



Preparative isolation of antioxidative compounds from *Dracocephalum heterophyllum* using off-line two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography guided by on-line HPLC-DPPH assay

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ABSTRACT

Traditional Tibetan medicine (TTM) has been valuable for the identification of new therapeutic leads. Nevertheless, reports about the chemical constituents of TTM are meager owing to the lack of suitable purification techniques. In this study, an off-line two-dimensional reversed-phase/hydrophilic interaction liquid chromatography (2D RP/HILIC) technique guided by on-line HPLC-DPPH has been established for the isolation of pure antioxidants from the extract of *Dracocephalum heterophyllum*. According to the chromatographic recognition outcome of the HPLC-DPPH system, the first-dimensional (1D) separation on the Megress C18 preparative column yielded 6 antioxidative fractions (61.4% recovery) from the ethyl acetate fraction (6.1 g). In the second-dimensional (2D) separation, a HILIC XAmide preparative column was employed. In total, 8 antioxidants were isolated from *D. heterophyllum* with a purity of > 95%, which indicated the efficiency of the developed method to prepare antioxidative compounds with high purity from plant extracts. In addition, this method was highly efficient for the preparation of structural analogues of the antioxidative polyphenols and could be applied for the purification of structural analogues from other resources.

1. Introduction

Dracocephalum heterophyllum is chiefly scattered across Qinghai, Tibet, Gansu, Xinjiang, Sichuan, Neimenggu and Western Sichuan [1]. It is a valuable Traditional Tibetan medicine and Traditional Chinese Medicine, with substantial therapeutic efficacy against icterohepatitis, swelling, bleeding and aching of gums, and dental ulcers [2]. It had also been reported that the herb possesses antiviral [3], antianoxic [4], antiasthmatic, antitussive and disinfectant activities [5], and its essential oil also has antimicrobial and antioxidant activities [2]. However, these bioactivity reports were based on the investigations of the crude extract, whereas its material basis and pharmacological activity are still unexplored.

Several phytochemical studies on *D. heterophyllum* have resulted in the isolation of several flavones, alkaloids, triterpenoids and phenylpropanoids [1,6,7]. Nevertheless, the complexity of the extract makes

the isolation so complicated that hardly any information has been reported about the chemical constituents responsible for the pharmacological activities. Hence, it is essential to develop a method for isolating pure compounds from *D. heterophyllum* for subsequent bioactivity research.

Even though oxidative stress is crucial for aerobic organisms, it might also be noxious under certain conditions and cause neurodegenerative diseases, inflammatory diseases, aging, tumors, and cardiovascular diseases [8–12]. Consequently, antioxidants might be vital agents for the deterrence or delaying of such diseases. Shi and co-workers [1] investigated the *in vitro* antioxidant activity of *D. heterophyllum* extracts, in which the ethyl acetate extract displayed potent free radical scavenging capacity. Nonetheless, the material basis for its antioxidative activity is still unexplored.

On-line HPLC-DPPH is a fast and highly efficient approach to discover antioxidants from natural resources. In this method, two HPLC

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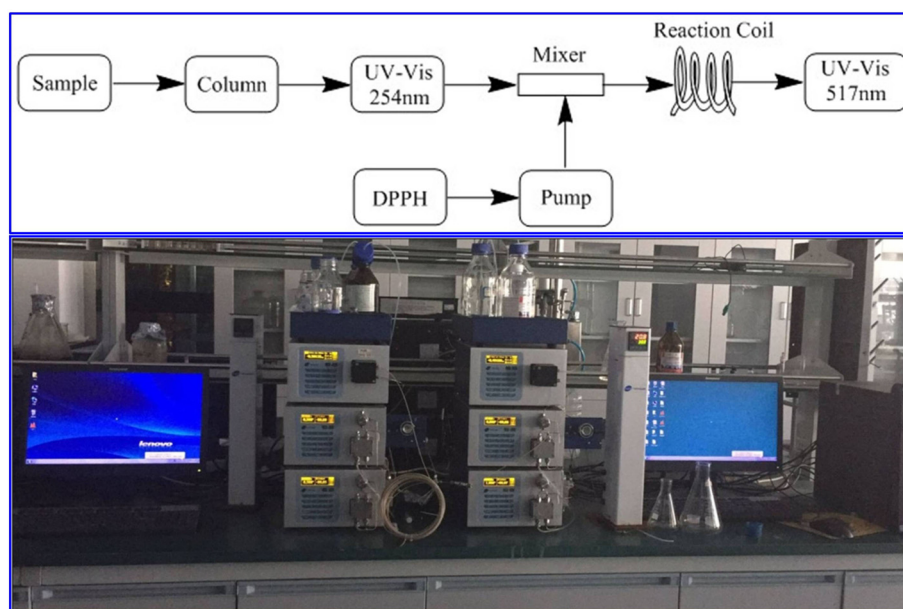


Fig. 1. The schematic diagram and actual picture of the on-line HPLC-DPPH assembly.

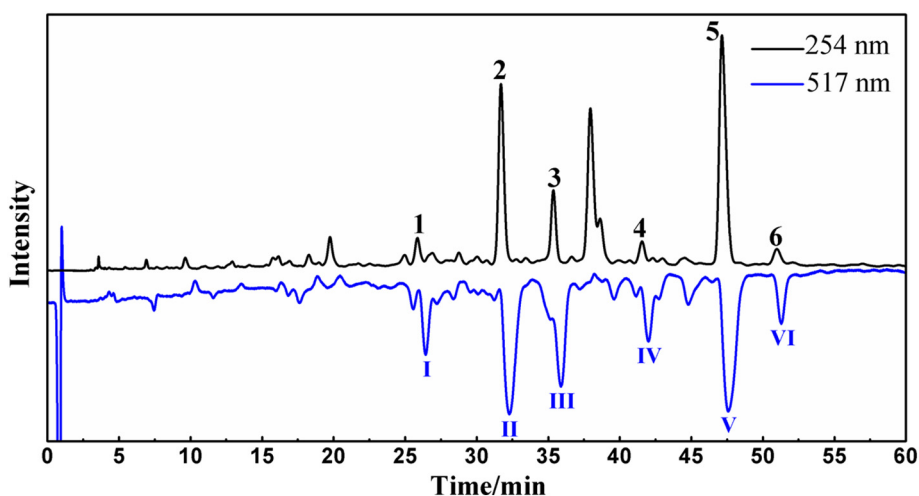


Fig. 2. The first-dimensional analytical chromatogram (254 nm) on Megress C18 analytical column and DPPH radical scavenging profile (517 nm) of ethyl acetate fractions pretreatment sample.

were connected by a mixer and reaction coil and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is added to the HPLC post-column flow and the antioxidants can be detected through a decline in absorbance at 517 nm. Previously, this technique has been applied for the discovery of antioxidants and other active compounds in many plants and foods [13–16]. Preparative HPLC is widely employed for the separation of single compounds from composite samples. It offers the advantages of superior performance, online detection, excellent reproducibility and automation [17–19]. In spite of its popularity, one-dimensional HPLC (1D HPLC) is not adequate for obtaining optimum separation for complex chemical components. To overcome this drawback, multi-dimensional HPLC methods based on different separation principles of stationary phases, have been developed. Consequently, two-dimensional HPLC (2D HPLC) has emerged as a valuable tool to isolate constituents from complex samples, owing to its superior separation selectivities and peak capacities [20–22]. Among the numerous combinations of 2D HPLC developed so far [17,19,23], reversed-phase liquid chromatography (RPLC) is extensively used, but the

orthogonality of RPLC/RPLC is low owing to the similar separation principles. In this context, RPLC coupled with hydrophilic interaction chromatography (HILIC) technique has been proved to be highly efficient for the isolation of bioactives from various natural resources [19].

Isolation of antioxidants usually depends on a microplate reader following each bioactivity-guided isolation, which is tedious and often the activity gets weaker or lost. Recently, HPLC-DPPH screening has emerged as a fast and highly efficient technique to detect antioxidants from natural resources. However, it is associated with many limitations like low resolution and peak capacity owing to only 1D analytical approach, which makes it considerably complicated to identify the compounds due to co-eluting effect. Moreover, this technique is merely appropriate for the screening of common compounds due to the structural characterization through MS only. In this work, we coupled an on-line HPLC-DPPH system with an off-line 2D RPLC/HILIC to investigate the antioxidative material basis of *D. heterophyllum* and established a new bioactivity-guided 2D separation technique for antioxidants.

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