Effects of ferulic acid on antioxidant activity in Angelicae Sinensis Radix, Chuanxiong Rhizoma, and their combination

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[ABSTRACT] The present study aimed at exploring different roles of the same compound in different environment, using preparative HPLC, and the significance to investigating bio-active constituents in traditional Chinese medicine (TCM) on the basis of holism. In this study, the depletion of target component ferulic acid (FA) by using preparative HPLC followed by antioxidant activity testing was applied to investigate the roles of FA in Angelicae Sinensis Radix (DG), Chuanxiong Rhizoma (CX) and their combination (GX). The antioxidant activity was performed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity testing. FA was successfully and exclusively depleted from DG, CX, and GX, respectively. By comparing the effects of the samples, it was found that FA was one of the main antioxidant constituents in DG, CX and GX, and the roles of FA were DG > CX > GX. Furthermore, the effects of FA varied at different doses in these herbs. This study provided a reliable and effective approach to clarifying the contribution of same compound in different TCMs to their bio-activities. The role of a constituent in different TCMs might be different, and a component with the same content might have different effects in different chemical environments. Furthermore, this study also suggested the potential utilization of preparative HPLC in the characterization of the roles of multi-ingredients in TCM.

[KEY WORDS] HPLC; Ferulic acid; Angelica sinensis; Ligusticum chuanxiong; Herb pair; Antioxidant activity

[CLC Number] R965

Introduction

As one of the oldest continuously practiced systems of traditional medicine in the world, herbal medicine has a history of several thousand years and their worldwide utilization has recently increased in both developing and developed countries. The World Health Organization estimated that 65%–80% of the world population used herbal medicines as the primary form of healthcare [1]. Traditional Chinese medicines (TCMs) are natural therapeutic remedies used under the guidance of traditional Chinese medical philosophy and have been prescribed by TCM practitioners in China and the Chinese community worldwide for thousands of years. Most TCMs are multi-ingredient formulae, and it is widely accepted that multiple constituents are responsible for their biological activities [2].

Angelicae Sinensis Radix (Chinese Danggui, DG) is the processed root of Angelica sinensis (Oliv.) Diels (Umbelliferae), which is widely used as one of the TCM materials in prescriptions and composite formulae to nourish blood, activate blood circulation, regulate menstruation, relieve pain, relax bowels, and so forth [3–4]. Chuanxiong (CX), the rhizome of Ligusticum chuanxiong Hort. (Umbelliferae), is one of the major clinically used cardiovascular-protective traditional Chinese herbs. Having a reputation for facilitating blood
circulation and dispersing blood stasis, this herb is commonly prescribed for the treatment of angina pectoris, cardiac arrhythmias, hypertension, and stroke \(^5\). When two herbs are used in combination, they may produce synergistic, additive, or antagonistic effects, based on TCM theory. The combination of DG and CX, called as Gui-Xiong (GX), is a well-known herbal pair recorded in many monographs \(^7\). DG, soft and moist in nature, can nourish blood, regulate menstruation, activate blood, relieve pain, resolve stasis, reduce swelling, and moisten the dryness to loosen the intestine. CX, acrid, fragrant and warm in nature, can promote the circulation of Qi and blood, dispel wind, and relieve pain. It is therefore believed that, when the two herbs are combined, they can activate, nourish, and promote blood at the same time. Moreover, DG can reduce the dryness of CX, while CX can prevent DG from greasy, thus resolving stasis without hurting healthy Qi and nourishing the blood without blood obstruction and Qi depression.

Both DG and CX contain many bio-active aromatic acids, especially ferulic acid (FA) \(^{3-11}\). FA is usually used to assess the quality of DG and CX \(^{12}\) and has been clinically used to treat angina pectoris and hypertension in China \(^{13}\). Previous investigations have suggested that it could significantly improve blood fluidity, inhibit platelet aggregation, decrease serum lipids, prevent thrombus formation, protect neurons, and exhibit anticancer and antioxidant activities \(^{14-20}\). FA also has anti-inflammatory action \(^{21}\), prevents ethanol-induced liver injury \(^{22}\), inhibits viral infections including AIDS \(^{23}\), suppresses the production of interleukin-8 (IL-8) which was the main cause of the local accumulation of neutrophils, and modulates various inflammatory reactions \(^{21}\). The content of FA in DG, CX and GX has been comparatively analyzed, and the results show that combination of DG and CX could demote the dissolution of FA \(^6\). It has been reported that FA is one of the main absorbed components in rat plasma \(^{24-25}\).

It is unknown whether the role of FA is same in DG and CX and whether its role will change when the two herbs are used in combination. In the present study, depletion of target component FA by preparative HPLC was applied to investigate the role of FA in DG, CX and GX (DG : CX = 1 : 1). Based on antioxidant activity testing. This study provided an effective approach to clarifying the contribution of same compound in different TCM preparations in relation to their bio-activities. Furthermore, this study demonstrated the utility of preparative HPLC in characterization of multi-ingredients in TCMs.

### Materials and Methods

#### Chemicals, reagents, and materials

Ferulic acid was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), with a purity of more than 98%. DPPH (1, 1-diphenyl-2-picrylhydrazyl) was purchased from Sigma-Aldrich (Shanghai, China). Methanol was HPLC-grade and obtained from Jiangsu Hanbon Science & Technology (HuaiAn, China) and deionized water was purified by an EPED Ultra-purification system (Eped, Nanjing, China). Other reagents and solutions such as ethanol and acetic acid were of analytical grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

Angelicae Sinensis Radix was collected at Min County, Gansu Province, China, in 2011. Chuanxiong Rhizoma was collected at Pengzhou, Sichuan Province, China, also in 2011. They were authenticated by Dr. YAN Hui (Department of Pharmacognosy, Nanjing University of Chinese Medicine, Nanjing, China). The voucher specimens (Nos. NJUTCM-2011081 and NJUTCM-2011082) were deposited in the Herbarium of Nanjing University of Chinese Medicine.

#### Apparatus and chromatographic conditions

**Preparative HPLC**

The depletion of FA from DG, CX or GX was performed on the Waters AutoPurification™ system (Waters Corp., Milford, MA, USA), including 2545 Binary Gradient Module, 2767 Sample Manager, column fluidics Organizer, 2489 UV/Visible Detector, Fractionlynx™ TM Software and XBridge™ prep C\(_{18}\) OBD™ column (30 mm × 150 mm, 5 μm). The mobile phase was composed of A (deionized water) and B (methanol) at the flow rate of 30 mL·min\(^{-1}\); the detection wavelength was set at 286 and 311 nm (\(λ_{\text{max}}\) of FA). The details are shown in Table 1; the two conditions differed in the gradient elution pattern.

#### Table 1 Gradient elution method for preparative HPLC

<table>
<thead>
<tr>
<th>Condition</th>
<th>Gradient Elution Method</th>
<th>Application</th>
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<tbody>
<tr>
<td>Condition I</td>
<td>0 min (20% B)—6 min (40% B)—11 min (65% B)—16 min (100% B)—18 min (20% B)—22 min (20% B)</td>
<td>DG, CX</td>
</tr>
<tr>
<td>Condition II</td>
<td>0 min (15% B)—16 min (27% B)—21 min (100% B)—23 min (15% B)—27 min (15% B)</td>
<td>GX</td>
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**Analytical HPLC-PDA analysis**

The analyses of the samples were performed on a Waters 2695 Alliance HPLC system (Waters Co., Milford, MA, USA), equipped with a quaternary pump solvent management system, an auto-sampler, and a non-line degasser. The separation was carried out on a Hypersil ODS2 column (4.6 mm × 250 mm, 5 μm) at a column temperature of 30 °C. The mobile phase was composed of A (Methanol) and B (0.1% aqueous acetic acid, \(V/V\)) with a gradient elution at the flow rate of 0.8 mL·min\(^{-1}\): 0–10 min, 10%–20% A; 10–25 min,
20%–40% A; 25–33 min, 40%–80% A; 33–34 min, 80%–10% A. Re-equilibration duration was 5 min between individual runs. A Waters 2998 photo diode array (PDA) was connected to the liquid chromatography for detection of elutes, with the full scan spectrum from 210 to 400 nm.

**Preparation of sample solutions for depletion of FA**

DG and CX were crushed into small pieces. DG, CX and GX (each 200 g) were weighed accurately and then extracted twice in 2.0 and 1.8 L of 50% aqueous ethanol (V/V), with refluxing times of 2 and 1.5 h, respectively. The decoction was combined and the solvent was removed below 60 °C till certain volume at the ratio of 1 : 1 (W/W, weight of all herbs and the extracted filtrates) under vacuum, and the samples of DG, CX and GX were obtained (1 mL was equivalent to 1.00 g of crude herbs).

The extracts were diluted with 20% aqueous methanol in ultrasonic machine. After centrifugation at 13 000 r·min⁻¹ for 10 min, the supernatants were prepared for the depletion of FA as the original solutions.

The sample solutions were injected onto the preparative HPLC system, and the injection volume was 500 μL each time. The condition I was used for the depletion of FA from DG and CX, and the condition II for the depletion of FA from GX. All fractions were collected according to the retention time of FA with 10 mL per fraction. The remnant solutions were collected, and then the solvent was removed at 60 °C under vacuum. Three new samples were obtained from the depletion solutions, i.e., DG without FA (DG − FA), CX without FA (CX − FA) and GX without FA (GX − FA).

![Fig. 1](image)

*Fig. 1 The preparative and analytical chromatograms of the depletion of FA from DG: (A1) preparative chromatogram at 286 nm; (A2) preparative chromatogram at 311 nm; (B1) analytical chromatogram of DG at 270 nm; (B2) analytical chromatogram of DG-FA at 270 nm; and (B3) analytical chromatogram of FA depleted from DG at 270 nm*

**Preparation of the DPPH solution for antioxidant activity test**

DPPH was weighed accurately and was dissolved in absolute ethanol to acquire the DPPH solution (0.1 mg·mL⁻¹). The solution was stored at 4 °C away from light.

**Preparation of sample solutions for antioxidant activity test**

FA was dissolved in DMSO (dimethyl sulfoxide, control) to obtain a stock solution (0.396 mg·mL⁻¹). In order to eliminate the effect of the solvent on the antioxidant assay, the final concentration of DMSO was fixed at 1% (V/V). The sample solutions including the original and depletion solutions were prepared by diluting the solution with 1% DMSO to a series of concentrations (every mL was equivalent...
to 10, 5, 2.5, 1.25 and 0.625 mg crude herbs, respectively).
FA was added into the depletion solutions to acquire a series of concentrations as the recovery solutions, and the concentrations of FA were the same as the extracts before the depletion. After all the sample solutions were centrifuged at 13 000 r·min⁻¹ for 10 min, the supernatants were collected and stored at 4 °C until analysis.

The depletion extracts were dissolved in DMSO to obtain three stock solutions of each crude herb (20 mg·mL⁻¹). The depletion solutions were prepared by diluting the stock solutions with 1% DMSO to two concentrations: 5 and 1.25 mg·mL⁻¹. FA was weighed accurately and was dissolved in 1% DMSO as standard stock solution. Then it was prepared by diluting the stock solutions with 1% DMSO to a series of concentrations (3.125, 12.5, 50, 100, and 200 μg·mL⁻¹). Deionized water or FA was added into the depletion solutions with a ratio of 1 : 1 (V/V) to gain a series of working solutions. After centrifugation at 13 000 r·min⁻¹ for 10 min, the supernatants were collected and stored at 4 °C until analysis.

**DPPH radical-scavenging activity assay**

The radical-scavenging activity was evaluated by a standard spectrophotometric assay using the DPPH radical in a 96-well microplate [26-27]. A 100 μL of working solutions and 100 μL of 0.1 mg·mL⁻¹ ethanolic DPPH solution were added to each well [28]. After 30 min incubation, the absorbance was determined at 517 nm (λmax). Inhibition of free radical in percent was calculated by using the following equation: \( I(\%) = \left( \frac{A_{DPPH} - A_{sample}}{A_{DPPH}} \right) \times 100 \), where \( A_{DPPH} \) is the absorption of the DPPH control solution against the blank of solvent, and \( A_{sample} \) is the absorption of the extract against the blank of sample solution [29]. Student’s \( t \)-test was used to determine the significance of differences between the different samples.

**Results and Discussion**

**The depletion results of preparative HPLC**

The preparation chromatograms for FA depletion from DG, CX and GX are showed in Figs. 1A, 2A and 3A, respectively. Analytical HPLC was used to analyze the depletion results and confirm its validity. Based on the comparison with standard substances and related literatures [30-33], the peak to be depleted was identified as the target component FA (\( t_R = 25.966 \) min), and their
purity was validated with wavelength scanning by analytical HPLC (Figs. 1B, 2B, and 3B). Their purity was over 90%, and FA was depleted from each sample. Using this method, three samples DG – FA, CX – FA and GX – FA were obtained. Thereafter, the antioxidant activities of DG, DG – FA, (DG – FA) + FA’ (sample DG – FA adding standard FA at same content as the original sample DG), CX, CX – FA, (CX – FA) + FA’ (sample CX – FA adding standard FA at same content as the original sample CX), GX, GX – FA and (GX – FA) + FA’ (sample GX – FA adding standard FA at same content as the original sample GX) were evaluated.

Fig. 3  The preparative and analytical chromatograms of the depletion of FA in GX: (A1) preparative chromatogram at 286 nm; (A2) preparative chromatogram at 311 nm; (B1) analytical chromatogram of GX at 270 nm; (B2) analytical chromatogram of GX-FA at 270 nm; (B3) analytical chromatogram of FA depleted from GX at 270 nm

Antioxidant activity

As shown in Fig. 4, the inhibitory activities of the tested samples were concentration-dependent, and the trend of antioxidant activity was as follows: DG ≈ (DG – FA) + FA’ > DG – FA, CX ≈ (CX – FA) + FA’ > CX – FA, GX ≈ (GX – FA) + FA’ > GX – FA. The inhibitory effects of the recovery samples ((DG – FA) + FA’, (CX – FA) + FA’ and (GX – FA) + FA’), in which the content of FA was the same as the original sample (DG, CX and GX), showed no obvious difference from their original samples. The results revealed that the depletion of FA reached the demand in the antioxidant activity evaluation, so the depletion samples could be used to evaluate the roles of FA in DG, CX and GX in their antioxidant activity.

The effects of all the depletion samples (DG – FA, CX – FA and GX – FA) were obviously lower than that of the corresponding original samples (DG, CX, and GX), which demonstrated that FA was one of the main antioxidant constituents in DG, CX, and GX. In addition, the effects of all the depletion samples still had certain antioxidant activity, indicating that there were other constituents contributing to the antioxidant activity in these herbs.

As shown in Fig. 5, the trends of relative inhibition ratio of FA on DPPH scavenging-activity were same in DG, CX, and GX. The effect increased as the concentration increased till 2.5 mg⋅mL⁻¹, but decreased above this concentration. The
Fig. 4 Comparing DPPH radical-scavenging activity of the original, depletion, and recovery samples (mean ± SD, n = 5). *P < 0.05, **P < 0.001, ***P < 0.001 vs the respective original solutions. Results might be related to other herbal constituents that might not show a certain effect or showed weak effect at lower s, especially below 2.5 mg·mL⁻¹, but might show a certain effect or potent effect at higher concentration. These results supported the well-known complexity of bio-active constituents in TCMs.

The main antioxidant constituents in DG are phenolic acids (such as FA and caffeic acid) and phthalides (such as Z-butylidenephthalide, Z-ligustilide, senkyunolide I and senkyunolide H); the main antioxidant constituents in CX are alkaloids (such as ligustrazine and perlolyrine) besides phenolic acids and phthalides [34-42]. Therefore, the bio-active components in GX are the combination of DG and CX; the role of a compound (such as FA) in a single herb (such as DG or CX) may differ from that in combined herb formulae (such as GX). The antioxidant constituents in DG were fewer than CX [5], and thus the role of FA in DG might be greater than that in CX. These results are also shown in Fig. 5. For further comparing the roles of FA in antioxidant activity in DG, CX, and GX, the effects of FA at different doses in DG, CX, and GX were also investigated on the basis of the depleted samples in the present study. As shown in Fig. 6, the roles of FA in DG, CX, and GX in their antioxidant activity showed in an apparent dose-dependent manner, and their dose-effect relationship curves had the same trend at the tested concentrations, which demonstrated that FA could produce a significant effect on antioxidant activity in DG, CX, and GX. The results also showed that the trends of FA action were DG > CX ≈ GX (Fig. 6), further demonstrating that the role of FA in DG was greater than that in CX and GX.

Our results revealed that the role of a constituent in different TCM preparations might be different, and a component with the same content level might have different effects in different chemical environments. Thus, the reasonable applications of TCMs need to consider their physicochemical and biological environments.

Conclusion

In the present study, a convenient and effective approach was established for studying complicated bio-active constituents of TCM with preparative HPLC followed by bioactivity evaluation. The role of the target component in different TCMs can be demonstrated by using this method. In the future, a constituent of interest could be depleted in more TCMs and more bioactivity tests could be designed for specific evaluation, providing more and
Fig. 6 Comparing the roles of FA at different doses on DPPH radical-scavenging activity in DG, CX and GX (mean ± SD, n = 5). P < 0.05, ***P < 0.001, ****P < 0.0001 vs GX: (A) each sample concentration was 10 mg·mL⁻¹; (B) each sample concentration was 2.5 mg·mL⁻¹; (C) each sample concentration was 0.625 mg·mL⁻¹; Relative inhibition ratio (%) = E_{sample - FA} • FA - E_{sample - FA}, E_{sample - FA} (DG - FA, E_{DG - FA}) and E_{DG - FA} radical-scavenging activity of the deletion samples (DG - FA, CX - FA and GX - FA), E_{sample - FA} • FA, E_{sample - FA} (DG - FA, E_{DG - FA} and E_{DG - FA}), DPPH radical-scavenging activity of the deletion samples including FA at different doses

stronger evidences for clarifying the roles of complicated bio-active constituents in TCM and the underlying mechanisms for the variability resulted from their compatibility.

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