Anthocyanins from the Red Juvenile Leaves of *Loropetalum Chinense* var. *rubrum* (Chinese Fringe Flower): Identification and pH Sensing Behaviors

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Abstract

Anthocyanins were extracted from the frozen juvenile leaves of *Loropetalum Chinense* var. *rubrum* with high efficiency using ethanol containing 0.1% HCl as extractant. After being purified with AB-8 resin, this extract was investigated with HPLC-DAD-MS/MS determination, and six anthocyanins were identified according to chromatogram, MS data and ratios of absorbance at 400-460 nm to that at absorption maximum (~520 nm), $A_{400\text{-}460}/A_{520}$. Besides cyanidin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, the most abundant malvidin 3,5-O-diglucoside was confirmed through preparative HPLC separation and ^{1}H NMR determination. However, two delphinidin- and petunidin-derived anthocyanins should be further clarified whether they are 3,5-O-diglucoside or 5-O-glucobioside. In addition, a novel anthocyanin, 2H-pyran[5,6]malvidin 3-O-glucobioside was also proposed yet need more data to confirm. Moreover, malvidin 3,5-O-diglucoside showed excellent pH sensing ability, displaying distinct color change from pink (pH 1.5) to colorlessness (pH 5.5), to blue (pH 6.8) and finally green (pH 8.8 to pH 10.5). All these should be helpful to evaluate this plant as natural pigment resource.

Keywords: anthocyanin, *Loropetalum Chinense* var. *rubrum*, HPLC, malvidin, absorption spectroscopy, pH indicator, color, extraction

Introduction

Modern food industry demands natural pigments of health benefits to replace artificial chemical colorants. Anthocyanins, the plant-sourced flavyliums (2-phenylchromenylium) with their 3-, 5-, 7-hydroxyl group being connected with saccharides via glycoside bond, show brilliant colors such as red, blue and purple and fine aqueous solubility.[1]Their polyphenolic structures provide still the ability of antioxidation.[2] All these suggest that anthocyanins might be potential food colorants. Their pH dependent color change provides the additional advantage to visualize food deterioration process,[3] which is always accompanied by pH change. However, anthocyanins have limited natural resource, this scarcity together with their poor stability in ambient conditions hamper their wide application in food industry. Therefore, exploring new plant resource to offer anthocyanins and clarifying their pH sensing ability are essential for their potential application. Most anthocyanins were extracted from fruits and flowers,[4,5] which disfavors the accessibility all through the year and constrains their production. Evergreen plants with anthocyanin-containing leaves might be reliable alternative to supply anthocyanins. *Loropetalum chinense* var. *rubrum,* the specific cultivars of Chinese fringe flower featuring showy reddish purple leaves (var. *rubrum*), is broad-leaved shrub of the witch hazel family and a typical landscape plant in china especially in roundabouts and median strips (Figure 1). These shrubs show alternate, ovate, pink/purple, evergreen leaves, and grows actively all through the year to offer large production of leaves. Additional advantage is that they are deer resistant and almost insect or disease free. High anthocyanin abundance (~0.3 mg/g) has been found in their leaves except for their flowers, [6,7,8,9,10,11] and our preliminary study has confirmed also the abundant anthocyanins in their fresh leaves.[12] Therefore, separation and identification of anthocyanins in their leaves may provide a new resource to supply nature food pigments.

Figure 1. (**a**) *Loropetalum Chinense* var. *rubrum* by the highway; (**b**) Leaves of *Loropetalum chinense* var. *rubrum* with pre-pruning; (**c**) Moon cakes cooked with different colors.

In this study, the frozen juvenile leaves from Chinese fringe flower were extracted with high efficiency by ethanol containing 0.1% hydrochloric acid (v/v). Then the extract was purified using macroporous resin AB-8 via elution with doubly distilled water, 0.01% HCl, ethyl acetate and 0.01% ethanol in sequence. Six anthocyanins were then identified by HPLC-DAD-MS, which demonstrated a main species of m/z 655. Further purification with preparative HPLC followed by ^{1}H NMR determination confirmed its assignment as malvidin-3,5-O-diglycoside. Investigating the colorimetric pH sensing ability of malvidin 3,5-O-diglycoside demonstrated a distinct pH-dependent color change, showing pink at pH 1.5, colorlessness at pH 5.5, blue at pH 6.8-7.4, green at pH 8.8-10.5. Colorimetric response demonstrated also that malvidin-3,5-O-diglycoside is more stable in acidic aqueous solution, and its instability in neutral and alkaline media made the blue and green colors disappear in less than 15 h in ambient condition.

Materials and Methods

Samples. The red juvenile leaves (the latest leaves with both sides in red were collected manually in Xianlin campus of Nanjing University and stored in refrigerator at -20°C immediately.

Anthocyanin extraction. The frozen leaves (52 g) were crushed into small species with a pestle, and then extracted with ethanol (500 mL) containing 0.1 % HCl (v/v)

via magnetic stirring at room temperature for 1.5 h. After filtration, the leaves were extracted again (500 mL) followed by filtration. The off-white solids were discarded, and combing filtrates afforded ~1000 mL deep magenta solution. Removing the solvent via evaporation with rotary evaporator at 35 ° C resulted in ~5.2 g brown residual. The residual was dissolved in aqueous solution (90 mL) containing 0.01% HCl (v/v), and then extracted with ethyl acetate (~80 mL × 15) till organic phase become almost colorless. The resulted purple aqueous phase then underwent further purification.

Anthocyanin purification with AB-8 resin. AB-8 resin (300 mL) was activated in a standard procedure: the resin soaking in acetone (2 BV, 24 h) in a column was rinsed with acetone (600 mL) in a rate of 2BV/h. Then the resin was soaked with 2BV ethanol for 5 h followed by rinse with ethanol (600 mL, 2BV/ h). The resin was then rinsed with deionized water till there is no smell of ethanol from the eluent. The resin was then eluted with 5% HCl in a rate of 2 BV/h followed by water rinse to make eluent neutral. Finally, the resin was rinsed by 2% NaOH in a rate of 2 BV/h followed by deionized water rinse to make eluent neutral.

Loading the purple extract onto the resin column, followed by elution with deionized water (800 mL), 0.01% water with 0.01% HCl (340 mL, v/v), and ethanol (460 ml, 0.01% HCl, v/v) in sequence in a rate of 2 BV/h. A purple fraction (~500 ml) was obtained upon ethanol elution. This fraction was then evaporated in vacuo with a rotatory evaporator to remove ethanol, affording 414 mg residual. The residual was dissolved in 0.01% HCl (110 mL, v/v) and stored in refrigerator at -20°C.

HPLC-DAD-MS/MS determination. The HPLC-diode array detection (DAD)-mass spectrometry (MS) determination was carried out using a Thermo Scientific Q Exactive LC-MS system, in which a Vanquish UHPLC with DAD detection was coupled to a hybrid quadrupole-orbitrap mass spectrometer equipped with an ESI (electrospray ionization) interfaces (Thermo Scientific). Chromatographic separation was carried out using a 150 mm × 4.6 mm i.d., 4 μm Agilent EC-C18 thermostated at 35 ° C. The mobile phase was composed of 0.1% TFA in water (solvent A) and

methanol (solvent B) at a flow rate of 1 mL/min. The gradient was utilized as follows: 0-5 min, 5% B; 10-15 min, 30% B; 16-26 min, 40% B; 30 min, end. Absorbance spectra were recorded every 0.2 s, between 190 and 700 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 520, 440, and 310 nm. MS data were recorded with a spray voltage of 3000 V, and the capillary temperature was 320 C, the sheath and aux gas (N_2) flow rates were 45 and 15 L/min, respectively. Aux gas heater temperature was 350 C, and fragmentation was realized with HCD (NCE@30). The determination was performed in the positive ion mode scanning from *m*/*z* 100 to 1500.

Separation with preparative HPLC. Purified extract by AB-8 resin was separated by preparative HPLC (Thermo scientific ultimate 3000 UHPLC) using a column of 30×250 mm i.d. 5 μm Venusil XBP C18(A) (Agela) via elution with a mobile phase composed by water (0.1% TFA, solvent A) and acetonitrile (0.1% TFA, solvent B) with a rate of 15 min/min. The gradient was set as: 0-2 min, 5% B; 25 min, 46% B; 30 min, 95% B; 35 min, 5% B; 40 min end. The sample (3-4 ml, ~ 3 mg/ml in 0.01% HCl) was injected manually into the injector. The UV/vis detector was set at 520 nm. The first three anthocyanins of high abundance were collected, and their purity was checked further by HPLC-DAD-MS analysis as previously described. In addition, Only the anthocyanin of retention time of 20.03 min showing the highest abundance was further lyophilized by Christ ALPHA 2-4 LSC plus (36 h) for ^{1}H NMR determination.

NMR Analysis. ^{1}H NMR determination was carried out on a Bruker AVANCE III HD 500 MHz NMR spectrometer in CD_3OD with TMS as internal standard. Sample temperature was stabilized at 25°C.

Colorimetric pH Sensing Behaviors and UV-vis Absorption Determination. The pH sensing behavior of the preparative HPLC-separated malvidin 3,5-O-diglucoside was determined in a series of tris-HCl buffers of pH 1.5, 2.5, 3.5, 4.5, 5.5, 6.8, 7.4, 8.8, 9.5, and 10.5. All the buffer concentration is 1.0 M, except for the buffer of pH 8.8 possessing a concentration of 1.5 M. Then 500 μL of ~0.13 mg/mL malvidin

3,5-O-diglucoside was added into 5 mL the buffers for UV-vis spectroscopic determination. For the AB-8-purified extract, 500 μL of extract solution (0.36 mg/mL) was added into the buffer for further determination. The photos of these mixtures were acquired before UV-vis determination.

Results and Discussion

Extraction and purification of anthocyanins from leaves of Chinese fringe flower

Our study found that anthocyanins in the leaves of Chinese fringe flower can be readily extracted from the frozen samples by ethanol or ethanol containing hydrochloric acid. The extraction efficiency will be distinctly decreased if the leaves were lyophilized and powdered,[6-8] and the reduced extraction production disfavors the anthocyanin application in daily life. The additional advantage of the direct extraction from the frozen sample is that the extraction time will be saved to 1.0-1.5 h even at ambient condition, much shorter than extraction from the lyophilized powders of leaves.

For the purification of preliminary extract from ethanol, the purification with silica gel chromatograph or preparative HPLC is not suitable for anthocyanin identification. Multiple monitoring at different wavelengths in HPLC-DAD-MS determination revealed that many impurities were eluted accompanying the desired anthocyanins. In addition, no distinct main species can be identified in MS spectra. However, a pre-purification with AB-8 resin would drastically enhance the purity of raw extract, especially when further purification with preparative HPLC was performed. In this case, there was no distinct impurity eluted simultaneously with anthocyanins according the multiple monitoring, and MS determination gave distinct signals for specific anthocyanins, which is helpful to identify these anthocyanins. Therefore, the purification of extract with AB-8 resin was essential to remove most impurities such as proteins and saccharides, which is comparable to the purification with C-18 Sep-Pak cartridge proposed by G. Vasapollo and coworkers.[13]

Anthocyanin profile in leaves of Chinese fringe flower

We used HPLC-DAD-MS/MS analysis to determine the anthocyanin composition in fringe flower leaves via recording the chromatogram at 520 nm (Figure 2a). There are 6 peaks in the chromatogram, showing retention time spanning from 11.89 to 19.09 min. Among these peaks, the most abundant compound appeared at retention time of 14.44 min, showing an area percentage of 77.22%. Positive ESI-MS determination disclosed a molecule weight of 655.1859, and its MS/MS signal of m/z 331.0804 indicated a malvidin structure in this anthocyanin (Table 1). All these inferred a structure of malvidin bearing 2 glucoses, i.e. malvidin 3,5-O-diglucoside, malvidin 3-O-glucobioside, or malvidin 5-O-glucobioside. In addition, MS determination indicated that the peak (7.22%) at 11.89 min can be assigned as delphinidin 3,5-O-diglucoside, delphinidin 3-O-gluobioside, or delphinidin 5-O-gluobioside; while the peak (11.35%) at 12.95 min can be assigned as petunidin 3,5-O-diglucoside, petunidin 5-O-glucobioside, or petunidin 3-O-glucobioside. The peak (2.01%) at 17.37 min can be easily assigned as cyanidin 3-O-glucoside according to its MS determination and UV-vis spectrum recorded by DAD detector. For the peak at 18.78 min, two anthocyanins were confirmed by MS determination, which inferred structure of peonidin 3-O-glucose and another anthocyanin species with a molecule ion m/z of 693.1624. This novel anthocyanin compound was proposed as 2H-pyran[5,6]malvidin 3-O-glucobioside according to its MS/MS signal showing two distinct signals of m/z 369.0568 and 531.1102 (Figure 2b). However, this proposed structure should be further confirmed by determining the ^{1}H- and ^{13}C NMR spectra of the purified sample.

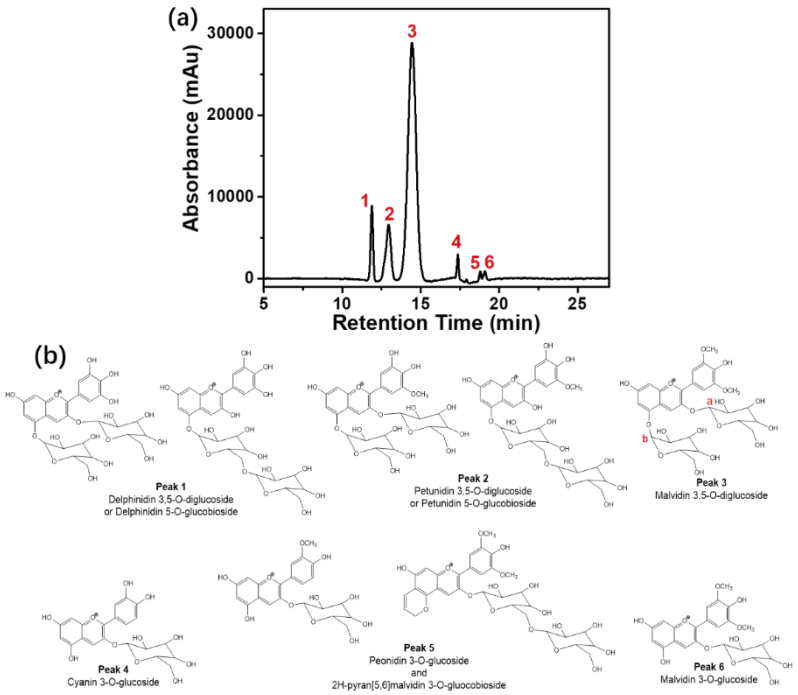

Figure 2. (a) HPLC-DAD chromatogram recorded at 520 nm for the AB-8-purified extract (ethanol, 0.01% HCl, v/v) from frozen leaves of Chinese fringe flowers; (**b**) assigned chemical structures corresponding to peaks in (**a**).

Table 1. Anthocyanins determined in leaves of Chinese fringe flower.

Peak number	Peak Area (%)	Retention time (min)	λ_{max} (nm)	$A_{460}/A_{\lambda max}$	M^+ (m/z)	MS^2 (M^+-X, m/z)	Assignment
1	7.22	11.89	522	0.11	627.1548	303.0493	dp 3,5-O-diglucoside or dp 5-O-glucobioside
2	11.35	12.95	524	0.06	641.1704	367.0649	pt 3,5-O-diglucoside or pt 5-O-glucobioside
3	77.22	14.44	524	0.229	655.1859	331.0804	mv 3,5-O-diglycoside
4	2.01	17.37	514	0.738	449.1054	287.0544	cy 3-glucoside
5	0.78	18.78	516	0.918	463.1218	301.0700	pn 3-O-glucoside
					693.1624	369.0568 531.1102	2H-pyran[5.6]malvidin 3-O-glucobioside
6	1.42	19.09	529	1.226	493.1332	331.0804	mv 3-O-glucoside

dp=delphinidin; pt=petunidin; mv=malvidin; cy=Cyanidin; pn=peonidin.

Harborne hypothesis suggests that anthocyanins with glycosidic substitutions at C-3 position normally exhibit a ratio of absorbance from 400-460 nm to that at absorption maximum (~520 nm), $A_{400\text{-}460}/A_{520}$, almost twice or even larger than that of anthocyanins with glycosidic substitutions at C-5 or C-3,5 positions.[14,15] Therefore, measuring ratio $A_{400\text{-}460}/A_{520}$ is helpful to discriminate C-3-substituted anthocyanins from C-5-subsitued and C-3,5-disubstitued anthocyanins. Taking the advantage of HPLC-DAD-MS method, the absorption spectra of these peaks have been obtained. According to known anthocyanins library and the determined ratios of $A_{400\text{-}460}/A_{520}$,

anthocyanins corresponding to peaks 4, 5 and 6 can be assigned as cyanidin 3-glucoside, pn 3-O-glucoside, and malvidin 3-O-glucoside (Table 1), besides MS signal of m/z 693.1624. These anthocyanins show $A_{400\text{-}460}/A_{520}$ ratios much larger than those for anthocyanins corresponding to peaks 1, 2 and 3. The smaller $A_{400\text{-}460}/A_{520}$ ratios for anthocyanins corresponding to peaks 1, 2 and 3 inferred that these anthocyanins might be substituted by glycoside at C-3,5- or C-5- position, i.e., 3,5-O-diglucosides or 5-O-glucobiosides (Table 1). Therefore anthocyanin correlated to peak 3 can be assigned as malvidin 3,5-O-diglucoside, or malvidin 5-O-glucobioside, and the structure of malvidin 3-O-glucobioside can be excluded.

Preparative HPLC separation and ^{1}H NMR determination.

Anthocyanins separation from the purified extract has been performed with preparative HPLC using an elution procedure described above. The chromatogram showed four main peaks with retention times of 17.81, 18.95, 20.03, 20.12 and 22.57 min (Figure 3), respectively. Three samples (**A**, **B** and **C**) were collected for the initial three peaks of higher abundance. Further determination for the three samples were carried out using HPLC-DAD-MS assay. Sample **A** showed a MS signal of m/z 627.1643. This anthocyanin is exactly identical to that corresponding to peak 1 in Figure 2a. Sample **B** showed a MS signal of m/z 641.1701, which matches to anthocyanin correlated to peak 2 in Figure 2a. For sample **C** (77.43%), its MS data are almost identical to those of peak 3 in Figure 2a. With its highest abundance among all the observed anthocyanins (77.43%) via preparative HPLC, sample **C** was further lyophilized. The resulted solids were then dissolved in CD_3OD for ^{1}H NMR determination. The result revealed that there were two doublets appeared respectively at 5.38 and 5.19 ppm, which can be assigned as the signal for proton ***a*** and ***b*** in malvidin 3,5-O-diglucoside (Figure 2b). On the other hand, the ^{1}H NMR spectrum of malvidin 3-O-glucoside showed only one doublet signal at 5.38 ppm. Since the proposed malvidin 5-O-glucobioside possesses also only one doublet around 5.40 ppm. Therefore the two doublets at 5.38 and 5.19 ppm confirmed the structure of malvidin O-3,5-diglucoside for sample **C** and peak 3 in Figure 2b. Since the

preparative HPLC method did not afford enough sample for anthocyanins corresponding to peaks 1 and 2, we still can not exclude anyone of the two proposed structures for both peaks.

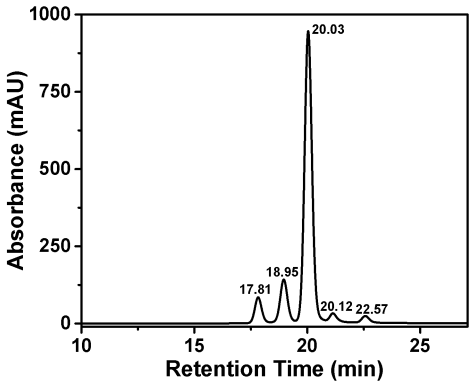

Figure 3. Preparative HPLC diagram for extraction sample after purification with AB-8 resin. Peak 1: RT, 17.81 min, 6.22%; peak 2: RT,18.95 min, 11.48%; peak 3: RT, 20.03 min, 77.43%; peak 4: RT, 20.12 min, 2.91%; peak 5: RT, 22.57 min, 1.97%.

pH sensing behavior of the separated malvidin 3,5-O-diglucoside and the AB-8-purified extract

As sample **C** (peak 3 in Figure 2) has been confirmed as malvidin 3,5-O-diglucoside, its pH sensing behavior was determined directly by measuring absorption spectra of the separated sample **C**. Therefore, solution of sample **C** (500 μL) was added into tris-HCl buffer (5 mL) followed by measurement. As shown in Figure 4c, malvidin 3,5-O-diglucoside possessed a typical absorption at 520 nm in medium of pH 1.5, showing the typical pink color. Increasing pH from 1.5 to 4.5 led to the distinct drop of absorbance at 520 nm, and this absorption finally disappeared at pH 5.5. In the meantime, the solution became almost colorless. Increasing medium pH to 6.8 resulted in a broad absorption band with a maximum at ~614 nm. In addition, a minor absorption band at 393 nm appeared, and the solution become blue. The subsequent pH increment from pH 6.8 to pH 9.5 enhanced both bands obviously, which made the solutions turned to cyan and finally green (Figure 4 a and c). However, absorbance at 614 nm underwent decrease from pH 9.5 to pH 10.5, suggesting a different structure change might occur in high alkaline medium. This result indicated that malvidin 3,5-O-diglucoside was an excellent pH indicator showing distinct color change from

pink (pH 1.5) to colorlessness (pH 5.5), to blue (pH 6.8) and finally green (pH 8.8 to pH 10.5). For the AB-8 purified extract, this anthocyanins mixture displayed a similar pH-dependent color change, except for magenta at pH 1.5 and pale pink at pH 5.5 (Figure 4b). This result indicated the pH sensing behavior of this mixture is mainly determined by malvidin 3,5-O-diglucoside. It should be noticed that the color (blue and green) observed at pH 6.8 to pH 10.5 disappeared after 15 h of exposure to ambient condition, suggesting these anthocyanins were not stable in near neutral and alkaline media.

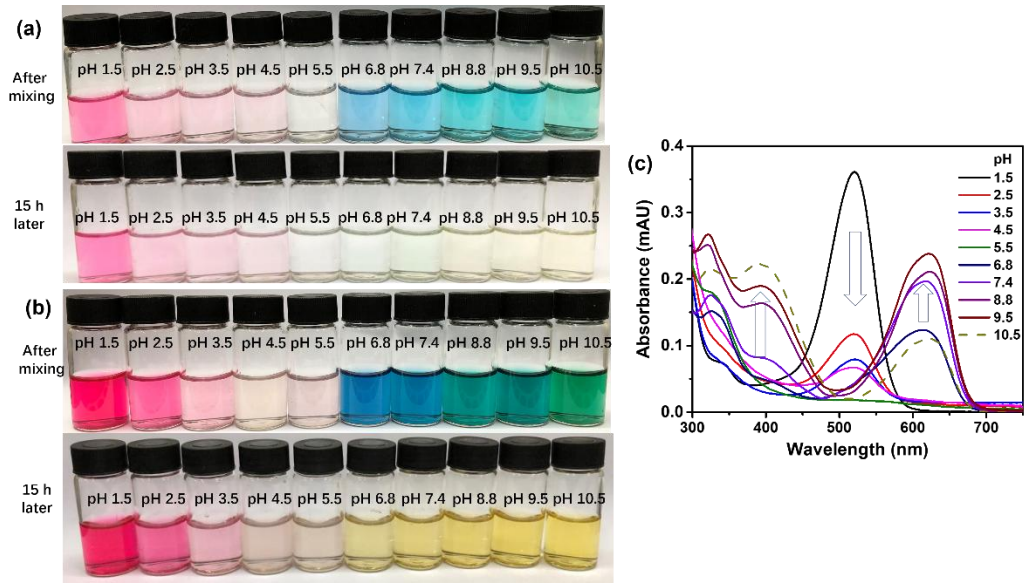

Figure 4. Photograph of malvidin 3,5-O-diglucoside (sample **C**, 0.026 mg/mL) separated via preparative HPLC (**a**) and AB-8-purified extract (**b**) in tris-HCl buffers (1.0 M) of different pH spanning 1.5 to 10.5. (**c**) Absorption spectra of solutions in (**a**).

Conclusions

In this study, anthocyanins from the frozen juvenile leaves of Chinese fringe flower was extracted with high efficiency using ethanol containing 0.1% HCl. Purifying the extract with AB-8 resin led to sample suitable for HPLC-DAD-MS/MS determination, which finally identified six anthocyanins based on MS and absorption determination. Besides cyanin 3-O-glucoside, peonidin 3-O-glucoside, malvidin 3-O-glucoside, the most abundant anthocyanin was further separated by preparative HPLC for ^{1}H NMR determination, and finally determined as malvidin 3,5-O-diglucoside. There are still two delphinidin- and petunidin-derived anthocyanins should be further determined whether they are 3,5-O-diglucoside or 5-O-glucobioside. Including the proposed

2H-pyran[5,6]malvidin 3-O-glucobioside, there are still three anthocyanins should be further confirmed in the future. Moreover, malvidin 3,5-O-diglucoside is an excellent pH indicator displaying distinct color change from pink (pH 1.5) to colorlessness (pH 5.5), to blue (pH 6.8) and finally green (pH 8.8 to pH 10.5), and it is instable in alkaline media in ambient condition. The current study revealed that leaves of Chines fringe flowers are fine resource of anthocyanins, and improving the related extraction and purification procedure might lead to stable supply of anthocyanins considering the stable leaf production of this evergreen plant.

Acknowledgements

We thank the financial support from the National Natural Sciences foundation and C
He thanks also the support from the National Talent Search Program for senior middle school students, and helpful training by Mr. Dongfan Song and Shankun Yao.

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