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Efficacy of orally administered *Lobelia chinensis* extracts on herpes simplex virus type 1 infection in BALB/c mice

Yuh-Chi Kuo[a,*], Yuh-Chi Lee[a], Yann-Lii Leu[b], Wei-Jern Tsai[c,d], Shi-Chuan Chang[e,f]

[a] *Institute of Life Science, Fu-Jen University, Taipei, Taiwan, ROC*
[b] *Graduate Institute of Natural Products, Chang-Gung University, Taipei, Taiwan, ROC*
[c] *National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC*
[d] *National Tai-Tung University, Taipei, Taiwan, ROC*
[e] *Institute of Emergency and Critical Care Medicine, National Yang-Ming University, Taipei, Taiwan, ROC*
[f] *Chest Department, Taipei Veterans General Hospital, Taipei, Taiwan, ROC*

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ABSTRACT

By a plaque reduction assay, methanolic extracts from *Lobelia chinensis* (LC) significantly blocked herpes simplex virus type 1 (HSV-1) replication in HeLa cells without apparent cytotoxicity. The 50% inhibitory concentration (IC_{50}) of LC on HSV-1 replication is 139.2 μg/ml. To elucidate LC anti-HSV-1 activity *in vivo*, BALB/c mice were injected subcutaneously with HSV-1 (2.5×10^6 PFU/50 μl), treated orally thrice a day with acyclovir (60 mg/kg/dose) or LC (20 and 50 mg/kg/dose) for 7 days, and inspected daily for signs of disease. Data from the scoring system indicated that animals infected with HSV-1, developed progressive zoster lesions starting 2 days postinfection (p.i.) and appeared the most serious syndromes at 4–5 days p.i. In marked contrast to the results with control mice, treatment with acyclovir or 50 mg/kg/dose LC resulted in a sustained protective effect. The HSV-1 titers and DNA levels in ground skin samples were significantly reduced by LC. No toxic effect of LC on liver and kidney functions was apparent. These results indicated that LC was a potent inhibitor of the *in vitro* and *in vivo* replication of HSV-1.

1. Introduction

Herpes simplex virus type 1 (HSV-1) causes a variety of infections in human (Roizman and Sears, 1996). The recipients of organ transplantation and immunocompromised or cancer patients are at high risk for increased severity of HSV-1 infection (Waggoner-Fountain and Grossman, 2004; Stranska et al., 2004). In addition, HSV-1 and HSV-2 have been shown to be factors for spreading of human immunodeficiency virus (Chen et al., 1998; Mann et al., 1984; Corey et al., 2004). Nucleoside analogues have been extensively investigated in the search for effective antiherpesvirus agents (Darby, 1994; Imai et al., 2004). Among these acyclovir is widely used for the systemic treatment of HSV infections. However, it has been observed that the acyclovir-resistant HSV infection has come from immunocompromised patients such as transplant and AIDS patients (Coen, 1996; Kimberlin and Whitley, 1996; Chibo et al., 2004). Therefore, it is of interest to develop new anti-HSV agents that substitute for or complement acyclovir.

Chinese herbs are potential sources of useful edible and medicinal plants. Parts of them are used as functional foods because of their immunomodulatory, antitumor, and antiviral functions (Chen et al., 2007; Kuo et al., 2001; Kuo et al., 2006). However, ethnopharmacology provides scientists with an alternative approach for the discovery of antiviral agents. There has been a promising result of a naturally occurring antiherpetic agent, *n*-docosanol, which has been approved by the US Food and Drug Administration as a topical treatment for herpes labialis (Alrabiah and Sacks, 1996; Pope et al., 1996; Sacks et al., 2001). We have proved that samarangenin B from *Limoninum sinense* blocks HSV-1 replication by the inhibition of viral macromolecular synthesis (Kuo et al., 2002a,b). These findings show that natural products are still potential sources in the search for new antiherpetic agents.

One successful replication cycle of HSV-1 is dependent upon the completion of a number of steps, including expression of viral genes such as glycoprotein B (gB) and gC, and DNA replication (Roizman and Sears, 1996). Inhibition of any of these stages blocks HSV-1 replication. In the present study, *Lobelia chinensis* (LC), which is a widely known folk medicine for the treatment of viral infection (Shibano et al., 2001), was selected for an anti-HSV-1 replication assay *in vitro* and *in vivo*. The plaque reduction assay offers an *in vitro* system to evaluate the effect of methanolic extracts from LC against HSV-1. In assessing the effectiveness of LC

* Corresponding author at: LS212, Laboratory of Molecular Pharmacology, Institute of Life Science, Fu-Jen University, No. 510, Chung-Cheng Road, Hsinchuang, Taipei Hsien 242, Taiwan, ROC. Tel.: +886 2 29053591; fax: +886 2 29052193.
E-mail address: 021553@mail.fju.edu.tw (Y.-C. Kuo).

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in vivo, here the protective effect of extracts in a murine model of subcutaneous HSV-1 infection was evaluated and compared with acyclovir.

2. Materials and methods

2.1. Preparation of crude extracts for L. chinensis

L. chinensis was purchased from Chinese medicine shops in Taipei. They were identified by Dr. Yann-Lii Leu, resident medicinal-plant expert of Graduate Institute of Natural Products, Chang-Gung University. The dried *L. chinensis* (100 g) was extracted with methanol (1 l × 3). After removing solvent, the methanolic extracts of LC were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml and stored at 4 °C until for use.

2.2. Cell culture and viruses

HSV-1 (KOS strain, VR-1493, ATCC) was propagated and titrated in HeLa cells as previously described (Kuo et al., 2006). The virus stocks were stored at −80 °C for use.

2.3. Plaque reduction assay

The assay followed procedures described previously (Kuo et al., 2001). HeLa cells (3.5×10^5 per dish) were overlaid with LC (9.4–200 μg/ml) or acyclovir (2 μg/ml) and 100 plaque forming units (PFU) of HSV-1 were added to each dish. Then 1% methylcellulose was added to each well at 1 h p.i. and the virus plaques formed in HeLa cells were counted by crystal violet staining at 5 days p.i. The activities of LC and acyclovir for inhibition of plaque formation were calculated.

2.4. Determination of cell viability

Approximately 3.5×10^5 HeLa cells were incubated with 0.1% DMSO, or 9.4–200 μg/ml of LC for 5 days. The cell viability was determined with the help of a hemocytometer following staining by trypan blue. The cell viability was also evaluated as lactate dehydrogenase (LDH) release according to the procedures described previously (Kuo et al., 2006). LDH activity in mU per ml, where 1 mU is the amount of enzyme required to transform 0.0167 nM nicotinamide adenine dinucleotide (NAD) per min, was determined.

2.5. Animals and virus inoculation

All animal studies were approved by the IACUC of Fu-Jen University. Six-week-old BALB/c mice were used in all studies and purchased from Experimental Animal Center of National Taiwan University, Taipei. Each group of animals contained six mice with an average weight of 19.5 ± 0.5 g/mouse at the beginning of the study. Mice were anesthetized by pentobarbital (50 mg/kg), clipped to remove hair, and subcutaneously injected with 50 μl suspension of HSV-1 containing 2.5×10^6 PFU at the left flank (Kurokawa et al., 1993). LC (20 or 50 mg/kg/dose) or acyclovir (60 mg/kg/dose; Calbiochem, Germany) were administered orally thrice a day for seven consecutive days, starting 4 h p.i. The dose intervals were equally spaced through 24 h. The mice were inspected daily, and disease severity was determined with the following scoring system: 0, no signs of infection visible; 1, vesicle formation; 2, slight zoster spread; 3, formation of large patches of zoster; 4, confluent zoster band; 5, hind limb paralysis; 6, death.

2.6. Titration of virus in skin tissues

The method was modified from the procedures described previously (Betz et al., 2002). Skin samples from control or HSV-1 infected BALB/c mice sacrificed at 7 days p.i. were ground with a glass homogenizer in 500 μl of MEM on ice and frozen-thawed for 3×. The debris was removed by centrifugation, and the supernatants were applied in serial dilutions to HeLa cell monolayers in 6-well plates. After adsorption for 1 h, the cells were covered with 1% methylcellulose. Virus plaques were counted after 5 days of incubation. The sensitivity for this assay is about from 10 to 10^9 PFU/ml.

2.7. Extraction of total cellular and tissue DNA

Cellular or tissue DNA was extracted by the method described previously (Kuo et al., 2006). HeLa cells (5×10^6) were infected with or without three multiplicity of infection (m.o.i.) of HSV-1 in the presence or absence of LC (9.4, 75, and 200 μg/ml) or acyclovir (2 μg/ml) and harvested at 24 h p.i. The DNA solutions were extracted with phenol–chloroform and their concentrations were determined by measuring the optical density at 260 nm.

2.8. Polymerase chain reaction (PCR)

The method has been described elsewhere (Kuo et al., 2002a,b). Briefly, 10 μl of total cellular DNA (100 ng) was mixed with 0.75 μM primers, 4 units of Taq polymerase, and 10 μl of reaction buffer in a total volume of 50 μl. The oligonucleotide primer pairs for HSV-1 ICP4 and β-actin were forward 5′-CCCGCCGATGCTGCCCTAAAC-3′; reverse 3′-TTCGCCAGACACCTACTCAAG-5′ (670 bp) and forward 5′-TTGAGACCTTCAACACCC-3′; reverse 3′-CTCTACTGAAGCTTTTCGACT (1300 bp), respectively (Cone et al., 1991). The amplified products were quantitated using laser scanning densitometer SLR-2D/1D (Biomed Instruments Inc., Fullerton, CA) within the linear range.

2.9. Real-time quantitative PCR

Total cellular or tissue DNA (100 ng) was added to SYBR Green Ready Start Taq polymerase mixture (Sigma–Aldrich) with forward and reverse primers for ICP4. The reaction conditions were 50 °C for 2 min following by 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Samples were analyzed on a StepOne™ real-time PCR System (Applied Biosystems, Foster City, CA). Viral DNA copies were quantified by a standard curve generated from purified HSV-1 DNA. The ICP4 primer set and real-time PCR system for detecting HSV-1 DNA, as determined by ABI sequence Detector Software v.1.7.

2.10. Determination of liver and kidney functions

After treatment for 1 week, serum samples were collected from BALB/c mice and assayed liver functions including asparate aminotransferase (AST) and alanine aminotransferase (ALT) concentration with GOT/AST-PIII and GPT/ALT-PIII kits (Fujifilm, Japan), respectively. The kidney functions, blood urea nitrogen (BUN) and creatinine (CRE), were determined by BUN-PIII and CRE-PIII kits (Fujifilm, Japan), respectively. The results were measured by Colorimetric Analyzer (DRI-CHEM 3000).

2.11. Statistical analysis

Data were presented as mean ± S.D. and the differences between groups were assessed with two-tailed Student's *t*-test. *P*-value of less than 0.05 was considered statistically significant.

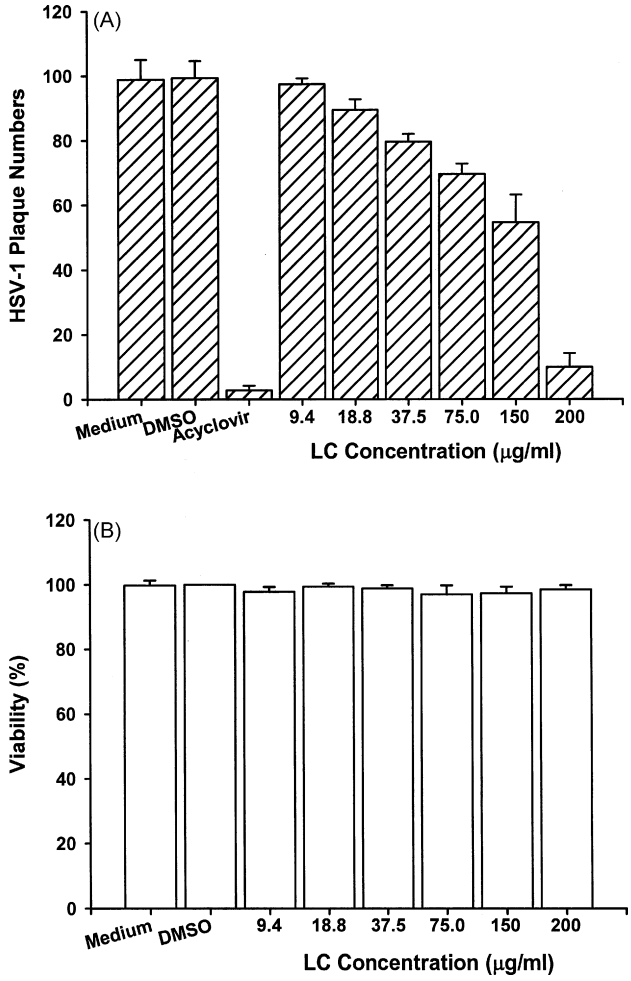

Fig. 1. Effects of LC on HSV-1 replication and HeLa cells viability. (A) Inhibitory effects of 9.4–200 μg/ml LC, 0.1% DMSO, and 2 μg/ml acyclovir on HSV-1 replication in HeLa cells were determined by a plaque reduction assay. Each bar represents the mean ± S.D. of three independent experiments. (B) HeLa cells (3.5×10^5 in 25 cm^2 flasks) were treated with medium, 0.1% DMSO, or indicated concentration of LC for 5 days. Then total, viable, and non-viable cell number were counted after stained by trypan blue.

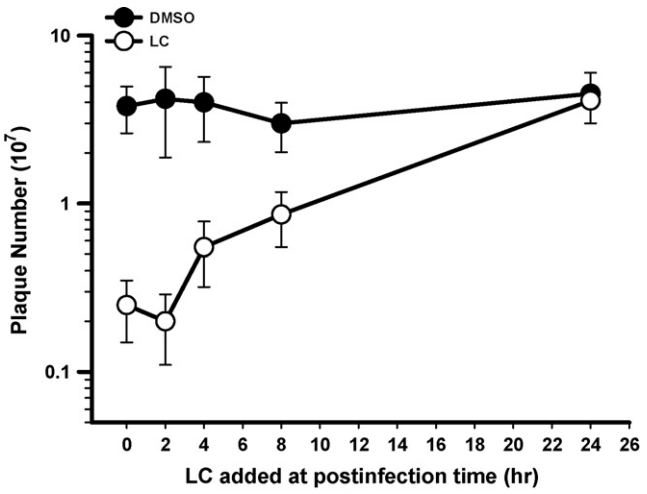

Fig. 2. Time of addition assay. HeLa cells (5×10^6) were infected with HSV-1 (3 m.o.i.) and LC (200 μg/ml) was added at the indicated time. Cell supernatants were collected at 25 h p.i. and HSV-1 titers were determined by plaque forming assay. Each point represents the mean ± S.D. of three independent experiments.

3. Results

3.1. The LC inhibits HSV-1 replication

To determine the antiviral activity of LC, different concentrations of LC (9.4–200 μg/ml) were evaluated for their effects on inhibiting HSV-1 plaque formation in HeLa cells. As shown in Fig. 1A, HSV-1 replication was not affected by DMSO treatment and acyclovir blocked HSV-1 plaque formation in HeLa. The results indicated that LC impaired HSV-1 replication in HeLa cells with a dosage-dependent manner. The 50% inhibitory concentration (IC_{50}) of LC on HSV-1 replication was 139.2 μg/ml.

3.2. Effects of LC on viability of HeLa cells

To delineate whether the inhibitory effect of LC on HSV-1 replication was related to cytotoxicity, we examined the viability of HeLa cells after treated with LC for 5 days. The DMSO did not affect the cell viability. Comparison with control groups, the viabilities of HeLa cells treated with various concentrations of LC were not significantly decreased (Fig. 1B). Moreover, the cytotoxic effect of higher concentration LC on HeLa cells was evaluated as LDH release. The data indicated that LDH release from HeLa cells was not different in the presence or absence of 200 and 400 μg/ml LC for 5 days (3.89 ± 1.0 mU/ml vs. 4.04 ± 1.8 mU/ml; 3.89 ± 1.0 mU/ml vs. 3.93 ± 0.9 mU/ml). These results demonstrated that inhibitory mechanisms of LC on HSV-1 replication were not due to cytotoxicity. Additionally, to evaluate whether LC had cytotoxic effects on normal diploid cells, human peripheral blood mononuclear cells (PBMC) were used as target cells and effects of LC on viability of PBMC were analyzed. Results showed that LC did not affect viability of PBMC and its therapeutic index was about 22.5.

3.3. Effects of time of LC addition on HSV-1 production

Time course experiments were performed to determine at what stage in replication process LC inhibited HSV-1 replication. LC (200 μg/ml) was added with HSV-1 at the same time (0 h p.i.) or added to the cultures at 2, 4, 8, and 24 h p.i. The cell supernatants were collected at 25 h p.i., and virus titers were determind by the plaque forming assay. Results showed that inhibitory effects of LC added to the cells at 2–4 h p.i. are similar to that obtained by adding LC and virus simultaneously (Fig. 2). A certain inhibition was also detected when LC was added to cells at 8 h p.i. The loss of inhibitory activity when LC was added at 24 h p.i. Results obtained demonstrated the addition of LC between 0 and 8 h significantly decreased HSV-1 replication.

3.4. LC decreased HSV-1 DNA synthesis in HeLa cells

We further defined whether LC (9.4, 75, 200 μg/ml) had any effects on HSV-1 DNA synthesis in HeLa cells. After HSV-1 adsorption, the cellular DNA was harvested at 24 h p.i. and viral DNA was analyzed by PCR. As shown in Fig. 3A, although HSV-1 DNA could not be detected in uninfected HeLa cells (Lane 1), the HSV-1 DNA synthesis was increased in HSV-1 infected cells (Lane 6). Acyclovir (Lane 10), 75 μg/ml (Lane 8) and 200 μg/ml (Lane 9) of LC treatment reduced the viral DNA synthesis in HeLa cells. Graphical representation of the ratio of HSV-1 to β-actin DNA showed that increasing of signal in HSV-1 infected cells was significantly reduced by 75 μg/ml ($P < 0.05$) and 200 μg/ml ($P < 0.01$) LC and acyclovir ($P < 0.01$). Furthermore, using real-time PCR, the copy number of HSV-1 DNA was evaluated in untreated cells and cells treated with acyclovir or LC. Fig. 3B shows that treatment with 75 μg/ml

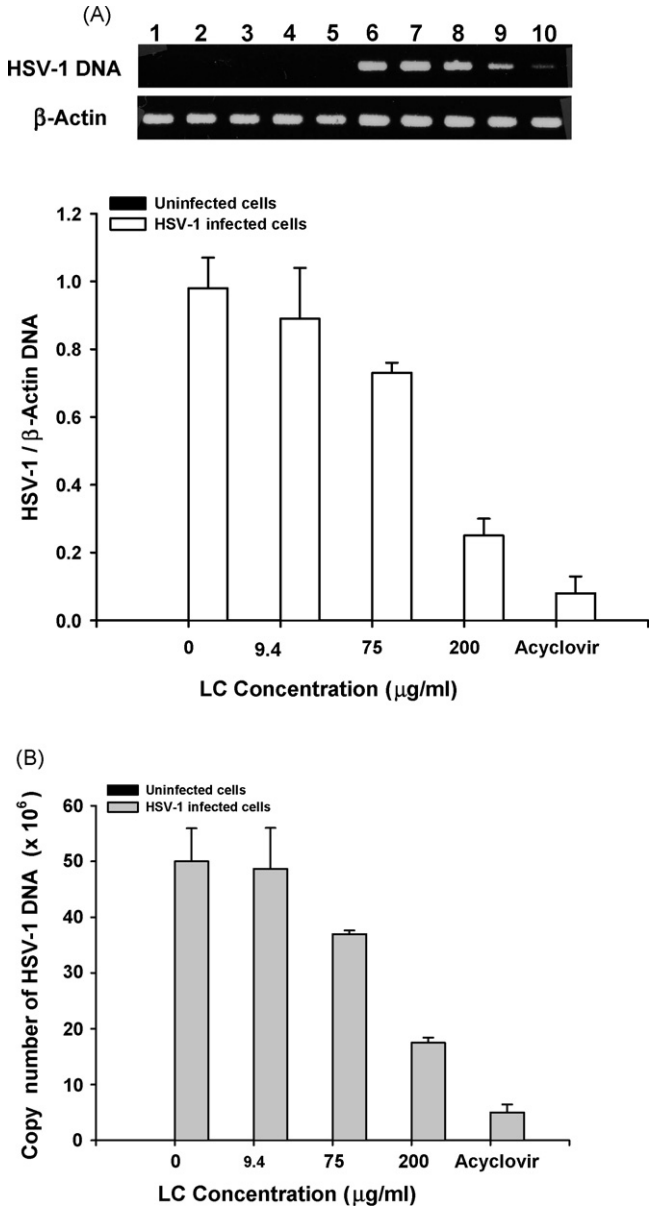

Fig. 3. LC-affected HSV-1 DNA synthesis in HeLa cells detected by PCR and real-time PCR. HeLa cells (5×10^6) were infected w/o HSV-1 (3 m.o.i.) in the absence or presence of LC (9.4, 75, 200 μg/ml) or acyclovir (2 μg/ml). Total cellular DNA was extracted at 24 h p.i. and the PCR was done as described in Section 2. (A) Following the reaction, the amplified products were run on 2% agarose gel. Lanes 1–5 indicated the data from uninfected cells incubated with DMSO (0.1%), LC (9.4, 75, 200 μg/ml), or acyclovir, respectively. Lanes 6–10 indicated the data from infected cells treated with DMSO, LC (9.4, 75, 200 μg/ml), or acyclovir, respectively. Each band was quantitated by densitometer and the ratio of HSV-1 DNA to β-actin DNA was calculated. (B) Real-time PCR was employed to calculate HSV-1 genome copy number in uninfected and LC or acyclovir treated cells. Each bar represents the mean ± S.D. of three independent experiments.

($P < 0.05$) and 200 μg/ml ($P < 0.01$) LC or acyclovir ($P < 0.01$) negatively affects HSV-1 DNA copy number. These results suggested that blocking of HSV-1 replication by LC impairments of HSV-1 DNA synthesis in HeLa cells could not be excluded out.

3.5. Effectiveness of LC time treatment compared to acyclovir in vivo

Initially, BALB/c mice were subcutaneously injected with HSV-1 at 5×10^5, 1.25×10^6, or 2.5×10^6 PFU and observed for 7 days for

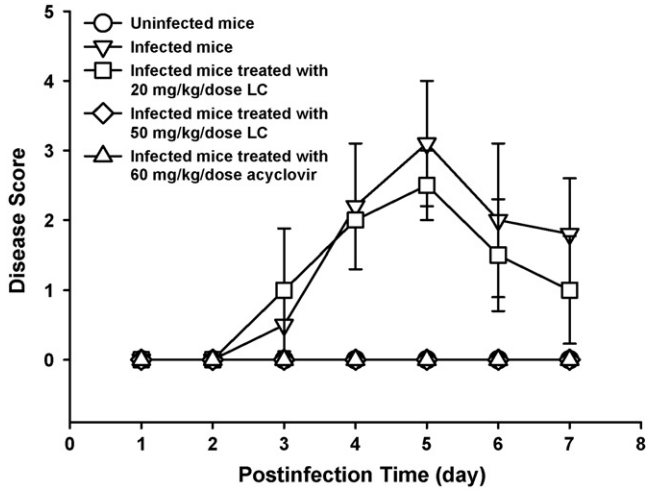

Fig. 4. Therapeutic effects of LC on HSV-1 skin lesions in BALB/c mice. The skin of 6-week BALB/c mice were infected with 2.5×10^6 PFU HSV-1 and oral treatment with LC (20, 50 mg/kg/dose) or acyclovir (60 mg/kg/dose) three times a day for 1 week. The severity of skin lesion was scored at various time points. Each point represents the average value of 10 mice per group ± S.D.

lesion development. When 5×10^5 PFU HSV-1 was used to initiate infection, mice did not exhibit herpes lesions in the skin within the 7-day time range. Typical herpes lesions significantly appeared on the 2.5×10^6 and 1.25×10^6 PFU HSV-1 infected skin on the 2nd and 4th day of infection, respectively, when compared to the control mice ($P < 0.05$). The lesions will be self-limited at 7-day p.i. (data not shown). We also used higher titer 1×10^7 PFU to infect mice but it easily caused mice death at 2nd day p.i. To assay the effectiveness of LC *in vivo*, mice were infected with a low challenge of HSV-1 (2.5×10^6 PFU) and treated orally with LC or acyclovir thrice a day for seven consecutive days and lesion development in the mice was scored daily. Results in Fig. 4 indicated that the lesion scores for mice that received 20 mg/kg/dose LC or no treatment (virus control group) were similar throughout the experiment. On day 7 p.i., lesions still could be found in these two groups. By contrast, lesions were almost completely disappeared in animals administered with 50 mg/kg/dose LC or acyclovir and showed similar results to the uninfected control. It suggested that high dose of LC (50 mg/kg/dose) and acyclovir were both effective in preventing lesion formation.

3.6. Effects of LC on HSV-1 titers in BALB/c mice

We next sought evidence of viral titers in the mice whether affected by LC. Skin tissues were collected from mice at 7 days p.i. and the plaque forming assay was applied in HSV-1 titration. As shown in Fig. 5, while uninfected tissues had no expression of HSV-1 titers below assay quantitation limits, increasing of HSV-1 titers was determined in untreated HSV-1 infected tissues and 20 mg/kg/dose treated infected tissues. However, high dose of LC (50 mg/kg/dose) and acyclovir decreased the levels of viral titers. The data suggest that LC can interrupt the HSV-1 replication in BALB/c mice.

3.7. Rare level of DNA in skin tissues from LC-treated BALB/c mice

To examine the correlation of the HSV-1 replication with the amount of HSV-1 DNA, we measured viral DNA and normalized it to cellular β-actin by using PCR analysis of the skin tissue DNA. Results indicated comparison with the uninfected group (Fig. 6A, Lane 1), levels of HSV-1 DNA increased in the HSV-1 infected tissue (Lane

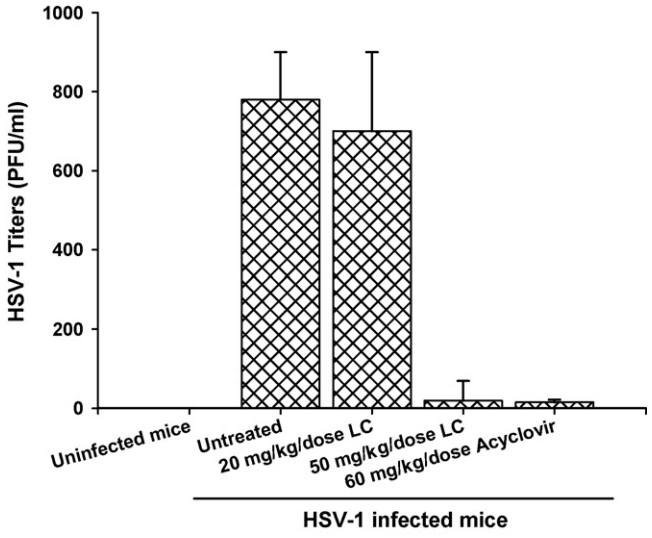

Fig. 5. The HSV-1 titers in skin tissues of BALB/c mice treated with LC. 6-week BALB/c mice were infected with 2.5×10^6 PFU HSV-1 and oral treatment with LC (20, 50 mg/kg/dose) or acyclovir (60 mg/kg/dose) three times a day for 1 week. After sacrificed at 7 days p.i., the skin tissue of the mice were removed and ground and the HSV-1 titers in supernatants were determined by a plaque forming assay as shown in Section 2. Each bar represents the average value of 10 mice per group ± S.D.

2). By contrast, the increasing of HSV-1 DNA in the viral infected tissue was decreased by 60 mg/kg/dose acyclovir and 50 mg/kg/dose (Lanes 4 and 5) but not by 20 mg/kg/dose LC. As judged by the intensity of the PCR bands, the level of HSV-1 DNA in skin tissues was significantly reduced by high dose of LC ($P < 0.01$). Fig. 6B presents the amounts of HSV-1 DNA copy number in 100 ng tissue DNA extracted from mice skin determined by real-time PCR. The studies demonstrated that the copy number of HSV-1 DNA was attenuated by 50 mg/kg/dose LC and acyclovir. We interpreted these results as reduction of HSV-1 DNA expression in mice by LC.

3.8. LC did not affect the liver and kidney functions in BALB/c mice

To realize whether LC had liver or kidney toxicity, the sera were used to determine AST, ALT, BUN, and CRE levels. As shown in Fig. 7A and B, whether uninfected or HSV-1-infected mice, the levels of AST, ALT, BUN, or CRE were no significant difference between vehicle control and LC-treated groups. These results suggested that LC-treatment for 1 week had no toxic effect on the liver and kidney functions of BALB/c mice.

4. Discussion

In the present study, we got the anti-HSV-1 extracts from *L. chinensis* and their antiviral activities were determined *in vitro* and *in vivo*. Results shown here indicated that LC suppressed HSV-1 multiplication in HeLa cells without significantly reducing the cells viability. To study its potential benefit on HSV-1 infection, we examined effects of LC on the viral infected BALB/c mice. The results revealed that LC decreased virus replication and disease in mice. *L. chinensis* belongs to Campanulaceae family and grows around China, Taiwan, Korea, and Japan (Shibano et al., 2001). The whole plants are applied to treat stomach cancer, hemorrhoid, poison, and oliguria in Chinese folk medicine (Elbein, 1987). It has been proved that *L. chinensis* has various biological activities including (1) Radicamines A and B from *L. chinensis* inhibit α-glucosidase activities (Shibano et al., 2001); (2) several alkaloids, including lobeline, lobelanine, and lobelanidine, have been isolated from *L. chinensis*

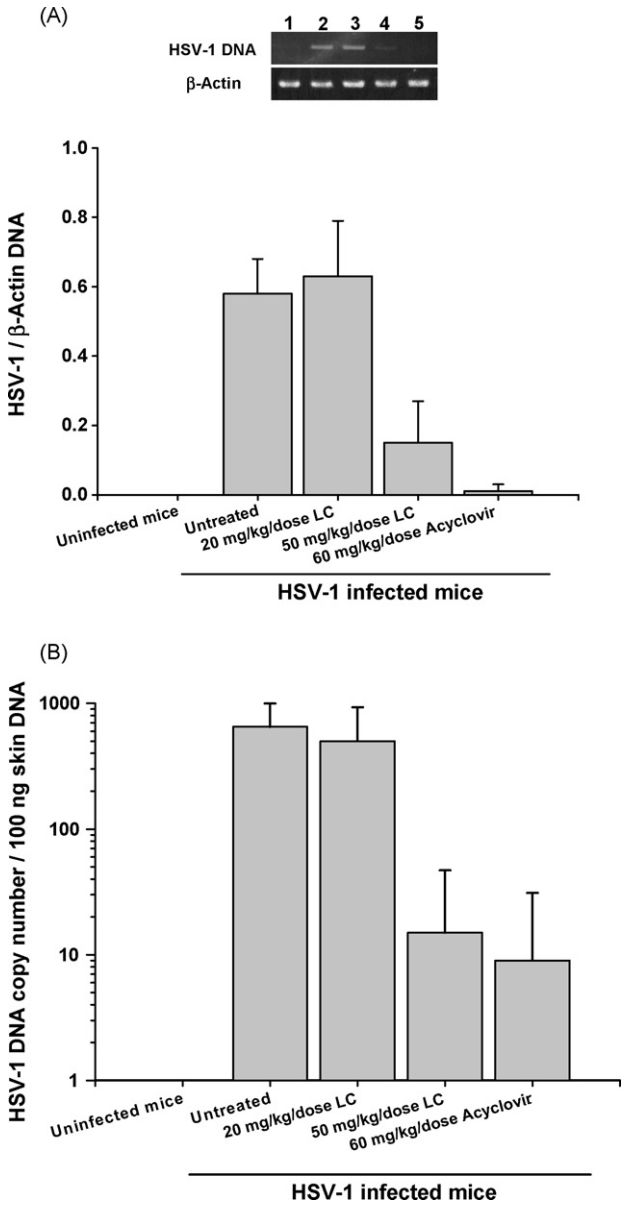

Fig. 6. LC-affected HSV-1 DNA synthesis in BALB/c mice detected by PCR and real-time PCR. 6-week BALB/c mice were infected with 2.5×10^6 PFU HSV-1 and oral treatment with LC (20, 50 mg/kg/dose) or acyclovir (60 mg/kg/dose) three times a day for 1 week. After sacrificed at 7-day p.i., the skin tissue of the mice were removed and ground and DNA was extracted with phenol–chloroform and analyzed by (A) PCR and (B) real-time PCR as described in the Section 2. (A) Lanes 1–5 indicated the results from uninfected mice, infected mice, and infected mice treated with low dose LC, high dose LC, and acyclovir, respectively. Each band was quantitated by densitometer and the ratio of HSV-1 to β-actin DNA was calculated. (B) The virus copy number per 100 ng DNA was determined by real-time PCR. Each bar represents the average value of 10 mice per group ± S.D.

(Shibano et al., 1997). Lobeline could be used as a treatment for drug addiction and neurotoxicity (Miller et al., 2007); (3) Lobelanine and lobelanidine bind to nicotinic acetylcholinergic receptors with an high affinity (Flammia et al., 1999); (4) Extracts from *L. chinensis* have anti-snake venom activities (Tian et al., 1996); (5) Hot water extracts from *L. chinensis* are inhibitors for tumor cells (Santosa et al., 1986). This is the first report of antiviral activities of *L. chinensis* *in vitro* and *in vivo* and the level of inhibition was compatible with that of the commonly used drug, acyclovir.

LC-alleviated HSV-1 replication in HeLa cells was not related to DMSO because cell viability and HSV-1 replication in HeLa cells

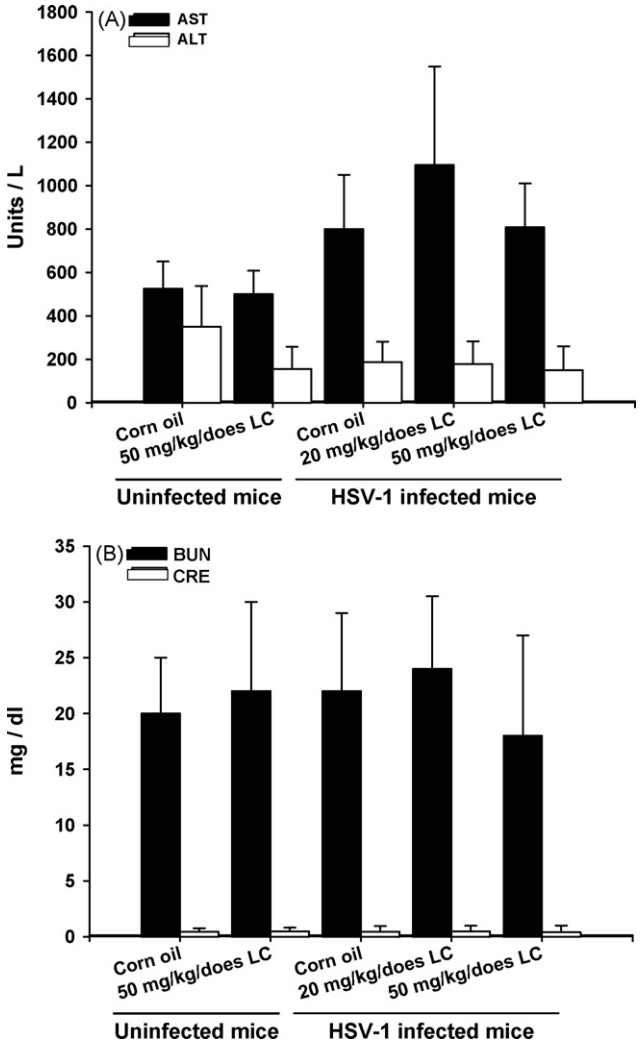

Fig. 7. Effects of LC on liver and kidney functions in BALB/c mice. 6-week BALB/c mice were infected with 2.5×10^6 PFU HSV-1 and oral treatment with LC (20, 50 mg/kg/dose) three times a day for 1 week. Sera were obtained from the mice and (A) the levels of AST (black bar) and ALT (white bar) were analyzed by GOT/AST-PIII and GPT/ALT-PIII kits, respectively. (B) The levels of BUN (black bar) and CRE (white bar) were determined by BUN-PIII and CRE-PIII kits, respectively. The results were measured by Colorimetric Analyzer (DRI-CHEM 3000). Data are shown as mean ± S.D. ($n = 10$).

were not changed by DMSO. In host cells, HSV-1 replication is coordinately regulated and sequentially ordered in a cascade and believed to proceed as synthesis of viral DNA and structural proteins such as gC and assembling and budding of viral particles (Roizman and Sears, 1996). To establish the step of the viral cycle affected by LC in HeLa cells, a time of addition assay was performed. The fact that LC was inhibitory when added between 0 and 8 h p.i., suggested that suppressive effects of LC might be not related to the blocking of viral attachment or entry. During this time frame, HSV-1 gradually expresses immediate-early and early gene and replicates progeny DNA (Roizman and Sears, 1996). Furthermore, we proved that LC impaired HSV-1 DNA copy number both *in vitro* and *in vivo*. Thus, the possibility that LC interrupted HSV-1 replication through disturbance of the viral DNA synthesis could not be excluded.

To elucidate LC *in vivo* activity, we established the titer and the time of application after HSV-1 infection that would most effectively cause skin lesions in BALB/c mice. Based on this initial result, it was determined that a low challenge dose 2.5×10^6 PFU HSV-1 was required to infect BALB/c mice and to obtain lesion development in the 7-day time range. After orally treated with effective drug acyclovir, the symptoms of HSV-1 infection were significantly ameliorated in BALB/c mice. We proved that LC is able to significantly reduce HSV-1-induced lesion formation in the skin of BALB/c mice. The *in vivo* effectiveness of LC is influenced by drug concentration and found to be as effective as acyclovir in lesion suppression. Furthermore, by RT-PCR, real-time PCR, and plaque formation assay, the levels of HSV-1 DNA and virus titers were obviously reduced in skin tissues of infected mice. We also demonstrated that LC and acyclovir decreased HSV-1 titers and DNA copy number in skin tissue. Thus, this subcutaneous HSV-1 infection mouse model could be applied as an antiviral-drug screening system.

It was shown that LC disrupted HSV-1 titers and the viral DNA synthesis in mice while it did not exhibit any liver and kidney toxicities. Although we did not determine whether LC directly acted on HSV-1 at the inoculation site, the data from *in vitro* study showed that the virus titer was not significantly decreased when viral particles were treated with 200 μg/ml LC at 37 °C for 1 h then infected cells (data not shown). Moreover, the results of time of addition assay indicated that added LC after viral adsorption, produced antiviral activity similar to HSV-1 and the extracts added at the same time. Recently, we are trying to orally treat animal with LC or acyclovir at 24 h p.i. The preliminary data demonstrated that both acyclovir and LC still showed efficacy. These results suggest that binding of LC to virion or destruction of HSV-1 structure may be unlikely a major inhibitory factor for virus replication. As such, decrease in herpes DNA synthesis could potentially account for the observed effects of LC *in vivo*. The normal ranges for mouse AST, ALT, BUN, and CRE are 59–550 U/L, 28–132 U/L, 18–29 mg/dl, and 0.2–0.8 mg/dl, respectively. In general, the unit of liver function is indicated as Units/L and the unit of kidney function is indicated as mg/dl. The results indicated that both liver functions and kidney functions did not change in mice orally treated with LC. LC has no liver and kidney toxicity. These data suggested that *L. chinensis* exhibited therapeutic efficacy in an animal infection model were possible candidates for anti-HSV-1 traditional medicine.

From the present results, we hypothesize that the impairment of HSV-1 multiplication in LC-treated HeLa cells or BALB/c mice, at least in part, was related to inhibition of viral DNA synthesis. Then LC decreased HSV-1 plaque formation in HeLa cells and skin tissues and improved skin diseases in BALB/c mice. On the other hand, LC lacked the elevated cytotoxicity. The polysaccharides (Marchetti et al., 1996), anthraquinones (Sydiskis et al., 1991), triterpenes (Simões et al., 1999), phloroglucinol (Arisawa et al., 1990), flavonoids (Kuo et al., 2002a,b), and catechin derivatives (Betz et al., 2002) isolated from medicinal plants are found to have inhibitory activities against the replication of HSV-1. Thus, Chinese herbs such as *L. chinensis* which act as inhibitors of HSV-1 infection may have the potential to impact clinical disease. Recently, we have identified four pure compounds including cycloeucalenol, β-amyrin, 5,7,4-trihydroxy-3′-methoxy flavone, and 5,7-methoxycoumarin from LC and used them in plaque reduction assay at 100 μM. The results indicated that their inhibitory activities are 15.5 ± 4.5, 32.2 ± 1.9, 2.63 ± 1.2, and 13.2 ± 8.6%, respectively. We also determined their activities at 100 μg/ml and results demonstrated their inhibitory activities are below 35%. If we mixed four compounds at equal ratio and determined its activity, the data showed that there was no additional effect for this mixture. The hot water extracted polysaccharides from LC had no anti-HSV-1 activity either (data not shown). We suggest that they are not major anti-HSV-1 components in LC and bioactive components remain to be determined. Currently, plans are underway for the isolation of bioactive principles from LC by a bioassay-guided fractionation procedure and study of detailed action mechanisms of LC.

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