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Medium- and High-Pressure Integrated Chromatographic Strategies for the Isolation and Purification of Free Radical Inhibitors from *Dracocephalum heterophyllum*

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Abstract: *Dracocephalum heterophyllum* has been reported as a traditional Tibetan medicine with diverse therapeutic benefits for the effective treatment of various diseases. However, only a few reports on its free radical inhibitors are limited due to its complex chemical composition and difficult isolation and purification processes. In this study, five free radical inhibitors are isolated by an integrated chromatographic method from *Dracocephalum heterophyllum*, followed by an investigation of the in vitro antioxidant activity utilizing 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Medium-pressure liquid chromatography was used for the pretreatment of the crude extract of *Dracocephalum heterophyllum* and targeted separation and purification of the free radical inhibitors using high-pressure liquid chromatography; the antioxidant peaks are recognized by the separation and purification process in combination with an online HPLC-DPPH system. Five free radical inhibitors with purity higher than 95% were obtained, namely xanthotoxol, 5-hydroxy-8-methoxypsoralen, luteolin, methyl rosmarinate, and ethyl rosmarinate. Finally, DPPH assays are performed, and their IC_{50} values for isolated compounds are 250.39 ± 13.32 μM, 26.91 ± 1.93 μM, 66.87 ± 14.33 μM, 21.99 ± 3.17 μM, and 36.96 ± 1.72 μM, respectively. This method is effective in isolating free radical inhibitors from *Dracocephalum heterophyllum*, and it has the potential to be adopted for the isolation of antioxidants from other plants of medicinal value as well.

Keywords: *Dracocephalum heterophyllum*; medium-pressure liquid chromatography; high-pressure liquid chromatography; online HPLC-1,1-diphenyl-2-picrylhydrazyl

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1. Introduction

Dracocephalum heterophyllum Benth is commonly used in Tibetan herbal medicine in ethnic areas, and its name is "Ji Zi Qing Bao" in Tibetan and "Bai Hua Zhi Zi Hua" in Chinese pharmacopeia [1]. It is a perennial herb of the Lamiaceae family, with the effect of liver diarrhea and clearing of heat [2]. It is commonly used in Tibetan medicine to treat jaundiced hepatitis and mouth ulcers. It is recorded in ancient texts that *D. heterophyllum* is effective in treating mouth ulcers and has anti-inflammatory properties [3,4]. The constituents of *D. heterophyllum* are mainly volatile oils, terpenoids, flavonoids, and phenylpropanoids [5–8]. Modern pharmacological studies have provided evidence for the pharmacological effects of *D. heterophyllum*, such as inhibition of anti-tumor, cardiac hypertrophy, anti-inflammatory, antioxidant and free radical scavenging, anti-viral, anti-hypoxia, anti-hypertensive, and cardio protection [9–12].

Antioxidants are chemicals which can regulate oxidative stress [13]. Antioxidant chemicals act through various mechanisms, such as transition metal chelation, single electron transfer (SET), and hydrogen atom transfer (HAT). Antioxidants exert their physiological effects by protecting cellular structures from damage caused by free radicals in chemical

reactions [14]. Previous data suggest that free radicals are significant in many important physiological activities, and oxidative stress determines the etiology of prevalent diseases, such as atherosclerosis, chronic renal failure, and diabetes. The common antioxidants nowadays are natural and synthetic [15]. Synthetic antioxidants are less harmful to the body, and it has become a trend to look for natural antioxidants [16].

The discovery and screening of antioxidants have shown significant progress due to online HPLC-1,1-diphenyl-2-picrylhydrazyl (HPLC-DPPH) screening. DPPH is coupled with the HPLC stream after the reduced absorption detected in the column, and the antioxidant contents at specific visible wavelengths are determined to effectively recognize free radical inhibitors in various extracts from natural products [17,18]. In traditional methods for isolating complex plant extracts, open-column chromatographic steps on polyamide, Sephadex LH-20 columns, or silica gel are required; however, they have limitations of complex processes, have irreversible adsorption, are time-consuming, and have poor reproducibility [19]. Preparative HPLC is a powerful separation and purification method, employing online detection and automatic control to purify target compounds efficiently. This approach has many advantages, including good reproducibility, high resolution, and high efficiency [20,21]. However, untreated samples that are separated directly with a high-pressure column can easily reduce the life of the stationary phase [22]. Therefore, samples are treated with medium-pressure chromatographic columns (MPCCs) before separation to extend the service life of high-pressure columns and save column costs. The traditional use of MPCCs is generally open, hindering online data monitoring. When MPCCs are applied to preparative HPLC, a variety of medium- and high-pressure columns combination could be obtained for realizing the isolation and purification of different types of natural products.

In previous studies, we successfully isolated four phenylethanols (decaffeoylverbascoside, rosmarinic acid, acteoside, and 2′-*O*-acetylplantamajoside) from *D. heterophyllum* by developing the MPCCs (silica gel × MCI GEL®CHP20P) combined with the high-pressure hilic/reversed-phase chromatography columns (Click XIon × Phenyl) [23]. Four kinds of MPCCs (silica gel × MCI GEL®CHP20P × diol × spherical C18) combined with high-pressure reversed-phase/reversed-phase chromatography columns (C18 AQ × Phenyl) were used for the separation of the four gingerols (5-methoxy-6-gingerol, 6-shogaol, 6-paradol, and diacetoxy-6-gingerdiol) [24]. Four furanocoumarins (isodemethylfuropinarine, demethylfuropinarine, alloimperatorin, and alloisoimperatorin) were isolated from the fractions of *D. heterophyllum* using three steps of MPCCs (silica gel × MCI GEL®CHP20P × diol) combined with the reversed-phase/reversed-phase (C18 AQ × 7-X10) high-pressure chromatography columns [25]. A lignin-like compound (Samwinol) was also isolated from the same subfraction with the MPCCs (silica gel × MCI GEL® CHP20P × diol × spherical C18) combined with a reversed-phase C18 high-pressure chromatography column (C18 AQ × C18 AQ) [11]. Different fraction samples cannot be separated using the same chromatographic strategy. A combination of different classes of medium- and high-pressure chromatography strategies should be developed for different compounds. For the bioactivity assessment and quality control, it is crucial for developing more reproducibility techniques to isolate and purify more free radical inhibitors from *D. heterophyllum*. This work used an online HPLC-DPPH system to recognize free radical inhibitors. An integrated medium- and high-pressure integrated chromatographic strategy were utilized to separate and purify different free radical inhibitors from *D. heterophyllum*. In addition, the antioxidant activity of isolated free radical inhibitors was tested using a DPPH assay.

2. Materials and Methods

2.1. Instrumentation, Separation Medium, and Reagents

LC-10AD HPLC device (Shimadzu Instruments, Co., Kyoto, Japan) and Essentia LC-16 (Shimadzu Instruments, Co., Shanghai, China) were used for online HPLC–DPPH systems, connected with the polyether ether ketone reaction coils (18.0 m × 0.25 mm i.d.) and a triple valve. The LC-10AD determined the DPPH screening profile, and HPLC analysis was

conducted utilizing LC-16. The medium- and high-pressure preparative isolations were performed on a nonstandard NP7000 preparative liquid chromatography and a maximum of 100 mL/min of each pump (Hanbon Science & Technology Co., Huaian, China). Agilent 6500 Q-TOF mass spectrometer acquired HRESI-MS spectra of samples. The Bruker Avance (600 MHz) was utilized to obtain ^{1}H and ^{13}C NMR spectra (Bruker, Karlsruhe, Germany). UV absorbance values for the DPPH assays were obtained by employing a Readmax 1900 microplate reader (Flash, Co., Shanghai, China). MCI GEL®CHP20P (120 μm) was acquired from Mitsubishi Chemical Corporation (Kyoto, Japan). The silica was obtained from Qingdao Ocean Chemical Corporation (Qingdao, China). Analytical and preparative columns (ReproSil-Pur C18 AQ) were provided by Maisch Corporation (Munich, Germany). ACCHROM Corporation (Beijing, China) supplied the Click XIon analytical and preparative columns. Sigma-Aldrich (Steinheim, Germany) provided the DPPH standard substance. Dichloromethane (CH_2Cl_2), methanol (MeOH), and acetonitrile (ACN) were obtained from Kelon Chemical Reagent Factory (Chengdu, China). HPLC grade H_2O was prepared using a water purifier from Moore (Chongqing, China). ACN and MeOH (HPLC grade) were supplied by Kelon Chemical Reagent Factory (Chengdu, China).

2.2. Sample Extraction and Medium-Pressure Liquid Chromatography Separation

The collection of the whole *D. heterophyllum* herb was carried out from North Mountain in Huzhu, Qinghai. The authentication of the herb was carried out by Prof. Lijuan Mei (Northwest Institute of Plateau Biology). The plant sample (voucher number: nwipb-2016-10-10) was stored in the Qinghai-Tibet Plateau Biological Museum. Utilizing 80.0 L MeOH, 10 kg *D. heterophyllum* (dry in the shade herb) was extracted at room temperature thrice for 12 h, each of which was then filtered (240.0 L) and concentrated to 5.0 L, and 1.5 kg of silica gel (amorphous) was mixed with the extract and dried at 40 °C. The final 2.8 kg of a dried mixture of a silica gel-sample was pretreated with an amorphous silica gel medium-pressure liquid chromatography (MPLC) column, using the mobile phases of MeOH and CH_2Cl_2 elution gradients of 0–30 min, 0% MeOH; 30–60 min, 0–100% MeOH; and 60–90 min, 100% MeOH at a 57.0 mL/min flow rate and the 65 g once loading sample. Through 43 repeated treatments, 205.5 g fraction Fr1 was obtained.

MeOH (2.0 L) was used to re-dissolve Fr1 (205.5 g); then amorphous silica (234.0 g) was added and dried at 40 °C. The pretreatment of 439.5 g of the dried mixed sample was carried out with MCI GEL®CHP20P MPCC (49 × 460 mm) utilizing a mobile phase (M.P) system of MeOH/H_2O and eluted with 0–150 min, 20–100% MeOH and 150–210 min, 100% MeOH with 57.0 mL/min flow rate followed by the recording of the elution chromatograms at 210 nm. After 8 repetitions, the fraction Fr1-3 was collected and concentrated, and 31.62 g of sample was obtained after drying and selected to elaborate the medium- and high-pressure integrated chromatographic strategies.

2.3. High-Pressure Liquid Chromatography Isolation and Purification of Free Radical Inhibitors from Fr1-3

Fraction Fr1-3 (31.6 g) was redissolved in MeOH (1.0 L), mixed with amorphous silica gel (30.0 g), and dried at 40 °C to obtain 61.6 g of silica gel mixed sample. Fr1-3 was separated using a preparative ReproSil-Pur C18 AQ column (20 × 250 mm, 5 μm). M.P A and B were HPLC-grade water and ACN, respectively. Conditions for elution were 0–60 min, 20–50% B with 19.0 mL/min flow rate followed by the recording the chromatogram at 210 nm. The single loading sample was 10 g. After 6 repeated preparations, Fr1-3-1 to Fr1-3-7 fractions were obtained. Under the recognition results of active peaks, the fractions Fr1-3-2 (100 mg), Fr1-3-3 (138 mg), Fr1-3-4 (219 mg), and Fr1-3-5 (216 mg) were selected as the target fractions.

Subsequently, the separation of Fr1-3-2 and Fr1-3-3 has also been performed on the ReproSil-Pur C18 AQ (20 × 250 mm, 5 μm) preparative column. The M.P and flow rate were identical to those used for preparing Fr1-3, and the only difference was the elution conditions, 15% acetonitrile isocratic for 60 min for Fr1-3-2 and 20% acetonitrile

isocratic for 60 min for Fr1-3-3. Finally, 2.55 mg of Fr1-3-2-1 and 5.00 mg of Fr1-3-3-1 were obtained, respectively.

Further isolation of Fr1-3-4 and Fr1-3-5 was performed on the preparative column Click XIon (20 × 250 mm, 5 μm). The M.P system was acetonitrile/water for 0–30 min, 100–85% acetonitrile at 19.0 mL/min, and 210 nm. The 16.96 mg active fraction Fr1-3-4-4 and 23.91 mg active fraction Fr1-3-5-4 were obtained, respectively. For Fr1-3-4-4 and Fr1-3-5-4, the final purification step was performed with the help of ReproSil-Pur C18 AQ while employing an ACN/water system. Fr1-3-4-4 was isocratically eluted with 18% acetonitrile for 70 min, and Fr1-3-5-4 was isocratically eluted with 22% acetonitrile for 55 min. Other conditions were the same as before. The final purification yielded 3.44 mg of Fr1-3-4-4-1, 6.85 mg of Fr1-3-4-4-2, and 8.40 mg of Fr1-3-5-4-1.

2.4. Free Radical Inhibitors Activity

The study utilized an online system of HPLC-DPPH to test the activity of Fr1-3-2-1, Fr1-3-3-1, Fr1-3-4-4-1, Fr1-3-4-4-2, and Fr1-3-5-4-1. While the analysis of the isolated antioxidants was carried out utilizing the analytical column of the ReproSil-Pur C18 AQ (4.6 × 250 mm, 5 μm), M.P A and B were HPLC-grade water and ACN, respectively. The conditions for elution were 0–60 min and 0–60% B at 1.0 mL/min and 210 nm. The DPPH (25 μg/mL) was used at a 0.8 mL/min flow rate and wavelength of 517 nm.

2.5. DPPH Assays

Appropriate amounts of Fr1-3-2-1, Fr1-3-3-1, Fr1-3-4-4-1, Fr1-3-4-4-2, and Fr1-3-5-4-1 were prepared as sample solutions over a range of (0.1, 1, 10, 50, 100, 500 μg/mL). DPPH was prepared with ethanol to 25 μg/mL concentration. The 96-well plate was added with a ratio of 3:7 sample solution and DPPH solution and incubated for 30 min in the dark, followed by the determination of the absorbance at 517 nm on a microplate reader. As a positive standard control, quercetin was utilized. The rate of DPPH radical scavenging was estimated as follows: Before calculation, the sample concentrations were converted to molar concentrations based on molecular weight.

$$\text{DPPH scavenging rate (\%)} = [1 - (A - A0)/A1] \times 100\%$$

where A, A0, and A1 were experimental, blank, and control groups absorbance, respectively.

2.6. Statistical Analysis

The IC_{50} of DPPH scavenging rate was calculated by SPSS 18.0 version (SPSS, Chicago, IL, USA). All experimental data are shown as the mean ± standard deviation in triplicate.

3. Results and Discussion

3.1. Sample Extraction and Application of Medium-Pressure Liquid Chromatography for Its Pretreatment

The proposed method is green and environment-friendly since methanol is chosen as the extraction. Air-dried *D. heterophyllum* whole grass (10.0 kg) yielded a crude sample of 1.3 kg at a 13.4% extraction yield. To eliminate polymers and sugars, the crude samples are pretreated with silica gel MPLC. Two medium-pressure columns of different sizes (49 × 100 mm and 49 × 460 mm) in the MPLC are connected into a single-column system to increase preparation efficiency and loading capacity. A baseline separation between two fractions is achieved (Figure 1A). Coarse separation of the crude sample into two fractions is easy to achieve. After 43 replicate separations, two fractions were acquired, with 205.5 g of the target fraction Fr1 (15.8% recovery).

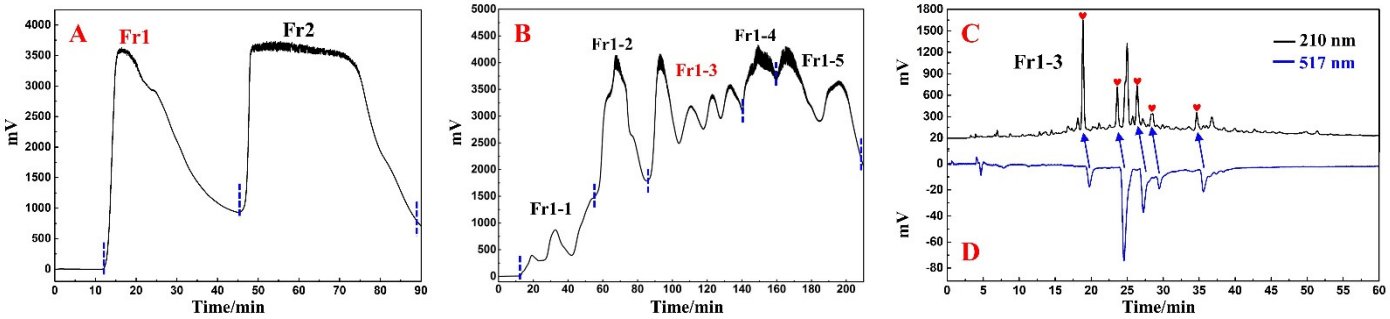

Figure 1. Chromatogram for separating *D. heterophyllum* extract (**A**) using silica gel MPLC system and the pretreatment chromatogram (**B**) of Fr1 from *D. heterophyllum* with MCI GEL®CHP20P MPLC. The analytical chromatogram (**C**) on the ReproSil-Pur C18 AQ column and DPPH radical scavenging profile (**D**) of fraction Fr1-3 sample.

The sample Fr1 also contains a large amount of chlorophyll, which may be deadened to the preparative column stationary phase, thus contaminating the column, reducing the column efficiency, and decreasing the column life. Therefore, chlorophyll removal before further preparative separations is necessary. Based on previous work in the laboratory [26], the pretreatment of Fr1 was conducted with MCI GEL®CHP20P MPCC to remove chlorophyll and enrich active components. Five fractions (Fr1-1 to Fr1-5) were collected after eight replicates (Figure 1B). To isolate more of the same categories of free radical inhibitors and to pave the way for the subsequent experiments to compare the structure-activity relationship of the same category compounds. In this paper, Fr1-3 (31.62 g) is chosen for the target free radical inhibitors separations and to evaluate the integrated chromatographic strategies.

Approximately 100 mg from fraction Fr1-3 was dissolved in MeOH (1.0 mL) followed by filtration via membrane filter (0.45 μm) to obtain a sample of fraction Fr1-3, recognized for antioxidant peaks in Fr1-3 by an online HPLC-DPPH system. The screening of antioxidant activity peaks was obtained after optimizing the analytical conditions of Fr1-3 on a ReproSil-Pur C18 AQ analytical column (Figure 1C,D). There are five main negative peaks at 517 nm in Fr1-3 (Figure 1D), indicating at least five antioxidant peaks in Fr1-3. Then, all main negative peaks at 517 nm were separated and purified.

3.2. Target Preparation of Free Radical Inhibitor of Fr1-3 with High-Pressure Liquid Chromatography

Figure 2A shows the preparative chromatogram of directly linearly amplified analytical conditions in Figure 1C of the Fr1-3 fraction. The preparation of this step subdivided the Fr1-3 fraction into a total of seven subfractions of Fr1-3-1 to Fr 1-3-7. The fraction Fr1-3 and its obtained subfractions were re-analyzed using the same chromatographic conditions to determine which subfraction the active peak was located, and the analytical comparison chromatograms were depicted in Figure 2B. The composition in subfractions Fr1-3-2 to Fr1-3-5 contains the time range in which negative peaks appear in Fr1-3. These four fractions were then used as the focal targets to purify the free radical inhibitors.

To further determine the reliability of the method, the Fr1-3-2 to Fr1-3-5 were identified on the online HPLC-DPPH system utilizing optimized conditions (Figure 3A–H). A negative peak in each fraction indicates that the target fractions were selected correctly and that at least one free radical inhibitor was present in each fraction. In the Fr1-3-2 fraction, peak 1 in Figure 3A corresponds to the negative peak I in Figure 3B, peak 2 in the Fr1-3-3 fraction in Figure 3C corresponds to negative peak II in Figure 3D, peak 3 in the Fr1-3-4 fraction in Figure 3E corresponds to negative peak III in Figure 3F, peak 3 is wrapped by two peaks, and peak 4 in the Fr1-3-5 fraction in Figure 3G corresponds to negative peak IV in Figure 3H. This is more relevant for the next step of purification and separation.

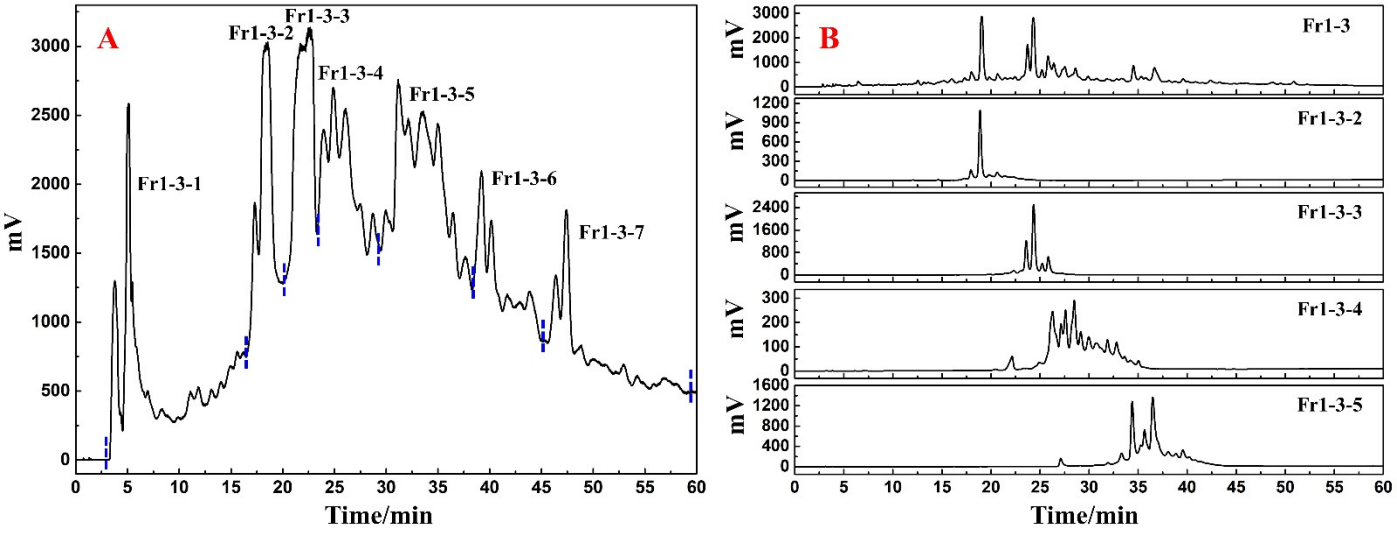

Figure 2. Preparation chromatograms (**A**) on the ReproSil-Pur C18 AQ preparative column of Fr1-3. Analytical comparison chromatograms (**B**) between fraction Fr1-3 and subfractions Fr1-3-2, Fr1-3-3, Fr1-3-4, and Fr1-3-5.

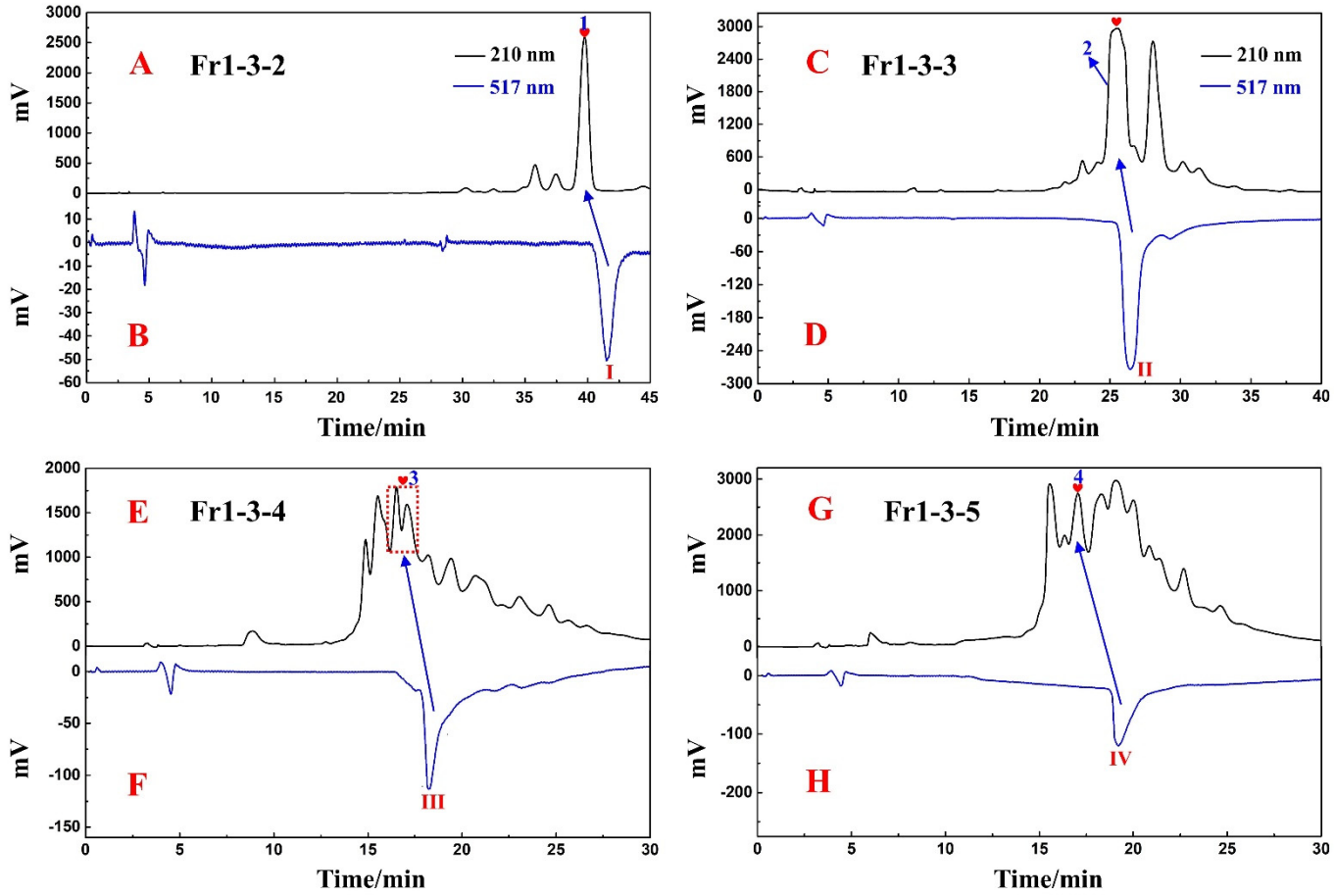

Figure 3. Chromatogram of the Fr1-3-2 verifying the DPPH inhibitory activity (**A**,**B**), Fr1-3-3 (**C**,**D**), Fr1-3-4 (**E**,**F**), Fr1-3-5 (**G**,**H**) on the ReproSil-Pur C18 AQ analytical column. Conditions for HPLC1 (**A**,**C**,**E**,**G**): M.P A: HPLC-grade water, B: acetonitrile (ACN); gradient: 20% ACN isocratic (**A**); 28% ACN (**C**,**E**); 32% ACN isocratic (**G**); wavelength: 210 nm; flow rate: 1.0 mL/min. Conditions for HPLC2 (**B**,**D**,**F**,**H**): DPPH solution flow rate: 0.8 mL/min; monitoring wavelength: 517 nm.

Figure 3A,C shows that the analytical column ReproSil-Pur C18 AQ can separate peaks 1 and 2 and other impurity peaks well; therefore, this column was selected for the next purification step. The chromatographic conditions were optimized in this work to obtain satisfactory separation curves based on the analytical conditions (Figure 3A,C). Figure 4A,C shows the optimized chromatograms; peaks 1 and 2 are well separated from the surrounding impurity peaks. However, Figure 3E,G shows that both fractions Fr1-3-4 and Fr1-3-5 were packed in a pile of peaks on the ReproSil-Pur C18 AQ analytical column with poor resolution for the next separation step. A different analytical strategy might be able to solve this problem. The separation mechanism of the hydrophilic column is different from the conventional reversed-phase column, and the hydrophilic column can be considered for the next step of purification as well as separation [27].

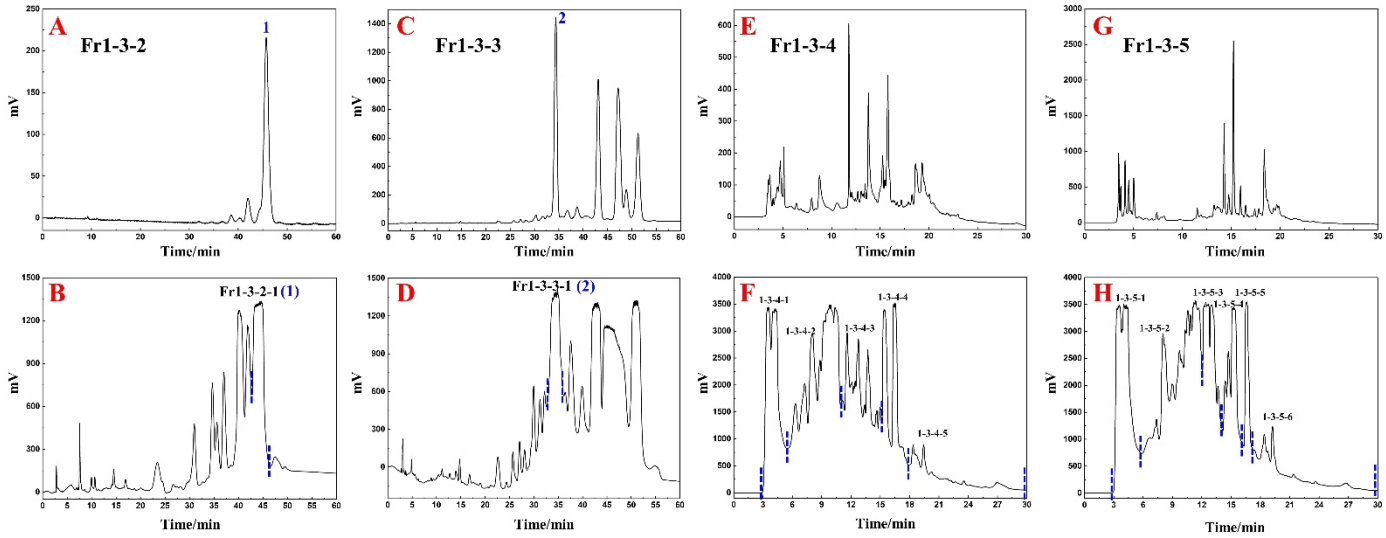

Figure 4. Fr1-3-2 and Fr1-3-3 in the analytical chromatograms (**A**,**C**) and preparative chromatograms (**B**,**D**) of the ReproSil-Pur C18 AQ column, respectively. Fr1-3-4 and Fr1-3-5 in the analytical chromatograms (**E**,**G**) and preparative chromatograms (**F**,**H**) of the Click XIon column, respectively.

Under optimized conditions, the analytical conditions were scaled up linearly for the Fr1-3-2 and Fr1-3-3, and the targeted preparation was performed on the ReproSil-Pur C18 AQ preparative column. Figure 4A,C depicts the preparative chromatograms of Fr1-3-2 and Fr1-3-3 fractions, respectively. Similar retention times for active peak 1 and active peak 2 were observed on the ReproSil-Pur C18 AQ preparative column (Figure 4B,D) and the analytical column (Figure 4A,C). Since the columns contained the same packing material, the retention of samples was the same; thus, the peaks appeared at similar times. After repeated chromatographic separations, 2.55 mg of Fr1-3-2-1 and 5.00 mg of Fr1-3-3-1 were purified from the two subfractions (Fr1-3-2 and Fr1-3-3), respectively.

For the Fr1-3-4 and Fr1-3-5, a Click XIon analytical column was chosen, and the final optimized profiles are shown in Figure 4E,G. Fr1-3-4 and Fr1-3-5 were evenly dispersed on the column rather than piled up in a mountain of peaks. Fr1-3-4 and Fr1-3-5 were suitable for this separation step with the Click XIon column. After linearly scaling up the optimized analytical conditions, the preparative chromatograms of Fr1-3-4 and Fr1-3-5 were obtained in Figure 4F,H, respectively. Fr1-3-4 was subdivided into five fractions, and Fr1-3-5 was subdivided into six fractions.

To investigate which subfractions the free radical inhibitors existed, each subfraction was re-analyzed by the ReproSil-Pur C18 AQ column under the same chromatographic conditions, and the composition of the subfractions was compared with Fr1-3-4 and Fr1-3-5, respectively. The final analytical comparison results are shown in Figure 5A–D, with peaks at 16-18 min (peak 3) corresponding to Fr1-3-4-4 and Fr1-3-4 and peaks at 17 min (peak 4) in Fr1-3-5-4 and Fr1-3-5. To determine the reliability of this result, the inverse peak recogni-

tion was performed on the online HPLC-DPPH system under the same chromatographic conditions. The recognition results are shown in Figure 5E–H, respectively. Negative peaks III (Figure 5F) and IV (Figure 5H) were present in Fr1-3-4-4 and Fr1-3-5-4, indicating that the free radical inhibitors in Fr1-3-4 and Fr1-3-5 were present in Fr1-3-4-4 and Fr1-3-5-4, respectively. These two subfractions became the target for the next purification step.

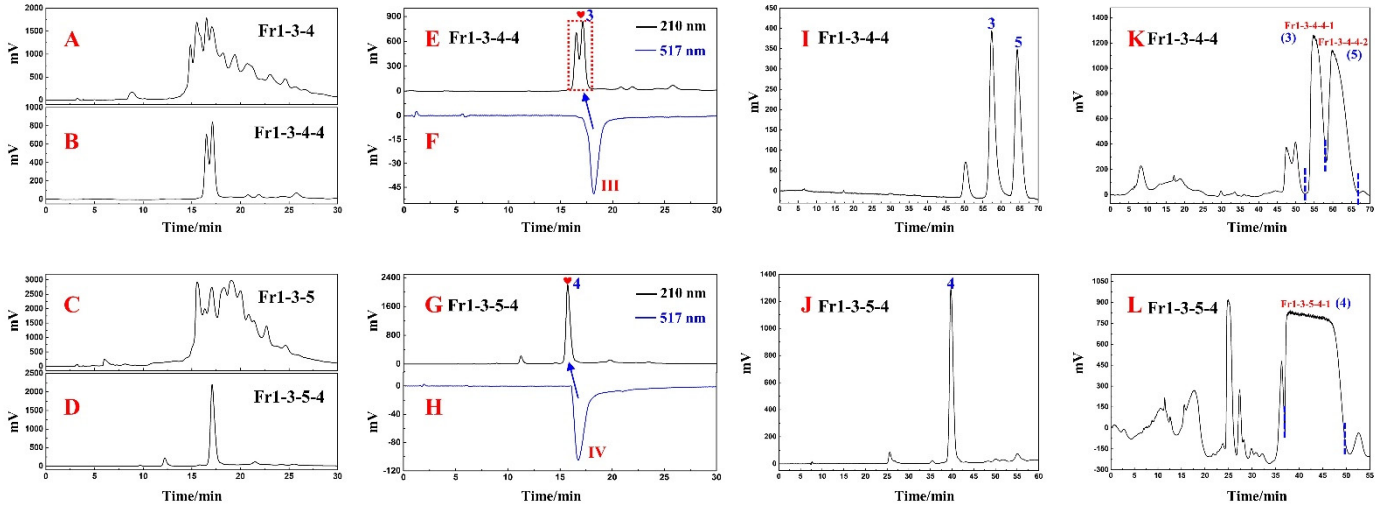

Figure 5. Analytical comparison chromatograms of Fr1-3-4 and Fr1-3-4-4 (**A**,**B**). Analytical comparison chromatograms of Fr1-3-5 and Fr1-3-5-4 (**C**,**D**). DPPH inhibitory activity verification chromatogram of the Fr1-3-4-4 (**E**,**F**), Fr1-3-5-4 (**G**,**H**) on the ReproSil-Pur C18 AQ analytical column. Fr1-3-4-4 and Fr1-3-5-4 in the analytical chromatograms (**I**,**J**) and preparative chromatograms (**K**,**L**) of the ReproSil-Pur C18 AQ column, respectively.

For Fr1-3-4-4 and Fr1-3-5-4, the chromatographic conditions were optimized to obtain the analytical chromatograms (Figure 5I,J). The previous hump was optimized (Figure 5I) into two single chromatographic peaks (peak 3 and peak 5) with good resolution. The main peak (peak 4) was well separated from the surrounding impurity peaks (Figure 5G) and conducive to the direct purification of the free radical inhibitor by linear amplification. Based on the analytical conditions, the analytical conditions were linearly amplified directly, and the final purification step for Fr1-3-4-4 and Fr1-3-5-4 was carried out using the preparative ReproSil-Pur C18 AQ column, and the preparative chromatograms are shown in Figure 5K,L. Fr1-3-4-4 was purified to obtain two free radical inhibitors (Fr1-3-4-4-1 and Fr1-3-4-4-2) with masses of 3.44 and 6.85 mg, respectively. Fr1-3-5-4 yielded a free radical inhibitor named Fr1-3-5-4-1 (8.40 mg).

3.3. Purity, Activity and Structural of Isolated Free Radical Inhibitors

The purity of the isolated Fr1-3-2-1, Fr1-3-3-1, Fr1-3-4-4-1, Fr1-3-4-4-2, and Fr1-3-5-4-1 were re-evaluated using the ReproSil-Pur C18 AQ analytical column and online HPLC-DPPH system. Figure 6A–J show that the purity of the five free radical inhibitors was much higher than 95% according to the calculation of the peak area percentage. Fr1-3-2-1, Fr1-3-3-1, Fr1-3-4-4-1, Fr1-3-4-4-2, and Fr1-3-5-4-1 were identified by HRESI-MS and NMR spectral data to elucidate the structures of the target free radical inhibitors with the literature data. Figures S1–S15 in the supplemental material showed the full spectra of the five free radical inhibitors structurally identified. The NMR and mass spectral data of Fr1-3-2-1, Fr1-3-3-1, Fr1-3-4-4-1, Fr1-3-4-4-2, and Fr1-3-5-4-1 were compared with xanthotoxol, 5-hydroxy-8-methoxypsoralen, luteolin, methyl rosmarinate, and ethyl rosmarinate, respectively (Figure 6K–O).

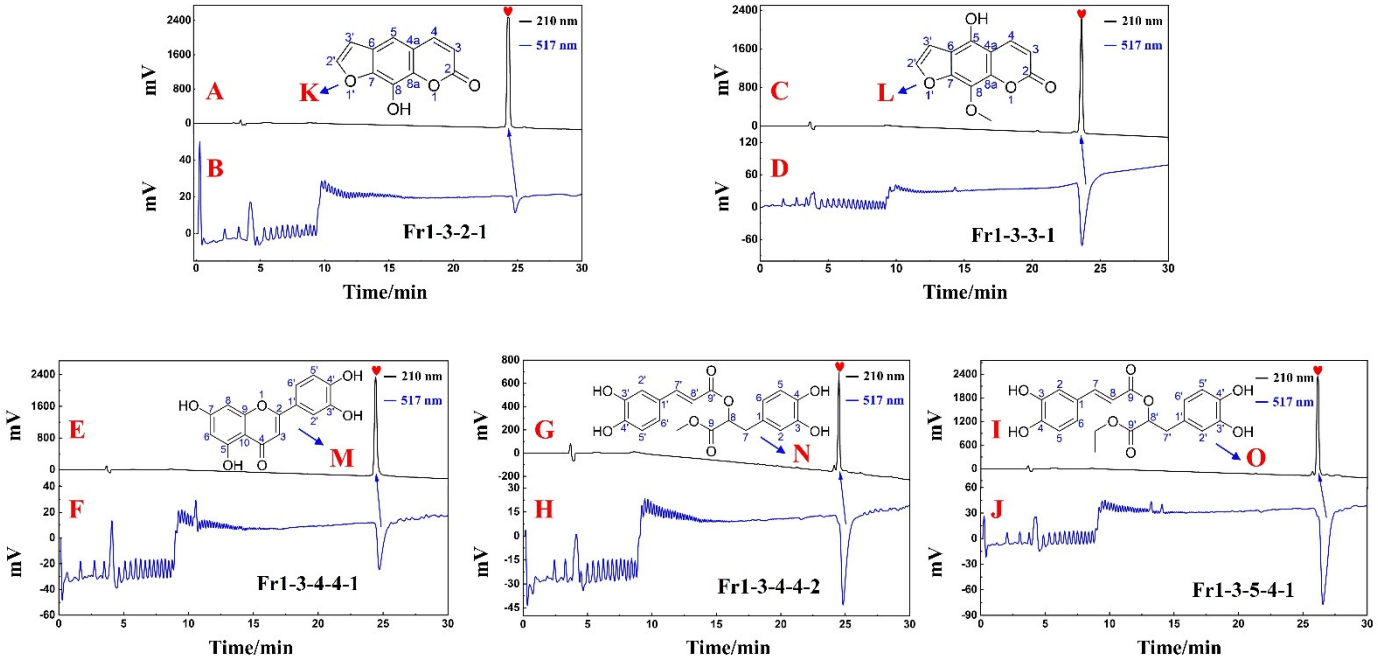

Figure 6. Purity and DPPH inhibitory activity verification chromatogram of the isolated Fr1-3-2-1 (**A**,**B**), Fr1-3-3-1 (**C**,**D**), Fr1-3-4-4-1 (**E**,**F**), Fr1-3-4-4-2 (**G**,**H**), and Fr1-3-5-4-1 (**I**,**J**). Chemical structures of Fr1-3-2-1 (**K**), Fr1-3-3-1 (**L**), Fr1-3-4-4-1 (**M**), Fr1-3-4-4-2 (**N**), and Fr1-3-5-4-1 (**O**) isolated from Fr1-3 of *D. heterophyllum*.

Fr1-3-2-1: (yellow powder, peak 1, xanthotoxol, 2.55 mg), HRESI-MS *m*/*z*: 201.0265 $[M-H]^-$. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.12 (1H, d, *J* = 9.5 Hz, 4-H), 8.07 (1H, d, *J* = 2.1 Hz, 2′-H), 7.45 (1H, s, 5-H), 7.04 (1H, d, *J* = 2.1 Hz, 3′-H), 6.40 (1H, d, *J* = 9.5, 3-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 160.1 (C-2), 147.5 (C-2′), 145.6 (C-7), 145.4 (C-4), 139.8 (C-8a), 130.1 (C-8), 125.2 (C-5), 116.3 (C-6), 113.8 (C-4a), 110.2 (C-3), 107.1 (C-3′), consistent with published data for xanthotoxol [28].

Fr1-3-3-1: (yellow powder, peak 2, 5-hydroxy-8-methoxypsoralen, 5.00 mg), HRESI-MS *m*/*z*: 231.0372 $[M-H]^-$. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 10.10 (1H, s, 5-OH), 8.17 (1H, d, *J* = 9.8 Hz, 4-H), 8.05 (1H, d, *J* = 2.3 Hz, 2′-H), 7.29 (1H, d, *J* = 2.3 Hz, 3′-H), 6.32 (1H, d, *J* = 9.8 Hz, 3-H), 4.09 (3H, s, 8-OCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 159.9 (C-2), 147.1 (C-2′), 146.2 (C-7), 141.2 (C-4), 139.9 (C-8a), 139.6 (C-8), 125.5 (C-5), 114.8 (C-6), 112.4 (C-4a), 107.1 (C-3), 105.4 (C-3′), 61.1 (C-9), consistent with the literature data for 5-hydroxy-8-methoxypsoralen [29].

Fr1-3-4-4-1: (yellow needles, peak 3, luteolin, 3.44 mg), HRESI-MS *m*/*z*: 309.0478 $[M+Na]^+$. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 12.97 (1H, s, 5-OH), 10.83 (1H, s, 7-OH), 9.92 (1H, s, 3′-OH), 10.83 (1H, s, 4′-OH), 7.41 (1H, dd, *J* = 8.3, 2.1 Hz, 6′-H), 7.39 (1H, d, *J* = 2.1 Hz, 2′-H) 6.89 (1H, d, *J* = 8.3 Hz, 5′-H), 6.67 (1H, s, 3-H), 6.44 (1H, d, *J* = 1.9 Hz, 8-H), 6.19 (1H, d, *J* = 1.9 Hz, 6-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 181.7 (C-4), 164.1 (C-7), 163.9 (C-2), 161.5 (C-5), 157.3 (C-9), 149.7 (C-4′), 145.7 (C-3′), 121.5 (C-1′), 119.0 (C-6′), 116.0 (C-5′), 113.4 (C-2′), 103.7 (C-10), 102.9 (C-3), 98.8 (C-6), 93.8 (C-8). The data supported the compound's identity as luteolin [30].

Fr1-3-4-4-2: (yellow powder, peak 5, methyl rosmarinate, 6.85 mg), HRESI-MS *m*/*z*: 397.1002 $[M+Na]^+$. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 9.66 (1H, s, 4′-OH), 9.16 (1H, s, 3′-OH), 8.81 (1H, s, 4-OH), 8.75 (1H, s, 3-OH), 7.48 (1H, d, *J* = 15.8 Hz, 7′-H), 7.06 (1H, d, *J* = 1.8 Hz, 2′-H), 7.02 (1H, dd, *J* = 8.2, 1.8 Hz, 6′-H), 6.77 (1H, d, *J* = 8.2 Hz, 5′-H), 6.65 (1H, d, *J* = 1.7 Hz, 2-H), 6.64 (1H, d, *J* = 8.0 Hz, 5-H), 6.50 (1H, dd, *J* = 8.0, 1.7 Hz, 6-H), 6.26 (1H, d, *J* = 15.9 Hz, 8′-H), 5.12 (1H, dd, *J* = 7.5, 5.1 Hz, 8-H), 3.63 (1H, s, 9-OCH_3), 2.96 (2H, m, 7-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 169.9 (C-9), 165.9 (C-9′), 148.8 (C-4′), 146.4 (C-7′), 145.6 (C-3′), 145.0 (C-3), 144.1 (C-4), 126.6 (C-1), 125.2 (C-1′), 121.7 (C-6′), 120.1 (C-6), 116.7 (C-2), 115.8 (C-5′),

115.4 (C-5), 115.0 (C-2′). 112.8 (C-8′), 72.8 (C-8), 52.0 (C-10), 36.2 (C-7). The compound was identified as methyl rosmarinate [31].

Fr1-3-5-4-1: (yellow powder, peak 4, ethyl rosmarinate, 8.40 mg,), HRESI-MS *m*/*z*: 411.1157 $[M+Na]^+$. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 9.65 (1H, s, 4′-OH), 9.15 (1H, s, 4-OH), 8.80 (1H, s, 3′-OH), 8.74 (1H, s, 3-OH), 7.49 (1H, d, *J* = 15.8 Hz, 7-H), 7.06 (1H, d, *J* = 2.0 Hz, 2-H), 7.02 (1H, d, *J* = 8.2, 2.0 Hz, 6-H), 6.77 (1H, d, *J* = 8.2 Hz, 5-H), 6.66 (1H, d, *J* = 2.0 Hz, 2′-H), 6.64 (1H, d, *J* = 7.9 Hz, 5′-H), 6.51 (1H, dd, *J* = 7.9, 2.0 Hz, 6′-H), 6.27 (1H, d, *J* = 15.8 Hz, 8-H), 5.08 (1H, dd, *J* = 7.0, 5.8 Hz, 8′-H), 4.09 (2H, q, *J* = 6.9 Hz, 10-H), 3.44 (1H, dd, *J* = 14.0, 7.0 Hz, 7′a-H), 2.95 (1H, t, *J* = 14.0, 5.8 Hz, 7′b-H), 1.14 (3H, t, *J* = 14.3, 6.9 Hz, 11-H), ^{13}C NMR (151 MHz, DMSO-d_6) δ: 169.4 (C-9′), 165.9 (C-9), 148.7 (C-4), 146.3 (C-7), 145.6 (C-3),145.0 (C-3′), 144.1 (C-4′), 126.6 (C-1′), 125.3 (C-1), 121.7 (C-6), 120.1 (C-6′), 116.7 (C-2′), 115.8 (C-5), 115.4 (C-5′), 115.0 (C-2), 112.9 (C-8), 72.9 (C-8′), 60.8 (C-10), 36.2 (C-7′), 14.0 (C-11). The above data were compared with those reported in the literature [31] and were consistent with ethyl rosmarinate.

A slight modification was conducted in the DPPH assay to quantify the antioxidant activity of the isolated free radical inhibitors [32], the DPPH radicals scavenging rate by free radical inhibitors was determined, and the IC_{50} values were calculated according to the formula. Their IC_{50} values were 250.39 ± 13.32 μM for xanthotoxol, 26.91 ± 1.93 μM for 5-hydroxy-8-methoxypsoralen, 66.87 ± 14.33 μM for luteolin, 21.99 ± 3.17 μM for methyl rosmarinate, and 36.96 ± 1.72 μM for ethyl rosmarinate, respectively (Figure 7). Compared with the positive control quercetin (IC_{50} value was 10.33 μM), Fr1-3-3-1 and Fr1-3-4-4-2 were slightly less effective in free radicals scavenging than quercetin; however, the overall effect was comparable. When comparing the IC_{50} values of Fr1-3-2-1 and Fr1-3-3-1, the antioxidant activity of Fr1-3-3-1 was stronger compared with Fr1-3-2-1 because of a lower IC_{50} value since it has substituents at both C5 and C8 positions, and the methoxylation of the C5 position significantly enhances its free radicals scavenging ability. The difference in the substitution groups ultimately leads to the difference in the ability to scavenge DPPH radicals.

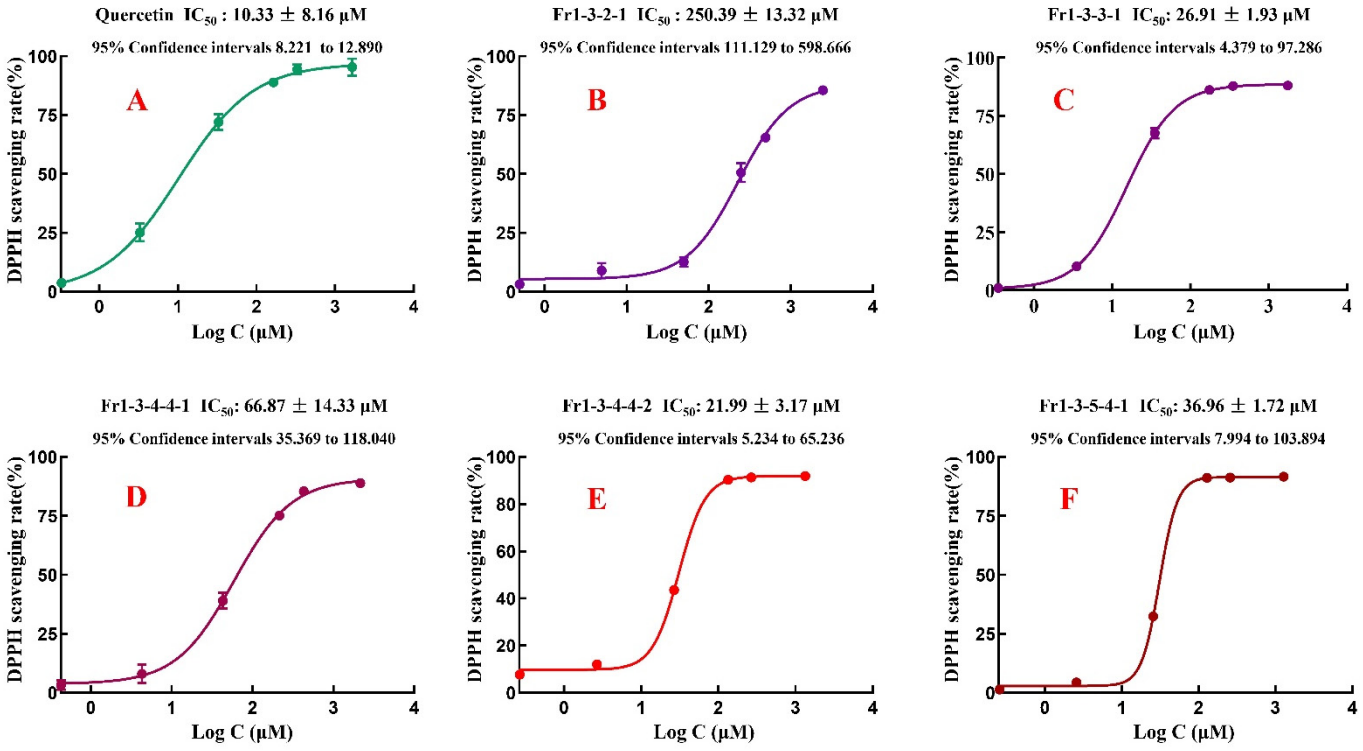

Figure 7. DPPH scavenging rate curves of quercetin (**A**), Fr1-3-2-1 (**B**), Fr1-3-3-1 (**C**), Fr1-3-4-4-1 (**D**), Fr1-3-4-4-2 (**E**), and Fr1-3-5-4-1 (**F**) and their IC_{50} values.

4. Conclusions

In this work, an online HPLC-DPPH system was used to recognize free radical inhibitors in *D. heterophyllum*, combining medium- and high-pressure integrated chromatographic methods for the separation and purification of five free radical inhibitors. Sample pretreatment was performed on the silica gel and MCI GEL®CHP20P MPCCs. Subsequently, the integration of reversed-phase and hydrophilic high-pressure liquid chromatography was applied in separating and purifying five free radical inhibitors, namely xanthotoxol, 5-hydroxy-8-methoxypsoralen, luteolin, methyl rosmarinate, and ethyl rosmarinate with purity higher than 95%. DPPH assays were also performed to evaluate these compounds' antioxidant capacity and finally concluded that these free radical inhibitors possess good antioxidant capacity. However, their mechanisms of action still need to be explored by different methods, such as ABTS, CUPRAC, and FRAP, to study various antioxidant mechanisms in cellular experiments. To the best of our knowledge, the described method lays the foundation for the isolation and purification of free radical inhibitors with good activity from various natural products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9120420/s1, Figure S1: High-resolution mass spectrum of xanthotoxol (Fr1-3-2-1). Figure S2: ^{1}H NMR Spectrum (600 MHz) of xanthotoxol (Fr1-3-2-1) (in DMSO-d_6). Figure S3: ^{13}C NMR Spectrum (151 MHz) of xanthotoxol (Fr1-3-2-1) (in DMSO-d_6). Figure S4: High-resolution mass spectrum of 5-hydroxy-8-methoxypsoralen (Fr1-3-3-1). Figure S5: ^{1}H NMR Spectrum (600 MHz) of 5-hydroxy-8-methoxypsoralen (Fr1-3-3-1) (in DMSO-d_6). Figure S6: ^{13}C NMR Spectrum (151 MHz) of 5-hydroxy-8-methoxypsoralen (Fr1-3-3-1) (in DMSO-d_6). Figure S7: High-resolution mass spectrum of luteolin (Fr1-3-4-4-1). Figure S8: ^{1}H NMR Spectrum (600 MHz) of luteolin (Fr1-3-4-4-1) (in DMSO-d_6). Figure S9: ^{13}C NMR Spectrum (151 MHz) of luteolin (Fr1-3-4-4-1) (in DMSO-d_6). Figure S10: High-resolution mass spectrum of methyl rosmarinate (Fr1-3-4-4-2). Figure S11: ^{1}H NMR Spectrum (600 MHz) of methyl rosmarinate (Fr1-3-4-4-2) (in DMSO-d_6). Figure S12: ^{13}C NMR Spectrum (151 MHz) of methyl rosmarinate (Fr1-3-4-4-2) (in DMSO-d_6). Figure S13: High-resolution mass spectrum of ethyl rosmarinate (Fr1-3-5-4-1). Figure S14: ^{1}H NMR Spectrum (600 MHz) of ethyl rosmarinate (Fr1-3-5-4-1) (in DMSO-d_6). Figure S15: ^{13}C NMR Spectrum (151 MHz) of ethyl rosmarinate (Fr1-3-5-4-1) (in DMSO-d_6).

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