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Hung-Chi Nian[a]; Ben-Zen Wu[a]; Bo-Jie Huang[b]; Chien-Ting Lo[c]; Jiunn-Guang Lo[a]; Ai-Yih Wang[b]; Kong-Hwa Chiu[d]
[a] Department of Biomedical Engineering and Environmental Sciences, National TsingHua University, HsinChu, Taiwan, ROC [b] Department of Radiological Technology, Yuanpei University, HsinChu, Taiwan, ROC [c] Department of Biological Science and Technology, National Chiao Tung University, HsinChu, Taiwan, ROC [d] Department of Applied Science, National DongHwa University, Hualien, Taiwan, ROC

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Pressurized CO_2 Extraction of Cantharidin from Mylabris for Anticancer Bioactive Component

Hung-Chi Nian,[1] Ben-Zen Wu,[1] Bo-Jie Huang,[2] Chien-Ting Lo,[3] Jiunn-Guang Lo,[1] Ai-Yih Wang,[2] and Kong-Hwa Chiu[4]

[1]*Department of Biomedical Engineering and Environmental Sciences, National TsingHua University, HsinChu, Taiwan, ROC*
[2]*Department of Radiological Technology, Yuanpei University, HsinChu, Taiwan, ROC*
[3]*Department of Biological Science and Technology, National Chiao Tung University, HsinChu, Taiwan, ROC*
[4]*Department of Applied Science, National DongHwa University, Hualien, Taiwan, ROC*

The use of pressurized liquid carbon dioxide to extract cantharidin from Mylabris was investigated. The study resulted in green extraction of bioactive ingredients from the insect, with extraction efficiency of 95% (three repetitions) obtained at a pressure of 1 MPa, temperature of 60°C, static extraction time of 30 minutes, and with 4 ml of acetonitrile as modifier. In addition to the identification of the extract, i.e., cantharidin, by chromatography, an in vitro assay for the effect of the extract on a cell was performed. The result reveals that cantharidin-extract is effective for primary medicinal use.

Keywords cantharidin; extraction; Mylabris; pressurized liquid carbon dioxide

INTRODUCTION

Traditional medicines taken orally or used for external applications are usually composite combinations, and most are prepared by decoction. Although they exhibit curative effects, their exact composition and certain other side effects are not clear in most cases. Therefore, isolation of the active ingredients in medicinal substances obtained from herbs or animals is scientifically significant. Recently, the application of green technology to this field is of increased interest. Usually, the extraction of ingredients of interest from animals is more difficult than from plants due to the complexity of the animal tissue. The beetle Mylabris phalerata Pallas (“Ban mao”) has a reputation in traditional Chinese medicine for reducing symptoms of central nervous system decline, including memory loss (1). Mylabris is the dried body of the Chinese blister beetle. In Western countries, Spanish fly, or cantharides, is often given to farm animals to incite them to mate. The species used in medicine are *Mylabris phalerata* and *M. cichorii*. The use of Mylabris as a traditional medicine in China can be traced back to more than 2000 years (its medical use in the West dates back to descriptions from Hippocrates). Mylabris was used externally to promote local blood circulation, to remove dead tissue and eliminate scrofula, and internally in small quantities for the treatment of swelling and rabies (2). In folk medicine, Mylabris has long been used to treat neoplastic diseases. If swallowed, it produces congestion of the urethra1 mucosa, which may result in priapism in men and pelvic congestion in women (3). Cantharidin has important antitumor properties (4). Recently, cantharidin has been identified as a strong inhibitor of protein phosphatases type 1 and 2A that are involved in the control of cell proliferation and activity of membrane-associated channels and receptors (5–7).

The active ingredient in Mylabris is cantharidin (Fig. 1). Cantharidin content in the beetle ranges from 0.6 to 5% of dry body weight (8). The acute LD_{50} in mice is 1.71 mg/kg (2). The estimated lethal dose of cantharidin is between 10 and 60 mg (9). The common method to extract cantharidin from beetles is soxhlet extraction (7). However, conventional extraction methods use large quantities of solvents, especially those classified as volatile organic compounds (VOCs) and harmful chlorinated solvents, such as dichloromethane, and the volume of solvent used compared to the amount of extractant isolated is extremely high. Therefore, using a green solvent is highly desirable in the pharmaceutical area. Green solvents such as water and CO_2 (both supercritical and liquid phase) are potential candidates to extract active ingredients from animals (insects). Sc-CO_2

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Address correspondence to Ai-Yih Wang, Department of Radiological Technology, Yuanpei University, HsinChu 300, Taiwan, Province of China. Tel.: 886 3 5381183 ext. 8914. E-mail: aypaiyi@mail.ypu.edu.tw; Kong-Hwa Chiu, Department of Applied Science, National DongHwa University, Hualien 970, Taiwan, Province of China. Tel.: 886 3 8235175; Fax: 886 3 8223635. E-mail: ckh@mail.ndhu.edu.tw

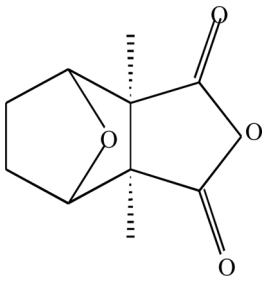

FIG. 1. Molecular structure of cantharidin, ($C_{10}H_{12}O_4$), molecular weight: 196.20 g.

fluid extraction (SFE) is a useful alternative to conventional solvent extraction because of its shorter extraction time and the reduced amount of organic solvent required. Sc-CO_2 can penetrate into the deeper parts of Mylabris tissue due to its zero surface tension. Carbon dioxide is a gas at normal temperatures and pressures and has a critical point at 31°C and 7.4 MPa. The advantages of Sc-CO_2 as a solvent are its purity (leaves no residue), the high solubility of organic compounds and the ease of recovery. Sc-CO_2 has the characteristics of either a liquid (strong solvating power) or a gas (fast exchange kinetics). When the extraction is completed, the liquid carbon dioxide can be removed by merely reducing the pressure, and allowing the gas to evolve. This can be performed at relatively low temperatures and thus many labile compounds are not decomposed. Carbon dioxide is safe, readily available and low-cost. It allows supercritical operations at relatively low pressures and at near-room temperatures (10). In addition to the common advantages of using Sc-CO_2 as an extraction solvent, the spent material is undamaged. It is capable of fractionating with or without additional distillation steps that may alter the chemical composition of the products (11–13).

To our knowledge, no data have been published related to the possible application of liquid carbon dioxide extraction (LCDE) to the extraction of active ingredients from animals, such as insects. The efficient extraction of active ingredients from animals is more difficult than from herbs, because the tissue matrix of animals is more complicated than that of herbs. The extraction method developed in this laboratory is similar to a combination of SFE and accelerated solvent extraction (ASE). ASE is usually performed at an elevated temperature between 50 and 200°C and at pressures between 10 and 15 Mpa. The extraction in this study is carried out under pressure to maintain the solvent in its liquid state at high temperatures and is considered an alternative for extraction of polar compounds.

MATERIALS AND METHODS

Chemicals and Standards

Methanol and acetonitrile (spectrometry grade) were purchased from J. T. Baker (USA). Cantharidin was obtained from Sigma (Japan). A standard stock solution of cantharidin was prepared by dissolving it in methanol at a concentration of 1 mg/mL. Working solutions were freshly prepared daily by diluting with methanol. All other chemicals were of analytical grade (Sigma, Japan) and were used as received. Carbon dioxide (99.5% purity) was purchased from Chiah Lung Enterprise Co.

Equipment

Carbon dioxide fluid extractions were performed using an ISCO Model 260D syringe pump and ISCO series-D pump controller (Teledyne, U.S.A.) (Fig. 2) to fashion an air-driven pump to deliver the CO_2 to the extraction cell (10 mL stainless steel vessel) housed within a temperature-controlled oven. The outlet of the extraction cell was connected to a thermally controlled variable restrictor, which maintained certain pressure conditions in the system. Ultrasonic bath (Delta Ultrasonic cleaner DC2000, 40 kHz, 200 W) was used for the ultrasonic extraction. The HPLC apparatus consisted of a modular chromatographic system (Varian Prep Star MODEL SD-1, U.S.A.) equipped with a photodiode-array detector operating at 237 nm.

Extraction Experiments

A variety of different modes of extraction, including conventional extraction, sonication vibration extraction, pressurized solvent extraction, and LCDE, were carried out in this study. LCDE was performed in an attempt to survey the optimum parameters, such as temperature, extraction time, and the number of extraction cycles, for the exhaustive extraction of cantharidine. A set amount of modifier was spiked directly onto the samples, i.e., Mylabris powder, prior to placing them in the extraction vessel.

Soxhlet Extraction

Mylabris powder, about 0.5 g, was extracted with 250 mL of methanol in a reflux for 65 hours at 230°C.

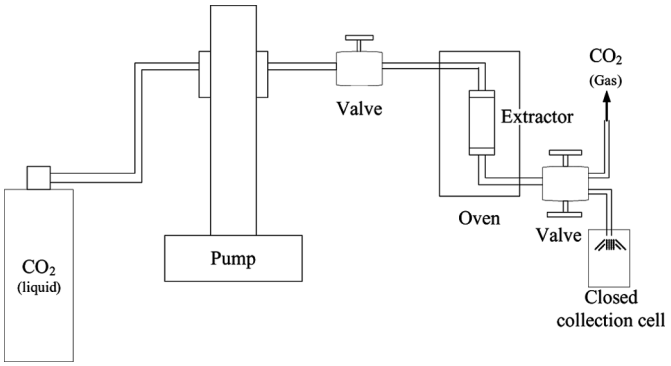

FIG. 2. Schematic diagram of the pressurized liquid CO_2 extraction system.

The extract was concentrated by a rotary evaporator to remove methanol and then redissolved in 5 mL of methanol for HPLC analysis. A second Soxhlet extraction was performed using acetonitrile as the extraction solvent at 230°C for 35 hours with the other conditions the same as those in the methanol extraction.

Sonication Vibration Extraction

Mylabris (0.5 g) was placed in a round flask with 10 mL of acetonitrile. The round flask was then placed in an ultrasonic bath and sonicated for 60 minutes at room temperature. The supernatant was concentrated by a rotary evaporator in an attempt to remove acetonitrile. The extract was redissolved in 5 mL of methanol for HPLC analysis.

Carbon Dioxide Extraction with Modifier

The dried Mylabris was milled to a powder (approximately 0.5 g) and mixed with the modifier, then loaded directly into the extraction cell. Extractions were carried out in the temperature range of 25–70°C and pressure range of 0.5–10 MPa. Extraction times were set between 5 and 40 minutes. As the CO_2 evaporated at the restrictor outlet due to decompression, the extract was collected in a collection vial followed by filtering through the PVTF filter (0.45 μm pore size). The collected extract was redissolved in 5 mL of methanol for HPLC analysis.

Pressurized Solvent Extraction

Mylabris (0.5 g) was packed in an extraction vessel (10 mL). Static extractions were carried out with an extraction time of 30 minutes. The temperature was fixed at 50°C, and the pressure ranged from 0.5 to 15 MPa. Acetonitrile was employed as the solvent. The extract was trapped in a collection vial. For sample trapping in the compressed CO_2 extraction mode, a stainless steel cylinder collector (SSCC) with a volume of 264 mL (inner radius of 6.7 cm and height of 7.5 cm), which could be screwed up tightly with a stainless steel cap, was specifically designed in this lab. A 100-mL glass beaker was placed inside this container, with the upper rim of the beaker in contact with the cap to avoid the possible loss of sample as it spurted from the outlet of the pipe mounted on the center of the cap and connected with the extractor. A tee connector between the extractor and the SSCC was used for either sample in or CO_2 out. After the desired extraction time, the valve on the connector was switched on for trapping the sample, while the pressure reached balance between the extractor and SSCC, releasing the CO_2. The sample along with CO_2 was adsorbed on the inner surface of the beaker. The adsorbent was then re-dissolved in methanol. The collected solution, i.e., Mylabris containing acetonitrile, was concentrated by a rotary evaporator to remove the acetonitrile. The extract was redissolved in 5 mL of methanol for HPLC analysis.

Identification and Recovery

Separations were performed on a Cosmosil C-18 column. The mobile phase was deionized water and methanol in gradient mode as follows: 0–20 minutes, methanol proportion from 0% to 91%; and 20–30 minutes, 91% methanol at a flow rate 0.7 mL/min. The identity of the cantharidin in the Mylabris extract was measured by chromatography using authentic standards. To evaluate the relative difference between different extraction methods, a 0.5 g of homogenized Mylabris powder was spiked with 1 mg cantharidin standard reagent. In order to calculate the recovery, the 0.5 g of Mylabris was used as a blank sample. Recovery was calculated as follows:

$$\text{Recovery}(\%) = \frac{\text{Measured cantharidin in spiked sample} - \text{cantharidin in blank sample}}{\text{spiked cantharidin}}$$

RESULTS AND DISCUSSION

Since enhanced diffusivity of solvents leads to an increase in extraction speed and efficiency (14), we employed the characteristics of pressurized liquids for the extraction of cantharidin in Mylabris. Carbon dioxide is a green solvent; however, it is rarely applied in the extraction of active ingredients from animals (15). To determine optimum conditions for the extraction of cantharidin from Mylabris, several parameters were investigated, i.e., modifier and its volume, extraction temperature, pressure, and time.

Modifier and Its Volume

Due to the limited solubility of cantharidin in carbon dioxide fluid, quantitative extraction of cantharidin using pure CO_2 fluid is very difficult. Therefore, the effect of a modifier on the extraction of cantharidin was studied. Four solvents, acetone ($C_T = 235$°C, $C_P = 4.8$ MPa), ethanol ($C_T = 243$°C, $C_P = 6.3$ MPa), methanol ($C_T = 240$°C, $C_P = 8.1$ MPa) and acetonitrile ($C_T = 275$°C, $C_P = 4.8$ MPa), were investigated due to their common use as the modifiers. Three milliliters of the modifier were added to the extractor containing the packed matrix and the

extraction conditions were set at 50°C and 10 MPa. The sample was subjected to static extraction for 40 mins. The results indicate that the extraction abilities vary with different modifiers. For 1 g of crude Mylabris powder, 9.91 mg of cantharidin were extracted using methanol as the modifier, 8.35 mg using acetonitrile, 8.00 mg using ethanol, and 7.48 mg using acetone. The two best modifiers in this study were methanol and acetonitrile. However, when methanol was used as the modifier, the chromatogram showed many unidentified peaks in addition to cantharidin (18.4 minutes) (Fig. 3a). In contrast, while using acetonitrile as the modifier cantharidin was the major component in the extract (Fig. 3b). In order to reduce the interference of matrices, acetonitrile was chosen as the modifier in this study. Furthermore, to achieve an optimal ratio between cantharadin and acetonitrile for better extraction efficiency, various volumes of acetonitrile were added in the extractor in which a fixed amount of Mylabris had been mounted. The results show that 4 mL of acetonitrile in 10 ml of extractor are most efficient. No obvious change resulted when more acetonitrile was added to the extractor. Therefore, 40% of acetonitrile (v/v) was chosen for further investigation.

Pressure and Temperature

Pressure and temperature are the two most important physical parameters in carbon dioxide fluid extraction. Together they define the density of carbon dioxide fluid, and the salvation power of carbon dioxide fluid is mainly related to its density (10). In this study, the results show no obvious affect between different pressures on the

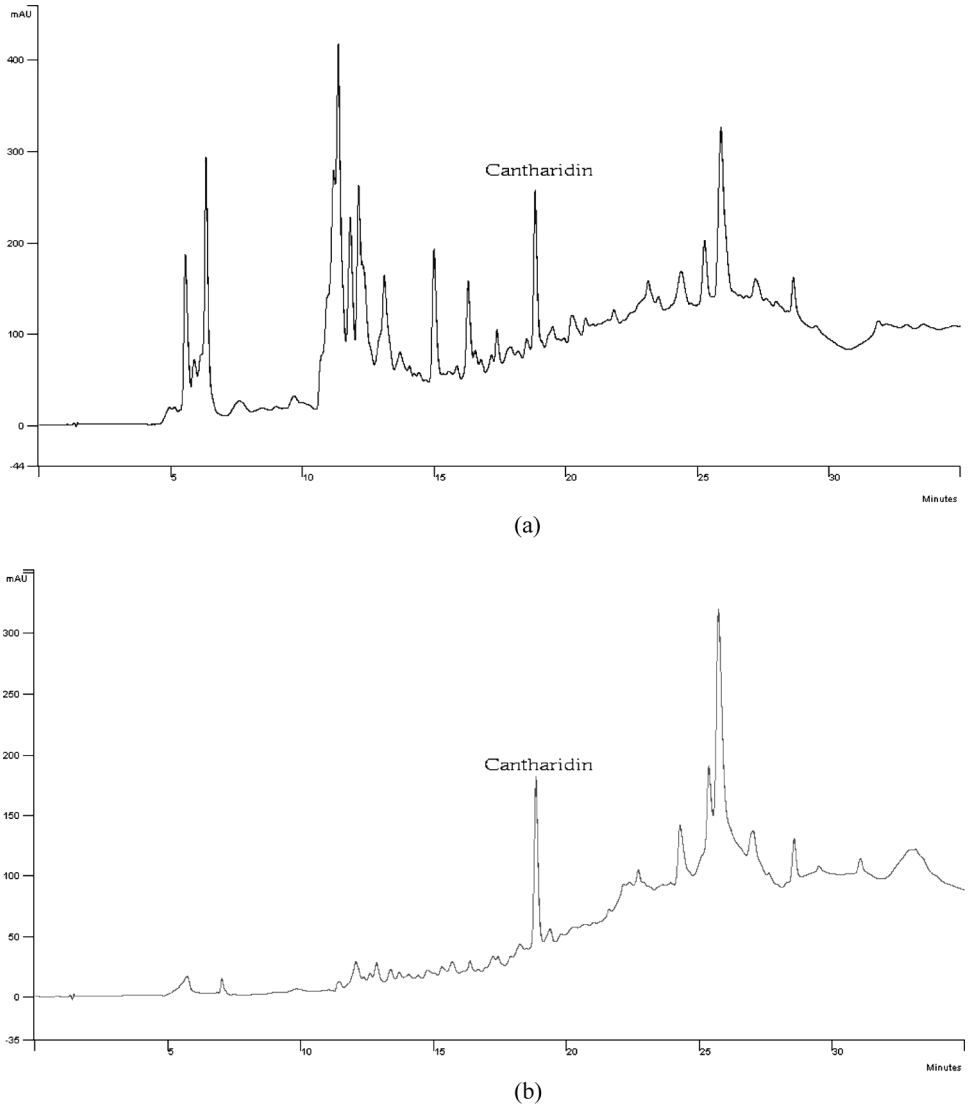

FIG. 3. The HPLC chromatogram of Sc-CO_2 extract. (a) 3 mL methanol as modifier during extraction. (b) 3 mL acetonitrile as modifier during extraction.

extraction efficiency; therefore, a pressure of 1.0 MPa was selected for both energy economy and safety. In carbon dioxide extraction, temperature coupled with pressure will affect not only the fluid density but also the volatility of the analytes of interest in the matrix. By increasing the temperature, the volatility of the analytes increases but the density of CO_2 fluid decreases. In this study, six different temperatures (25, 30, 40, 50, 60, and 70°C) were evaluated to optimize the extraction temperature. The results indicate that 60°C is the optimum temperature for extraction.

Extraction Time

The length of the extraction time influenced the extraction efficiency and the selectivity of the fluid. The static mode permitted better penetration of the matrix by the fluid than did the dynamic mode (16). This study shows that the most efficacious extraction time was 30 minutes during which about 8.50 mg of cantharidin were obtained from 1 g crude Mylabris powder.

Optimization of Extraction Condition

After evaluating the above various parameters, the optimal extraction conditions were found to be $P = 1.0$ MPa, $T = 60°C$, $t_{static} = 30$ minutes, and $V_{modifier} = 4$ mL in 10 mL of extractor (i.e., 40% (v/v)). Under these conditions, carbon dioxide fluid was in the liquid phase. To achieve complete extraction, samples were submitted to three cycles of static extraction at the same conditions. Extracts from each cycle were collected in separate vials and assayed individually. The quantitative assay of total cantharidin according to the above mentioned procedure indicated that 87.7% of cantharidin was extracted by the first cycle; the second cycle donated 7.0%, and the third cycle afforded only minimal additional amounts of cantharidin. This indicates that the extraction efficiency was around 95% after triple extractions. A total of 9.18 mg of cantharidin were obtained from 1 g crude Mylabris powder by three-cycle extraction. In comparison with other extraction methods, it is apparent that green solvent liquid CO_2 offers advantages (Table 1).

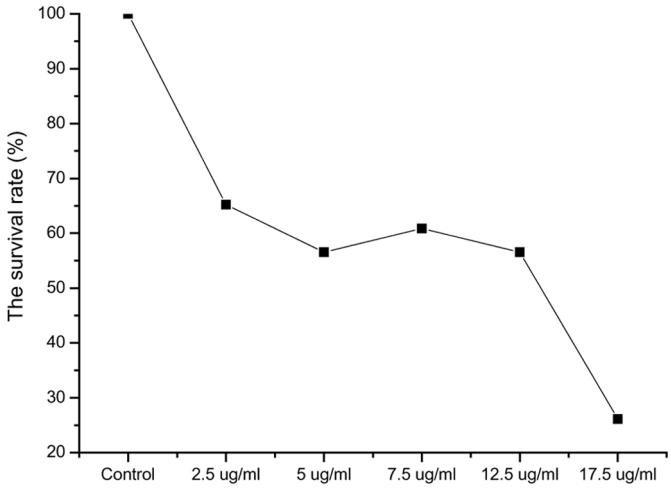

FIG. 4. The survival rate of liver cancer cell lines (Huh-7) under the treatment of various concentrations of cantharidin.

Cell Assay

Liver cancer cells (Huh-7), which were obtained from Professor J. L. Tsai of the Graduate Institute of Occupational Safety and Health, Kaohsiung Medical University were placed in 12 wells of a microtiter, with approximately 10^5 cells in each well. After attaching for 24 hours, each well was treated with cantharidin extract in various concentrations. After 24 hours, the results indicated that the liver cancer cell lines were killed and LD_{50} is around 5 μg/mL (Fig. 4). Using a microscope (Inverted Microscope, Nikon TMS-F, Magnification × 100), apoptosis was observed in the liver cancer cell lines (Fig. 5). The amount of apoptosis in the cell lines was proportional to the amount of cantharidin added; therefore, we propose that cantharidin can inhibit the growth of liver cancer cells. In medicinal research, the identification and purity of bioactive ingredients obtained from herbs or from animals, e.g., insects such as the beetle, are critical; however, bioactive ingredients prepared by using extraction techniques will contain some unwanted but minimal amounts of other components. Nevertheless, since the extract has shown a positive

TABLE 1
The extraction ability of various extraction methods in the isolation of cantharidin from Mylabris

Extraction method	Extraction efficacy	Relative efficiency based on CO_2 fluid extraction
Soxhlet extraction (methanol)	7.35 mg/g	80.0%
Soxhlet extraction (acetonitrile)	9.49 mg/g	103.0%
Sonication vibration	7.18 mg/g	78.2%
High pressure solvent extraction[a]	8.39 mg/g	91.4%
CO_2 fluid (modifier)[b]	9.18 mg/g	100.0%

[a]Acetonitrile as the solvent.
[b]Three extraction cycles were carried out.

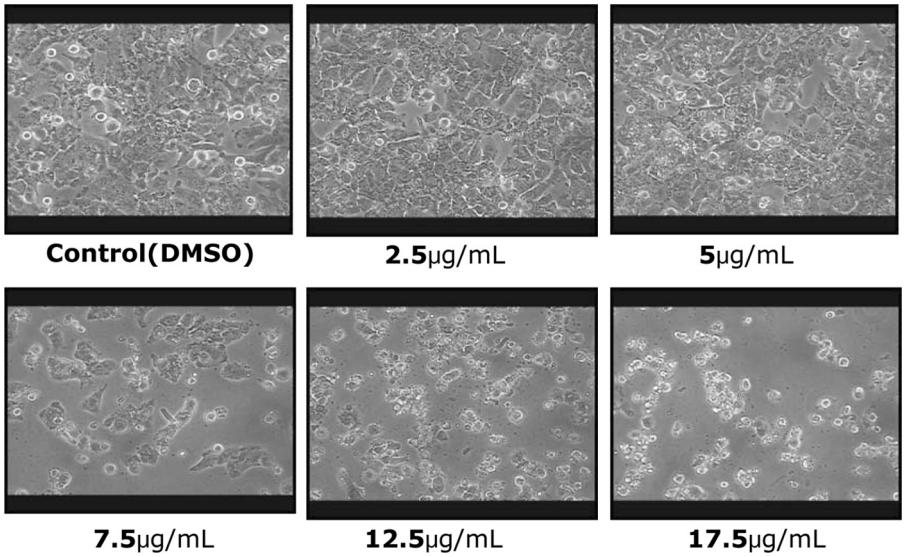

FIG. 5. Apoptosis of liver cancer cell lines treated with different concentrations of cantharidin.

therapeutic effect on cancer cells, we suggest that the secondary aim of this study has been achieved, in addition to the primary goal to identify the structure of the extract.

CONCLUSIONS

The carbon dioxide fluid extraction method has the advantages of needing less solvent, and extraction time and being less costly and more environmentally friendly. While applying this extraction approach in the separation of cantharidin from Mylabris, our research has also shown that the extract, i.e., the active component, is of medical benefit. The optimization of the extraction mode was fast and straightforward, and our experiment showed that three extraction cycles of 30 minutes each afforded almost exhaustive extraction. An investigation of the various parameters affecting the extraction behavior of constituents from complex herbal matrices will be carried out in future studies.

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