

Duchesnea Phenolic Fraction Inhibits *In Vitro* and *In Vivo* Growth of Cervical Cancer Through Induction of Apoptosis and Cell Cycle Arrest

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Duchesnea indica (Andr.) Focke has been commonly used to treat cancer in Asian countries for centuries, and recently has been shown to possess anticancer properties *in vitro* and *in vivo*. But the underlying mechanism of the anticancer action is unclear, especially in *in vivo* studies. In this study, we investigated the anticancer effect and associated mechanisms of Duchesnea phenolic fraction (DPF) on cervical cancer *in vitro* and *in vivo*. Our results showed that DPF significantly inhibited cervical cancer cell proliferation in dose- and time-dependent manners. DPF induced apoptosis as determined by AO/EB staining, DNA fragmentation and flow cytometry. Some apoptosis correlated proteins were altered following DPF treatment. Bax was up-regulated while Bcl-2 was down-regulated, and the expression ratio of Bax/Bcl-2 was increased. These resulted in the translocation of Bax to mitochondria, the release of cytochrome *c* from the mitochondria to the cytosol and caspase-3 activation. Concurrently, DPF provoked S phase arrest along with significant down-regulation of S phase-associated proteins, such as cyclin A, cyclin E, cyclin D1 and cdk2. Transplanted U14 cervical cancer mouse model was used to evaluate the antitumor effect of DPF *in vivo*. Compared with control, DPF treatment markedly prolonged survival of tumor-

bearing mice and dose-dependently reduced the tumor weight. DPF could induce apoptosis in tumor tissues as evidenced by increased TUNEL-positive cells, activation of caspase-3, up-regulation of Bax and down-regulation of Bcl-2. In addition, DPF significantly decreased the expression of cell proliferation markers PCNA and ki67 in tumors. All together, these data sustain our contention that DPF has anticancer properties and merits further investigation as a potential therapeutic agent. *Exp Biol Med* 234:74–83, 2009

Key words: *Duchesnea indica* (Andr.) Focke; cervical cancer; apoptosis; cell cycle

Introduction

Cervical cancer comprises approximately 12% of all cancers in women. It is the second most common malignancy after breast cancer for women worldwide, while the commonest cancer in developing countries (1). According to the global estimates, it had an incidence of 493,000 new cases and accounted for 274,000 deaths in the year 2002, despite the availability and reliability of the Pap smear (2). Approximately 83% of new cases and 85% of deaths from cervical cancer occur in developing countries (3–6), and more than 75% of cervical cancer cases in these countries are diagnosed in advanced stages (7). The standards of treatment include radiation therapy, chemotherapy and surgery (8). Although patient survival is favorable in early-stage cervical cancer, patients in advanced stages suffer greatly resulting in a 5-year survival rate of about 20–40% (9). Therefore, searching for new alternative strategies for the prevention and treatment of cervical cancer is essential.

One such strategy is to consider natural product modification. Traditional Chinese Medicine (TCM) has

Financial support for this study was provided by the National Key Basic Research and Progress Project Fund (973), Ministry of Sciences and Technology of China (2004CB72030).

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Received June 20, 2008.

Accepted September 10, 2008.

DOI: 10.3181/0806-RM-204

1535-3702/09/2341-0074\$15.00

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accumulated rich clinical experience and a number of folk recipes in cancer prevention and therapy in Chinese 5000-year civilization history. Plants and plant-derived drugs play dominant roles in cervical cancer chemotherapy. *Duchesnea indica* (Andr.) Focke and *D. chrysantha* (Zoll. et Mor.) Miq, with the common name Indian-mock strawberry (IMH), have been documented as anti-inflammatory, astringent and anticancer folk medicines in the Chinese ancient medical works such as *Mingyi Bielu* and *Compendium of Chinese Materia Medica*. It is often used for cancer therapy alone or as a main ingredient in the formulas with traditional reputed benefits for the treatment of cancer in China and Japan. The aqueous extract of *D. indica* was reported to have antiproliferative activity *in vitro* against many different types of cancer cells (10, 11) and shows anti-neoplastic activity against S180, H22, and S37 *in vivo* (12). Previous phytochemical and pharmacological studies demonstrated that triterpenoids, phenolic compounds, polysaccharides and biologically active lectin contributed to the anticancer (13–16), antioxidative (17) and immunostimulatory (18) properties of *Duchesnea*. Furthermore, combined treatment with *Ganoderma lucidum* and *D. chrysantha* extracts caused a synergistic induction of apoptosis in HL-60 cells (18). Our previous study demonstrated that the phenolic extract of *D. indica* triggered apoptosis in ovarian cancer SKOV-3 cells through the mitochondria-dependent pathway (11). These studies suggest that IMH possesses anticancer properties as a potential therapeutic agent. However, scarce data are available regarding the efficacy and *in vivo* mechanisms of the action of *Duchesnea* on cervical cancer.

Taking into account the above, the present study focused on the anticancer activity and related mechanisms of *Duchesnea* phenolic fraction (DPF), the most effective anticancer fraction of *Duchesnea*, against cervical cancer both in cell culture and in a transplanted tumor mouse model. The results showed that DPF inhibited cancer cell growth *in vitro* and *in vivo*, and the mechanisms were associated with cell cycle arrest and induction of apoptosis.

Materials and Methods

Plant Material and Preparation of DPF. *D. indica* (Andr.) Focke, cultivated in Anhui province of China, was supplied by the company of Chinese Materia Medica in Beijing. The species was identified by Professor Ben-Gang Zhang from the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. A voucher specimen (number 20050443) is available in the herbarium of the institute.

Aerial part (150 g) of the herb was soaked in 2 liters of boiling water for 1 hr, following filtration with filter paper. The extraction step was repeated 3 times. The combined extract solution was applied to a polystyrene AB-8 resin column (The Chemical Plant of NanKai University, Tianjin, China, 50 × 8 cm i.d.), which was preconditioned by washing with acetone followed with 90% ethanol and then

equilibrated with 0.1% HCl aqueous solution. Non-phenolic impurities including sugars, amino acids, proteins, and minerals were washed exhaustively with 10 liters of water. Phenolic compounds were eluted from the resin with 6 liters of 90% ethanol. The ethanol eluant was concentrated to dryness by a rotary evaporator under reduced pressure, to give a brown powder (9.43 g), namely *Duchesnea* phenolic fraction (DPF). The total amount of major phenolics in DPF was quantified using Folin-Ciocalteu assay and the total phenolic content of DPF was 48.03 ± 0.07 g of catechin equivalents/100 g DPF.

Cell Lines and Culture. The Chinese hamster ovary (CHO) cells and human cervical cancer HeLa and C33A cells were obtained from American Type Culture Collection. The mouse cervical carcinoma U14 cells, Chinese hamster lung fibroblast cell line V79 and normal human liver L02 cells were obtained from the Cell Bank of Institute of Basic Medical Sciences (Peking Union Medical College, Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone).

Cytotoxicity Assays. Cytotoxicity of DPF on HeLa, C33A and U14 cells was assessed using MTT assay as described previously (19). Briefly, cells were seeded in 96-well plates at density of 5,000–10,000 cells/well. After treatment with different concentrations of DPF and 20 μ M cisplatin for 24–72 hrs, MTT was added to each well at a final concentration of 0.5 mg/ml and further incubated for 4 hrs at 37°C. To stop the reaction, the medium was removed and 100 μ l DMSO was added. The absorbance at 570/650 nm was detected using a microplate ELISA reader (SpectraMax 190, Molecular Devices, USA). Results were expressed as the mean percentage of cell growth inhibition $[(OD_{\text{control}} - OD_{\text{treated}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100\%]$. The IC₅₀ value was expressed as the concentration of DPF that inhibited the growth of cells by 50%.

Morphological Study with Fluorescence Microscope. The AO/EB (acridine orange/ethidium bromide) staining method was used to observe the apoptotic morphological changes (20). This method combines the differential uptake of fluorescent DNA binding dyes AO (green fluorescence) and EB (red fluorescence). Briefly, AO/EB mixture [25 μ l, containing 4 μ g/ml AO and 4 μ g/ml EB in PBS (pH 7.4)] was added to cells treated with 80 μ g/ml DPF for 36 hrs. Then the cells were observed under the fluorescence microscope (Nikon ECLIPSE TE2000U, Nikon Corporation, Tokyo, Japan).

Detection of Internucleosomal DNA Fragmentation by Electrophoresis. DNA fragmentation was analyzed by the gel electrophoresis method according to Herrmann *et al.* (21). Briefly, cells were harvested and then treated with 100 μ l lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). The supernatant was collected by centrifugation for 5 mins at 1600 g. The supernatant was brought to 1% SDS and treated for 2 hrs with RNase A (final concentration 5 mg/ml, Sigma) at 56°C

followed by digestion with proteinase K (final concentration 2.5 mg/ml, Merck) for at least 2 hrs at 37°C. After addition of 1/2 volume 10 M ammonium acetate, the DNA was precipitated with 2.5 volume cold absolute ethanol. The DNA pellet was obtained by centrifugation at 12,000 *g* for 20 mins at 4°C, and the DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and fractionated by electrophoresis on a 2% agarose gel.

Flow Cytometry Analysis. Cells were harvested by centrifugation, washed with PBS, and fixed with ice-cold 70% ethanol overnight. Fixed cells were treated with 25 µg/ml RNase A at 37°C for 30 mins and then stained with propidium iodide (PI) (50 µg/ml, Sigma) solution for 30 mins in the dark. DNA content was determined by flow cytometer (Coulter Epics XL, USA) and the analysis was based on at least 10,000 events. The percentages of cells in the apoptotic sub-G1 and different cell cycle phases were analyzed using the Multicycle software (Phoenix Flow Systems, USA).

Caspase-3 Activity Assay. The activation of caspase-3 was investigated by the caspase-3 colorimetric assay kit (Promega) according to the supplier's manual.

Protein Extraction and Western Blot Analysis. HeLa and C33A cells, at 50% confluency, were treated with 20–160 µg/ml of DPF for 48 hrs. At the end of treatment, total cell extract was prepared in lysis buffer. Cytosolic and mitochondrial proteins were prepared as described previously (11). After measuring the protein concentration by the Bradford method (22), Western blot analysis using equal amounts of denatured proteins per sample was done as published previously (11).

Animal Studies. The U14 mouse cervical carcinoma cells were used in animal experiments to study the *in vivo* antitumor efficacy of DPF. Female CD-1 mice (20–24 g) were obtained from Beijing Vital Laboratory Animal Technology (Beijing, China) and maintained in pathogen-free conditions, with food and water *ad libitum*. All procedures involving animals and their care were approved by the Animal Ethics Committee at Peking Union Medical College (Beijing, China) and conducted in compliance with *Guide of the Care and Use of Laboratory Animals* (NIH publication No. 86–23, revised 1996).

For analysis of therapeutic efficacy in the intraperitoneal model, U14 cells (2×10^6 cells in 200 µl of PBS) were ip injected. After 24 hrs, mice were randomly divided in four groups ($n = 15$ mice per group) and gavaged with sterile water (control group) or various doses of DPF (0.25 g/kg, 0.5 g/kg, 1.0 g/kg) in sterile water (treatment groups) once a day until all mice died. Tumor induced mortality “events” were recorded.

In the subcutaneous model, mice were implanted sc with U14 cells (2×10^6 cells in 200 µl of PBS) into the right flank. After 24 hrs, mice were randomly divided in four groups ($n = 15$ mice per group) and gavaged with sterile water or various doses of DPF (0.25 g/kg, 0.5 g/kg and 1 g/kg) in sterile water, once a day for 20 days. At the end of

treatment, mice were humanely sacrificed and tumors were collected and weighed. Half of the tumor tissue was fixed in 4% paraformaldehyde in PBS and embedded in paraffin for TUNEL and immunohistochemical analysis. The other half was homogenized with dounce homogenizer in lysis buffer, and the resulting total cell lysate was analyzed for caspase-3 activity and protein expression by Western blot as described above.

In Situ Apoptosis Detection by Terminal Deoxynucleotidyl Transferase-Mediated Nick-End Labeling Staining. Tumors were fixed in 4% paraformaldehyde in PBS, followed by paraffin embedding for generation of 5-µm-thick sections. All tumor sections were de-waxed and processed conventionally. Apoptosis of tumor sections was detected by TUNEL assay using the In Situ Cell Death Detection Kit, POD (Roche), according to the manufacturer's instructions. DAB was used as chromogen and sections were counterstained with Hematoxylin.

Immunohistochemical Analysis. Tumor sections were incubated with rabbit polyclonal anti-Bax antibody, anti-Bcl-2 antibody (1:200 dilution, Santa Cruz) and anti-ki67 antibody, anti-PCNA antibody (1:100 dilution, Zymed), then subsequently reacted with the biotinylated secondary antibody. The histostain-SP kit (Zymed) was used to visualize the immune complexes according to the manufacturer's instructions.

Statistical Analysis. Data were expressed as mean $\pm$ SEM. All statistical analyses were performed using GraphPad Prism 4.0 Software. Statistical significant differences between control and DPF-treated samples were determined by One-way ANOVA followed by Dunnett's test or Bonferroni test for post-hoc comparisons. The survival analysis was performed using the Kaplan-Meier survival curve and log-rank test. In each case, *P* value < 0.05 was considered statistically significant.

Results

DPF Dose-Dependently Inhibited the Cell Proliferation of Cancer Cells. The cytotoxic effect of DPF on the growth of cervical cancer cells was determined by MTT assay. DPF inhibited cell proliferation at dose- and time-dependent manners, and IC₅₀ values in cervical cancer cells (HeLa, C33A and U14) ranged from 20–60 µg/ml after 72 hrs of treatment (Table 1). In HeLa and C33A cells, DPF treatment at 5–160 µg/ml concentrations significantly inhibited cell proliferation for 48 and 72 hrs. Compared with HeLa and C33A cells, U14 cells were more sensitive to DPF at the same concentrations (Fig. 1C). In addition, normal cells were much less susceptible to the cytotoxic effect of DPF with higher IC₅₀ values for 72 hrs (Table 1).

DPF Induced Apoptosis in Cervical Cancer Cells. The morphological changes of the cells treated with 80 µg/ml of DPF for 36 hrs were observed by AO/EB staining. DPF-treated HeLa and C33A cells showed significant morphological apoptotic changes, including cell

Table 1. Concentration Producing 50% Growth Inhibition (IC₅₀) of DPF on Cervical Cancer or Normal Cell Lines at 72 Hrs^a

Cell lines	IC ₅₀ (μg/ml)
HeLa	51.02 ± 0.48
C33A	51.35 ± 1.39
U14	27.72 ± 1.97
V79	168.39 ± 1.28
L02	160.79 ± 2.08
CHO	220.46 ± 2.64

^a Results are expressed as the mean ± SEM of three independent experiments.

shrinkage, membrane blebbing, nuclear condensation and fragmentation, and formation of apoptotic bodies (Fig. 2A). Characteristic DNA ladder was observed apparently in HeLa cells incubated with DPF at 160 or 320 μg/ml concentrations for 48 hrs (Fig. 2B). Additionally, DPF treatment at 20–160 μg/ml concentrations for 24 or 48 hrs led to a significantly dose-dependent accumulation of sub-G1 group (Fig. 2C).

Based on the increase of apoptosis in DPF-treated cells, our next aim was to examine the effect of DPF on the molecules that play major roles in execution of apoptotic events. In both HeLa and C33A cells, treatment with 20–160 μg/ml of DPF for 48 hrs caused a dose-dependent increase in caspase-3 activity (Fig. 2D). Figure 3A showed that Bax expression was significantly up-regulated whereas Bcl-2 expression was substantially down-regulated, and the ratio of Bax/Bcl-2 was also markedly increased in protein levels (Fig. 3C). In addition, mitochondrial Bax protein levels markedly increased in HeLa cells upon exposure to DPF for 48 hrs, and DPF caused a significant increase in cytosolic cytochrome *c*, which was due to a concomitant decrease in mitochondrial cytochrome *c* (Fig. 3B).

DPF Induced S Phase Arrest and Modulated the Associated Cell Cycle Regulators in Cervical Cancer Cells. After treatment with DPF at 20–160 μg/ml concentrations for 48 hrs, C33A and HeLa cells showed dose-dependent accumulation in S phase accompanied by a concurrent decrease of G0/1 phase. In each case, DPF did not change the proportion of cells in G2/M phase (Fig. 4).

To evaluate the effect of DPF on cell cycle regulatory molecules involved in S phase arrest, protein expression changes in DPF-treated HeLa and C33A cells were detected by Western blot analysis. Cells treated with DPF at 20–160 μg/ml concentrations showed a significantly dose-dependent reduction in expression of cdk2, cyclin A, cyclin D1 and cyclin E proteins (Fig. 3A).

Antitumor Effects of DPF on the Intraperitoneal and Subcutaneous Tumor Models. *In vivo* efficacy of DPF against cervical carcinoma was studied in the tumor transplanted mouse models. In mice bearing intraperitoneal U14 tumors, treatment with 0.25 g/kg, 0.5 g/kg, 1.0 g/kg

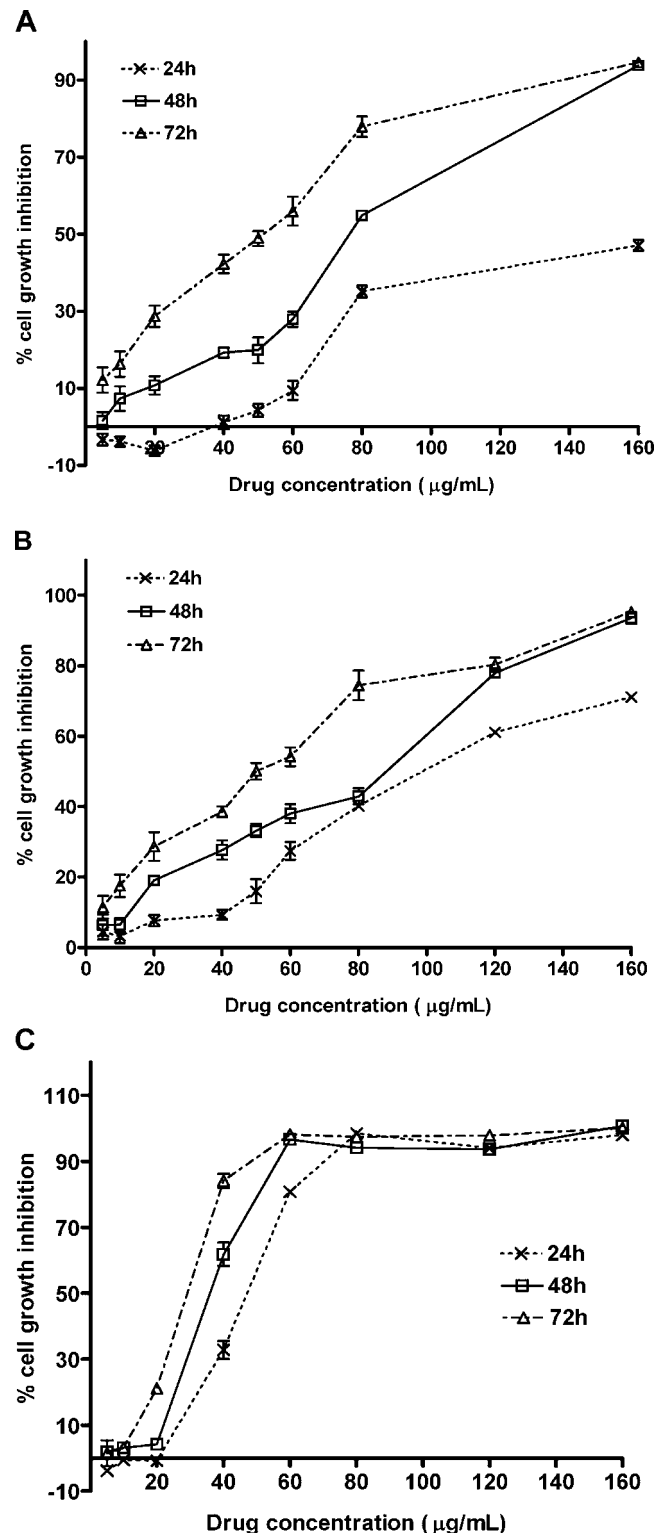


Figure 1. DPF inhibited cell proliferation of HeLa (A), C33A (B) and U14 (C) cervical cancer cell lines after treatment with various concentrations of DPF (5–160 μg/ml) for 24, 48, and 72 hrs. Results were from one of three independent experiments with similar results.

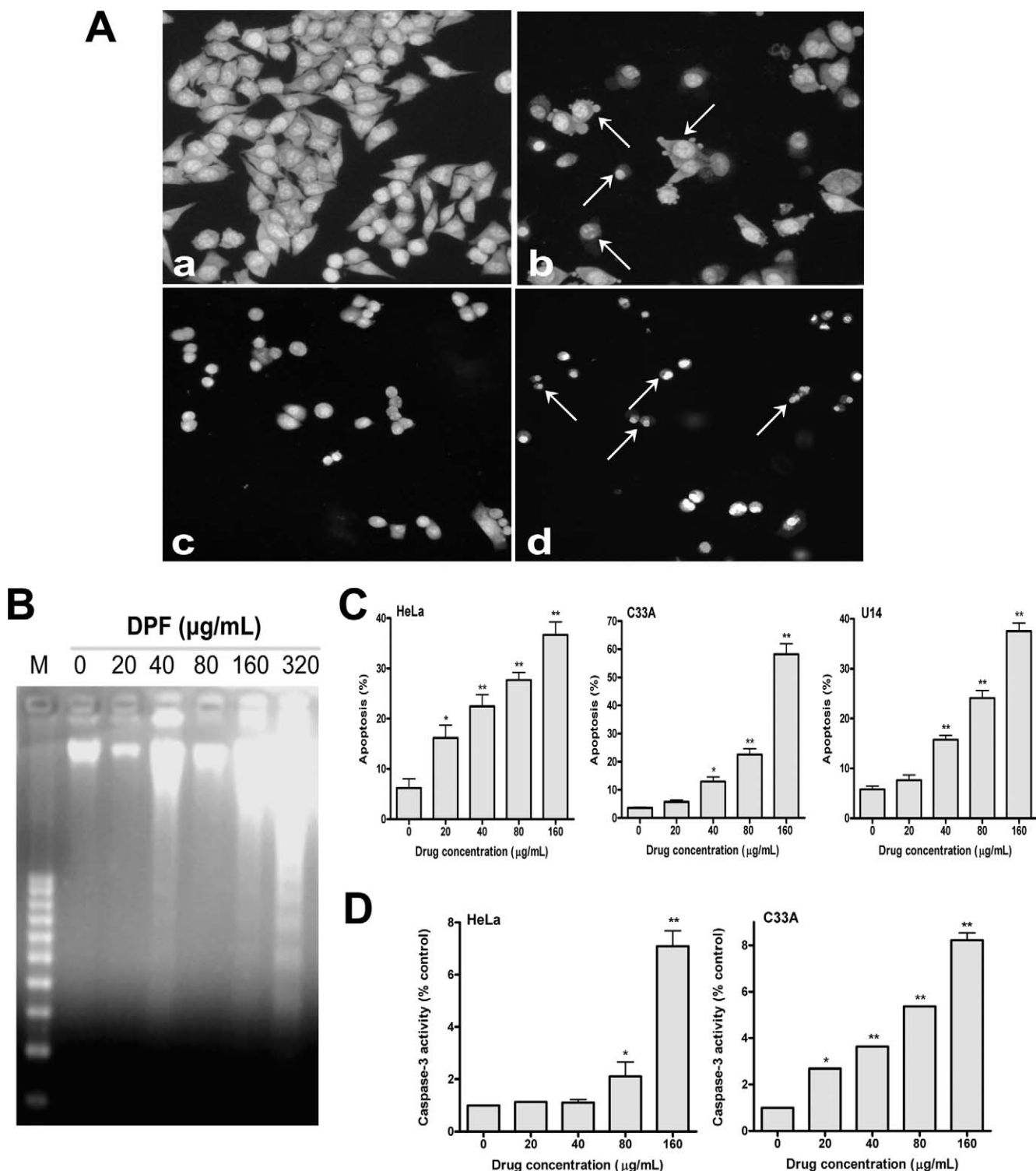


Figure 2. DPF induced apoptosis in cervical cancer cells. A, Representative photos of AO/EB staining in 80 µg/ml of DPF treated HeLa (b) and C33A (d) cells and untreated control cells (a, c) were taken under $\times 400$ magnification. Viable cells were determined by the uptake of AO and the exclusion of EB stain. Early and late apoptotic cells were identified by perinuclear condensation of chromatin stained by AO and EB, respectively. Necrotic cells were identified by uniform labeling with EB. The control cells had uniform bright green nuclei and orange cytoplasm, while cells treated with DPF exhibited the characteristic changes of apoptosis, with cell shrinkage, membrane blebbing, nuclear condensation and fragmentation, and formation of apoptotic bodies (arrows). B, Agarose gel of electrophoresis of DNA from HeLa cells cultured with either 0.16% DMSO or different concentrations of DPF (20–320 µg/ml) for 48 hrs. Marker was the 100 bp DNA ladder, results were from one representative experiment out of three that gave similar results. C, The percentage of apoptotic cells was determined by flow cytometry analysis. Cells were treated with either 0.08% DMSO or 20–160 µg/ml of DPF for 24 hrs (U14 cells) or 48 hrs (HeLa and C33A cells). D, Caspase-3 activity was analyzed after cells were treated with either 0.08% DMSO or DPF for 48 hrs. * $P < 0.05$; ** $P < 0.01$ versus control.

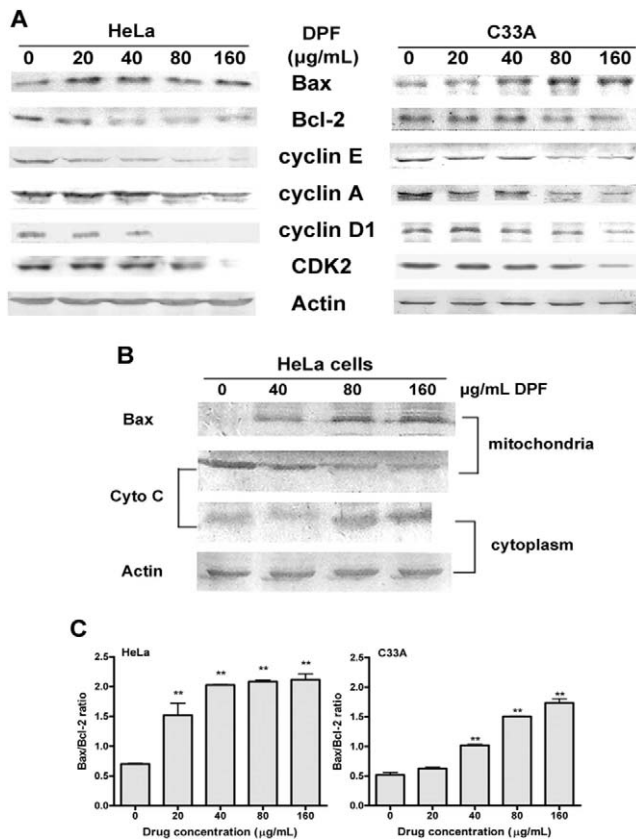


Figure 3. Expression of apoptosis-associated proteins and cell cycle regulators in cervical cancer cells treated with DPF. Representative blots were shown from three independent experiments with identical results. Whole cell lysates (30 µg/lane) were analyzed by Western blot for Bax, Bcl-2, cyclins and cdk2 expression (A). The ratio of Bax/Bcl-2 protein expression was determined from three separate experiments. ** $P < 0.01$ versus control (C). Mitochondrial translocation of Bax and cytochrome *c* release upon DPF treatment were analyzed by Western blot, and equal amounts of the protein (20 µg) were loaded on each lane (B).

doses of DPF significantly improved survival ($P < 0.05$) (Fig. 5A). Water-treated animals had a mean survival time of 12.23 days, whereas DPF-treated animals markedly prolonged the survival time with mean survival time of 14.46, 15.73 and 14.47 days, respectively ($P < 0.05$). The effect of DPF in the subcutaneous U14 model was shown in Figure 5B. By day 3 after tumor implantation, more than 80% of the mice had palpable flank tumors. During the course of the study, no mice died. Oral gavage feeding of DPF at 0.25 g/kg, 0.5 g/kg and 1 g/kg doses for 20 days significantly reduced the tumor weight by 35.20%, 49.05% and 69.60%, respectively (1.665 ± 0.242 g in control group versus 1.079 ± 0.183 g in 0.25 g/kg group, 0.850 ± 0.136 g in 0.5 g/kg group and 0.506 ± 0.077 g in 1 g/kg group).

DPF Induced Apoptosis and Suppressed Cell Proliferation in U14 Transplanted Mouse Model. To consider the possibility that DPF induced apoptosis *in vivo*, paraffin-embedded sections of tumors were analyzed by TUNEL assay. As shown in Figure 5C, the percentage of TUNEL-positive cells (brown) markedly increased, suggest-

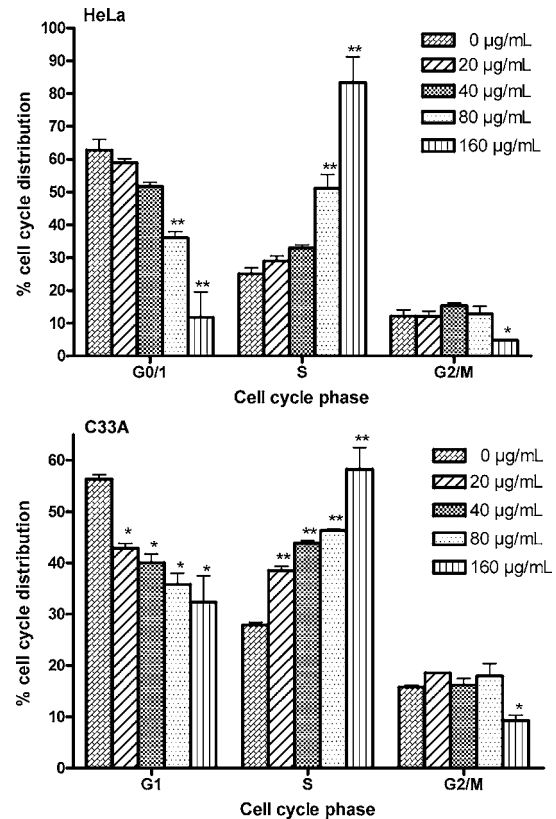


Figure 4. DPF induced cell cycle arrest in human cervical cancer HeLa and C33A cells. Cells were treated with either 0.08% DMSO or 20–160 µg/ml of DPF for 48 hrs. * $P < 0.05$; ** $P < 0.01$ versus control.

ing the increase in apoptotic tumor cells after DPF intake. Tumor samples with DPF treatment displayed a significant increase in caspase-3 activity (Fig. 5D). The expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 were studied by immunohistochemical and Western blot analysis. After treatment with DPF for 20 days, Bax expression was up-regulated whereas Bcl-2 expression was down-regulated in U14 tumors, and the Bax/Bcl-2 ratio was significantly increased in a dose-dependent manner (Fig. 6A, 6C). These findings were in accord with *in vitro* observations. In addition, PCNA and ki67 proteins were markedly decreased in DPF-treated tumors (Fig. 6B).

Discussion

In this study, we evaluated the anticancer efficacy and associated mechanisms of DPF on cervical cancer in cell culture and translated the *in vitro* findings into an *in vivo* animal model. The cytotoxicity of DPF *in vitro* was detected by MTT assay, and DPF showed strong inhibitory effect on cervical cancer cells (Fig. 1). These results are in agreement with previous studies on various cancer cells representing different tissues, including lung, breast, ovary, pancreas, stomach and prostate (10–13). From our previous study, DPF had a proportionately greater cytotoxic effect on cervical and ovarian cancer cells than other tumor cells

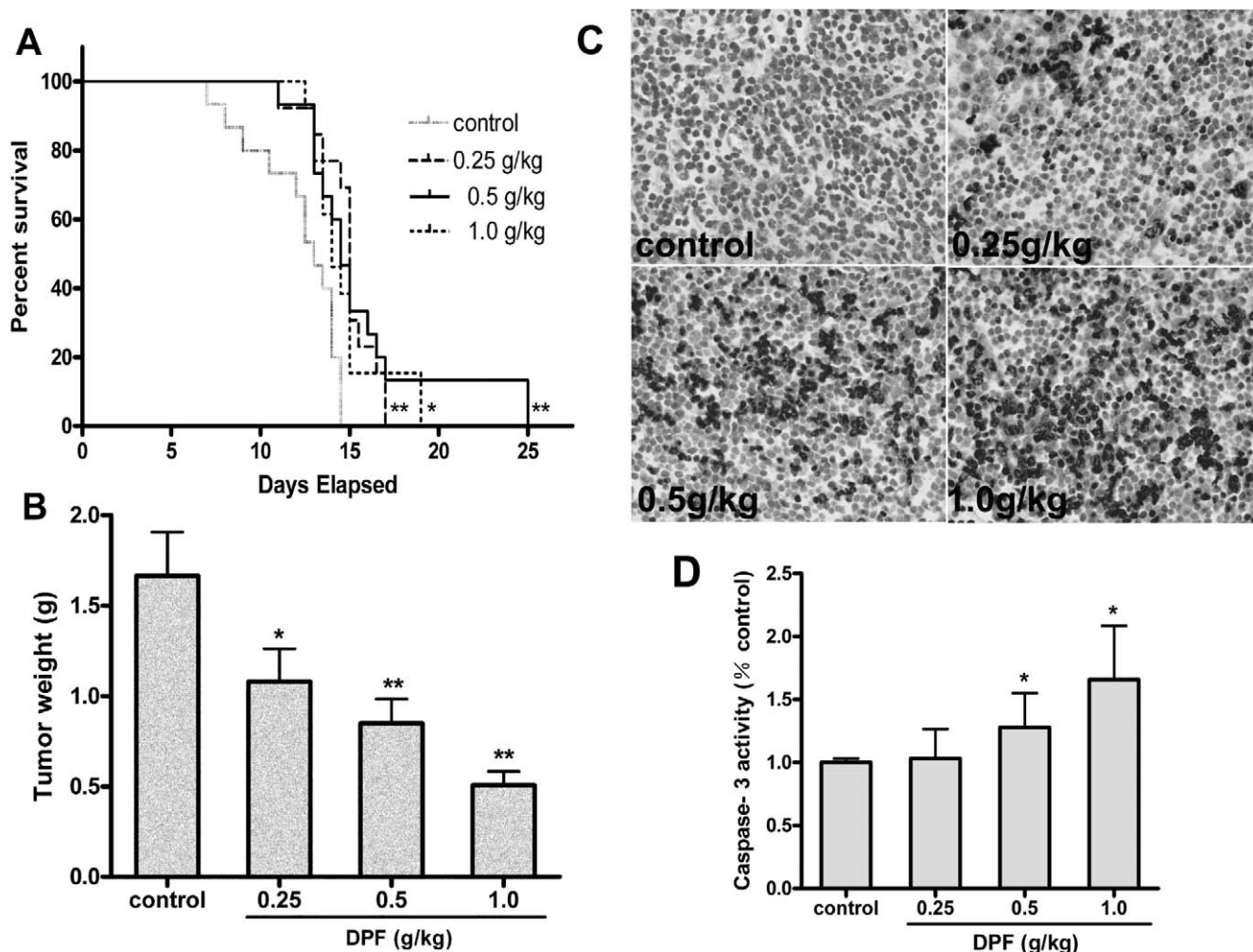


Figure 5. Antitumor effect of DPF in mice bearing cervical U14 tumors. Mice were implanted ip (A) or sc (B, C, D) with 2×10^6 U14 cells. After 24 hrs, mice were treated with water (control) or 0.25–1 g/kg doses of DPF. A, Kaplan-Meier survival curves. Statistical analysis done using log-rank test for survival curves gave the following *P* values: 0.001 (0.25 g/kg DPF vs control), 0.003 (0.5 g/kg DPF vs control), 0.014 (1.0 g/kg DPF vs control). B, After treatment for 20 days, tumors were harvested and weighed. Immunohistochemical staining for apoptosis was carried out on sections of control and DPF-treated tumors by TUNEL assay (C), and TUNEL-positive cells stained brown ($\times 400$). Caspase-3 activity of U14 tumors was analyzed (D). * *P* < 0.05; ** *P* < 0.01 versus control.

based on *in vitro* cytotoxic results (11). In the present study, DPF inhibited HeLa, C33A and U14 cells in dose- and time-dependent manners. Furthermore, the cytotoxic activity of DPF was characterized by a tumor-selective manner, as reflected by the comparatively high IC_{50} values on normal cells for 72 hrs (Table 1). The *in vivo* study conducted on mice sc transplanted cervical U14 cancer cells demonstrated that 0.25–1 g/kg DPF markedly inhibited tumor growth in a dose-dependent manner (Fig. 5B). In mice bearing intra-peritoneal U14 tumors, different doses of DPF significantly increased survival and prolonged the survival time compared with control (*P* < 0.05, Fig. 5A), but no statistically significant differences were found among DPF-treated groups (*P* > 0.05). The longest survival was obtained in mice treated with 0.5 g/kg DPF (the mean survival time: 15.73 days). This effect of DPF did not parallel the effect on the tumor weight of the mice. Despite the greater inhibition

of tumor growth in the subcutaneous tumor model, 1.0 g/kg DPF was less efficient than 0.5 g/kg DPF in prolonging the survival time of the mice. This was probably due to the toxic side effect of high dose of DPF. Additionally, in the *in vitro* studies, the time course studies of HeLa and C33A cells showed that exposure to DPF (5–60 μ g/ml in HeLa cells, 5–50 μ g/ml in C33A cells) for 24 hrs did not show significant inhibitory effects on cell growth, whereas, at the same concentrations, the cell proliferation decreased markedly after 48 hrs and 72 hrs (Fig. 1A, 1B). This delay raised a possibility that DPF did not have an immediate toxic effect on these two cells and DPF might induce a series of cellular events, which lead to inhibition of cell growth and/or induction of cell death.

Our next aim was to understand whether the inhibition of cell proliferation in the presence of DPF is brought about by induction of apoptosis. Apoptosis is an important

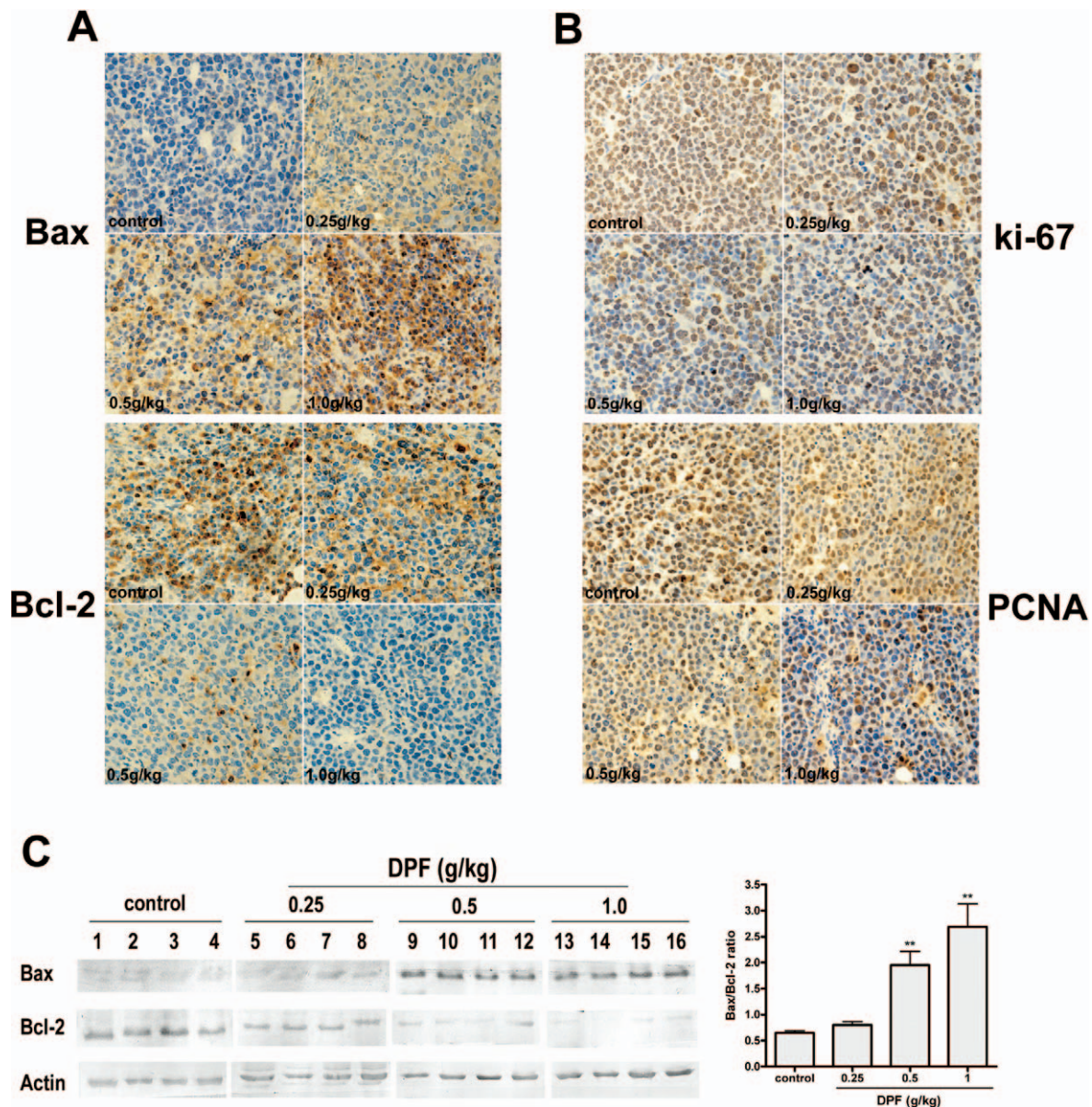


Figure 6. DPF feeding induced apoptosis and inhibited tumor proliferation *in vivo*. Representative photos of tumor sections immunohistochemically stained with anti-Bax and anti-Bcl-2 (A), anti-ki-67 and anti-PCNA (B) demonstrated the expressions of cell apoptosis molecular markers and cell proliferation markers (the positive cells stained brown, $\times 400$). C, Parts of the four randomly selected tumors each from four individual mice in control and DPF-fed groups were used for total cell lysate preparation and analyzed by Western blot, and actin was used as loading control. The ratio of Bax/Bcl-2 protein expression was shown. ** $P < 0.01$ versus control. A color version of this figure is available in the online journal.

homeostatic mechanism that balances cell division and cell death and maintains the appropriate cell number in the body (23). To date, many therapeutic natural phytochemicals were demonstrated to promote apoptosis in cancer cells, including phenolics, alkaloids, saponins and flavonoids (24–27). In present study, DPF induced apoptosis in cervical cancer cells, as evidenced by the typical cellular morphology, the internucleosomal DNA fragmentation and dose-dependent sub-G1 population accumulation (Fig. 2A–C). In addition, increased TUNEL-positive cells in U14 tumors of DPF-treated mice also showed that the anticancer effect of

DPF *in vivo* was mediated through induction of apoptosis (Fig. 5C).

Further investigations were focused on the mechanisms of the DPF-induced apoptosis in cervical cancer cells. The mitochondrial pathway is an important mechanism of apoptosis. The key element in the pathway is the efflux of cytochrome *c* to cytosol, which is regulated mainly through alteration in Bax and Bcl-2 protein levels (28–29). In present *in vitro* and *in vivo* studies, the up-regulation of Bax expression and the reduction of Bcl-2 expression in DPF-treated cells (Fig. 3) and tumor tissues (Fig. 6) led to an

increase in the ratio of Bax/Bcl-2. Because of Bax/Bcl-2 imbalance, Bax was found to be translocated to mitochondria, followed by the release of cytochrome *c* from mitochondria to the cytosol (Fig. 3), sequential activation of caspase-3 (Fig. 2D) and induction of mitochondrial-dependent apoptosis. Our results on the mechanisms of DPF-induced apoptosis in cervical cancer cells were in agreement with our previous research in ovarian cancer cells, but contrary to the data in HL-60 cells (18). In that study, Duchesnea extract exerted minimal effects on the apoptosis-associated proteins (Bax, Bcl-2) and caspase-3 activity. Thus, the extract by itself could not activate the mitochondria-dependent apoptotic pathway. One reason for the discrepancy between those results and our findings might be cell-type-specific variations. Another reason might be the different nature between the two extracts. Previous report indicated that the responsible component was polysaccharide, which is different in nature from the active phenolic components in our DPF extract. In the case of all these results, it is possible that *D. indica*, an herb containing phenolic compounds and other bioactive components, triggered apoptosis via mitochondria-dependent and mitochondrial-independent pathway.

Our data clearly showed that, in DPF-treated HeLa and C33A cells, the significant increase of S phase cells was accompanied by a decrease of G0/1 phase cells and no change of G2/M phase cells (Fig. 4). Thus, the increase of cell numbers in S phase was clearly due to the decrease of cells in the G0/1 phase, and the blockage effect of DPF occurred at the G1-S transition. The changes of multiple regulators associated with cell cycle were investigated in order to further elucidate the mechanism of action of DPF on cell cycle. The progression through various phases of cell cycle is governed by sequential activation of different cdk, which is mediated by their interaction with activating partners (cyclins) (30). The association of cdk2 with cyclin E/A regulates the G1-S transition (30). Cyclin D expression is deregulated frequently in human neoplasms, and agents that can down-regulate cyclin D1 expression could be helpful in the prevention as well as treatment of human neoplasms (31). Our data showed that DPF down-regulated the levels of cyclin A, E, D1 and cdk2 proteins in a dose-dependent manner (Fig. 3A), thus providing a possible explanation for the observed S phase arrest induced by DPF treatment. As shown in Figure 2 and Figure 4, the apoptosis induced by DPF was mediated via arresting cell cycle in S phase; that is to say, DPF could induce apoptosis in a cell cycle-dependent manner in cervical cancer HeLa and C33A cells. However, the association between the down-regulation of cell cycle regulatory molecules and changes of expression of pro-apoptotic or anti-apoptotic regulators in DPF-treated cells is not fully understood and warrants further investigation.

Additionally, tumor tissues derived from DPF-treated mice showed that DPF inhibited tumor proliferation with reductions in the proliferation markers PCNA and ki67 (Fig.

6B). Combined with MTT assay and immunostaining of PCNA and ki67, it is possible that suppression of tumor proliferation may also be a predominant mechanism responsible for the anticancer effect of DPF.

The extra mechanisms of cell growth-inhibitory effects of DPF have yet to be elucidated. Our previous phytochemical study demonstrated that DPF contained high concentrations of ellagic acid and brevifolin carboxylic acid (data not shown), and other compounds (including ursolic acid, quercetin and kaempferol, etc.) (15). Ellagic acid (32), quercetin (33) and ursolic acid (34) have been reported to induce apoptosis and block cell cycle in human cervical cancer cells with different HPV status. Furthermore, HPV-18 E6/E7 gene expression significantly decreased after ursolic acid treatment in HeLa cells (34). Our present and previous studies showed that DPF demonstrated inhibitory effect on both HPV-negative C33A cells, HPV-positive HeLa and ME180 cells. These results indicated that the anticancer effect of DPF was irrespective of HPV status. More work is required to determine the effect of DPF on HPV gene expression, and this is the focus of ongoing studies.

In conclusion, pronounced *in vitro* and *in vivo* studies suggested that DPF, a mixture of plant polyphenols, had potent anticancer effects and caused both cell cycle arrest and apoptosis. It is tempting to speculate that DPF might be utilized as a potential therapeutic agent against cervical and ovarian cancer. Specifying active components of DPF and examining the mechanisms of their anticancer actions are in process; these works may lead to new therapeutic options and improve understanding of the interaction of phenolic compounds with gene regulation in human cancer.

1. Stewart BW, Kleihues P. World Cancer Report. Lyon: IARC Press, 2003.
2. Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide. Lyon: IARC Press, No. 5 Version 2.0, 2004.
3. Ashford L, Collymore Y. Preventing cervical cancer worldwide. Washington: Population Reference Bureau, 2005.
4. Sankaranarayanan R. Worldwide burden of gynaecological cancer: the size of the problem. Best Pract Res Clin Obstet Gynaecol 20:207–225, 2006.
5. Schiffman M, Castle PE. The promise of global cervical-cancer prevention. N Engl J Med 353:2101–2104, 2005.
6. Sherris J, Herdman C, Elias C. Beyond our borders: cervical cancer in the developing world. West J Med 175:231–233, 2001.
7. Reproductive Health Outlook. Cervical cancer prevention. <http://www.rho.org/html/cxca.htm> [accessed January 11, 2006].
8. Dreyer G. Operative management of cervical cancer. Best Pract Res Clin Obstet Gynaecol 19:563–576, 2005.
9. American Cancer Society. Cervical cancer: how is cervical cancer stage? <http://www.cancer.org> [reviewed January 18, 2007].
10. Shoemaker M, Hamilton B, Dairkee SH, Cohen I, Campbell MJ. *In vitro* anticancer activity of twelve Chinese medicinal herbs. Phytother Res 19:649–651, 2005.
11. Peng B, Chang Q, Wang LW, Hu Q, Wang YQ, Tang JT, Liu XM. Suppression of human ovarian SKOV-3 cancer cell growth by

- Duchesnea phenolic fraction is associated with cell cycle arrest and apoptosis. *Gynecol Oncol* 180:173–181, 2008.
12. Duan JY, Liu XP, Li Q. Research on the effect of *Duchesnea indica* (Andr.) Focke. *Pharmacol Clinics Chinese Materia Medica* 14:28, 1998 (in Chinese).
 13. Lee IR, Yang MY. Phenolic compounds from *Duchesnea chrysantha* and their cytotoxic activities in human cancer cell. *Arch Pharm Res* 17: 476–479, 1994.
 14. Ye L, Yang JS. New ellagic glycosides and known triterpenoids from *Duchesnea indica* Focke. *Acta Pharm Sin* 31:844–848, 1996 (in Chinese).
 15. Kim JM, Jang DS, Lee YM, Yoo JL, Kim YS, Kim JH, Kim JS. Aldose-reductase- and protein-glycation-inhibitory principles from the whole plant of *Duchesnea chrysantha*. *Chem Biodivers* 5:352–356, 2008.
 16. Peng JN, Lu YR, Chen DH. Studies on the chemical constituents of Indian mockstrawberry. *Chin Tradit Herb Drugs* 26:338–341, 1995 (in Chinese).
 17. Kim IG, Jung IL, Oh TJ, Kim KC, Shim HW. Polysaccharide-enriched fraction isolated from *Duchesnea chrysantha* protects against oxidative damage. *Biotech Lett* 24:1299–1305, 2002.
 18. Kim KG, Kim JS, Son JK, Kim IG. Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells by the *Ganoderma lucidum* and *Duchesnea chrysantha* extracts. *Cancer Lett* 246:210–217, 2007.
 19. Cheng YL, Chang WL, Lee SC, Liu YG, Lin HC, Chen CJ, Yen CY, Yu DS, Lin SZ, Harn HJ. Acetone extract of *Bupleurum scorzonifolium* inhibits proliferation of A549 human lung cancer cells via inducing apoptosis and suppressing telomerase activity. *Life Sci* 73: 2383–2394, 2003.
 20. McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, Nishioka WK, Green DR. The end of the (cell) line: methods for the study of apoptosis *in vitro*. *Methods Cell Biol* 46:13–85, 1995.
 21. Herrmann M, Lorenz HM, Voll R, Grunke M, Woith W, Kalden JR. A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Res* 22:5506–5507, 1994.
 22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976.
 23. Martin SJ, Green DR. Apoptosis and cancer: the failure of controls on cell death and cell survival. *Crit Rev Oncol Hematol* 18:137–153, 1995.
 24. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. *Lancet Oncol* 4:721–729, 2003.
 25. Liu RH. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* 134:3479–3485, 2004.
 26. Nichenametla SN, Taruscio TG, Barney DL, Exon JH. A review of the effects and mechanisms of polyphenolics in cancer. *Crit Rev Food Sci Nutr* 46:161–183, 2002.
 27. Dragsted LO, Strube M, Larsen JC. Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacol Toxicol* 72:116–135, 1993.
 28. Mehmet H. Apoptosis: caspase find a new place to hide. *Nature* 403: 29–30, 2002.
 29. Oltvai ZN, Millman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74:609–619, 1993.
 30. Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res* 60: 3689–3695, 2000.
 31. Sausville EA, Johnson J, Alley M, Zaharevitz D, Senderowicz AM. Inhibition of CDKs as a therapeutic modality. *Ann N Y Acad Sci* 910: 207–222, 2000.
 32. Narayanan BA, Geoffroy O, Willingham MC, Re GG, Nixon DW. p53/p21 (WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. *Cancer Lett* 136:215–221, 1999.
 33. Yoshida T, Maeda A, Horinada M, Shiraishi T, Nakata S, Wskada M, Yogosawa S, Sakai T. Quercetin induces gadd45 expression through a p53-independent pathway. *Oncol Rep* 14:1299–1303, 2005.
 34. Yim EK, Lee MJ, Lee KH, UM SJ, Park JS. Antiproliferative and antiviral mechanisms of ursolic acid and dexamethasone in cervical carcinoma cell lines. *Int J Gynecol Cancer* 16:2023–2031, 2006.