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Short communication

Tanshinone IIA and tanshinone I production by *Trichoderma atroviride* D16, an endophytic fungus in *Salvia miltiorrhiza*

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ABSTRACT

In this study the isolation of an endophytic fungus from the root of the medicinal herb *Salvia miltiorrhiza* Bunge is reported for the first time. The fungus produced tanshinone I and tanshinone IIA in rich mycological medium (potato dextrose broth) under shake flask and bench scale fermentation conditions. The fungus was identified as *Trichoderma atroviride* by its morphology and authenticated by ITS analysis (ITS1 and ITS2 regions and the intervening 5.8S rDNA region). Tanshinone I and tanshinone IIA were identified by HPLC and LC-HRMS/MS and confirmed through comparison with authentic standards. This endophytic fungus has significant scientific and industrial potential to meet the pharmaceutical demands for tanshinone I and tanshinone IIA in a cost-effective, easily accessible and reproducible way.

Introduction

Tanshinone I and tanshinone IIA (Fig. 1) are abietane-type norditerpenoid quinone natural products found in the Chinese medicinal herb *Salvia miltiorrhiza* Bunge. Specifically, together with tanshinone IIB and cryptotanshinone, they are found as bioactive lipophilic pigments in the intensely red rhizome (root), which is called Danshen in Chinese Traditional Medicine.

Tanshinone I and tanshinone IIA are reported to display a variety of biological activities. Tanshinone I is reported to induce apoptosis of the leukemia cells (Liu et al. 2010), human colon cancer Colo 205 cells (Su et al. 2008) and activated Hepatic Stellate Cells (Kim et al. 2003). It displays anticancer effects in human non-small cell lung cancer (Lee et al. 2008) and human breast cancer (Nizamutdinova et al. 2008). Meanwhile, tanshinone IIA affects the cardiovascular action (Li et al. 2007), including anti-cardiomyocyte hypertrophy (Yang et al. 2007), anti-atherosclerosis (Fang et al. 2008), antihypertension (Chan et al. 2009) and anti-ischaemic heart diseases (Bi et al. 2008). In addition, tanshinone IIA is a potent, natural anticarcinogenic agent for the management of systemic malignancies (Kapoor 2009).

Currently, tanshinone I and tanshinone IIA are mainly extracted from the root of the perennial plant *Salvia militiorrhiza*. However, this wild plant is in short supply because of its over-collection. This plant can be cultivated in china, but it needs a long time to maturate and the cultivar degenerates easily. Furthermore, as the pharmacologiocal properties of tanshinone I and tanshinone IIA are established, their demand is expected to increase. Consequently, the projected demands of tanshinone I and tanshinone IIA are unlikely to be met from the nature sources. Therefore, it is essential to find other sources of tanshinone I and tanshinone IIA to meet pharmaceutical demand.

In 1993, a significant discovery was made in that an endophytic fungi was isolated from extinct wild plant, *Taxomyces andreanae* and this produced the bioactive compounds, taxol and taxane, just like the host plant (Stierle et al. 1993). The potential of using endophytes as an effective alternative or novel source for therapeutic compounds is well recognized. Several workers have reported the use of endophytes for the production of pacitaxel, camptothecin, podophyllotoxin, hypericin, and gentiopicrin isolated from the hosts *Taxus cuspidata*, *Nothapodytes foetida*/*Camptotheca acuminata*, *Podophyllum peltatum*, *Hypericum perforatum*, and *Gentiana macrophylla*, respectively, although there are no reports of successful industrial scale-ups (Zhao et al. 2011).

It is well known that a microbial source of a valuable product is usually easier and more economical to produce. Therefore, if an endophytic fungus isolated from *Salvia miltiorrhiza* can produce the same bioactive compounds by fermentation, such as tanshinone I and tanshinone IIA, just like its host plant, this will provide an effective alternative or novel source for the bioactive compounds. Based on this theory, this study was carried out to isolate endophytic fungi from *Salvia miltiorrhiza* and to find out whether any of these fungi can produce tanshinone I and/or tanshinone IIA. If this is the case,

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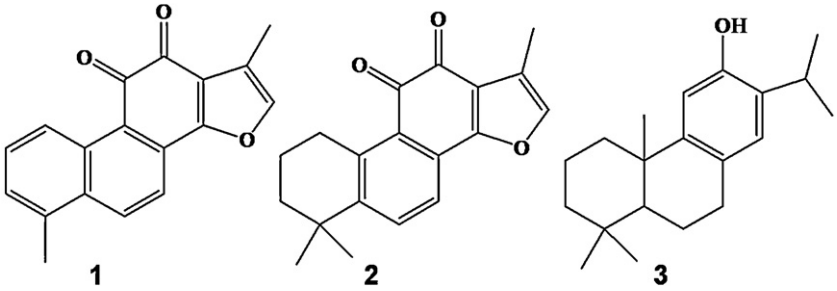

Fig. 1. The structures of tanshinone I (1), tanshinone IIA (2) and ferruginol (3).

then the isolated fungi can be processed by fermentation to produce the bioactive compounds, thus meeting the pharmaceutical demand.

Materials and methods

Materials and media

Fresh roots of *Salvia miltiorrhiza* Bunge were collected from the *Salvia miltiorrhiza* GAP planting base of TASLY in the city of Shangluo (Shaanxi Province), People's Republic of China. The roots were removed from the soil and transported to the School of Pharmacy, Second Military Medical University, Shanghai, China, for processing within 24 h of collection.

The reference standards of tanshinone I and tanshinone IIA used were from Chengdu Mansite Pharmacetical CO. LTD., Chengdu (Sichuan Province), People's Republic of China.

Potato dextrose agar (PDA) medium (200 g potato, 20 g D-glucose, 15 g agar, 1000 ml deionized water) was used for isolating and culturing endophytic fungi. Potato dextrose broth (PDB) medium (200 g potato, 20 g D-glucose, 1000 ml deionized water) was used for the fermentation of endophytic fungi. Both media were sterilized by autoclaving for 30 min.

Isolation and culture of endophytic fungus

The roots of *Salvia miltiorrhiza* were washed thoroughly in running tap water followed by deionized (DI) water to remove any soil and dirt adhering to the roots. Surface sterilization of the roots was carried out essentially as reported by Kusari et al. (2009) with slight modifications. Briefly, small root segments of approximately 15 mm length were cut with the aid of a flame-sterilized razor blade and were surface-sterilized by sequential immersion in 75% ethanol for 30 s, 1.3 M sodium hypochlorite (3–5% available chlorine) for 3 min, and 75% ethanol for 30 s. These were then rinsed three times in sterile water to remove any excess surface sterilants with the excess moisture being blotted on a sterile filter paper. Finally, these surface-sterilized small root segments were cut into pieces of approximately 2 mm thickness and were evenly spaced in Petri dishes containing PDA medium amended with penicillin ($100\,mg\,l^{-1}$) to eliminate any bacterial growth. The petri dishes were sealed using Parafilm (Pechiney, Chicago, IL) and incubated at $26 \pm 2\,^{\circ}C$ in an incubator until fungal growth was observed and the cultures were monitored on a daily basis to check for the growth of endophytic fungal colonies. The hyphal tips, which grew from sample segments over 2–3 weeks, were isolated and subcultured onto a new rich mycological medium, PDA medium, and brought into pure culture.

Preparation of mycelia and broth extracts

Endophytes obtained were inoculated into 250-ml erlenmeyer flasks, each containing 100 ml PDB medium. Biomass was removed by filtration after incubation for 10 days at 28 °C on rotary shakers at 180 rpm; the mycelia and broth were treated separately. The mycelia were washed 3 times in deionized water (DI), and were dried in an oven at 40 °C to obtain the dry weight. The dry mycelia were then suspended in methanol, homogenised, and then sonicated in an ultrasonicator at room temperature. This homogenate was then filtered through three pieces of filter paper under vacuum. The methanol was removed after each filtration by rotary evaporation under vacuum at 50 °C, thus yielding the methanol extract of mycelia. The water of spent broth was also removed by rotary evaporation under vacuum at 75 °C and the extraction was then dissolved in methanol and then filtered through two pieces of filter paper under vacuum. Finally, the methanol was removed after each filtration by rotary evaporation under vacuum at 50 °C, thus yielding the methanol extract of the broth.

HPLC analysis of the mycelia and broth extracts

The methanol extractions of mycelia and broth were dissolved in methanol for prior to HPLC analysis. This was performed on an Agilent-1100 system with a ZORBAX SB-C18 chromatographic column(250 mm × 4.6 mm, 5 μm) at 30 °C with a H_2O (+0.5% HCOOH) (A)/acetonitrile (B) gradient, a sample injection volume of 20 μl, flow rate of $0.8\,ml\,min^{-1}$, and a detection wavelength 280 nm. Samples were analyzed by using a gradient program as follows: run was commenced with 20% B, linear gradient to 40% B within 20 min, and then linear gradient to 80% B in 1 min, followed by linear gradient to 90% B in 19 min, and finally linear gradient to 20% B within 5 min.

LC-HRMS/MS analysis of the selected extracts

The mass spectrometer was equipped with an Agilent HPLC system 1290 consisting of pump, flow manager, and auto sampler (injection volume 2 μl). Nitrogen was used as sheath gas (Gas Temp: 350 °C, Gas Flow 11 l/min, Nebulizer 50 psi). The separations were performed by using a Column Technology C18 column (5 μm, 2.1 × 150 mm) with a H_2O (+0.5% HCOOH) (A)/acetonitrile (B) gradient (flow rate $0.4\,ml\,min^{-1}$). Samples were analyzed by using a gradient program as follows: run was commenced with 5% B, linear gradient to 30% B within 5 min, followed by linear gradient to 50% B in 3 min, and then linear gradient to 70% B in 12 min, followed by linear gradient to 90% B within 5 min, and then linear gradient to 95% B within 5 min, finally a gradient of 95% B for 3 min. The spectrometer was operated in positive mode ($1.5\,spectrum\,s^{-1}$; mass