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Full Length Article

Analysis of Flavonoids in *Rhododendron pulchrum* Flowers by HPLC-MS/MS

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Abstract

Rhododendron plants with bright colors and long flowering periods have a high appreciation value. In addition, *Rhododendron* is rich in flavonoids and has many biological activities. *Rhododendron pulchrum* can be cultivated all over the world with rich resources, which can make up for the shortage of wild *Rhododendron* resources. Therefore, it has high development and application value. In this study, the flavonoids in *R. pulchrum* flower were analyzed and identified by HPLC-ESI-MS/Ms. The gradient elution was carried out with methanol (B)-0.1% formic acid solution (A). The flow rate was 0.7 mL min^{-1}. The column temperature was 35°C. The ESI ion source was used to collect data under the negative ion mode. Six flavonoids were identified as malva-3-arabinoside, myricetin 3-rhamnoside, quercetin-3-galactoside, quercetin-3-O-arabinoside, quercetin-3-rhamnoside and quercetin. During the flowering process, the content of flavonoids constituents reached the highest peak at the bud stage, and decreased from the bud stage to the late flowering stage. HPLC-ESI-MS/MS method can be used as a rapid detection method for the quality of *R. pulchrum* flowers. © 2020 Friends Science Publishers

Key words: *Rhododendron pulchrum* sweet; Flower; flavonoids; HPLC-MS/MS

Introduction

Flavonoids are the important natural small molecule organic compounds, abundantly existing in plants. These are polyphenolic compounds from plant secondary metabolites (Wang and Yang 2016). Their structures are a series of C6-C3-C6 compounds with 2-phenylchromone as the basic mother nucleus (Tan 2002). Natural flavonoids are mostly derivatives of this basic structure and often exist in the form of glycosides in plants (Laggoune *et al*. 2011). Flavonoid glycosides are generally miscible with water, methanol, ethanol and other solvents, but difficult to dissolve in organic solvents such as ether, chloroform, benzene and so on (Kavita *et al*. 2018; Oluwaseun *et al*. 2018). The molecular structure of flavonoids is related to its biological activity, so various flavonoids have different pharmacological properties such as strong antioxidant, antibacterial, anti-inflammatory, anticancer and anti-aging (Cao *et al*. 2003; Roy *et al*. 2014; Zhang 2017).

Rhododendron pulchrum Sweet., a semi-evergreen shrub with bright colors and long flowering periods, has a high appreciation value and can be cultivated worldwide (Editorial Committee of Flora of China, Chinese Academy of Sciences 2004). A large number of research data have shown that wild *Rhododendron* are rich in flavonoids, and have many medicinal functions such as cough expectorant, antibacterial, anti-inflammatory, analgesic, etc. (Liu *et al*. 2010; Mittal *et al*. 2012; Sun *et al*. 2019). However, due to excessive picking, the resources of wild *Rhododendron* are getting lost gradually. In order to better protect such wild plant resources, we must find a plant source containing a lot of abundant flavonoids to replace wild *Rhododendron*. Therefore, the research team turned its attention to the *R. pulchrum* that can be cultivated everywhere. In the early stage of the project through preliminary analysis of the flavonoids in the *R. pulchrum* leaves, we find that it is an alternative resource for flavonoids with great potential and has high development and application value. Therefore, in this paper further analysis and research on the flavonoids of *R. pulchrum* has been reported.

At present, multi-level liquid chromatography / mass spectrometry technology has been widely used for the qualitative and quantitative analysis of natural compounds, such as the identification of compound fragments and the rapid analysis of unknown chemical components (Liu *et al*. 2017; Sun *et al*. 2018; Li *et al*. 2018). The research team has used HPLC-MS technology to analyze and identify 5 flavonoids in *R. pulchrum* leaves (Zhang *et al*. 2012). On this basis, HPLC-ESI-MS was used to identify the flavonoids and determine the content change of flavonoids

in the flowering process in *R. pulchrum*, which will provide a theoretical basis for further application of flavonoids in *R. pulchrum*.

Materials and Methods

Experimental materials and treatments

From late march to late April 2019, the purple flowers were collected from the excellent *R. pulchrum* cultivated in Minjiang University. They were collected from four flowering stages, namely bud stage, initial flowering stage, full flowering stage, and late flowering stage. After picking in the morning, it was brought back to the laboratory and dried at 50℃. After crushing, it was passed through a 60-mesh sieve and collected for further analysis.

HPLC-MS analysis

Chromatographic conditions: Waters C18 column (4.6 mm×250 mm, 5 μm). Flow rate: 0.7 mL/min, initial column temperature is 35℃; injection volume is 20 μL, detection wavelength: 356 nm. Mobile phase A was 0.1% formic acid and mobile phase B was methanol. Elution procedure: 0 min, 31% B; 24 min, 43% B; 30 min, 50% B; 35 min, 60% B.
Mass spectrometry conditions: ion trap analyzer, electrospray ionization (ESI), negative ion detection mode, mass scan range (m/z): 200 to 700, capillary voltage: 3.5 kV, capillary outlet voltage: 100 V, drying gas temperature: 350℃, drying gas volume flow (N2): 10 L/min, atomizing gas pressure: 40 psi.

Standard curve drawing

Quercetin-3-galactoside, quercetin-3-O-arabinoside, quercetin-3-rhamnoside, and quercetin were measured by reversed-phase high-performance liquid chromatography under the above conditions, the peak area y is the ordinate, and the injection concentration x (μg/μL) is the abscissa. Linear regression analysis was performed. The standard curve equation for quercetin-3-galactoside is y = 3616171.61x-38716.31, r = 0.9998, and the linear range is 0.02~1.00 μg/μL. The standard curve equation of quercetin-3-O-arabinoside is y = 4046254.32x-36624.35, r = 0.9996, the linear range is 0.01~0.80 μg/μL. The standard curve equation of quercetin-3-rhamnoside is y = 4613441.22x-63649.51, r = 0.9995; the linear range was 0.02~1.00 μg/μL. The standard curve equation of quercetin is y = 3654072.63x-79893.53, r = 0.9995, the linear range is 0.01 ~ 0.80 μg/μL.

Preparation and determination of sample solutions

A 0.5 g of powder of *R. pulchrum* flowers was weighed accurately, then placed in a centrifuge tube and 80% ethanol solution was added to make the liquid material ratio of 120 mL/g. The mixture was put into the ultrasonic extraction system, and the extraction time was set at 60 min and the extraction temperature was 40°C. Then, the extract solution was centrifuged at a speed of 5000 r/min for 15 min (Shen *et al*. 2016). Finally, the supernatant was collected and passed through the 0.22 μm filter membrane to obtain the sample solution for analysis. The peak areas of quercetin-3-galactoside, quercetin-3-o-arabinoside, quercetin-3-rhamnoside and quercetin were calculated and substituted into the standard curve equation of the above reference, and the contents of each component were calculated.

Data analysis

Agilent Chem Station workstation and data processing software were used to analyze the total ion chromatogram and mass spectrometry data of each sample. The flavonoids in *R. pulchrum* flowers were identified by comparing the retention time of each component in *R. pulchrum* flowers, the data information of primary and secondary mass spectrometry, chemical composition database and related literature (Adam *et al*. 2004; Filip and Magda 2004; Li *et al*. 2009; Xu *et al*. 2010; Wu *et al*. 2011; Lv *et al*. 2015).

The content of each flavonoid component in the sample is determined by drawing a standard curve, the calculation formula was: "content of flavonoid component = (content of flavonoid component × total volume of flower sample extraction) × 100 / (quality of flower sample × injection volume)". Values indicating significant difference were analyzed for LSD and compared by the *t* test at P=0.05 between the bud stage and other flowering stages. The data are shown as mean ± standard deviation (SD) of three repetitions. All data were analyzed using SPSS 22.0 software.

Results

The separation of flavonoids in *Rhododendron pulchrum* flowers

HPLC-ESI-MS/Ms negative ion mode was used to analyze flavonoids in *R. pulchrum* flowers extracted with 80% ethanol. The (-) ESI-MS mass spectrometry total ion current (TIC) is shown in Fig. 1. The separation of flavonoids in *R. pulchrum* flowers were obtained by gradient elution with methanol solution as mobile phase (Fig. 2). Because the flavonoids *R. pulchrum* flowers are relatively polar and thermally unstable, ESI ion sources are used. In addition, the hydroxyl groups in the molecule easily form stable oxygen anions, so the total ion current (TIC) obtained by the analysis of negative ion mode has a better signal-to-noise ratio. The total ion chromatogram of the mass spectrum obtained is basically consistent with the UV chromatogram at 356 nm, but the baseline noise of the total ion chromatogram is large.

The identification of flavonoids in *R. pulchrum* flowers

HPLC-ESI-MS/Ms was used to analyze the molecular ion peaks in the chromatogram of flavonoids in *R. pulchrum*

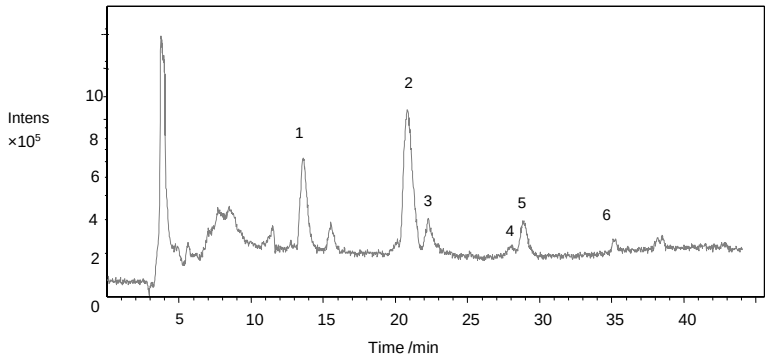

Fig. 1: TIC of the flavonoids in *R. pulchrum* flowers

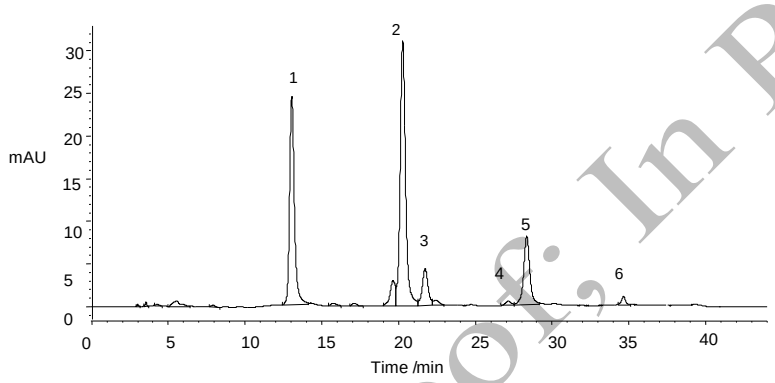

Fig. 2: HPLC chromatogram of the flavonoids in *R. pulchrum* flowers

flowers by primary and secondary ion trap mass spectrometry, respectively. By comparing the corresponding ion peak information of flavonoids components in liquid chromatography with the information of the total ion current mass spectrum, and combining with the literature reports, the chemical structure of the six main peaks separated from flavonoids in *Rhododendron pulchrum* flowers by HPLC was deduced (Table 1).

Content of flavonoid components in *R. pulchrum* flowers at different flowering stages

Results showed that the content of flavonoids in *R. pulchrum* flowers decreased from bud stage to terminal flowering stage, especially from bud stage to initial flowering stage, indicating that the content of flavonoids reached a peak before flower opening (Table 2). The analysis of LSD showed there were the significant difference in the content of flavonoids between the bud stage and other flowering stages (Table 2). Among them, the content of quercetin-3-rhamnoside was 2.123 ± 0.081 mg/g at the flower bud stage, the content decreased by 27.65% ($P<0.01$) at the initial flowering stage, then decreased by 38.44% ($P<0.01$) at the full flowering stage, and finally decreased by 43.85% ($P<0.01$) at the end of the flowering period. However, there was no significant difference in the content of Quercetin-3-O-arabinoside between the bud stage and other flowering stages ($P>0.05$). The pigment of rhododendrons is mainly composed of flavonoids compounds. Thus, with the extension of the flower opening period, the color of flowers gradually fades, which may be related to the decrease in the content of flavonoids.

Discussion

The flower composition of *Rhododendron* is very complex. The flavonoids of *Rhododendron* flowers are mainly quercetin glycosides, malvidin glycoside and myricetin glycosides (Swiderski *et al*. 2004; Zhang *et al*. 2017). Quercetin glycosides are the most widespread flavonoids in *Rhododendrons*, followed by myricetin glycosides, which are auxiliary pigments of mallow glycosides (Li *et al*. 2008). HPLC-MS/MS technology was used to preliminarily identify six flavonoids in *R. pulchrum* flowers (Zhang *et al*. 2012; Lou *et al*. 2015). This result is consistent with the chromatographic data under the same separation conditions (Mok and Lee 2013). Mallow is a purplish red pigment in plants, and *R. pulchrum* are rich in mallow-3-arabinoside, which may be the main reason for the purple color of the flowers. The main reason of *R. pulchrum* flowers' purple color may also be the co-color effect of flavonoids on anthocyanins, which stabilizes the dehydrogenation base of purple in the solution and prevents it from transforming into a colorless chalcone structure (Li *et al*. 2010; Oh *et al*. 2017).

Table 1: Mass spectrometric analysis of flavonoids in *R. pulchrum* flowers

Peak No	Retention time (min)	MS (m/z)	MS^2 (m/z)	Relative molecular mass	Compound
1	13.0	480.2 [M - H]$^-$	330.4 [(M - H)- 150]$^-$	481.3	malva -3- arabinoside
2	20.4	463.3 [M - H]$^-$	317.1 [(M - H)- 146]$^-$	464.4	myricetin 3- rhamnoside
3	22.3	463.2 [M - H]$^-$	300.9 [(M - H)- 162]$^-$	464.4	quercetin -3- galactoside
4	27.0	433.2 [M - H]$^-$	301.1 [(M - H)- 132]$^-$	434.4	quercetin -3-O-arabinoside
5	28.3	447.2 [M - H]$^-$	301.0 [(M - H)- 146]$^-$	448.4	quercetin -3- rhamnoside
6	35.2	301.1 [M - H]$^-$	301.1 [M - H]$^-$	302.2	quercetin

Table 2: Content of flavonoid components in *R. pulchrum* flowers at different flowering stages

Flowering stage	Content of flavonoid components (mg/g)			
	quercetin -3- galactoside	Quercetin-3-O-arabinoside	Quercetin-3- rhamnoside	quercetin
Bud stage	1.435 ± 0.033	0.255 ± 0.023	2.123 ± 0.081	0.414 ± 0.053
Initial flowering stage	1.074 ± 0.027a	0.184 ± 0.011	1.536 ± 0.013a	0.285 ± 0.038b
Full flowering stage	0.881 ± 0.048 a	0.158 ± 0.025	1.307 ± 0.077a	0.263 ± 0.035b
Late flowering stage	0.815 ± 0.022 a	0.147 ± 0.043	1.192 ± 0.059a	0.253 ± 0.023b

Mean ± standard deviation. Different letters indicate significant difference according to T test at 0.05 level (a: P< 0.01, b: P < 0.05)

Rhododendron not only has high appreciation value, but also high medicinal value. It is reported that flavonoids are widely distributed in *Rhododendron* plants, and they have many biological properties (Malkoc *et al*. 2016). This is the first report on antimicrobial activity of flavonoids of *R. arboreum* flowers (Sonar *et al*. 2012). The flavonoids in *Rhododendron* flower have antioxidant activities (Jung *et al*. 2007; Dede *et al*. 2019). Total favones of *R. simsii* Planch fower have a significant protective effect against cerebral ischemia-reperfusion injury (Chen *et al*. 2018). *R. luteum* is a great source of antioxidant and antitumor natural agents due to their capability of decreasing cancer cells proliferation (Demir *et al*. 2016). The results showed that the content of flavonoids was the highest in the flower bud period, and subsequently showed a significant decreasing trend. Therefore, in order to better develop and utilize the commercial utilization value of *Rhododendron* pigments and flavonoids, the flower bud period should be selected as the best harvesting time.

Conclusion

In this study, a rapid and sensitive HPLC-ESI-MS/MS method has been developed and was used to preliminarily identify six flavonoids in *R. pulchrum* flowers, which are malvacein-3-arabinoside, myricetin 3-rhamnoside, quercetin-3-galactoside, quercetin-3-O-arabinoside, quercetin-3-rhamnoside, and quercetin. This method was successfully applied to the determination of flavonoid content in *R. pulchrum* flowers at different flowering stages. In the future, the method can also be used as an efficient and reliable quality control method for other plant species.

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Author Contributions

MZ and QL planned the experiments, MZ, YZ and YH interpreted the results, MZ and BL made the write up and analyzed the data, YL made illustrations.

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