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Inhibition of the Ethanolic Extract from *Polygonum cuspidatum* Root on the Functions of Epstein-Barr Virus Latent Membrane Protein 1

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ABSTRACT

Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1), which is expressed during viral latency, has oncogenic properties that are associated with several malignant diseases. The purpose of this study is to examine how the ethanolic extract from *Polygonum cuspidatum* root (PcE) inhibits the functions of latent membrane protein 1. We found that the viability of EBV-positive cells, B95-8 cells decreased in a time- and dose-dependent manner after the cells were treated with PcE. Furthermore, PcE inhibited the transcription of the LMP1 gene, *BNLF1*. This was attributed to the inhibition of the expression of Rta, which are known to activate the transcription of *BNLF1*. Flow cytometry analysis showed that PcE caused an increase in the number of B95-8 cells that were arrested at the sub-G1 phase and displayed typical features of apoptosis. In other words, PcE promoted B95-8 cells apoptosis due to its ability to inhibit the expression of latent membrane protein 1 and deplete NF- κ B in the nucleus. These results indicated that PcE may be a useful therapeutic drug to kill cells that are infected by EBV.

Key words: Epstein-Barr virus, *Polygonum cuspidatum*, latent membrane protein 1

INTRODUCTION

Epstein-Barr virus (EBV) is an oncogenic human herpesvirus, which infects B lymphocytes and epithelial cells⁽¹⁾. Although the infection is often asymptomatic, it causes infectious mononucleosis and is associated with several human malignancies, including Burkitt's⁽²⁾ and Hodgkin's lymphomas⁽³⁾ nasopharyngeal carcinoma (NPC)⁽⁴⁾ and gastric carcinoma⁽⁵⁾. EBV gene expression differs among malignancies associated with the virus. EBV in Burkitt's lymphoma tissues usually have a type-I latency pattern with expression of EBNA-1 but not the other latency-associated proteins. EBV in tissues from patients with Hodgkin's lymphomas and nasopharyngeal carcinoma has a type-II latency pattern with expression of EBNA (EBV nuclear antigen) 1, LMP (latent membrane protein) 1 and LMP2. EBV in immunocompromised patients with EBV lymphoproliferative disease generally have a type-III latency pattern and express EBNA1, EBNA2, EBNA3A, EBNA3C, LMP1, LMP2A and LMP2B⁽⁶⁾. The EBV-encoded LMP1 is an integral membrane protein that has been known to have oncogenic properties during latent infection⁽⁷⁾.

Transcription of the LMP1 gene, *BNLF1*, is initiated from a proximal promoter, ED-L1, and a distal promoter, TR-L1⁽⁸⁾. These two promoters are regulated by EBNA1⁽⁹⁾ and lytic protein, Rta⁽¹⁰⁾. Meanwhile, the ED-L1 promoter is activated by EBNA2⁽¹¹⁾ and NF- κ B⁽¹²⁾. LMP1 also activates the function of NF- κ B, which is a key modulator of cell growth and relevant to the oncogenic role of EBV in some malignant tumors⁽¹³⁾. NF- κ B that is bound to its inhibitory protein I κ Bs is retained in the cytosolic compartment⁽¹⁴⁾. Following activation, I κ Bs are phosphorylated by I κ B kinases and subsequent degradation of phosphorylated I κ Bs allows translocation of NF- κ B into the nucleus and binding to their cognate DNA-binding sites to regulate the transcription of a large number of genes including antimicrobial peptides, cytokines, chemokines, stress response proteins, and anti-apoptotic proteins⁽¹⁴⁾. In other words, NF- κ B plays a critical role in LMP1-mediated signal transduction, involves in the regulation of cell apoptosis, cell cycle and carcinogenic transformation. Although the treatment for some EBV-associated malignancies has improved in recent years, new approaches to therapy are needed.

Polygonum cuspidatum Sieb. et Zucc. (*P. cuspidatum*) is used for the treatment of various inflammatory diseases, hepatitis and tumors in eastern Asian countries⁽¹⁵⁾. It had

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been reported that the methanolic extract of *P. cuspidatum* contains various compounds including piceid, resveratrol, anthraglycoside B, emodin and physcion⁽¹⁶⁾. Our earlier work revealed that the ethanolic extract of *P. cuspidatum* (PcE) inhibited the expression of EBV lytic proteins, including, Rta, Zta and EA-D⁽¹⁷⁾. Moreover, resveratrol, the major component of PcE, has the antiviral activity to inhibit the expression of EBV lytic proteins, including Rta, Zta and EA-D⁽¹⁸⁾. This study found that PcE inhibits the expression of LMP1 and triggers EBV-positive cells toward apoptosis.

MATERIALS AND METHODS

I. Materials

P. cuspidatum was collected from the San-Dei-Men area in Pingtung County, Taiwan and verified by Prof. C. S. Kuoh. The specimen was deposited in the herbarium of the National Cheng Kung University, Tainan, Taiwan.

II. Extraction of *P. cuspidatum*

Ten grams of dried powder from the *P. cuspidatum* root was extracted three times in 100 mL of ethanol by refluxing at 85°C for 2 h. After each extraction, the ethanol fraction was collected by filtration. The ethanol was then removed by rotary evaporation and a yield of 21.6% was obtained. The resulting residues were finally dissolved in dimethyl sulfoxide (DMSO).

III. Cell Culture and Lytic Induction of EBV

B95-8 is a cotton-top tamarin B cell line immortalized by an EBV strain from a patient with infectious mononucleosis. P3HR1 is a Burkitt's lymphoma cell line which is latently infected by EBV, showing no detectable LMP1. These cells were obtained from Prof. S. T. Liu of Chang-Gung University. All cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (Biological Industries, Israel). Cells were treated with 3 mM of sodium butyrate (SB) or 30 ng/mL of TPA to induce the EBV lytic cycle⁽¹⁹⁾.

IV. Cell Viability Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay was used to assess the effects of PcE on B95-8 cell viability. The assay relies on the production of a colored formazan by the action of mitochondrial dehydrogenase on MTT⁽²⁰⁾. Briefly, 0.5 mg/mL solution of MTT in RPMI 1640 medium was added into 1×10^5 /mL B95-8 cells for 4 h at 37°C. The dark blue crystals were dissolved by DMSO. The plates were read on an ELISA reader using a wavelength of 492 nm. Cell survival was calculated as a percentage of MTT inhibition by the following formula: Relative Cell Viability (%) = (Mean Experimental Absorbance/Mean Control Absorbance) \times 100%

V. Immunoblot Analysis

Cell lysate was prepared from 3×10^6 B95-8 cells with 100 μ L of lysis buffer that contained 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and 0.5% NP40 using a method described elsewhere⁽²¹⁾. SDS-polyacrylamide gel electrophoresis and immunoblot analysis using anti-Rta and anti-LMP1 antibodies, which were purchased from Argene (Varilhes, France) and Thermo Scientific (CA, USA), respectively, were performed according to the methods described elsewhere⁽²²⁾. The intensities of the bands were quantitated by scanning densitometry.

VI. Transient Transfection and Luciferase Assay

P3HR1 cells (5×10^6) were transfected with 10 μ g of *TR-L1-Luc* with a Bio-Rad electroporator using the method of Chang *et al.*^(23,24) Cells were harvested at 24 h after transfection, and luciferase activity was determined using a luminometer (Berthold, Germany).

VII. Indirect Immunofluorescence Analysis and DAPI Staining of Apoptotic Cells

B95-8 cells were plated on poly-L-lysine-coated over-slips and fixed with 4% paraformaldehyde at 4°C for 15 min, followed by treatment with PBS containing 0.5% Triton X-100 for 10 min at room temperature. After blocking with 1% BSA in PBS at room temperature for 30 min, monoclonal anti-NF- κ B p65 antibodies (Cell signaling, Beverly, MA) were applied at a dilution of 1 : 50 and incubated at room temperature for 1 h. Next, the cells were washed with PBS and incubated with the CY3-conjugated anti-IgG (Sigma) for 1 h. After counterstaining with 4',6'-diamidino-2-phenylindole (DAPI), the cells were examined under a fluorescence microscope. The cells were stained with DAPI to reveal apoptotic cells. Nuclear morphology was visualized at 400x under a fluorescence microscope.

VIII. Flow Cytometric Analysis

Flow cytometry was performed to quantitatively detect the apoptotic rate. To determine the stages of the cell cycle, the cells after treatment of PcE were harvested, washed and fixed in 70% ethanol overnight at 4°C, and stained with propidium iodide. The sub-G1 peak was measured with a flow cytometer (Model FACScanTO, BD Biosciences) and analyzed using Cell Quest software (BD Biosciences).

IX. Statistical Analysis

Data were analyzed statistically by one-way analysis of variance (ANOVA) using the SAS JMP 6.0 software package. Values were presented as means \pm S.D. and a *p* value of less than 0.05 was regarded as significant.

RESULTS

I. Inhibitory Effect of PcE on the Growth of B95-8 Cells

To determine the effects of PcE on B95-8 cells, which is an EBV-positive B cell line with a type III latency pattern. The cells were treated with various concentrations of PcE for one, three or six days. The viability of the cells was measured by the MTT assay. PcE at the concentration of 100 $\mu\text{g}/\text{mL}$ killed 24% of B95-8 cells after one day of culturing. Three days after treatment, the viability of B95-8 cells decreased when they were treated with PcE (range, 25-100 $\mu\text{g}/\text{mL}$) in a dose-dependent manner. PcE decreased cell viability to 50% (CC_{50}) at 76 $\mu\text{g}/\text{mL}$. PcE exhibited a more cytotoxic effect on B95-8 cells on six days after treatment. These results indicated that the cell viability decreased in dose and time-dependent fashions (Figure 1).

II. Inhibition of the Expression of EBV Encoded Rta and Latent Membrane Protein 1 by PcE

Previous studies showed that PcE inhibited the expression of EBV lytic genes in P3HR1 cells⁽¹⁷⁾. To determine whether PcE inhibited the expression of Rta in B95-8 cells, B95-8 cells ($6 \times 10^5/\text{mL}$) were treated with PcE before lytic induction for 24 h. The results showed that PcE at concentrations of 12.5 and 50 $\mu\text{g}/\text{mL}$ inhibited the expression of Rta by 50 and 90%, respectively (Figure 2A). EBV-encoded LMP1 affected cell proliferation, transformation, anti-apoptosis and immortalization⁽¹³⁾. PcE had a cytotoxic effect towards B95-8 cells and inhibited the expression of Rta. Moreover, it had been documented that the lytic protein, Rta, promoted the transcription of LMP1 TR-L1 promoter and induced the expression of LMP1⁽¹⁰⁾. Therefore, we will elucidate whether PcE affected the expression of LMP1. After treating the

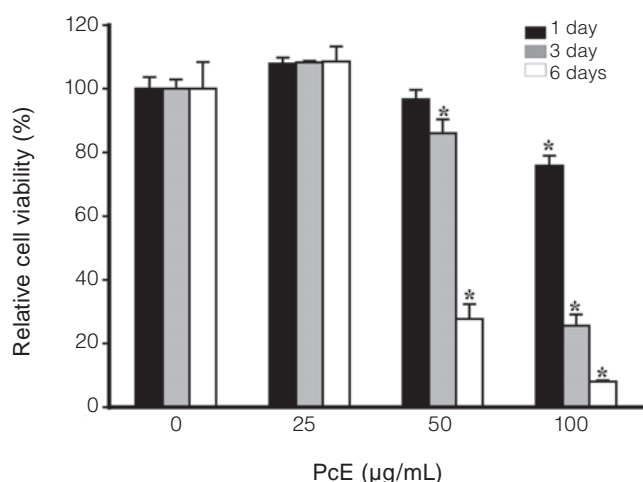


Figure 1. Effect of PcE on the growth of B95-8 cells. The B95-8 cells were treated with PcE for 1, 3 and 6 days. Relative cell viability was determined by MTT assay. The data were presented as mean \pm S.D. from three independent experiments. * $p < 0.05$, which represents a significant difference from the values of the control group.

B95-8 cells ($6 \times 10^5/\text{mL}$) with various concentrations of PcE for 24 h, the expression of LMP1 was performed by immunoblots. The results showed that 50 $\mu\text{g}/\text{mL}$ of PcE reduced the expression of LMP1 by 40%, and 100 $\mu\text{g}/\text{mL}$ of PcE completely inhibited the expression of LMP1 (Figure 2B).

III. Inhibition of the Transcriptional Activity of LMP1 Promoter, TR-L1

Transient transfection was performed to explore whether PcE influenced the transcription of the LMP1 promoter. Reporter plasmid, *TR-L1-Luc*, which contained a firefly luciferase gene transcribed from the LMP1 promoter, TR-L1, were transfected into P3HR1 cells, and then treated with PcE before lytic induction for 24 h. Upon analyzing the luciferase activity exhibited by the cells, it was observed that PcE at 25 $\mu\text{g}/\text{mL}$ suppressed the transcriptional activity of the LMP1 promoter which was induced by sodium butyrate, by 70%, and PcE at at 50 $\mu\text{g}/\text{mL}$ inhibited the transcriptional activity of the LMP1 promoter by 90% (Figure 3).

IV. Translocation of NF- κB Inhibited by PcE

It is well documented that LMP1 can activate the NF- κB signal pathway. NF- κB is a transcriptional factor, which can regulate cell growth and is also an anti-apoptotic protein⁽¹⁴⁾. In this study, we demonstrated that PcE reduced the expression of LMP1. Thus, we will investigate the subcellular location of transactivated p65 subunit at PcE treatment by indirect immunofluorescence assay. An antibody specific to the p65 subunit of NF- κB was detected with high immunoreactivity in the cytoplasm and nucleus. PcE at 50 $\mu\text{g}/\text{mL}$ depleted NF- κB (p65) in the nuclei of B95-8 cells after 24 h

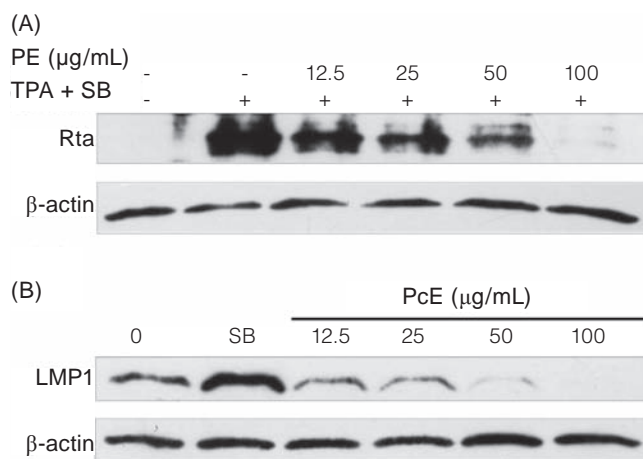


Figure 2. Inhibition of latent membrane protein 1 (LMP1) and lytic protein Rta expression by PcE. For the analysis of EBV lytic protein Rta expression (A), PcE was added to B95-8 cells before lytic induction with TPA (30 ng/mL) and SB (3 mM). After 24 h of culturing, the immunoblotting assay was performed using anti-Rta and β -actin monoclonal antibodies. For LMP1 expression assay (B), B95-8 cells were treated with PcE (A) for 24 h. The immunoblotting assay was performed using anti-LMP1 and β -actin monoclonal antibodies.

of treatment (Figure 4). The results indicated that the p65 subunit was barely detectable in the nuclei following 24 h of incubation with PcE.

V. PcE Induces B95-8 Cells Apoptosis

It has been demonstrated that PcE reduced the expression of LMP1 and depleted the NF- κ B in the nuclei of B95-8 cells. To determine whether PcE induced B95-8 cells apoptosis, B95-8 cells were treated with various concentrations of PcE

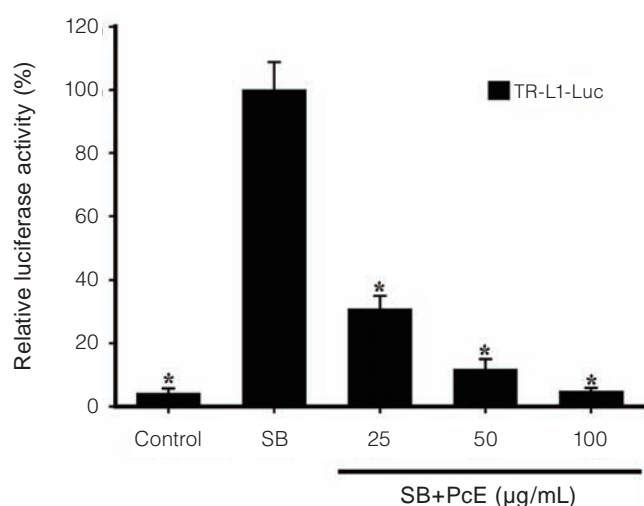


Figure 3. Transcriptional inhibition of LMP1 promoter by PcE in P3HR1 cells. P3HR1 cells were transfected with TR-L1-Luc plasmid, and then treated with sodium butyrate (SB) and PcE (25 - 100 μ g/mL). Luciferase activity was measured by a luminometer at 24 h after treatment. The data were presented as mean \pm S.D. from three independent experiments. * $p < 0.05$, which represents a significant difference from the values of the SB group.

for 72 h. DAPI staining was performed to detect apoptosis. Under fluorescence microscopy, the cells without PcE treatment showed normal spherical nuclei and uniform chromatin distribution, and the cells treated with 50 and 100 μ g/mL PcE exhibited typical features of apoptosis—chromatin condensation and nuclear fragmentation (Figure 5).

Further, we quantified the apoptotic cell after PcE treatment. B95-8 cells were treated with various concentrations of PcE for 72 h, and apoptotic nuclei were identified in the subdiploid region (sub-G1) of the histograms by flow

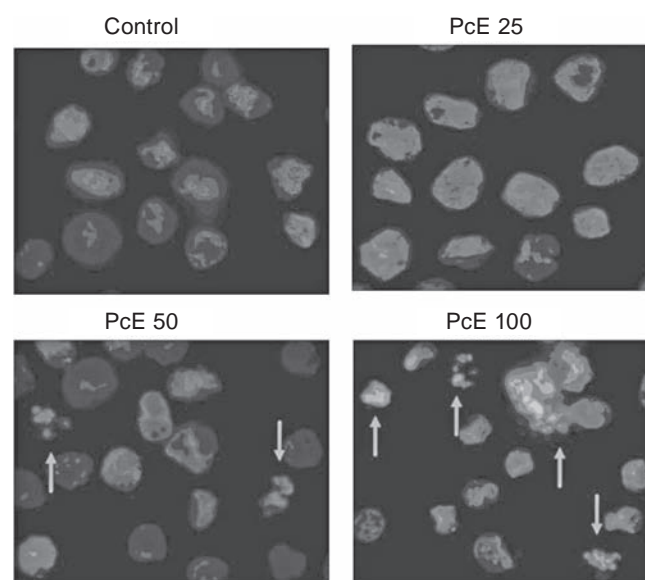


Figure 5. Apoptosis characteristic of B95-8 cells induced by PcE. B95-8 cells were treated with PcE (25-100 μ g/mL) for 3 days. The nuclei were characterized by using DAPI staining and then photographed by fluorescent microscopy.

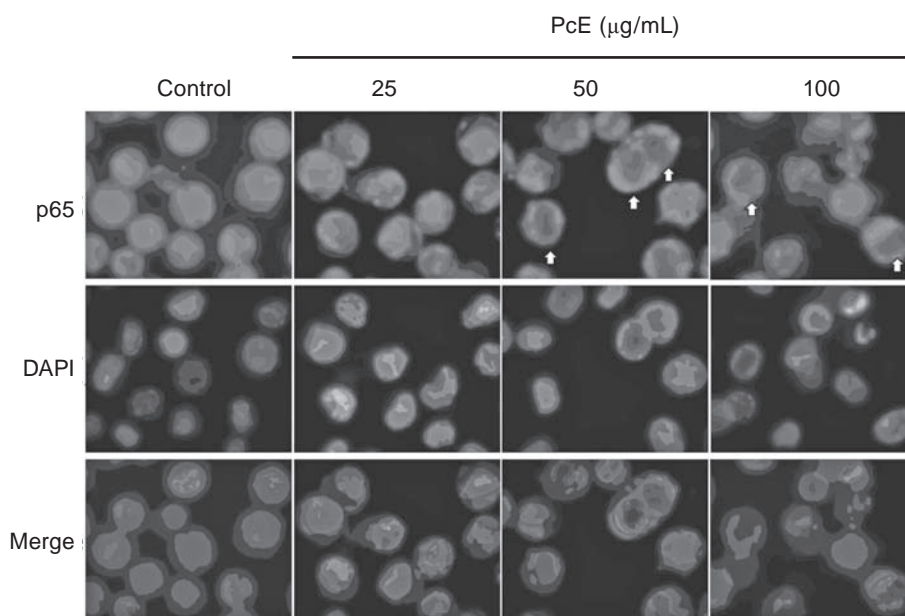


Figure 4. Inhibition of nuclear localization of activated p65. B95-8 cells were treated with PcE (25 - 100 μ g/mL) for 24 h. NF- κ B localization was detected using a fluorescence-labeled anti-NF- κ B p65 antibody and nuclear staining by DAPI.

cytometry using PI staining. The rate of cells undergoing apoptosis (sub-G1) in B95-8 cells treated with 50 and 100 $\mu\text{g}/\text{mL}$ of PcE increased from 2.6 to 12.07% and 15.62%, respectively (Figure 6).

DISCUSSION

It has been well established that the combination of lytic EBV infection and certain antiviral agents can enhance the killing of EBV-positive tumor cells, such as using sodium butyrate^(25,26) or NF- κB inhibitor, aspirin⁽²⁷⁾ and combined with GCV to induce lytic cytotoxicity in EBV-positive cells. On the other hand, interfering with LMP1 expression by siRNA^(28,29), DNazymes⁽³⁰⁾ and arsenic trioxide⁽³¹⁾ have also been demonstrated to induce apoptosis of EBV-positive lymphoma cells and NPC cells. Additionally, Epigallocatechin-3-gallate not only inhibits the growth of nasopharyngeal carcinoma cells, but also blocks LMP1-caused NF- κB activation mediated *via* interfering with the phosphorylation of I $\kappa\text{B}\alpha$ ⁽³²⁾. This study showed that PcE inhibits the expression

of latent membrane protein 1 and triggers EBV-positive cells toward apoptosis.

For B95-8 cells treated with PcE (range, 25-100 $\mu\text{g}/\text{mL}$), the cell viability was decreased in a dose-dependent manner on the third day after treatment. At 50 $\mu\text{g}/\text{mL}$, PcE significantly inhibited the expression of LMP1 and transcriptional activity of LMP1 promoter by decreasing the expression of Rta protein. Furthermore, PcE reduced the translocation of NF- κB from the cytoplasm to the nuclei and promoted chromatin condensation, marginal and nuclear fragmentation. The rate of cells undergoing apoptosis in B95-8 increased to five fold. In other words, PcE depleted NF- κB in the nuclei of B95-8 cells by decreasing the expression of LMP1 and induced apoptosis of EBV-positive cells. In previous studies, we had demonstrated that PcE inhibited the expression of EBV lytic proteins including, Rta, Zta and EA-D and reduced the production of virus particles⁽¹⁷⁾. Therefore, PcE may represent a novel strategy for the treatment of EBV-associated lymphomas.

Moreover, PcE contains resveratrol and emodin⁽¹⁷⁾. Previous studies have documented that resveratrol has

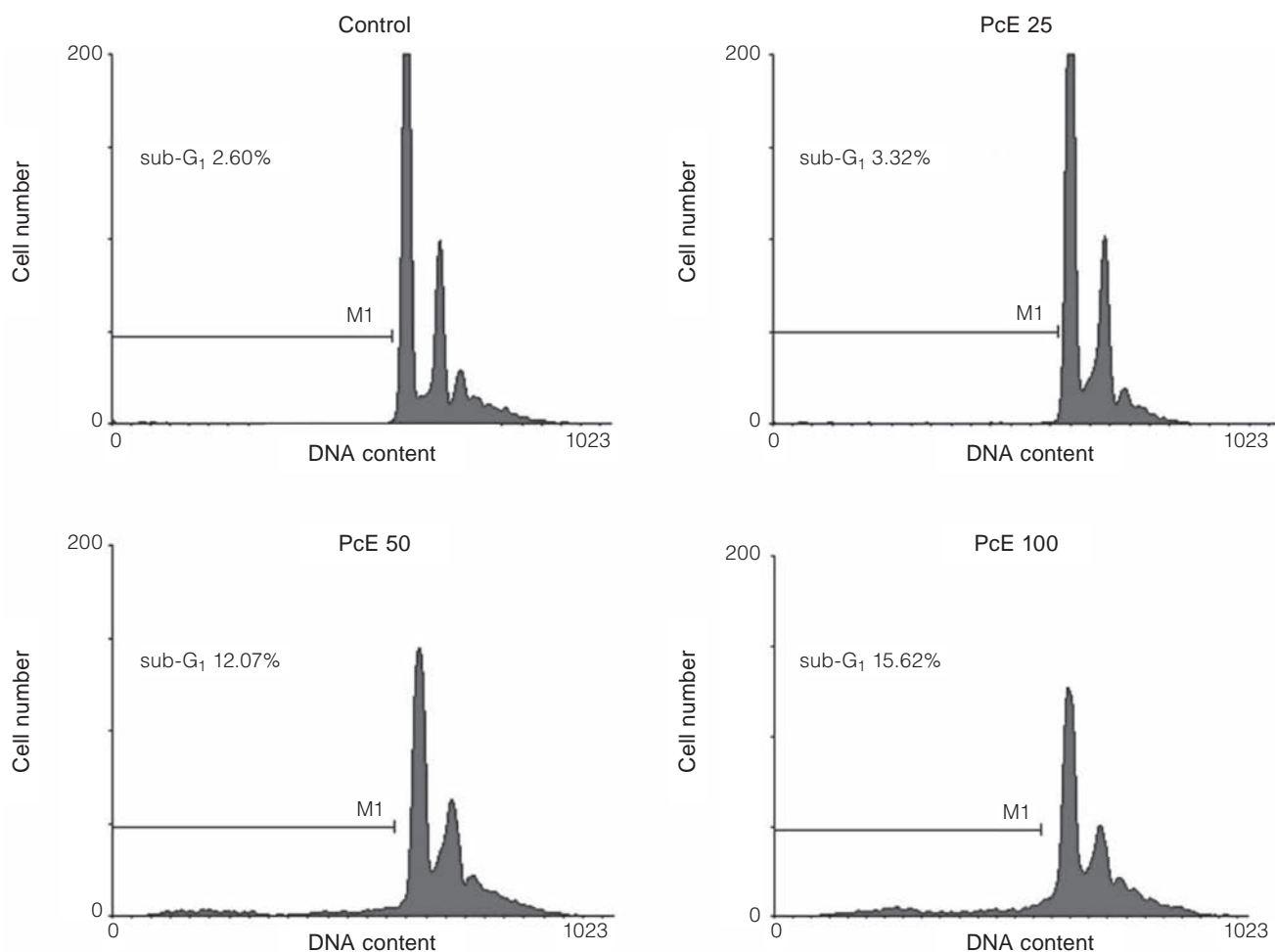


Figure 6. Cell cycle distributions of B95-8 cells were analyzed by flow cytometric analysis. The cells were treated with PcE for 3 days, and then stained with PI solution to assess the cell cycle distribution pattern. The percentage of sub-G1 phase over total cells was calculated and expressed on each histogram.

the antiviral activity to inhibit the expression of EBV lytic proteins, including Rta, Zta and EA-D⁽¹⁸⁾. It also inhibits the DNA replication of the human cytomegalovirus⁽³³⁾ and herpes simplex virus⁽³⁴⁾, and promotes the apoptosis of human oral epidermoid carcinoma KB cells⁽³⁵⁾ and Burkitt's lymphoma cells⁽³⁶⁾ by blocking NF- κ B transcription factor activation. Emodin induces the apoptosis of human lung adenocarcinoma cells⁽³⁷⁾, gallbladder cancer cells⁽³⁸⁾, prostate cancer cells⁽³⁹⁾ and hepatocellular carcinoma cells⁽⁴⁰⁾ through reactive oxygen species (ROS) production. Therefore, we propose that the major component, resveratrol, may be involved in PcE-induced apoptosis of B95-8 cells through decreasing the expression of LMP1 and depleting NF- κ B in nuclei of B95-8 cells. Besides, emodin may have participated in PcE and promoted the B95-8 cells apoptosis mediated *via* the generation of ROS.

CONCLUSIONS

This study demonstrated that PcE inhibited the expression of latent membrane protein 1 and induced the apoptosis of B95-8 cells. The result indicated that PcE may be a useful therapeutic drug for EBV-positive lymphoma cells.

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