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Antitumor Effects of Osthol from *Cnidium monnieri: An In Vitro* and *In Vivo Study*

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***Cnidium monnieri* (L.) Cusson is a Chinese medicine which is used widely by traditional medicine doctors. Osthol is a major bio-activity compound of the herb. In this study, osthol was isolated from *C. monnieri* and its *in vitro* and *in vivo* antitumor effects studied. The results of the *in vitro* study showed: that osthol inhibited the growth of HeLa, in a time- and concentration-dependent manner, with IC_{50} values of 77.96 and 64.94 μM for 24 and 48 h, respectively; that osthol had lower cytotoxic effects in primary cultured normal cervical fibroblasts; and that increased DNA fragmentation and activated PARP in HeLa after treatment with osthol which could induce apoptosis. The results of the *in vivo* model showed that the survival days of the P-388 D1 tumor-bearing CDF_1 mice were prolonged (ILS% = 37) after osthol (30 mg/kg) was given once a day for 9 days. Based on these results, it is suggested that osthol could inhibit P-388 D1 cells *in vivo* and induce apoptosis in HeLa cells *in vitro*, and that osthol is good lead compound for developing antitumor drugs. However, *C. formosanum* Yabe of Taiwan's endemic plants contained little osthol, with no imperatorin, and its major components were different from that of *C. monnieri*. Therefore, it is suggested that *C. formosanum* also may possess economic worth. Copyright © 2006 John Wiley & Sons, Ltd.**

Keywords: osthol; *Cnidium monnieri*; Umbelliferae; *Cnidium formosanum*; P-388 D1-bearing CDF_1 mice model; apoptosis.

INTRODUCTION

The dried fruits of *Cnidium monnieri* (L.) Cusson (Umbelliferae) have been used widely in Chinese herbal prescriptions such as *She Chuang Zi*. In Taiwan *C. monnieri* is imported from China. But *C. formosanum* Yabe, an endemic plant in Taiwan, is not commonly used as a Chinese medicine (Xu, 1972). However, the root of *C. formosanum* is used as a folk medicine in Taiwan to treat bone pain disease (Xu, 1972). Osthol and imperatorin were isolated from *C. monnieri* (Yang *et al.*, 2003a). These two compounds have been shown to induce the death of several types of tumor cells and apoptosis in HL-60 and P-388 D1 cells (Yang *et al.*, 2003a). Furthermore, osthol has been shown to have more cytotoxicity than imperatorin in HeLa cells – human cervical tumor cells (Yang *et al.*, 2003a). Baba *et al.* reported that the main constituents of *C. formosanum* are angular-type dihydrofurocoumarins (Baba *et al.*, 1985). In contrast, Sagara also reported that *C. formosanum* fructus does not contain imperatorin and coumarin constituents (Sagara *et al.*, 1987). Therefore, osthol and imperatorin have been used as bio-substance markers to separate *C. monnieri* and *C. formosanum* by HPLC analysis. Hopefully, a good economic plant from the endemic plants of Taiwan can be identified and the cytotoxic mechanism of osthol can be shown in HeLa cells *in vitro* and the antitumor effect in P-388 D1-bearing mice.

Osthol induces apoptosis in P-388 D1 cells (Yang *et al.*, 2003a). The antitumor effects of osthol are continually being explored in the P-388 D1-bearing CDF_1 mice model. The leukemia P-388 D1 *in vivo* animal model is popularly used to assess the antitumor effects of cytotoxic compounds (Arsenoua *et al.*, 2004). CDF1 mice are from a hybrid fertilization of female BALB/c and male DBA/2. After CDF1 mice were given intraperitoneally lymphocytic P-388 D1 cells, the mice developed ascites with increased body weights in 10 days. Osthol was orally administered to the P-388 D1-bearing CDF_1 mice once a day for 9 days and their survival days observed to evaluate the antitumor effects of osthol. Osthol also induces tumors and the death of vascular smooth muscle cells (Yang *et al.*, 2003a; Kawaii *et al.*, 2001; Hitotsuyanagi *et al.*, 1996; Guh *et al.*, 1996; Fujioka *et al.*, 1999). But the mechanism of cytotoxicity is still unclear. In this paper, it was intended to show whether osthol can induce apoptosis or necrosis in HeLa cells. Anticancer drugs induce tumor cell death through necrosis during chemotherapy, but produce several side effects such as inflammation (McConkey, 1998; Debatin and Krammer, 2004). In the apoptotic mode, the affected cells participate in a self-destruction cascade to cause DNA degradation through endonuclease activation, nuclear disintegration and the formation of apoptotic bodies, and rapid clearance by macrophages (McConkey, 1998; Debatin and Krammer, 2004; Roos *et al.*, 2004). This process of the cell destruction is through a regulated process to remove unwanted or damaged tissues. Therefore, anticancer drugs that induce tumor cell death via

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apoptosis during chemotherapy are closer to the normal physiological reaction. Thus, compounds from Chinese medicine that induce apoptosis in tumor cells are thought to be good candidates for development as anticancer drugs. In this study, it was intended to assess the cytotoxic effects of osthol through apoptosis.

MATERIALS AND METHODS

Identification of *C. monnieri* (L.) Cusson and *C. formosanum* Yabe. As in previous studies, osthol and imperatorin were isolated from *C. monnieri* (L.) Cusson and their purity (exceeding 99.5%) determined with HPLC (Yang *et al*., 2003a). In this study, osthol and imperatorin were dissolved in methanol as bio-marker substances. *C. monnieri* (L.) Cusson (CM) was purchased from a traditional Chinese medicinal market in Taipei, Taiwan and was identified by Dr Hsing-Chang Chang. *C. formosanum* Yabe (CF) was collected in Chia Yi, Taiwan and was identified by Dr Lih-Geeng Chen. A voucher of the plant material (CM-1013 and CF-001) is deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University. The above two samples were pulverized and filtered through a no 20 mesh. Two grams of pulverized sample was extracted with 10 mL methanol for 30 min in an ultrasonic bath. The solution was filtered through a 0.45 μm syringe filter, and a 10 μL volume was injected directly into the HPLC system. The HPLC system consisted of a Shimadzu LC-10AS pump system (Tokyo, Japan) equipped with a Shimadzu SPD-10AV detector, a C-R8A recorder, an SIL-9A autoinjector and a CTO-10A oven. The mobile phase was composed of acetonitrile: H_2O (40: 60 v/v). The solvents were filtered through a 0.45 μm FP Vericel (PVDF) membrane filter from the Pall Corporation (Ann Arbor, MI, USA). A LiChrospher 100 RP-18e, 5 μm, 4 mm i.d. × 250 mm column (Merck, Darmstadt, Germany) was used. The flow-rate was 1.0 mL/min with UV absorbance detection at 320 nm. The operation was carried out at 40°C (Sagara *et al.*, 1987).

***In vivo* antitumor assays.** P-388 D1 cells (1×10^6 cells/mouse) were transplanted intraperitoneally (i.p.) into 5-week-old CDF_1 female mice (DBA/2 male × BALB/c female) on day 0. Osthol was dissolved in 10% soybean oil, which was administered orally once a day on days 0–8. The antitumor effect was defined as the percent increase in life span (% ILS) calculated according to the following equation:

$$\%\,\mathrm{ILS} = [(T - C)/C] \times 100\%$$

where T and C are the median survival times (day) of the osthol group and 10% soybean oil (vehicle control) group, respectively). Student's t-test was used to compare the survival time (day) between the test and control groups (Filipski *et al.*, 1999; Yang *et al.*, 2003b).

Cell cultures. The human cervical carcinoma (HeLa) and murine leukemia (P-388 D1) cell lines were obtained from American Type Cell Culture (ATCC, Rockville, MD, USA) and maintained in DMEM (Gibco) supplemented with 10% FBS, 100.0 mg/L streptomycin and 100 IU/mL penicillin (Gibco). All cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Primary culture. Normal cervical fibroblasts (NCF) were isolated from human cervical tissues. NCF was cultured with DMEM containing 10% FBS, 100 mg/L streptomycin and 100 IU/mL penicillin at 37 °C with 5% CO_2 and used for experimental protocols from passages 1 to 3 (Wang *et al.*, 2001).

Cytotoxicity assays. A stock solution of test samples (50 mM) was prepared by dissolving the test samples in dimethyl sulfoxide (DMSO) and then storing it at 4 °C until use. Serial dilutions of the stock solution were prepared in the culture medium in 96-well microtiter plates, and test samples were added at the appropriate concentrations to cell cultures for 24 h and 48 h without renewal of the medium. The number of surviving cells was then counted using the tetrazolium (MTT) assay (Bruggisser *et al.*, 2002). The cytotoxicity index (CI%) was calculated according to the following equation:

$$\mathrm{CI}\% = [1 - (T/C)] \times 100\%$$

where T and C are the mean optical density of the treated group and vehicle control group, respectively). In accordance with the CI% of the dose-response curve, the concentration of the test compound was estimated giving 50% of cell growth inhibition (IC_{50} value). The selective index (SI) was calculated by dividing the IC_{50} value of the HeLa cells with the IC_{50} value of the NCF cells.

Agarose gel electrophoresis. HeLa cells (5×10^5 cells/well) were exposed to osthol for 48 h and collected into tubes and then washed with PBS. The cells were incubated in 200 μL of lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% Sarkosyl, 250 μg/mL proteinase K) overnight at 56 °C. The DNA was extracted with one volume of chloroform/phenol/isoamyl alcohol (25:24:1), and the extent of DNA fragmentation assessed with 1.5% agarose gel electrophoresis (Wang *et al.*, 2001).

Flow cytometry analysis. HeLa cells (5×10^5 cells/well) were exposed to osthol for 48 h and harvested in PBS. The cells were fixed in ice-cold 80% ethanol, treated with 1.0 mg/mL RNase A, and stained with 50 μg/mL propidium iodide. The stained-cells were run through a FACScan (Becton Dickinson, San Jose, CA, USA). The results are presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence (Wang *et al.*, 2001).

Western blot analysis. HeLa cells (5×10^5 cells/well) were exposed to osthol for 48 h and lysed with lysis buffer containing 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40 and protease inhibitors. The protein samples resolved by denaturing with 10% SDS-polyacrylamide gels using standard methods (Wang *et al.*, 2001). Total proteins (30 μg) were used for Western blot analysis and the protein was transferred to a nitrocellulose membrane by electroblotting. The membranes were probed with anti-PARP (a rabbit polyclonal antibody), and visualized using a BCIP/NBT kit (BCIP/NBT, Gibco) according to the manufacturer's instructions. As

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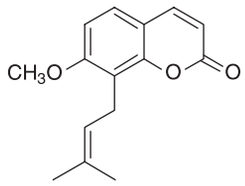

Figure 1. Chemical structure of osthol isolated from the fruit of *Cnidium monnieri* Cusson.

a loading control, anti-α-tubulin (a mouse monoclonal antibody) was used.

RESULTS AND DISCUSSION

The distribution of *Cnidium monnieri* (L.) Cusson was in the northern and southern China (Zhu, 1998). However, *Cnidium monnieri* from different origins in China will have different contents of coumarins. The fruit collected in the southern China mainly contains linear furocoumarins while that of the north predominantly yields angular furocoumarins (Zhu, 1998). Osthol is a simple coumarin, and imperatorin is a linear furocoumarin. Both coumarins have several pharmacological functions: antiallergic (Matsuda *et al.*, 2002), antiinflammatory (Liu *et al.*, 1998; Wang *et al.*, 2000), antiproliferation (Yang *et al.*, 2003a; Kawaii *et al.*, 2001; Hitotsuyanagi *et al.*, 1996; Guh *et al.*, 1996), vasorelaxing effect (Chiou *et al.*, 2001) and preventing prophylactic effects in hepatitis (Okamoto *et al.*, 2001). According to the above references, osthol was more antiproliferative in tumor cells than the other coumarins and the isopentenyl group in coumarins was necessary. It is suggested that growing Fructus Cnidii in the South of China is a good location. The geographical location and weather of Taiwan and southern China are alike. As mentioned previously *C. formosanum* Yabe is an endemic plant in Taiwan and its root is used as folk medicine for arthritis (Xu, 1972). However, the fruits of *C. formosanum* are not used as much as *C. monnieri* fruits. Sometimes, the Fructus Cnidii is mixed with *C. monnieri* and *C. formosanum* in traditional medicine marketing (Sagara *et al.*, 1987). According to our HPLC analysis, the fruits of *C. formosanum* contained little osthol but no imperatorin (Fig. 2). The HPLC profiles of the two species Fructus Cnidii were similar, but the ratio of the principal constituents is different. Therefore, *C. formosanum*, which should be to an economical plant, needs to be explored further.

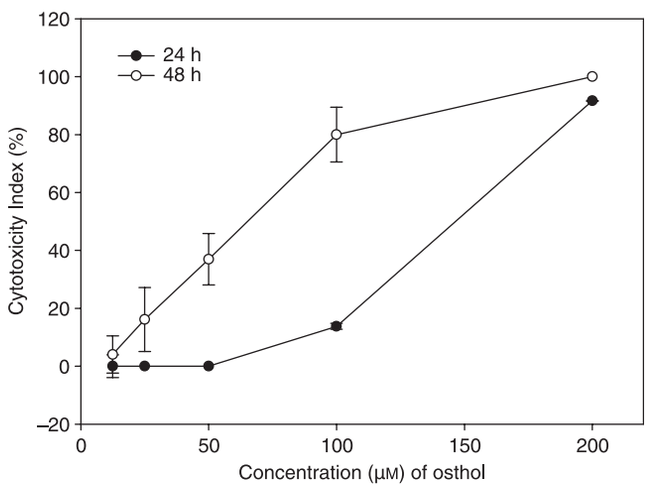

Figure 3. Cytotoxic effects of osthol against HeLa cells, in time and concentration-dependent manner. Data were means of results from three separate experiments.

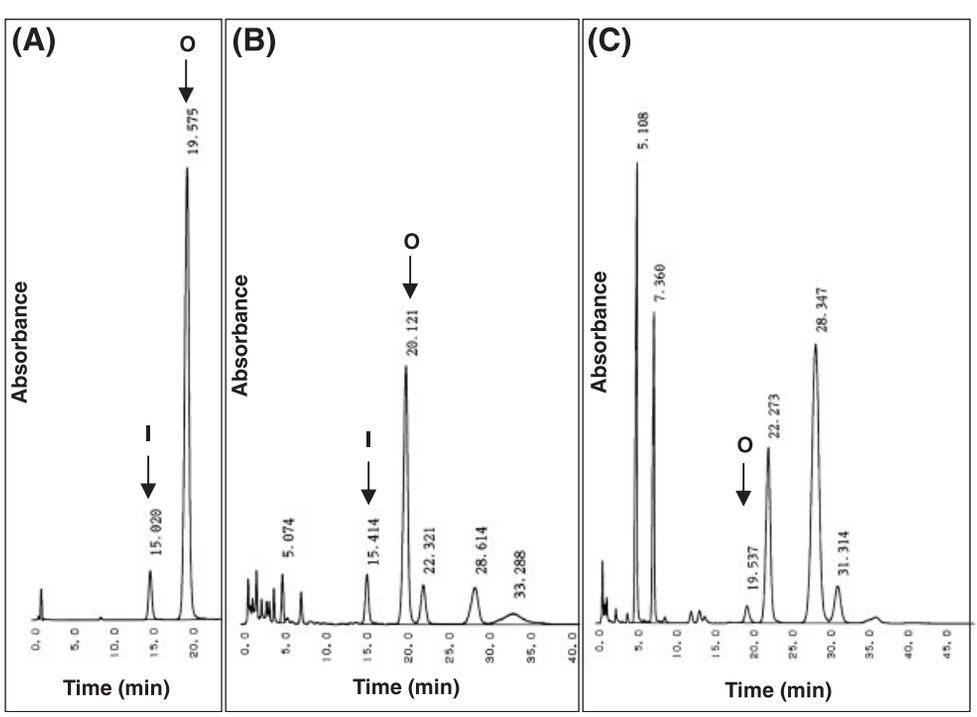

Figure 2. The HPLC chromatogram profile of (A) bio-marker substances of imperatorin (**I**) and osthol (**O**); (B) Fructus of *C. monnieri*; (C) Fructus of *C. formosanum*. The retention times of imperatorin and osthol were 15.0 and 19.5 min, respectively.

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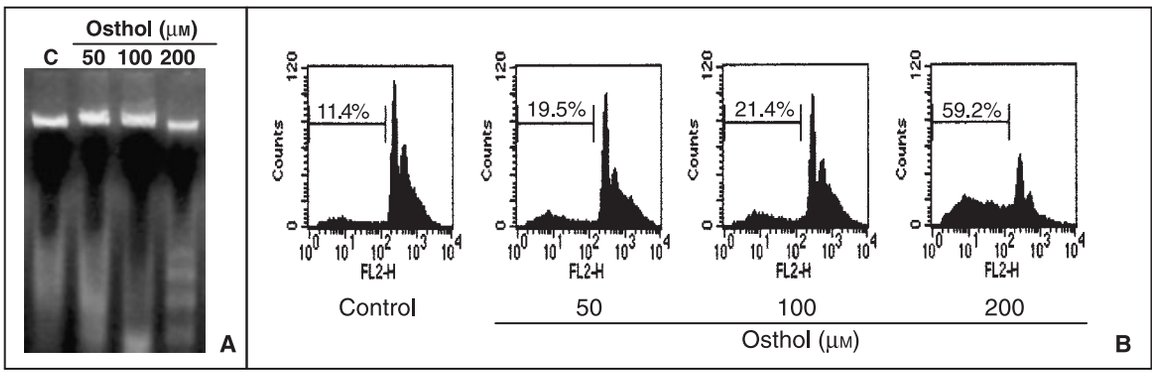

Figure 4. DNA fragmentation were measured by agarose gel electrophoresis (A) and flow cytometry (B) in HeLa cells treated with 50, 100 and 200 μM of osthol for 48 h. C: solvent control (0.5% DMSO).

Table 1. Percent increase in life span of osthol-treated P-388 D1-bearing CDF_1 mice

Group	Mice No. 1	2	3	4	5	Middle	ILS %
Control[a]	21	22	24	28	35	24	–
30 mg/kg	23	24	33	33	>60	33	37.5

[a] Solvent control was 10% soybean oil.

Table 2. IC_{50} values of osthol and adriamycin on HeLa and NCF after 24 and 48 h of treatment

	IC_{50} (μM) 24 h HeLa	48 h HeLa	NCF[a]	SI[b]
Osthol	80.0	64.9	168.0	2.59
Adriamycin	1.91			

Adriamycin was used as a positive control.
[a] NCF, primary cultured normal cervical fibroblasts.
[b] SI, selectivity index, IC_{50} for NCF/IC_{50} for HeLa after treatment 48 h.
Data were obtained from three separate experiments.

In vivo antitumor assays of osthol

The antitumor effects of osthol were evaluated using the murine P-388 D1 leukemia *in vivo* model. Osthol was administered orally to P-388 D1 leukemia in CDF_1 mice once a day for 9 days. Osthol at a dose of 30 mg/kg b.w. prolonged the life span of P-388 D1 tumor bearing CDF_1 mice by more than 37.5% compared with the solvent control mice (Table 1). One mouse of the treated group survived more than 60 days. The murine P-388 D1 leukemia *in vivo* model was established in our laboratory. Adriamycin was used as a positive control and defined the potency of the antitumor effects. If the test sample can prolong the survival days of P-388 D1 bearing mice by more than 60 days, it has potential as an antitumor drug (Huang *et al.*, 2005). The data showed that osthol inhibited the growth of P-388 D1 cells in CDF_1 mice.

Induced apoptosis of HeLa cells by osthol

Osthol inhibited the growth of a human cervical tumor cell line, HeLa, in a time- and concentration-dependent manner, with IC_{50} values of 77.96, and 64.94 μM for 24 and 48 h, respectively. The primary cultured human normal cervical fibroblasts (NCF) were used to evaluate the toxicity of osthol. Osthol was less cytotoxic in NCF than HeLa, and the selective index (SI) was about 2.6 (Table 2).

A characteristic feature of apoptosis is DNA fragmentation. Increased DNA fragmentation was apparent in HeLa cells treated with 100 μM osthol for 48 h and it showed dose dependence. A typical experimental result of agarose gel electrophoresis is shown in Fig. 4A. Cell apoptosis from osthol was also confirmed by flow cytometric analysis of DNA-stained cells. Apoptotic cells with degraded DNA, mostly located below the G_1 peak in the DNA histogram (M1), were estimated from Fig. 4B to be 11.4% in control cells, while the percentage of apoptotic cells increased in a dose-dependent manner after 48 h. On the other hand, apoptosis produced a pattern typical of apoptotic PARP cleavage: a catalytically active band of intact PARP at 116 kDa, and an active band at 85 kDa corresponding to the apoptotic cleavage product of PARP. When the HeLa cells were treated with osthol for 48 h, the 116 kDa band activity diminished progressively, while the 85 kDa signal increased dose-dependently (Fig. 5). As the above results show, osthol could induce apoptosis in HeLa cells after treatment for 48 h.

Among several pharmacologic functions, osthol has been found to inhibit P-388 D1 cells *in vivo* and to induce apoptosis in HeLa cells in this study. *C. formosanum*, an endemic plant in Taiwan, is a good natural resource which needs to be developed for its active compounds.

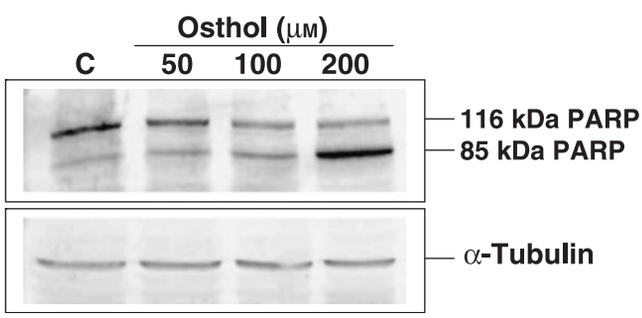

Figure 5. Western blot analysis of PARP proteins in osthol-treated HeLa cells for 48 h. C: solvent control (0.5% DMSO).

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