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Bioactive components of the roots of *Salvia miltiorrhizae*: Changes related to harvest time and germplasm line

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ABSTRACT

Field-grown Danshen (*Salvia miltiorrhizae*) has been used for the preparation of phytopharmaceutical products. The changes in bioactive components in the roots of *S. miltiorrhizae* related to harvest time and germplasm were investigated in trials during 2008 and 2009. The content of bioactive components in the roots was closely related to germplasm and harvest time, resulting in different optimum harvest dates for these three Danshen germplasm varieties in order to obtain the highest content of bioactive components. As each medicinal product has its own content requirement for different bioactive compounds, the best harvest time might be identified according to the accumulation dynamics of target compounds in a selected Danshen germplasm. This may be critical for the quality control of Danshen cultivation.

1. Introduction

Danshen, the roots and rhizomes of *Salvia miltiorrhizae* Bunge, is one of the best known traditional Chinese medicines (TCM). It is a cylindrical succulent root with slight curvature. The name Danshen refers to the crimson or reddish-brown colour in the root cortex which resembles Panax. Because of its good performance and few side effects as confirmed in long-term clinical application (Hu and Tian, 2004; Liang, 2008; Su and Zhan, 2009; Yuan and Fan, 2009), Danshen has been widely used either alone or in combination with other herbal ingredients in China to treat coronary heart disease (Lam et al., 2006), neuroasthenic insomnia (Sze et al., 2005), hepatocirrhosis (Lin et al., 2006; Wu et al., 2007), cancer (Zhang and Wang, 2006), hepatitis, dysmenorrheal hypertension, bone loss and chronic renal failure (Tan et al., 2005; Yang et al., 2006).

Chemical investigation has shown that the bioactive components of Danshen are classified into two groups: hydrophilic phenolic acids such as salvianolic acid, rosmarinic acid, lithospermic acid, and caffeic acid, and lipophilic tanshinones (diterpenoid quinones) which impart the reddish-brown colour to the roots, including tanshinone IIA, tanshinone I, tanshinone IIB, and cryptotanshinone (Li, 1996; Min, 2000). Because of its pharmacological importance, demand for Danshen has increased steadily in recent years. About 20 million kg of Danshen are required for prescriptions or export from China each year (Xing and Xing, 2009). Hence, production protocols for the cultivation of high-yielding and high-quality Danshen are needed in order to meet the strong market demand for the medicinally important compounds.

An important aspect of Danshen production is the timing of harvest to maximize yields and content of desirable compounds. The root swelling stage has long been taken as the harvest time (Liao et al., 2004; Lin et al., 2008) because this provides the highest root yields and the best marketing attributes (Sun et al., 2005; Zhang et al., 2007). However, there are large variations in the quantities of the major bioactive components in this stage, resulting in different optimal harvest times (Deng et al., 2009; Yan et al., 2009; Zhao, 2009; Zhao and Zhao, 2009). In previous studies variations due to different germplasm and cultivation patterns, such as planting date and growth years, have received little interest (Li et al., 2005; Tang et al., 2006; Jiang et al., 2008), or only the yield (i.e. root dry matter) or content of single active components was considered (Wang et al., 2005; Li and Yu, 2007; Zhang et al., 2007), resulting in different harvesting dates. In addition, Danshen used in some studies was wild material from different regions, instead of cultivated varieties, and growth conditions such as germplasm, growth years and cultivation methods, were not clearly recorded, leading to inconsistent results (Hu et al., 2003; Duan et al., 2006).

It was therefore necessary to study the variations in content of major bioactive compounds in the roots of *Salvia miltiorrhizae* in relation to harvest time and germplasm. It was also important to determine the optimal harvest time of desirable compounds for growers. In order to study these factors the accumulation dynamics

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of several kinds of active components were investigated simultaneously during the root swelling stage. Three cultivated Danshen germplasm varieties were selected and planted at the same location under identical conditions to avoid the potential effects of environmental, geographical, soil and climatic conditions.

2. Materials and methods

2.1. Plant materials

Varieties from three production areas in China, namely Shaanxi violet flower Danshen (SXV), Shandong white flower Danshen (SDW), and Henan violet flower Danshen (HNV), were authenticated by Professor Wei Jianhe of the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences (CCAMS) and Peking Union Medical College. All three germplasm lines were planted in a field at IMPLAD, CAMS, Beijing, China using conventional commercial cultivation methods. SXV and HNV were transplanted in April 2008, and SDW was transplanted in April 2007. After seed ripening in the late August 2008, all of these original Danshen varieties entered the root swelling stage which lasted until the aerial parts wilted in the middle of November. As late September (Ran et al., 2007; Zhao, 2009; Zhao and Zhao, 2009), late October (Jiang et al., 2008; Deng et al., 2009; Yan et al., 2009), and late November (Deng et al., 2009) was reported to be the best harvest time for Danshen, respectively, considering the highest content of some bioactive components, each germplasm line was harvested on three fixed dates, e.g. September 25, October 22 and November 20. The experiment was conducted again in 2009 using the same cultivation method and harvest dates but all the *S. miltiorrhizae* materials were transplanted at the same time. Roots were oven dried to the constant weight at 60 °C and weighed, and then were ground to pass through a 0.3 mm sieve. Contents of cryptotanshinone, tanshinone IIA, danshensu and salvianolic B were analyzed by HPLC, and the content of total tanshinones was determined by UV spectrophotometry.

2.2. Analysis of active components

2.2.1. Chemicals and standards

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (NJ, USA). Deionized water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). Analytical grade phosphoric acid and ethanol were from Beijing Beihua Fine Chemicals Co. Ltd. (Beijing, China). Four authentic standards of danshensu, salvianolic B, tanshinone IIA and cryptotanshinone were purchased from the National Institute for Control of Biological and Pharmaceutical Products (Beijing, China).

2.2.2. Apparatus and analytical conditions

A Waters 1525 HPLC system (Waters Technologies, Milford, MA, USA) comprised a binary solvent delivery system, an on-line degasser, a Waters 717plus auto-sampler, a column temperature controller and a Waters 2457 dual λ absorbance detector coupled with an analytical workstation. The column configuration was a Waters Symmetry C18 reserved phase column (5 μm, 250 mm × 4.6 mm). The sample injection volume was 10 μl.

Chromatographic conditions for lipophilic components: detection wavelength was set at 270 nm for analysis. The mobile phase consisting of methanol and deionized water (75:25, v/v) was used at the flow rate of 1.0 ml min^{-1}, and the column temperature was maintained at 25 °C.

Chromatographic conditions for hydrophilic components: detection wavelength was set at 288 nm, the flow rate was 1.0 ml min^{-1}, and the column temperature was maintained at 25 °C. A gradient elution of A (acetonitrile) and B (0.026% aqueous phosphoric acid, v/v) was used as follows: initial 0–20 min, linear change from A–B (5:95, v/v) to A–B (23:77, v/v); next 20–33 min, linear change to A–B (29:71, v/v), and then this ratio was maintained for 10 min; 43–50 min, linear change to A–B (5:95, v/v), and this ratio was maintained for 20 min.

An UV 1100 UV–vis spectrophotometer from Shanghai Tianmei Instrument Co. Ltd. (Shanghai, China) was used to analyze the content of total tanshinones. The ultraviolet spectrophotometer was used at a detection wavelength of 270 nm.

2.2.3. Preparation of sample solutions

Cryptotanshione, tanshinone IIA, danshensu and salvianolic acid B. Individual samples were accurately weighed (0.300 g for cryptotanshione and tanshinone IIA, 0.200 g for danshensu and salvianolic acid B), suspended in methanol (cryptotanshione and tanshinone IIA) and 75% methanol (danshensu and salvianolic acid B) (50 ml), then extracted under reflux for 1 h. After cooling to room temperature and preparation by the method of weight relief, the extracted solution was filtered through a membrane (0.45 μm) and then 10 μl injected into the HPLC.

Total tanshinones: individual samples (0.500 g) were accurately weighed and soaked in 5 ml ethanol at 4 °C overnight and then extracted in an ultrasonic bath at room temperature for 20 min. After centrifugation at 2856 × *g* for 20 min, 0.5 ml supernatant was diluted with 3.5 ml ethanol and analyzed with an ultraviolet spectrophotometer at a detection wavelength of 270 nm.

3. Results and discussion

Contents of cryptotanshione, tanshinone IIA, total tanshinones, danshensu, and salvianolic acid B in the roots of three Danshen germplasm collected at different harvest time are presented in Tables 1 and 2. The results indicate that the contents of these major bioactive compounds in the roots of Danshen varied with year, harvest time and germplasm.

For SXV and SDW, contents of cryptotanshione, tanshinone IIA and total tanshinones in 2008 were significantly lower than in 2009, while for HNV they were higher in 2008 than in 2009 and most differences reached significance (Table 1). Danshensu content was higher in 2008 than in 2009 in all three germplasm lines (Table 2). Salvianolic acid B content of SDW was lower in 2008 than in 2009 but there were no uniform changes among each harvest date for SXV and HNV (Table 2). In 2008, the content of each compound in HNV was the highest among the three varieties, with cryptotanshione, tanshinone IIA, and total tanshinones contents about 230–352% higher than in SXV and 15–52% higher than in SDW (Table 1). Danshensu and salvianolic acid B content were about 30–40% higher than in SDW (Table 2). However, in 2009, the content of each compound in SDW was the highest among the three varieties. Cryptotanshione, tanshinone IIA, and total tanshinones contents were about 290–525% higher than in HNV and 56–96% higher than in SXV (Table 1), and salvianolic acid B content was about 17% higher than in HNV (Table 2). All of these Danshen were harvested in the first growth year except SDW in 2008 which was harvested during its second growth year. Li et al. (2005) reported that the contents of tanshinone IIA and salvianolic acid B in the roots of Danshen harvested in the second growth year were the lowest comparing to that harvested in the first and third growth year. Except that, there were few studies related to the content differences of bioactive components in the roots of Danshen harvested at different growth years because it was normally harvested in the first full growth year in the conventional commercial production. The age of plant maybe one of the possible reasons for these lower contents of bioactive components in the roots of SDW in 2008 com-