

Original Article

Inhibition of the cervical cancer growth by cidan and cisplatin combination through induction of apoptosis

Hui Wang¹, Xiang Liu¹, Ju-Xiang Sun¹, Jin-Hua Liu¹, Na Liu¹, Xin-Qun Wang², Ai-Hua Li¹

¹Department of Obstetrics and Gynecology, People's Hospital of Linyi City, Linyi 276000, Shandong Province, China; ²Department of Obstetrics and Gynecology, Traditional Chinese Medicine Hospital of Linyi City, Linyi 276003, Shandong Province, China

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Abstract: Aim of the present study was to investigate the anti-tumor effect of cidan and cisplatin alone or in combination in the Hela cervical cancer cell and mice tumor xenograft. MTT assay was used for analysis of cell proliferation and Annexin-V/PI FACS assay for the induction of apoptosis. The results from MTT assay revealed a significant reduction in viability of Hela cells by cidan and cisplatin in dose and time dependent manner. Cidan and cisplatin reduced the viability of Hela cells at 48 h to 43.4 and 39.2%, respectively ($P < 0.05$). Incubation of with cidan and cisplatin alone or in combination induced apoptosis in 46.54, 52.27 and 74.69%, ($P < 0.05$) respectively at 48 h. Western blot analysis revealed a significant increase in the expression of pro-apoptotic factor caspase-3 in Hela cells on treatment with the combination of cidan and cisplatin. In mice tumor xenograft model, tumor growth was significantly inhibited in the group treated with combination of cidan and cisplatin after 14 days compared to 30 days in the cisplatin treatment group. The results from TUNEL assay revealed that proportion of apoptotic cells was significantly higher in the mice treated with cidan and cisplatin combination compared to cidan or cisplatin treatment groups alone ($P < 0.05$). Thus, cidan enhances the anti-tumor effect of cisplatin both *in vitro* and *in vivo*, therefore the combination can be used as efficient treatment strategy for cervical cancer.

Keywords: Proliferation, apoptosis, xenograft, tumor volume, inhibition

Introduction

Cervical cancer is one of the commonly detected cancers in females which affects more than 510,000 women and leads to 288,000 deaths every year [1]. The estimated 5-year survival rate of the cervical carcinoma patients is more than 70% and the prognosis of advanced or recurrent cervical cancer is very poor [2]. Currently, the treatment strategies for cervical carcinoma include, radiation therapy and chemotherapy alone or in combination with herbal medicines [3, 4]. Combination with herbal constituents from traditional Chinese medicine (TCM) have resulted development of the efficient treatment strategies for cancers [5, 6]. One of the potent chemotherapeutic agents for treatment of cervical cancer is cisplatin and its use has led to more than 20% response rate [7]. Studies have demonstrated that use of single chemotherapeutic agent has limited success in inhibiting cancer particularly, metasta-

sis. Thus, combination of the various chemotherapeutic agents investigated to develop efficient treatment strategies.

Traditional Chinese Medicine (TCM) either alone or in combination with standard chemotherapeutic agents has been used for the treatment of various types of cancers. TCMs exhibit their effect through inhibition of the carcinoma cell growth [8-11] and by improving the immune system [12]. Cidan, the mixture of 10 plant extracts is used for the treatment of cancer without inducing any side effects. The major component of the cidan is β -elemene which is reported to exhibit dose dependent inhibitory effect on the growth of hepatocellular carcinoma cells, HepG2. Studies have revealed that β -elemene induces apoptosis, arrests cell cycle and promotes expression of Fas and Fas ligand [13, 14]. Furthermore, in pulmonary carcinoma cells β -elemene in combination with other chemotherapeutic agents improved the efficacy of

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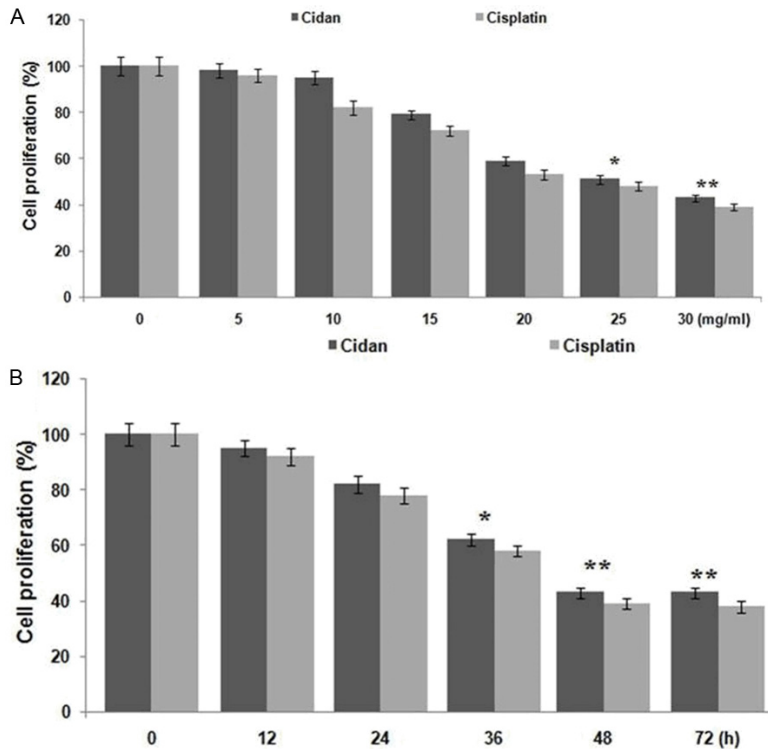


Figure 1. Cidan and cisplatin exhibit dose and time-dependent inhibitory effect on the proliferation of HeLa cells. The cells were treated with 5 to 50 mg/ml doses of cidan and cisplatin and then analyzed by MTT assay. (* $P < 0.05$) compared to the control cell cultures.

treatment therapy [15]. In the present study, antitumor effect of cidan alone or in combination with cisplatin against cervical carcinoma was investigated. It was observed that cidan treatment inhibited cell proliferation and induced apoptosis in cervical carcinoma cells. Moreover, cidan enhanced the anti-tumor activity of cisplatin significantly.

Materials and methods

Chemicals and reagents

Cidan and other common chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM medium was obtained from Gibco (Gaithersburg, MD, USA) and 10% fetal bovine serum from Gibco-BRL (Grand Island, NY, USA).

Animals

Twenty nude mice (BALB/cAnNCrj-nu/nu) five-week-old were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were acclimatized to the laboratory environment for one week before the experiments

were conducted. The mice were housed under laminar airflow conditions, temperature-controlled and 12-h/12-h light/dark cycle. All the animal experiments were performed according to the guidelines for animal care and use Animal Ethics Committee of the College of Medicine at Inje University.

Cell lines and culture

Human cervical cancer cell line HeLa was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin. Incubation of the cells was performed in a 5% CO₂ incubator at 37°C.

MTT assay

Effect of cidan or cisplatin on the proliferation of HeLa cells was analyzed by MTT method. In this procedure, the cells were distributed at a density of 2.5×10^5 cells per ml into the 96-well flat-bottom plates in 100 μ l DMEM medium. Various concentration of cidan or cisplatin were added to each well of the plate and incubated for 48 h. After incubation, 150 μ l MTT (5 mg/ml PBS) solution was added to each well of the plate and incubated for 4 h. Then the supernatant was decanted and 50 μ l DMSO was added to each well of the plate. Absorbance for each well of the plate was recorded at 570 nm three times.

Apoptosis assay

Effect of cidan or cisplatin alone or in combination on the induction of apoptosis in HeLa cells was analyzed using fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection kit I (BD Biosciences, San Jose, CA, USA). The cells at a density of 2×10^5 cells per well were distributed in 100 mm culture dishes, and incubated for 48 h with cidan or cisplatin alone or in combina-

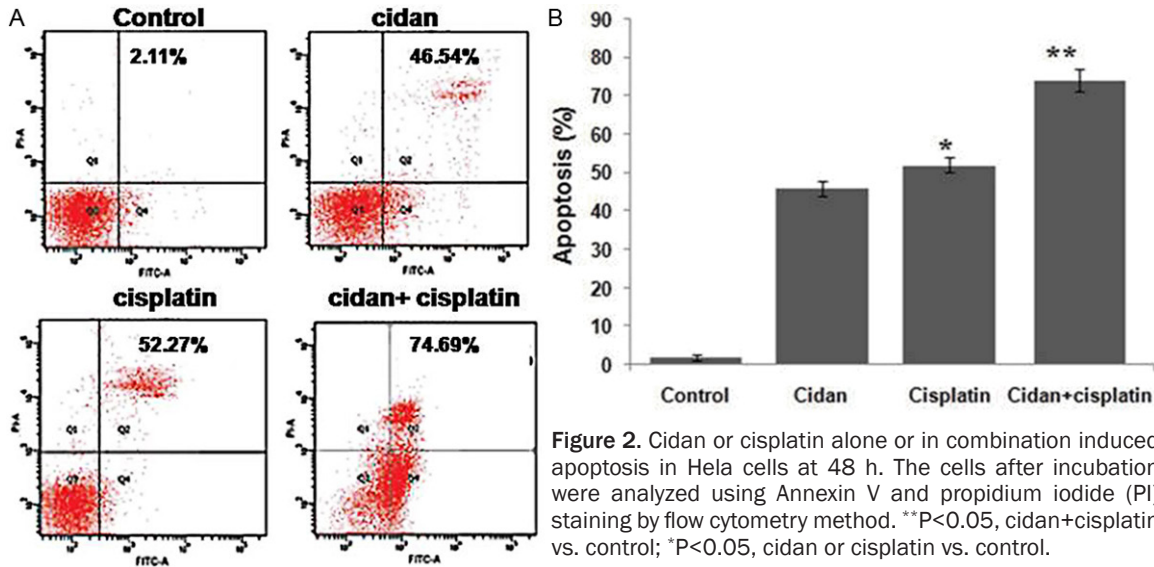


Figure 2. Cidan or cisplatin alone or in combination induced apoptosis in HeLa cells at 48 h. The cells after incubation were analyzed using Annexin V and propidium iodide (PI) staining by flow cytometry method. **P<0.05, cidan+cisplatin vs. control; *P<0.05, cidan or cisplatin vs. control.

tion. The cells were then harvested, centrifuged for 10 min at 500 × g and the cell pellets were treated with Annexin V binding buffer (40 mM CaCl₂, 10 mM HEPES/NaOH and 2.5 mM MgCl₂). Then to each of the plate was added FITC-conjugated Annexin V (5 μl) and propidium iodide (PI; 5 μl) and incubation was continued for 20 min under dark conditions. Fluorescence-Activated Cell Sorting (FACScan) instrument (Becton-Dickinson, Franklin Lakes, CA, USA) was used for the analysis of the apoptotic cells.

Western blot assay

HeLa cells after incubation for 48 h with cidan or cisplatin alone or in combination were harvested and lysed to extract the total proteins. Bio-Rad protein assay kit (Bio-Rad, Shanghai, China) was used for the determination of concentration of the proteins. The protein samples (30 μg) were isolated using 12% SDS-PAGE and were then transferred onto PVDF membranes. The membranes were washed three times with Tris-buffered saline and Tween (TBST; Probe Co. Ltd., Guangzhou, China) followed by incubation for 45 min in blocking buffer (5% dried milk in PBS). Then the membrane was incubated for overnight with primary antibodies at 4°C. The primary antibodies used were against caspase-3, cleaved caspase-3 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1,000-fold dilution). The membranes were washed twice with TBST followed by incubation with anti-mouse-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz,

CA, USA, 1,000-fold dilution) for 1 h more. For the visualization of blots enhanced chemiluminescence detection system (Amersham) was used. β-actin was used as the internal loading control.

Treatment strategy

The mice were divided randomly into four groups of 5 each; control, cidan, cisplatin and cidan+cisplatin groups. The animals in the cidan, cisplatin and cidan+cisplatin groups were anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture followed by administration of HeLa cells (2 × 10⁵) subcutaneously. The mice in the cidan, cisplatin and cidan+cisplatin groups were injected 10, 5 and 10+5 mg/ml doses of cidan, cisplatin and cidan & cisplatin daily for 30 days. The animals in the control group received same volume of normal saline. Tumor size was recorded for each of the animal on the alternative days to calculate the tumor volume.

Histological examination

On day 31, the animals were sacrificed to extract the tumor tissues from each mice after anesthetization. The tissue samples were then analyzed by using a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay. This was performed using In Situ Cell Death Detection kit conjugated with horseradish peroxidase (Roche Applied Science, Indianapolis, IN, USA), as per the manual protocol and a Leica DMI microscope (Leica,

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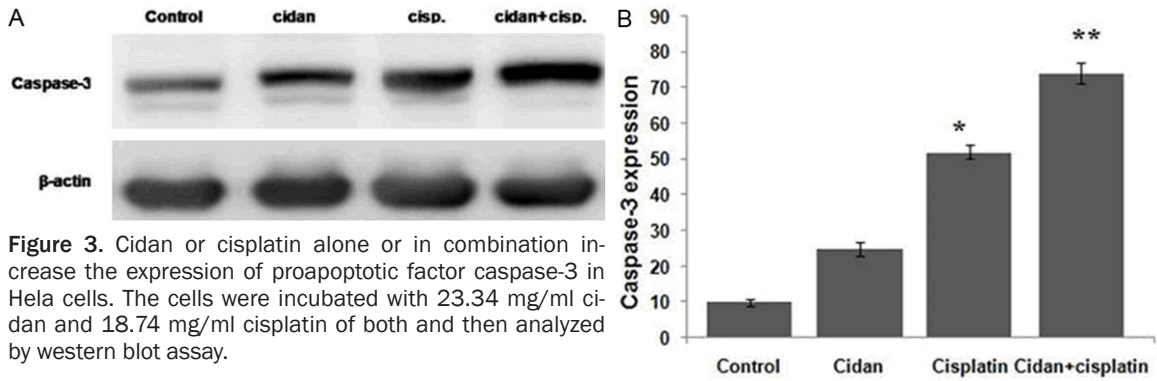


Figure 3. Cidan or cisplatin alone or in combination increase the expression of proapoptotic factor caspase-3 in HeLa cells. The cells were incubated with 23.34 mg/ml cidan and 18.74 mg/ml cisplatin of both and then analyzed by western blot assay.

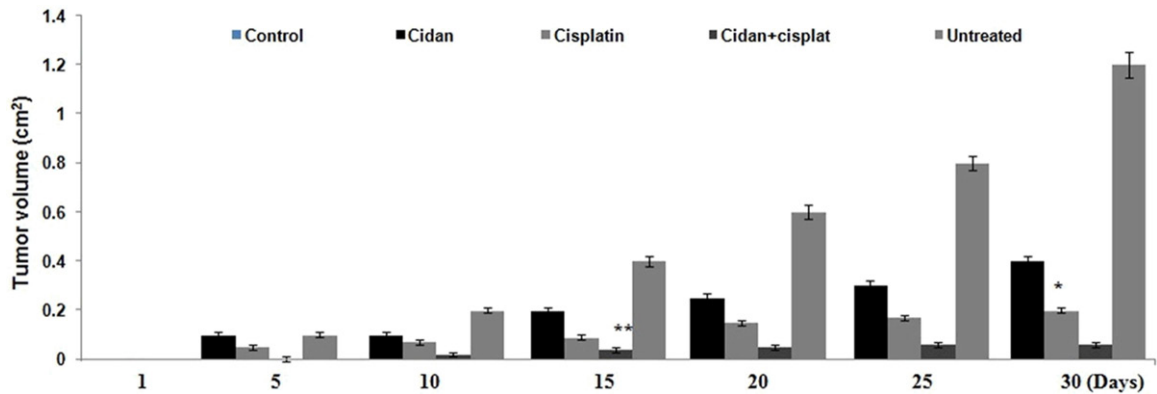


Figure 4. Cisplatin and combination of cidan and cisplatin inhibited tumor growth in the HeLa tumor xenograft model. The mice were treated with the cidan, cisplatin and cidan+cisplatin groups were injected 10, 5 and 10+5 mg/mml doses of cidan, cisplatin and cidan & cisplatin daily for 30 days. The animals in the control group received same volume of normal saline. On alternative days tumor volume for each animals was determined. **P<0.05 cidan+cisplatin vs. control; cisplatin vs. control.

Wetzlar, Germany) was used for the tissue analysis.

Statistical analysis

The data expressed are the mean \pm standard deviation (SD). The data obtained for various groups was compared by using Student's t-test and was analyzed using Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA, USA). Differences were considered statistically significant at P<0.05.

Results

Cidan treatment exhibits inhibitory effect on HeLa cell proliferation

The results from MTT assay showed a concentration and time dependent reduction in the proliferation rate of HeLa cells on treatment with cidan and cisplatin (Figure 1). Increase in the concentration of cidan from 10 to 30 mg/

mlled to decrease in the proliferation rate of HeLa cells from 95.6 to 43.4% at 48 h (P<0.05). Treatment of the HeLa cells with cisplatin at 5 and 30 mg/ml reduced cell proliferation to 82.7 and 39.2%, respectively at 48 h. For cidan and cisplatin the IC₅₀ values were 23.34 and 18.74 mg/ml, respectively at 48 h.

Induction of apoptosis in HeLa cells by treatment with cidan

Apoptosis induction in HeLa cells on treatment with cidan and cisplatin was analyzed using Annexin V/PI double staining by flow cytometric method. The cells were incubated with IC₅₀ concentration of cidan and cisplatin alone or in combination for 48 h. In HeLa cells, cidan and cisplatin treatment at 23.34 and 18.74 mg/ml concentrations induced apoptosis in 46.54 and 52.27%, respectively at 48 h (Figure 2A). However, treatment of HeLa cells with cidan and cisplatin combination for 48 h increased the

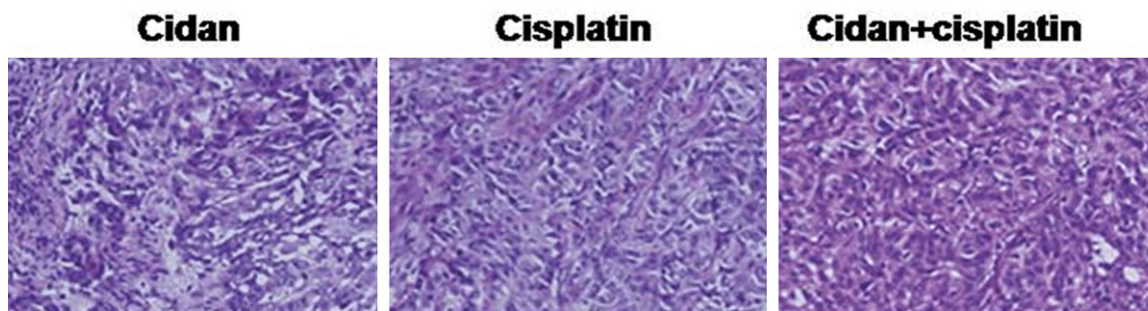


Figure 5. Analysis of the apoptosis in tumor cells of mice tumor xenograft following one month treatment with cidan, cisplatin and their combination. The tumor samples were extracted and then analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.

proportion of apoptotic cells to 74.69%, ($P < 0.05$) (Figure 2B).

Effect of cidan and cisplatin on the activation of caspase-3 in Hela cells

The results from western blot analysis revealed a significantly ($P < 0.05$) higher level of caspase-3 expression in Hela cells treated with the combination of cidan and cisplatin compared to the cells treated with cidan or cisplatin alone (Figure 3).

Effect of cidan and cisplatin on the Hela cell tumor xenograft growth

Tumor growth in the cisplatin treatment group of mice was reduced significantly after 1 month compared to the control ($P < 0.05$). Cidan treatment exhibited inhibitory effect on the tumor growth in mice but the reduction in growth was lower compared to the cisplatin treatment group. However, the growth of tumor in mice treated with combination of cidan and cisplatin was significantly inhibited at day 14 of the treatment ($P < 0.05$) (Figure 4).

Effect of cidan and cisplatin on tumor cells in vivo

Analysis of the effect of cidan and cisplatin combination on tumor xenograft mice model was performed using TUNEL assay. The results revealed that cidan and cisplatin combination treatment induced apoptosis in the tumor xenograft cells (Figure 5). The proportion of apoptotic cells was significantly higher in the mice treated with cidan and cisplatin combination compared to cidan or cisplatin treatment groups alone ($P < 0.05$).

Discussion

TCMs exhibit their effect through inhibition of the carcinoma cell growth [8-11] and by improving the immune system [12]. Cisplatin is a standard antitumor drug which inhibits carcinoma cell proliferation by damaging cellular DNA and activating pro-apoptotic factors [16]. Effect of the cisplatin on cervical carcinoma in combination with various other drugs has been investigated [17-19]. The present study was aimed to investigate the effect of cidan alone and in combination with cisplatin on the cervical carcinoma. Results from the present study revealed a concentration and time dependent reduction in the proliferation rate of Hela cells on treatment with cidan and cisplatin. The IC_{50} values for cidan and cisplatin were determined to be 23.34 and 18.74 mg/ml, respectively. Treatment with cidan or cisplatin induced apoptosis significantly in Hela cells compared to the control cells at 48 h. However, combination of the cidan and cisplatin markedly increased the proportion of apoptotic Hela cells compared to cidan or cisplatin alone. Analysis of the pro-apoptotic factors using western blot assay revealed higher expression in cells treated with a combination of cidan and cisplatin compared with cidan or cisplatin alone. Cisplatin exhibited significantly higher inhibitory effect on the tumor growth in Hela tumor xenograft mice model compared to the cidan. However, combination of the cidan and cisplatin showed synergistic effect on the inhibition of tumor growth in the mice model. Combination of cidan and cisplatin significantly inhibited tumor growth in the mice after 14 days compared to 30 days in the cisplatin treatment group. Cidan alone could not significantly inhibit the tumor growth in the

tumor xenograft mice model. In the present study the mechanism underlying interaction between cidan and cisplatin was not fully understood. Cidan efficiently enhanced the antitumor effect of cisplatin against Hela cancer cells and in tumor xenograft mice model.

In summary, cidan enhances the anti-tumor effect of cisplatin both *in vitro* and *in vivo*, therefore the combination can be used as efficient treatment strategy for cervical cancer. Further studies to understand the mechanism of action of cidan and cisplatin combination are under process.

Disclosure of conflict of interest

None.

Address correspondence to: Ai-Hua Li, Department of Obstetrics and Gynecology, People's Hospital of Linyi City, 27 Jiefang Road, Lanshan District Linyi City, Linyi 27600, Shandong Province, China. Tel: 0086-539-8224013; Fax: 0086-539-8224013; E-mail: liaihuaah@tom.com

References

- [1] Saslow D, Castle PE, Cox JT, Davey DD, Einstein MH, Ferris DG, Goldie SJ, Harper DM, Kinney W, Moscicki AB, Noller KL, Wheeler CM, Ades T, Andrews KS, Doroshenk MK, Kahn KG, Schmidt C, Shafey O, Smith RA, Partridge EE; Gynecologic Cancer Advisory Group, Garcia F. American Cancer Society Guideline for human papillomavirus (HPV) vaccine use to prevent cervical cancer and its precursors. *CA Cancer J Clin* 2007; 57: 7-28.
- [2] Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007; 57: 43-66.
- [3] Leitao MM Jr and Chi DS. Recurrent cervical cancer. *Curr Treat Options Oncol* 2002; 3: 105-111.
- [4] Yang J, Li J, Sun M and Chen K. Studies of traditional Chinese medicine monomer on HeLa cell of cervical cancer. *Pak J Pharm* 2014; *Sci* 27 Suppl: 1063-1068.
- [5] Cohen I, Tagliaferri M and Tripathy D. Traditional Chinese medicine in the treatment of breast cancer. *Semin Oncol* 2002; 29: 563-574.
- [6] Li X, Yang G, Li X, Zhang Y, Yang J, Chang J, Sun X, Zhou X, Guo Y, Xu Y, Liu J, Bensoussan A. Traditional Chinese medicine in cancer care: a review of controlled clinical studies published in Chinese. *PLoS One* 2013; 8: e60338.
- [7] Bonomi P, Blessing JA, Stehman FB, DiSaia PJ, Walton L and Major F. Randomized trial of three cisplatin dose schedules in squamous-cell carcinoma of the cervix: a Gynecologic Oncology Group study. *J Clin Oncol* 1985; 3: 1079-1085.
- [8] Wang WX, Li TX, Ma H, Zhang JF, Jia AQ. Tumoral cytotoxic and antioxidative phenylpropanoid glycosides in *Smilax riparia* A. DC. *J Ethnopharmacol* 2013; 149: 527-532.
- [9] Liang QL, Dai CC, Jiang JH, Tang YP and Duan JA. A new cytotoxic casbanediterpene from *Euphorbia pekinensis*. *Fitoterapia* 2009; 80: 514-516.
- [10] Fang R, Houghton PJ and Hylands PJ. Cytotoxic effects of compounds from *Iris tectorum* on human cancer cell lines. *J Ethnopharmacol* 2008; 118: 257-263.
- [11] Duan JA, Wang L, Qian S, Su S and Tang Y. A new cytotoxic prenylated dihydrobenzofuran derivative and other chemical constituents from the rhizomes of *Atractylodes lancea* DC. *Arch Pharm Res* 2008; 31: 965-969.
- [12] Lin H, Liu J and Zhang Y. Developments in cancer prevention and treatment using traditional Chinese medicine. *Front Med* 2011; 5: 127-133.
- [13] Dai ZJ, Tang W, Lu WF, Gao J, Kang HF, Ma XB, Min WL, Wang XJ, Wu WY. Antiproliferative and apoptotic effects of β -elemene on human hepatoma HepG2 cells. *Cancer Cell Int* 2013; 13: 27.
- [14] Lu JJ, Dang YY, Huang M, Xu WS, Chen XP, and Wang YT. Anti-cancer properties of terpenoids isolated from *Rhizoma Curcumae* - a review. *J Ethnopharmacol* 2012; 143: 406-411.
- [15] Wang B, Peng XX, Sun R, Li J, Zhan XR, Wu LJ, Wang SL, Xie T. Systematic review of β -elemene injection as adjunctive treatment for lung cancer. *Chin J Integr Med* 2012; 18: 813-823.
- [16] Mandic A, Hansson J, Linder S and Shoshan MC. Cisplatin induces endoplasmic reticulum stress and nucleus-independent apoptotic signaling. *J Biol Chem* 2003; 278: 9100-9106.
- [17] Robati M, Holtz D and Dunton CJ. A review of topotecan in combination chemotherapy for advanced cervical cancer. *Ther Clin Risk Manag* 2008; 4: 213-218.
- [18] Bonomi P, Blessing J, Ball H, Hanjani P and DiSaia PJ. A phase II evaluation of cisplatin and 5-fluorouracil in patients with advanced squamous cell carcinoma of the cervix: a Gynecologic Oncology Group study. *Gynecol Oncol* 1989; 34: 357-359.
- [19] Daghestani AN, Hakes TB, Lynch G and Lewis JL. Cervix carcinoma: treatment with combination cisplatin and bleomycin. *Gynecol Oncol* 1983; 16: 334-339.