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Supercritical fluids extraction of cinnamic acid derivatives from Brazilian propolis and the effect on growth inhibition of colon cancer cells

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ABSTRACT

This work examined supercritical carbon dioxide ($SC\text{-}CO_2$) extractions of an anti-cancer compound, 3,5-diprenyl-4-hydroxycinnamic acid (DHCA) from propolis lumps and studied the inhibitive effect of the $SC\text{-}CO_2$ extracts on the growth of two cancer cells. The maximum amount of DHCA, 91.9 mg/g, was obtained by Soxhlet ethyl acetate extraction and the 41.2 wt.% pure DHCA was recovered using $SC\text{-}CO_2$ at 20.7 MPa and 323 K with 6 wt.% ethyl acetate addition. The effects of temperature and the addition of ethyl acetate on the DHCA purity, examined using a two factorial central composite response surface methodology, indicated that both factors were significant. A normal phase column adsorption approach was directly employed to purify the DHCA, and the purity was increased to 95 wt.%. The cell concentrations following growth inhibition ranged from 10 μg/mL to 500 μg/mL, indicating that the $SC\text{-}CO_2$ extracts and 95 wt.% DHCA effectively inhibited the growth of human leukemia (HL-60) and colon (colo 205) cancer cells, but did not suppress the growth of two human normal cells.

1. Introduction

Propolis is a resinous product that is gathered by honeybees from various plant exudates and is collected in beehives. With various botanical and geographical origins, more than 200 compounds have been isolated and identified from propolis (Marcucci, 1995; Marcucci *et al.*, 2000). Propolis has been used in folk medicine for many years, especially in European and Asian countries, because of its therapeutic properties, such as being antimicrobial, being antiviral, being an anti-inflammatory, causing immunomodulation, free radical scavenging, and having hepato-protectivity (Banskota *et al.*, 2000; Burdock, 1998; Kujumgiev *et al.*, 1999; Kumazawa *et al.*, 2004; Marcucci *et al.*, 2001; Nakanishi *et al.*, 2003; Oršolić *et al.*, 2004).

Recently, considerable research has focused on the anti-cancer activity of propolis. Two major anti-cancer compounds isolated from propolis are caffeic acid phenethyl ester (CAPE) and 3,5-diprenyl-4-hydroxycinnamic acid (DHCA), which excellently inhibit the growth of cancer cells (Akao *et al.*, 2003; Kimoto *et al.*, 1998, 2001; Matsuno *et al.*, 1997). The DHCA compound was first isolated by German scientists (Bohlmann and Jakupovic, 1979; Bohlmann *et al.*, 1981). DHCA has been purified by normal phase column chromatography (Aga *et al.*, 1994). A few studies of the extraction of DHCA using organic solvents have been published (Matsuno *et al.*, 1998; Park *et al.*, 2004). DHCA has also been produced by a total synthesis method (Uto *et al.*, 2002) and quantified by high-performance liquid chromatography (Shimizu *et al.*, 2004).

In recent years, supercritical fluid extraction has been successfully applied to produce highly valuable products from plant materials. Most active compounds in propolis are fat-affinitive materials and their solubilities in CO_2 are relatively low. This fact limits the application of supercritical fluid extraction in the apicultural industry. You *et al.* (2002) increased the amount of flavonoids in water-soluble propolis by $SC\text{-}CO_2$ extraction. Wang *et al.* (2004) extracted antioxidant compounds from propolis by $SC\text{-}CO_2$ fractionation. Catchpole *et al.* (2004) extracted flavonoids and essential oils from propolis tincture using supercritical carbon dioxide. They have also removed compounds with high molecular weight by supercritical antisolvent precipitation. Lee *et al.* (2007) used supercritical carbon dioxide-modified ethyl acetate to extract DHCA from Brazilian propolis, and obtained highly pure DHCA by column chromatography followed by $SC\text{-}CO_2$ extraction.

This study investigates the supercritical carbon dioxide extraction of DHCA from Brazilian propolis. The addition of ethyl acetate during the extraction of DHCA is examined and $SC\text{-}CO_2$ extraction based on response surface methodology is discussed. Growth inhibition tests of human leukemia, hepatoma, and colon

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cancer cells were performed to verify the anti-cancer ability of these SC-CO_2 extracts.

2. Materials and methods

2.1. Materials and reagents

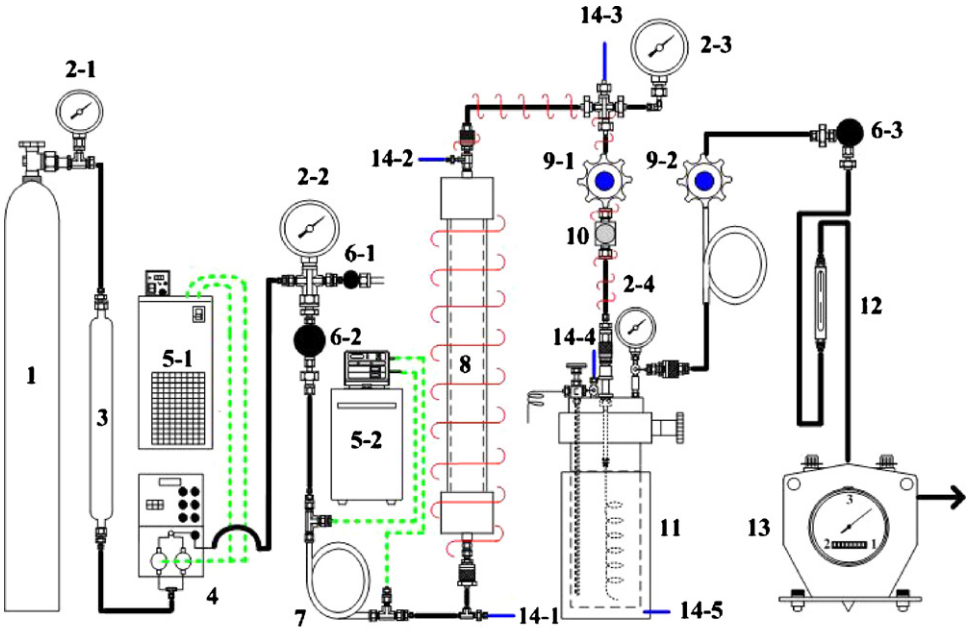
Several kilograms of de-waxed Brazilian propolis lumps (Ali-Green grade) were kindly donated by Bio-Joint Natural Co., Ltd. (Taipei, Taiwan). Liquid CO_2 (99.5%) was purchased from Toyo gas company (Taichung, Taiwan). Ethyl acetate (99.9%) and *n*-hexane (99.9%) were purchased from a local reagent chemical supplier (Mallinckrodt, USA). HPLC grade 99.9% methanol (Mallinckrodt, USA), de-ionized water prepared using a Milli-Q system (Millipore, USA), 99.8% acetic acid (Merck, Germany), and 60 M silica gel (Macherey-Nagel, Germany) were used without further purification.

2.2. SC-CO_2 extraction of propolis

Propolis lumps were ground into 2 mm particles using a blade-type grinder, then collected by sieving through a 10-mesh international-type stainless steel screen before use. The maximum recovery of DHCA in Brazilian propolis was obtained by Soxhlet ethyl acetate extraction over a period of 16 h. Fig. 1 presents the SC-CO_2 extraction that was used by You *et al.* (2002). Before extraction, 10 g of ground propolis powder and 30 g of steel beads were uniformly loaded into an extractor (75 mL, L/D = 30) (8); 5 cm thickness of glass wool was placed on both the top and the bottom of the extractor to prevent the entrainment of propolis particles, and 200 mL of 95% ethanol was loaded into the absorber (750 mL, L/D = 10) (11), which acted as an absorbent. Then, liquid CO_2 was pumped using a high-pressure pump (CM-3200, Thermo Separation Products, USA) and flowed into the extractor at a constant flow rate of 10 mL/min after preheating. The extraction pressure varied from 13.8 MPa to 27.6 MPa was regulated using a back-pressure regulator (26-1721, Tescom, USA) (9-1), and the extraction temperature ranged from 308 K to 333 K, controlled by a PI type controller. The pressure of the absorber was set to 5 MPa, and another back-pressure regulator (9-2) was used to separate CO_2 from the extracts that were collected at ambient temperature. The consumed CO_2 volume was measured using a wet gas meter (TG3, Ritter, Germany) (13). Furthermore, the amount of ethyl acetate, from 0 to 6 wt.%, was weighed and preloaded in the extractor as a co-solvent.

Following study of the effects of temperature and co-solvent addition on a few preliminary SC-CO_2 extractions, two-factor central composite response surface methodology (RSM) software (Stat-Ease, USA) was adopted to study the effect of the operating conditions of SC-CO_2 extractions on the purity of the DHCA in the extracts as well as to search for the optimum conditions in this procedure. The extraction temperature and the addition of the co-solvent were selected as two factors that influence the recovery and purity of DHCA. The temperature ranged from 313 K to 333 K and 2 wt.% to 6 wt.% ethyl acetate (EtOAc) was added. The recovery and purity of DHCA were calculated by Eqs. (1) and (2), respectively.

$$R_i = \frac{\text{weight of } i \text{ component in the extracts}}{\text{weight of } i \text{ component in Soxhlet extract}} \times 100\%,$$
$$i = \{\text{DHCA}\} \quad ; \text{recovery}. \tag{1}$$



1. CO_2 Cylinder
2-1~2-4. Pressure gauge
3. Gas dryer
4. High-pressure pump
5-1~5-2. Circulator
6-1~6-3. Metering valve
7. Heat exchanger
8. Extractor
9-1~9-2. Back pressure regulator
10. Micro-metering valve
11. Absorber
12. Float flow meter
13. Wet gas meter
14-1~14-5. Thermocouple

Fig. 1. Schematic diagram of SC-CO_2 extraction of propolis.