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Inhibitory Effects of *Polygonum cuspidatum* on the Epstein-Barr Virus Lytic Cycle

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ABSTRACT

Polygonum cuspidatum is widely used as a medicinal herb in Asia. In this study, we examined the ethanolic extract of *Polygonum cuspidatum* roots (PcE) for their capacity to inhibit the EBV lytic cycle. The cell viability was determined by the MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method. The expression of Epstein-Barr virus (EBV) lytic proteins was analyzed by flow cytometric assay. Real-time quantitative PCR was used to measure the EBV virion production. Transient transfection analysis was subsequently performed to assess the transcription of lytic genes, including BRLF1 and BZLF1. Results showed that the PcE inhibited the transcription of EBV immediate early genes, the expression of EBV lytic proteins, including Rta, Zta, and EA-D and reduced the production of virus particles, showing that PcE is useful for preventing the proliferation of the virus.

Key words: *Polygonum cuspidatum*, antiviral activity, EBV

INTRODUCTION

Epstein-Barr virus (EBV) is a gammaherpesvirus, which infects human lymphoid cells and epithelial cells[(1)]. Infection by this virus is associated with a number of human cancers, including Burkitt's lymphoma[(2)], nasopharyngeal carcinoma (NPC)[(3)] and Hodgkin's disease[(4)]. The reactivation of EBV from latency to the lytic cycle is necessary for the virus to produce virions to establish infections[(5,6)]. At the onset of the lytic cycle, EBV expresses two transcription factors, Rta and Zta, which are transcribed from BZLF1 and BRLF1, respectively[(7)]. These two proteins trigger an ordered cascade of the expression of viral lytic genes, including that of BMRF1 and BALF5, which encoded diffused early antigen (EA-D) and DNA polymerase[(8)]. Rta and Zta can also activate IL-6 transcription in lytically-infected B cells which leads to immortalized B cells[(9)]. Therefore, an effective strategy to block the viral lytic cycle is of value to reduce the risk of the disease and to improve the clinical outcome. Earlier studies have established that lytic EBV replication is inhibited by acyclovir and ganciclovir, which inhibit specifically the function of viral-encoded DNA polymerase[(10)]. An earlier study showed that epigallocatechin gallate (EGCG) inhibits the expression of the EBV immediate-early genes transcription at a concentration of 50 μM[(11)]. Ethanolic extract from *Andrographis paniculata* and andrographolide also inhibit the expression of Rta, Zta and EA-D at 25 μg/mL and 5 μg/mL, respectively[(12)].

Polygonum cuspidatum is an herbal medicine, which is commonly used for the treatment of atherosclerosis, as well as other medical ailments including cancers, asthma, hypertension and cough[(13)]. The methanolic extract of *P. cuspidatum* roots contains anthraglycoside B, physcion, piceid, emodin and resveratrol[(14)]**.** Previous studies showed that the ethanolic extract of *P. cuspidatum* inhibits hepatitis B virus replication[(15)]. In addition, resveratrol, the major compound of *P. cuspidatum*, showed antiviral ability against human cytomegalovirus (HCMV)[(16)], herpes simplex virus type 1 (HSV-1)[(17)], influenza virus[(18)], vaccinia virus[(19)], EBV[(20)] and Varicella-zoster virus (VZV)[(21)]. Furthermore, resveratrol synergistically enhances the anti-HIV-1 activity of the nucleoside analogues zidovudine (AZT), zalcitabine (ddC) and didanosine (ddI)[(22)]. Emodin has an inhibitory effect on HBV[(23)] and HSV[(24)] replication. Moreover, emodin blocks the SARS coronavirus spike protein and angiotensin-converting enzyme 2 interaction[(25)]. However, no study

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has been reported on the inhibition of EBV lytic cycle by *P. cuspidatum*. Thus, our study is to evaluate the ethanolic extract of *P. cuspidatum* roots (PcE) on the inhibition of the transcription of EBV immediate early genes, the expression of EBV lytic proteins, including Rta, Zta, and EA-D and virion production.

MATERIALS AND METHODS

I. *Material*

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 DMSO.

III. *HPLC Analysis*

The components in PcE were analyzed by high performance liquid chromatography (HPLC) using a LiChrospher 100 RP-18e (250 × 4.6 mm i.d., 5 μm) column (Merck, Darmstadt, Germany). The mobile phase consisted of (A) water and (B) methanol. Gradient elution was performed as follows: 30 - 50% B in 0 - 15 min, 50 - 90% B in 15 - 35 min, and 95% B in 35 - 45 min. The flow rate was set at 1 mL/min. The effluent was monitored from 210 to 500 nm by a diode array detector.

IV. *Cell Culture and Lytic Induction of EBV*

P3HR1, a Burkitt's lymphoma cell line which was latently infected by EBV, was cultured in RPMI 1640 medium containing 10% fetal calf serum (Biological Industries, Israel). Cells were treated with 3 mM of sodium butyrate (SB) to induce the EBV lytic cycle[26].

V. *Cell Viability Assay*

A 1 mg/mL solution of [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) in RPMI 1640 medium was added into 1×10^5 P3HR1 cells. The dehydrogenase activity of the viable cells was measured using the method of Carmichael *et al*[27].

VI. *Flow Cytometric Analysis*

Flow Cytometric Analysis was performed as described[20], using mouse monoclonal anti-Rta, anti-Zta (Argene, Varilhes, France), and anti-EA-D antibodies (Chemicon, Temecula, CA, USA). Secondary antibodies used in the study included Alexa Fluor 488-conjugated goat anti-mouse IgG from Invitrogen. Finally, the cells were resuspended in 1% paraformaldehyde and analyzed using a flow cytometer (Model FACScanTO, BD Biosciences)

VII. *Transient Transfection and Luciferase Assays*

P3HR1 cells (5×10^6) were transfected with 10 μg of pRLUC and pZLUC with a Bio-Rad electroporator using the method of Chang *et al*[28,29]. The cells were harvested at 24 h after transfection, and luciferase activity was determined using a luminometer (Berthold, Germany)[28].

VIII. *Quantification of EBV Particles Produced by P3HR1 Cells*

P3HR1 cells were cultured for 5 days. EBV particles released into the culture medium were harvested by centrifugation. Viral DNA was extracted and the amount of EBV DNA was determined by real-time PCR using an iCycleriQ multicolor real-time PCR detection system (BioRad, CA, USA) with primer and a probe that were specific to the EBNA1 gene[30]. The copy number of the EBV genome was calculated using maxi-EBV DNA extracted from *E. coli* as a reference. The molecular weight of maxi-EBV is about 1.2×10^7 and 1 ng of maxi-EBV equals to 5.05×10^6 copies of the maxi-EBV genome.

IX. *Statistical Analysis*

Data were analyzed statistically by one-way analysis of variance (ANOVA) using the SAS JMP 6.0 software package. Data are presented as means ± SD and a p value of < 0.05 was regarded as significant.

RESULTS

I. *Identification of Resveratrol and Emodin in PcE*

The PcE was analyzed by HPLC and the identification of the target compounds in PcE (resveratrol and emodin) was based on retention time and UV spectra, compared against those of the pure standards of resveratrol and emodin. The peak eluting at 10.99 min and 33.01 min were ascribed to resveratrol and emodin, respectively (Figure 1).

II. *Determining the Toxicity of PcE to P3HR1 Cells*

PcE was added into 1×10^5 cells/mL. After 24 h of treatment, cell viability was determined by MTT assay. The results showed that PcE affected the viability of P3HR1 cells in a dose-dependent manner. PcE decreased cell viability to 50% (CC_{50}) at 84.56 μg/mL (Figure 2).

Journal of Food and Drug Analysis, Vol. 19, No. 2, 2011

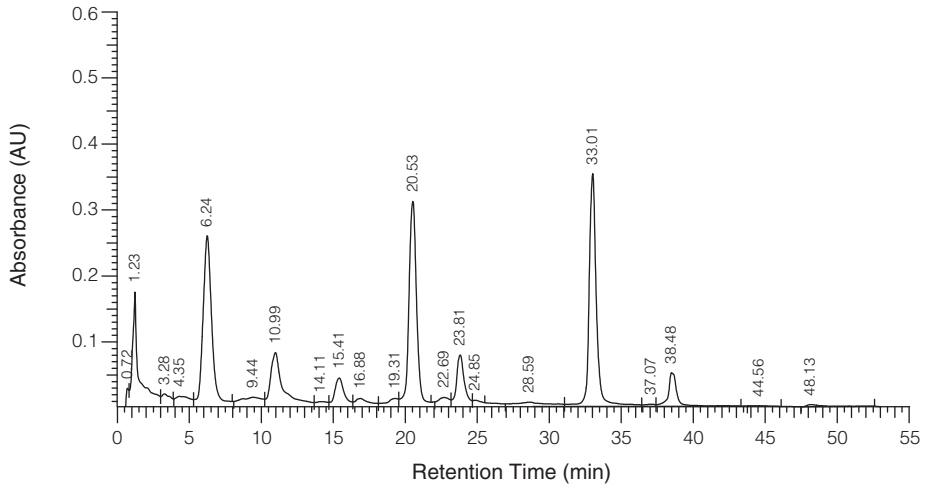

Figure 1. Chromatogram of the ethanolic extract of *Polygonum cuspidatum* root (PcE) by HPLC. Mobile phase: methanol-water (methanol: 0-15 min, 30-50%; 15-35 min, 50-90%; 35-45 min, 95%); flow rate: 1.0 mL/min; detection wavelength: 280 nm.

III. *Flow Cytometric Analysis of the Expression of EBV Lytic Proteins*

The presence of EBV lytic proteins in P3HR1 cells was further analyzed by flow cytometric analysis. The P3HR1 population that was untreated with SB expressed Rta, Zta and EA-D was 2.9%, 2.9% and 4.8%, respectively (Figure 3A). The population that expressed three proteins after SB treatment increased to 49.2%, 51.4% and 43%, respectively. When 12.5 μg/mL of PcE was added before lytic induction, the population that expressed Rta, Zta and EA-D decreased to 34.5%, 29.4% and 38.6%, respectively (Figure 3A). The population that expressed Rta, Zta, and EA-D further decreased when the concentration of PcE increased to 25 and 50 μg/mL. At 50 μg/mL, the population that expressed Rta, Zta, and EA-D decreased to 7.8%, 7.3%, and 1%, respectively (Figure 3A). These results showed that PcE inhibited the expression of Rta, Zta and EA-D in a dose-dependent manner (Figure 3B). The concentration of PcE required to inhibit EBV immediate-early proteins expression by 50% (EC_{50}) is approximately 29 μg/mL.

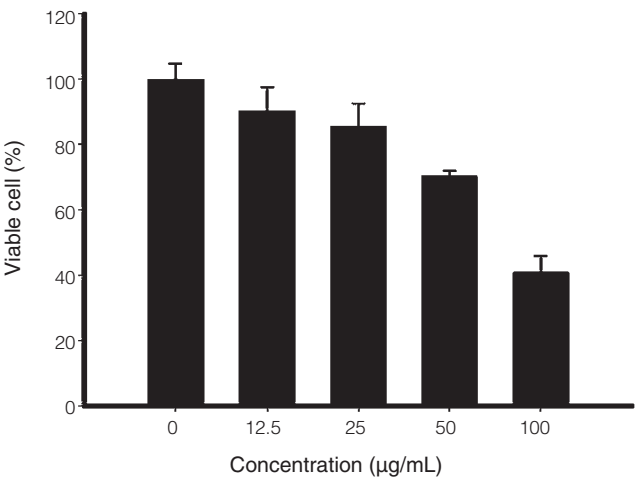

Figure 2. Effect of PcE on cell viability. P3HR1 cells (1×10^5 cell/mL) were treated with PcE. Cell numbers and viability were monitored by MTT assay after 24 h of treatment. The data are presented as mean ± SD from three independent experiments. At 50 and 100 μg/mL, $p < 0.05$ *vs.* control.

IV. *Inhibiting the Transcription of EBV Immediate-Early Genes*

The activity of the BRLF1 and BZLF1 promoters was analyzed by transient transfection assay in P3HR1 cells using reporter plasmids, pRLUC and pZLUC, respectively. The results showed that 12.5, 25 and 50 μg/mL of PcE inhibited the BRLF1 promoter activity (35%, 54% and 77%, respectively). At these concentrations, PcE also inhibited BZLF1 promoter activity (45%, 63% and 70%, respectively) (Figure 4).

V. *Inhibition of the Production of EBV Particles*

P3HR1 cells were treated with 12.5 - 50 μg/mL of PcE before lytic induction. After culturing for five days, EBV particles released into the medium were isolated. Real-time qPCR was used to determine the amount of EBV DNA purified from the viral particles. The results showed that PcE at a concentration of 25 μg/mL decreased virus production by 76% (Figure 5). The effective concentration of PcE that inhibited EBV genome copy numbers by 50% (EC_{50}) is 17.9 μg/mL.

DISCUSSION

Previous studies showed that lytic EBV proteins actually induce the expression of B-cell growth factor, IL-6, cellular IL-10 and viral IL-10, allowing B cells to grow

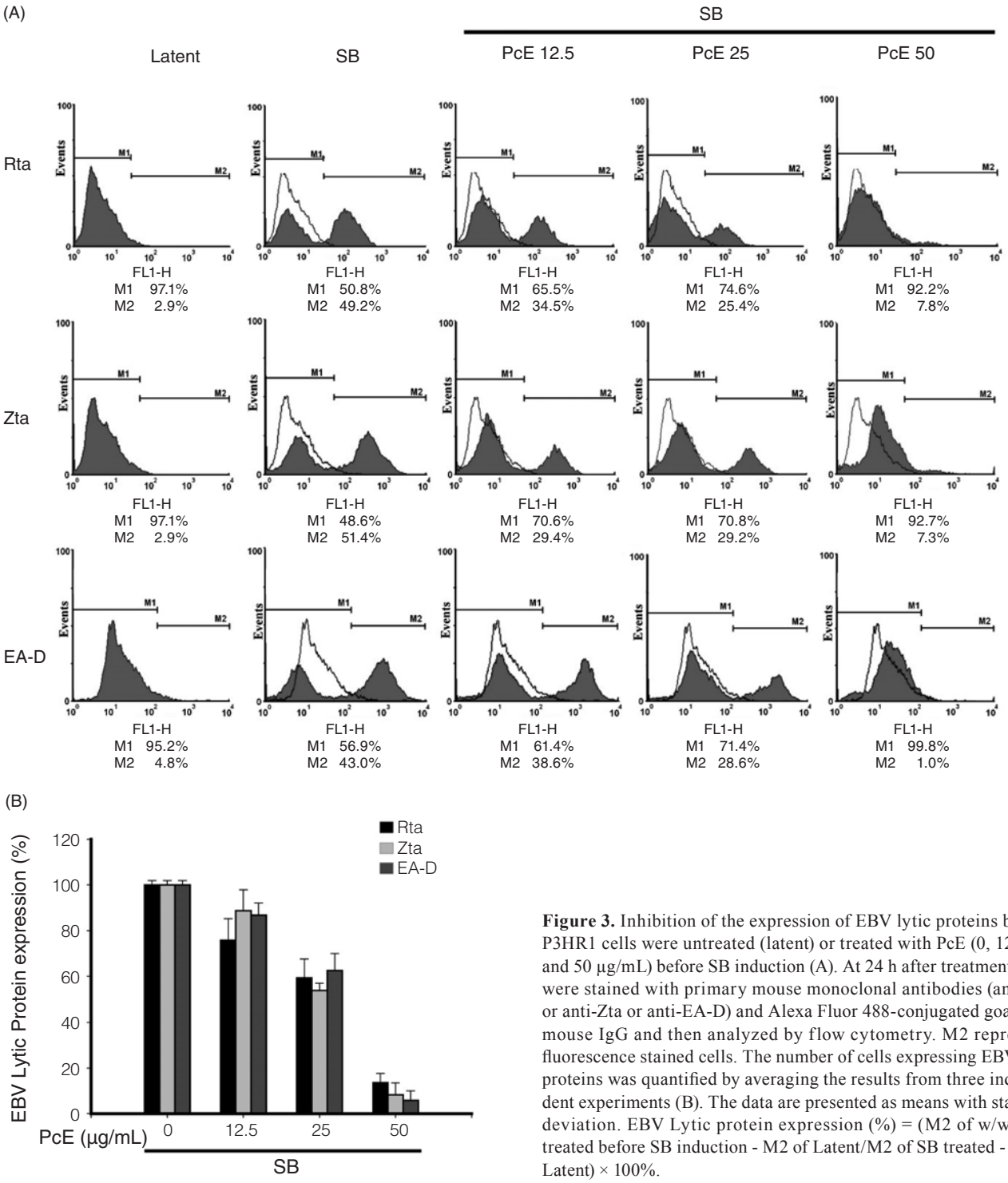

Figure 3. Inhibition of the expression of EBV lytic proteins byPcE. P3HR1 cells were untreated (latent) or treated with PcE (0, 12.5, 25 and 50 μg/mL) before SB induction (A). At 24 h after treatment, cells were stained with primary mouse monoclonal antibodies (anti-Rta or anti-Zta or anti-EA-D) and Alexa Fluor 488-conjugated goat anti-mouse IgG and then analyzed by flow cytometry. M2 represents fluorescence stained cells. The number of cells expressing EBV lytic proteins was quantified by averaging the results from three independent experiments (B). The data are presented as means with standard deviation. EBV Lytic protein expression (%) = (M2 of w/wo PcE treated before SB induction - M2 of Latent/M2 of SB treated - M2 of Latent) × 100%.

more efficiently[31,32]. Lytically-infected cells also produce VEGF and thus contribute to angiogenesis in both B-cell and epithelial-cell malignancies[32]. Therefore, new treatment strategies aimed at completely suppressing the expression of all lytic viral proteins are useful in controlling early EBV-associated malignancies.

In this study, it was found that PcE significantly reduces the expression of EBV immediate-early proteins, Rta, Zta and EA-D in a dose-dependent manner (Figure 3). In other words, PcE interferes with an early step of the EBV replication cycle. The concentration of PcE required to inhibit EBV immediate-early proteins expression by 50% (EC_{50}) is approximately 29 μg/mL. Moreover, PcE exhibits cytotoxicity in P3HR1 cells and the CC_{50} is 84.56 μg/mL. Our study also demonstrates that PcE inhibits the transcriptional activity of BRLF1 and BZLF1 promoters, which in turn,

Journal of Food and Drug Analysis, Vol. 19, No. 2, 2011

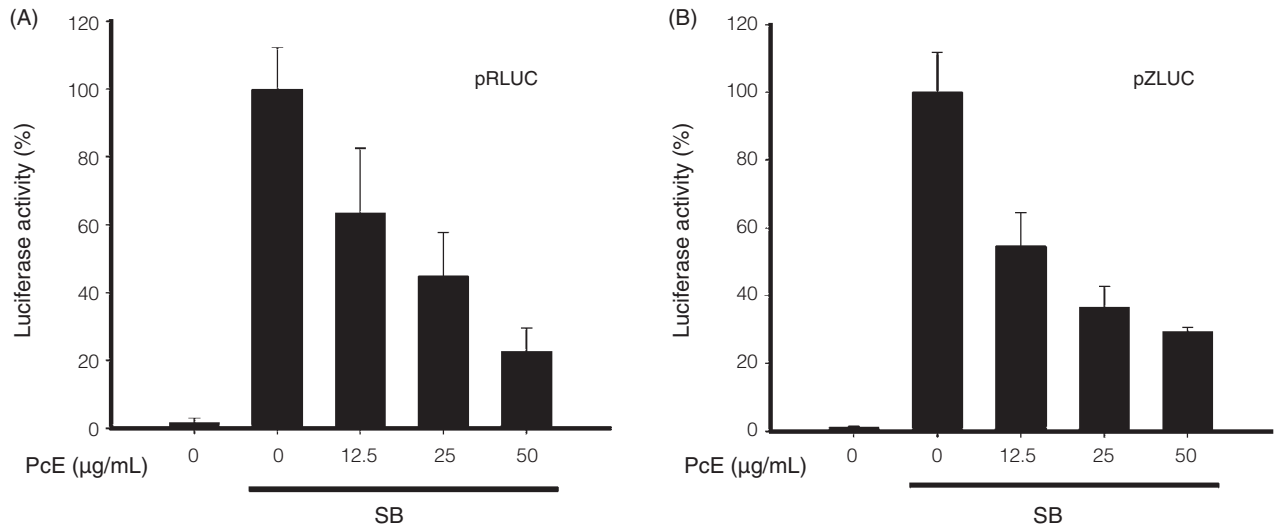

Figure 4. Inhibition of the transcription of the BRLF1 and BZLF1 promoters by PcE. P3HR1 cells were transfected with pRLUC or pZLUC and treated with PcE before lytic induction. At 24 h after transfection, luciferase activity was measured using a luminometer. Each transfection experiment was performed three times, and each sample in the experiment was prepared in duplicate.

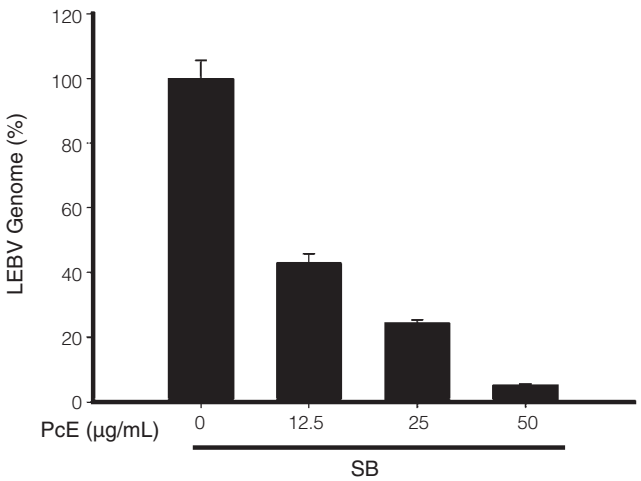

Figure 5. Effect of PcE on EBV particles production. P3HR1 cells were treated with PcE in the presence of sodium butyrate (SB). Five days after the treatment, EBV particles were harvested by centrifugation. The amounts of EBV DNA isolated from the particles were determined by real-time qPCR. The copy number of EBV genome was calculated by using maxi-EBV. EBV Genome (%) = Log of copy number (PcE treated before SB induction – PcE and SB untreated) / log of copy number (SB treated only – PcE and SB untreated) × 100%. The data are presented as mean ± SD from at least three independent experiments.

affects viral lytic proteins expression. In addition, the inhibition actually decreases the production of mature viral particle. Real-time qPCR indicates that the effective concentration of PcE that inhibits EBV genome copy numbers by 50% (EC_{50}) is 17.9 μg/mL (Figure 5), compared to that obtained by flow cytometry (29 μg/mL). Besides Rta and Zta proteins, the activation of EBV lytic cycle requires other factors, such as the MBD1-containing chromatin-associated factor 1 (MCAF1) [33]. These data implied that PcE may inhibit other lytic genes involved in EBV lytic cycle. However, the inhibitory profile remains unchanged, indicating that flow cytometry is as reliable as the real-time qPCR method for initial drug screening.

The molecular mechanism underlying the inhibition of EBV early gene expression by PcE is unclear. Results showed that PcE contains resveratrol and emodin (Figure 1). Previous studies showed that emodin and resveratrol inhibit the activation of p38 MAPK, ERK and JNK signaling and affect the activation of the promoters that are activated by AP-1 or ATF2[34], as both the BRLF1 and BZLF1 promoters are strongly activated by AP-1 and ATF2[35-37]. The transcriptional inhibition of BRLF1 and BZLF1 promoters may be due to resveratrol that activates the deacetylase activity of sirtuin protein[20]. Therefore, we suggest that emodin and resveratrol may be involved in the inhibition of PcE in the EBV lytic cycle.

CONCLUSIONS

In this study, the results clearly demonstrated that PcE inhibits the transcription of lytic genes and the lytic cycle of EBV to reduce the production of viral particles. PcE could potentially be used for the development of anti-EBV drugs.

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