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Original Article

Anticancer effect of PP31J isolated from *Physalis pubescens* L. in human cervical carcinoma cells

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Abstract: Extracts derived from *Physalis pubescens L.* may function as cancer therapies. The pharmacological effects of PP31J on human cervical carcinoma cells (HeLa cells) were investigated in this study. HeLa cells were treated with PP31J, and then cell proliferation, apoptosis, and cell cycle distribution were measured using a cell counting kit-8 (CCK-8) assay and flow cytometry. Protein expression levels of regulators of cell apoptosis and cell cycle were also examined using western blotting. Our data show that PP31J inhibited the growth of HeLa cells. Significant growth inhibition compared to the vehicle-treated group was observed using a concentration of 5 μM PP31J at 24, 48, and 72 h. PP31J also selectively arrested cell cycle progression in the G1 phase at 40 μM ($P < 0.05$) and in the G2/M phase at 20 μM ($P < 0.01$) and 40 μM ($P < 0.001$). Our results further demonstrate a significant increase in cell apoptosis ($P < 0.001$) following PP31J treatment (10, 20, and 40 μM). Immunoblotting data show that PP31J downregulated ($P < 0.01$) the expression of Bcl-xL and decreased ($P < 0.05$) the expression of Survivin and Cyclin D1 at 20 and 40 μM. This study shows the anti-tumor activity of PP31J in HeLa cells and that the effects of PP31J on cell cycle distribution and apoptosis induction were partially attributed to the regulation of Cyclin D1, Survivin, and Bcl-xL.

Keywords: HeLa cell, cell cycle, apoptosis, cyclin D1, G2/M arrest

Introduction

Although the development of screening programs has reduced the incidence and mortality of cervical cancer, the disease remains a leading cause of cancer-related death in women [1, 2]. There are estimated to be more than 5×10^5 new cervical cancer cases annually, with more than 2.5×10^5 cancer-related deaths [3]. Recent evidence has demonstrated that concurrent chemoradiotherapy using a cisplatin-based regimen can significantly improve both overall survival and progression-free survival [4]. Unfortunately, platinum-based therapies are commonly associated with side effects, such as myelosuppression, leukopenia, ototoxicity, neurotoxicity, and nephrotoxicity. The development of drug resistance also limits treatment. Thus, identifying novel chemotherapeutic agents remains a critical challenge in cancer medicine.

Michael reaction acceptors containing electrophilic moieties are functional molecules that are directly or indirectly involved in various life processes [5]. Recently, a compound isolated from the plant *Physalis pubescens* L. Known as PP31J, or physapubescin B, has been tested as a Michael reaction acceptor [6, 7]. Physalis plants are distributed mainly in China and Vietnam. The major chemical constituents of these plants, physalins, have been used in traditional Chinese medicines to detoxify patients. These chemicals demonstrate important physiological activities, including cancer prevention and anti-inflammatory, anti-bacterial, and analgesic effects [8-12]. Furthermore, *in vitro* treatment of several cell lines with *Physalis* derivatives have demonstrated significant anti-tumor activities for this compound [7]. To further investigate the anti-cancer potential of *Physalis* derivatives, we evaluated the selectivity and efficiency of physalin-based anti-tumor agents. Our results show that PP31J inhibited cellular proliferation and induced apoptosis in human cervical cancer HeLa cells.

Materials and methods

Materials and reagents

The HeLa human cervical cancer cell line was obtained from the American Type Culture Collection (ATCC). The cells were cultured in 1640 medium (Hyclone, Logan, UT, USA) supplemented with fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel). The cell counting kit-8 (CCK-8) and trypsin-EDTA solution were obtained from Beyotime Institute of Biotechnology (Shanghai, China). The primary antibodies to Bcl-xl, Cyclin D1, and Survivin were purchased from Proteintech (Chicago, IL, USA). The Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from Nanjing Key Gen Biotech (Nanjing, China). PP31J was a gift from Zhongjun Ma (Ocean College, Zhejiang University, China).

Cell culture

HeLa cells were cultured in 1640 medium with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged twice weekly to maintain logarithmic growth.

CCK-8 assay

The anti-proliferative effects of PP31J were determined by CCK-8 assay. Cells were treated with 0.25% trypsin and then seeded into 96-well plates at a density of 5×10^5 cells/well. Various concentrations of PP31J (0, 10, 20, and 40 μM) were added and the cells were incubated for 24, 48, or 72 h before 20 μL of CCK-8 solution was added to each well. The plates were further incubated for 3 h at 37°C. The absorbance values were detected at 450 nm. Cell proliferation was measured using microscopy at the IC50 (50% inhibitory concentration).

Cell cycle analysis

Cell cycle analysis was performed using flow cytometry. The cells were treated with 0.25% trypsin, and then 5×10^5 HeLa cells were seeded into a 24-well plate. PP31J was added at various concentrations (0, 10, 20, 40 μM), and the cells were incubated at 37°C with 5% CO_2 for 48 h. Cells were washed with PBS and treated with 0.25% trypsin. After an additional wash with PBS, cells were fixed in chilled alcohol for 2 h at 4°C. Cells were stained with PI (50 μg/mL) in the dark at room temperature for 45 min and washed with PBS. Each treatment was performed in triplicate. Cell cycle analysis was performed using an AccuriC6 flow cytometer (BD Biosciences). The percentage of cells in each phase of the cell cycle was calculated using CELL Quest software (BD Biosciences).

Detection of cell apoptosis

Cells were trypsinized and seeded in 24-well plates at a density of 5×10^5 cells/well. Cells were treated with various concentrations of PP31J (0, 10, 20, and 40 μM) and incubated at 37°C with 5% CO_2 for 48 h. Treated cells were washed with PBS, harvested in 0.25% trypsin, washed twice with PBS, and then suspended in binding buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added to the cells according the manufacturer's instructions. Each treatment was performed in triplicate. Cells were incubated in the dark for 20 min, washed with PBS, and then promptly analyzed by flow cytometry.

Western blotting

Cells were lysed in cold RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM sodium chloride, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) and incubated for 3 h. Lysates were centrifuged at 12,000 g for 10 min, and the protein concentration in the supernatant was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Next, 40 μg of protein were separated using 10% SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% skim milk for 1 h at room temperature and then incubated with primary antibodies (Bcl-xl, Cyclin D, and Survivin) overnight at 4°C with agitation. Membranes were washed in tris-buffered saline with 0.05% Tween 20 (TBST) three times for 5 min each. Membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,000) for 1 h at 37°C. Next, membranes were washed three times with TBST, and the proteins were visualized using enhanced chemiluminescence. The experiments were performed in triplicate, and the densitometry analysis was performed using ImageJ 1.44 software (NIH, USA).

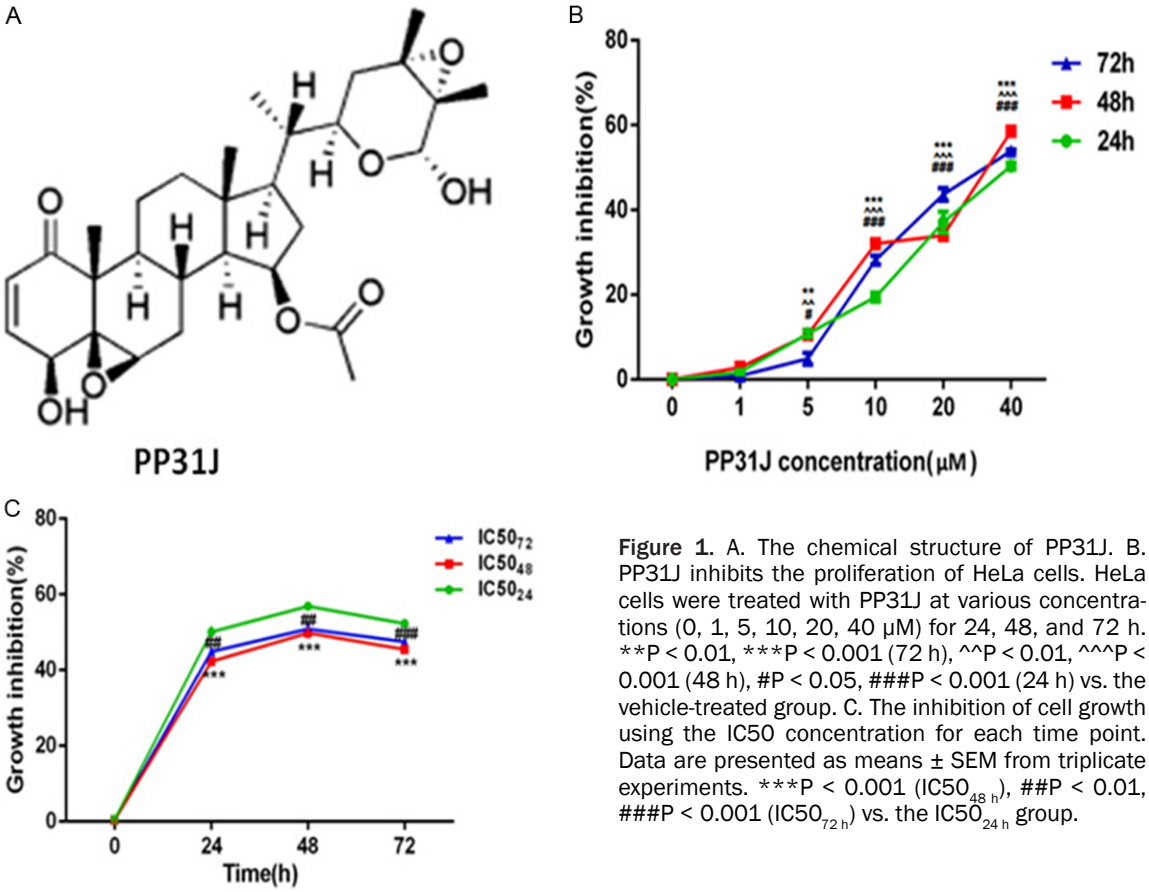

Figure 1. A. The chemical structure of PP31J. B. PP31J inhibits the proliferation of HeLa cells. HeLa cells were treated with PP31J at various concentrations (0, 1, 5, 10, 20, 40 μM) for 24, 48, and 72 h. **P < 0.01, ***P < 0.001 (72 h), ^^P < 0.01, ^^^P < 0.001 (48 h), #P < 0.05, ###P < 0.001 (24 h) vs. the vehicle-treated group. C. The inhibition of cell growth using the IC50 concentration for each time point. Data are presented as means ± SEM from triplicate experiments. ***P < 0.001 ($IC50_{48\ h}$), ##P < 0.01, ###P < 0.001 ($IC50_{72\ h}$) vs. the $IC50_{24\ h}$ group.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM) and were analyzed for statistical significance using two-tailed Student's t tests using Graphpad prism 6 software. A level of P < 0.05 was regarded as statistically significant in all experiments.

Results

PP31J inhibits the proliferation of HeLa cells

The chemical structure of PP31J is shown in **Figure 1A**. The cytotoxicity of PP31J in the HeLa cell line was assessed after 24, 48, and 72 h of drug treatment [7]. PP31J inhibited the growth of HeLa cells in a concentration-dependent fashion (**Figure 1B**). The growth inhibition was significant when the concentration of PP31J exceeded 5 μM compared to the vehicle-treated group at each time point. The IC50 values at 24, 48, and 72 h were 29.41, 23.13, and 24.35 μM, respectively. The effect of growth inhibition increased at 24 h and reached its peak at 48 h. These data indicate that PP31J potently inhibited HeLa cell proliferation at all doses (**Figure 1C**).

PP31J promotes G2/M-phase arrest in HeLa cells

Flow cytometry was used to examine whether the proliferation of treated cells decreased because of cell cycle arrest at a specific phase or if cells died by apoptosis. Treatment with PP31J for 48 h induced G2/M-phase arrest of the cell cycle in a dose-dependent manner. This effect was significant at PP31J concentrations of 20 μM (**Figure 2**, P < 0.01) and 40 μM (P < 0.001). Additionally, the percentage of cells in the S-phase was significantly reduced at treatment concentrations of 20 μM (P < 0.05) and 40 μM (P < 0.01).

PP31J induces apoptosis in HeLa cells

The induction of apoptosis was evaluated in HeLa cells following 48 h of PP31J treatment (**Figure 3**). The treated cells were stained with

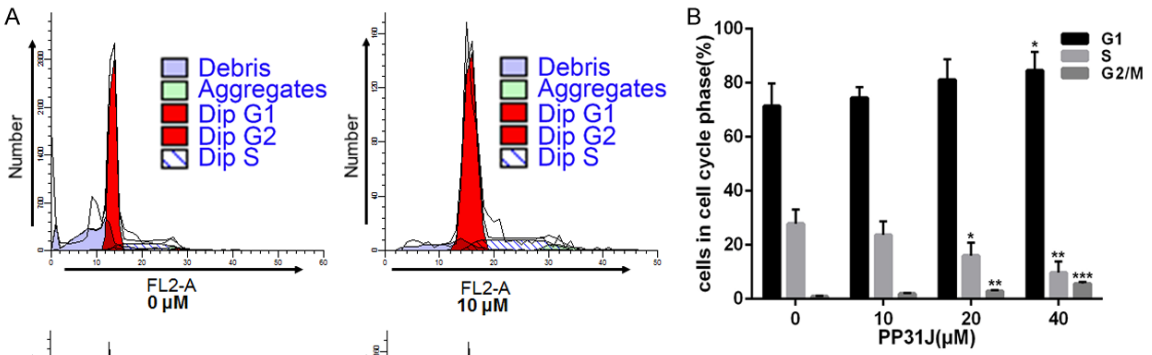

Figure 2. Effects of PP31J on the cell cycle arrest of HeLa cells. HeLa cells were treated with PP31J at various concentrations (0, 10, 20, and 40 μM) for 48 h. A. Representative flow cytometry histograms for each experimental group. B. The percentages of each cell cycle phase in response to various PP31J concentrations. Data are presented as mean ± SEM from triplicate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the vehicle-treated group.

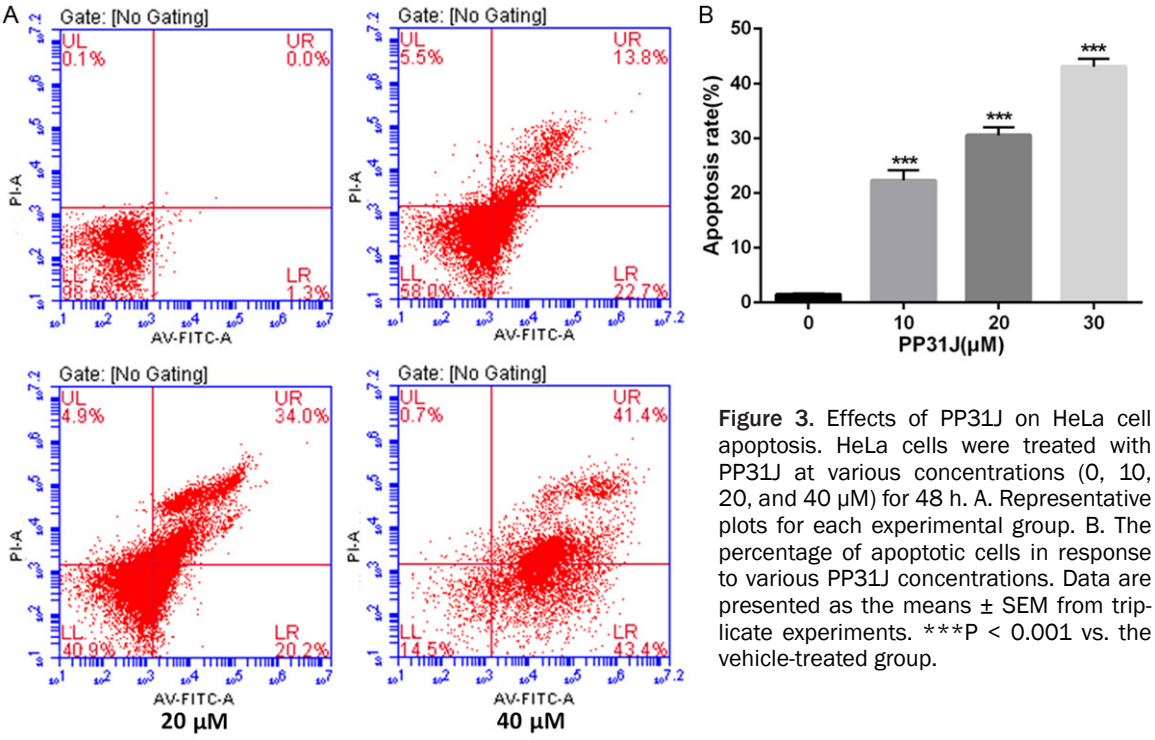

Figure 3. Effects of PP31J on HeLa cell apoptosis. HeLa cells were treated with PP31J at various concentrations (0, 10, 20, and 40 μM) for 48 h. A. Representative plots for each experimental group. B. The percentage of apoptotic cells in response to various PP31J concentrations. Data are presented as the means ± SEM from triplicate experiments. ***P < 0.001 vs. the vehicle-treated group.

Annexin V/PI and then analyzed by flow cytometry. The percentage of apoptotic cells increased (P < 0.001) from 1.3% without PP31J treatment to 22.7%, 40.2%, and 43.4% at PP31J concentrations of 10, 20, and 40 μM, respectively.

PP31J alters the expression of cell cycle and apoptosis regulatory proteins

The underlying mechanism of cell cycle arrest and apoptosis induced by PP31J was investigated (**Figure 4**). Western blot assays were per-

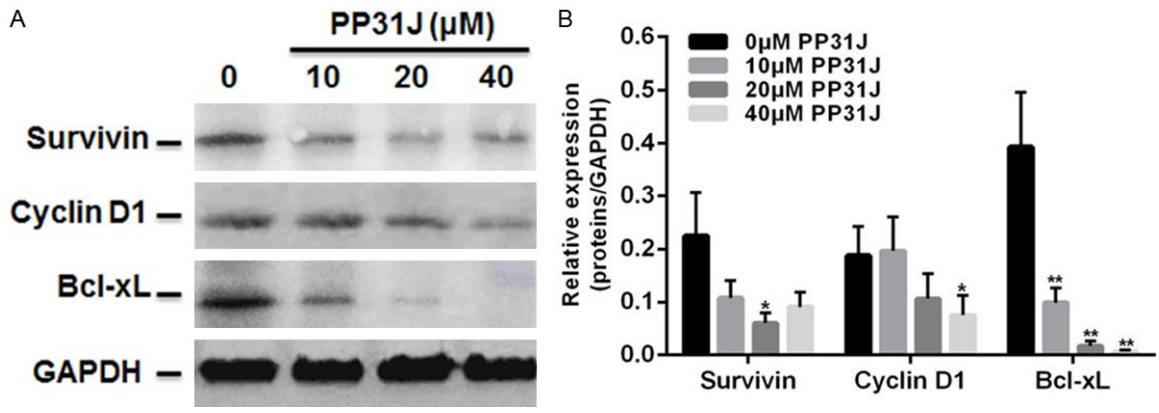

Figure 4. Effects of PP31J on the protein expression of Survivin, Cyclin D1, and Bcl-xL in HeLa cells. HeLa cells were treated with PP31J at various concentrations (0, 10, 20, and 40 μM) for 48 h. A. Western blot results for each experimental group. B. Densitometric quantification of protein expression levels in A. Data are presented as means ± SEM from triplicate experiments. *P < 0.05, **P < 0.01 vs. the vehicle-treated group.

formed to determine the expression levels of several proteins that regulate the cell cycle and apoptosis, including Survivin, Cyclin D1, and Bcl-xL. Incubation of HeLa cells with PP31J significantly ($P < 0.01$) downregulated the expression of Bcl-xL in a dose-dependent manner. The expression levels of Survivin and Cyclin D1 were also decreased ($P < 0.05$) at PP31J concentrations of 20 and 40 μM, respectively.

Discussion

Physalis pubescens L. (Solanaceae), which is cultivated in China, is the plant source of a traditional Chinese medicine that has been used for a long period of time to reduce fever and detoxify patients. Previous studies have demonstrated that the dichloromethane extract of *Physalis pubescens* L. has cancer-preventing, anti-inflammatory, anti-bacterial, and analgesic properties [11, 13, 14]. Physalin and physapubescin B are both natural compounds extracted from *Physalis pubescens* L. that possess anti-tumor activities [15, 7]. PP31J is another major bioactive substance isolated from *Physalis pubescens* L. The present study was performed to explore whether PP31J also possesses anti-tumor effects.

In this study, we used the CCK-8 assay to detect the cytotoxicity of PP31J in HeLa cells. The results indicate that PP31J concentrations ranging from 5 to 40 μM significantly inhibited the proliferation of HeLa cells in a dose-dependent manner. These findings suggest a potential role for PP31J in cervical carcinoma prevention. Similarly, physapubescin B, another active compound isolated from *Physalis pubescens* L., demonstrates anti-tumor activity against prostate cancer cells *in vitro* [7].

We next investigated the functional effects of PP31J treatment on HeLa cells. Our data indicate that PP31J treatment significantly increased the accumulation of cells in the G1 and G2/M phases and decreased the proportion of cells in S phase. The cell cycle arrest in the G1 phase was partially due to the downregulation of Cyclin D1 (CDK1), which drives cell cycle progression at the G1/S transition and has a role in tumorigenesis [16]. Similar findings have been reported for benzoylureas, which are physalin analogues. These compounds show antitumor activity *in vivo* and *in vitro* by downregulating CDK1 expression in human cancer cells [17]. However, the underlying mechanism for the cell arrest in the G2/M stage requires additional research. The results in this study are consistent with the effect of physapubescin B on G2/M arrest in PC-3 cells [7].

The present study demonstrates that PP31J at concentrations of 10, 20, and 40 μM induced HeLa cell apoptosis. This effect on cell apoptosis may have been the result of expression changes in Survivin and Bcl-xL, which are expressed in many solid tumors and are correlated with cell apoptosis [18-20]. Our results also demonstrate that the *Physalis pubescens* L. extract PP31J had anti-tumor activity in HeLa cells. In particular, HeLa cells treated with PP31J showed decreased proliferation, cell

cycle arrest in the G1 and G2/M phases, and increased apoptosis. These effects of PP31J were attributed to its regulation of CDK1, Survivin, and Bcl-xL. Thus, this study provides evidence for the potential application of PP31J or *Physalis pubescens* L. in the treatment of human cervical carcinoma.

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Disclosure of conflict of interest

None.

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References

[1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55: 74-108.

[2] Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 2006; 24: 2137-2150.

[3] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

[4] Kuzuya K. Chemoradiotherapy for uterine cancer: current status and perspectives. Int J Clin Oncol 2004; 9: 458-470.

[5] Umayahara K, Takekuma M, Hirashima Y, Noda SE, Ohno T, Miyagi E, Hirahara F, Hirata E, Kondo E, Tabata T, Nagai Y, Aoki Y, Wakatsuki M, Takeuchi M, Toita T, Takeshima N, Takizawa K. Phase II study of concurrent chemoradiotherapy with weekly cisplatin and paclitaxel in patients with locally advanced uterine cervical cancer: the JACCRO GY-01 trial. Gynecol Oncol2016; 140: 253-258.

[6] Wang W, Wang J, Li N, Zhang X, Zhao W, Li J, Si Y. Chemopreventive flavonoids from Millettia pulchra Kurz var-laxior (Dunn) Z. Wei (Yulangsan) function as Michael reaction acceptors. Bioorg Med Chem Lett 2015; 25: 1078-1081.

[7] Ding W, Hu Z, Zhang Z, Ma Q, Tang H, Ma Z. Physapubescin B exhibits potent activity against human prostate cancer in vitro and in vivo. J Agric Food Chem 2015; 63: 9504-9512.

[8] Ji L, Yuan Y, Ma Z, Chen Z, Gan L, Ma X, Huang D. Induction of quinone reductase (QR) by withanolides isolated from Physalis pubescens L. Steroids 2013; 78: 860-865.

[9] de Haan LH, Pot GK, Aarts JM, Rietjens IM, Alink GM. In vivo relevance of two critical levels for NAD(P)H:quinone oxidoreductase (NQO1)-mediated cellular protection against electrophile toxicity found in vitro. Toxicol In Vitro 2006; 20: 594-600.

[10] Dinkova-Kostova AT, Talalay P. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch Biochem Biophys 2010; 501: 116-123.

[11] Qiu L, Zhao F, Jiang ZH, Chen LX, Zhao Q, Liu HX, Yao XS, Qiu F. Steroids and flavonoids from Physalis alkekengi var. franchetii and their inhibitory effects on nitric oxide production. J Nat Prod 2008; 71: 642-646.

[12] Januário AH, Filho ER, Pietro RC, Kashima S, Sato DN, França SC. Antimycobacterial physalins from Physalis angulata L. (Solanaceae). Phytother Res 2002; 16: 445-448.

[13] Franco LA, Matiz GE, Calle J, Pinzón R, Ospina LF. Antiinflammatory activity of extracts and fractions obtained from Physalis peruviana L. calyces. Biomedica 2007; 27: 110-115.

[14] Agata K, Kusiak J, Stępień B, Bergier K, Kuźniak E. Bioactive secondary metabolites produced by plants of the genus Physalis. Postepy Hig Med Dosw (Online) 2010; 64: 665-673.

[15] Yu Y, Hamza A, Zhang T, Gu M, Zou P, Newman B, Li Y, Gunatilaka AA, Zhan CG, Sun D. Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochem Pharmacol 2010; 79: 542-551.

[16] Zuryń A, Litwiniec A, Gagat M, Drzewucka J, Gackowska L, Grzanka A. The influence of arsenic trioxide on the cell cycle, apoptosis and expression of cyclin D1 in the Jurkat cell line. Acta Histochem 2014; 116: 1350-1358.

[17] Coker-Gurkan A, Arisan ED, Obakan P, Ozfiliz P, Kose B, Bickici G, Palavan-Unsal N. Roscovitine-treated HeLa cells finalize autophagy later than apoptosis by downregulating Bcl-2. Mol Med Rep 2015; 11: 1968-1974.

[18] Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 2003; 22: 8581-8589.

[19] Geng D, Song X, Ning F, Song Q, Yin H. MiR-34a inhibits viability and invasion of human papillo-

mavirus-positive cervical cancer cells by targeting E2F3 and regulating survivin. Int J Gynecol Cancer 2015; 25: 707-713.

[20] Um HD. Bcl-2 family proteins as regulators of cancer cell invasion and metastasis: a review focusing on mitochondrial respiration and reactive oxygen species. Oncotarget 2016; 7: 5193-5203.