and (B) acetonitrile: water (1:1 v/v) containing 2% formic acid. The gradient was from 6 to 10% B for 4 min, from 10 to 25% B for 8 min, isocratic 25% B for 1 min, from 25 to 40% for 7 min, from 40 to 60% for 15 min, from 60 to 100% for 5 min, from 100 to 6% for 5 min, at a flow rate of 1.0 ml/min. Injection volumes were 20 μl, and the detection wavelength was 516 nm[9].

6. ESI-MS analysis
Agilent Zorbax SB-C18 column was used. The condition of MS were as follows: ESI interface, positive ion model, 35 psi nebulizer pressure, 10 L/min dry gas flow rate, 350°C dry gas temperature and scans at m/z 150 to 1000. All analyses were duplicated[8].

RESULTS AND DISCUSSION

1. Extraction of anthocyanin
Solvent extraction processes is the first step for isolation of anthocyanin pigment from plants[10]. Anthocyanins are polar molecules and consequently more soluble in polar solvents, however extraction conditions are also key factors in their overall solubility[11,12]. Alcoholic extraction is suitable for extracting anthocyanins from fruits and vegetables it shown in the previous research in study of anthocyanin from purple-fleshed sweetpotato powder, purple corn, red and black currants, and grapes. In the extraction process of anthocyanin from particular corps in plants solvent type, solvent concentration, solid–liquid ratio (solid loading), incubation temperatureand incubation time are important for the stability and concentration of anthocyanins[12-17]. Methanol is the most suitable solvent for the anthocyanin extraction process , but it has more toxic and hazardous to handle comparing with the other alcohols. Ethanol is a good option for replacing methanol because it is has less toxicity and can also recover anthocyanins with good quality characteristics[10]. The use of acid at the exctraction process is to stabilize anthocyanins in the flavylium cation form, which is red at low pH[18]. Hydrochloric acid is commonly used for solvent acidified but it may hydrolyze acylated anthocyanins, to avoid or at least minimize the breakdown of acylated anthocyanins, organic acids such as acetic, citric or tartaric acids, which are easier to eliminate during anthocyanin concentration, have been preferred[19].

2. Test for anthocyanins
The red, purple and blue colors found in many plants are due to two classes of water soluble pigments: anthocyanins and betacyanins. The anthocyanins are flavonoids, a class of phenolic molecules that are synthesized through the Shikimic acid pathway and are widespread in the plant kingdom. Betalains, a group of pigments that includes the betacyanins are indole-derived alkaloids and contain nitrogen. The extracts in acidied ethanol were tested for the presence of anthocyanins by observing pigment color under acidic conditions by adding HCl. 3 ml of extract and 3 ml HCl were mixed in a test-tube and then placed in vessel with boiling water for 5 min. The mixture was stable and did not lose color when boiled indicated the presence of anthocyanins in the extracts.

3. Characterization of anthocyanin with the spectrophotometer
The UV-Visible absorption spectra of anthocyanins from *Ficus Padana* Burm.f fruit (pH = 1,5) were recorded between 200 and 800 nm with a double-beam scanning UV-Visible spectrophotometer (Figure 2). The result indicated that the anthocyanin pigment from *Ficus Padana* Burm.f has two absorption peaks (acidified ethanol with citric acid): one is λmax = 278 nmin UV range and another is λmax = 526 nm in visible range.

The emergence of two absorption peaks obtained from sample extracts showed that the presence of anthocyanin compounds. As a group of the flavonoid, anthocyanin has two cluster groups which provide local absorption at UV-Vis absorption that is the benzoyl and sinamoil groups. Anthocyanin compounds have two characteristic absorption at a wavelength region that is UV (260-280 nm) and visible (490-550 nm).

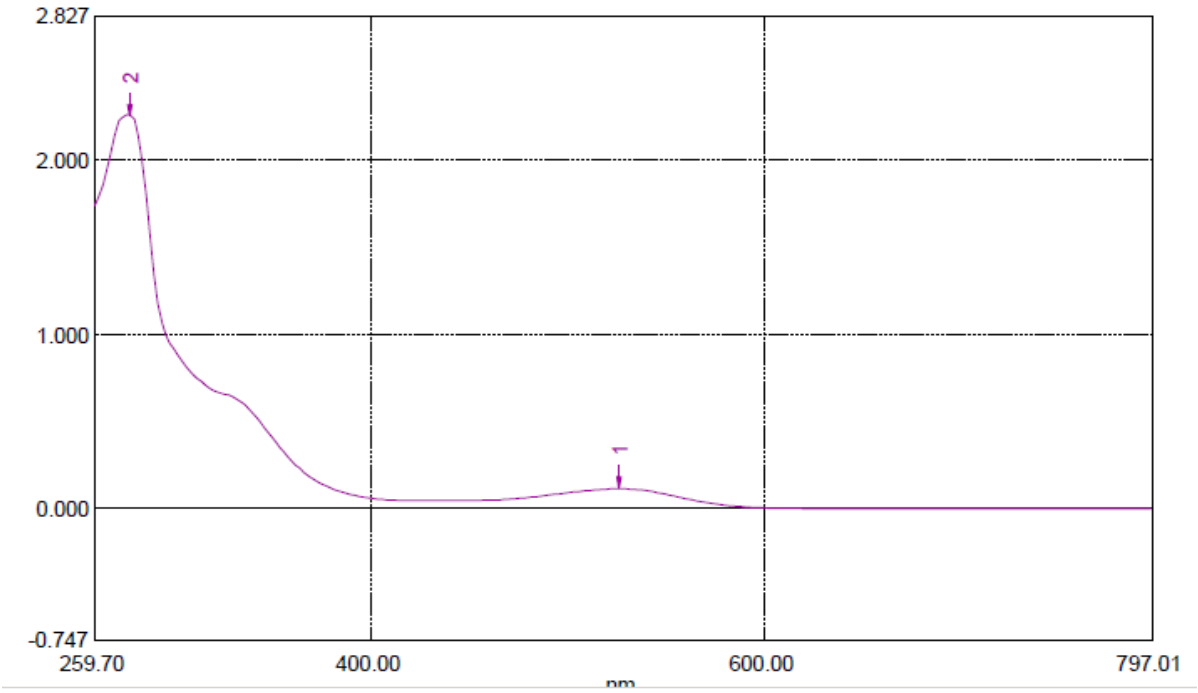

Figure 2. The UV-Visible absorption spectra of anthocyanins from *Ficus padana* Burm.f fruits (pH = 1,5)

4. Characterization of anthocyanin by measurement of the stability of anthocyanin extract

Anthocyanin extracts obtained testing the effect of pH stability . Treatment given pH is the pH of 1 , 3 , 5 , 7 , and 9. The color of anthocyanin extracts at different pH conditions give different colors , which also followed different wavelengths of maximum absorbance of each extract at each pH condition (table 1).

Table 1 .Observation of the color of each treatment pH and UV-Visible absorption spectra of each treatment pH

No	pH	Color	UV-Visible absorption spectra			
			λ1(nm)	Abs	λ2 (nm)	Abs
1	1	red	526	0.115	278	2.261
2	3	slightly red	510	0.097	276	2.242
3	5	colorless	504	0.043	276	2.242
4	7	colorless	-	-	276	2.424
5	9	slightly red	514	0.055	276	2.271

Absorbance values and the color of anthocyanin fruit *Ficus padana* Burm.fpada pH 1.5 showed high values and red , this is due to a pH ≤ 2 anthocyanins are in the most stable condition , the main anthocyanin structures are in the form of red flavilium cations[20]. At pH 3, red color of anthocyanins from *Ficus Padana* Burm.f start to fade , there is a trend that the absorbance value decreased with increasing pH, up to pH 5 and pH 7 . The absorbance values sharply decline occurred in anthocyanin fruit *Ficus Padana* Burm.f especially at pH 5 and pH 7. Buffer system contained in the extractsof anthocyanin of *Ficus Padana* Burm.f in the range of pH 5 and 7, which becomes colorless extracts showed degradation of anthocyanin structure , in this condition , red flavilium hydrated cations into the structure forms colorless (carbinol)[20].

Extract mixed conditions with pH 9 buffer back gives a slightly red color , by providing in the region of visible light absorption at a wavelength of 514 nm , this may be related to the bond acylation of anthocyanin compounds that exist.

5. HPLC-DAD and ESI-MS analysis

Figure 3 showed the anthocyanin profile of the extract using the HPLC-DAD chromatograms at 516 nm.As can be seen, there are two peaks in the chromatogram at the retention time range of 15–20 min, indicating the presence of two different anthocyanin in fruit of *Ficus padana* Burm.f.The chromatogram of the product afteracid hydrolysis of the anthocyanin extract, also recordedat 516 nm,showed that only one aglycones could be obtained from the two anthocyanins in *Ficus Padana* Burm.ffruit (figure 4). These two anthocyanins and their correspondingaglycones, the structures of were identified by spectrometric data ESI-MS .

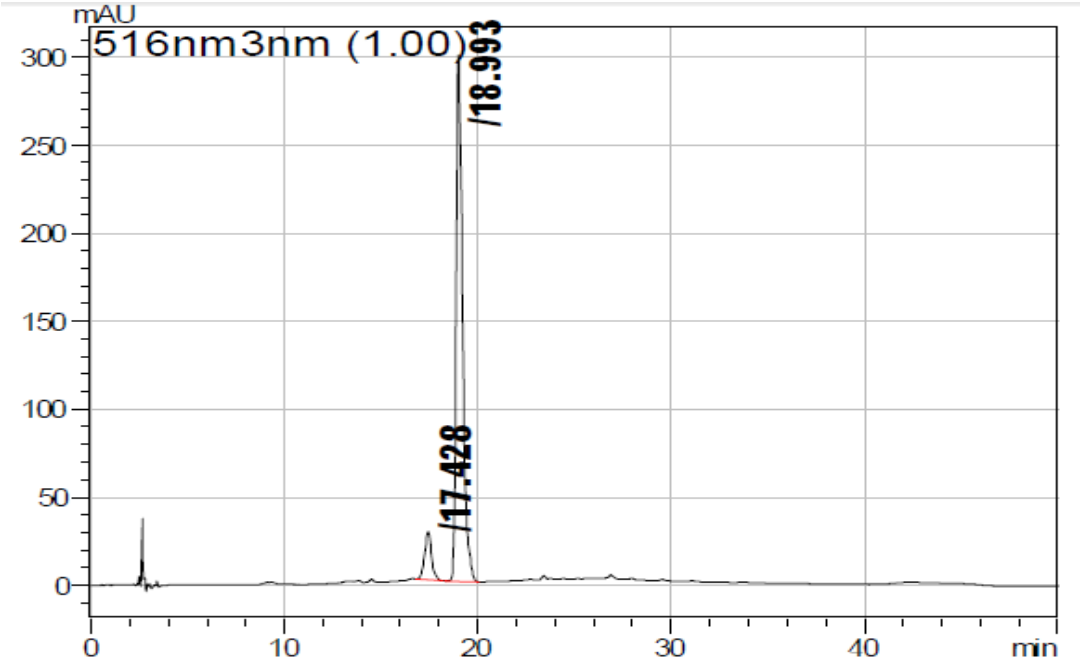

Figure 3. HPLC chromatograms of extract of *Ficus Padana* Burm.f fruits (DAD, 516 nm)

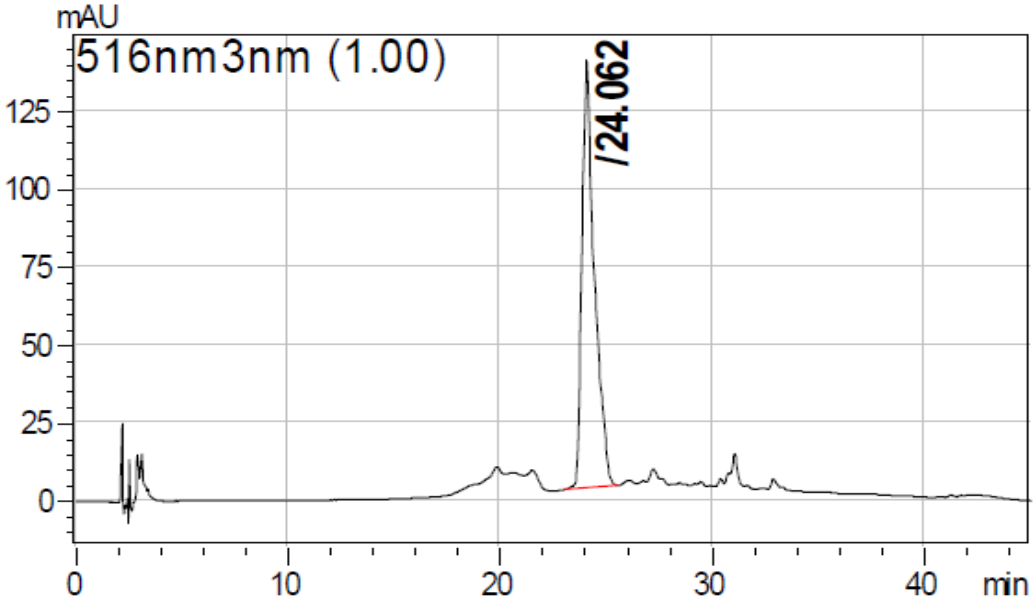

Figure 4. HPLC chromatograms of extract of *Ficus Padana* Burm.f fruits after acid hydrolysis (DAD, 516 nm)

A total of two anthocyanin compounds were identified by their elution order and by comparing the m/z of each anthocyanin molecule and its fragmentation to prepared database. Peak 1 and 2 showed fragment ions at m/z 271 in MS analyses that could be tentatively identified as pelargonidin derivatives. Peaks 1 and 2 showed identical molecular ions at m/z 828 and 519, which concurs with that found in on line database[8]used for confirmation purposes. Peak 1at *m/z* 828 and MS2 fragments at 519 and 271 due to loss of coumarylglucoside (–309 amu) and malonylglucoside (–3248 amu), respectively, was identified as due to pelargonidin 3-(6”-p-coumarylglucoside)-5-(4”’-malonylglucoside) (Figure 5).

Peak 2 as the major anthocyanin in extract of *Ficus padana* Burm.f fruitswas attributed to pelargonidin 3-(6"-malonylglucoside), by HPLC-DAD and HPLC-MS (*m*/z 519, MS2 fragments at 271, corresponding to a first loss of 248 amu (malonyl moiety)(Figure 6).

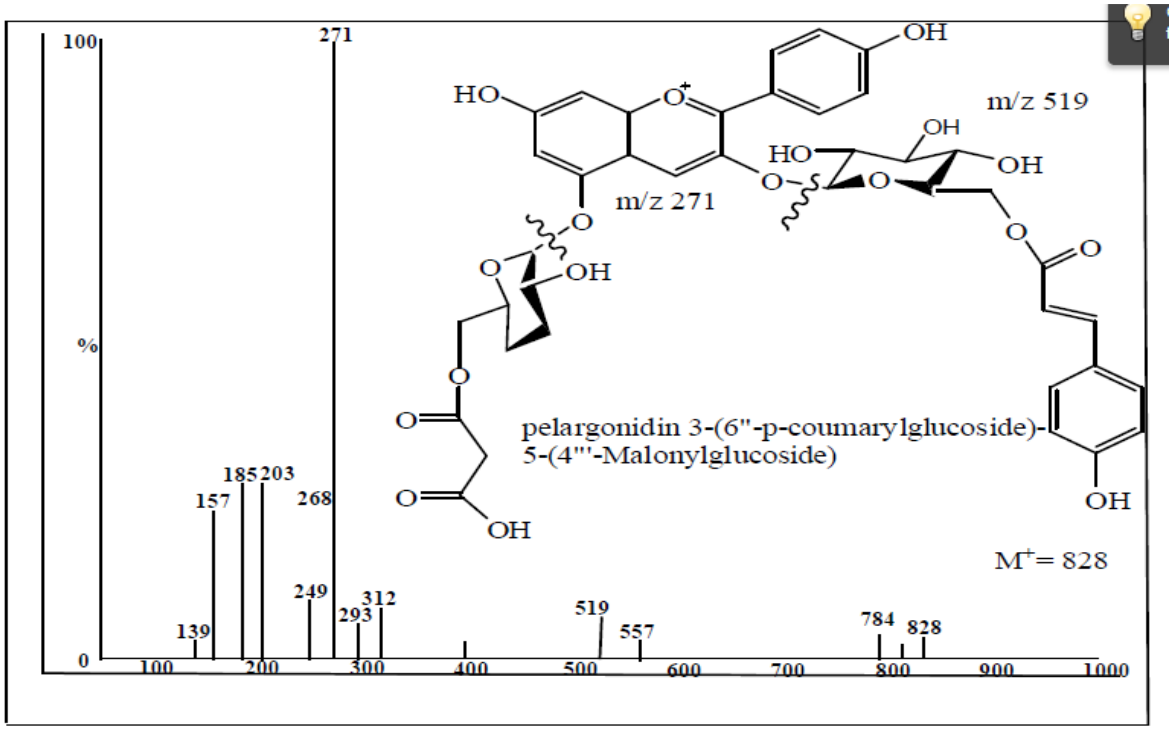

Figure 5. Structure and MS data of anthocyanin peak 1

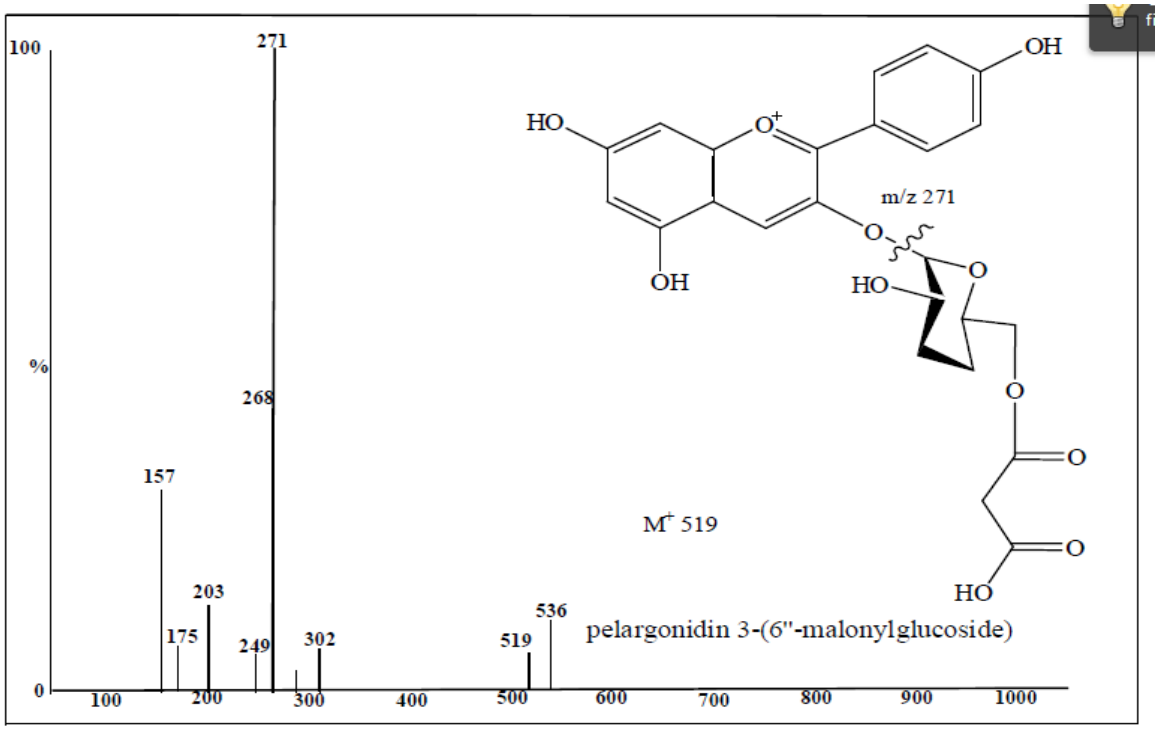

Figure 6. Structure and MS data of anthocyanin peak 2.

CONCLUSION

The pigment in fruits of *Ficus Padana* Burm.f is a kind of natural pigment forfood processing and has potential medical and commercial values. This study focused on thesimple characterisation of anthocyanins from*Ficus Padana* Burm.f. The pigment was identified by means of UV-Visible spectroscopy, HPLC-DAD and LC-MS. The anthocyanins showed pelargonidin aglycon with compounds arepelargonidin 3-(6”-p-coumarylglucoside)-5-(4”’-Malonylglucoside) and pelargonidin 3-(6’’-Malonylglucoside). The second one is a major compound that taking up

to 91% of the total anthocyanin content in *Ficus padana* Burm.f. The results confirmed that *Ficus Padana* Burm.f contained acylated anthocyanin pigments.

Acknowledgement

The authors would like to thank Professor Fauzan Azima for giving granted to use the laboratory instrumental, Mr. Ario Betha Juanssilfero,M.Sc CPE and Mr.Harmizal for helpfull discussion on laboratory research.

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