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Cimicifuga foetida extract inhibits proliferation of hepatocellular cells via induction of cell cycle arrest and apoptosis

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Abstract

The purpose of this study is to determine whether the ethyl acetate fraction (EAF) from the aerial part of *Cimicifuga foetida* Linnaeus possesses the anti-tumor action on hepatoma, and therefore, provide evidence for the traditional use of the plant as a detoxification agent. EAF was extracted and its cytotoxicity was evaluated on a panel of Hepatocytes by MTT assay. The IC_{50} values of EAF on HepG2, R-HepG2 and primary cultured normal mouse hepatocytes were 21, 43 and 80 μg/mL, respectively. Morphology observation, Annexin V-FITC/PI staining, cell cycle analysis and western blot were used to further elucidate the cytotoxic mechanism of EAF. EAF induced G_0/G_1 cell cycle arrest at lower concentration (25 μg/mL), and triggered G_2/M arrest and apoptosis at higher concentrations (50 and 100 μg/mL, respectively). An increase in the ratio of Bax/Bcl-2, activation of downstream effector Caspase 3, and cleavage of poly-ADP-ribose polymerase (PARP) were implicated in EAF-induced apoptosis. In addition, EAF inhibited the growth of the implanted mouse H_{22} tumor in a dose-dependent manner with the growth inhibitory rate of 63.32% at 200 mg/kg. In conclusion, EAF may potentially find use as a new therapy for the treatment of hepatoma.

Keywords: *Cimicifuga foetida* Linnaeus; EAF; Cell cycle; Apoptosis; H_{22} hepatoma

1. Introduction

Tumorigenesis is the manifestation of a delicate balancing act gone awry (Mathew and White, 2006). Deregulated cell growth and suppressed cell death together provide the underlying platform for neoplastic progression (Evan and Vousden, 2001). Cell cycle progression and apoptosis are two pivotal signaling mechanisms used to maintain homeostasis in healthy tissue. Cell proliferation is governed by cell cycle, which is an order of events that is tightly regulated by a number of cyclin-dependent kinases (CDK) and cyclins (Buecher et al., 2003). A shift in favor of proliferative signals, for example, over-activation of cyclin/CDK, often leads to uncontrolled cell division and malignancy. Necrosis and apoptosis are two distinct mechanisms of cell death. Unlike necrosis, apoptosis, also known as programmed cell death, is an expedient way of eliminating superfluous abnormal cells by phagocytosis without an inflammatory response. Multiple lines of evidence suggest that the loss of control of cell cycle or apoptosis is responsible for cancer initiation and progression (Tu et al., 1996; Vitale-Cross et al., 2004). In turn, one essential strategy for cancer therapy is focused on target proteins that suppress cell cycle progression and apoptosis in the tumor cells. It has been found that many anti-cancer agents arrest the cell cycle at the G_1, S or G_2/M phase and then induce apoptotic cell death (Fujimoto et al., 1999; Cheng et al., 2005; Qi et al., 2005; Yang et al., 2005; Wang et al., 2007). In the search for new cancer therapeutics, the herbs used in traditional medicines for cancer treatment are promising candidates.

The genus *Cimicifuga* (Ranunculaceae) consists of more than 18 species and many rhizomes of these plants have been widely used in traditional medicine worldwide. Rhizomes of *Cimicifuga foetida* Linnaeus and *Cimicifuga dahurica*, two of the most common Asiatic species, have been employed as cooling and detoxification agents by Chinese people since ancient times. As we know, tumor is a kind of toxin in the theory of Chinese Medicine, so it is of interest to investigate the anti-tumor activity of Cimicifuga plants. *Cimicifuga racemosa*, a

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famous European species has been shown remarkable antitumor activities in recent studies. Several kinds of extracts from rhizomes of *Cimicifuga racemosa* were demonstrated to kill estrogen receptor-positive (MCF-7), estrogen receptor negative (MDA-MB231 and MDA-MB-453) human breast carcinoma and androgen-sensitive LNCaP human prostate cancer-derived cell lines. The cytotoxic mechanisms of these extracts were related to the induction of cell cycle arrest, apoptosis and degradation of cytokeratin (CK) 18 (Einbond et al., 2004; Hostanska et al., 2004, 2005; Jarry et al., 2005; Seidlova-Wuttke et al., 2006). The standard *Cimicifuga racemosa* extract BNO 1055 also inhibits the formation and/or proliferation of tumors induced by subcutaneous inoculation of LNCaP cells in immunodeficient nu/nu mice (Seidlova-Wuttke et al., 2006). Recently, two cycloartane triterpenoid glycosides isolated from the rhizomes and aerial part of *Cimicifuga foetida* Linnaeus have demonstrated the cytotoxicity (Tian et al., 2006a; Sun et al., 2007). However, up till now, the aerial part of *Cimicifuga foetida* has been discarded and few reports been conducted studying its pharmacological effects.

In the present study, we reported the antitumor activity and mechanisms of action of EAF from the aerial part of *Cimicifuga foetida* Linnaeus to provide evidence for its traditional use as a detoxification agent.

2. Materials and methods

2.1. Extraction and concentration measurement

The plant material was collected in Ankang, Shannxi Province, China, in August 1998 and was identified by Professor Ruile Pan of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College. A voucher specimen has been deposited in the Herbarium of the same Institute (No. 19980816-1). The powdered aerial part of the plant was extracted exhaustively with 80% ethanol under refluxing. After combination, the solvent was evaporated under vacuum to obtain the crude extract, which was chromatographed on a silica gel column and eluted with *n*-hexane and acetic acetate, respectively. After the solvent had been removed under vacuum, EAF was the ethyl acetate fraction.

Twenty-one triterpene glycosides including 25-anhydrocimigenol-3-O-β-D-xylopyranoside, 23-O-aceytalcimigenol-3-O-β-D-xylopyranoside, together with several phenolic acids, such as ferulic acid and isoferulic acid were isolated from EAF. The total triterpene glycosides concentration in EAF was estimated by a colorimetric method as we have described previously (Q.Chang et al., 1995), with slight modifications. Prior to the assay, the sample was cleaned of the sugar by a Waters Oasis HLB solid-phase extraction cartridge. The concentration of total percentage of triterpene glycosides, determined at 544 nm, was 53.65 ± 2.81 expressed as cimigenol xylopyranoside equivalent. The concentration of phenolic compounds in EAF was estimated at 750 nm using Folin–Ciocalteu assay (Gahler et al., 2003). The total percentage of phenolic concentration in EAF was 13.41 ± 0.28 expressed as Ferulic acid (Sigma) equivalent. All the assays for concentration determination were conducted in triplicate.

2.2. Cell culture and drug treatment

HepG2 (ATCC, Rockville, MD, USA) cells were maintained in RPMI 1640 containing 10% FBS (Gibco, BRL), 2 mg/mL sodium bicarbonate, 100 μg/mL penicillin sodium salt and 100 μg/mL streptomycin sulfate. R-HepG2 (City University of Hong Kong) cells were maintained in the presence of 1.2 μM doxorubicin (Sigma). Cells were grown to 70% confluence, trypsinized with 0.25% trypsin-2 mM EDTA, and plated in 96 well plates. Mouse hepatocytes were isolated from normal CD-1 (ICR) mice (Beijing Vital Laboratory Animal Technology, Beijing, China) using the enzymatic perfusion technique as we have described previously (Tian et al., 2005). The viability of the mouse hepatocytes, tested with trypan blue was about 80%. In all experiments, cells were grown in RPMI-1640 medium with 10% FBS for 24 h prior to treatment.

EAF was dissolved in DMSO at a concentration of 250 mg/mL, and diluted in tissue culture medium before use. The concentration of DMSO (0.1%) did not affect the cell viability.

2.3. Cytotoxicity assay

Cells were seeded in 96 well plates and treated with EAF or vehicle at various concentrations and incubated for 48 h followed by MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay (Won et al., 2006). Betulinic acid, a known selective cytotoxic triterpene was used as positive control. IC_{50} of EAF on different cell lines were derived from the dose response curves.

2.4. Cell cycle analysis

HepG2 cells were treated with EAF at different concentrations (25, 50 and 100 μg/mL for 48 h) and time points (at 50 μg/mL for 12, 24 and 48 h). The supernatant was then collected and the cells were trypsinized, collected and fixed in 70% cold ethanol (4 °C) overnight. After washing twice with PBS, cells were resuspended in PBS. RNase A (0.5 mg/mL) and PI (2.5 μg/mL) were added to the fixed cells for 30 min. The DNA content of cells was then analyzed with a FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA) and Coulter (Fullerton, CA, USA) Epics XL. The percentage of cells in different cell cycle phases was calculated by ModFit 3.0 (Verity Software House, Inc.).

2.5. Morphology observation

AO/EB (acridine orange/ethidium bromide) fluorescence staining method was used to observe apoptotic morphology of individuals in cell population. Briefly, HepG2 cells were treated with vehicle or EAF for 48 h at 50 μg/mL. Photographs were taken under an inverted Leica fluorescence 40 × 10 microscopy after AO/EB staining.