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Efficacy of traditional herbal medicines in combination with acyclovir against herpes simplex virus type 1 infection in vitro and in vivo

Masahiko Kurokawa [a], Kazuhiko Nagasaka [a], Tatsuji Hirabayashi [a], Shin-ichi Uyama [a], Hideki Sato [a], Takashi Kageyama [a], Shigetoshi Kadota [b], Haruo Ohyama [c], Toyoharu Hozumi [c], Tsuneo Namba [b], Kimiyasu Shiraki [a,*]

[a] *Department of Virology, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan*

[b] *Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan*

[c] *Central Research and Development Laboratory, Showa Shell Sekiyu K.K., Atsugi, Kanagawa 243-02, Japan*

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Abstract

Traditional herbal medicines have been safely used for the treatment of various human diseases since ancient China. We selected 10 herbal extracts with therapeutic antiherpes simplex virus type 1 (HSV-1) activity. Among these, *Geum japonicum* Thunb., *Rhus javanica* L., *Syzygium aromaticum* (L.) Merr. et Perry, or *Terminalia chebula* Retzus showed a stronger anti-HSV-1 activity in combination with acyclovir than the other herbal extracts in vitro. When acyclovir and/or a herbal extract were orally administered at doses corresponding to human use, each of the 4 combinations significantly limited the development of skin lesions and/or prolonged the mean survival times of infected mice compared with both acyclovir and the herbal extract alone ($P < 0.01$ or 0.05). These combinations were not toxic to mice. They reduced virus yields in the brain and skin more strongly than acyclovir alone and exhibited stronger anti-HSV-1 activity in the brain than in the skin, in contrast to acyclovir treatment by itself. Combinations of acyclovir with historically used herbal medicines showed strong combined therapeutic anti-HSV-1 activity in mice, especially reduction of virus yield in the brain.

* Corresponding author. Fax: +81 (764) 34 5020.

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1. Introduction

Herpetic infection is common and a major cause of morbidity in humans. Acyclovir (ACV) is a selective antiherpetic agent (Elion et al., 1977; Fyfe et al., 1978; Furman et al., 1984; Shiraki et al., 1984) and has been widely used for the treatment of herpes simplex virus type 1 (HSV-1) and varicella zoster virus infection (Dunkle et al., 1991; Meyers et al., 1982; Whitley et al., 1986; Whitley et al., 1991; Whitley et al., 1992). The combined treatment of two drugs with different modes of anti-HSV-1 action has been shown to increase the anti-HSV-1 activity expected from the treatment with each drug alone (Schinazi et al., 1982; Smith et al., 1982; Fraser-Smith et al., 1985; Ayisi et al., 1986; Hilfenhaus et al., 1987; Ellis et al., 1989; Lobe et al., 1991; Pancheva, 1991). We have previously reported that 12 traditional herbal medicines have therapeutic anti-HSV-1 activity in mice cutaneously infected with HSV-1 (Kurokawa et al., 1993). These medicinal herbs can be easily obtained in Japan and China. Their extracts have been directly and orally used for the treatment of various human diseases (Jiangxu New Medical College, 1978; Terasawa, 1993). The application and dosage of these herbal extracts for each disease have been historically selected and established by their efficacies and adverse reactions in human use. Thus the herbal extracts that exhibited anti-HSV-1 activity in vivo may supplement the anti-HSV-1 activity of ACV and clinically augment the therapeutic efficacy of ACV at conventional doses, without major adverse reactions. The combination of ACV with a herbal extract may be beneficial for the treatment of human HSV-1 infection. In this study, we examined the anti-HSV-1 activity of ACV with each of 10 herbal extracts in vitro and specifically selected 4 herbal extracts that augmented the anti-HSV-1 activity of ACV. Furthermore, we confirmed that ACV, in combination with each selected extract, augmented the therapeutic efficacy of ACV alone and herbal extract alone in a cutaneous HSV-1 infection mouse model and observed that these combinations strongly reduced the virus yield in the brain of infected mice.

2. Materials and methods

2.1. Viruses and cells

HSV-1 strains used were the 7401H strain (Kumano et al., 1987; Kurokawa et al., 1993), the thymidine kinase deficient (TK^-) B2006 strain (Dubbs and Kit, 1964), and phosphonoacetic acid-resistant (PAA^r) strain. The PAA^r strain was plaque-purified from 7401H strain-infected Vero (E6 strain) cell cultures in the presence of 150 μg/ml of PAA in our laboratory. Virus stocks were prepared from HSV-1-infected Vero cells as reported previously (Shiraki and Rapp, 1988; Kurokawa et al., 1993). Vero cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5 and 2% calf serum, respectively.

2.2. Preparation of herbal extracts

Herbal extracts were prepared from the following 10 dried traditional herbal medicines as described previously (Kurokawa et al., 1993): *Alpinia officinarum* Hance. (*A. officinarum*) (rhizome); *Geum japonicum* Thunb. (*G. japonicum*) (whole plant); *Paeonia suffruticosa* Andrews (*P. suffruticosa*) (root bark); *Phellodendron amurense* Ruprecht (*P. amurense*) (bark); *Polygala tenuifolia* Willd. (*P. tenuifolia*) (root); *Polygonum cuspidatum* Sieb. et Zucc. (*P. cuspidatum*) (root and rhizome); *Rhus javanica* L. (*R. javanica* L.) (gall); *Syzygium aromaticum* (L.) Merr. et Perry (*S. aromaticum*) (flower bud); *Terminalia arjuna* Wight et Arn. (*T. arjuna*) (bark); *Terminalia chebula* Retzus (*T. chebula*) (fruit). These traditional herbal medicines have been authenticated and stocked at the Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University, Japan. Briefly, the dried materials of authenticated medicinal herbs were boiled under reflux and the aqueous extracts were then filtered and dried by lyophilization. The lyophilized materials were suspended in distilled water at the indicated concentrations. The suspension was boiled for 10 min and centrifuged at 3000 rpm for 15 min and the supernatant was used as a herbal extract in this study. Different lots of each herbal extract were prepared and the following experiments were repeated by using the various lots.

2.3. Acyclovir

ACV was supplied by Nippon Wellcome K.K. Japan and used for the in vitro assays. Tablets of ACV were purchased from Nippon Wellcome K.K. for oral administration to mice. A tablet (200 mg) was powdered and suspended in distilled water or herbal extracts.

2.4. Plaque-reduction assay

The combined effects of ACV with a herbal extract were examined for anti-HSV-1 activity in the plaque reduction assay. Duplicate cultures of Vero cells in 60 mm plastic dishes were infected with 100 plaque-forming units (PFU)/0.2 ml of HSV-1 for 1 h. The cells were overlaid with 5 ml of nutrient methylcellulose (0.8%) medium containing various concentrations of ACV and/or a herbal extract, and then cultured at 37°C for 2 days. The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue solution, and the number of plaques were counted under a dissecting microscope (Shiraki et al., 1991; Kurokawa et al., 1993). The effective concentrations for 50% plaque reduction (EC_{50}) and 75% plaque reduction (EC_{75}) were determined from a curve relating the plaque number to the concentration of drugs.

When ACV and a herbal extract were combined at concentrations corresponding to each of their EC_{50} values, the interaction between them was analyzed by comparing the observed values for the combined treatment with the expected values for theoretically combining the two agents. The expected value for the sum of the plaque inhibition for ACV and herbal extract was calculated using the following formula proposed by Webb, (1963):

Expected total plaque inhibition = (plaque inhibition produced by ACV) + (plaque inhibition produced by herbal extract) × (1 − plaque inhibition produced by ACV)

The combined action of ACV with a herbal extract was evaluated by constructing an isobologram and calculating the corresponding functional inhibitory concentrations (FIC) (Biron and Elion, 1982; Fraser-Smith et al., 1985). The FIC indexes were calculated as follows:

FIC index = (EC_{50} or EC_{75} of herbal extract in combination with ACV/EC_{50} or EC_{75} of herbal extract alone) + (EC_{50} or EC_{75} of ACV in combination with herbal extract/EC_{50} or EC_{75} of ACV alone)

FIC indexes less than 1 and more than 1 were considered to be synergistic and antagonistic, respectively. When FIC indexes were 1, the combined effects were considered to be additive.

2.5. Effect of drugs on virus growth

The combined effects of ACV with a herbal extract on the growth of HSV-1 were examined by the growth inhibition assay using concentrations greater than the EC_{50} value for each drug. Monolayers of Vero cells in 25 cm^2 plastic flasks were infected with HSV-1 at 0.01 PFU/cell for 1 h. The infected cells were washed three times with MEM and incubated in maintenance medium containing ACV and/or a herbal extract. After 24 h incubation at 37°C, the cultures were frozen and thawed three times and centrifuged at 3000 rpm for 15 min. Virus titers in their supernatants were determined by the plaque assay on Vero cells (Shiraki et al., 1992). Interaction between ACV and a herbal extract was evaluated by a mathematical technique proposed by Webb (1963) as described above.

2.6. Cytotoxicity assay of drugs in cell culture

Cytotoxicity was examined by the growth inhibition of Vero cells as described previously (Kurokawa et al., 1993). The cytotoxicity of ACV and/or a herbal extract was examined at concentrations higher than those used in the plaque reduction assay and the growth inhibition assay. Briefly, Vero cells were seeded at a concentration of 2.5×10^4 cells/well in 24-well plates and grown at 37°C for 2 days. The culture medium was replaced by fresh medium containing ACV and/or herbal extract at various concentrations, and cells were further grown for 2 days. The cells were treated with trypsin and the number of viable cells was determined by the trypan blue exclusion test. The concentrations of herbal extracts reducing cell viability by 50% (CC_{50}) were determined from a curve relating percent cell viability to the concentration of drugs.

2.7. Mouse HSV-1 infection

Female BALB/c mice weighing about 20 g (6 weeks old, Sankyo Laboratory Service Co., Ltd., Tokyo, Japan) were infected with HSV-1 strain 7401H (1×10^6 PFU/mouse) after scarification of the shaved right midflank with a 27-gauge needle as described

previously (Kumano et al., 1987; Kurokawa et al., 1993). ACV and/or a herbal extract was orally administrated once at 8 h prior to and three times (approximately 8-h intervals) daily for 7 successive days after viral inoculation. The dose (250 mg/kg) of herbal extract corresponds to the conventional doses of dried traditional herbal medicines for human use (Jiangxu New Medical College, 1978). The dose (5 mg/kg) of ACV corresponds to a clinical dose for humans. We also used 50 and 125 mg/kg of herbal extracts and 2.5 mg/kg of ACV to evaluate the combined effects of ACV with a herbal extract at their subclinical doses. The development of skin lesions and death were observed three times a day and the severity of the lesions was assessed as described previously (Kumano et al., 1987; Kurokawa et al., 1993): 0, no lesion; 2, vesicles in local region; 4, erosion and/or ulceration in local region; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; 10, severe zosteriform lesion; 12, death. The infected mice were fed and observed for at least a month to determine their mortality. Student's *t*-test was used to evaluate the significance of differences between the two groups treated with different drugs in mean survival times and mean times at which skin lesions were initially scored as 2 (vesicles in local region) or 6 (zosteriform lesion) after infection. Two-way analysis of variance (ANOVA) was used to analyze the interaction between ACV and a herbal extract in the development of skin lesions and in mean survival times. Statistical differences in the mortality were evaluated using the Kaplan–Meier method and the Wilcoxon test. A *P*-value of less than 0.05 was statistically defined as significant.

2.8. Determination of virus yields in brain and skin

Virus yields of the brain and skin were determined according to the methods reported by Minagawa et al. (1988) with minor modification. The mice were cutaneously infected with 7401H strain. ACV and/or herbal extract was orally administrated for 6 days following the same schedule. Most of the infected mice in the water-administered control group were dead after 6 days postinfection in our cutaneous HSV-1 infection model. Therefore the mice were killed under ether anesthesia at 6 days after infection. Their brain and skin (whole lesions that include the area (5×5 mm) encompassing the inoculation site) were simultaneously removed and homogenized in 2 ml of phosphate-buffered saline. The homogenate was centrifuged at 3000 rpm for 15 min and the virus yield in the supernatant was determined by the plaque assay on Vero cells (Shiraki et al., 1992).

2.9. Toxicity assay in mice

ACV and herbal extract alone and in combination were examined for toxicity in uninfected mice. The 5 mice in each group were administered 5 mg/kg ACV and/or 250 mg/kg herbal extract for 7 days following the same schedule used in infected mice. The uninfected mice were weighed on 1–7, 10, 15, and 25 days after initial administration on day 0. The mortality of the uninfected mice was calculated on day 25.

3. Results

3.1. Effects of ACV with herbal extract on anti-HSV-1 activity in vitro

Combinations of ACV with each of 10 herbal extracts were examined for their anti-HSV-1 activity in the plaque reduction assay at concentrations corresponding to the EC_{50} value of each drug (Table 1). Among these combinations, the combinations of ACV with *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* reduced percent plaque formation to less than 10% of control. Their anti-HSV-1 activities were stronger than those of ACV with *A. officinarum*, *P. suffruticosa*, *P. amurense*, *P. tenuifolia*, *P. cuspidatum*, or *T. arjuna*. The former 4 herbal extracts potentiated the anti-HSV-1 activity of ACV (B/A values in Table 1: 3.7–164). The combinations of ACV with each of 10 herbal extracts were also examined for anti-HSV-1 activity by the plaque reduction assay using various concentrations less than the EC_{50} value for each drug. When these data were analyzed by isobolograms using EC_{50} and EC_{75} values (Fig. 1), the range of FIC indexes for these combinations was 0.58–1.07 and 0.33–1.27 at the EC_{50} and EC_{75} levels, respectively. These combinations were not synergistic.

The combined anti-HSV-1 activity of ACV with *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* was examined by the growth inhibition assay using concentrations greater than those used in the plaque reduction assay (Table 2). The virus

Table 1
Combined anti-HSV-1 effects of ACV and a herbal extract in vitro

Treatment (EC_{50}, μg/ml)	Percent plaque formation [a]			B/A [d]
	Herbal extract alone (A)	Observed value for combination [b] (B)	Expected value for combination [c]	
Alpinia officinarum (200)	58.0 ± 5.6	30.6 ± 16.3	31.1	1.0
Geum japonicum (120)	52.8 ± 4.8	1.1 ± 1.9	28.3	25.7
Paeonia suffruticosa (400)	59.5 ± 8.4	26.0 ± 23.7	31.9	1.2
Phellodendron amurense (400)	61.5 ± 6.9	24.5 ± 7.5	33.0	1.3
Polygala tenuifolia (400)	55.2 ± 8.7	15.7 ± 17.5	29.6	1.9
Polygonum cuspidatum (200)	59.4 ± 5.6	20.6 ± 11.6	31.8	1.5
Rhus javanica (50)	55.7 ± 7.1	0.0	29.9	> 29.9
Syzygium aromaticum (60)	61.2 ± 5.6	0.2 ± 0.3	32.8	164
Terminalia arjuna (50)	52.8 ± 7.2	24.8 ± 6.5	28.3	1.1
Terminalia chebula (65)	58.5 ± 2.6	8.4 ± 1.8	31.4	3.7

Note: Combined effects of ACV with a herbal extract were examined for antiviral activity against 7401H strain in the plaque reduction assay using concentrations corresponding to their EC_{50} values as described in the text. Percent plaque formation at 0.35 mg/ml of ACV was 53.6 ± 4.4 (mean ± S.D.).

[a] The range of plaque numbers of untreated controls was 50–100. The values represent the mean percentage ± S.D. compared to untreated controls of 3–4 independent experiments.

[b] Herbal extracts and ACV (0.35 μg/ml) were simultaneously treated for anti-HSV-1 activity. The dose (0.35 mg/ml) of ACV used is the EC_{50} of 7401H strain.

[c] Expected mean values for combination were calculated by the method of Webb (1963) as described in the text.

[d] Ratio of expected mean value to observed mean value.

solution was mixed with ACV and/or herbal extracts at drug concentrations used in this assay and then virus titers in the mixtures were determined by the plaque assay. The dilutions, more than 10-fold of their mixtures, did not prevent plaque-forming ability. Therefore, virus yield in the culture supernatants was determined by the dilutions more than 10-fold to prevent the carryover of input ACV and/or herbal extracts. The results obtained for these combined treatments are shown in Table 2. The extract of *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* potentiated the anti-HSV-1 activity of ACV at concentrations more than their EC_{50} values (B/A values in Table 2: 10.1–37.7). Thus we examined the combined effects of ACV with *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* in the following studies.

ACV and herbal extract alone and in combination were examined for cytotoxicity against Vero cells (Table 3). ACV alone was not cytotoxic at concentrations used (0.5–5 mg/ml). The CC_{50} values of herbal extracts were 2.3- to 3.3-fold higher than their EC_{50} values (see Tables 3 and 4). The concentrations of herbal extracts used in the plaque reduction and growth inhibition assays were lower than their CC_{50} values and not cytotoxic. When each herbal extract was combined with ACV at 0.5–5 μg/ml, CC_{50} values for the combinations were similar to those for herbal extract alone. None of the combinations of ACV and herbal extract was more cytotoxic to Vero cells than herbal extract alone.

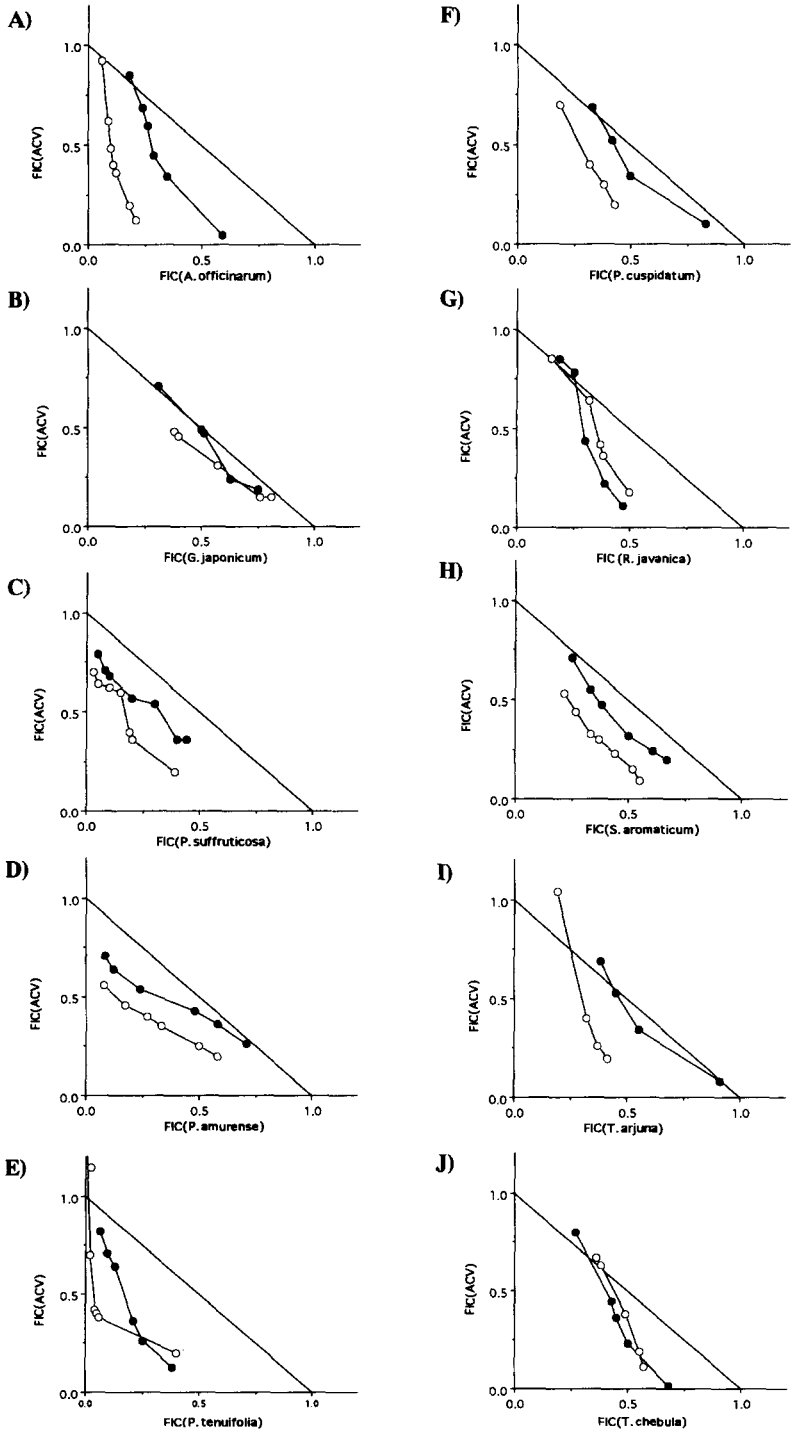
3.2. Susceptibility of ACV-resistant strains to herbal extracts

Susceptibility of HSV-1 TK^-, PAA^r, and wild-type 7401H strains to 4 herbal extracts (*G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula*) was examined to evaluate their mechanisms of anti-HSV-1 action. As shown in Table 4, the EC_{50} values of ACV for TK^- strain and PAA^r strain were 85.7- and 21.1-fold greater than that for 7401H strain, respectively. The EC_{50} values of PAA for TK^- strain and PAA^r strain were 1.5-and 6.8-fold greater than that for 7401H strain, respectively. However, the EC_{50} values of the 4 herbal extracts for TK^- and PAA^r strains were similar to those for the wild-type 7401H strain. The susceptibility of ACV-resistant strains to the 4 herbal extracts was similar to that of the wild-type strain.

3.3. Effects of ACV with herbal extract on anti-HSV-1 activity and toxicity in vivo

Therapeutic anti-HSV-1 efficacies of ACV with *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* were examined in a cutaneous HSV-1 infection model in mice (Table 5). Each of 4 herbal extracts (250 mg/kg) corresponding to the doses for human use showed significant therapeutic efficacy in the development of skin lesions and/or mean survival times as compared with controls in this model, but ACV (2.5 mg/kg) did not (Kurokawa et al., 1993). The subclinical dose (2.5 mg/kg) of ACV was combined with 250 mg/kg of herbal extract in this experiment to evaluate the mechanism of the combined therapeutic anti-HSV-1 efficacy.

The combinations of ACV (2.5 mg/kg) with *G. japonicum*, *S. aromaticum*, or *T. chebula* (250 mg/kg) significantly delayed the development of skin lesions and/or prolonged mean survival times as compared with ACV alone and herbal extract alone


A)
FIC(ACV)
FIC(A. officinarum)
B)
FIC(G. japonicum)
C)
FIC(P. suffruticosa)
D)
FIC(P. amurense)
E)
FIC(P. tenuifolia)
F)
FIC(P. cuspidatum)
G)
FIC (R. javanica)
H)
FIC(S. aromaticum)
I)
FIC(T. arjuna)
J)
FIC(T. chebula)
0.0
0.5
1.0

($P < 0.05$ or $P < 0.01$ by Student's t-test), but the combination of ACV with *R. javanica* did not. In the case of combination of ACV with *G. japonicum*, there was a significant interaction in the development of vesicles and in mean survival times and this combination enhanced therapeutic efficacy of ACV alone and *G. japonicum* alone ($P < 0.05$ by 2-way ANOVA). The progression of disease was dose-dependently examined in mice treated with ACV and/or *G. japonicum*. When ACV (2.5 mg/kg) was dose-dependently combined with *G. japonicum* of different preparation (Expt. 4 in Table 5), these combinations arrested the progression of skin lesions depending on the increase of combined dose of *G. japonicum* (Fig. 2). The combination of ACV (2.5 mg/kg) with *G. japonicum* (250 mg/kg) showed significant interaction and significantly prolonged mean survival times as compared with ACV alone and *G. japonicum* alone ($P < 0.05$ by 2-way ANOVA). This combination again significantly delayed the development of skin lesions and prolonged mean survival times as compared with ACV alone and *G. japonicum* alone ($P < 0.01$ by Student's t-test). The combinations of ACV (5 mg/kg) with each of 4 herbal extracts (250 mg/kg) showed significant therapeutic efficacy in the development of skin lesions and/or mean survival times as compared with ACV alone and herbal extract alone ($P < 0.01$ or $P < 0.05$ by Student's t-test, data not shown). Similar therapeutic efficacy was confirmed in the repeated experiments using different preparations of herbal extracts. Therefore the combinations of ACV with each herbal extract, especially *G. japonicum*, augmented the therapeutic efficacy of ACV at doses corresponding to human use.

The toxicity of ACV and herbal extract, alone and in combination, was examined in mice at the clinical doses of ACV (5 mg/kg) and herbal extracts (250 mg/kg) as used for mice infected with HSV-1 cutaneously (Table 6). When 5 mg/kg of ACV was combined with 250 mg/kg of each herbal extract, no lethal toxicity was observed in uninfected mice for at least 25 days after initial administration. There was no significant difference in the average weights between treated and untreated mice on days 7 and 25 ($P > 0.05$). Similar results were observed on days 1–6, 10 and 15 (data not shown). No treatment with ACV and/or herbal extract was significantly toxic.

3.4. Effects of ACV with herbal extract on virus yields in the skin and brain of infected mice

The combined anti-HSV-1 activity of ACV with *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* was evaluated in the skin and brain of HSV-1-infected mice. The subclinical dose (2.5 mg/kg) of ACV was combined with the clinical dose (250 mg/kg) of herbal extracts in this experiment to examine their combined anti-HSV-1 activity in detail. This clinical dose (250 mg/kg) of herbal extracts in mice corresponds

Fig. 1. Isobolograms showing the anti-HSV-1 action of ACV with herbal extracts (*A. officinarum* (A), *G. japonicum* (B), *P. suffruticosa* (C), *P. amurense* (D), *P. tenuifolia* (E), *P. cuspidatum* (F), *R. javanica* (G), *S. aromaticum* (H), *T. arjuna* (I), or *T. chebula* (J)). The FIC value of 1.0 corresponds to the EC_{50} or EC_{75} (μg/ml) of each drug. The straight lines between FIC values of 1.0 for two drugs represent the dose for combinations that would be needed to produce an EC_{50} or EC_{75} value if the interaction of the two drugs was additive. The actual doses for combinations that produced an EC_{50} and EC_{75} value were shown by closed circles and open circles, respectively.

Table 2
Combined effects of ACV and a herbal extract on growth inhibition of HSV-1 in Vero cells

Treatment	Concentration (μg/ml)	Virus yield [a] (PFU/ml)	Percent virus yield (A)	Expected percent virus yield for combination [b] (B)	B/A [c]
Expt. 1					
Control	0	2.61×10^7	100		
G. japonicum	100	8.75×10^6	33.52		
	140	1.15×10^6	4.41		
ACV	0.3	5.46×10^5	2.09		
	0.6	9.25×10^4	0.35		
G. japonicum / ACV	100/0.3	4.24×10^4	0.16	0.70	4.4
	100/0.6	1.40×10^3	0.0053	0.13	24.5
	140/0.3	1.10×10^4	0.042	0.092	2.2
	140/0.6	2.53×10^3	0.0097	0.018	1.9
Expt. 2					
Control	0	4.88×10^7	100		
R. javanica	20	2.08×10^6	4.26		
ACV	0.3	2.31×10^5	0.47		
	0.6	3.75×10^4	0.077		
R. *javanica* / ACV	20/0.3	$<10^3$	<0.0020	0.022	>11.0
	20/0.6	$<10^2$	<0.00020	0.0033	>16.5
Expt. 3					
Control	0	2.25×10^7	100		
S. aromaticum	50	2.55×10^6	11.33		
	70	3.13×10^6	13.91		
ACV	0.3	4.43×10^5	1.97		
	0.6	1.38×10^5	0.61		
S. aromaticum / ACV	50/0.3	2.50×10^4	0.11	0.23	2.1
	50/0.6	1.50×10^3	0.0067	0.068	10.1
	70/0.3	2.00×10^3	0.0089	0.28	31.5
	70/0.6	5.00×10^2	0.0022	0.083	37.7
Expt. 4					
Control	0	1.18×10^7	100		
T. chebula	70	6.50×10^5	5.51		
	90	2.75×10^5	2.33		
ACV	0.6	3.00×10^4	0.25		
	0.9	1.25×10^4	0.11		
T. chebula / ACV	70/0.6	3.57×10^2	0.0030	0.014	4.7
	70/0.9	5.75×10^2	0.0049	0.0061	1.2
	90/0.6	4.00×10^2	0.0034	0.0058	1.7
	90/0.9	<10	<0.000085	0.0025	>29.4

Note: ACV and herbal extract alone and in combination were examined for the antiviral activity against 7401H strain in the growth inhibition assay as described in the text.

[a] Mean of triplicate cultures.

[b] Expected mean values for combination were calculated by the method of Webb as described in the text.

[c] Ratio of expected mean value to observed mean value.

Table 3
Cytotoxicity of ACV and herbal extract alone and in combination to Vero cells

Treatment	CC_{50} (μg/ml) [a]			
	Combined with ACV (μg/ml)			
	0	0.5	1.0	5.0
G. japonicum	350 (1.00)[b]	374 (1.07)	378 (1.08)	327 (0.93)
R. javanica	130 (1.00)	110 (0.85)	155 (1.19)	165 (1.27)
S. aromaticum	230 (1.00)	185 (0.80)	220 (0.96)	175 (0.76)
T. chebula	235 (1.00)	180 (0.77)	220 (0.94)	230 (0.98)

Note: The effects of ACV and herbal extract alone and in combination were examined for cytotoxicity in the growth inhibition of Vero cells in 24-well plates as described in the text. Untreated control had 3.4×10^5 cells/well. ACV (0.5–5.0 μg/ml) did not inhibit the growth of Vero cells.

[a] The concentrations of herbal extracts reducing cell viability by 50%.

[b] Numbers in parentheses represent FIC values which were calculated by the following formula: FIC = (CC_{50} of herbal extract in combination with ACV)/(CC_{50} of herbal extract alone).

to about 12-fold of the dose (mg/kg) for humans based on body surface area (Freireich et al., 1966). Table 7 shows the virus yields of the skin and brain removed from infected mice on day 6 after infection. As virus yields in the homogenates of skin and brain were determined by their 10- to 10^3-fold dilutions, there was no problem with carryover of administered ACV and/or herbal extracts in the plaque assay. ACV reduced virus yields in the skin and brain to 18.7% ($P < 0.05$ by Student's t-test) and 56.5% of controls, respectively. *G. japonicum* also reduced virus yield in the skin and brain to 77.6 and 34.6% of controls, respectively. The virus yield for *R. javanica* or *T. chebula* was similar to the control in the skin (104.0 or 99.0%, respectively) but was reduced to 72.5 or 14.9% of control in the brain, respectively. The virus yield for *S. aromaticum* was similar to control in the skin and brain (94.1 and 113.7%, respectively). The combinations of ACV with each herbal extract reduced virus yields in the skin to 8.32–17.0% of control ($P < 0.05$ by Student's t-test) and in the brain to 1.04–6.13% of control.

Table 4
Susceptibility of 7401H, TK^- (B2006), or PAA-resistant 7401H strains to ACV, PAA, and herbal extracts

	EC_{50} (μg/ml) [a]		
	7401H strain	TK^- strain	PAA-resistant strain
ACV	0.35 ± 0.05	> 30	7.4 ± 0.26
PAA	24.4 ± 2.6	37.1 ± 1.8	166.8 ± 6.3
G. japonicum	137.4 ± 29.5	155.8 ± 35.9	153.3 ± 24.7
R. javanica	56.7 ± 6.0	60.7 ± 3.8	49.3 ± 7.1
S. aromaticum	70.3 ± 3.7	75.3 ± 13.5	88.8 ± 3.3
T. chebula	81.2 ± 8.4	81.3 ± 15.9	82.8 ± 22.3

Note: ACV, PAA, and herbal extracts were examined for the antiviral activity against 7401H wild type strain, TK^- strain, and PAA-resistant strain in the plaque reduction assay and EC_{50} values were determined from 3 independent experiments as described in the text.

[a] Effective concentration for 50% plaque reduction.

Table 5
Combined effects of ACV with a herbal extract on cutaneous HSV-1 infection in BALB/c mice

Treatment	Mean time (days ± S.D.)			Mortality [c]
	Score 2 [a]	Score 6 [a]	Survival [b]	
Expt. 1				
Control	3.39 ± 0.51	5.15 ± 0.38	6.92 ± 0.64	13/14
ACV (2.5 mg/kg)	3.62 ± 0.51	5.62 ± 0.87	7.73 ± 1.49	11/14
G. japonicum (250 mg/kg)	3.69 ± 0.63	6.00 ± 0.91 [d]	7.00 ± 0.82	10/13
ACV (2.5 mg/kg) + *G. japonicum* (250 mg/kg)	4.71 ± 0.47 [d,e,f]	6.11 ± 0.33 [d]	9.36 ± 1.03 [d,g,f]	10/13
Expt. 2				
Control	3.00 ± 0.00	5.10 ± 0.32	6.60 ± 0.97	10/10
ACV (2.5 mg/kg)	3.30 ± 0.48	5.30 ± 0.48	7.30 ± 0.82	10/10
S. aromaticum (250 mg/kg)	3.50 ± 0.53 [d]	5.80 ± 0.42 [d]	6.80 ± 0.79	10/10
ACV (2.5 mg/kg) + *S. aromaticum* (250 mg/kg)	3.80 ± 0.42 [d,g]	5.80 ± 0.42 [d,g]	8.30 ± 1.25 [d,g,f]	10/10
Expt. 3				
Control	3.20 ± 0.42	5.30 ± 0.48	7.20 ± 0.92	10/10
ACV (2.5 mg/kg)	3.60 ± 0.70	5.44 ± 0.53	7.67 ± 1.23	9/10
Herbal extracts (250 mg/kg)				
R. javanica	3.90 ± 0.57 [d]	5.90 ± 0.32 [d]	6.60 ± 0.52	10/10
T. chebula	3.30 ± 0.48	5.60 ± 0.42	6.70 ± 0.68	10/10
ACV (2.5 mg/kg) + Herbal extracts (250 mg/kg)				
R. javanica	3.90 ± 0.57 [d]	6.00 ± 0.82 [h]	7.78 ± 1.09 [f]	9/10
T. chebula	4.00 ± 0.67 [d,i]	6.11 ± 0.33 [d,e,i]	7.56 ± 1.33 [i]	9/10
Expt. 4				
Control	3.13 ± 0.35	5.23 ± 0.46	6.50 ± 0.54	8/8
ACV (2.5 mg/kg)	3.38 ± 0.52	5.75 ± 0.71	7.12 ± 0.64	8/8
G. japonicum				
(62.5 mg/kg)	3.56 ± 0.53	5.75 ± 0.71	6.89 ± 0.60	9/9
(125 mg/kg)	3.67 ± 0.71	5.57 ± 0.54	6.78 ± 0.44	9/9
(250 mg/kg)	3.71 ± 0.49 [h]	5.83 ± 0.75	7.00 ± 0.00 [h]	8/8
ACV (2.5 mg/kg) + *G. japonicum*				
(62.5 mg/kg)	3.89 ± 0.60 [d]	6.11 ± 0.78 [h]	7.78 ± 0.83 [d,i]	9/9
(125 mg/kg)	4.00 ± 0.50 [d,g]	6.00 ± 0.87 [h]	8.13 ± 1.89 [h]	8/9
(250 mg/kg)	4.44 ± 0.53 [d,e,f]	6.75 ± 0.46 [d,e,i]	8.60 ± 0.55 [d,e,f]	6/9

Note: Therapeutic anti-HSV-1 efficacy of ACV with a herbal extract was examined in a cutaneous HSV-1 infection model in mice, and the development of skin lesions and death in 7401H strain-infected mice were determined as described in the text. Underlined numbers were statistically significant by 2-way ANOVA test ($P < 0.05$).

[a] Mean time at which score 2 or 6 was first observed after infection.

[b] Surviving mice were not included for the calculation of mean survival times.

[c] Number of dead mice/number of mice tested.

[d] $P < 0.01$ vs control by Student's t-test.

[e] $P < 0.01$ vs ACV by Student's t-test.

[f] $P < 0.01$ vs HW-extract alone by Student's t-test.

[g] $P < 0.05$ vs ACV by Student's t-test.

[h] $P < 0.05$ vs control by Student's t-test.

[i] $P < 0.05$ vs HW-extract alone by Student's t-test.

The ratio (S/B value in Table 7) of percent virus yields in skin to brain was calculated to compare the anti-HSV-1 activity of each treatment with each organ. The percent virus yield in the brain of ACV-treated mice was 3.0-fold greater than that in the skin. The percent virus yields in the brain of *G. japonicum*-, *R. javanica*-, or *T. chebula*-treated mice were 2.2-, 1.4-, or 6.6-fold less than those in the skin, respectively. When the ratio of S/B in ACV-treated mice was 100% as shown in Table 7, the ratio of S/B in *G. japonicum*-treated mice was 660%. Similarly, the ratios in *S. aromaticum*-, *R. javanica*-, or *T. chebula*-treated mice were 250, 420, or 2000%, respectively. The 4 herbal extracts showed 2.5- to 20-fold stronger anti-HSV-1 activity in the brain than in the skin, when compared with ACV-treated mice. In contrast, ACV showed stronger anti-HSV-1 activity in the skin than in the brain.

The percent virus yields in the brain of mice treated with the combinations of ACV with *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* were 1.5- to 8.0-fold less than those in the skin (S/B in Table 7). When the ratio of S/B in ACV-treated mice was 100% (Table 7), the ratio of S/B in mice treated with the combination of ACV with *G. japonicum* was 2420%. Similarly, the ratios in mice treated with combinations of ACV with *S. aromaticum*, *R. javanica*, or *T. chebula* were 790, 450, or 850%, respectively. Thus, these herbal extracts augmented the anti-HSV-1 activity of ACV in the brain more strongly than in the skin (4.5- to 24.2-fold). Alternatively, when the ratio of S/B in each herbal extract-treated mouse was 100% as shown in Table 7, the ratios of S/B in mice treated with combinations of ACV with *G. japonicum*, *S. aromaticum*, or *R. javanica* were 360, 310, or 110%, respectively. The ratio of S/B in mice treated with ACV and *T. chebula* was 42%. Thus the combinations of ACV with *G. japonicum*, *S. aromaticum*, or *R. javanica* augmented the anti-HSV-1 activity of these herbal extracts in the brain more strongly than in the skin. Similar results were obtained on repeating the experiment. Therefore the combinations of ACV with each of the former

Table 6
Toxicity of ACV and herbal extract alone and in combination to uninfected mice

Treatment	Dose (mg/kg)	Mean weight ± S.D. on day: [a]		Mortality [b]
		7	25	
Control (water)		19.5 ± 1.1	20.5 ± 0.5	0/5
ACV	5	19.8 ± 1.4	19.8 ± 1.7	0/5
G. japonicum	250	19.9 ± 1.4	20.7 ± 1.1	0/5
S. aromaticum	250	18.3 ± 0.8	19.5 ± 1.0	0/5
R. javanica	250	20.0 ± 0.7	20.0 ± 0.9	0/5
T. chebula	250	19.2 ± 0.9	19.4 ± 1.2	0/5
ACV/*G. japonicum*	5/250	18.5 ± 1.3	19.5 ± 1.0	0/5
ACV/*S. aromaticum*	5/250	20.1 ± 0.5	20.3 ± 1.0	0/5
ACV/*R. javanica*	5/250	18.7 ± 1.0	19.9 ± 0.7	0/5
ACV/*T. chebula*	5/250	19.6 ± 1.3	19.9 ± 0.7	0/5

Note: ACV and herbal extract alone and in combination were examined for toxicity to uninfected mice as described in the text. The range of the initial weight of mice on day 0 was 17–19 g.

[a] Mean weight ± S.D. of 5 mice in each group.

[b] Number of dead mice/number of mice tested. Mortality was calculated at 25 days after the first administration of drugs.

Table 7
Effects of ACV and herbal extract alone and in combination on virus yields in the skin and brain of HSV-1-infected mice

Treatment	Dose (mg/kg)	Virus yield [a] (mean ± S.D., PFU/organ) × 10^{-4}		Skin/Brain [b] (S/B)	Percent to S/B of ACV [c]	Percent to S/B of herbal extract
		Skin	Brain			
Control (water)	303 ± 124 (100) [d]	6.07 ± 4.78 (100)	1			
ACV	2.5	56.6 ± 24.5 (18.7) [e]	3.43 ± 2.05 (56.5)	0.33	100	
G. japonicum	250	235 ± 71.0 (77.6)	2.10 ± 1.69 (34.6)	2.2	660	100 [f]
S. aromaticum	250	285 ± 127 (94.1)	6.90 ± 3.64 (113.7)	0.83	250	100 [g]
R. javanica	250	315 ± 206 (104.0)	4.40 ± 6.58 (72.5)	1.4	420	100 [h]
T. chebula	250	300 ± 134 (99.0)	0.906 ± 0.713 (14.9)	6.6	2000	100 [i]
ACV/*G. japonicum*	2.5/250	25.2 ± 8.30 (8.32) [e,j]	0.063 ± 0.05 (1.04) [k]	8.0	2420	360 [f]
ACV/*S. aromaticun*	2.5/250	29.3 ± 12.9 (9.76) [e,l]	0.225 ± 0.187 (3.71) [l]	2.6	790	310 [g]
ACV/*R. javanica*	2.5/250	27.8 ± 29.6 (9.17) [e]	0.372 ± 0.307 (6.13)	1.5	450	110 [h]
ACV/*T. chebula*	2.5/250	51.4 ± 18.1 (17.0) [e,l]	0.363 ± 0.260 (5.98)	2.8	850	42 [i]

Note: ACV and herbal extract alone and in combination were examined for antiviral activity against 7401H strain in the skin and brain of the infected mice on day 6 after infection as described in the text.

[a] Virus yields represent the geometric mean ± S.D. per organ of three mice in each group.

[b] S/B represents the ratio of percent virus yields of the skin over the brain for each treatment.

[c] Values represent the percentages of S/B ratio in ACV-treated mice.

[d] Numbers in parentheses represent percent virus yields.

[e] $P < 0.05$ vs control by Student's t-test.

[f,g,h,i] Values represent the percentage of S/B ratio in *G. japonicum*-, *R. javanica*-, *S. aromaticum*-, or *T. chebula*-treated mice, respectively.

[j] $P < 0.01$ vs herbal extract by Student's t-test.

[k] $P < 0.05$ vs ACV by Student's t-test.

[l] $P < 0.05$ vs herbal extract by Student's t-test.

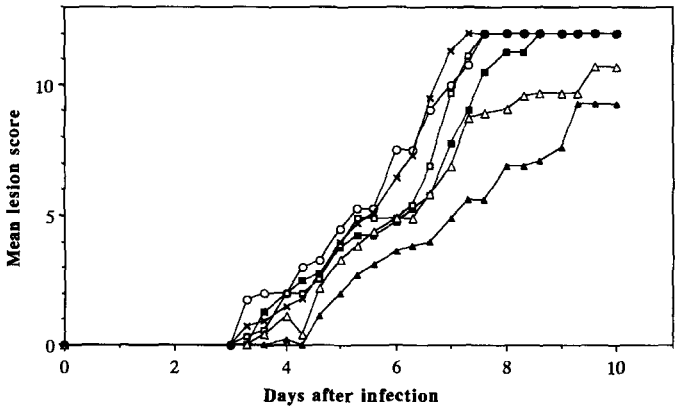


Fig. 2. Combined effects of ACV (2.5 mg/kg) in combination with varying doses of *G. japonicum* on mice cutaneously infected with HSV-1. Eight to 9 mice in each group were infected. ACV and/or herbal extracts were orally applied three times daily for 7 days after infection. ○, control (water treatment); ■, 2.5 mg/kg ACV; ×, 125 mg/kg *G. japonicum*; □, 250 mg/kg *G. japonicum*; △, 2.5 mg/kg ACV + 125 mg/kg *G. japonicum*; ▲, 2.5 mg/kg ACV + 250 mg/kg *G. japonicum*.

three herbal extracts strengthened the anti-HSV-1 activity of ACV alone or herbal extract alone in the brain more than in the skin.

4. Discussion

Many investigators have examined the combined treatments of ACV with the other drugs having different modes of anti-HSV-1 action from ACV and have shown that the combinations augmented the anti-HSV-1 activity of each drug in vitro and in vivo (Schinazi et al., 1982; Hilfenhaus et al., 1987; Ellis et al., 1989; Lobe et al., 1991; Pancheva, 1991). Such treatments may shorten duration of the clinical symptoms of HSV-1 infection (Ellis et al., 1989; Lobe et al., 1991) and may be effective against ACV-resistant variants that may occur during ACV therapy (Sibrack et al., 1982; Norris et al., 1988; Ellis et al., 1989; Erlich et al., 1989; Birch et al., 1990; Lobe et al., 1991; Nugier et al., 1992). Many compounds which exhibit varied antiviral actions have been found in herbal extracts (Okada and Kim, 1972; Amoros et al., 1987; Ito et al., 1987; Chang and Yeung, 1988; Fukuchi et al., 1989; Hudson, 1989; Tabba et al., 1989; Yamamoto et al., 1989; Tang et al., 1990; Sydiskis et al., 1991; Hayashi et al., 1992; Yao et al., 1992). ACV and herbal extracts have been historically used for humans and the combinations of these may improve the treatment of HSV-1 infection.

In this study we showed that *G. japonicum*, *R. javanica*, *S. aromaticum*, or *T. chebula* augmented therapeutic anti-HSV-1 efficacy of ACV in mice cutaneously infected with HSV-1 (Table 5). These 4 herbal extracts have been directly and orally administered for the treatment of various diseases in humans such as bruising, empyema, gastric and duodenal ulcer, acute gastroenteritis, diarrhea, chronic pharyngolaryngitis, etc. as described in Jiangxu New Medical College, 1978. The dosages of these herbal

extracts have been established in traditional therapy and used safely for humans for several thousand years. Cutaneous infection with 7401H HSV-1 strain is a lethal model and causes the development of skin lesions and death in mice. We used 15 mg/kg/day of ACV and 750 mg/kg/day of herbal extracts for oral administration to mice as the clinical doses in this HSV-1 infection model. The dose (mg/kg/day) of herbal extracts in mice is calculated from the dose (mg/kg/day) in humans and was not toxic to mice as was expected from experience with human use (Table 6). Thus, the conventional use of 4 herbal extracts would be applicable as anti-HSV-1 medicines for augmenting therapeutic anti-HSV-1 efficacy of ACV in the human. The combination of ACV with higher doses of herbal extracts than 750 mg/kg/day may show more effective therapeutic anti-HSV-1 efficacy in mice

We examined the combined anti-HSV-1 activity of ACV with herbal extracts at various concentrations lower than the CC_{50} value of each drug and selected 4 herbal extracts which showed strong combined anti-HSV-1 activity with ACV. Ten herbal extracts used exhibited additive anti-HSV-1 activity in combination with ACV in the plaque reduction assay at concentrations lower than their EC_{50} values (Fig. 1). However *G. japonicum*, *R. javanica*, *S. aromaticum*, and *T. chebula* strongly potentiated anti-HSV-1 activity of ACV in the plaque reduction assay at their EC_{50} values (Table 1). Since it was difficult to examine the effect of combinations at higher doses than the EC_{50} value of each drug using plaque reduction assay (Schinazi et al., 1982), we also examined the combined effects of ACV and herbal extracts in the growth inhibition assay. In this assay, 4 herbal extracts potentiated anti-HSV-1 activity of ACV at concentrations higher than their EC_{50} values (Table 2). The difference of the combined anti-HSV-1 activities between these assays may be caused by the difference of doses used in these assays or detection systems rather than the difference in the mechanism. The susceptibility of ACV-resistant strains to the 4 herbal extracts was similar to that of the wild-type strain (Table 4). These herbal extracts may have different mechanisms of anti-HSV-1 action from ACV. Thus the combination of ACV with herbal extracts might have worked synergistically. When the therapeutic anti-HSV-1 activity of these 4 combinations was examined in mice cutaneously infected with HSV-1, they showed significant therapeutic efficacy (Table 5) and no toxicity (Table 6). These combinations reduced the virus yields in the brain of infected mice more than those in the skin compared with ACV alone (Table 7). Thus the strong anti-HSV-1 activity observed in the brain might be due to the synergistic action of ACV and herbal extract as observed in the growth inhibition assay.

G. japonicum, *R. javanica*, *S. aromaticum*, and *T. chebula* showed stronger anti-HSV-1 activity in the brain than in the skin, but in contrast, the anti-HSV-1 activity of ACV was weaker in the brain than in the skin (Table 7). Thus the strong anti-HSV-1 activity of each herbal extract in the brain may be responsible for the strong combined anti-HSV-1 activity of ACV with each herbal extract in the brain. When ACV was combined with *G. japonicum*, *R. javanica*, or *S. aromaticum*, the anti-HSV-1 activity of ACV was strengthened in the brain more than in the skin and the anti-HSV-1 activity of each of the herbal extracts was similarly strengthened (Table 7). However when ACV and *T. chebula* were combined, the yield reduction for *T. chebula* was greater in the skin than in the brain. On the contrary, the yield reduction for ACV was greater in the

brain than in the skin in combination with *T. chebula*. In any case, *T. chebula* augmented anti-HSV-1 activity of ACV in vivo. Thus the former three combinations may be more effective in reducing the growth of HSV-1 in the central nervous system than the latter combination. We expect that these extracts are beneficial in preventing central nervous system complications.

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