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Ethanol modified supercritical fluids extraction of scopoletin and artemisinin from *Artemisia annua* L.

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Abstract

This work studied the supercritical carbon dioxide (SC-CO_2) extractions with adding 16.25% ethyl alcohol as a co-solvent to obtain scopoletin and artemisinin from *Artemisia annua* L. A two-factor central composite experimental design was adopted to determine the optimal operation conditions in extracting the maximal amount of these bioactive compounds. Experimental results indicated that the 289 μg of scopoletin/g of the feed in dry basis and the 11.6 mg of artemisinin/g of the feed had been obtained at these optimal conditions. Two hours ethanol modified SC-CO_2 extractions were superior to 16 h Soxhlet *N*-hexane extractions in producing more pure artemisinin and scopoletin and the amount of the extracts increased with the density of SC-CO_2. A normal phase silica-gel column chromatography to purify artemisinin from the SC-CO_2 extract presented that the purity of artemisinin attained 98.2% but with a low recovery of 54.4%. This reduced recovery may be caused by an occurrence of the bridging peroxide atoms of artemisinin reacted with silica-gel molecules during the column purification.
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Keywords: *Artemisia annua* L.; Artemisinin; Scopoletin; Supercritical carbon dioxide; Column purification

1. Introduction

Artemisia annua L. is a famous medicinal herb that has commonly been used in Chinese traditional therapy for the treatment of fever and malaria. Chinese chemists have devotedly isolated several effective bioactive compounds from this plant since the Vietnam War started 1971. A substance called artemisinin is a sesquiterpene lactone having a peroxide group is responsible for its famous anti-malaria activity. Artemisinin has been successfully used in curing thousands of malaria patients in Asia, including those with both chloroquine-sensitive and chloroquine-resistant strains of Plasmodium falcipaurum [1]. Thermal stability studies have indicated that artemisinin is stable in organic solvents heated up to 150 °C. However, when heated over 180 °C above its melting point (156–157 °C) for 2.5 min, artemisinin degrades into three small compounds [2–4].

Following the standard protocols of National Cancer Institute, bioassays *in vitro* presents that artemisinin exhibits strong cytotoxicity to the P-388, A-549 and HT-29 tumor cells [5]. Research has demonstrated that artemisinin is about 10 times as strong in inhibiting the growth of the 3T3 kidney fibroblasts as in inhibiting the Nb2a cells. On the other hand, one study indicates that Ehrlich ascites tumor cells are less susceptible to the artemisinin toxicity [6].

Scopoletin (6-methoxy-7-hydroxy coumarin) is one of the coumarins that is present in *A. annua*. Pure scopoletin is a yellow to beige crystalline powder; the molecular weight is 192; and the melting point is 204–206 °C [7]. Ojewole and Adesina [8] indicated that scopoletin suppressed acetylcholine-induced contractures of the toad rectus abdominis muscle [8]. Cassady et al. [9] demonstrated that scopoletin has anti-tumor activity. Kang et al. [10] showed that scopoletin inhibits the nitric oxide synthesis in a dose-dependent manner in murine macrophage-like RAW 264.7 cell stimulated with interferon-y (IFN-y) plus lipo-polysaccharide [10]. Scopoletin is considered as an anti-inflammatory medicine [11] and as a Hevea hytoalesin [12,13]. Some studies evidenced that scopoletin has antifungal synergistic activity [14] and it is an antispasmodic component [15]. However, artificial syntheses of artemisinin and scopoletin are costly and infeasible in comparing with Soxhlet solvent extractions of them from nature materials [16]. Supercritical fluid

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extractions applied to extract bioactive compounds from natural plant materials and to examine their anti-oxidative abilities had recently been reported by Chang et al. [17] and by Lee et al. [18]. Artemisinin and its major precursor artemisinic acid have been extracted by supercritical fluid extraction and analyzed by supercritical fluid chromatography [19]. A study has been conducted to enhance the dissolution rate of artemisinin in order to improve the intestinal absorption characteristics by means of supercritical fluid technology [20]. Quispe-Condori et al. (2005) have reported that the maximum artemisinin global yield (0.70% in dry basis) was obtained at the 50 °C and 300 bar SC-CO_2 extraction [21]. Ethanol modified supercritical carbon dioxide extraction of scopoletin from stinging nettle roots by Sajfrtová et al. [22] and *N*-hexane modified supercritical carbon dioxide extraction of artemisinin from *A. annua* by Lin et al. [16] have been investigated recently. Studies of ethanol modified supercritical carbon dioxide extraction of both scopoletin and artemisinin from *A. annua* have not yet been reported. Therefore, this work studied supercritical carbon dioxide extraction with added ethyl alcohol to obtain scopoletin and artemisinin from *A. annua* and examined the purification of artemisinin from the SC-CO_2 extract using the column chromatography.

2. Experimental approach

2.1. Reagents and materials

Ten kilograms of dried *Artemisia* whole plant imported from China were kindly supplied by Kaiser Pharmaceutical Company (Kaiser, Tainan, Taiwan) with the moisture content of $9.7 \pm 0.1\%$, as measured by an infrared meter (A & D, AD-4714A, Japan). This material was then ground by a blade type grinder by every 2-s period and the powder was screened through a 10 mesh stainless steel sieve (0.2 cm opening) ready to use. A detailed preparation procedure was reported by Lin et al. [16]. De-ionized water was obtained from a Milli-Q reverse osmosis purification system. 99% scopoletin (sigma, USA), 98% artemisinin (Sigma, USA), 99.9% *N*-hexane (J.T. Backer, USA), 99.8% absolute ethanol (Merck, Germany), 99.5% ethyl acetate (Sigma, USA), 99.9% methanol (J.T. Backer, USA), analytical grade of F_{254} silicagel 60 resin and thin layer plate (Merck, Germany) were purchased from a local supplier used without further purification.

2.2. Classical Soxhlet solvent extractions

A batch of 20 g of *Artemisia* powder loaded in a cellulose timber of the 250 ml reflux type Soxhlet column, was continuously extracted by 300 mL de-ionized water, methanol, ethanol (EtOH), ethyl acetate and *N*-hexane for 16 h, respectively. The Soxhlet EtOH extraction was further extended to the 20 h. All these extracts were collected, filtered, dried and weighted. Then, the total amount, the recovery and the purity of artemisinin and scopoletin in the extracts were determined.

2.3. Supercritical fluid extractions

Fig. 1 depicts a schematic flow diagram of the SC-CO_2 extraction equipment used in this study. In experiments, a batch of 65 g of *Artemisia* powder added with added 7% ethyl alcohol evenly loaded inside a 250 ml stainless steel tubular extractor, packing intermittently with seven 1cm-thickness glass wool layers. This powder was soaked for 2 h in the extractor prior to the desired extractive condition reached. The rest ethyl alcohol was continuously added into a 10 mL on-line cartridge and flowed into the extractor carried by the SC-CO_2 fluid. Lin et al. [16] and Chiu et al. [23] described this co-solvent modified supercritical fluid extraction in detail. In briefly, liquid CO_2 from a siphon-tube type cylinder (1) went through a cooling bath at 283 K and then compressed to the desired working pressure by a syringe pump (4) and heated up to the supercritical condition by a coil-type heat exchanger (7). The supercritical CO_2 flowed into an extractor (8), contacted with powders, and extracted artemisinin and scopoletin into the CO_2 phase. Temperature controllers (5), temperature detector (20), and pressure gauge (5) were used to manipulate and control the desired temperature and pressure. During the extraction, the extract laden CO_2 gas led into the first absorber (13) filled with 500 mL ethanol by a pressure decrease. Then, the extract was further collected in the second separator filled with 500 mL ethanol controlled at 5 MPa and 303 K. Finally, the expanded low-pressure CO_2 gas released from the extract passed through a wet gas flow meter (16) and went to the ambient condition. The precipitates inside the back pressure regulator was washed out; mixed with the extract absorbed in ethanol; dried out the solvent; dissolved in 2 ml absolute ethanol ready for the GC and HPLC analyses of artemisinin and scopoletin. The 50 ml samples were taken in triplexes; the solvent was dried out in a force circulation oven at 60 °C (333 K); the residue was weighed by a Mettler balance ready for the determination of the purity, the recovery and the yield. Then, concentration factors of artemisinin and scopoletin were calculated.

Influential parameters perhaps affecting the extracted amount of artemisinin and scopoletin are temperature, pressure, the CO_2 usage and the co-solvent addition ratio. A few sensitivity tests ranged from 2500 psig (17.34 MPa) to 4500 psig (31.13 MPa) and from 40 °C (313 K) to 60 °C (333 K), employing 1–12 h of the immerse time, 240–600 L of the CO_2 usage and 7.0–16.25% of the addition ratio, were executed to determine the most important parameters for supercritical fluid extractions. A two-factor center composite scheme comprising 4-axial point and 4-factor point was then designed to find the optimal extraction conditions to obtain the maximal amount of artemisinin and scopoletin in the extracts.

2.4. Quantification of artemisnin and scopoletin

The amount of artemisinin and scopoletin in the extracts had been quantified individually by GC and HPLC methods. A solvent gradient HPLC system comprising a LiChroCart C18–5 μm (4 mm × 125 mm) reverse phase column (Merck, Germany), Hitachi L-4200H UV detector, L-7100 pump and D7000 control

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