Inhibition of the Epstein–Barr Virus Lytic Cycle by Andrographolide

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***Andrographis paniculata* Nees is a medicinal plant that is commonly used in Asia. This work demonstrates that 25 μg/ml of ethanolic extract from *A. paniculata* (EEAP) and 5 μg/ml of andrographolide, a bioactive compound in EEAP, effectively inhibit the expression of Epstein–Barr virus (EBV) lytic proteins, Rta, Zta and EA-D, during the viral lytic cycle in P3HR1 cells. Transient transfection analysis revealed that the lack of expression of Rta, Zta and EA-D is caused by the inhibition of the transcription of BRLF1 and BZLF1, two EBV immediate-early genes that encode Rta and Zta, respectively. This study finds that the inhibition prevents the virus from producing mature viral particles. Meanwhile, andrographolide is not toxic to P3HR1 cells when the concentration is below 5 μg/ml, indicating that the compound is potentially useful as an anti-EBV drug.**

Key words Epstein–Barr virus; antiviral activity; andrographolide; *Andrographis paniculata*

The Epstein–Barr virus (EBV) is a human herpesvirus that infects lymphoid and epithelial cells.[1)] Infection by this virus causes infectious mononucleosis and is closely related to several malignant diseases, including Burkitt's lymphoma,[2)] T-cell lymphoma,[3)] Hodgkin's disease,[4)] gastric cancer[5)] and nasopharyngeal carcinoma.[6)] After infecting B lymphocytes, the virus is typically latent.[7)] However, the virus has to enter a lytic cycle to proliferate.[8)] At the onset stage of the EBV lytic cycle, the virus expresses two immediate-early genes, BRLF1 and BZLF1, which encode transcription factors, Rta and Zta, respectively.[9)] These two transcription factors often cooperate or act alone to activate various sets of lytic genes, including BMRF1 and BALF5, which encode diffused early antigen (EA-D) and DNA polymerase,[10)] respectively. Without Rta or Zta, the virus cannot complete its lytic cycle.[11)] As it is generally known, acyclovir and ganciclovir, two nucleoside analogs that specifically inhibit the function of herpesvirus DNA polymerases, have been used clinically to treat herpes infections.[12)] Compounds isolated from *Artemisia lactiflora*, *Camelia sinesis*, *Glycyrrhiza radix*[13)] and *Oryzae sativa* also inhibit the EBV lytic cycle.[14—17)] Of these compounds, epigallocatechin gallate (EGCG) from green tea, *C. sinesis*, has been shown to inhibit the transcription of BRLF1 and BZLF1.[15)]

Andrographis paniculata is a medicinal plant that is commonly used to treat a range of illnesses, including bacterial infections, inflammations and high blood pressure. Earlier works have identified many important ingredients in the plant, including diterpenes, flavonoids and stigamasterols. One of the diterpenoids, andrographolide, is particularly important because of its anti-inflammatory,[18)] antithrombotic,[19)] anticancer[20)] and immunostimulatory activities.[21)] Furthermore, andrographolide exhibits a neutralizing activity against the human immunodeficiency virus (HIV)[22,23)] and herpes simple virus 1 (HSV-1).[24)] This investigation demonstrates that the ethanolic extract from *A. paniculata* (EEAP) and andrographolide inhibit the transcription of EBV immediate-early genes and the production of EBV virions. Andrographolide is potentially useful as an anti-EBV drug.

MATERIALS AND METHODS

Materials *A. paniculata* was collected from the San Dei Men area in the Pingtung County, Taiwan and verified by Prof. C. S. Kuoh. The specimen of this plant is deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan. Andrographolide (Aldrich Chemical Company) was dissolved in dimethylsulfoxide (DMSO) before use.

Extraction of *A. paniculata* Ten grams of dried powder from the aerial parts of *A. paniculata* was extracted three times in 100 ml of ethanol by refluxing at 85 °C for 2 h. Following each extraction, the ethanol fraction was collected by filtration. The ethanol was then removed by rotary evaporation and yield was 0.53%. The resulting residues were dissolved in DMSO for *in vitro* studies.

Cell Culture and EBV Lytic Induction P3HR1 cells, a Burkitt's lymphoma cell line that is latently infected by EBV, were cultured in RPMI 1640 medium that was supplemented with 10% fetal calf serum (Biological Industries, Israel). After culturing for 24 h, the cells were treated with EEAP and andrographolide. After incubating for 1 h, cells were treated with 30 ng/ml of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 3 mM of sodium butyrate (SB) to induce the EBV lytic cycle.[25,26)]

Analysis of Expression of EBV Lytic Proteins Cell lysate was prepared from 3×10^6 P3HR1 cells with 100 μl 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.[15)] SDS-polyacrylamide gel electrophoresis and immunoblot analysis with anti-Rta, anti-Zta and anti-EA-D antibodies, which were purchased from Argene (Varilhes, France), were performed using methods described previously.[27)]

Indirect Immunofluorescence Analysis P3HR1 cells were plated on poly-L-lysine-coated overslips and fixed with 4% paraformaldehyde at 4 °C for 15 min, followed by treatment with PBS containing with 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-Rta,

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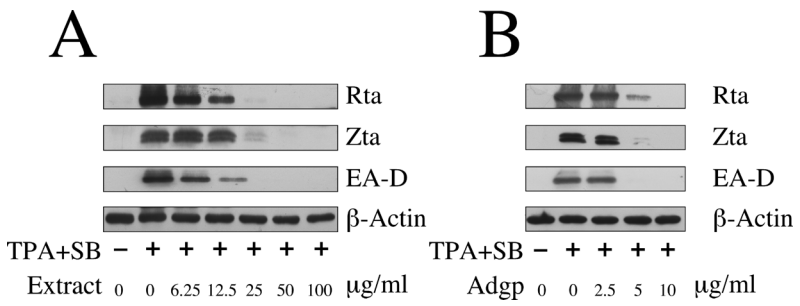

Fig. 1. Inhibition of Rta, Zta and EA-D Expression by EEAP and Andrographolide

EEAP (Extract) (A) or andrographolide (Adgp) (B) was added to P3HR1 cells 1 h before lytic induction with TPA and sodium butyrate (SB). Syntheses of Rta, Zta and EA-D by EBV was studied by immunoblot analysis at 24 h after lytic induction with anti-Rta, anti-Zta, anti-EA-D and anti-β-actin antibodies.

anti-Zta, and anti-EA-D antibodies were applied at a dilution of 1 : 200 and incubated at room temperature for 1 h. Next, the cells were washed with PBS and incubated with the FITC-conjugated goat anti-mouse IgG for 1 h. After counterstaining with 4′,6′-diamidio-2-phenylindole (DAPI), the cells were examined under a fluorescence microscope.

Flow Cytometry Analysis P3HR1 cells were treated with antibodies as described for indirect immunofluorescence analysis, except that the cells were fixed with 4% paraformaldehyde. Finally, cells were resuspended in 1% paraformaldehyde and analyzed with a flow cytometer (model FACScanTO, BD Biosciences).

Transient Transfection Assay 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] Lysate was prepared at 24 h after transfection and luciferase activity was determined using a luminometer (Berthold, Germany) according to a method described elsewhere.[28]

Determining the Number of EBV Particles Formed by P3HR1 Cells After lytic induction, P3HR1 cells were cultured for 5 d. EBV particles released into the culture medium were harvested by treatment with polyethylene glycol (PEG) 6000 and centrifugation at $9400\times\boldsymbol{g}$ for 1 h. The pellet was treated with DNase I and proteinase K, before being extracted with phenol. EBV in the aqueous fraction was precipitated with ethanol and the amount of EBV DNA was determined by real-time PCR, using the method of Chiu *et al.*[30]

Cell Viability Test A 5 mg/ml 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) solution in RPMI 1640 medium was added into 6×10^5 P3HR1 cells. The dehydrogenase activity of the viable cells was measured using the method of Carmichael *et al.*[31]

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±S.D. and a p value of <0.05 was regarded as significant.

RESULTS

Inhibition of the Expression of EBV Lytic Proteins P3HR1 cells (3×10^6) were treated with TPA and SB for 24 h to induce the EBV lytic cycle. As expected, immunoblot analysis revealed that three EBV lytic proteins, Rta, Zta and EA-D, were expressed after lytic induction (Figs. 1A, B). Meanwhile, adding 6.25 μg/ml of EEAP prior to lytic induction had little effect on the synthesis of these three proteins (Fig. 1A). However, the synthesis of Rta and EA-D was partially inhibited by 12.5 μg/ml of EEAP (Fig. 1A), although that of Zta was unaffected at this concentration (Fig. 1A). At 25 μg/ml, EEAP significantly reduced the expression of Zta and completely suppressed the expression of Rta and EA-D (Fig. 1A); at 50 μg/ml, the EEAP completely inhibited the expression of Zta (Fig. 1A). Meanwhile, 5 μg/ml of andrographolide significantly decreased the expression of Rta and entirely suppressed the expression of Zta and EA-D (Fig. 1B). None of these three proteins was expressed after 10 μg/ml of andrographolide was added to the culture medium (Fig. 1B). These results indicated that EEAP and andrographolide inhibit the expression of EBV lytic proteins.

Indirect Immunofluorescence Analysis of EBV Proteins Expression The inhibitory effects of EEAP and andrographolide on the expression of EBV proteins were also assessed by indirect immunofluorescence analysis. The staining results showed that Rta, Zta and EA-D were expressed at 24 h after TPA and SB treatment (Fig. 2). The treatment with 25 μg/ml EEAP or 5 μg/ml andrographolide prior to lytic induction significantly inhibited the expression of these three proteins at 24 h after treatment (Fig. 2).

Quantitative Flow Cytometry Analysis of the Inhibitory Effects of EEAP and Andrographolide on EBV Proteins Expression The presence of lytic proteins was further confirmed by flow cytometry analysis. The population of P3HR1 cells that expressed Rta, Zta and EA-D was 52.8%, 60. 8%, and 53.5%, respectively, following TPA and SB treatment (Fig. 3A). Adding 25 μg/ml EEAP before lytic induction decreased the population that expressed Rta, Zta and EA-D decreased to 21.1%, 27.1% and 8.6%, respectively (Fig. 3A); treating with 2.5 μg/ml andrographolide, 18.6%, 22.5% and 15.5%, respectively. Moreover, treating the cells with 5 μg/ml andrographolide decreased the expression to 1.2%, 3.1% and 0.3%, respectively (Fig. 3B). The expression of these proteins was undetected if the cells were treated with 25 μg/ml EEAP or 5 μg/ml andrographolide.

Inhibition of the Transcription of EBV Immediate-Early Genes Transient transfection assay was performed to determine whether EEAP and andrographolide influenced the transcription of BRLF1 and BZLF1. Reporter plasmids, pRLUC and pZLUC, which contained a firefly luciferase gene transcribed from the BRLF1 and BZLF1 promoter, were transfected into P3HR1 cells after EBV lytic induction. Analyzing the luciferase activity exhibited by the cells indicated that adding 12.5 μg/ml of the EEAP into the culture medium inhibited the activity of the BRLF1 promoter by 58%. However, the same concentration of the extract did not

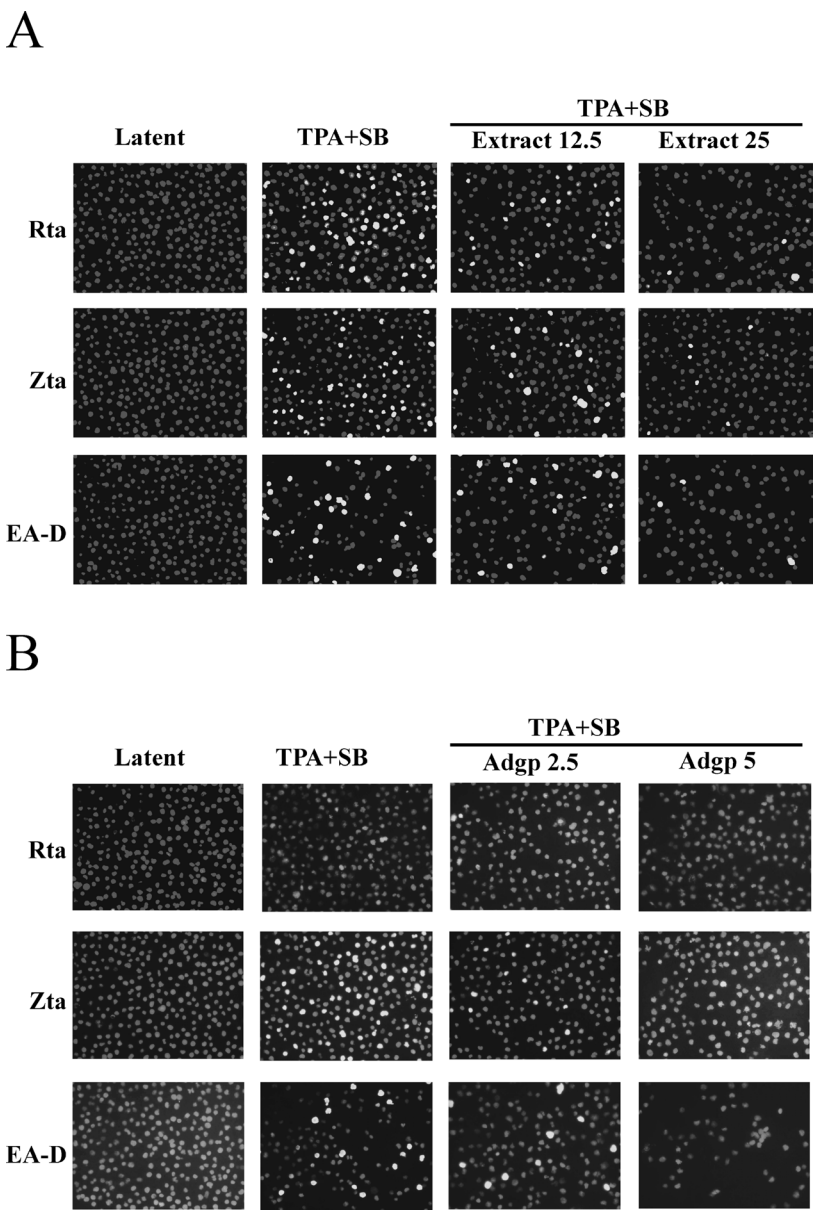

Fig. 2. Indirect Immunofluorescence Analysis of the Inhibitory Effects of EEAP and Andrographolide on the Expression of EBV Lytic Genes

P3HR1 cells were treated with TPA and SB to stimulate the expression of lytic genes, including Rta, Zta, and EA-D. Cells untreated with TPA and SB (Latent) were used as a negative control. Cells treated with TPA and SB were also pre-treated with 12.5 and 25 μg/ml EEAP (Extract-12.5 and Extract-25) (A) or 2.5 and 5 μg/ml andrographolide (Adgp-2.5, Adgp-5) (B) to examine the inhibitory effects of EEAP and andrographolide on the expression of EBV lytic proteins. At 24 h after lytic induction, proteins expressed by the cells were detected by indirect immunofluorescence analysis using anti-Rta, Zta, and EA-D antibodies.

influence the activity of the BZLF1 promoter (Fig. 4). At 25 μg/ml, the EEAP inhibited the activity of the BRLF1 and BZLF1 promoter by 79% and 84% respectively (Fig. 4). Meanwhile, andrographolide at 5 μg/ml inhibited the activity of the BRLF1 and BZLF1 promoter by 62% and 92%; at 2.5 μg/ml, by 39% and 83%, respectively (Fig. 4).

Inhibition of EBV Virion Production Numbers of EBV virion produced by P3HR1 cells were determined at the fifth day after lytic induction. As expected, treating P3HR1 cells with TPA and SB produced about 4.2×10^7 viral particles (Fig. 5). Treating the cells with 25 μg/ml of EEAP or 5 μg/ml of andrographolide reduced the numbers of viral particles to the background level (Fig. 5), indicating that the EEAP and andrographolide inhibited EBV lytic replication.

Effect of EEAP and Andrographolide on the Viability of P3HR1 Cells The toxicity of EEAP and andrographolide to P3HR1 cells was determined using the MTT method.[31] The EEAP at concentrations of 25 μg/ml and 50 μg/ml killed 10% and 22% of P3HR1 cells after 24 h of culturing (Fig. 6A). The extract at 100 μg/ml killed 60% of the cells (Fig. 6A). This study also demonstrated that andrographolide did not influence the cell viability at concentrations under 5 μg/ml. The compound killed about 20% of the cells at 10 μg/ml (Fig. 6B).

DISCUSSION

As it is generally known, many clinical symptoms of herpesvirus infections, for example, cold sores, genital herpes, chicken pox, infectious mononucleosis, are associated with

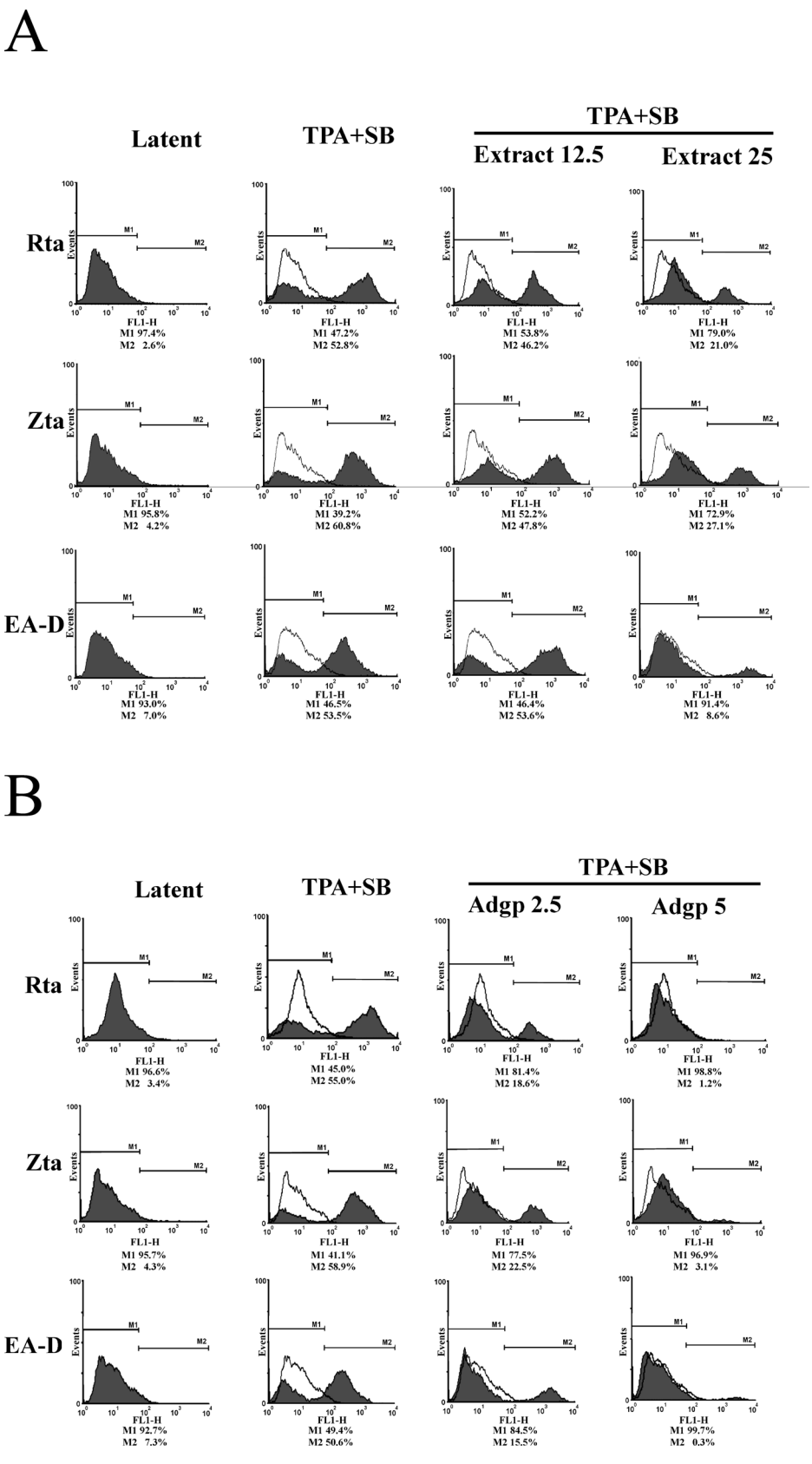

Fig. 3. Determining the Expression of Rta, Zta and EA-D in P3HR1 by Flow Cytometry Analysis

P3HR1 cells were treated with EEAP or andrographolide before lytic induction. At 24 h after lytic induction, cells were incubated with monoclonal anti-Rta, anti-Zta and anti-EA-D antibodies and stained with secondary anti-mouse IgG-FITC-conjugated antibody. M2 represents stained P3HR1 cells.

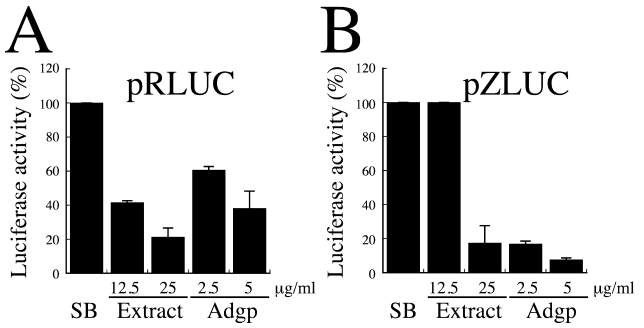

Fig. 4. Inhibition of BRLF1 and BZLF1 Transcription by EEAP and Andrographolide

EEAP or andrographolide (Adgp) was added to P3HR1 cells 1 h before transfection with pRLUC (A) and pZLUC (B) and lytic induction with sodium butyrate (SB). Luciferase activity exhibited by the cells was monitored using a luminometer at 24 h after lytic induction. Each transfection experiment was performed three times and each sample in the experiments was prepared in duplicate. The data are presented as means± S.D. from three independent experiments.

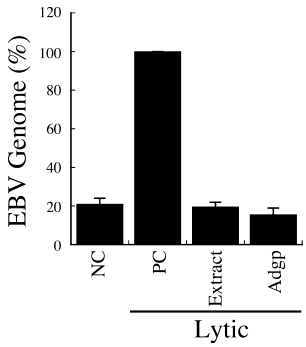

Fig. 5. Inhibition of EBV Virion Production by EEAP and Andrographolide

P3HR1 cells were treated with 25 μg/ml of EEAP and 5 μg/ml of andrographolide 1 h before lytic induction. EBV particles that were released into the cultured were harvested by PEG6000 precipitation. The amount of EBV DNA was determined by real-time PCR after the DNA was extracted from the viral particles. Cells that had not been treated with TPA and sodium butyrate were used as a negative control (NC). The data are presented as mean±S.D. from three independent experiments.

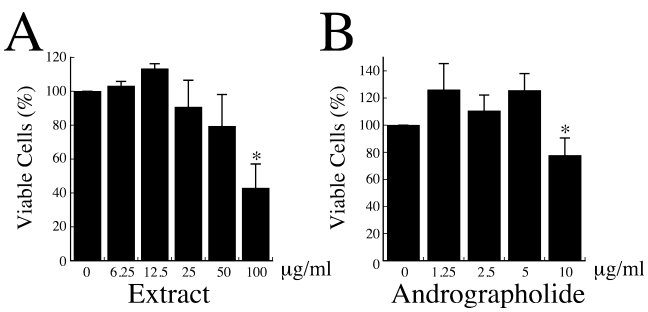

Fig. 6. Toxicity of EEAP and Andrographolide to P3HR1 Cells

Cell viability was determined using the MTT method.[31] P3HR1 cells were treated with EEAP (Extract) and andrographolide after lytic induction. The data are presented as mean±S.D. from three independent experiments. $*p<0.05$, which represents a significant difference from the values of the control group.

viral lytic reactivation.[32,33] Hence, anti-herpes drugs that are commonly used such as acyclovir and ganciclovir, are nucleoside analogs, suppress the function of herpes DNA polymerase and viral lytic DNA replication.[12,34,35] An earlier study demonstrated that EBV virions that are produced by B lymphocytes exhibit a tropism by which they preferentially infect epithelial cells.[33] Therefore, the lytic reactivation of EBV in B lymphocytes may be an important step toward the oncogenesis of epithelial cell that is associated with EBV, including nasopharyngeal carcinoma.[36] Therefore, development of an effective strategy to inhibit the lytic cycle may be valuable in reducing the disease risk.

This study found that 12.5 to 25 μg/ml of the EEAP markedly reduced the expression of the EBV immediate-early proteins, Rta and Zta and EA-D (Figs. 1A, 2A, 3A). The inhibition is probably attributable to the active ingredient in the plant, andrographolide, since this compound at 5 μg/ml (14 μM) also effectively inhibits the expression of these proteins (Figs. 1B, 2B, 3B). An earlier study showed that EGCG from green tea inhibits the EBV lytic cycle at a concentration of 50—70 μM,[15] which is substantially higher than the dose of andrographolide that effectively inhibits the EBV lytic cycle at 14 μM (5 μg/ml) (Figs. 1, 2, 3, 4), suggesting that andrographolide is more effective than EGCG in inhibiting EBV reactivation.

This investigation establishes that EEAP and andrographolide inhibit the transcription of BRLF1 and BZLF1 (Fig. 4), explaining why the EBV cannot complete its lytic cycle to produce EBV particles (Fig. 5). Exactly how andrographolide inhibits the transcription of BRLF1 and BZLF1 is currently unknown. It is plausible that andrographolide inhibits the functions of the transcription factors that activate the transcription of BRLF1 and BZLF1. The transcription of BRLF1 and BZLF1 is also known to be affected by the activation of various signaling pathways, including p38 and JNK[37]; further work must be conducted to determine whether andrographolide inhibits the signaling pathways that are known to activate the transcription of EBV immediate-early genes. Chen *et al.*[38] found that andrographolide not only suppresses the mitochondrial pathway of apoptosis but also activates the phosphatidyl inositol-3-kinase/Akt signaling pathway. However, whether this pathway influences the expression of BRLF1 and BZLF1 is unknown.

As it is generally known, EBV in P3HR1 cells does not enter the lytic cycle unless the cells are treated with compounds such as TPA and SB.[25,26] However, this work identified a low level of EBV DNA in the culture medium after the cells had been cultured for 5 d (Fig. 5). EBV is known to reactivate spontaneously, especially under stress conditions.[39] After culturing for 5 d, important growth factors in the culture medium may be depleted and insufficient to support normal cell growth. Under such stress conditions, EBV may enter the lytic cycle, explaining why low numbers of EBV particles were detected (Fig. 5).

A. paniculata, which is rich in andrographolide, is extensively used as a remedy with anti-inflammatory, antipyretic and antimicrobial activities.[18,22—24,40,41] This study finds that andrographolide has an anti-EBV activity. Furthermore, the result also revealed that andrographolide has low cytotoxicity toward P3HR1 cells under the concentration of 5 μg/ml. Thus, it is potentially useful in preventing the lytic development of EBV and other related herpes viruses.

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