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Improved isolation procedure for shikonin from the root of the Chinese medicinal plant *Lithospermum erythrorhizon* and its solubilization with cyclodextrins

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ABSTRACT

In this study, we improved the method for the isolation and purification of the bioactive compound shikonin from the dried root of the traditional Chinese medicinal plant *Lithospermum erythrorhizon* without a tedious chromatographic separation. Furthermore, we also attempted the solubilization of shikonin in water with cyclodextrins (CDs) using a solid phase "high-speed vibration milling". Among the various CDs, sulfobutylether-β-CD (SBEβ-CD) showed a highest solubilizing ability and 52.5% of shikonin was solubilized with equimolar amount of SBEβ-CD. In addition, the SBEβ-CD·shikonin complex showed higher apoptogenic activity than that of the same concentration of free shikonin against murine primary peritoneal macrophages. These results indicate that SBEβ-CD is a suitable excipient for clinical application of shikonin.

1. Introduction

The bioactive compound shikonin [5,8-dihydroxy-2-(ll-hydroxy-14-methyl-13-pentenyl)-l,4-naphthoquinone, (Fig. 1) is a naphthoquinone derived from the roots of the Chinese medicinal plant *Lithospermum erythrorhizon* Siebold & Zucc. The (*S*)-isomer alkannin is found mainly in the roots of the Boraginaceae family, especially *Alkanna tinctoria*, which is widely distributed plant in China (Papageorgiou, 1977). Both shikonin and alkannin exist mainly as their 11-*O*-acyl derivatives in the plant roots. Shikonin has been known to show various bioactivities such as wound healing effect (Papageorgiou et al., 2008, 2006a, 1999), antibacterial (Al-Mussawi, 2010) and antitumor (Gong and Li, 2011). Recently, Shi and Cao (2014) demonstrated that shikonin promotes autophagy in BXPC-3 human pancreatic cancer cells by the inhibition of PI3K/Akt signaling pathway. Yang et al. (2014) showed that shikonin reduces the levels of inflammatory cytokines (IL-6, NO and TNF-α) and high mobility group box 1 (HMGB1) in lipopolysaccharide (LPS)-stimulated murine macrophage-like RAW264.7 cells.

Shikonin is prepared mainly from above-mentioned plant materials (Papageorgiou et al., 2006b), or produced through bioprocess engineering of *Echium italicum* L. (Zare et al., 2011, 2010). However, many previous studies show that one or several steps of chromatographic procedures were required for isolation and purification of shikonin from those materials. For example, Lu et al. (2004) reported the method using preparative high-speed counter-current chromatography for purification of crude shikonin obtained from the roots of *L. erythrorhizon*. Singh and Sharma (2014) demonstrated the separation procedure of shikonin extracted from tissue cultures of *Arnebia hispidissima* with column chromatography and preparative TLC.

Here, we report the simple method for the isolation of shikonin from the root of *L. erythrorhizon* without a tedious chromatographic separation. Furthermore, we also investigate the preparation of water-soluble complexes of shikonin with cyclodextrins (CDs) and their apoptotic effects against murine primary peritoneal macrophages.

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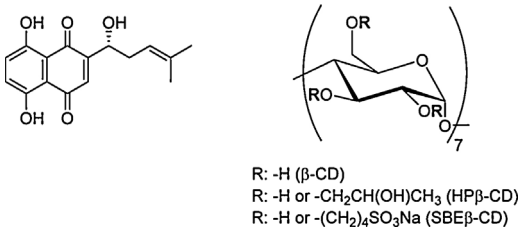

Fig. 1. Structures of shikonin and β-cyclodextrins.

2. Materials and methods

2.1. Materials

All materials obtained commercially (guaranteed reagent grade) were used. Standard grades of shikonin and alkannin were purchased from Wako (Japan). Both the dried roots of Japanese and Chinese *L. erythrorhizon* were gifted from Pharma Crea Kobe Co., Ltd. (Japan). β-CD, γ-CD, 2-hydroxypropyl-β- and γ-CD (HPβ-CD and HPγ-CD) were purchased from Sigma Chemical Co. (USA). Sulfobutylether-β-CD sodium salt (SBEβ-CD, captisol®) was gifted from A Ligand Technology (USA). Grinding of the dried roots were performed by an universal mill (M20, Ika). ^{1}H NMR spectra were determined on Bruker AVANCE300. UV–vis absorbance of shikonin solution was measured on Shimadzu UV-2500PC spectrophotometer. Preparation of the water-soluble complexes were performed by a high-speed vibration mill (MM200, Retsch). Female C57/BL6 mice at 7 weeks of age were obtained from Japan SLC, Inc. (Japan). The mice were injected intraperitoneally with thioglycolate medium. Murine primary peritoneal macrophages were isolated by peritoneal lavage of stimulated C57/BL6 mice and cultured in RPMI1640 containing 10% heat-incubated fetal bovine serum (FBS) supplemented with streptomycin and penicillin at 37 °C with 5% CO_2 atmosphere. The experiments were performed with the permission of the Animal Ethics Committee of Osaka City University in accordance with the Declaration of Helsinki.

2.2. Isolation of shikonin from the dried roots of L. erythrorhizon

The dried roots of *L. erythrorhizon* were frozen by liquid nitrogen and grinded into flour using the mill. The obtained powder (40.0 g) was dispersed in hexane (400 mL) and stirred overnight at room temperature in the dark. The solvent was filtrated and the residue was added with 50 mL of hexane and filtrated. The extraction procedure was repeated four times and all the filtrates were combined and the solvent was concentrated until a volume of about 100 mL had been remained. The dark red solution was cooled in ice bath and added with 200 mL of 1 M NaOH solution. After stirring for 1 h at 0 °C, the organic layer was removed. The blue aqueous layer was washed with toluene (200 mL × 2). After filtration, 300 mL of 1 M citric acid solution was add to the filtrate. The muddy red aqueous layer was extracted with $CHCl_3$ (100 mL × 3). The organic layers were collected and concentrated. The residue was subsequently dissolved in 100 mL of acetone. After filtration, the filtrate was slowly poured into 200 mL of ice-cold water with stirring. A large amount of precipitate was observed and the mixture was stored overnight in a refrigerator. The precipitate was collected by filtration and washed with a large volume of water. After drying under reduced pressure using P_2O_5 as a desiccating agent, 800 mg of shikonin was obtained in 2% yield from the dried powder of the roots.

mp 138–141 °C {mp 135–138 °C (Afzal and Al-Oriquat, 1986)}; $[\alpha]^{20}{}_D$ = +400.0 (c 0.063, acetone); IR (KBr) 3256, 2929, 1606, 1572, 1454, 1342, 1231, 1204, 1114, 1064, 778 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$); δ 1.67 (s, 3H), 1.77 (s, 3H), 2.30–2.45 (m, 1H), 2.60–2.75 (m, 1H), 4.93 (ddd, 1H, *J* = 1.1, 4.2, 7.9 Hz), 5.22 (m, 1H), 7.18 (d, 1H, *J* = 1.3 Hz) , 7.21 (d, 2H, *J* = 1.3 Hz), 12.51 (s, 1H), 12.61 (s, 1H); Anal. Calcd: C, 66.66; H, 5.59; O, 27.75. Found: C, 66.71; H, 5.68; O, 27.61. ^{1}H NMR is an identical with the previous report (Afzal and Al-Oriquat, 1986).

HPLC analysis of the obtained shikonin was performed with a Daiso SP-120-5 ODS-BP column. Enantiomeric excess value for the prepared shikonin was also determined by chiral HPLC analysis with a Daicel CHIRALPAK AD-H column (Assimopoulou and Papageorgiou, 2004a). Elutions were carried out with MeCN/H_2O/AcOH (70:29:1, v/v, for an ODS column) or *n*-hexane/2-propanol (90:10, v/v, for a chiral column) at a flow late of 1 mL/min, respectively, and the eluents were monitored at 254 nm. Retention times of the standard shikonin and alkannin by chiral HPLC analysis were 10.5 and 11.4 min, respectively. Enantiomeric excess value of the obtained shikonin was calculated as 82%ee from each peak area (shikonin/alkannin = 91.2:8.8).

2.3. Purity determination of shikonin by UV spectrometric analysis

The purity of shikonin was estimated by the modified method described in the reference (The Japanese Cosmetic Ingredients Codex 1993, 1994). Briefly, about 2 mg of shikonin was weighed precisely and put into a 2 mL volumetric flask and dissolved in 2 mL of ethanol. The part of the solution (0.25 mL) was added to a 5 mL volumetric flask and diluted to 5 mL with ethanol. The diluted solution (0.5 mL) was poured into another 5 mL volumetric flask and mixed with 25 g/L KOH aqueous solution (2.5 mL). The mixture was stored for 1 min at room temperature and then diluted to 5 mL with ethanol (sample solution). Blank solution was also prepared using aq. KOH and ethanol. The absorbance of the sample solution at 624 nm was measured and the purity of the isolated shikonin was calculated by the Eq. (1).

$$\text{Purity}\,(\%) = \frac{A_{624} \times 740}{\text{Weighed shikonin}\,(\text{mg})} \quad (1)$$

2.4. General method for the solubilization of shikonin in water

Shikonin (1.00 mg, 3.47 μmol) and two molar equivalents of HPβ-CD (MW: ~1500, 10.4 mg) were placed in an agate capsule together with two agate mixing balls and were vigorously mixed by shaking at a rate of 25 Hz for 60 min by the use of a high-speed vibration mill. The solid mixture was dissolved in 10 mL of water and centrifuged at 10,000 × *g* for 10 min. The resulting supernatant was filtered by a membrane filter (0.22 μm) to obtain the solution of HPβ-CD·shikonin complex. The solutions of other CD·shikonin complexes were also prepared by the similar procedures using β-CD (7.9 mg), γ-CD (9.0 mg), HPγ-CD (MW: ~1580, 11.0 mg) and SBEβ-CD (MW: ~1800, 12.5 mg), respectively.

2.5. Isolation of murine peritoneal macrophages

Female C57/BL6 mice were injected intraperitoneally with 2 mL of 4% thioglycollate medium (Difco). Four days later, mice were sacrificed. Peritoneal lavage in each mouse was performed with 5 mL of ice-cooled PBS. The recovered cells were centrifuged at 300 × *g* at 4 °C for 5 min. After removing the supernatant, the cell pellet was resuspended in RPMI-1640 medium. Cells were counted and 3×10^5 cells (for CellTiter-Glo™ assay) or 1×10^6 cells (for immunoblot analysis) were plated.