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Chemical Components of *Polygonum cuspidatum* Ethylacetate Subfraction and their Effects on Epstein-Barr Virus Lytic Genes Expression

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Abstract

The study highlights the effect of ethylacetate subfraction F1 from *Polygonum cuspidatum* root and piceid on the inhibition of Epstein-Barr virus (EBV) lytic gene expression. The MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide] method was employed to assess the effect of F1 and piceid on the cell survival of P3HR1. The immunoblotting method and flow cytometry were applied to analyze the expression of EBV lytic proteins. The results showed that the EC_{50} (effective concentration required to inhibit 50% EBV lytic proteins) of F1 and piceid inhibiting the lytic proteins of EBV were 23.3 μg/mL and 119.6 μM, respectively. For P3HR1 cytotoxicity, the CC_{50} (cytotoxic concentration that decreased cell viability by 50%) of F1 and piceid were 93.1 μg/mL and 507.9 μM, respectively. In other words, F1 and piceid effectively inhibited the expression of EBV lytic proteins at a non-cytotoxic concentration. qPCR was performed to analyze the effect of F1 and piceid on the transcription of lytic genes, including BRLF1, BZLF1, and DNA replication of EBV. The results showed that F1 and piceid inhibited the transcription of lytic genes and reduced DNA replication of EBV with EC_{50} of 55.5 μg/mL and 89.9 μM, respectively. The results of this study confirmed that F1 and piceid could effectively inhibit the expression of EBV lytic proteins and EBV DNA replication, indicating that F1 and piceid are potentials for use as anti-EBV drugs.

Keywords: Piceid, Antiviral activity, Epstein-Barr virus, *Polygonum cuspidatum*

Introduction

Epstein-Barr virus (EBV) is a human herpesvirus that belongs to the γ-herpes virus subfamily. EBV mainly infects human B lymphocytes and epithelial cells causing infectious mononucleosis (Murata *et al*., 2021) and many malignant diseases such as Burkitt's lymphoma (Heslop, 2020), Hodgkin's disease, gastric cancer, T-cell lymphomas and nasopharyngeal carcinoma (Wong *et al*., 2022). When cells are infected with EBV, virion production is reduced during the latent cycle, presenting the coexistence of virus and host (Murata *et al*., 2021). The EBV is reactivated by ultraviolet light (Chen *et al*., 2020), sodium butyrate (SB) (Koike *et al.*, 2020), 12-O-tetradecanoyl phorbol 13-acetate (TPA) (Dochi *et al.*, 2022), trichostatin A (TSA), and valproic acid (VPA) (Anderson *et al*., 2019) allowing the BZLF1 and BRLF1 genes to express immediate-early proteins Zta and Rta, respectively. Zta and Rta are transcription factors that can activate the transcription of BMRF1 and BALF5, which encode the diffused early antigen (EA-D) and DNA polymerase and promote EBV's entering the lytic cycle (Murata *et al*., 2021). The viral DNA replicates by a rolling circle mechanism and assembles into virus capsid antigens and membrane proteins to produce mature virus particles; EB virions then infect other B lymphocytes and epithelial cells.

Some studies have found that EBV entering the lytic cycle can induce cytokines and cause tumorigenesis. For example, the EBV immediate early lytic protein Zta induces the production of multiple oncogenic and inflammatory factors (Jangra *et al.*, 2019). Therefore, inhibiting the virus entry into the lytic cycle can effectively treat EBV-related diseases. Nucleoside analogs, such as acyclovir and ganciclovir, are often used to treat EBV infection and are effective in treating infectious mononucleosis (Zhang *et al*., 2021), post-transplant lymphoproliferative disorder (Dugan *et al.*, 2018). Earlier studies have shown that acyclovir and ganciclovir can affect EBV replication by inhibiting virus-encoded DNA polymerase (Pagano *et al.*, 2018) but not the expression of EBV's immediate early and early lytic proteins. In addition to chemical substances, ingredients extracted from plants can inhibit the lytic cycle, such as epigallocatechin gallate (EGCG), resveratrol, curcumin, sulforaphane, protoapigenone, luteolin, andrographolide, moronic acid, emodin, and glycyrrhizic acid (Yiu *et al.*, 2010; Yiu *et al.*, 2014; Andrei *et al*., 2019; Wu *et al.*, 2019; Benedetti *et al*., 2020; Li *et al.*, 2021; Shao *et al*., 2022). They prevent EBV from entering the lytic cycle by blocking the expression of EBV early proteins.

Earlier studies exhibit that the ethanolic extract of *P. cuspidatum* and resveratrol inhibit EBV lytic cycle and induce EBV-positive cell apoptosis (Yiu *et al.*, 2010; Yiu *et al.*, 2014; Wu *et al*., 2018; Yiu *et al.*, 2021). The ethanolic extract of *P. cuspidatum* root contains anthraglycoside B, physcion, piceid, emodin, and resveratrol (Yun-Ting *et al.*, 2020). Piceid (3,5,4-trihydroxystilbene-3-O-β-D-glucopyranoside) also called polydatin, is a resveratrol derivative, which retains the biological

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activity of resveratrol; it has the functions of anti-inflammation, regulating the immune system, antioxidation, inducing tumor cell apoptosis, inhibiting genetic mutations, preventing cardiovascular disease, protecting myocardial and liver cells, lowering cholesterol in the blood, inhibiting lipid peroxidation, and preventing shock (Karami *et al.,* 2022). It can be found in plants such as peanuts, *Polygonum cuspidatum*, mulberry, grape skin, and cocoa beans. To date, no studies have been conducted on the inhibition of immediate-early gene expression and DNA replication of EBV by piceid. This study will explore whether *P. cuspidatum* root subfraction F1 and piceid can affect the DNA replication of EBV by blocking early gene transcription of EBV and expression of lytic proteins.

Materials and Methods

Plant Material

P. cuspidatum was collected from the San-Dei-Men area in Pingtung County in Taiwan and verified by Prof. C. S. Kuoh. The specimen was deposited in the herbarium of the National Cheng Kung University, Tainan, Taiwan. Piceid was purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Sample Preparation and HPLC Analysis

Using the Yiu method (Yiu *et al.,* 2014), the subfraction F1 of ethyl acetate isolated from *P. cuspidatum* root was prepared and analyzed by high-performance liquid chromatography with a diode array detector ranging from 210 to 500 nm. A yield of 1.81% was obtained. The resulting residues were finally dissolved in dimethyl sulfoxide (DMSO).

Cell Culture and Lytic Induction of EBV

P3HR1, a Burkitt's lymphoma cell line 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 (Yiu *et al.,* 2010).

Cytotoxicity Assay

P3HR1 cells were treated with *Polygonum cuspidatum* root subfraction F1 or piceid. After 24 h incubation, a 0.5 mg/mL [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) solution was added into RPMI 1640 medium. The dehydrogenase activity representing the viable cells was measured.

Immunoblot Analysis

The cell lysate was prepared from 3 x 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 (Yiu *et al.,* 2010). SDS-polyacrylamide gel electrophoresis and immunoblot analysis with anti-Rta, anti-Zta, and anti-EA-D antibodies purchased from Argene (Varilhes, France) were performed using methods described previously.

RNA and DNA Extraction

For qPCR, RNA was extracted from 3 × 10^6 cells using the conventional Trizol method (Invitrogen, Carlsbad, CA). DNA removal was performed using the RNase-Free DNase Set (promega, Madison, WI). Total RNA was added to the High capacity cDNA reverse transcription kit (Applied Biosystems) to produce cDNA. Briefly, 10 µL of treated RNA (2 µg) was mixed with 0.8 µL 25 × dNTP mix (100 mM) and 2 µL 10 × RT Random Primers, followed by adding 2 mL 10 × RT buffer, 4.2 µl 0.1% DEPC water and 1 µL MultiScribe™ Reverse Transcriptase (50 U/µL). The components in the tube were mixed gently and incubated for 10 minutes at 25°C, for 120 minutes at 37°C, and 5 minutes at 85°C. DNA was extracted using a Quick-gDNA miniPrep kit (Zymo Research).

Real-Time Quantitative PCR

For mRNA analysis, Quantitative PCR (qPCR) was done with the SYBR green (Applied Biosystems); DNA analysis using Taqman probe was performed (5′-6-FAM-GGAGACACATCTGGACCAG-MGBNFQ-3′) on the ABI StepOneTM qPCR system with StepOneTM software v 2.1. All reactions were run in triplicate. Mean cycle threshold (C_T) values were normalized to β-actin, yielding a normalized C_T (ΔC_T). The ΔΔCT value was calculated by subtracting respective control from the ΔCT, and the expression level was then calculated by 2 raised to the power of the respective $-\Delta\Delta C_T$ value. Relative mRNA (DNA) level (%) = $2\text{-}\Delta\Delta C_T$ (SB and sample treatment)/ $2\text{-}\Delta\Delta C_T$ (SB treatment) × 100. Primers for mRNA analysis include the following: BRLF1 forward (5′-TCACTACACAAACAGACGCAGCCA-3′) and reverse (5′-AATCTCCACACTCCCGGCTGTAA-3′); BZLF1 forward (5′-AGAAGCACCTCAACCTGGAGACAA-3′) and reverse (5′-CAGCGATTCTGGCTGTTGTGGTTT-3′); and β-actin forward (5′-CGTCTTCCCCTCCATCG) and reverse (5′-CTCGTTAATGTCACGCAC-3′). Primers for DNA analysis, EBNA1 forward (5′-TACAAGACCTGGAAAGGCC-3′) and reverse (5′-TCTTTGAGGTCCACTGCC -3′).

Statistical Analysis

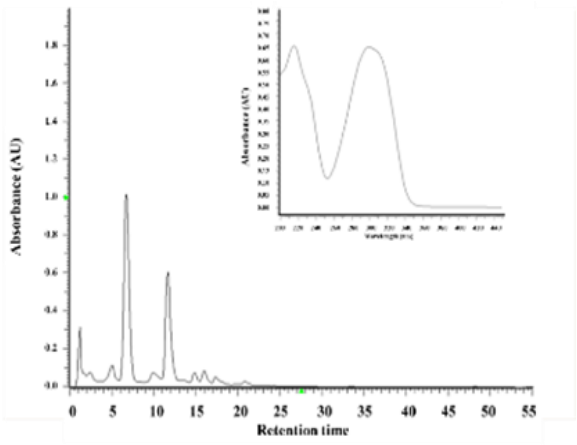
One-way analysis of variance (ANOVA) was used to analyze the data followed by Dunnett's *post hoc* test using SAS JMP 8.0 software. Values are expressed as mean ± SD of three replicates and a *p*-value of <0.05 is regarded as significant.

Results and Discussion

Composition Analysis of Polygonum cuspidatum Ethylacetate Subfraction F1

The ethanol extract of *Polygonum cuspidatum* root was subject to partition into hexane and ethyl acetate, and the collected ethyl acetate fraction was separated by a semi-preparative high-pressure liquid chromatography analyzer to obtain the ethyl acetate subfraction F1 and F1a. Reverse-phase high-performance liquid chromatography was used to analyze the composition of F1 and F1a; values obtained were compared with the retention time and ultraviolet spectrum of pure standards of piceid and resveratrol to determine the target compounds in F1 and F1a. As shown in **Figure 1**, the peak eluting at 5.13 min was piceid and at 11.6 min

was resveratrol. The content of piceid and resveratrol in F1 was 20.49 and 10.4% and in F1a were 51.76% and 1.17%, respectively.



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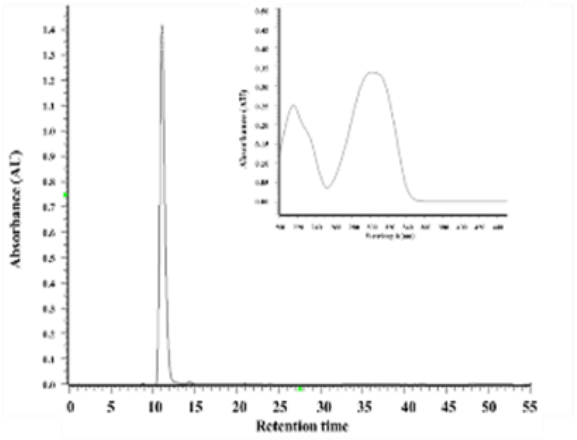

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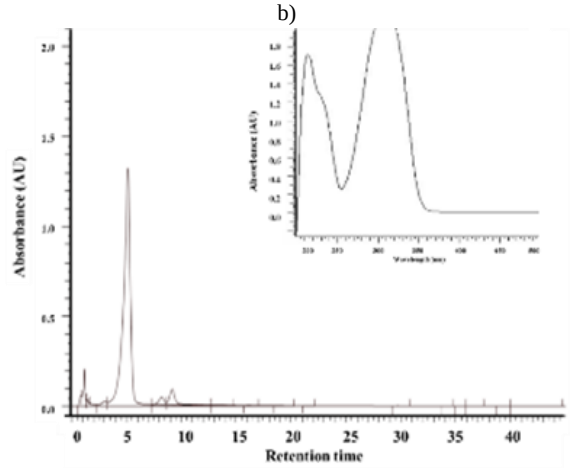

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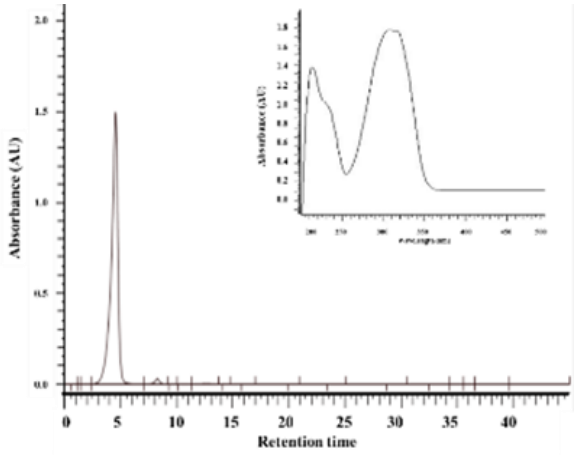

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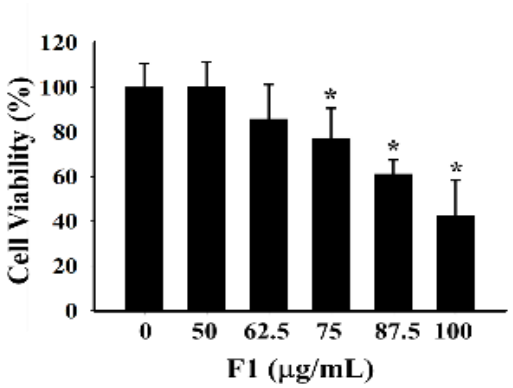
Figure 1. HPLC chromatograms and UV spectra of the ethylacetate subfraction F1 (a) and F1a (c) isolated from *Polygonum cuspidatum root* (a) and pure standard of resveratrol (b) and piceid (d)

Effect of F1 and Piceid on the Growth of P3HR1 Cells

To understand whether the ethyl acetate subfraction F1 from the roots of *Polygonum cuspidatum* and piceid have antiviral activity without affecting cell survival, the MTT method was proposed to analyze the survival rate of P3HR1 cells. After 24 hours of adaptation, P3HR1 cells (1×10^5 cells/mL) were added to different concentrations of F1 (0-100 μg/mL) or piceid (0-512 μM); after 24 hours of reaction, the MTT method was then performed to determine the cell survival rate. The results showed that F1 and piceid did not affect the survival rate of P3HR-1 cells. When concentrations of F1 and piceid were below 62.5 μg/mL and 128 μM, respectively, the cell survival rate was higher than 85%. When the F1 concentration was increased to 100 μg/ml, the cell survival rate was only 40%. Piceid affected the survival rate of P3HR1 cells at the concentration of 256 μM and 512 μM, showing 72.9% and 50.4%, respectively. The median cytotoxic concentration (CC_{50}) of F1 and piceid were 93.1 μg/mL and 507.9 μM, respectively, which is shown in **Figure 2**.



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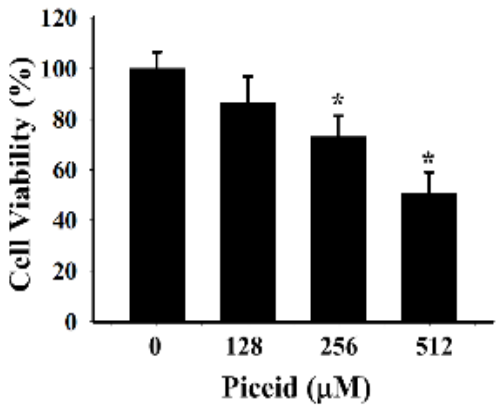

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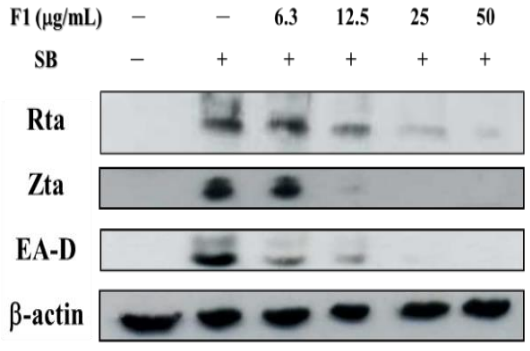
Figure 2. Toxicity of F1 and piceid on P3HR1 cells

Use of Immunoblotting to Assess the Effect of F1 and Piceid on EBV Lytic Proteins Expression

EBV reactivation can be induced by sodium butyrate to express the immediate-early proteins, Zta and Rta, which are transcription factors involved in the promotion of the expression of other lytic cycle genes, such as activation of the translation of the BRLF1 gene to produce EA-D protein. EBV is reactivated from the latent to lytic cycle and infects other cells. Therefore, EBV lytic protein expression represents that EBV is in the DNA replication stage. 6 × 10^5/mL of P3HR1 cells were treated with the ethylacetate subfraction F1 from *Polygonum cuspidatum* root and piceid. Before the induction of the EBV lytic cycle, SB was added. After 24-hour treatment, the cells were harvested. The lytic proteins, Rta, Zta, and EA-D of EBV in the cells were determined by immunoblot assay. **Figure 3** indicates that F1 and piceid significantly inhibited the expression of Rta, Zta, and EA-D at the concentration of 12.5 μg/mL and 32 μM, respectively. The F1 concentration increasing to 25 μg/mL and 64 μM for piceid completely inhibited the expression of Zta and EA-D. The performance of Rta, Zta, and EA-D is no longer detectable at 50 μg/mL for F1.



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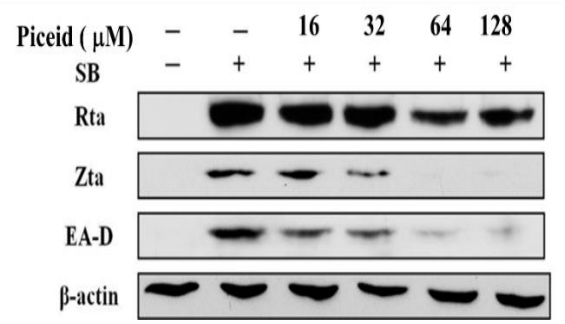

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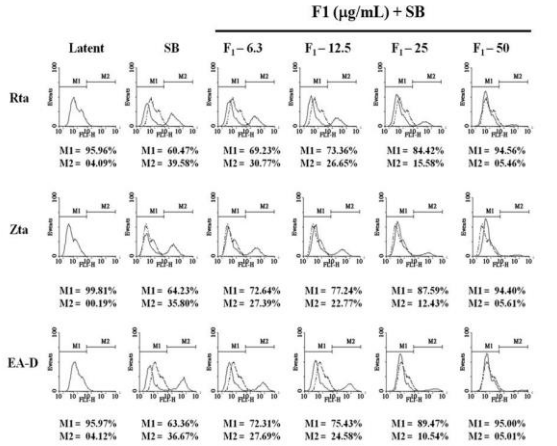
Figure 3. Inhibitory effects of F1 and piceid on the expression of EBV lytic proteins, including Rta, Zta and EA-D

Quantification of Cells Expressing EBV Lytic Protein by Fow Cytometry

Figure 3 showed that F1 and piceid had the effect of inhibiting the lytic proteins Rta, Zta, and EA-D. Therefore, we further used flow cytometry to quantify cells expressing EBV lytic proteins. P3HR1 cells (6 × 10^5/mL) were treated with SB for 24 hours, and the percentages of cells expressing Rta, Zta, and EA-D lytic proteins were analyzed by flow cytometry showing the results being 39.6%, 35.8%, and 36.7%, respectively. When treated with an F1 concentration of 25 μg/mL before SB induction, the percentage of cells expressing Rta, Zta, and EA-D decreased to 15.6%, 12.4%, and 10.5%, respectively; when the concentration was increased to 50 μg/mL, it further decreased to 5.5%, 5.6%, and 5.0%, respectively **(Figure 4a)**. The number of cells (M2) expressing Rta, Zta, and EA-D induced by SB was regarded as total expression, and then the data from three independent flow cytometry analyses were quantified. The results showed significant differences between cells treated with SB and 12.5 μg/ml of F1 before SB induction in Rta, Zta, and EA-D protein expression, with only 65.2%, 66.6 %, and 57.8% left, respectively; when the concentration was increased to 25 μg/mL, it decreased to 39.2%, 35.5%, and 28.0%. At 50 μg/mL, the expressions (%) were only 24.3%, 20.7%, and 17.9%, showing that F1 had dose-dependent effects on the inhibition of lytic protein expression. Further assessment on the concentration required for F1 to inhibit 50% of the expressions of Rta, Zta, and EA-D was conducted, and EC_{50} was 25.9 μg/mL, 25.0 μg/mL, and 19.0 μg/mL, respectively **(Figure 4c)**.

The evaluation of the inhibitory effects of piceid showed that the number of cells expressing Rta, Zta, and EA-D lytic proteins in the SB group was 34.27%, 44.1%, and 34.9%, respectively. At concentrations of 32 μM, 64 μM, and 128 μM, piceid demonstrated the ability to inhibit EBV lytic protein expression. At 128 μM, the percentages of Rta, Zta, and EA-D-positive cells decreased to 14.83%, 22.03%, and 19.27%, respectively **(Figure 4b)**. Cells expressing Rta, Zta, and EA-D lytic proteins were quantified, and the results showed that the number of Zta-positive cells was significantly different from that of the SB control group at a concentration of 16 μM, with a decrease of about 25.8%. There was also a significant reduction in the number of cells expressing Rta and EA-D at a piceid concentration of 32 μM, compared with

the SB control group, with a reduction by approximately 20.0% and 17.7%, respectively; at 128 μM, Rta, Zta, and EA-D lytic protein cells were reduced by 56.7%, 50.0%, and 44.8%, respectively. Piceid has a dose-dependent activity on inhibiting EBV lytic protein expression. EC_{50} values for Rta, Zta, and EA-D expressions were 111.0 μM, 109.1 μM, and 138 μM, respectively **(Figure 4d)**.



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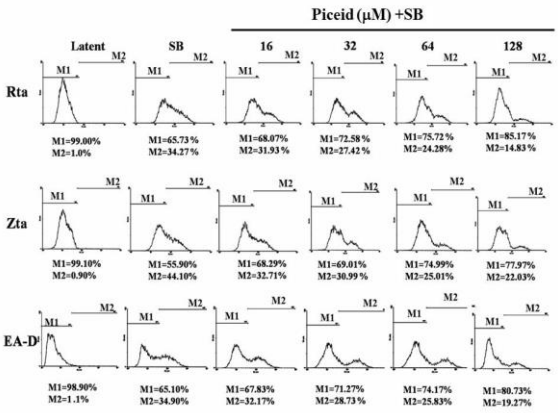

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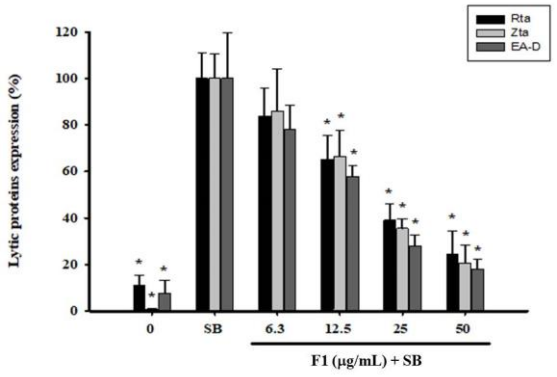

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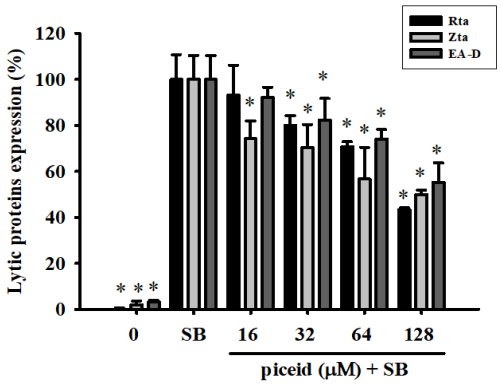

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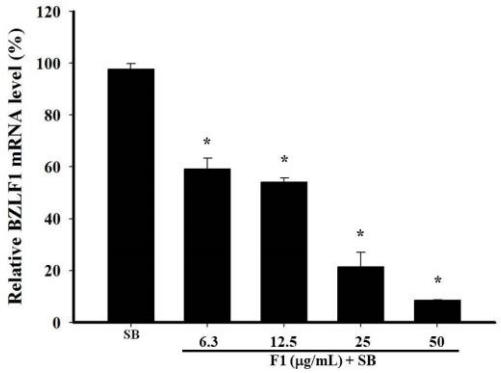
Figure 4. Quantification of cells expressing EBV lytic proteins, including Rta, Zta, and EA-D treated with F1 and piceid.

Inhibition of the Transcription of EBV Immediate-Early Genes

The expression of EBV immediate-early genes, BRLF1, and BZLF1 mRNA, in P3HR1 cells, was analyzed by reverse transcription and quantitative PCR (qPCR). **Figure 5a** and **Figure 5b** show that the ethyl acetate subfraction F1 from *Polygonum cuspidatum* root significantly reduced the transcription of BZLF1 and BRLF1 compared with the SB induction group. The mRNA expression levels of BZLF1 and BRLF1 treated with SB were 97.7 ± 2.1% and 99.8 ± 0.2%, respectively. When 6.3 μg/mL of F1 was added before lytic induction, BZLF1 and BRLF1 expressions were significantly reduced, with levels being only 59.1 ± 4.2% and 51.3 ± 18.7%. The levels of BZLF1 and BRLF1 mRNA were lowered to 21.3 ± 5.65% and 16.5 ± 4.9%, respectively, at 25 μg/mL. When increased to 50 μg/mL, the levels significantly reduced the transcription of BZLF1 and BRLF1 genes, being only 8.6 ± 0.2% and 9.1 ± 4.6%, respectively. **Figure 5c** and **Figure 5d** indicate that piceid inhibited EBV lytic gene transcription induced by SB and showed dose effects. When 32 μM and 64 μM piceid were added to the concentration, the expression of BZLF1 mRNA decreased to 60.3 ± 2.3% and 38.1 ± 5.1%, respectively. When the concentration was increased to higher than 64 μM, piceid significantly inhibited the expressions of BRLF1 mRNA; at 64 μM, the expression of BRLF1 mRNA was only 48.5 ± 7.7%.



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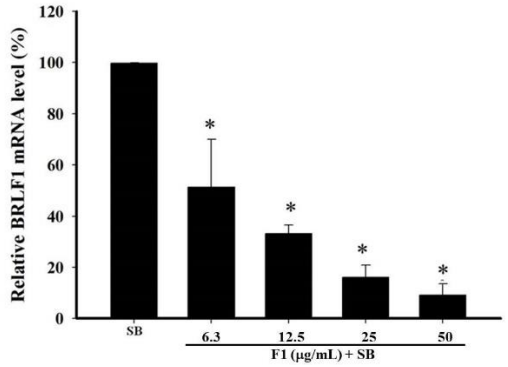

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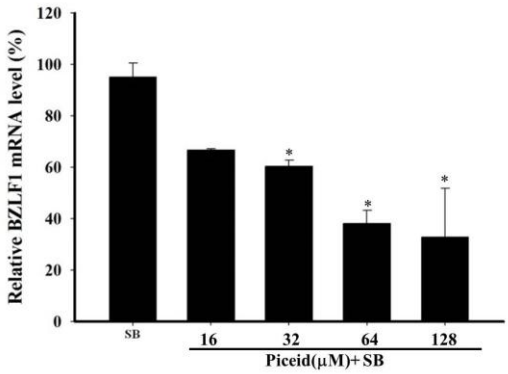

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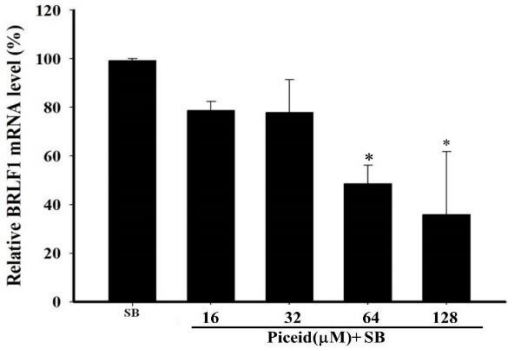
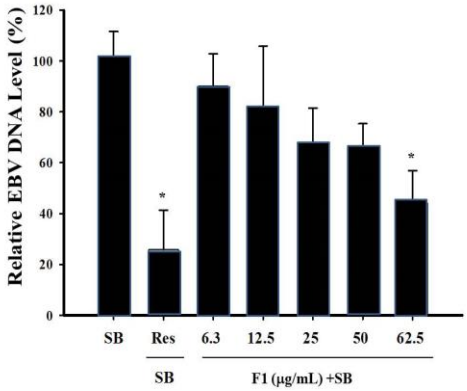


d)

Figure 5. Effects of F1 and piceid on the EBV BZLF1 and BRLF1 mRNA expression.

Inhibition of EBV DNA Replication

Results from immunoblot and flow cytometry analysis found that F1 and piceid effectively inhibited the expressions of EBV lytic proteins Rta, Zta, and EA-D induced by SB at a non-cytotoxic concentration. We further explored whether F1 and piceid reduce EBV replication. P3HR1 cells were treated with 6.3-62.5 μg/mL of F1 or 16-128 μM piceid before lytic induction. After culturing for two days, EBV DNA in P3HR1 cells was isolated by qPCR to determine the amount. From **Figure 6a**, compared with the group induced by SB alone, F1 at 12.5 μg/mL inhibited EBV DNA replication in the group pretreated with SB followed by the F1 treatment, with a decrease of about 18%. When the concentration was increased to 62.5 μg/mL, there was a significant difference ($p<0.05$) among groups, with a reduction in EBV DNA replication by 56% and the EC_{50} being 55.5 μg/mL. In addition, the group pretreated with 55 μM of resveratrol and SB addition, which served as the positive control group, showed a reduction in EBV DNA replication by 74%. Compared with the SB group, at a concentration of 32 μM, piceid was shown to significantly inhibit EBV DNA replication with a reduction of 33%; at the concentrations of 64 μM and 128 μM, the inhibition rates were 38% and 64%, respectively, shown in **Figure 6b**. The EC_{50} was 89.9 μM.



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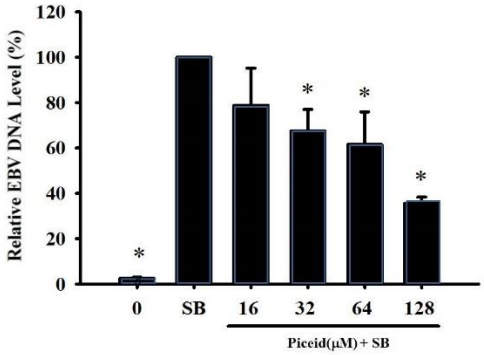

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Figure 6. Inhibition Effects of F1 and piceid on EBV DNA replication

Drugs currently used clinically to treat EBV lytic infection are mostly anti-herpes virus agents, such as acyclovir (ACV), a guanosine analogue, and ganciclovir (GCV). The main mechanism of action is to monophosphorylate ACV and GCV by EBV-encoded protein kinase, and then use the host thymidine kinase to make triphosphorylated ACV and GCV that further inhibits EBV DNA polymerase and replication of viral DNA (Pagano *et al.*, 2018). Although these drugs can inhibit EBV DNA replication, they are highly toxic to patients and show negative effects. They are likely to cause decreased white blood cell or platelet counts in patients and increased side effects such as anemia, skin rash, and abnormal liver function. Therefore, in recent years, there have been numerous studies on natural Chinese herbal medicines aiming to

find agents that can effectively inhibit EBV entry into the lytic cycle by environmental factors resulting in subsequent infection or diseases.

Previous literature has confirmed that natural products and Chinese herbal medicines have anti-EBV lytic cycle activity such as EGCG (Chang *et al.*, 2003) and andrographolide (Lin *et al.*, 2008) at a concentration of 100 μM and 14 μM, respectively in P3HR-1 cells that are not cytotoxic and can completely inhibit transcription and expression of EBV lytic proteins in very early stage. Glycyrrhizic acid, at 40 μM, can inhibit early EBV lytic replication with an EC_{50} of 30 μM (Lin, 2003). Moronic acid inhibits the production of Rta, Zta, and EA-D proteins at 10-20 μM, reduces the expression of BZLF1 and BRLF1 mRNA at 5-15 μM and inhibits the EC_{50} of Rta at 3.2 μM (Chang *et al.*, 2010).

This study proved that the main component of F1 contained 20.5% of piceid and 10.4% of resveratrol, which can inhibit the expression of EBV lytic proteins, with EC_{50} of F1 being 23.3 μg/mL and EC_{50} of piceid being 119.6 μM (46.7 μg/mL). Yiu *et al.* reported that resveratrol could inhibit EBV lytic proteins with an EC_{50} of 24 μM (5.5 μg/mL) (Yiu *et al.*, 2010). It is speculated that resveratrol, piceid, and other compounds may be involved in the F1 inhibitory effect on the expression of EBV lytic proteins. Furthermore, it was found that piceid significantly inhibited EBV lytic protein expression, with an EC_{50} of 119.6 μM. In the cytotoxicity test of P3HR1, CC_{50} of piceid was 507.9 μM, and the therapeutic index (TI) was 4.2. Piceid and resveratrol have similar structures, with an additional sugar group. In terms of efficacy, resveratrol inhibited 50% of EBV lytic protein expression with an EC_{50} being 24 μM, CC_{50} of P3HR1 cells being 137.6 μM, and TI being 5.7. Both substances can inhibit EBV lytic cycle at a nontoxic concentration. In inhibiting the performance of Epstein-Barr virus lytic protein, resveratrol is more effective than piceid. Previous literature pointed out that rutin's glycosidic bonds can be hydrolyzed through the enzyme system of bacteria in the gastrointestinal tract, and then transform into quercetin and then enter the cell to exhibit better biological activity (Yang *et al*., 2012). It is further speculated that piceid and resveratrol may also have the same effects as rutin and quercetin; piceid may exhibit a greater inhibitory effect on the EB virus lytic cycle after biological metabolism than resveratrol.

The possible mechanism for the resveratrol and piceid to activate the deacetylation activity of sirtuin is by inhibiting p38 MAPK, ERK, and JNK transduction that affects AP-1 and ATF2 activation, preventing activation of EBV immediate-early genes, and thereby inhibiting EBV lytic cycle (Yiu *et al*., 2010).

Conclusion

Our study results demonstrate that F1 and piceid could inhibit lytic gene transcription, prevent EBV lytic cycle, and reduce viral DNA replication. The ability of F1 and piceid to affect EBV reactivation has the potential to be used to develop anti-EBV drugs.

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