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Systematic separation and purification of 18 antioxidants from *Pueraria lobata* flower using HSCCC target-guided by DPPH–HPLC experiment

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ABSTRACT

A combinative method using DPPH–HPLC and HSCCC has been developed to screen and separate antioxidants from ethyl acetate fraction of *Pueraria lobata* flower. Under the target-guidance of DPPH–HPLC experiment, 18 isoflavones were isolated by HSCCC using several elution modes, such as classical elution, stepwise elution, extrusion elution and recycling elution. Following an additional clean-up step by crystallization, the purities of all the antioxidant compounds were all over 98.0% as determined by HPLC, and their structures were elucidated by UV, MS/MS and NMR analysis. Compounds glycitin (**2**), tectoridin (**4**), daidzin (**10**), 3′-hydroxydaidzein (**13**) and tectorigenin (**14**) were found to be the major antioxidants in *P. lobata* flower based on the relative peak areas in the HPLC chromatogram. Ten compounds including one new compound were first isolated from *P. lobata* flower. New compound, irisolidone-7-*O*-β-D-glucopyranpsyl-(1→6)-β-D-glucopyranoside (**6**), showed moderate DPPH antioxidant activity with IC_{50} value of 110.3 μg/ml. Results of the present study indicated that the combinative method using DPPH–HPLC and HSCCC was a speedy, efficient and reproductive technique to systematically isolate antioxidant compounds from complex natural products.

1. Introduction

Natural products have been used for medical purposes for centuries, which are good exogenous candidates to provide antioxidants. Antioxidants are considered important phytonutrients on account of their many health benefits [1]. Many natural products, rich in flavonoids and phenolic acids, have been demonstrated in previous studies to have higher antioxidant activity [2–7]. Surprisingly, in spite of the fact that the antioxidant activities of many natural products have been reported, few studies have been conducted to screen and identify antioxidants from crude extract of natural products because of their complexity [8–10].

Pueraria lobata is a common traditional Chinese medicine, which belongs to *Leguminosae* family [11]. The *P. lobata* root (Gegen) is beneficial for cardiovascular diseases [12], while the *P. lobata* flower (Gehua) is used to treat intoxication, hepatic and gastrointestinal tract lesion induced by alcohol [13]. Studies have shown that the active constituents in the extract of *P. lobata* are isoflavones, including puerarin, daidzein, genistin, and their glycosides [14–16]. Isoflavones are a group of compounds having the aromatic heterocyclic skeleton of flavan, which exhibited various biological activities, such as anti-inflammatory activity, anti-thrombotic activity, anti-hypertensive activity, anti-arrhythmic activity, etc. [17]. Moreover, the well-known beneficial effects of isoflavones in cancer prevention and osteoporosis treatment have been linked to their antioxidant and phytoestrogenic properties [18]. The previously conducted work mainly focused on the systematical isolation of compounds by repeated column chromatography over silica gel [14–16], or development of an HPLC–MS/MS method for the quantification and identification of some commercially available isoflavones [19,20]. Therefore, further studies on pharmacological and clinical effect of *P. lobata* flower necessitated the development of an efficient screening and preparative separation methods to elucidate the active compounds. Different solvent fractions of *P. lobata* flower were actively screened based on the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and its ethyl acetate fraction exhibited considerable antioxidant effect. Therefore, the ethyl acetate fraction might be a good candidate for further development as antioxidant remedies, which prompted us to perform a detailed target-guided chemical investigation on ethyl acetate fraction of *P. lobata* flower.

The conventional activity-guided fractionation of complex plant extracts is a time-consuming and labor intensive process, which often leads to the loss of activity during the isolation and purification procedures due to dilution effects or decomposition [21]. In order to avoid the above-mentioned problems, simple,

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rapid and effective methods to screen and purify potential antioxidants from complex plant extract are essential. Recently, HPLC methods coupled on-line with antioxidant activities using post-column reaction have been reported to be successfully applied to screen antioxidants from complex mixtures [22–24]. However, this method could not be adopted widely because it needs special equipment and technical skill for analysis. Afterward, thin layer chromatography (TLC) bioautography assay against DPPH radical has been developed [9,25]. However, the separation resolutions of TLC were relatively small, and sometimes several compounds might be co-eluted as one spot. Recently, a useful approach by spiking complex extract with DPPH test has been successfully developed [8,26]. As for preparative method, high-speed counter-current chromatography (HSCCC) is an optimal choice, which is a continuous liquid–liquid partition chromatography based on partitioning of compounds between two immiscible liquid phases without support matrix, no irreversible adsorption, low risk of sample denaturation, total sample recovery, large load capacity, and low cost [27], and has been successfully applied to isolate and purify many bioactive compounds from natural products [28–31]. Cao and co-works have successfully isolated six isoflavones from *P. lobata* by HSCCC [32]. In general, the split between screening methods and preparative means were disadvantageous to the fast isolation of active compounds from natural products.

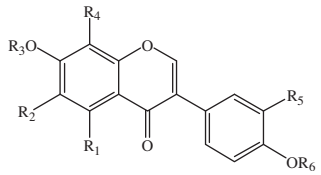
As part of our ongoing efforts on rapid screening and isolating antioxidants from natural products [8,23], the objective of the present study was to develop a systematic HSCCC method combining several elution modes for preparative isolation of antioxidants from ethyl acetate fraction of *P. lobata* flower, guided by DPPH–HPLC experiment. The results yielded 18 active isoflavones, puerarin-4′-*O*-β-D-glucopyranoside (**1**), glycitin (**2**), tectorigenin-7-*O*-β-D-xylosyl-(1→6)-β-D-glucopyranoside (**3**), tectoridin (**4**), genistein-8-*C*-β-D-glucopyranoside (**5**), irisolidone-7-*O*-β-D-glucopyranpsyl-(1→6)-β-D-glucopyranoside (**6**), puerarin (**7**), biochanin A-7-*O*-β-D-glucopyranoside (**8**), kakkalide (**9**), daidzin (**10**), 3′-methoxydaidzin (**11**), ononin (**12**), 3′-hydroxydaidzein (**13**), tectorigenin (**14**), daidzein (**15**), genistein (**16**), 3′-methoxydaidzein (**17**) and irisolidone (**18**) (Fig. 1). Among them, compound **6** was a new compound, and compounds **1**, **5**, **7**, **8**, **10**–**13** and **17** were first reported from the *P. lobata* flower. And the antioxidant activity of new compound was estimated. This is the first report on screening and separation of 18 antioxidant compounds from *P. lobata* flower by DPPH–HPLC combined with HSCCC.

2. Experimental

2.1. Chemicals and reagents

Petroleum ether (60–90 °C), ethyl acetate, *n*-butanol, 95% ethanol and methanol used for active fractions preparation and HSCCC separation were of analytical grade and obtained from Chemical Reagent Factory of Hunan Normal University (Hunan, China). Acetonitrile and acetic acid used for HPLC analysis were of chromatographic grade (Merk, Darmatadt, Germany). All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Millipore, Bedford, MA, USA). DPPH (95%) was bought from Sigma-Aldrich (Shanghai Division), and DPPH radical solutions were freshly prepared in methanol every day and kept protected from light. Multi-well plates (Greiner) and multi-well plates readers (Bio-Tek Instruments, USA) were used in the antioxidant activity experiments.

The *P. lobata* flower was collected from Guilin, Guangxi Province, China, in 2008, and identified by Prof. Mijun Peng, Key Laboratory of Hunan Forest Products and Chemical Industry Engineering, Jishou University. A voucher specimen (FPL200804) is



No.	Compounds	R_1	R_2	R_3	R_4	R_5	R_6
1	puerarin-4'-*O*-β-D-glucopyranoside	H	H	H	Glc	H	glc
2	glycitin	H	OMe	Glc	H	H	H
3	tectorigenin-7-*O*-β-D-xylosyl-(1→6)-β-D-glucopyranoside	OH	OMe	Glc^6-Xyl	H	H	H
4	tectoridin	OH	OMe	Clc	H	H	H
5	genistein-8-*C*-β-D-glucopyranoside	OH	H	H	Glc	H	H
6	irisolidone-7-*O*-β-D-glucopyranpsyl-(1→6)-β-D-glucopyranoside	OH	OMe	Glc^6-Glc	H	H	Me
7	puerarin	H	H	H	Glc	H	H
8	biochanin A-7-*O*-β-D-glucopyranoside	OH	H	Glc	H	H	Me
9	kakkalide	OH	OMe	Glc^6-Xyl	H	H	Me
10	daidzin	H	H	Glc	H	H	H
11	3'-methoxydaidzin	H	H	Glc	H	OMe	H
12	ononin	H	H	Glc	H	H	Me
13	3'-hydroxydaidzein	H	H	H	H	OH	H
14	tectorigenin	OH	OMe	H	H	H	H
15	daidzein	H	H	H	H	H	H
16	genistein	OH	H	H	H	H	H
17	3'-methoxydaidzein	H	H	H	H	OMe	H
18	irisolidone	OH	OMe	H	H	H	Me

Fig. 1. Structures of antioxidants isolated from ethyl acetate fraction of *P. lobata* flower.