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Dysregulation of KSHV Replication by Extracts from *Carthamus tinctorius* L.[§]

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***Carthamus tinctorius* L. (CT) is traditionally used to reduce ailments from diseases of the musculoskeletal system and connective tissue and diseases of blood circulation and the cardiovascular system. Flower extracts from CT are known to have antibacterial activity, anti-inflammatory activity, and to inhibit tumor promotion in mouse skin carcinogenesis. In order to discover new antiviral agents from CT extracts, we tested whether CT extracts contain antiviral activity against gammaherpesvirus infection. This study demonstrated that treatment with CT extracts disrupted KSHV latency in the viral-infected host cells, iSLK-BAC16. n-Hexane and EtOH fractions of CT extracts critically affected at least two stages of the KHSV life-cycle by abnormally inducing KSHV lytic reactivation and by severely preventing KSHV virion release from the viral host cells. In addition to the effects on KSHV itself, CT extract treatments induced cellular modifications by dysregulating cell-cycle and producing strong cytotoxicity. This study demonstrated for the first time that CT extracts have antiviral activities that could be applied to development of new anti-gammaherpesviral agents.**

***Keywords*:** *Carthamus tinctorius* L., Kaposi's sarcoma-associated herpesvirus, extracts, latency, lytic reactivation

Introduction

Carthamus tinctorius L. (CT) is a member of the family Compositae or Asteraceae and is mainly cultivated to harvest its seed and flowers for use as edible oils, dyes and medicines (Asgary *et al*., 2012). In Korea, CT has traditionally been used to cure diseases of the musculoskeletal system, connective tissue, blood circulation and cardiovascular system (Chang *et al*., 1996; Park *et al*., 2001). Flower extracts from CT exhibit effective antibacterial activity, anti-inflammatory action, and inhibit tumor promotion in mouse skin carcinogenesis (Hiramatsu *et al*., 2009). In particular, CT extracts display stronger antimicrobial effects than other plant extracts such as *Rubia tinctorum* and *Juglans regia* (Mehrabian *et al*., 2000). Microorganisms most sensitive to the aqueous extract of CT include *Bacillus mycoides*, *Bacillus subtilus*, *Bacillus cereus*, *Geotrichum candidum*, *Aspergillus nigra*, and *Penicillium expamsum*.

Kaposi's sarcoma herpesvirus (KSHV) is the eighth human gammaherpesvirus discovered from Kaposi's sarcoma lesion of an AIDS patient in 1994 (Ganem, 2006). Through strong molecular and epidemiological studies, KSHV was associated with Kaposi's sarcoma (KS) and certain lymphoproliferative disorders (Chang *et al*., 1994). As the loss of KSHV genome causes cell death, KSHV is actually required for development of these malignancies. KSHV displays two patterns of infection: latent and lytic replications. During latent replication, KSHV expresses only a restricted set of genes whose products contribute to maintain viral latency and transform the viral host cells. Upon induction of KSHV lytic replication, KSHV activates to express whole viral lytic genes whose products are later packaged and released as KSHV virions to infect neighboring host cells.

Most KS is now treated as a human sarcoma rather than a viral disease (Jarviluoma and Ojala, 2006). HAART (highly active anti-retroviral therapy) was introduced to treat HIV infection, and it has appeared highly effective in controlling AIDS-related KS. Indeed, HAART has significantly decreased the incidence of AIDS-related KS in the USA. However, there are some drawbacks to treating AIDS with HAART. HAART usually takes 3 months to restore the patient's immune system. Thus AIDS-relative lymphoma (ARD) was not completely cured by HAART because even an unsatisfying treatment of ARD takes 3-6 months. In an exemplary case of primary effusion lymphoma (PEL) caused by KSHV infection, the cure rate of PEL by HAART was 42% and the median survival of patients suffering from PEL was 6 months. Another drawback is the frequent occurrence of mutations in the HIV genome, which enhances HIV resistance against HAART. Because of these features, lower efficacy of HAART

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has recently been reported in KS treatment (Stallone *et al.*, 2005).

Interests are increasing in traditionally bioactive natural products due to both (1) the low success rate in developing new drugs from synthetic compounds and (2) the loss of efficacy by existing antiviral agents. CT is a good source from which new antiviral drugs can be discovered because it produces numerous bioactive compounds such as carthamin, safflomin A, safflor yellow A-B, carthamidin, cartorimine, tracheloside, kinobeon A, and cartormin (Korean Pharmacognosy Committee, 2006). These compounds appear to produce a variety of biological effects that alleviate illnesses. In the context of investigating such new antiviral agents, our study was dedicated to investigating whether CT extracts prevent gammaherpesvirus infection and, if so, the inhibitory mechanism of CT extracts against viral infection.

Materials and Methods

Preparation of the crude extract of *C. tinctorius* L.

C. tinctorius L. was extracted by liquid-liquid extraction using solvent polarity with n-Hexane, ethanol (EtOH), 60% Ethanol, and sterile distilled water. CT extracts were dried to powder and used for further studies. Stock solutions of CT extracts were made by dissolving them in Dimethylsulfoxide (DMSO) at a final concentration of 200 mg/ml and used at working concentrations of 0.5 or 1 mg/ml.

Cell cultures

iSLK-BAC16 (KSHV genome integrated cell line) and iSLK-Puro (GFP-negative controlled cell) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Cellgrom, USA) supplemented with 10% Fetal Bovine Serum (Omega, Canada), antibiotics/antimycotics (Gibco, USA), and Glutamax (Gibco) at 37°C, 5% CO_2, 95% humidity in a CO_2 incubator (Brulois *et al.*, 2012). iSLK-BAC16 cells were selected with hygromycin B (450 μg/ml), G418 (120 μg/ml), and puromycin (1 μg/ml), and iSLK-puro cells were selected with G418 (240 μg/ml) and puromycin (1 μg/ml). iSLK cells were incubated for 48 h after treated with each fraction of CT extracts. Vero rKSHV.219 cells (a gift from Jae J. Jung) were cultured in the same DMEM used for iSLK cells under selection of puromycin (5 μg/ml).

FACS analysis

To evaluate the effect of CT extracts on KSHV latency, FACS (Fluorescence-Activated Cell Sorting ARIA III; BD Biosciences, USA) analysis was conducted. iSLK-BAC16 and iSLK-puro cells were treated with each CT extract fraction. The resultant 0.5×10^6 cells were fixed with 2% paraformaldehyde in PBS solution and subjected to FACs analysis. Events from 1×10^4 cells were acquired by gating live cells based on forward and side scatter profiles, and expression of Green Fluorescence Protein (GFP) was measured with FITC filter. Blank-treated iSLK-puro and iSLK-BAC16 cells were used as negative and positive controls, respectively.

Fluorescent microscopy

Fluorescent microscope assays were conducted to determine the effect of each CT extract fraction on KSHV latency in Vero rKSHV.219. Vero rKSHV.219 cells produce GFP during KSHV latent replication and produce RFP during KSHV lytic replication (Vieira and O'Hearn, 2004). Vero rKSHV.219 cells were treated with each CT extract fraction at 0.5 mg/ml and analyzed for KSHV latency at 48 h post-treatment using a fluorescent microscope.

RT-qPCR

RNA was extracted from iSLK-BAC16 cells treated with CT extract fractions using RNeasy Mini Kit (QIAGEN, USA), then synthesized into cDNA using Superscript II Reverse Transcriptase (Invitrogen, USA). The resultant cDNA was diluted 1/50 (for latent genes) and 1/25 (for lytic genes). The effect of CT extract treatment on KSHV gene expression was evaluated by Real-time quantitative PCR (qPCR). Latent gene primers were for ORF73, ORF72, ORF71, miRNA, and K12, lytic gene primers were for ORF50, ORF68, ORF69, ORFK14, and ORF74, and Internal control gene primers were for GAPDH and GFP. qPCR was conducted using iQ SYBR green reagent (Bio-Rad, USA) in Real-Time qPCR CFX96 (Bio-Rad). Each sample was analyzed for KSHV gene expression in triplicate. Although all primer set sequences were previously published (Kang and Lieberman, 2011), these sequences are available upon request.

Quantification of intracellular and extracellular KSHV genomic DNA

Following lysis and sonication using the Bioruptor (5 min with 30 sec on/off), genomic DNA (gDNA) was extracted from iSLK-BAC16 cells treated with each CT extract fraction. 50 ng of the resultant gDNA was subjected to Real-time qPCR assay, and the relative amount of KSHV gDNA was determined with an internal control such as GAPDH. Intracellular KSHV copy number was considered the relative amount of KSHV gDNA in total gDNA. To measure the relative extracellular KSHV copy number, 20 ml of each culture medium was collected from iSLK-BAC16 cells treated with each CT extract fraction. The culture media was filtered through 0.45-nm syringe filter, loaded on a 20% sucrose cushion in PBS solution, and subjected to ultracentrifugation (CP100WX, Hitachi, Japan) at 27,000 rpm for 90 min. The viral pellet was lysed in 100 μl of FA lysis buffer [EDTA (1 mM, pH 8.0), HEPES-KOH (50 mM, pH 7.5), NaCl (140 mM)], sonicated in bioruptor for 5 min with 30 sec on/off, and applied to general DNA extraction process. Final viral DNA was resolved in 100 μl of RNase-free water and subjected to qPCR analysis with KSHV ORF50 to quantify viral DNAs.

Cell-cycle analysis

To assess the effect of CT extracts on cell-cycle progress of KSHV latently infected host cells, iSLK-BAC16 cells were treated with each fraction of CT extracts, stained with propidium iodide (PI) solution for 48 h post-treatment, and then applied to cell cycle analysis using FACs ARIA III. In detail, 3×10^6 cells were seeded in 60-mm culture dishes. On

the following day, when cell confluency reached 70%, iSLK-BAC16 cells were treated with each CT extract fraction at 0.5 mg/ml. iSLK-BAC16 cells were harvested by trypsin at 48 h post-treatment, washed with cold PBS, fixed in 95% ethanol for at least 1 h, treated with 300 µg of RNase A to remove all traces of RNAs, stained in 10× PI solution, and finally analyzed for cell-cycle progress with FACs Aria III (BD Bioscience).

Cytotoxicity assay

To evaluate the cytotoxic effects of CT extracts on iSLK-BAC16 cells, cellular cytotoxicity assay was conducted with Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). Briefly (Ishiyama *et al.*, 1997), 100 µl of 1×10^4 cell suspension were seeded per well and then treated with each CT extract fraction at 0.5 mg/ml on the following day. The treatments lasted for 24 h or 48 h. 10 µl of Cell Counting Kit-8 (CCK-8) (Dojindo) solution was added to each sample, further incubated for 3 h, and then the samples were applied to an ELISA reader to read the absorbance of cell suspensions. All steps followed the manufacturer recommended protocol.

Western blotting analysis

To assess the effects of CT extracts on KSHV protein synthesis, Western blotting assays were conducted with iSLK-BAC16 cells treated with each CT extract fraction at 0.5 mg/ml. Treated iSLK-cells were harvested with trypsin at 48 h post-treatment. 1×10^6 cells were lysed in reporter lysis buffer (Promega, USA) supplemented with 1 µl of proteinase inhibitor and 10 µl of phenylmethysulfonyl fluoride (PMSF). The cell lysates were further fractionated by sonication with Bioruptor sonicator for 5 min with 30 sec on/off. If necessary, the cell lysates were snap frozen in liquid nitrogen and stored at -80°C. After quantitation using Protein Assay (Bio-Rad), 3 µg of cell lysates were loaded onto 7% SDS-PAGE gel and KSHV RTA was detected by Western blotting analysis with rat monoclonal LANA antibody (KSHV ORF73), with GAPDH detection as an internal control. HRP-conjugated sheep anti-mouse IgG was used as the secondary antibody in Western blotting assays.

Apoptosis analysis

To determine if treatment with CT extracts caused apoptosis in iSLK-BAC16, the caspase 3 activities in iSLK-BAC16 cells were measured at 0, 24, and 48 h post-treatment using Caspase Glo 3/7 kit (Promega). Briefly, 100 µl of 1×10^4 cell suspension was seeded per well and treated with each CT extract fraction at 0.5 mg/ml the following day. At 48 h post-treatment, caspase Glo 3/7 reagent was equilibrated to room temperature prior to adding 100 µl of reagent per well. At 3 h post further incubation, luminescence was measured at 560 nm.

Results

Disruption of KSHV latency by extracts from *C. tinctorius* L. (CT)

SLK cells are known to allow efficient KSHV infection, maintain tight and stable KSHV latency, and support robust viral

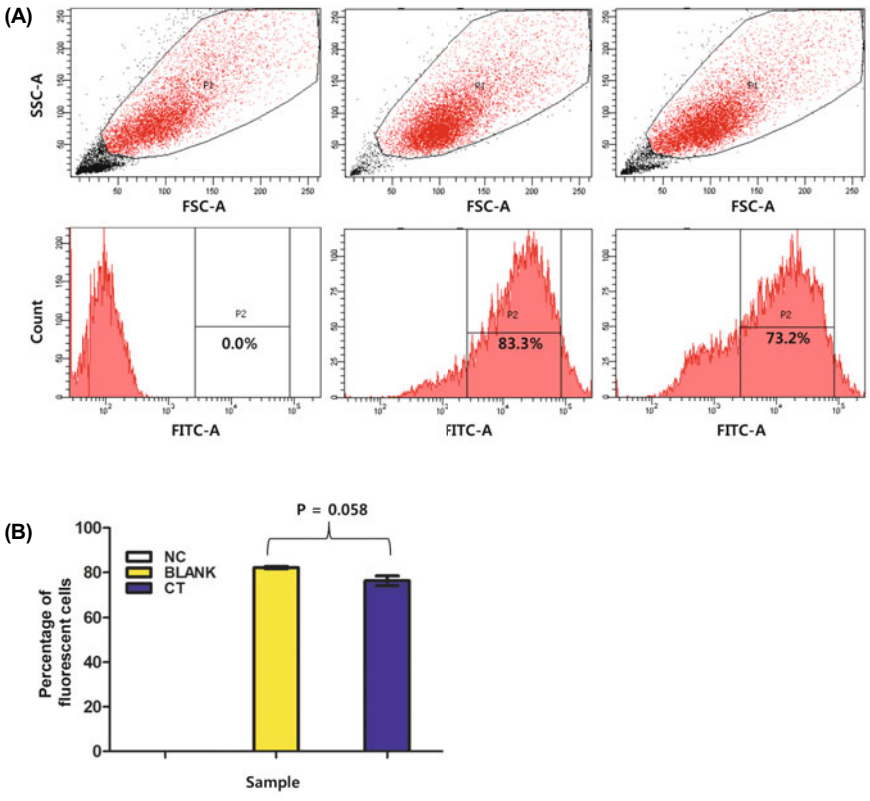

Fig. 1. Cytometric profile of KSHV latency in iSLK-BAC16 cells treated with CT extracts. iSLK-BAC16 cells were treated with CT extracts at 0.5 mg/ml and subjected to FACs analysis. (A) Fluorescein isothiocyanate (FITC) emission representing GFP expression in iSLK-BAC16 cells was measured four times by FACs Aria III. A representative FACs plot is shown in Fig. 1A. (B) Although it was statistically insignificant at $P<0.05$, the treatment with CT extracts in four trials reduced the average FITC emission from iSLK-BAC16 cells by 5.95%, indicating that KSHV latency was disturbed by the CT extract treatment. Blank-treated iSLK-puro and iSLK-BAC16 cells were used as negative and positive controls, respectively.

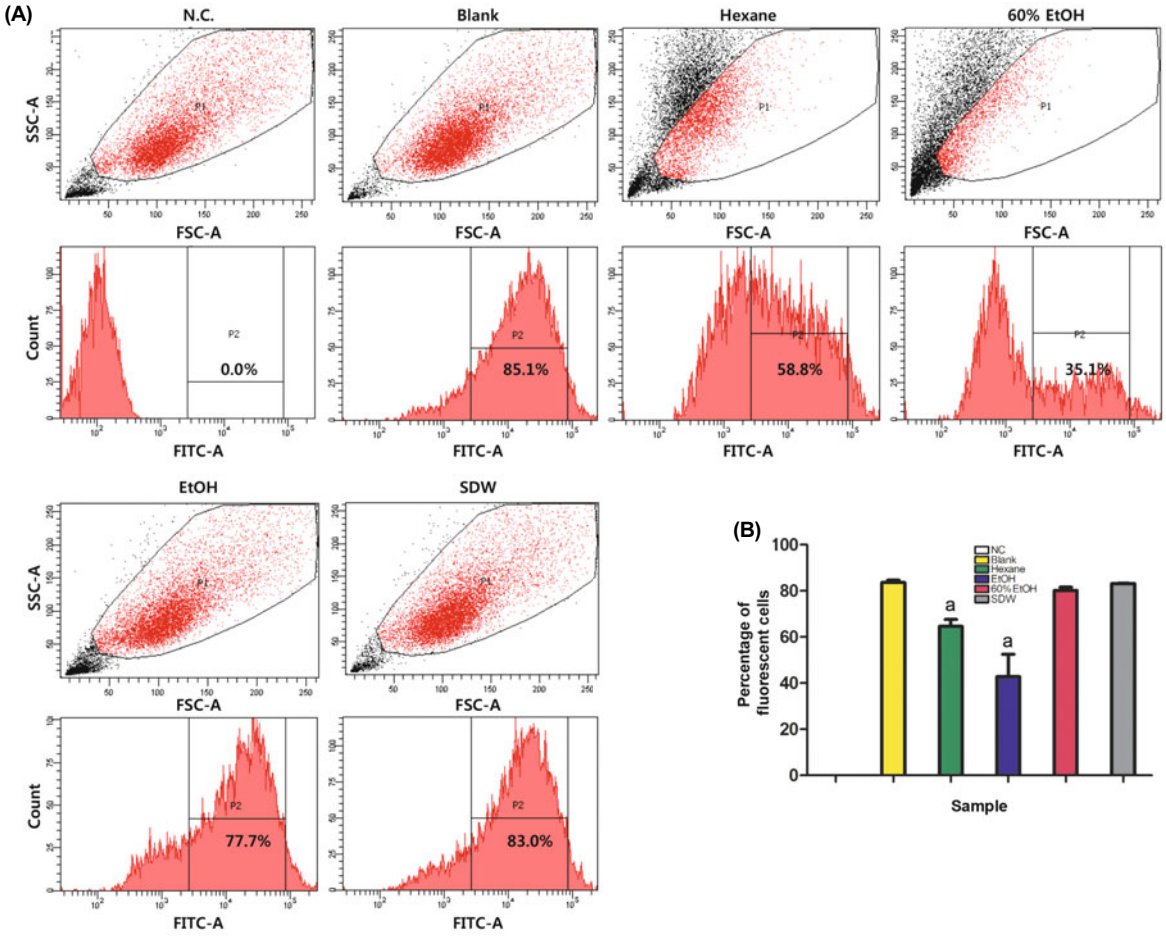

Fig. 2. Cytometric profile of KSHV latency in iSLK-BAC16 cells treated with each CT extract fraction. iSLK-BAC16 cells were treated with several CT extract fractions at 0.5 mg/ml and subjected to FACs analysis. The CT extract was fractionated by solvents such as n-Hexane, EtOH, 60% EtOH and water. iSLK-BAC16 cells were treated with each CT extract fraction and subjected to FACs analysis. FITC emission representing GFP expression was measured by FACs Aria III. (A) A representative FACs plot is shown in Fig. 2A. (B) FITC emission from iSLK-BAC16 cells was severely decreased by treatment with n-Hexane and EtOH fractions of CT extracts. These reductions were statistically significant at $P<0.05$. [a] Significantly different from those of the control group treated with blank at $P<0.05$.

reactivation upon viral lytic induction (Myoung and Ganem, 2011). iSLK cells, a derivative of SLK were generated by transfecting SLK cells with a doxycycline-inducible RTA transgene (Myoung and Ganem, 2011). KSHV viruses were derived from iSLK cells transfected with KSHV-BAC16 by inducing KSHV lytic replication. The resultant KSHV viruses could then infect iSLK cells, eventually generating the iSLK-BAC16 cell line (Brulois *et al.*, 2012). An intensive previous study with iSLK-BAC16 cells demonstrated that KSHV can establish its latency upon infection into iSLK cells and produce a high titer of KSHV virions after sodium butyrate treatment for lytic induction. Therefore, iSLK-BAC16 is a representative *in vitro* KSHV infection model. By measuring FITC emission from iSLK-BAC16 cells using FACS Aria III, the cytometric profile of KSHV latency was determined in iSLK-BAC16 cells treated with CT total extracts. Although it was not statistically significant at $P<0.05$, treatment with CT extracts slightly decreased KSHV latency by 5.97% compared to blank treatment (Fig. 1). Such decrease was also observed in the treatment with CT extracts at higher concentration (1.0 mg/ml) (Supplementary data Fig. S1). Next, in order to see which CT extract fraction had antiviral effects on the regulation of KSHV latency, CT extracts were fractionated using solvents such as n-Hexane, EtOH, 60% EtOH, and water. iSLK-BAC16 cells were treated with the resultant fractions, followed by FACS analysis. Interestingly, treatment with n-Hexane and EtOH fractions caused severe loss of FITC emission, representing GFP expression, in iSLK-BAC16. These losses were 19.1% with the n-Hexane fraction and 40.9% with the EtOH fraction with statistical significance, respectively. These results indicated that KSHV latency was strongly disrupted by the n-Hexane and EtOH fractions (Fig. 2). Even greater disruption was observed following treatment with n-Hexane and EtOH fractions of CT extracts at higher concentration (1.0 mg/ml) (Supplementary data Fig. S2). Vero rKSHV.219 cells were used in the fluorescent microscopic study of CT effect on KSHV latency. Vero rKSHV.219 cells, a Vero cell line latently infected with KSHV, tend to express GFP (green fluorescence protein) during latent replication and RFP (red fluorescence protein) during lytic replication, which provides a good indication

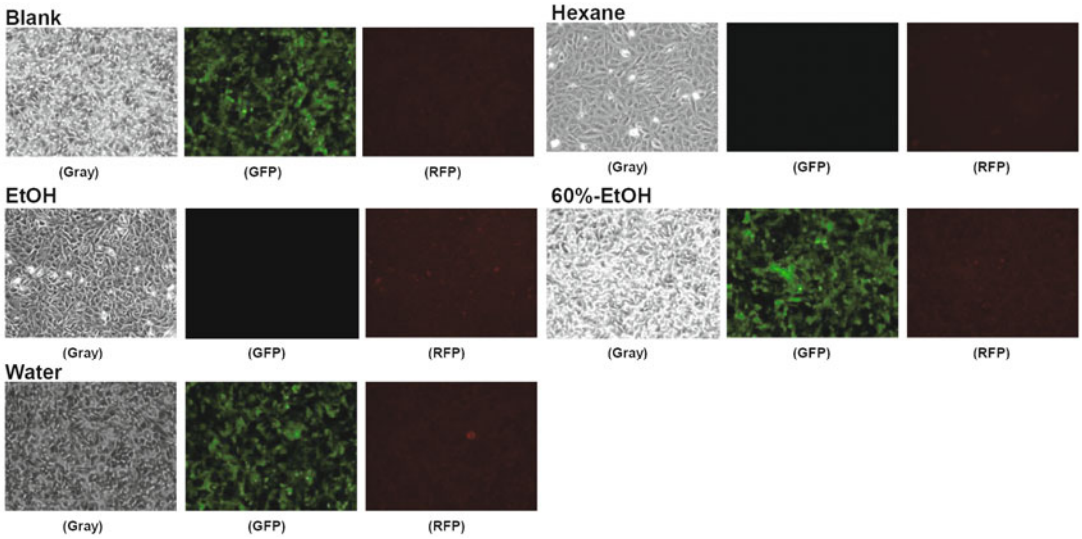

Fig. 3. Fluorescent microscopic assay of KSHV latency in Vero rKSHV.219 treated with each CT extract fraction. Vero rKSHV.219 cells produce GFP during KSHV latent replication and produce RFP during KSHV lytic replication (Vieira and O'Hearn, 2004). Vero rKSHV.219 cells were treated with each CT extract fraction at 0.5 mg/ml and analyzed for KSHV latency at 48 h post-treatment using fluorescent microscopy. N-Hexane and EtOH fractions significantly decreased GFP expression levels in Vero rKSHV.219 cells, indicating that KSHV latency was almost completely disrupted by the n-Hexane and EtOH fractions. RFP expression was not detected in any CT treatments.

of KSHV replication status (Vieira and O'Hearn, 2004). Vero rKSHV.219 cells were treated with each CT extract fraction to see which fractions produced the strongest damage to KSHV latency. As in iSLK-BAC16 cells, highly expressed GFP signals from KSHV latency in Vero rKSHV.219 were almost abolished by treatment with n-Hexane and EtOH fractions (Fig. 3). In contrast, other fractions did not induce any loss of GFP intensity. These results indicated that the n-Hexane and EtOH fractions of CT total extracts may contain biologically active antiviral components.

Induction of abnormal KSHV lytic reactivation by extracts from *C. tinctorius* L.

A previous study suggested that there is a strong co-relationship between GFP expression and KSHV latent replication (Vieira and O'Hearn, 2004). To investigate what CT extract fraction affects KSHV gene expression in iSLK-BAC16 cells, RT-qPCR assays were conducted with RNAs extracted from iSLK-BAC16 cells treated with each CT extract fraction. Interestingly, n-Hexane and EtOH fractions strongly increased the expression of KSHV lytic genes such as OFR50 (encoding RTA), ORF68, ORF69, ORFK14 (encoding vOX2), and ORF74 (encoding vGPCR). However, they moderately increased the expression of KSHV latent genes such as ORFK12 (encoding Kaposin), ORFmiRNA (encoding miRNA), ORF71 (encoding vFLIP), ORF72 (encoding vCyclin), and ORF73 (encoding LANA) (Fig. 4). The KSHV lytic gene induction seemed to be independent of KSHV RTA expression. The induction of LANA production was confirmed by

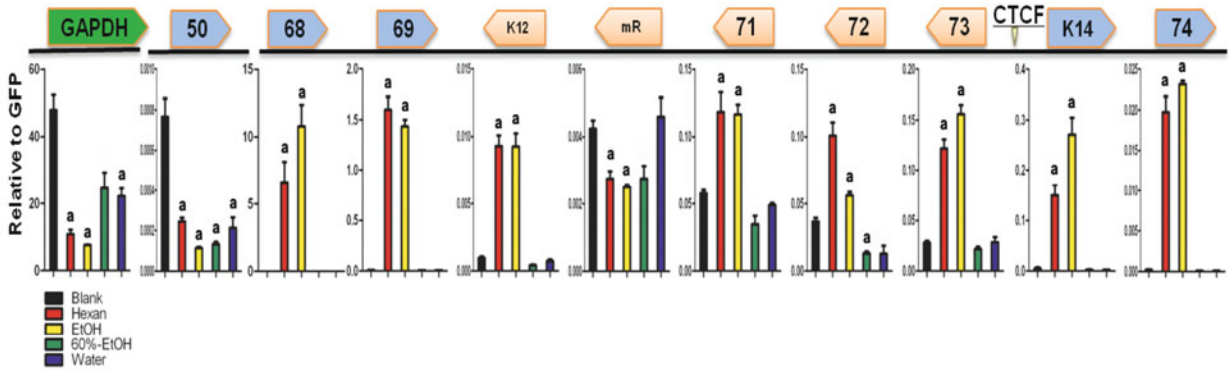

Fig. 4. Evaluation of effect of CT extract fractions on KSHV gene transcription in iSLK-BAC16 cells. iSLK-BAC16 cells were treated with each CT extract fraction at 0.5 mg/ml and the KSHV gene transcription profile was determined by RT-qPCR assay in 48 h post-treatment. N-Hexane and EtOH fractions vigorously induced transcription of KSHV lytic genes such as ORF68, ORF69, ORFK14 (Kaposin), and ORF74 (vGPCR) regardless of KSHV RTA induction. Transcription of KSHV latent genes such as ORF71 (vFLIP), ORF72 (vCyclin) and ORF73 (LANA) were also significantly enhanced, although the latent genes were not induced as strongly as lytic genes. [a]Significantly different from those of the control group treated with blank at $P<0.05$.

Western blotting analysis. Approximately 198 kDa LANA was highly induced by treatment with n-Hexane (Fig. 5). These results indicated that the n-Hexane and EtOH fractions contain antiviral ingredients that vigorously interrupt the KSHV gene expression.

Extracts from *C. tinctorius* L. prevent KSHV release from infected cells

It was also interesting to determine if the dysregulation of KSHV lytic gene expression caused any defect in production of viral progeny because KSHV lytic replication is required to produce KSHV virion. To identify which step of the KSHV life-cycle was disrupted by treatment with CT extract fractions, we measured intracellular and extracellular KSHV copy numbers in iSLK-BAC16 cells at 48 h post-treatment with CT extracts. Intracellular KSHV copy numbers were not affected by any CT extract fraction. In contrast, extracellular KSHV copy numbers were almost completely reduced by the EtOH or Water fractions, and approximately 50% reduced by the n-Hexane fraction (Fig. 6). These data suggest that EtOH or water fractions of CT extracts contain antiviral components capable of severely disrupting the release of KSHV virions from iSLK-BAC16 cells.

Disturbance of cell-cycle by extracts from *C. tinctorius* L.

In addition to antiviral effect on KSHV, it was necessary to test if treatments with CT extract fractions significantly affected the viral host cells because the removal of KSHV from its host cells leads to death of host cells (Ganem, 2006). In order to determine which CT extract fraction affected the cell-cycle of iSLK-BAC16 cells, we analyzed the cell-cycle progress of iSLK-BAC16 cells treated with each fraction. The analysis revealed that the n-Hexane and EtOH fractions caused arrest at S and G2/M phase of cell-cycle (Fig. 7). Other fractions appeared to have no effect on the cell-cycle progress of iSLK-BAC16 cells. These results suggested that the n-Hexane and EtOH fractions contain bioactive compounds that dysregulate the cell-cycle of KSHV latently infected iSLK-BAC16 cells.

Cytotoxicity caused by extracts from *C. tinctorius* L.

FACs analysis revealed that treatment with the n-Hexane and EtOH fractions of CT extracts caused significant cytotoxicity in iSLK-BAC16 cells. Thus it was necessary to quantitatively determine the cellular cytotoxic effects caused by CT

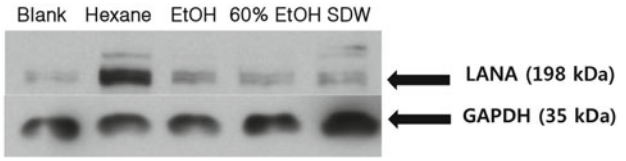

Fig. 5. Evaluation of effect of CT extract factions on KSHV protein production in iSLK-BAC16 cells. iSLK-BAC16 cells were treated with each CT extract fraction at 0.5 mg/ml and KSHV protein production was assessed at 48 h post-treatment by Western blotting assay. N-Hexane fraction vigorously induced 198 kDa LANA protein, relative to 35 kDa GAPDH (internal control) production.

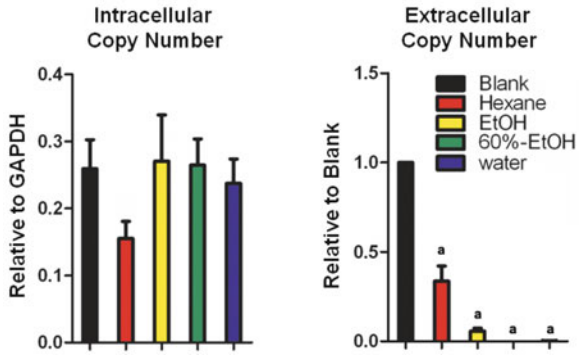

Fig. 6. Evaluation of effect of CT extract fractions on KSHV virion production in iSLK-BAC16 cells. iSLK-BAC16 cells were treated with each CT extract fraction at 0.5 mg/ml, and KSHV virion production was assessed at 48 h post-treatment. Relative intracellular and extracellular KSHV genome copy numbers were measured as previously described. Briefly, intracellular KSHV genome copy number and total genomic DNA (gDNA)s were extracted from iSLK-BAC16, and the relative proportion of KSHV viral genome in gDNAs was assessed using a quantitative PCR (qPCR) assay. C_T values from qPCR assay with a GAPDH primer set indicate relative amounts of total gDNA and those with a KSHV ORF50 primer set represent relative KSHV viral genome amounts. Intracellular KSHV genome copy numbers were not significantly affected by treatment with each CT extract fraction except n-Hexane. KSHV virion genomic DNAs in culture media released from iSLK-BAC16 cells were considered as extracellular KSHV genome copy numbers and were determined by qPCR with KSHV ORF50. The extracellular KSHV genome copy numbers from iSLK-BAC16 cells treated with each fraction were defined relative to that of blank-treated iSLK-BAC16 cells. All fractions except n-Hexane showed strong reduction of extracellular KSHV virion amounts. [a] Significantly different from those of the control group treated with blank at $P<0.05$.

extracts. The n-Hexane and EtOH fractions showed strong cytotoxicity in iSLK-BAC16 cells, while other fractions did not (Fig. 8). These results indicate that the n-Hexane and EtOH fractions contain compounds that induce cell death in iSLK-BAC16 cells.

Induction of apoptosis by extracts from *C. tinctorius* L.

It was reasonable to test if apoptosis contributes to the severe cytotoxicity upon treatment with the n-Hexane and EtOH fractions of CT extracts. Caspase 3 activity was measured to see if each CT extract fraction induced apoptosis in iSLK-BAC16 cells (Fig. 9). Caspase 3 activity was significantly increased when iSLK-BAC16 cells were treated with the 60% EtOH and water fractions, but it was significantly decreased when iSLK-BAC16 cells were treated with the n-Hexane and EtOH fractions. This suggests that the n-Hexane and EtOH fractions do not contain bioactive compounds, but that the 60% EtOH and water fractions contain compounds that are capable of inducing apoptosis.

Discussion

In our study, we demonstrated that CT extracts have antiviral activity against KSHV infection. CT extracts not only disrupted KSHV latency by interrupting the regulation of KSHV latent and lytic gene expression, but also inhibited

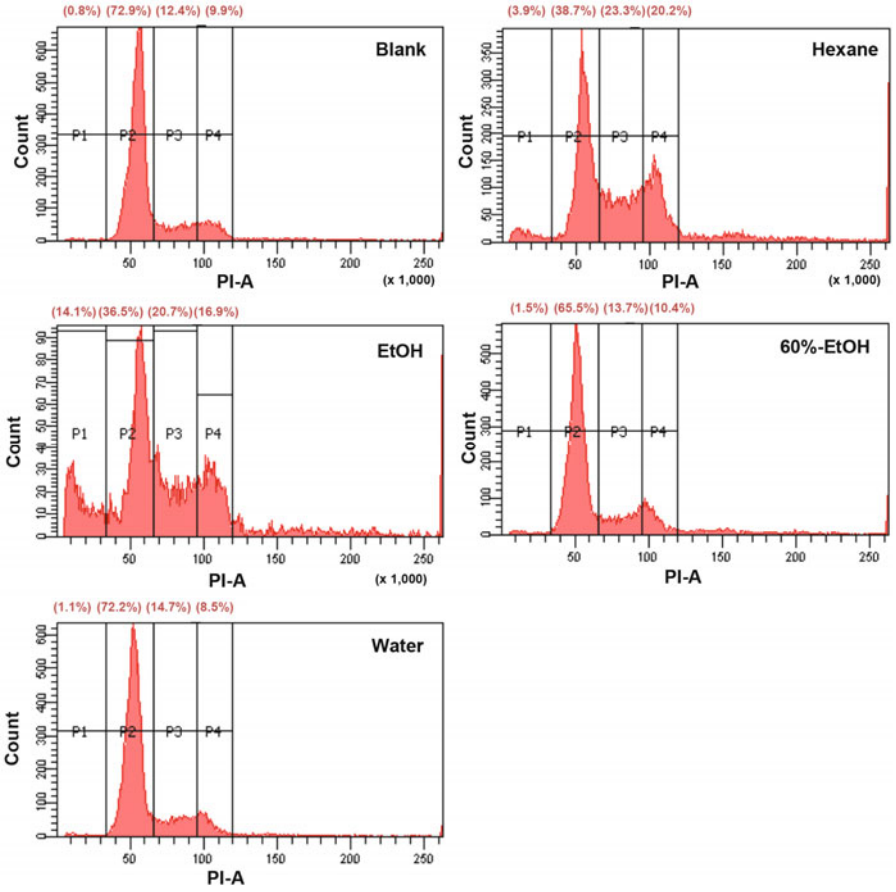

Fig. 7. Evaluation of effect of CT extract fractions on cell cycle progress of KSHV latently infected host cells. iSLK-BAC16 cells were treated with each CT extract fraction at 0.5 mg/ml, stained with propidium iodide (PI) solution at 48 h post-treatment, and then applied to cell cycle analysis using FACS ARIA III. N-Hexane and EtOH fractions caused severe dysregulation of cell cycle progress in iSLK-BAC16 cells.

KSHV release from infected host cells. In addition, host cells infected with KSHV were also affected by CT extracts. The cell-cycle of the infected host cells was disturbed by extending S and G2/M phase upon treatment with CT extract. The cytotoxicity in host cells was also significantly enhanced, and cellular apoptosis was also affected by CT extracts. We further analyzed which fractions of CT extracts exhibited antiviral activities and found that n-Hexane and EtOH fractions had high antiviral activities.

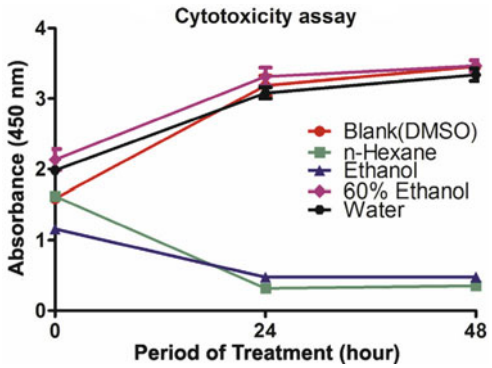

Fig. 8. Evaluation of effect of CT extract fractions on cytotoxicity of KSHV latently infected host cells. Cytotoxicity assays in iSLK-BAC16 cells treated with CT extract fractions were conducted with Cell Counting Kit-8 (CCK-8) (Dojindo). Briefly, 100 μl of 1×10^4 cell suspension was seeded per well and the cells were treated the following day with each CT extract fraction at 0.5 mg/ml. The treatment was extended for 24 h or 48 h. 10 μl of Cell Counting Kit-8 (CCK-8) (Dojindo) was added to each sample, further incubated for 3 h, and then samples were applied to an ELISA reader to read the absorbance of cell suspensions. N-Hexane and EtOH fractions showed strong cytotoxicity in iSLK-BAC16 cells relative to other fractions, including internal control.

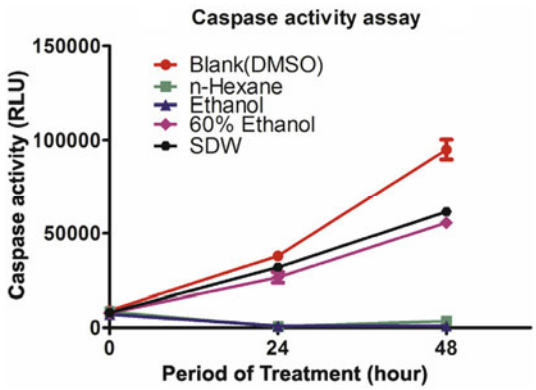

Fig. 9. Evaluation of apoptosis induction by CT extract fractions in KSHV latently infected host cells. To determine if treatment with CT extracts at 0.5 mg/ml causes apoptosis in iSLK-BAC16 cells, the caspase 3 activity in iSLK-BAC16 cells was measured at 0 h, 24 h and 48 h post-treatment using Caspase Glo 3/7 kit (Promega). Treatments with the n-Hexane and EtOH fractions suppressed apoptosis induction, while those of 60% EtOH and water fractions enhanced apoptosis induction.

CT extracts vigorously induced KSHV lytic gene expression rather than latent replication, which seemed to be independent of KSHV RTA induction. Many lytic genes were strongly expressed, but few latent genes were. Such lytic gene induction did not lead to the production of KSHV progeny virions, which suggested that lytic gene expression in the absence of RTA induction poorly orchestrates KSHV virion assembly. Thus, functional progeny virions would not be produced. The KSHV gene induction by CT extracts may generate factors that are required to maintain the intracellular KSHV episome in the host nucleus, but not enough to produce all other factors necessary to make infectious KSHV virion that are capable of extracellular release. We propose that one possible mechanism of antiviral activity by CT extract is disruption of the KSHV gene expression system through abnormal induction of KSHV lytic genes.

Glycyrrhizic acid (GA) is known to prevent KSHV from expressing the viral latent genes, but does not cause any defect in KSHV lytic gene expression (Curreli *et al*., 2005; Kang and Lieberman, 2011). Treatment with GA on BCBL1 cells (KSHV latently infected B-cell lymphoma) disturbs the production of KSHV major latent transcripts and in turn blocks the establishment of KSHV latency. One important feature of GA treatment is to remove KSHV genome from host cells that are latently infected with KSHV. On the other hand, treatment with n-Hexane and EtOH fractions strongly induced the expression of most KSHV lytic genes, including some latent genes, and caused severe defects in the release of KSHV virions from host cells. Thus, n-Hexane and EtOH fractions contain important antiviral compounds that are similar to GA, which can eradicate functional KSHV genomes from latently infected host cells.

Cellular populations at S and G2/M phases were significantly increased by treatment with CT extracts. Based on previous reports (Cook and Martin, 1986), this increase is likely due to the malfunction of G1, G2 and metaphase checkpoints. There are at least three possible mechanisms of cell-cycle dysregulation by CT extracts (Ciosk *et al*., 1998; Peters, 1998). First, the n-Hexane and EtOH fractions of CT extract might prevent the CDK (cyclin-dependent kinase) inhibitor p16 from inhibiting another CDK from binding to its cyclin (D), which leads to S phase entry without going through the G1 checkpoint. Secondly, the n-Hexane and EtOH fractions might disrupt the action of a maturation promoting factor (MPF), which results in accumulation of cell population at G2 phase. Third, the n-Hexane and EtOH fractions might disturb the inhibitory role of anaphase-promoting complex and hold the cell population at metaphase without reentering the G1 phase. Among these three potential mechanisms, the treatment of n-Hexane and EtOH fractions is likely to result in both (1) functional resistance to cell cycle inhibitors and (2) pRb inactivation at G1 checkpoint due to induction of LANA and vCyclin. LANA is able to inactivate pRb by binding it, and vCyclin complexed with CDK6 (cycline dependent kinase 6) also inactivates pRb by phosphorylating it (Chang *et al*., 1996; Ballestas *et al*., 1999). However, further study is required to dissect the mechanism of cell-cycle dysregulation by CT extracts.

The treatment with n-Hexane and EtOH fractions of CT extract also enhanced cytotoxicity in KSHV latently infected host cells. This might be due to KSHV abnormal lytic reactivation, which leads to the production of some viral gene products that stimulate host cell-death by either necrosis or apoptosis. Lytic infection often triggers apoptosis in host cells by activating both extrinsic and intrinsic apoptotic pathways. KSHV encodes a negative regulator of NF-kB pathways (Brown *et al*., 2003; Seo *et al*., 2004). vIRF3 is reported to bind and inhibit the IKKβ that activates the NF-kB pathway (Seo *et al*., 2004). This inhibition prevents KSHV anti-apoptotic factors from blocking apoptosis of KSHV-infected host cells. CT extracts might prevent host cells from producing such anti-apoptotic factors, which eventually results in anti-apoptosis and increases the cytotoxicity in the host cells. However, it is difficult to exclude other possible mechanisms for CT extracts that may increase cytotoxicity in iSLK-BAC16 cells. Indeed, due to the suppression of caspase 3 activity, direct killing by the n-Hexane and EtOH fractions may also significantly contribute to cellular cytotoxicity. Further studies should be conducted to determine the cause of cellular cytotoxicity by CT extracts.

In summary, our study found that CT extracts effectively disturb KSHV latency and induce cytotoxicity in KSHV-infected host cells. In particular, the n-Hexane and EtOH fractions showed strong antiviral activity as well as cytotoxicity, suggesting that these fractions contain naturally bioactive compounds that inhibit gammaherpesviral infections.

Future studies will focus on identifying single compounds from the n-Hexane and EtOH fractions of CT extract. Upon purification, such compounds will be tested for anti-gammaherpesviral activities using both *in vitro* and *in vivo* models.

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