Cirsilineol inhibits proliferation of cancer cells by inducing apoptosis via mitochondrial pathway

Xia Sheng, Yang Sun, Ye Yin, Ting Chen and Qiang Xu

Abstract

Cirsilineol (4′,5-dihydroxy-3′,6,7-trimethoxyflavone) is a compound isolated from the herb of *Artemisia vestita* Wall (Compositae). In this study, we aimed at examining the anti-proliferative activity of cirsilineol against multiple types of cancer cells and the underlying mechanisms. Cirsilineol significantly inhibited proliferation of Caov-3, Skov-3, PC3 and Hela cells in a concentration-dependent manner. The compound also dose-dependently induced apoptosis in Caov-3 cells, as determined by annexin V/propidium iodide staining. Besides, cirsilineol induced a remarkable change in mitochondrial membrane potential and caused release of cytochrome *c* to cytosol. Furthermore, the compound caused a marked activation of capase-3, caspase-9 and poly (ADP-ribose) polymerase (PARP). These results suggested that the induction of apoptosis via the mitochondrial pathway was involved in the anti-proliferative activity of cirsilineol against cancer cells.

Introduction

In modern pharmacological studies extracts of, or principles from, plants have been shown to have various bioactivities such as anti-inflammatory and anti-mutagenic effects (Schinella et al 1998; Nakasugi et al 2000; Seo et al 2002). Cirsilineol (4′,5-dihydroxy-3′,6,7-trimethoxy-flavone) is isolated from the herb of *Artemisia vestita* Wall (Compositae), which has been widely used in traditional Tibetan and Chinese medicine for a variety of inflammatory diseases, such as rheumatoid arthritis, contact dermatitis and sepsis (Qiangba et al 2002; Wang et al 2005). Although this compound was reported more than 20 years ago (Maruhenda et al 1987), its biological activity has not been explored much except for antibacterial and antioxidant activity (Kelm et al 2000; Heo et al 2001; Isobe et al 2006). It has been reported that some flavonoids showed broad-spectrum anti-tumour effects (Kim et al 2004, 2005; Katayama et al 2007; Nam et al 2008). These findings drive us to explore a possible similar effect of cirsilineol. In this study, we have first demonstrated that cirsilineol has anti-proliferative activity against various cancer cells by inducing apoptosis.

Materials and Methods

Drugs and reagents

Cirsilineol (Figure 1) was isolated from the herb of *Artemisia vestita* Wall. The following reagents were purchased as follows: 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sunshine Biotechnology, China); antibodies to poly (ADP-ribose) polymerase (PARP), caspase-3, caspase-9, cytochrome *c* oxidase subunit IV (COX-IV) and cytochrome *c* (Cell Signaling Technology, Beverly, MA, USA); antibody to tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); peroxidase-labelled anti-rabbit antibody, peroxidase-labelled anti-mouse antibody (KPL, Gaithersburg, MD, USA); 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR, USA).

Extraction and isolation

The dried aerial part of *Artemisia vestita* was extracted with 75% ethanol. Then the extract was applied on a macroporous adsorption resin HP-20 (250–850 μm; Mitsubishi Chemical, Japan), eluted by water, 30% ethanol, 60% ethanol and 90% ethanol, respectively, and

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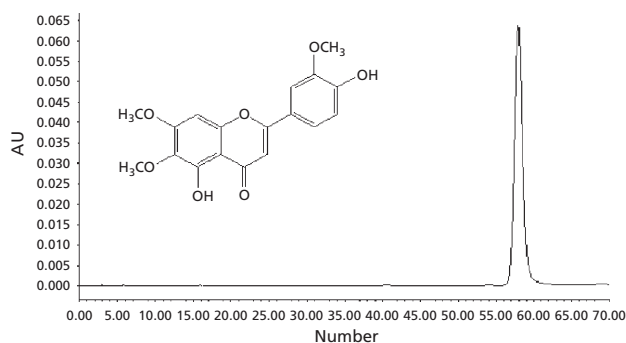E-mail: molpharm@163.com

Figure 1 HPLC analysis of J03 isolated from *Artemisia vestita* Wall and the structure of cirsilineol.

evaporated to dryness under reduced pressure to yield four fractions, Fr. 1–Fr. 4 (Fr. 1, 30.5 g; Fr. 2, 60.6 g; Fr. 3, 90.6 g; and Fr. 4, 33.1 g). Then Fr. 3 was introduced onto a silica gel column and eluted with CH_2Cl_2–MeOH (100:0 to 30:70) step gradient to give 11 fractions (Fr. 3-1 to Fr. 3-11). Fr. 3-2 was rechromatographed over silica gel column (100–200 mesh; Qingdao Oceanic Chemical Plant, China) eluted with CH_2Cl_2–MeOH (49:1) to yield J03 (cirsilineol, 98% purity by HPLC).

HPLC analysis and structural elucidation

All the content tests were applied on a Waters 600 pump, with a 2487 UV-Vis detector, an online degasser, and a 5-μL injection loop. J03 was applied to a C18 column (Kromasil, 4.6 × 250 mm, 5 μm) and eluted with CH_3OH–H_2O (55:45). The effluents were detected at 275 nm. Column temperature was set up at 25°C and the flow rate was 1 mL min^{-1}. NMR and ES-MS were used for structure elucidation. The ^{1}H and ^{13}C NMR measurements were carried out in a Bruker DPX-300 spectrometer operating at 300 and 100 MHz, respectively. ES-MS experiments were recorded on an ABI Mariner ESI-TOF mass spectrometer.

Cell lines

Human cancer cells, Caov-3, Skov-3, PC3 and Hela cells, were obtained from ATCC. These cells were maintained in DMEM (Life Technologies Inc., Grand Island, NY, USA); human liver cancer cell line HepG2 and normal liver cell line L02 (ATCC) were cultured in RPMI-1640 medium (Life Technologies Inc.). All the media were supplemented with 10% fetal bovine serum (FBS; Life Technologies Inc.), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in the air. All the cell lines used were adherent cells.

MTT assay

Cancer cells (5 × 10^3 cells per well) were seeded in a 96-well plate with different concentrations of cirsilineol (0, 10, 20 and 40 μL), which was dissolved in RPMI-1640 medium with less than 1% dimethyl sulfoxide (DMSO). After incubation at 37°C for 48 h, MTT was dissolved in

phosphate-buffered saline (PBS) at 5 mg mL^{-1} and added to culture media at a final concentration of 0.5 mg mL^{-1}. After 4 h, media were removed, and 100 μL DMSO was added to each well to dissolve purple crystals of formazan. The plate was shaken for 10 min to allow complete solubilization. Spectrophotometric absorbance at 540 nm was read on a 96-well plate reader.

Assay for cell apoptosis by annexin V/propidium iodide (PI) double staining

Caov-3 cells (2 × 10^5 cells per well) were incubated in a 12-well plate for 24 h in the presence of indicated concentrations of cirsilineol. After the incubation, the cells were washed with PBS and used for determining apoptosis. Apoptotic cell death was identified by double supravital staining with recombinant FITC (fluorescein isothiocyanate) conjugated annexin V and propidium iodide, using the Annexin V-FITC Apoptosis Detection kit (Becton Dickinson, Frankly Lakes, NJ, USA) according to the manufacturer's specifications. Flow cytometric analysis was performed immediately after the staining. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software.

Assay for mitochondrial membrane potential

The JC-1 dye assay was used for determination of mitochondrial membrane potential according to the manufacturer's instructions. Briefly, cells were incubated in a 12-well plate for 24 h in the presence of indicated concentrations of cirsilineol. After culture, the cells were fixed in 0.5% paraformaldehyde (PFA) for 30 min and stained with 1 μL JC-1 at 1 mg mL^{-1} in DMSO for 15 min at 37°C. Then, the changes in red-orange fluorescence were analysed. Data acquisition and analysis were performed in a Becton Dickinson FACSCalibur flow cytometer using CellQuest software.

Gel electrophoresis and western blot analysis

Cells were incubated for 24 h in the presence of indicated concentrations of cirsilineol. After incubation, cells were harvested and lysed. Mitochondrial protein and cytosolic protein were isolated using a Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Proteins were quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's specifications. For western blot analysis, the proteins were electrophoresed on a 7.5–10% SDS-PAGE (SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis) gel, followed by immunoblotting on PVDF (polyvinylidene fluoride) membrane. Then, the membrane was blocked with 5% nonfat dry milk in TBS (Tris-buffered sulfate) with 0.1% Tween 20 for 1 h at room temperature. Finally, proteins were visualized using the enhanced chemiluminescence detection system (Cell Signaling Technology, MA, USA) after incubation with antibodies to PARP (mouse anti human antibody; Sigma, MO, USA), caspase-3 (mouse anti human antibody; Sigma), caspase-9 (rabbit anti human antibody; Santa Cruz

Biotechnology), cytochrome *c* (mouse anti human antibody; Cell Signaling Technology), COX-IV (mouse anti human antibody; Cell Signaling Technology) or tubulin (mouse anti human antibody; Sigma) overnight at 4°C. Then they were incubated with peroxidase-labelled anti-rabbit antibody or peroxidase-labelled anti-mouse antibody (Cell Signaling Technology) for 1 h at room temperature. Peroxidase activity was visualized on X-ray film in darkroom.

Statistical analysis

Results were expressed as mean ± s.d. of three independent experiments and each experiment included triplicate sets. Data were statistically evaluated by one-way analysis of variance followed by Dunnett's test between control group and multiple dose groups, with the level of significance chosen as $P < 0.05$.

Results

Identification of cirsilineol

J03 isolated was subjected to HPLC analysis and structure determination. The purity of J03 was confirmed to be 98% by

HPLC (Figure 1). The structure of J03 was identified as cirsilineol (Figure 1) by MS and NMR spectral analyses and compared with the reported data (Liu & Mabry 1981). ES-MS: $[M+H]^+$ 345.1534. ^{1}H NMR (DMSO-*d*6, 500 Hz) δ: 3.73 (3H, s, C6-OCH$_3$), 3.90 (3H, s, C7-OCH$_3$), 3.94 (3H, s, C3′-OCH$_3$), 6.93 (1H, s, C3-H), 6.96 (1H, s, C8-H), 6.98 (1H, d, C5′-H), 7.60 (1H, d, C2′-H), 7.63 (1H, dd, C6′-H), 10.02 (1H, s, C4′-H), 12.95 (1H, s, C5-H).

Cirsilineol inhibits proliferation of cancer cell lines Caov-3, Skov-3, PC3, Hela and HepG2 cells in a concentration-dependent manner, but not normal liver cell line L02 cells

To demonstrate the effects of cirsilineol on different cancer cells, exponentially growing cells were cultured over a range of concentrations of cirsilineol for 48 h. Treatment with cirsilineol significantly reduced proliferation of all these 4 kinds of cancer cells in a dose-dependent manner (Figure 2A). In addition, two liver cell lines, cancer cell line HepG2 and normal cell line L02, were used for comparing the effect of cirsilineol, and Caov-3 cells were used as positive control. As a result, Caov-3 and HepG2 cells
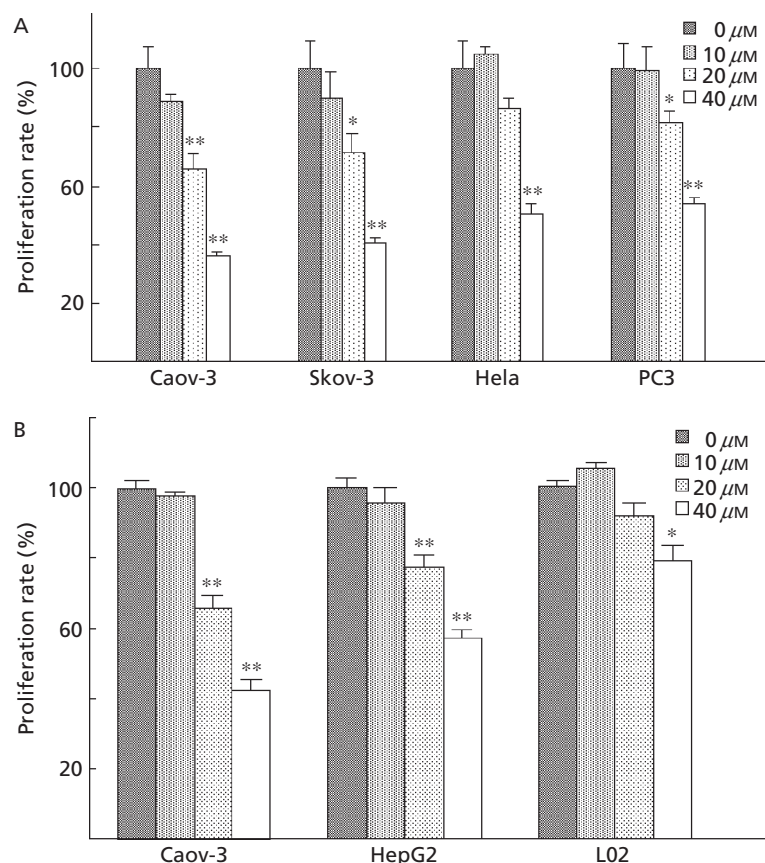

Figure 2 A. Cirsilineol inhibits proliferation of Caov-3, Skov-3, PC3 and Hela cells. B. Cirsilineol inhibits proliferation of HepG2 but not L02 cells. Cells were first cultured in a 96-well plate for 24 h. Then they were treated with different concentrations of cirsilineol for a further 48 h. The MTT assay was used to evaluate the proliferation rate. Each datum indicated the mean ± s.d. of three independent experiments. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test).

showed significant reduction of proliferation, while the proliferation of L02 was much less affected by cirsilineol (Figure 2B).

Cirsilineol induces apoptosis in Caov-3 cells

As shown in Figure 3A, B, Caov-3 cells were treated with different concentrations of cirsilineol for 24 h. Annexin V positive cells were early and late apoptotic populations. Treatment with cirsilineol induced apoptosis in Caov-3 cells in a dose-dependent manner.

Cirsilineol activates caspase-9, -3 and PARP to promote apoptosis

To examine the status of the caspase-3 protein, we performed western blot analysis by using an anti-caspase-3 antibody, which recognizes both procaspase-3 and p17 cleaved

caspase-3. The p17 cleavage product appeared in the lysates of Caov-3 cells treated with 50 μM cirsilineol or 10 μM quercetin (Figure 4). Cirsilineol at 25 μM induced a slight cleavage of caspase-3. In addition, cirsilineol at 50 μM and quercetin at 10 μM also cleaved PARP to a band of 85 kDa and increased the amount of cleaved caspase-9 (Figure 4).

Cirsilineol induces loss of mitochondrial membrane potential (MMP)

The mitochondrial apoptosis pathway is one of the upstream pathways of caspase-3. Alterations in MMP were examined upon cirsilineol treatment. Caov-3 cells were incubated with cirsilineol or quercetin for 24 h. As shown in Figure 5A, a reduction in MMP was detected in both cirsilineol- and quercetin-treated groups and we clearly observed a dose-dependent effect of cirsilineol.
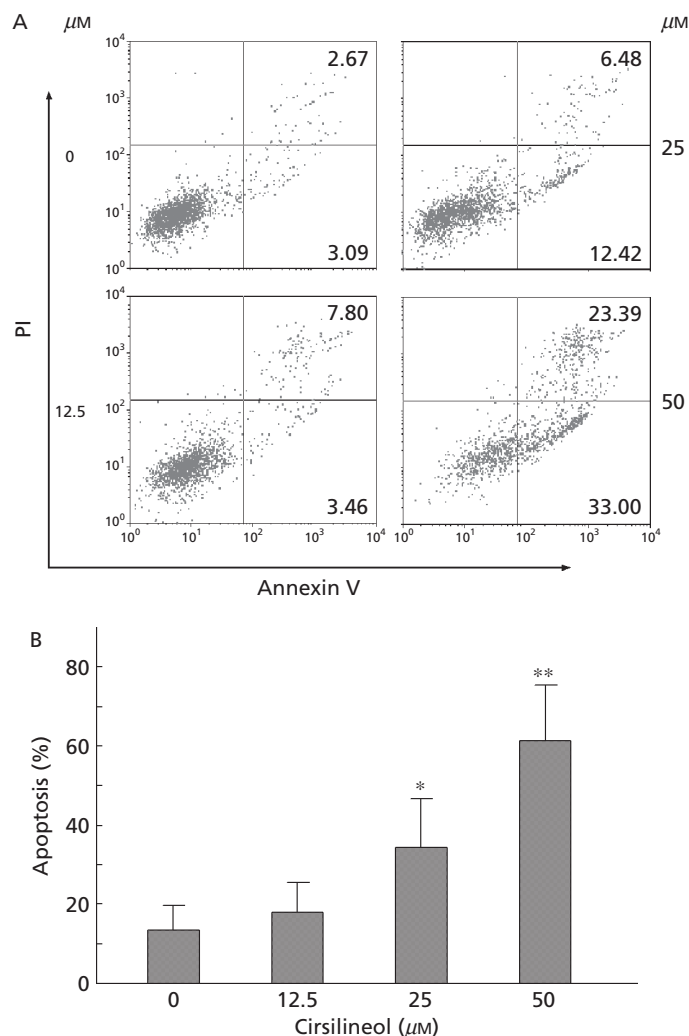

Figure 3   Cirsilineol induces apoptosis of Caov-3 cells. Cells were seeded in a 12-well plate overnight, and then were treated with different concentrations of cirsilineol for 24 h. Annexin V/PI staining was used to measure the apoptosis rate. The results in A are representative of three separate experiments. Data in B are expressed as histograms of mean ± s.d. of three independent experiments. *$P < 0.05$, **$P < 0.01$ vs control (Dunnett's test).
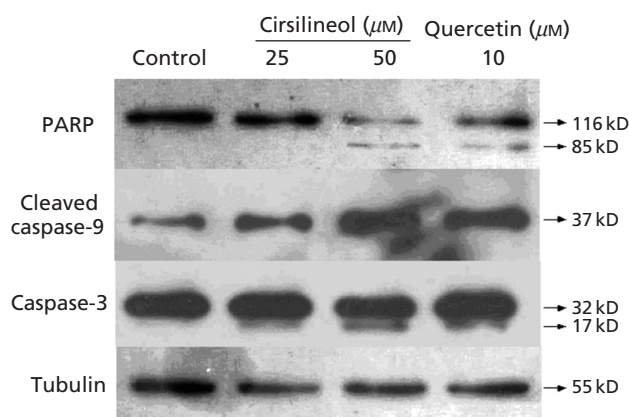
Figure 4 Cirsilineol activates caspase-9, -3 and PARP to promote apoptosis. Whole cell lysates were prepared from Caov-3 cells, which had been incubated with 25 or 50 μM cirsilineol or 10 μM quercetin for 24 h, respectively. The proteins were subjected to western blot analysis. Data are representative of three independent experiments.

Cirsilineol induces cytochrome *c* release from mitochondria to cytosol

Caov-3 cells were incubated with cirsilineol for 24 h. After incubation, mitochondrial protein and cytosolic protein was isolated from the cells, respectively, and 30 μg of each protein fraction was subjected to western blotting. Equal protein loading was confirmed by immunodetection of COX-IV for mitochondrial protein or tubulin for cytosolic protein. As shown in Figure 5B, cirsilineol greatly increased the cytosolic cytochrome *c* and decreased the mitochondrial cytochrome *c* as compared with the control group.

Discussion

This study provides insights into the molecular mechanisms of the anti-tumour activity of cirsilineol. First, we found that cirsilineol inhibited the proliferation of several human cancer cell lines in a dose-dependent manner (Figure 2A),
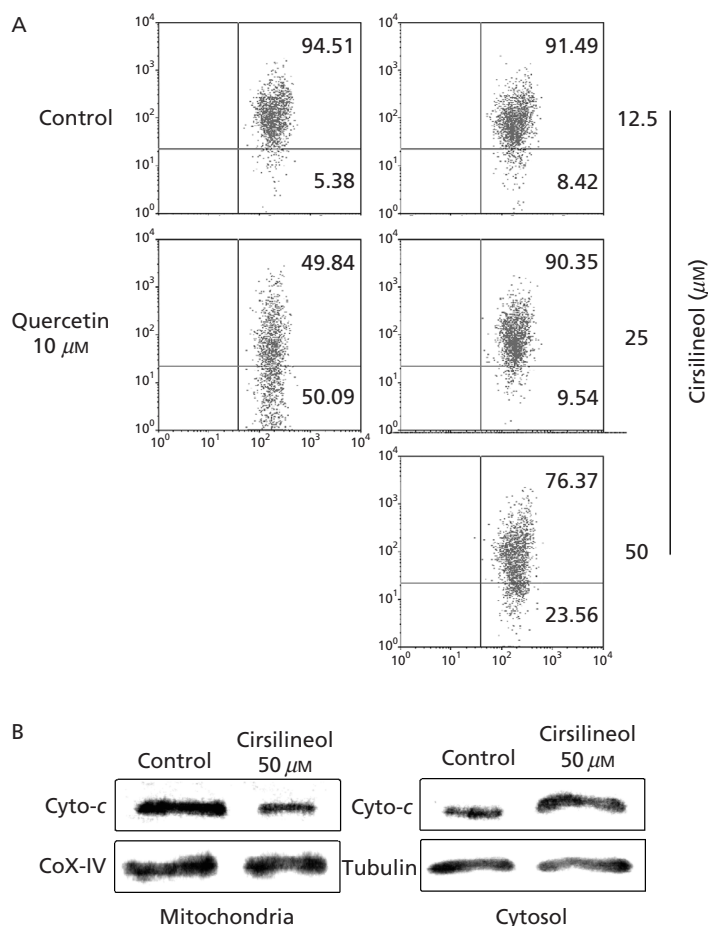

Figure 5 A. Cirsilineol dose-dependently induces loss of mitochondrial membrane potential. Caov-3 cells were cultured for 24 h in the presence of cirsilineol or quercetin. Then they were harvested for measuring mitochondrial membrane potential by flow cytometry using JC-1 staining. Dots on the lower side indicate cells with lower membrane potential. One of three independent experiments is shown. B. Cirsilineol induces cytochrome *c* release from mitochondria to cytosol. Mitochondrial and cytosolic proteins were prepared from cells treated with 50 μM cirsilineol. They were subjected to western blotting using indicated antibodies.

suggesting a relatively ubiquitous anti-tumour activity of this compound. Further comparison between malignant liver cell line HepG2 and normal liver cell line L02 shows that cirsilineol could selectively reduce proliferation in cancer cells but less in normal cells (Figure 2B). This finding seems in accordance with the report by Gupta et al (2001) that apigenin, a flavonoid, had a proliferation-inhibitory effect on cancer cells rather than normal ones. Since the Caov-3 cell line seemed to be more susceptible to cirsilineol treatment in the above anti-cancer assays, we used the cell line in subsequent experiments to maximize the proapoptotic effect of cirsilineol. To investigate the mechanisms underlying the anti-proliferative activity, we observed the apoptosis-inducing effect of cirsilineol on Caov-3 cells, which occurred in a concentration-dependent manner (Figure 3), as did its anti-proliferative effect (Figure 2). This finding suggests a role of apoptosis induction in the anti-proliferative effect of cirsilineol against Caov-3 cells.

Apoptosis is a form of cell death that permits the removal of damaged, senescent or unwanted cells in multicellular organisms, without damage to the cellular microenvironment. Defective apoptosis represents a major causative factor in the development and progression of cancer (Millan & Huerta 2007). All the criteria used to describe apoptotic cells are morphological, including the presence of typical DNA fragments, caspases activation and a phosphatidylserine shift toward the outer leaflet of the cell membrane (Hail et al 2006). Caspases are responsible for crucial aspects of apoptosis-induced cell death. Among the (at least) 11 known members of the caspase family, caspase-3 is an executioner in caspase cascades and is a main player in apoptosis (Lavrik et al 2005). As an important substrate of caspase-3, PARP cleaveage is considered to show caspase-3 activity (Decker & Muller 2002). Considering that quercetin, another widely used flavone, has a strong proapoptotic effect on cancer cells (Granado-Serrano et al 2006), we used it as a control in the apoptosis assay for comparison. Figure 4 shows that caspase-3 and PARP were both cleaved in the cells treated with cirsilineol and quercetin. This result confirmed the role of apoptosis induction in the anti-tumour mechanisms of cirsilineol.

Mitochondria are known as the bioenergetic and metabolic centres of eukaryotic cells. During the process of apoptosis, mitochondria suffer specific damage, including perturbation of mitochondrial membrane permeability. As a result cytochrome c is released to the cytosol. Once released, cytochrome c, in interaction with apoptotic protease activating factors (Apaf 1), initiates the activation of caspase-9 that leads to the subsequent apoptosis (Zou et al 1999). The release of cytochrome c, as a component of the electron transfer chain, can potentially halt the electron transfer, leading to failure in maintaining the mitochondrial membrane potential and ATP synthesis. Moreover, because cytochrome c carries electrons from cytochrome c reductase to cytochrome c oxidase, by which oxygen molecules are reduced to water, a blockade at this step would increase the production of reactive oxygen species with subsequent lipid peroxidation (Hockenbery et al 1993; Cai et al 2000). To know how cirsilineol induces apoptosis, we further observed

several cellular events relating to the induction of apoptosis. Cirsilineol dose-dependently disrupted the mitochondrial membrane potential (Figure 5A), and caused release of cytochrome c to cytosol (Figure 5B). Cirsilineol also triggered caspase-9 activation (Figure 4). All these findings indicated the involvement of the mitochondrial pathway in the apoptosis.

In the case of apoptosis induction, quercetin (3,3′,4′,5,7-pentahydroxyflavone) showed obviously better efficacy than cirsilineol (4′,5-dihydroxy-3′,6,7-thimethoxyflavone). Comparison of the structure of these compounds suggested that the 3-hydroxyl group might be very important for the function of flavone compounds.

Conclusions

Taken together, our data showed that cirsilineol inhibited the proliferation of human cancer cells by inducing apoptosis. As the mechanism involved apoptosis induction, the mitochondrial pathway via release of cytochrome c may be mainly considered. Furthermore, the activation of caspase-9, -3 and PARP was also included in the cirsilineol-induced apoptosis. These findings explored here may be helpful for understanding the properties of this interesting compound.

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