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# Antioxidant profiling of vine tea (*Ampelopsis grossedentata*): Off-line coupling heart-cutting HSCCC with HPLC–DAD–QTOF-MS/MS

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ABSTRACT

Vine tea with strong antioxidant activity is commonly consumed as healthy tea/beverage. However, detailed information about its antioxidants is incomplete. Here, off-line hyphenation of heart-cutting high-speed countercurrent chromatography (HSCCC) with high performance liquid chromatography–diode array detector–quadrupole time-of-flight tandem mass spectrometry (HPLC–DAD–QTOF-MS/MS) were described for systematic profiling antioxidants in vine tea. At first, antioxidants were rapidly screened by 1,1-diphenyl-2-picryl-hydrazyl radical–high performance liquid chromatography (DPPH–HPLC). Subsequently, stepwise HSCCC using petroleum ether–ethyl acetate–methanol–water (4:9:4:9, v/v/v/v) and (4:9:5:8, v/v/v/v) as solvent systems was optimized to fractionate and enrich antioxidants from ethyl acetate fraction of vine tea. Finally, heart-cutting mode was used to collect five interesting HSCCC fractions for HPLC–DAD–QTOF-MS/MS analysis. Desirable orthogonality between HSCCC and HPLC led to identification of fifteen antioxidant flavonoids, while four minor flavonoids were first reported in vine tea. Results showed that the developed system is efficient to comprehensively explore antioxidants from complex natural herbs.



## Introduction

*Ampelopsis grossedentata*, a plant belonging to Vitaceae family, is distributed widely in mountainous areas of southern China. The tender leaves and stems of *A. grossedentata*, also called vine tea, have been commonly consumed as healthy tea, beverage and herbal medicine for hundreds of years. Pharmaceutical investigations show that vine tea exhibits significant bioactivities of antioxidant (Hou et al., 2014; Ye, Wang, Duncan, Eigel, & O'Keefe, 2015), anti-inflammatory (Chen et al., 2015; Hou et al., 2015), antiviral (Yan & Zheng, 2009), antitumor (Zhou et al., 2014), anti-diabetic and anti-hyperglycemic properties (Chen, Wu, Zou, & Gao, 2016). Antioxidant activity has sparked interest due to its relevant to some metabolic diseases (Hu et al., 2016; Zhou et al., 2015). Flavonoids, a kind of phenolic antioxidants existed in many foods and herbs (Karabin, Hudcova, Jelinek, & Dostalek, 2015; Mattila, Hellström, Karhu, Pihlava, & Veteläinen, 2016), are found to be the major metabolites in vine tea (Du, Cai, Xia, & Ito, 2002; Du, Chen, Jerz, & Winterhalter, 2004; Gao et al., 2009; Wang, Zheng, Xu, & Zheng, 2002). Dihydromyricetin, a dihydroflavonol with higher 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) scavenging activity than butylated hydroxytoluene, accounts for around 20% (w/w) on dry weight of vine tea (Du, Chen, Jerz, & Winterhalter, 2004). Thus, most reports focused on the bioactivity assays of dihydromyricetin or crude extract of vine tea. Nevertheless, the therapeutic efficacies of natural products are achieved by combinatorial compounds rather than single or two major compounds. Moreover, some minor compounds (less than 0.1% (w/w) on dry weight of herbs) are found to present significant biological activities and have been developed as clinical drugs or lead compounds for drug discovery (e.g. paclitaxel, vincristine) (Oberlies & Kroll, 2004). Then it is important to comprehensively investigate antioxidant flavonoids in vine tea.

Compounds in natural products exist with different abundances and structures. DPPH, a paramagnetic compound with an odd electron, would capture one or more hydrogen atoms of antioxidants after spiking with them. Then higher performance liquid chromatography (HPLC) peak areas of antioxidants would reduce or disappear. Therefore, DPPH–HPLC technology has been developed to rapidly and effectively screen antioxidants from complex natural products by comparing their HPLC peak areas before and after

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spiking with DPPH (Hu et al., 2015; Qiu et al., 2012; Zhao et al., 2015). Notably, no sample pretreatment was contained. HPLC–diode array detector–quadrupole time-of-flight tandem mass spectrometry (HPLC–DAD–QTOF-MS/MS) provided ultraviolet (UV) spectra, high-resolution MS and MS/MS spectra, which were useful for structural identification even when standard compounds were not available (Abu-Reidah, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2016; Pihlava & Kurtelius, 2016; Sarah et al., 2016; Zhang et al., 2016). High-abundant compounds in vine tea (e.g. dihydromyricetin, myricetin, and myricitrin) have been analyzed and characterized by HPLC system in detail (Du et al., 2004; Gao et al., 2009). From Fig. 1a, four major peaks (I–IV) existed, in addition, some minor compounds were observed when HPLC chromatogram was zoomed for 13 folds (Fig. 1b). Then, it was necessary to develop some methods to identify minor compounds, especially for those overlapping with major compounds. Recently, two-dimensional (2D) HPLC was developed. Orthogonal columns used in 2D HPLC would provide high peak capacity and resolution for comprehensive compounds analysis (Li et al., 2016; Yang et al., 2016). However, for compounds at very low abundance, some preparative methods were still irreplaceable to enrich them.

High-speed countercurrent chromatography (HSCCC), a unique liquid–liquid partition chromatography method based on partitioning of compounds between two immiscible liquid phases with a support-free matrix, no irreversible adsorption, low risk of sample denaturation and total sample recovery, is an optimal choice for purify major compounds and enrich minor compounds from complex matrix (Esatbeyoglu, Wray, & Winterhalter, 2015; Zhao et al., 2015; Zhou et al., 2015). Major compounds were knocked out from *M. doumeri* by HSCCC, and then thirty minor antioxidants were enriched and identified by HPLC–DAD–QTOF-MS/MS (Zhao et al., 2015). To make full use of the orthogonality between HSCCC and HPLC, off-line comprehensive HSCCC × HPLC–MS/MS was developed, and eighty-five compounds were detected in *Citrus limetta* (Rodríguez-Rivera, Lugo-Cervantes, Winterhalter, & Jerz, 2014). However, heart-cutting mode is effective by only collection of interesting HSCCC effluents for HPLC analysis.

Thus, the aim of this research is to investigate the antioxidant activity and antioxidant compounds in vine tea. In order to achieve antioxidant information, total flavonoid contents and DPPH scavenging activities of three fractions with different polarities were firstly evaluated, and DPPH–HPLC was then used to rapidly screen antioxidants from the fraction with strongest activity. Co-eluted and minor antioxidants could be successfully separated, enriched and then identified by making full use of the orthogonality between HSCCC and HPLC. The resultant approach found fifteen antioxidants in ethyl acetate fraction of vine tea, and four minor ones were reported in vine tea for the first time.

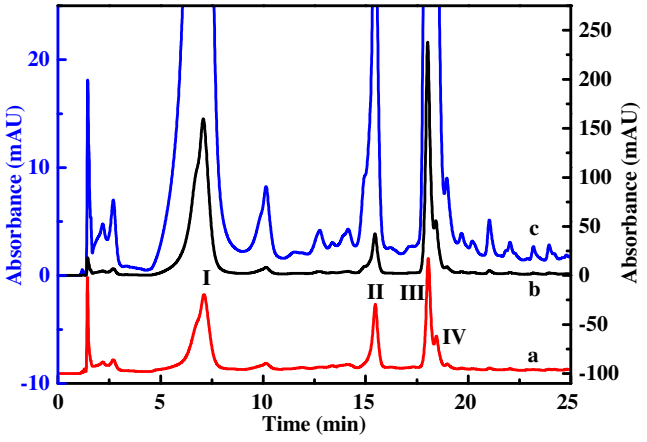


**Fig. 1.** HPLC full chromatogram of *n*-butanol fraction (a) and ethyl acetate fraction (b) of vine tea, and *y*-axis zoomed-in chromatogram (c) of ethyl acetate fraction of vine tea.

## Experimental

### *Chemicals and reagents*

All organic solvents used for extraction and separation were of analytical grade (Chemical Reagent Factory of Hunan Normal University, Hunan, China). Methanol and formic acid (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) were used for HPLC analysis. Deionized water (18.2 MΩ) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ascorbic acid (VC) and DPPH (95%) were bought from Sigma-Aldrich (Shanghai Division). Nine flavonoid standards, dihydromyricetin, myricetin, dihydroquercetin, quercetin, hesperitin, kaempferol, myricitrin, quercetin-3-*O*-α-L-rhamnoside, and kaempferol-3-*O*-α-L-rhamnoside, were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Phloridzin and phloretin with purities over 98% were purified from *Malus doumeri* by two-step HSCCC in our laboratory. Firstly, phloridzin was purified by HSCCC using ethyl acetate–*n*-butanol–methanol–25 mM ammonium acetate solution (3.5:1.5:1:4, v/v/v/v) as solvent system. Then, extruded sample from the first HSCCC separation was injected into the second HSCCC to purify phloretin using petroleum ether–ethyl acetate–methanol–water (1:2:1.5:1.5, v/v/v/v) as solvent system. Their structures were identified by UV, MS and nuclear magnetic resonance (NMR) (Zhao et al., 2015).

### *Preparation of vine tea extract*

Vine tea was collected in May 2015 from Jianghua (Hunan province of China, Northern altitude 25°15′, longitude 112°46′, altitude: 610 m). The plant material was identified using a species identification key (Editorial committee of flora of China, 1998) by Prof. Zhaoming Xie, Research Institute of Chinese Medicine, Hunan Academy of Chinese Medicine, Changsha, China. After collection, vine tea was immediately dried at 40 °C in an oven with air circulation. Dry vine tea was ground and sieved, and materials between 180 and 250 μm was used for extraction. Vine tea powder (25.0 g) was extracted with 70% ethanol (250 mL, three times) at 85 °C (each for 3 h). After filtration, organic solvent was removed from the combined extracts under reduced pressure to yield a total crude extract (11.4 g). Crude extract, suspended in water (100 mL), was then extracted successively with 3 × 100 mL of petroleum ether, ethyl acetate and *n*-butanol. After extraction, solvents were removed under reduced pressure, and then petroleum ether fraction (0.2 g), ethyl acetate fraction (1.9 g) and *n*-butanol fraction (1.4 g) were stored at 4°C for further experiments (Liang et al., 2015).

### *Determination of total flavonoid contents*

Total flavonoid contents were estimated by $NaNO_2$–$Al(NO_3)_3$ colorimetric method (Zheng, Xia, & Lu, 2015). Vine tea extract (1.0 mg/mL, 0.4 mL) was mixed with $NaNO_2$ solution (4%, 0.4 mL). After standing for 6 min, $Al(NO_3)_3$ solution (9%, 0.4 mL) were added and incubated for another 6 min at room temperature. Then NaOH solution (1.6 mol/L, 2.0 mL) was added and kept for 12 min. Finally, absorbance was measured at 510 nm against the control. Total flavonoids contents were calculated using a standard calibration of quercetin (4.0–14.0 μg/mL) and expressed as mg of quercetin equivalent (QE) per g of dry weight (DW) sample (mg