CHAPTER IV

RESULTS

1. Cytotoxicity of lipoic acid, lipoamide, and ACV on Vero cells

In this study, cellular toxicity of lipoic acid, lipoamide, and ACV was determined as CC_{50}. All CC_{50} values in this study were calculated by regression analysis (see appendix). The maximal concentration of DMSO that did not affect the cytotoxicity to Vero cells was 2% (Lipipun *et al.*, 1999). In addition, the result of cytotoxicity of DMSO on Vero cells determined with MTT reduction assay was shown in Figure 10. The CC_{50} value of DMSO was 4.712%. Therefore, the final concentration of DMSO in each drug stock solution was not more than 2%, and the concentration would be further diluted by growth medium before used in this study. Finally, the maximum concentration of DMSO used in all antiviral tests was 0.25% which did not show any effect to cell growth.

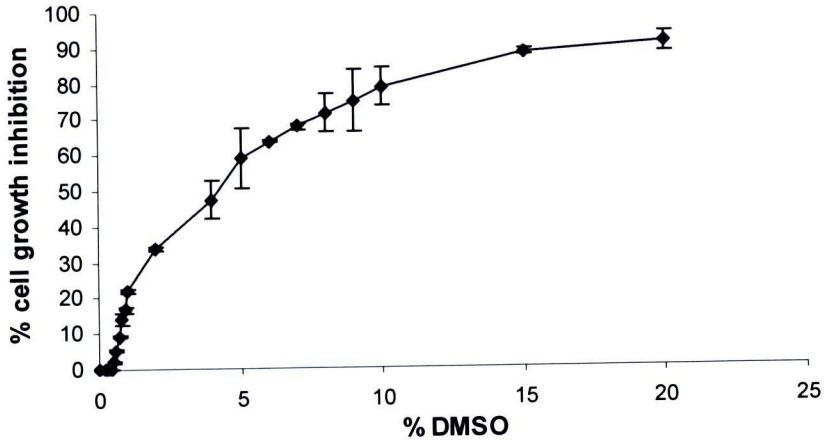

Figure 10. Cytotoxicity of DMSO on Vero cells determined by MTT reduction assay. Each bar of point represents the percentage of cell growth inhibition compared with the controls. Data were reported as the mean ± S.D. from three independent experiments.

Cytotoxicity of lipoic acid, lipoamide, and ACV was examined by trypan blue exclusion test and MTT reduction test. Trypan blue exclusion method, in which dead cells were stained blue while living cells remained clear, showed the total viable cell numbers in treated Vero cells as compared with untreated control Vero cells. The results were shown in Figure 11. Lipoic acid,

lipoamide, and ACV exhibited CC_{50} values of 242.69, 239.03, and 1,650.59 μg/ml, respectively.

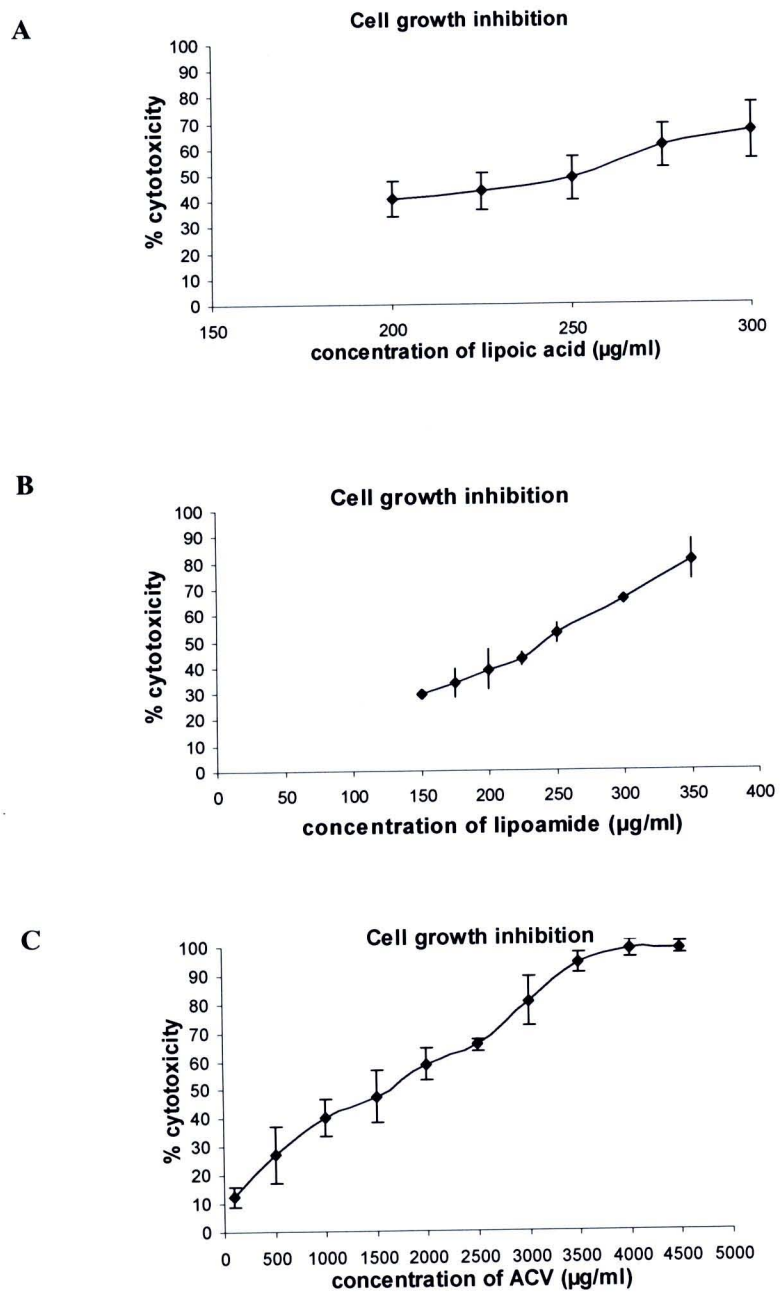

Figure 11. Cytotoxicity of lipoic acid (A), lipoamide (B) and ACV (C) determined by trypan blue exclusion method. Vero cells were incubated with various concentrations of test substances for 5 days, then the cells were counted after staining with trypan blue. The data were reported as mean ± S.D. from at least three experiments.

In the second method, MTT reduction assay, which measured cellular enzymes activity that correlated with cell viability, showed the similar results. Cytotoxicity of lipoic acid, lipoamide, and ACV on Vero cells performed with this method was shown in Figure 12. The CC_{50} values of lipoic acid, lipoamide, and ACV were 292.19, 271.10, and 1,602.81 μg/ml, respectively.

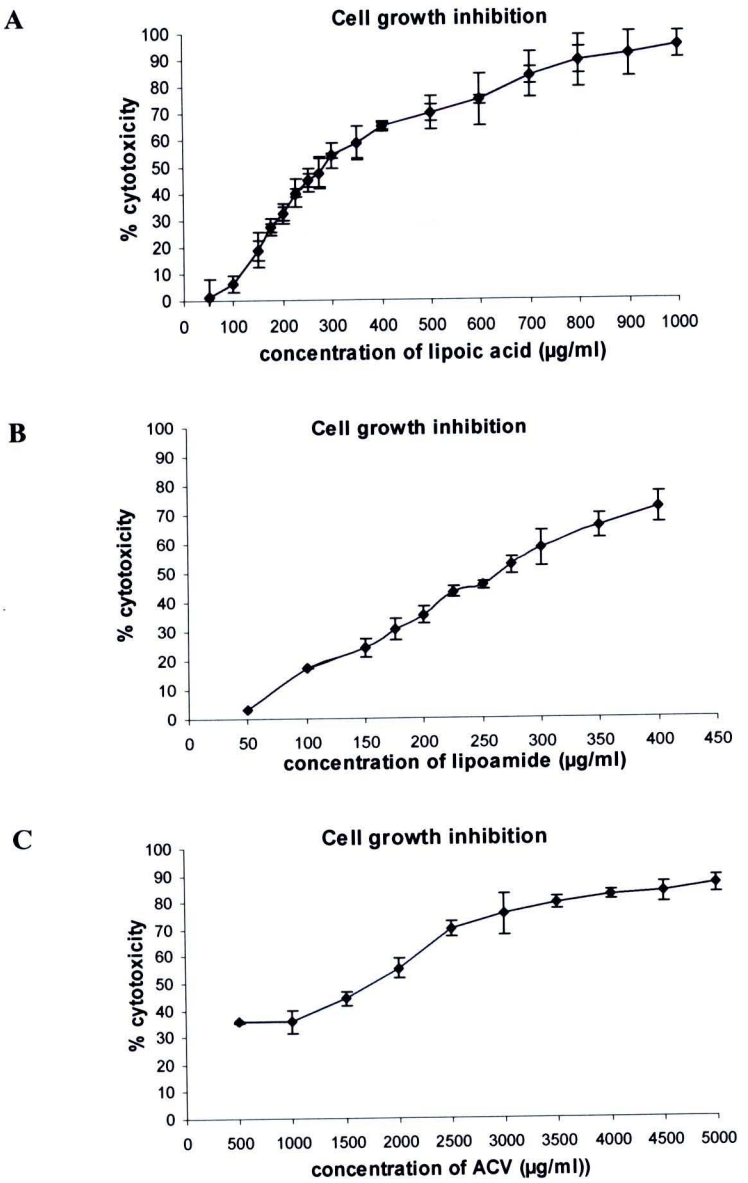

Figure 12. Cytotoxicity of lipoic acid (A), lipoamide (B), and ACV (C) determined by MTT reduction assay. Vero cells were incubated with various concentrations of test compounds in media for 72 hours. The media were replaced with MTT solution. After incubation at 37°C for 4 hours, acid-alcohol was added and optical density was read at 595 nm. The data were reported as mean ± S.D. from at least four independent experiments

The CC_{50} of all test substances on Vero cells performed by both methods were summarized in Table 1. The CC_{50} values of lipoic acid, lipoamide, and ACV determined by the trypan blue exclusion test were similar to those obtained by the MTT test.

Table 1 Cytotoxicity of lipoic acid, lipoamide, and ACV on Vero cells.

	CC_{50} (μg/ml)	
Antiherpetic substances	Trypan blue exclusion method	MTT reduction assay
Lipoic acid	242.69	292.19
Lipoamide	239.03	271.10
Acyclovir	1650.59	1602.81

50% cytotoxic concentration (CC_{50}) values represent concentrations of the compounds required to reduce the viability of the cells by 50%. The reported values were derived from at least three independent assays of both methods.

2. Anti-HSV-1 and HSV-2 activity of lipoic acid and lipoamide

Antiviral activities of lipoic acid, lipoamide, and ACV (as a positive control) were determined as 50% inhibitory concentration (IC_{50}) (see appendix).

2.1 Effect on viral inactivation

Inactivation assay was used to investigate the inhibitory activity of lipoic acid and lipoamide to HSV-1 and HSV-2 on Vero cells, and ACV was used as a positive control. The anti-HSV-1 and anti-HSV-2 activities of each substance were shown in Figure 13. The IC_{50} values of lipoic acid, lipoamide, and ACV against HSV-1 were 96.34, 41.28, and 0.26 μg/ml, respectively. For anti-HSV-2 activity, the IC_{50} values were 111.75, 50.15, and 0.32 μg/ml for lipoic acid, lipoamide, and ACV, respectively. The IC_{50} values of each substance required for inactivation of HSV-2 was higher than the value required for inactivation of HSV-1. When the concentration of both lipoic acid and lipoamide was increased to 200 μg/ml, the inhibitory activities of these substances were almost 100%.

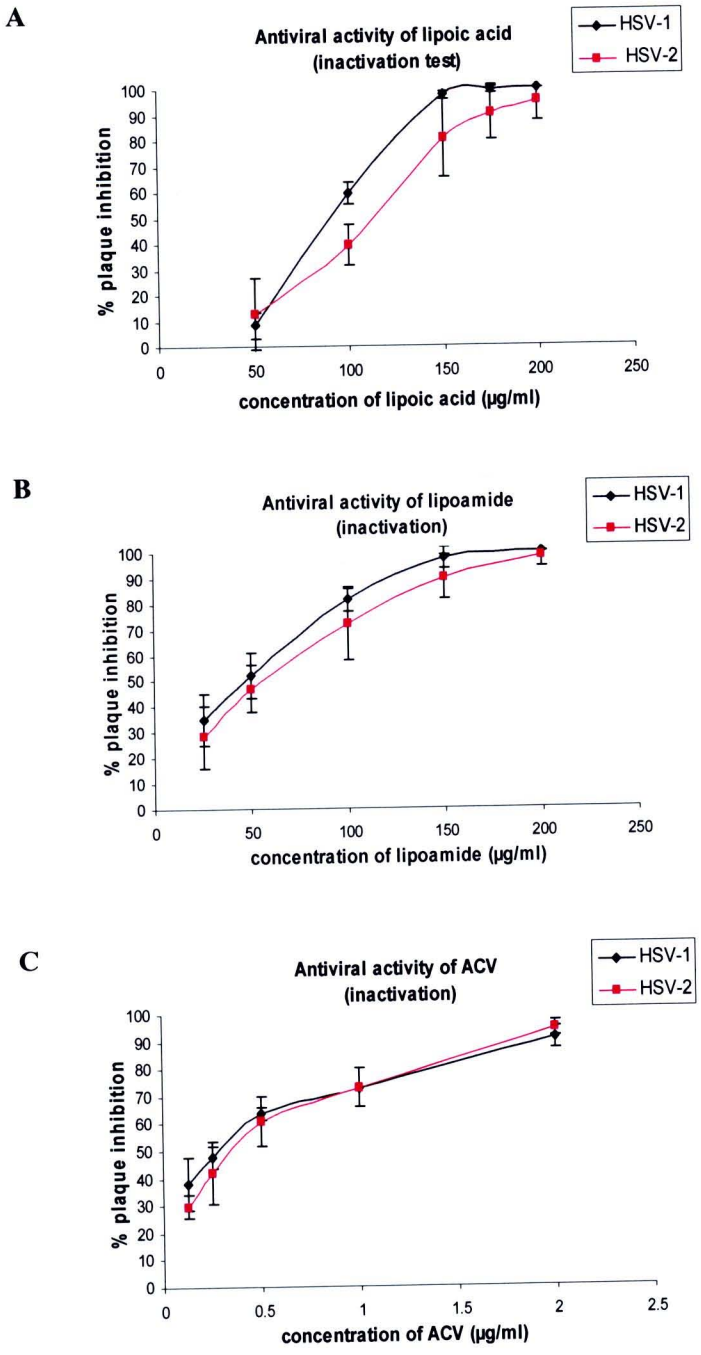

Figure 13. Anti-HSV activity of lipoic acid (A), lipoamide (B), and ACV (C) as determined by inactivation assay. Percentages of plaque inhibition were determined using infected cells as control. The data were reported as mean ± S.D. derived from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection was significantly different ($P<0.05$).

2.2 Effect on plaque formation

The antiviral activity of lipoic acid, lipoamide, and ACV (as positive control) obtained from plaque reduction assay or post-treatment was shown in Figure 14. The IC_{50} values of lipoic acid against HSV-1 and HSV-2 were 115.49 and 130.13 µg/ml, respectively. The IC_{50} of lipoamide were 60.47 µg/ml for HSV-1 inhibition, and 83.49 µg/ml for inhibition of HSV-2. ACV inhibited HSV-1 and HSV-2 plaque formation on Vero cells with IC_{50} of 0.34 and 0.60 µg/ml, respectively. The results showed that at the same concentration the efficiency of plaque inhibition of all test substances to HSV-1 infected cells is higher than to HSV-2 infected cells. When the concentration is as high as 200 µg/ml, lipoic acid and lipoamide almost completely inhibited the HSV-1 and HSV-2 plaque formation.

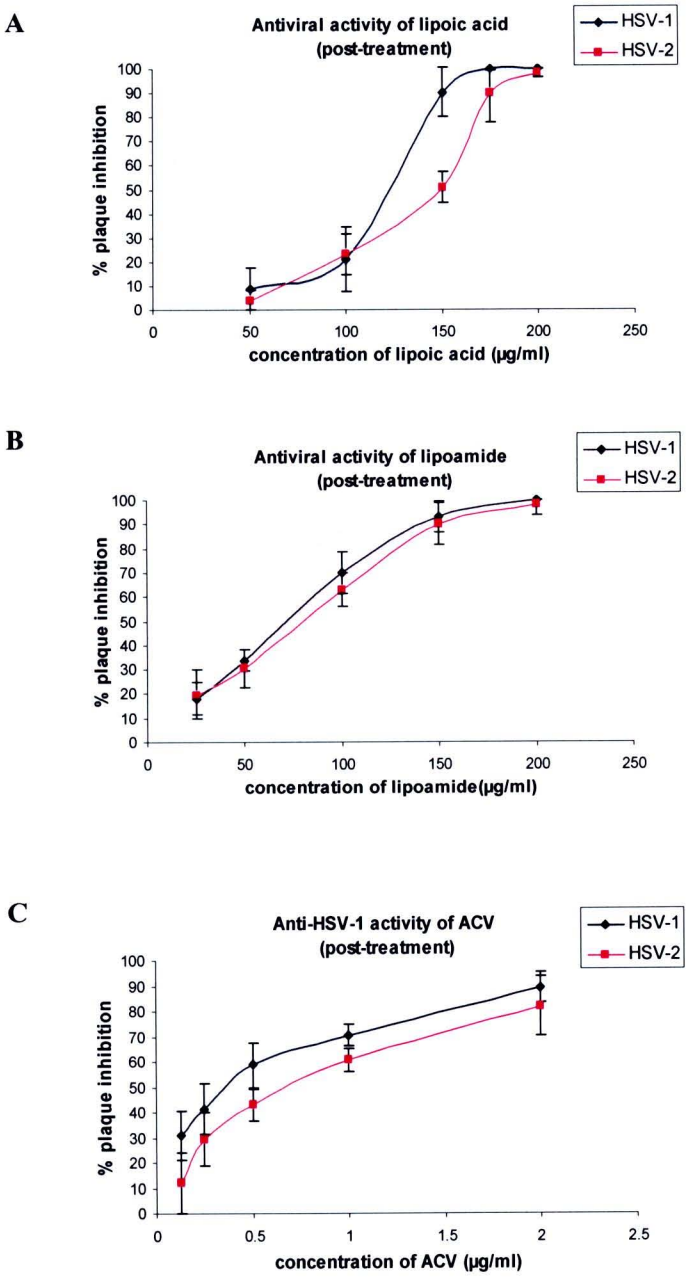

Figure 14. Anti-HSV activity of lipoic acid (A), lipoamide (B), and ACV (C) as determined by plaque reduction assay. The data were reported as mean ± S.D. from at least four independent experiments. Percent of plaque inhibition between treated and untreated cells after infection was significantly different ($P<0.05$).

2.3 Inhibition of HSV cytopathic effect (CPE)

The inhibition of the cytopathic effect was measured by MTT reduction assay. The results of viral inhibition were shown in Figure 15. In the inhibiton HSV-1 effect on Vero cells, the IC_{50} values of lipoic acid, lipoamide, and ACV were 126.85, 104.90, and 0.46 μg/ml, respectively. For HSV-2 effect on Vero cells, the IC_{50} values of lipoic acid, lipoamide, and ACV were 135.06, 107.48, and 0.59 μg/ml, respectively. The inhibition of HSV-2 effect by all three substances was less than that obtained with HSV-1.

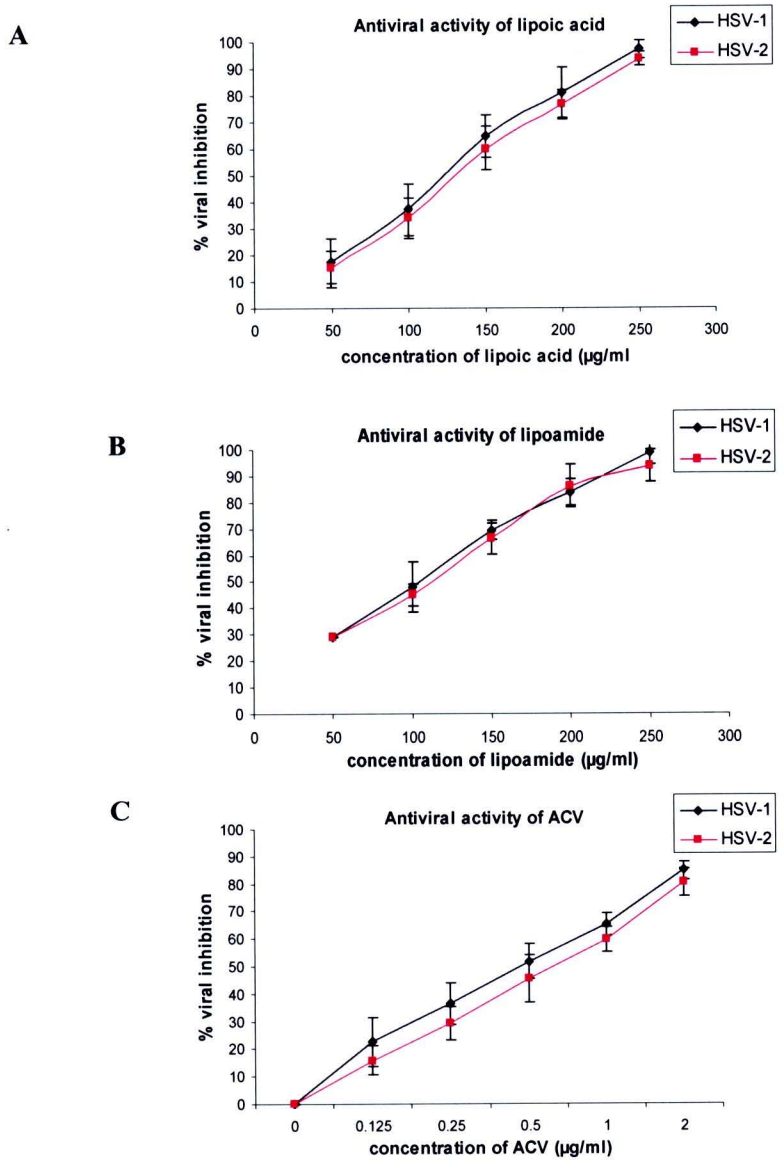

Figure 15. Anti-HSV activity of lipoic acid (A), lipoamide (B), and ACV (C) determined by MTT reduction assay. The data were reported as mean ± S.D. from at least four independent experiments. Percent of virus inhibition between treated and untreated cells after infection was significantly different ($P<0.05$).

The IC_{50} and SI (selective index) values against both HSV-1 and HSV-2 of lipoic acid, lipoamide, and ACV obtained form inactivation, plaque reduction, and MTT reduction assays on Vero cells were summarized in Table 2 and Table 3. In all cases, SI were more than 2 according to the CC_{50} of each drug higher than its IC_{50}. There were observed differences in the degree of viral inhibition depending on the assay used. The IC_{50} obtained from inactivation test was less than the IC_{50} obtained from the other two methods.

Table 2 Antiviral activity of lipoic acid, lipoamide, and ACV on HSV-1.

		HSV-1					
Antiherpetic substances	Host cells	IC_{50} (μg/ml) [a]	SI[d]	IC_{50} (μg/ml) [b]	SI[d]	IC_{50} (μg/ml) [c]	SI[d]
Lipoic acid	Vero	96.34	3.04	115.49	2.54	126.85	2.31
Lipoamide	Vero	41.28	6.57	60.47	4.48	105.17	2.58
Acyclovir	Vero	0.26	>100	0.34	>100	0.46	>100

a: obtained by inactivation assay

b: obtained by plaque reduction assay

c: obtained by MTT reduction assay

d: selective index of each drug calculated from CC_{50} / IC_{50} in each treatment and the results were determined from at least four independent experiments.

Table 3 Antiviral activity of lipoic acid, lipoamide, and ACV on HSV-2.

		HSV-2					
Antiherpetic substances	Host cells	IC_{50} (μg/ml) [a]	SI[d]	IC_{50} (μg/ml) [b]	SI[d]	IC_{50} (μg/ml) [c]	SI.[d]
Lipoic acid	Vero	111.75	2.62	130.13	2.25	135.06	2.17
Lipoamide	Vero	50.15	5.41	83.49	3.25	108.42	2.50
Acyclovir	Vero	0.32	>100	0.60	>100	0.59	>100

a: obtained by inactivation assay;

b: obtained by plaque reduction assay;

c: obtained by MTT reduction assay;

d: selective index of each drug calculated from CC_{50} / IC_{50} in each treatment and the results were determined from at least four independent experiments.

3. Effect of lipoic acid on HSV infectivity

The virucidal activity of lipoic acid against HSV-1 and HSV-2 was displayed in Table 4. The HSV-1 and HSV-2 titers after cell were treated with increasing concentration of lipoic acid slightly decreased compared with untreated control cells in condition of 37°C, 1 hour incubation. However, this difference on viral titer was not significant ($P>0.05$) even when treated with higher concentration of lipoic acid (500 μg/ml). It could be concluded that lipoic acid did not have direct effect on HSV-1 and HSV-2 infectivity.

Table 4 Effect of lipoic acid on HSV infectivity[a].

concentration of lipoic acid (μg/mL)	HSV-1 titer (x 10^5 PFU/mL)	HSV-2 titer (x 10^6 PFU/mL)
control	5.76 ± 0.68	7.65 ± 0.64
50	5.43 ± 0.57	5.73 ± 0.88
100	4.50 ± 0.60	7.60 ± 0.60
150	4.68 ± 0.54	5.65 ± 0.62
200	4.36 ± 0.61	7.64 ± 0.99
250	4.39 ± 0.29	6.31 ± 0.49
300	4.03 ± 0.22	6.74 ± 0.67
350	5.57 ± 0.34	5.66 ± 0.47
400	4.99 ± 0.35	5.77 ± 0.56
450	4.48 ± 0.30	6.36 ± 0.29
500	3.84 ± 0.14	7.76 ± 0.60

[a]Virucidal assay was performed by incubation of virus and lipoic acid at 37°C for 1 hour. The residual virus infectivity was titrated by plaque assay and reported as mean ± S.D. of three independent experiments.

4. Mode of anti-HSV activity of lipoic acid

Preliminary tests for possible mechanisms of action of lipoic acid in anti-HSV-1 and HSV-2 infection were performed using post-binding assay, penetration assay, virus growth inhibition assay, and pre-treatment assay.

4.1 Effect on viral adsorption

The effect of lipoic acid on HSV-1 and HSV-2 attachment to Vero cells was analyzed by post-binding assay. The results were shown in Figure 16. Citrate buffer pH 4 was used as a positive control and no plaque was detected. No statistically significant difference ($P>0.05$) in the amount of adsorbed HSV-1 and HSV-2 was observed between cells treated with lipoic acid and untreated control cells. The data indicated that HSV-1 and HSV-2 attachment was not affected by lipoic acid.

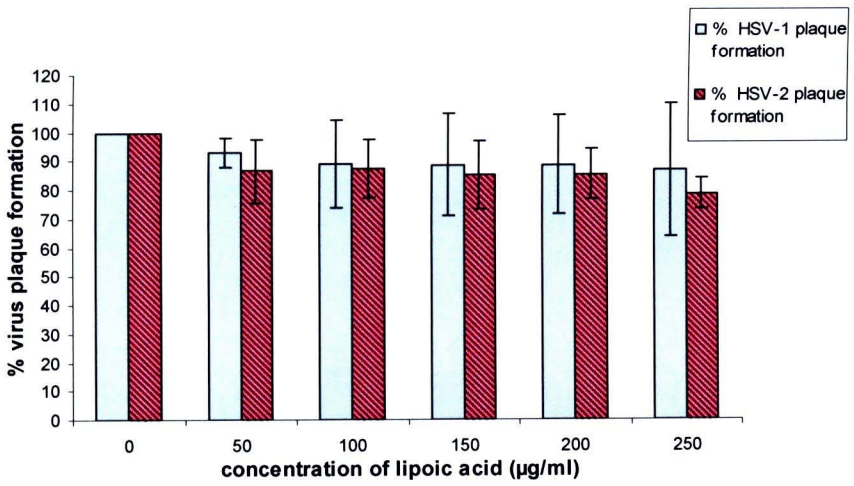

Figure 16. Effect of lipoic acid on HSV-1 and HSV-2 adsorption as determined by post-binding assay. HSV-1 and HSV-2 were attached to Vero cells and then incubated with lipoic acid at 4°C. The number of plaque was reported as mean ± S.D. from four independent experiments. Low pH citrate buffer was used as a positive control and no plaque was detected.

4.2 Effect on viral penetration

In penetration assay modified from Piret *et al.* (2002), no viral inhibition was observed when Vero cells were infected with HSV-1 or HSV-2 at 4°C. The penetration of viruses was prevented by increasing the temperature to 37°C and treating the infected cells with lipoic acid for different period of time. The result was shown in Figure 17. The number of both HSV-1 and HSV-2 plaque formation between treated and untreated infected control cells was not significantly different ($P>0.05$). Another method modified from De Logu *et al.* (2000) that increase the incubation time of lipoic acid with the infected cells after temperature shift showed the similar results (Figure 18). The quantities of HSV-1 and HSV-2 in treated cells at any time point were not significantly different ($P>0.05$) from those observed from untreated infected control cells. Altogether, these results suggested that lipoic acid did not interfere with HSV penetration into Vero cells.

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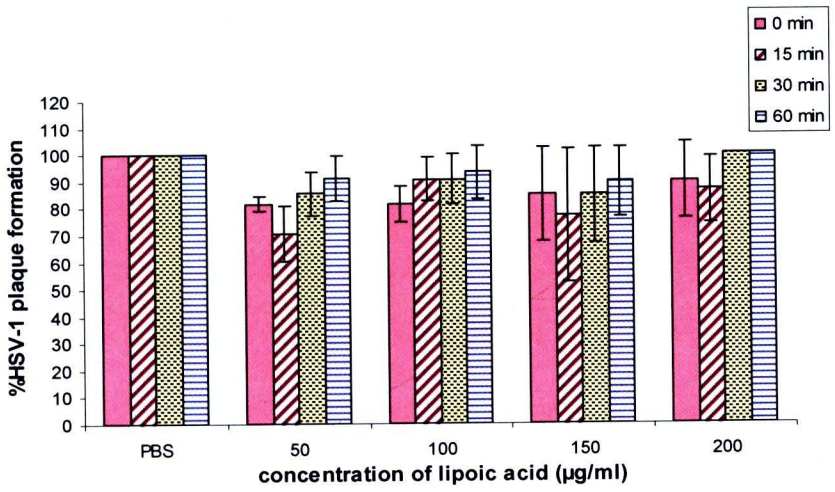

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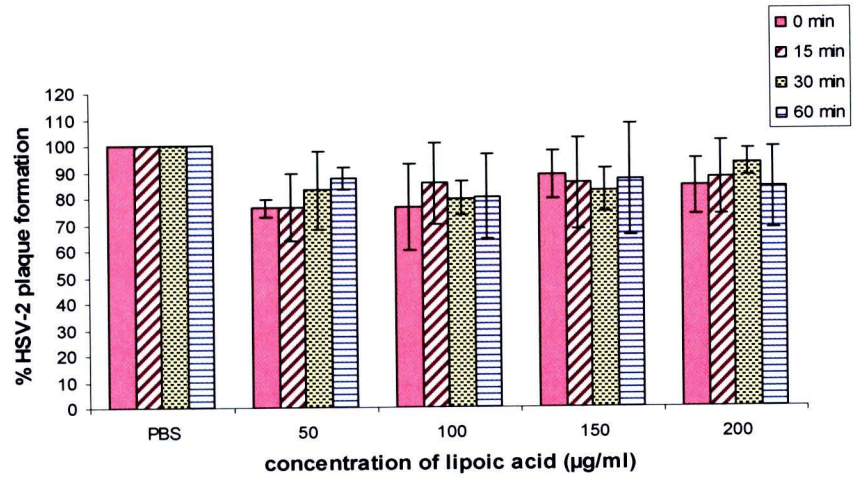
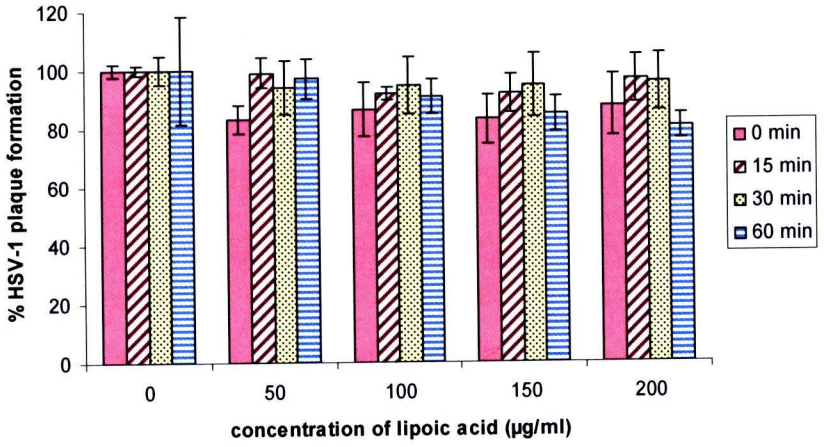


Figure 17. Effect of lipoic acid on HSV-1 (A) and HSV-2 (B) penetration determined by the assay derived from Piret *et al.* (2002). Penetration assay was performed on Vero cells. After viral adsorption at 4°C, the temperature was shifted to 37°C for 0, 15, 30, and 60 minutes to allow penetration of bounded virus into treated and untreated control cells. The number of plaques was reported as mean ± S.D. from four independent experiments.

A



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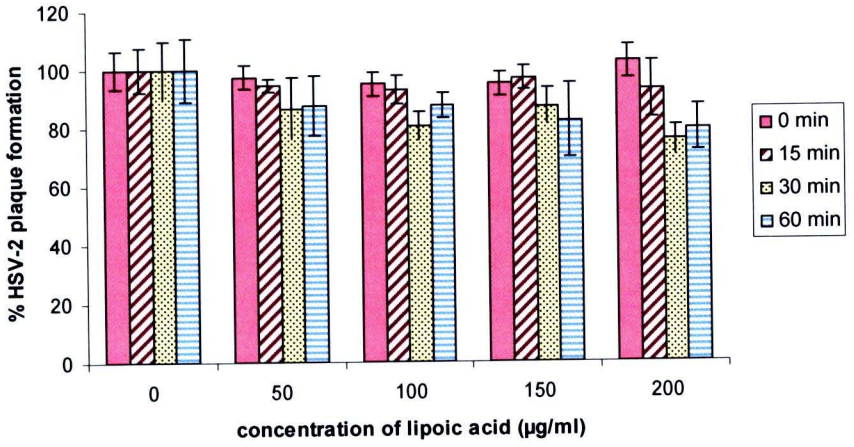

Figure 18. Effect of lipoic acid on HSV-1 (A) and HSV-2 (B) penetration determined by the assay derived from De Logu *et al.* (2000). Penetration assay was performed on Vero cells. After viral adsorption at 4°C, the temperature was shifted to 37°C for 0, 15, 30, and 60 minutes to allow penetration of bounded virus into treated and untreated control cells. The number of plaques was reported as mean ± S.D. from four independent experiments.

4.3 Effect on virus growth

To study the inhibitory effect of lipoic acid on the stages of HSV-1 and HSV-2 infection, a time of addition experiment was performed. Lipoic acid was added to Vero cells at various time points including before and after virus infection. The results shown that lipoic acid treatment both before and after virus inoculation potentially suppressed HSV-1 and HSV-2 infection as displayed in Figure 18. When lipoic acid was added to the cells as 1 hour pre-treatment at 37°C and then washed out before viral infection, the reduction of virus yield was more than 80% for both HSV-1 and HSV-2 as compared with untreated control cells. Moreover, the addition of lipoic acid to pretreated cells at 1 and 3 hour post infection exhibited HSV inhibitory activity higher than 98% for HSV-1 and 94% for HSV-2. The extent of inhibition of HSV-1 and HSV-2 production was observed when the treatment with lipoic acid was started either 1 or 3 hour after virus inoculation and removed at 3 and 9 hour post infection, respectively. Indeed, the inhibition of HSV-1 and HSV-2 replication was greater than when lipoic acid was given to the cell at 1 hour after virus infection, and the compound was maintained in the culture until the end of the experiment. However, all three conditions in which lipoic acid was added to the cells after virus challenge resulted in reduction of HSV-1 and HSV-2 infectivity by more than 50% as compared to untreated infected cells. The result indicated that lipoic acid affected all steps of HSV replication. In addition, it probably affected the susceptibility of the target cells to infection by HSV.

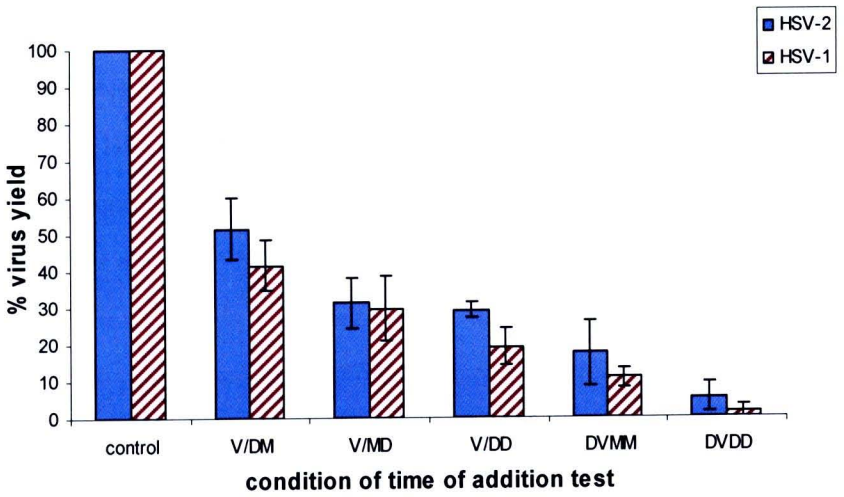

Figure 18. Inhibition of HSV growth by lipoic acid as determined by time of addition assay. Control: untreated infected control cells; V/DM: lipoic acid added at 1 hour after viral adsorption and then withdrawn and replaced by growth medium at 3 hour post infection; V/MD: lipoic acid added at 3 hour after viral adsorption and then withdrawn and replaced by growth medium at 9 hour post infection (the end of experiments); V/DD: lipoic acid added at 1 hour after viral adsorption and then withdrawn and replaced by growth medium at 9 hour post infection (the end of experiments); DVMM: lipoic acid given to uninfected cells for 1 hour and removed before viral infection; DVDD: lipoic acid given to uninfected cells for 1 hour and removed before viral infection and then added again at 1 hour after viral adsorption and then withdrawn and replaced by growth medium at 9 hour post infection (the end of experiments). The time of addition experiments were performed on Vero cells as described in chapter III. Each bar represented the percentage of virus yield (PFU/ml) compared with controls. Data were reported as the mean $\pm$ S.D. from three independent experiments. The difference in virus yield of all cases was statistically significant ($P<0.05$).

4.4 Prophylactic activity of lipoic acid

Prophylactic activity assay or pretreatment assay was used to determine the activity of lipoic acid in preventing HSV-1 and HSV-2 infection on Vero cells. Pretreatment with lipoic acid before virus adsorption showed dose-dependent inhibitory activity on plaque formation as shown in Figure 19. HSV-1 and HSV-2 plaque formation was almost completely inhibited by the added lipoic acid at all time periods including before, during, and after virus infection. For each condition, only when uninfected cells were pretreated with lipoic acid both HSV-1 and HSV-2 plaque formation was reduced by more than 80%. Antiviral activity was higher when lipoic acid was added to the cells again either during or after virus adsorption. The addition of lipoic acid to pretreated cells during 1 hour of virus adsorption produced less antiviral activity in the inhibition of plaque formation than the addition after viral infection.

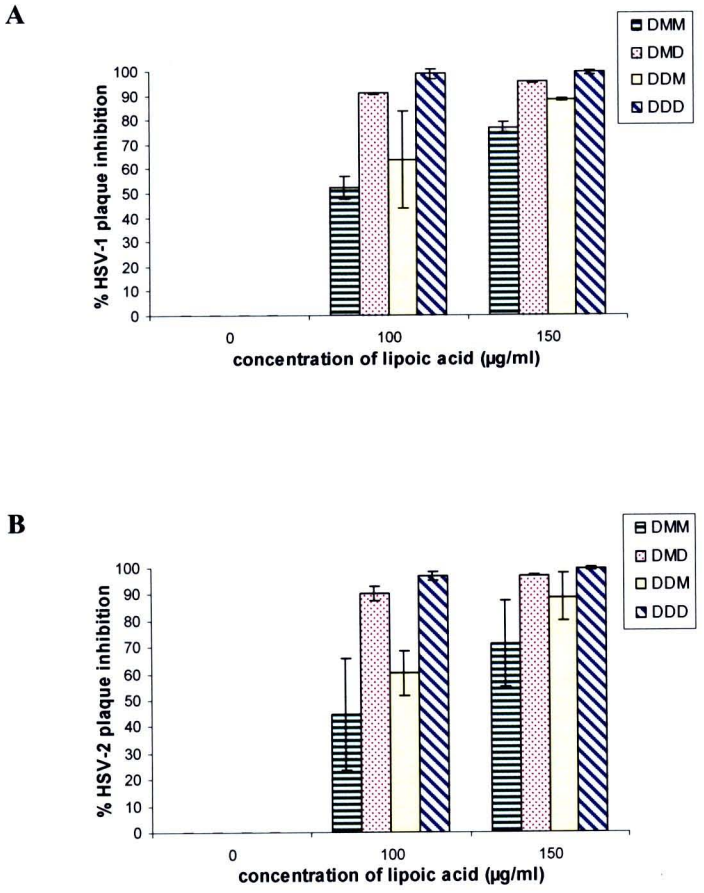

Figure 19. Anti-HSV activity of lipoic acid against HSV-1 (A) and HSV-2 (B) determined by pre-treatment assay. DMM: lipoic acid given to uninfected cells for 1 hour and removed before viral infection; DMD: lipoic acid given to uninfected cells for 1 hour and removed before viral infection and the test substance added again at 1 hour post infection and maintain during 48 hours of incubation; DDM: lipoic acid given to uninfected cells for 1 hour and maintained during 1 hour of viral adsorption; DDD: lipoic acid given to uninfected cells for 1 hour and maintained until the end of experiments (48 hour). Each bar represented the percent inhibition of plaque formation with respect to the untreated infected control cells. Data were reported as the mean $\pm$ S.D. from four separate experiments. The difference in inhibition of plaque formation of all cases was statistically significant ($P<0.05$).

5. Anti-HSV-1 and anti-HSV-2 activities of lipoic acid in various cell types

Effect of lipoic acid against HSV-1 and HSV-2 infection on cervix epithelium HeLa cell culture and Normal human dermal fibroblast NHDF CC-2511 cell culture were determined.

5.1 Effect of lipoic acid on viability of cells

Cytotoxicity of lipoic acid was examined by means of MTT reduction assay. The results were presented in Figure 20. Similar to Vero cells, lipoic acid had an effect on the proliferation of HeLa cells, and the CC_{50} value of this substance in this cell line was 259.05µg/ml as shown in Table 5. On the contrary, lipoic acid affected NHDF CC-2511 cell proliferation less than that on Vero cells and HeLa cells. The CC_{50} of lipoic acid in NHDF CC-2511 was more than 500 µg/ml. The data suggested that variation in the toxicity of lipoic acid depended on cell types used in this study.

Table 5 Cytotoxicity of lipoic acid on different cell types

Host cells	CC_{50} (µg/ml)[a]
Vero	292.19
HeLa	259.10
NHDF CC-2511	569.14

[a]50% cytotoxicity concentration (CC_{50}) values represent concentration of lipoic acid that shows 50% cytotoxicity in MTT reduction test. The data were determined from at least four independent experiments.

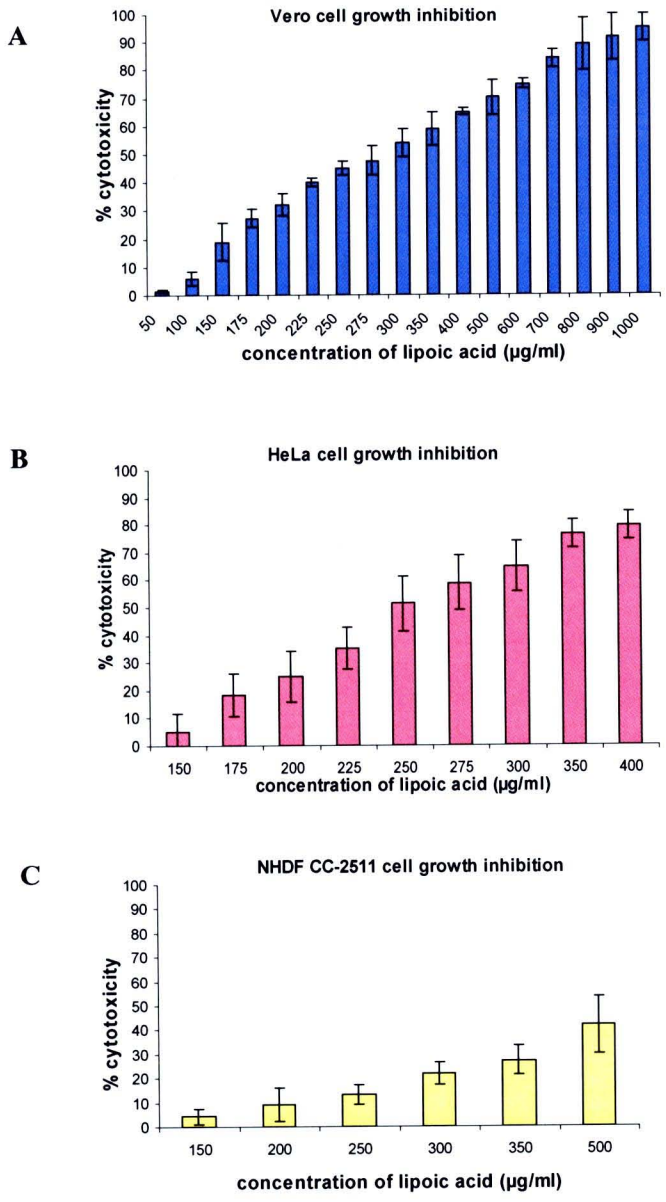

Figure 20. Cytotoxicity of lipoic acid on Vero cells (A), HeLa cells (B), and NHDF CC-2511 (C) as determined by MTT reduction method. Cells were incubated with various concentrations of test compounds in media for 72 hours. Cell viability was determined by MTT assay. The data were reported as mean ± S.D. from at least four independent experiments and the difference in % cytotoxicity between treated cells and control in each cell type was statistically significant ($P<0.05$).

5.2 Inhibition of HSV-1 and HSV-2 infection by lipoic acid

MTT reduction assay was used to determine antiviral activity of lipoic acid in HeLa cells and NHDF CC-2511 cells. The IC_{50} values of lipoic acid against both HSV-1 and HSV-2 on Vero cells, HeLa cells, and NHDF CC-2511 cells were summarized (Table 6). In HSV-1 inhibition, the IC_{50} values were 109.61 and 58.20 μg/ml for HeLa cells and NHDF CC-2511 cells, respectively. The IC_{50} values of lipoic acid against HSV-2 infection in HeLa cells and NHDF CC-2511 cells were 125.90 and 92.26 μg/ml, respectively. These IC_{50} values were lower than the IC_{50} obtained from Vero cells. These IC_{50} and SI values were shown in Figure 21.

Table 6 Anti-HSV activity of lipoic acid on different cell types.

	HSV-1		HSV-2	
Host cells	IC_{50} (μg/ml)[a]	SI	IC_{50} (μg/ml)	SI[b]
Vero	126.85	2.30	135.06	2.16
HeLa	109.61	2.36	125.90	2.06
NHDF CC-2511	58.20	9.78	92.26	5.80

[a]50% inhibitory concentration (IC_{50}) was the concentration of lipoic acid required to inhibit 50% of virus-induced CPE.

[b]SI: selective index of each drug calculated from CC_{50} / IC_{50}. of at least four independent experiments.

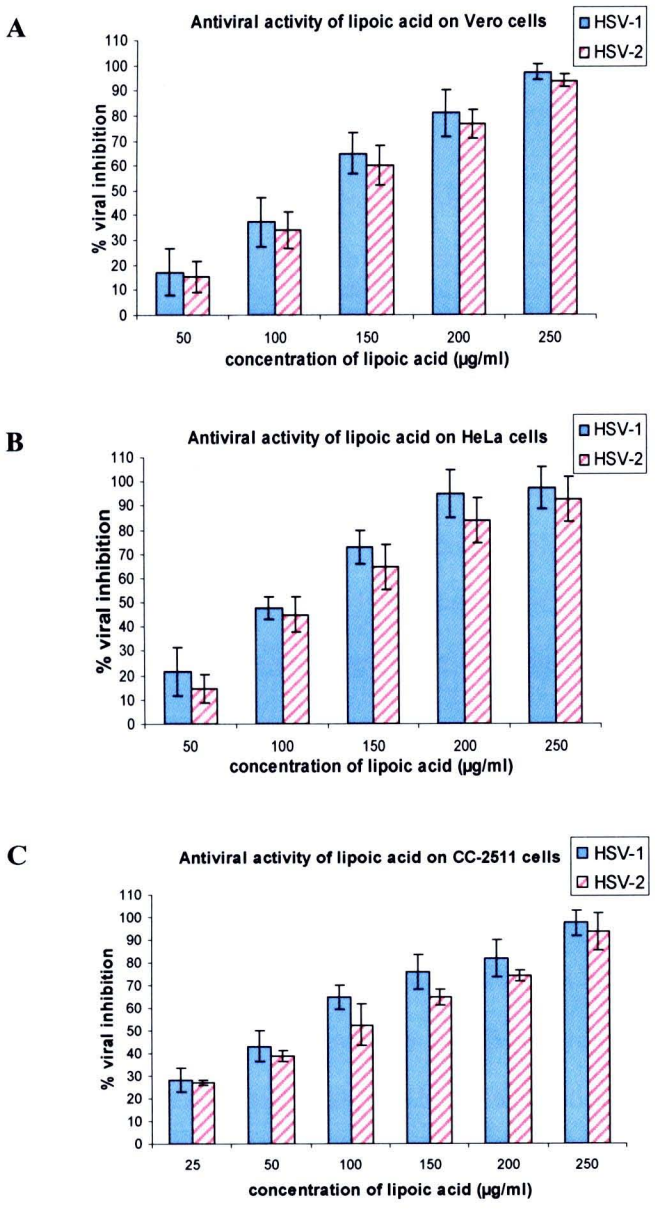

Figure 21. Anti-HSV activity of lipoic acid on different cell types. A: Vero cells; B: HeLa cells; C: NHDF CC-2511. Virus inhibition was examined by MTT reduction assay. The data were reported as means ± S.D. from at least four independent experiments. The viral inhibition on each cell type was significantly different ($P<0.05$).