Preparative isolation, quantification and antioxidant activity of dihydrochalcones from Sweet Tea (*Lithocarpus polystachyus* Rehd.)

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ABSTRACT

Dihydrochalcones are the main active components of *Lithocarpus polystachyus* Rehd. (Sweet Tea), they are directly related to the sweet tonic beverage and traditional herb. In this work, two runs of preparative high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane/ethyl acetate/ethanol/water (1:4:3:4, v/v) were employed to separate three dihydrochalcones (phloridzin, trilobatin and phloretin) from Sweet Tea. About 6.4 mg of phloridzin, 48.4 mg of trilobatin, and 4.7 mg of phloretin with purities of 96.7%, 98.4% and 98.1% were obtained from 130 mg of the crude Sweet Tea extract. Phloridzin, trilobatin, and phloretin had effective radical scavenging activities, with IC_{50} values of 866.80, 20.16 and 179.47 μg/mL, respectively, in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method. The contents of phloridzin, trilobatin and phloretin in dried old leaves and tender leaves of tea were in the range of 10.1–18.0, 113.7–128.8, 3.6–4.3 mg/g and 9.3–9.8, 82.9–103.1, 1.9–2.5 mg/g, respectively. The results indicated that the HPLC had good precision, accuracy and repeatability for the determination of three dihydrochalcones in samples.

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1. Introduction

Lithocarpus polystachyus Rehd., a perennial shrub, is widely distributed throughout the mountainous regions of southern China. Its leaves, called Sweet Tea (ST) in southern China, can be harvested two or three times a year and have been commonly used as a sweet food source and traditional oriental medicine [1,2]. The ST extract was demonstrated to significantly decrease the levels of serum lipids, attenuate body weight gain, lower circulating leptin and insulin levels, ameliorate the state of oxidative stress, increase serum adiponectin levels, decrease circulating C-reactive protein and resistin levels, and depress the expression of PPARγ and C/EBPα in the epididymal adipose tissue of obese rats [3]. During investigations seeking anti-obesity and sweet compounds in the leaves, abundant dihydrochalcones were isolated, with contents of up to 7% [4–6]. Among them, three dihydrochalcones, trilobatin,

phloridzin, and phloretin, were identified as the major bioactive compounds in *L. polystachyus*. Phloridzin, an isomer of trilobatin, was demonstrated to inhibit glucose intestinal absorption and renal resorption, resulting in the normalization of blood glucose and overall reduction of glycaemia in animal models [7,8]. In addition, trilobatin showed strong inhibitory activity against α-glucosidase and moderate inhibitory activity against α-amylase with fewer side effects. Accumulating evidence showed that trilobatin isolated from *L. polystachyus* is a potential anti-diabetic compound [9,10]. Phloretin exhibits anti-tumor activity and inhibits human leukemia cell growth [11,12]. Phloridzin and phloretin were also effective in preventing bone resorption in an ovariectomized rat model with chronic inflammation due to their phytoestrogenic and putative anti-inflammatory activities [13].

Additionally, ST was found to be a potential rich and cheap source of sweetener for diabetic patients. However, since dihydrochalcones often exist in ST along with many other polyphenolic compounds with similar properties [1,14,15], isolation of the massive, high-purity dihydrochalcones from ST is a challenge. Full characterization requires isolation of individual constituents as a prerequisite to structure elucidation and further bioactive evaluation. In this study, an effective separation and purification of the complex matrix dihydrochalcones from ST was achieved using the high-speed counter-current chromatography (HSCCC) method. The structures of the three isolated dihydrochalcones (trilobatin, phlo-

Table 1
The K values of the three components in different two-phase solvent systems.

Solvent system (v/v)	Ratio	K Value		
		Phloridzin	Trilobatin	Phloretin
n-Hexane–ethyl acetate–ethanol–water	0:6:2:6	6.23	8.65	9.36
n-Hexane–ethyl acetate–ethanol–water	1:6:2:6	4.35	6.24	7.64
n-Hexane–ethyl acetate–ethanol–water	1:6:3:6	2.69	3.47	4.68
n-Hexane–ethyl acetate–ethanol–water	1:4:3:4	1.14	1.99	3.33
n-Hexane–ethyl acetate–ethanol–water	1:7:1:7	0.34	0.64	1.75
n-Hexane–ethyl acetate–ethanol–water	2:2:1:2	0.21	0.22	1.26
n-Hexane–ethyl acetate–ethanol–water	1:4:3:8	0.12	0.17	0.37

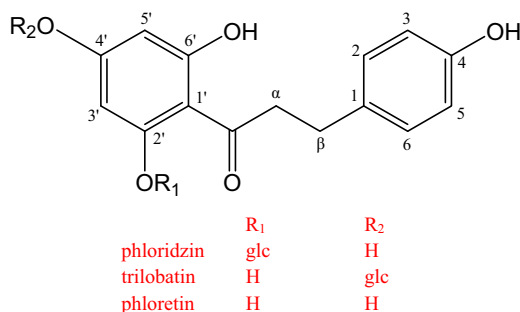|	R_1	R_2
phloridzin	glc	H
trilobatin	H	glc
phloretin	H	H

Fig. 1. Chemical structures of phloridzin (1), trilobatin (2) and phloretin (3).

ridzin and phloretin) are shown in Fig. 1. The antioxidant capacities of the purified dihydrochalcones were also determined by measuring the scavenging activities in a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. In addition, quantitative analysis of their content in tender and older tea leaves was performed.

2. Materials and methods

2.1. Chemicals and reagents

n-Hexane, ethyl acetate, methanol, and ethanol used for fraction preparation and HSCCC separation were of analytical grade, obtained from the Tianjin Chemical Factory, Tianjin, China. HPLC grade acetonitrile was obtained from Fisher Scientific, USA. Water was redistilled and passed through a 0.22 μm filter prior to use in all studies. Dimethyl sulfoxide (DMSO-d_6) was used as the solvent in nuclear magnetic resonance spectroscopy (NMR) determination.

Leaves of *L. polystachyus* Rehd. were collected from the Xuefeng Mountain, Huaihua, China in 2012 and were identified by Dr. Yinan Zheng (College of Chinese Material Medicine, Jilin Agricultural University).

2.2. Preparation of crude extract

Dried and powdered tender leaves of *L. polystachyus* (200 g, 80 mesh) were extracted by reflux in 95% ethanol (2 L) for 3 h. The extraction was repeated three times, then combining the ethanol extracts and evaporating *in vacuo* to yield a crude extract (33 g). The crude extract was suspended in distilled water (500 mL) and extracted successively with light petroleum (b.p. 60–90 °C) and ethyl acetate (500 mL × 3). The ethyl acetate extracts were combined and concentrated, and the resultant crude mixture (21 g) stored in the refrigerator for the subsequent HSCCC separation.

2.3. Selection of two-phase solvent system

HSCCC separation was performed with a Model GS-10A high-speed counter-current chromatography (Beijing Institute of New Technology Application, Beijing, China) equipped with a polyte-

trafluoroethylene multilayer coil (total volume: 230 mL) according to the method of Sun et al. [16]. In the present study, *n*-hexane/ethyl acetate/ethanol/water was used as the two-phase solvent system in HSCCC. The solvent system ratios were selected according to the partition coefficients (K) of the three target compounds in the ethyl acetate extracts. The K values of the target components were determined, according to the literature [17,18] by HPLC analysis as follows: about 5 mL of each phase of the equilibrated two-phase solvent system was added to approximately 10 mg of the ethyl acetate extract. The test tube was capped and shaken vigorously for 1 min. After the upper phase and the lower phase had separated fully, 1 mL of each layer was removed and evaporated to dryness *in vacuo* separately. The residues was dissolved in 1 mL methanol and analyzed by HPLC. The peak areas of the upper phase were recorded as X_1, the peak areas of the lower phase as X_2. The K values of the three components were obtained by the equation: $K = X_1/X_2$.

2.4. HSCCC separation procedure

The coil column was filled first with the upper phase of the solvent. While the apparatus was rotated forward at 850 rpm, the lower phase was pumped into the head end of the HSCCC coil column at a flow-rate of 1.5 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 5 mL sample solution, containing 130 mg of the ethyl acetate extracts, was injected into the injection valve. The effluent from the column outlet was continuously monitored with a UV detector at 280 nm. Fractions 1–3 of HSCCC were collected according to the elution profile and evaporated *in vacuo*. Dried fraction 1 was subjected to a second HSCCC separation performed under the same conditions. The entire HSCCC separation experiment was conducted at room temperature.

2.5. DPPH radical scavenging activity

DPPH radical scavenging activities of compounds 1–3 were determined by the method of Brand-Williams et al. [19], and Tapia et al. [20], with slight modifications. Briefly, a solution of DPPH radical in ethanol (0.2 mM) was prepared, of which 0.2 mL was added to antioxidant sample solutions in ethanol (9.8 mL) at different concentrations. The decrease in absorbance at 517 nm was determined after 30 min for all samples at 30 °C. Absorbance measurements were recorded on a SHIMADZU UV-2450 spectrophotometer throughout the experiment. Rutin and Vc were used as positive controls. All determinations were performed in triplicate. The DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity}(\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control and A_1 the absorbance of target compounds and positive control.