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Removal of toxic compounds from *Acer tegmentosum* using supercritical fluid extraction

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초임계유체 추출을 이용한 산겨릅나무로부터 독성성분들의 제거

표동진★ · 진정은

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Abstract: *Acer tegmentosum* is a tree used to treat various liver diseases in Korea. There have been some concern regarding the safety of *Acer tegmentosum* due to some toxic chemical compounds in its stems. Supercritical fluid extraction (SFE) was employed to develop a removing method of toxic compounds from *Acer tegmentosum*. The toxic compounds were effectively extracted with ethanol modified supercritical fluid CO_2.. The optimum condition of SFE was 100 bar of pressure, 40°C of extraction temperature, 3 mL/min of CO_2 flow rate, 0.2 mL/min of modifier (ethanol) flow rate.

요 약: 산겨릅나무(*Acer tegmentosum*)는 지방간, 가염, 간경변증, 간암에 뚜렷한 치료 작용이 있으며, 특히 간에 쌓인 독을 풀고 간세포를 살리는 효능이 있어 약용으로 많이 사용된다. 그러나 산겨릅나무 줄기에는 독성물질이 있어 안전성, 오남용 등의 문제점을 가지고 있다. 그래서 산겨릅나무 줄기의 독성물질을 제거하고 독성 물질에 대한 연구를 활발히 하기 위해 먼저 초임계유체 추출기법(SFE, Supercritical Fluid Extraction)을 이용하였다. 초임계유체 추출에서 최적의 실험 조건은 압력 100 bar, 추출온도 40°C로 구성하고, 초임계 CO_2의 유속 3 mL/min과 modifier인 에탄올의 유속 0.2 mL/min 이다.

Key words : Acer tegmentosum, supercitical fluid extraction

1. Introduction

Acer tegmentosum is a tree used to treat various liver diseases in Korea.

Its Korean popular names are “Beolnamu” and Sancheongmok”. It is a 10~15 m tall tree belonging to the Acereaceae and grows at 500~1,000 m above sea level. There have been some concern regarding

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the safety of *Acer tegmentosum* due to some toxic chemical compounds[1,2] in its stems. In this paper, supercritical fluid extraction (SFE) was employed to develop a removing method of toxic compounds from *Acer tegmentosum*.

Extraction with supercritical fluids as solvents have received wide attention recently. A number of potential advantages including more rapid extraction rate, more efficient extractions, increased selectivity, and potential for combined analyte fraction in conjunction with extraction are possible with supercritical fluid extraction.

These advantages of SFE accrue from the properties of a solvent at temperatures and pressures above its critical point. At elevated pressure, this single phase will have properties that are intermediate between those of the gas and the liquid phases and are dependent on the fluid composition, pressure, and temperature. The compressibility of supercritical fluids is large just above the critical temperature, and small change in pressure result in large changes in the density of the fluid.[3] The density of a supercritical fluid is typically 10^2-10^3 times that of gas. Consequently, molecular interactions increase due to shorter intermolecular distances. However, the diffusion coefficients and viscosity of the fluid, although density dependent, remain more like that of a gas.[3] The "liquidlike" behavior of a supercritical fluid results in greatly enhanced solubilizing capabilities compared to the corresponding liquid. These properties allow similar solvent strengths to liquids but with greatly improved mass-transfer properties which provide the potential for more rapid extraction rates and more efficient extraction due to better penetration of the matrix.

Among a wide variety of supercritical fluids, CO_2 has been the most commonly employed due to its comparatively low critical temperature (31.1°C) and pressure (78.3 atm) together with its another advantages such as environmental acceptance and non toxicity to human health.[4]

Because CO_2 is a nonpolar compound, analytes to be extracted with supercritical fluid CO_2 are limited to relatively nonpolar compounds. Therefore, a solvent modifier is generally added to supercritical fluid CO_2 to facilitate the extraction of more polar compounds. The addition of polar modifiers can increase the polarity of supercritical CO_2, and significantly enhance extraction efficiency, resulting in faster extractions.[5]

Recently, many studies have been performed to extract and separate biologically active components from natural products using supercritical CO_2 modified by various polar co-solvents such as methanol and ethanol etc.[6] It has also been found that modifiers help to increase the interior volume and surface area of plant matrices by destroying or swelling them, thus resulting in significant increases in extraction efficiency.[7,8]

As examples of supercritical fluid extraction, Sugiyama[9] *et al*. was successfully performed to extract caffeine from the green beans and Schneiderman[10] *et al*. was successfully performed to extracte menadione (vitamin K_3) from animal food.

The advantages of supercritical fluid extraction for the removal of toxic compounds from *Acer tegmentosum* compared conventional liquid extraction are the followings; (a) more selective extraction is possible (b) it is less expensive in terms of solvent cost and laboratory time, (c) carbon dioxide is available, to be used as a pure or modified solvent, with its convenient critical temperature, its non-toxicity.

2. Experimental

2.1. Materials and reagents

Acer tegmentosum samples were collected at local mountains in Jeongseon, Korea. Dried and powdered *Acer tegmentosum* stems (5 g) were used for supercritical fluid extraction. Supercritical fluid extractions were performed with CO_2 (Scott co., USA, 99.99%). All solvents were HPLC grade from Fisher Scientific Korea Ltd.

2.2. SFE System

Supercritical fluid extractions of *Acer tegmentosum* were performed using a JASCO (Tokyo, Japan) LC-900 SFE system. The schematic diagram of the system is shown in *Fig.* 1. This system consisted of

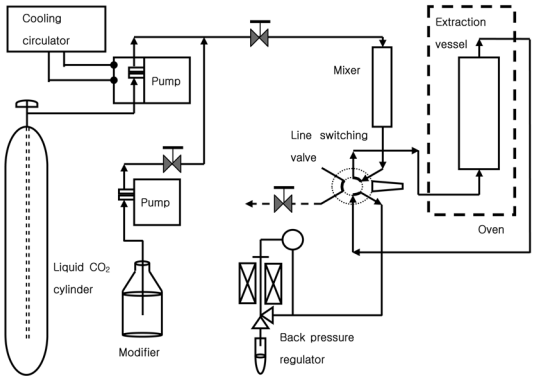

Fig. 1. Schemetic flow diagram of SFE modular system.

three sections: fluid delivery, extraction, and collection. The fluid delivery section included two pumps, which delivered liquid CO_2 and a modifier solvent separately. In the extraction section, supercritical fluid extractions were performed with CO_2 modified aqueous ethanol. The collection section included a back pressure regulator, which kept the pressure of an extraction vessel at the desired value. The effluent flowing through the back pressure regulator reduced its pressure to atmospheric and thereby solutes in the effluent reduced their solubility to virtually zero. In this way, the solutes were deposited and collected in a collection vessel. Since we used aqueous ethanol modified CO_2 as a extracting solvent, the extracts were collected in a liquid solvent in the collection vessel.

Acer tegmentosum sample (2.5 g) was loaded into a extraction vessel and both CO_2 and a modifier were supplied to the extractor by each pump.

Supercritical fluid extracts of *Acer tegmentosum* and residues were examined against cytotoxic activity and analyzed by high performance liquid chromatography (HPLC).

2.3. MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

HaCaT cells were maintained in a humidified 37°C incubator at 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL of streptomycin. Cytotoxicity experiments were initiated by plating 10^5 cells in 100 μL of cell suspension into each well of a 96-well tissue culture plate. The plate was incubated for 24 hours.

Medium was removed from the wells and replaced with 100 μL of medium containing test agent at various concentration, or with fresh medium for control wells. After 24 hours incubation, MTT assays were carried out.

Medium was removed and a MTT solution in PBS was added to each well. At the end 4 hours incubation periods at 37°C, the plate was centrifuged, and the untransformed MTT removed by carefully inverting, flicking, and blotting the plate. Iso-propanol was added to each well and the plate was then vigorously shaken. The optical density of each well was measured using ELISA reader (Victor3, Perkin Elmer, Singapore) with a 570 nm test wavelength.

2.4. HPLC analysis

The HPLC system consisted of a JASCO PU-980 pump, an UV-2075 UV detector and a Waters Spherisorb S5 ODS2 (4.6×150 mm) column. An aliquot of 10 μL was injected into the HPLC system. The mobile phase was changed from water:acetonitrile =85:15 to 35:65 during chromatographic separation. The flow rate was 1 mL/min, and UV detector operating at 280 nm.

3. Results and Discussion

SFE conditions for the optimization of the extraction of toxic compounds from *Acer tegmentosum* were evaluated. The amount of extraction of toxic compounds were quantitatively measured by MTT assay described in the earlier section.

3.1. Optimization of temperature and pressure

Extraction efficiency of toxic compounds was investigated as a function of extraction temperature and pressure. Extraction temperatures of 30, 40, 50, and 60 were chosen, and pressures corresponding to 90, 100, 110, and 120 bar were set at the chosen temperatures. As shown in *Fig.* 2, increased temperature at same pressure resulted in decreased

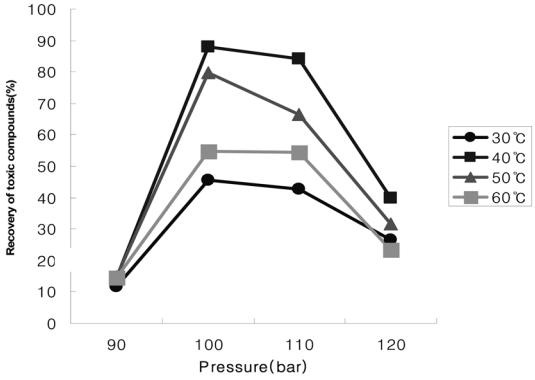

Fig. 2. Effect of pressure and temperature on the extractions of toxic compounds from *Acer tegmentosum*.

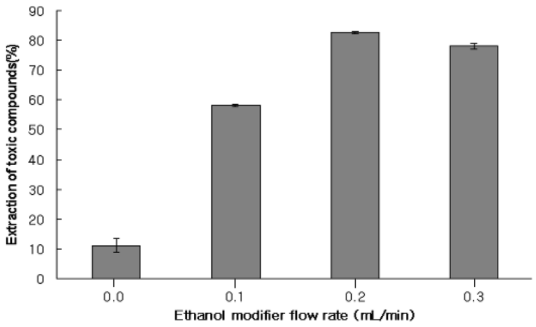

Fig. 3. Effect of modifier flow rate on the extractions of toxic compounds from *Acer tegmentosum*. Each values represent the mean values of five independent experiments.

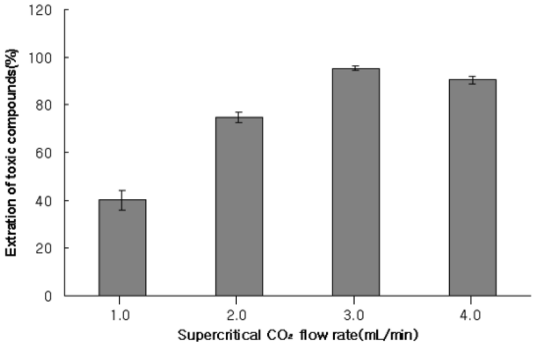

Fig. 4. Effect of supercritical CO_2 flow rate on the extractions of toxic compounds from *Acer tegmentosum*. Each values represent the mean values of five independent experiments.

extractions of toxic compounds. The only exception is 30°C. In the case of 30°C, the extraction efficiency was very low because 30°C is lower than the critical temperature of supercritical fluid carbon dioxide. These results seemed to be caused by the instability of toxic compounds at high temperature. The pressure of 100 bar showed the highest extraction efficiency. Therefore, optimum temperature and pressure for the extraction of toxic compounds were set at 40°C and 100 bar, respectively.

3.2. Optimization of supercritical CO_2 flow rate

In order to determine the optimal flow rate of supercritical CO_2, its effect on the extraction efficiency was assessed between 1.0 and 4.0 mL/min in 1.0 mL/min increments under optimum temperature and pressure. *Fig.* 4 shows that an increase in the flow rate of supercritical CO_2 led to an increased recovery. The highest recovery of 90% was obtained at the flow rate of 3.0 mL/min.

3.3. Optimization of modifier (ethanol) flow rate

Because CO_2 is a nonpolar compound, analytes to be extracted with supercritical CO_2 are limited to relatively nonpolar compounds. Therefore, a solvent modifier is generally added to supercritical CO_2 in order to facilitate the extraction of more polar compounds. The target analytes, toxic compounds, usually possesses high polarity. Thus, an experiment was carried out to evaluate the effect of the addition of ethanol as a modifier to supercritical CO_2. *Fig.* 3 shows that the addition of 0, 0.1, 0.2, and 0.3 mL/min of ethanol at optimum temperature and pressure (40°C, 100 bar) significantly improved the extraction efficiency as compared to the extraction without ethanol addition. The modifier flow rate of 0.2 mL/min showed the highest extraction efficiency.

3.4. HPLC analysis of toxic compounds

To establish chromatographic parameters allowing the gradient HPLC analysis of toxic compounds in the *Acer tegmentosum*, the composition of mobile phase and the wavelength for maximum absorbance were investigated. Mixtures of water-acetonitrile were examined in different ratios, and the most suitable

mobile phase was found to be 85:15 to 35:65 (v/v) mixture. Ultraviolet spectrum range showed that the absorbance was maximal at 280 nm.

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