CHAPTER IV

RESULTS

1. Preparation of crude extracts of medicinal plants

Five extracts for each medicinal plant of *G. pentaphylla* , *I. maxima*, and *W. edulis* were performed according to the figure 4. The percent yield of the most active extracts were 2.38, 4.04 and 3.24 for F4/ *G. pentaphylla,* F3/ *I. maxima*, and F4/ *W. edulis* , respectively, as indicated in table 4.

2. Cytotoxicity test

The cytotoxicity of the extracts were determined using uninfected Vero cells to obtain the 50% cytotoxic concentration (CC_{50}). The cells were treated with various concentrations of the extracts for 72 h. Then the cells were fixed and stained with methylene blue and the OD at 550 was measured. All the CC_{50} were determined by as described in appendix. The dimethyl sulfoxide (DMSO) was used to solubilize the extracts. The maximal concentration of DMSO that did not affect the cytotoxicity to cell culture was 2%. Therefore, in this study, the final concentration of DMSO in each plant extract solution tested was not more than 2%.

Five medicinal plant extracts from each of 3 plants: *G. pentaphylla, I. maxima* and *W. edulis* were used. All extracts were tested for cytotoxicity assay in Vero cell, the results were indicated in table 5. The selective index determined by the ratio of CC_{50} and EC_{50} were demonstrated in table 6-8. The selective index of 80.93 (95.17), 15.46 (13.97) and 71.36 (56.91) were observed for the most active fraction of each medicinal plant , F4 /*G. pentaphylla* , F3 / *I. maxima* and F4 / *W. edulis*, respectively against HSV-1 (HSV-2). The CC_{50} of ACV in Vero cells was also determined and the value was 2750±250 µg/ml.

3. Antiviral activity of medicinal plant extracts against HSV-1 and HSV-2

Five extracts from G*lycosmis pentaphylla, Ipomoea maxima, and Willughbeia edulis* prepared and tested for antiviral activity. Antiherpes simplex virus activity of the extract was performed in Vero cells infected with HSV-1 and HSV-2 to elucidate the mode of action of the inhibition of virus replication, the effects of the extracts were studied in inactivation, prophylactic activity and plaque reduction or post-treatment assay.

As indicated in Table 6-8 , it was revealed that the extracts from ethanol fraction (F1), chloroform fraction (F2), methanol fraction (F3), hexane fraction (F4) and aqueous fraction (F5) elicited different extent of antiviral activities at various concentrations.

The possibility that the extracts directly interfered with virus infectivity, thus preventing adsorption of virus particles to host cells, was iinvestigated. In inactivation assay, HSV was pretreated with various concentrations of the extracts for 1h at 37°C before adding to the host cells and the virus was detrermined by plaque assay. Figure 5-7 showed that *G. pentaphylla* (F4) , *I maxima* (F3) and *W. edulis* (F4) decreased, in concentration-dependent manner(r=0.71-0.97), their infectivities for Vero cells. The concentrations of extracts of *G. pentaphylla* , *I maxima* and W. edulis which inhibit 50% (EC_{50}) of the infectivity of HSV-1 (and HSV-2) were 11.36 ±0.51(9.66±0.49), 9.14±0.49 (10.11±0.21) and 15.05±0.46 (18.87±0.86) µg/ml, respectively.

In prophylactic activity assay, Vero cells were pretreated with various concentrations of the extracts for 1h at 37°C before cell infection. The virus was determined by plaque assay after the incubation for 48 h. As indicated in figure 5-7, it was showed that *G. pentaphylla* (F4) , *I maxima* (F3) and *W. edulis* (F4) for 1h at 37°C before HSV-1 and HSV-2 infection, decreased, in concentration-dependent manner (r=0.73-0.99), their infectivities for Vero cells. The concentrations of extract of *G. pentaphylla* , *I maxima* and *W. edulis* which inhibit 50% (EC_{50}) of the infectivity of HSV-1

(HSV-2) were 258.44±2.88 (313.67±6.93), 37.06 ±0.76 (48.92±0.38) and 263.48±2.83(328.56±0.99) µg/ml, respectively.

In plaque reduction or post-treatment assay, Vero cells were infected with HSV for 1 h and the cells were treated with various concentrations of the extracts for 1 h. Then the number of virus was determined by plaque assay after the incubation for 48 h. The result was demonstrated in figure 5-7. It revealed that the extracts of *G. pentaphylla* (F4) , *I maxima* (F3) and *W. edulis* (F4) , decreased, in concentration-dependent manner(r=0.95-0.99), their infectivities for Vero cells. The concentrations of extracts of *G. pentaphylla* , *I maxima* and *W. edulis* which inhibited 50% (EC_{50}) of the infectivities of HSV-1 (and HSV-2) were 154.66±2.00 (159.09±8.59), 44.34±0.50(49.14±1.42) and 226.45±0.38 (236.24±1.64) µg/ml, respectively.

Effective concentration for 50% virus growth inhibition (EC_{50}) of acyclovir, as a control against HSV-1 (HSV-2) in inactivation, prophylactic activity and plaque reduction assay as indicated in table 9 were 0.082±0.002 (0.45±0.002), 0.103±0.004 (0.58±0.005), and 0.16±0.002 (0.69±0.01) µg/ml, respectively.

Table 4 Percent Yield of crude extracts

No.	Plants [Thai name]	Fresh plant weight (g)	Fraction	Extract weight (g)	Yield (%)
1	*Glycosmis pentaphylla* (Retz).DC [เขยตาย]	859.00	F1	94.50	11.00
			F2	40.98	4.77
			F3	21.70	2.52
			F4	20.44	2.38
			F5	10.95	1.27
2	*Ipomoea maxima* (linn.f.).Don [สะอึก]	556.80	F1	60.94	10.94
			F2	31.42	5.64
			F3	22.50	4.04
			F4	23.21	4.17
			F5	15.70	2.81
3	*Willughbeia edulis* roxb [คุย]	780.50	F1	78.92	10.11
			F2	35.73	4.58
			F3	21.49	2.75
			F4	25.28	3.24
			F5	11.59	1.48

F1: Ethanol extract; F2: Chloroform extract; F3: Methanol extract; F4: Hexane extract; F5: Aqueous extract

Table 5 *In vitro* cytotoxic concentration (CC_{50}) of medicinal plant extracts.

No.	Plants (Thai name)	Fraction	CC_{50}(ug/ml)
1	*Glycosmis pentaphylla* (Retz).DC (เขยตาย)	F1	840.47 ±9.79
		F2	805.73 ±1.26
		F3	898.34 ±2.92
		F4	919.32 ±8.14
		F5	1738.91±3.83
2	*Ipomoea maxima* (linn.f.).Don (สะอึก)	F1	99.49 ± 0.85
		F2	103.00± 0.91
		F3	141.27± 0.76
		F4	90.49± 0.56
		F5	154.05± 0.24
3	*Willughbeia edulis* roxb. (คุย)	F1	845.95 ± 1.54
		F2	823.81± 1.72
		F3	944.24± 1.51
		F4	1073.90± 0.97
		F5	1487.75 ±0.29

F1: Ethanol extract; F2: Chloroform extract; F3: Methanol extract; F4: Hexane extract; F5: Aqueous extract

Table 6 Tests for antiviral activities of *Glycosmis pentaphylla* against HSV-1 and HSV-2 using inactivation assay, prophylactic activity assay and plaque reduction assay.

Treatments	Extracts	[a]EC_{50} µg/ml (mean ± SD)		[a]CC_{50} µg/ml (mean±SD)	Selective Index (CC_{50}/EC_{50})	
		HSV-1	HSV-2		HSV-1	HSV-2
Inactivation assay	F1	46.89 ± 0.59	51.25 ± 1.15	840.47 ±9.79	17.89	16.40
	F2	24.19 ± 1.27	26.88 ± 0.36	805.73 ±1.26	33.31	29.96
	F3	17.37 ± 0.16	17.63 ±0.59	898.34 ±2.92	51.72	50.96
	F4	11.36 ± 0.51	9.66 ± 0.20	919.32 ±8.14	80.93	95.17
	F5	34.53 ± 0.86	58.58 ± 0.43	1738.91±3.83	50.36	29.68
Proohylactic activity assay	F1	383.2± 4.60	412.98± 9.81	840.47 ±9.79	2.19	2.04
	F2	447.05± 2.80	784.10± 23.61	805.73 ±1.26	1.80	1.03
	F3	354.04 ±4.69	639.52 ±21.81	898.34 ±2.92	2.54	1.40
	F4	258.44± 2.88	313.67± 6.93	919.32 ±8.14	3.56	2.93
	F5	549.53± 8.06	815.70 ±2.90	1738.91±3.83	3.16	2.13
Plaque reduction assay	F1	198.80± 2.30	267.11± 5.40	840.47 ±9.79	4.23	3.15
	F2	219.67± 8.58	226.5± 8.09	805.73 ±1.26	3 67	3.56
	F3	324.47± 10.20	302.01± 8.45	898.34 ±2.92	2.77	2.97
	F4	154.66± 2.00	159.09± 8.59	919.32 ±8.14	5.94	5.78
	F5	1300.00 ±16.93	1500.6±8.63	1738.91±3.83	1.34	1.15

[a] EC_{50} or CC_{50} was deterined by three independent experiments.

Table 7 Antiviral activities of *Ipomoea maxima* against HSV-1 and HSV-2 using inactivation assay , prophylactic activity assay and plaque reduction assay.

Treatments	Extracts	[a]EC_{50} µg/ml (mean ± SD)		[a]CC_{50} µg/ml (mean±SD)	Selective Index (CC_{50}/EC_{50})	
		HSV-1	HSV-2		HSV-1	HSV-2
Inactivation assay	F1	10.13 ± 0.25	10.29 ± 0.33	99.49 ± 0.85	9.82	9.67
	F2	10.38 ± 1.47	11.33 ± 0.83	103.00± 0.91	9.92	9.09
	F3	9.14 ± 0.49	10.11 ± 0.21	141.27± 0.76	15.46	13.97
	F4	13.02 ± 0.36	13.49 ± 1.37	90.49± 0.56	6.95	6.70
	F5	55.37 ± 0.33	59.85 ± 0.42	154.05± 0.24	2.78	2.57
Prophylactic activity assay	F1	43.15 ± 0.51	49.26 ± 0.60	99.49 ± 0.85	2.30	2.02
	F2	65.10 ± 0.68	66.38 ± 1.65	103.00± 0.91	1.58	1.55
	F3	37.06 ± 0.76	48.92 ± 0.38	141.27± 0.76	3.81	2.89
	F4	74.93 ± 0.46	82.77 ± 2.49	90.49± 0.56	1.21	1.09
	F5	103.99 ± 2.43	106.38 ± 1.47	154.05± 0.24	1.48	1.45
Plaque reduction assay	F1	48.45 ± 0.14	54.00 ± 0.07	99.49 ± 0.85	2.05	1.84
	F2	52.48 ± 2.35	60.69 ± 1.41	103.00± 0.91	1.96	1.70
	F3	44.34 ± 0.60	49.14 ± 1.42	141.27± 0.76	3.19	2.87
	F4	63.44 ± 1.34	85.99 ± 1.42	90.49± 0.56	1.43	1.05
	F5	127.24 ± 1.87	129.77 ± 1.31	154.05± 0.24	1.21	1.19

[a] EC_{50} or CC_{50} was deterined by three independent experiments.

Table 8 Antiviral activities of *Willughbeia edulis* against HSV-1 and HSV-2 using inactivation assay, prophylactic activity assay and plaque reduction assay.

Treatments	Extracts	[a]EC_{50} µg/ml (mean ± SD)		[a]CC_{50} µg/ml (mean±SD)	Selective Index (CC_{50}/EC_{50})	
		HSV-1	HSV-2		HSV-1	HSV-2
Inactivation assay	F1	36.85 ± 1.89	37.41 ± 0.96	845.95 ± 1.54	22.96	22.61
	F2	31.78 ± 1.55	35.77 ± 0.59	823.81± 1.72	25.92	23.03
	F3	19.81 ± 0.30	26.88 ± 2.57	944.24± 1.51	47.66	35.13
	F4	15.05 ± 0.46	18.87 ± 0.86	1073.90± 0.97	71.36	56.91
	F5	231.15 ± 2.09	318.89 ± 4.23	1487.75 ±0.29	6.44	4.67
Prophylactic activity assay	F1	429.49 ± 1.60	480.42 ± 1.59	845.95 ± 1.54	1.97	1.76
	F2	428.77 ± 2.18	479.95 ± 0.88	823.81± 1.72	1.92	1.72
	F3	356.90 ±2.11	430.81 ± 1.83	944.24± 1.51	2.65	2.19
	F4	263.48 ± 2.83	328.56 ± 0.99	1073.90± 0.97	4.08	3.26
	F5	478.59 ± 2.36	686.38 ± 1.85	1487.75 ±0.29	3.11	2.17
Plaque reduction assay	F1	254.06 ± 1.15	263.85 ± 0.79	845.95 ± 1.54	3.33	3.21
	F2	251.77 ± 0.80	257.63 ± 2.29	823.81± 1.72	3.27	3.20
	F3	244.63 ± 0.31	249.91 ± 0.87	944.24± 1.51	3.86	3.78
	F4	226.45 ± 0.38	235.24 ± 1.64	1073.90± 0.97	4.74	4.57
	F5	575.33 ± 1.74	608.44 ± 2.81	1487.75 ±0.29	2.56	2.45

[a] EC_{50} or CC_{50} was deterined by three independent experiments

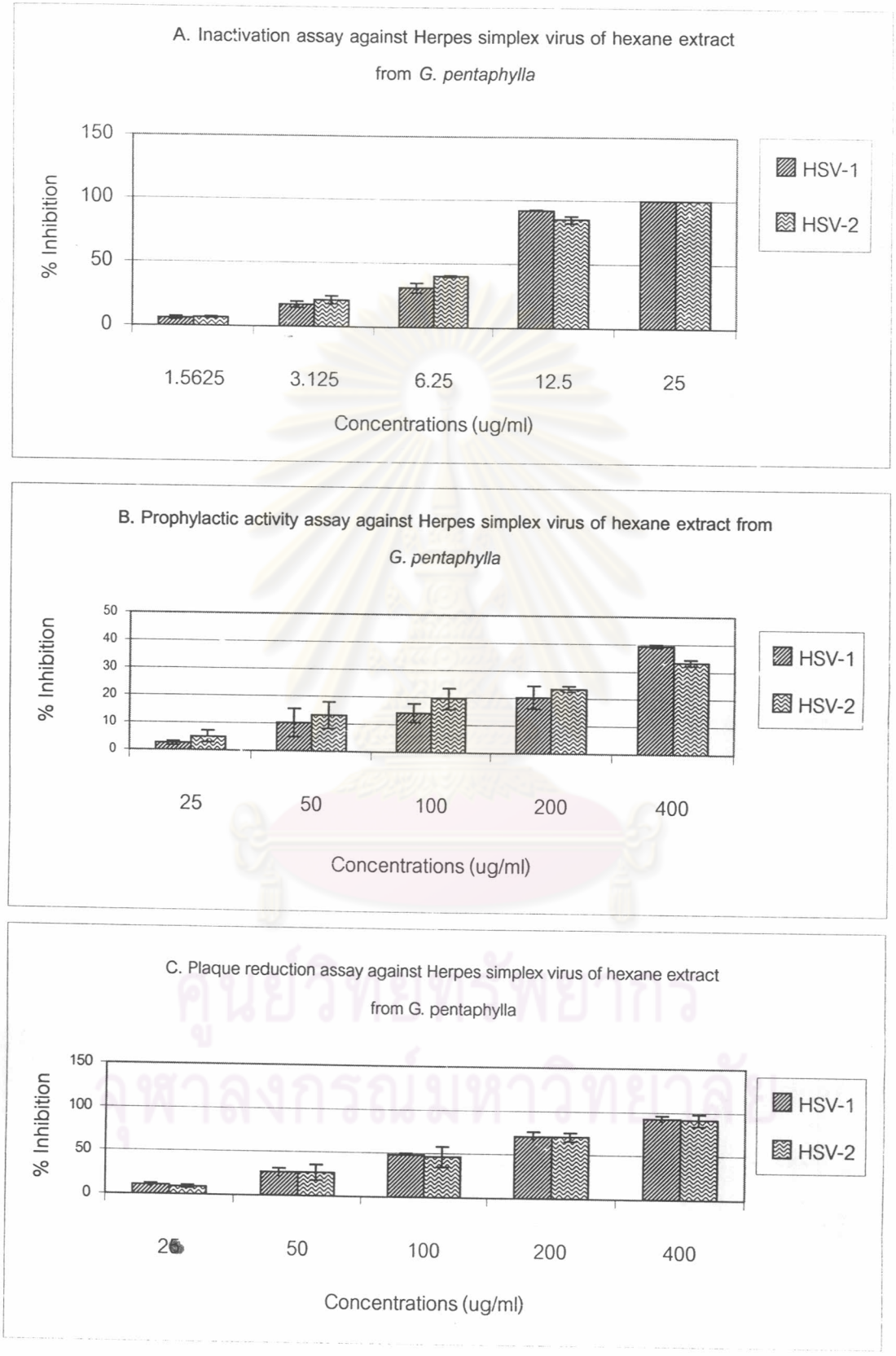


Figure 5 Antiviral acitvity of hexane extract from *Glycosmis pentaphylla* against herpes simplex virus. A. Inactivation assay, B. Prophylactic activity assay, C. Plaque reduction assay. bar represented SD from three independent experiments.

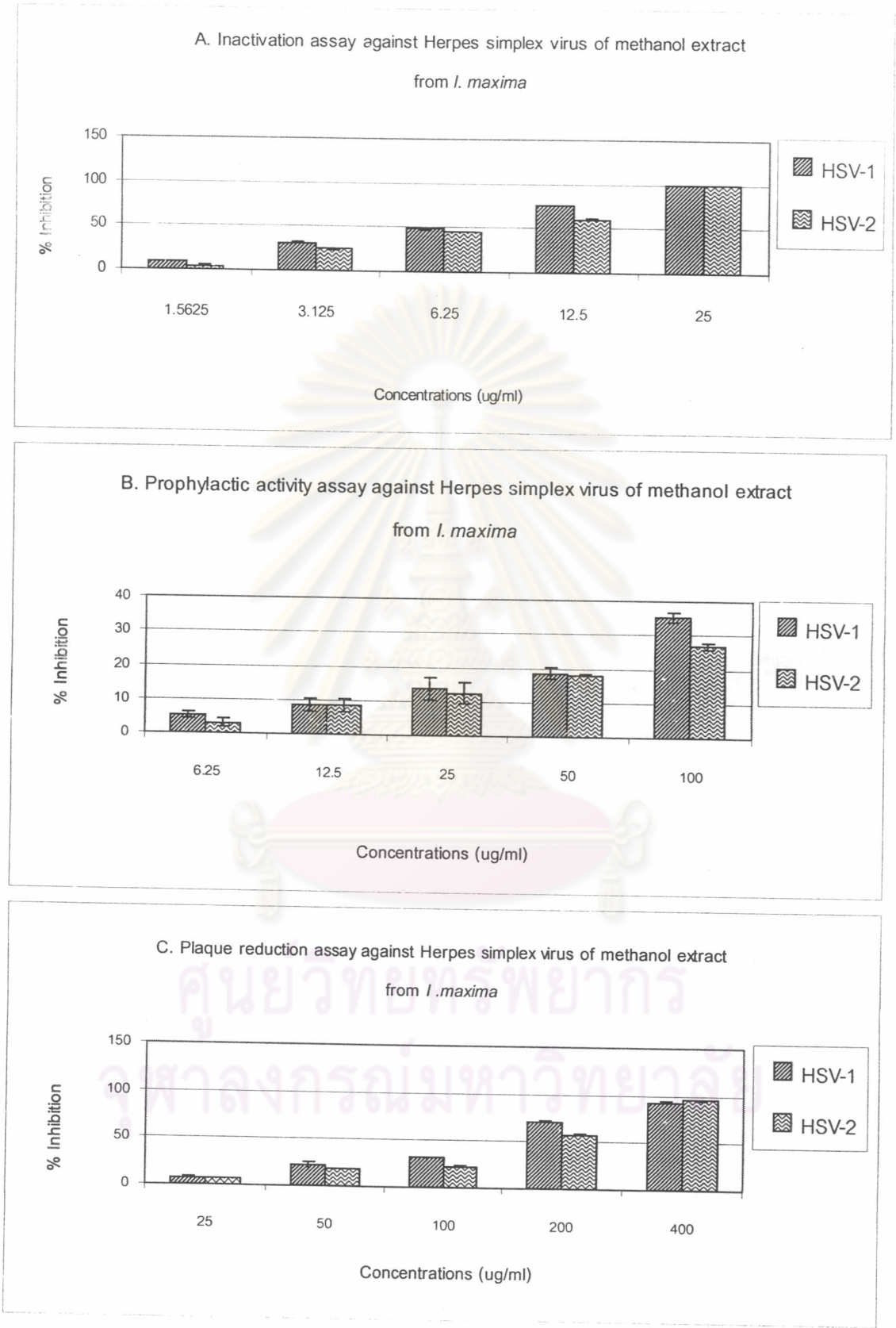
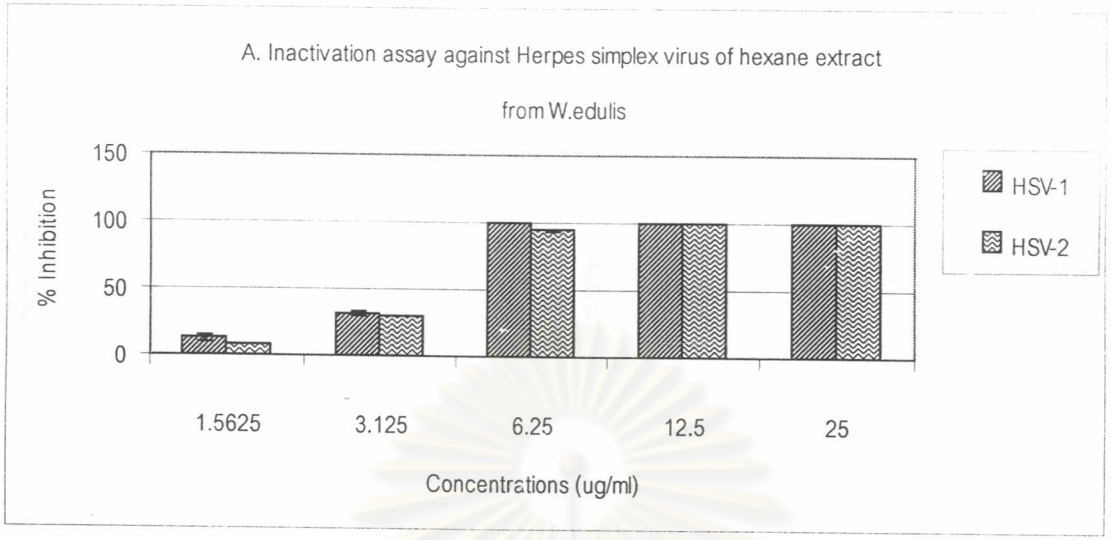
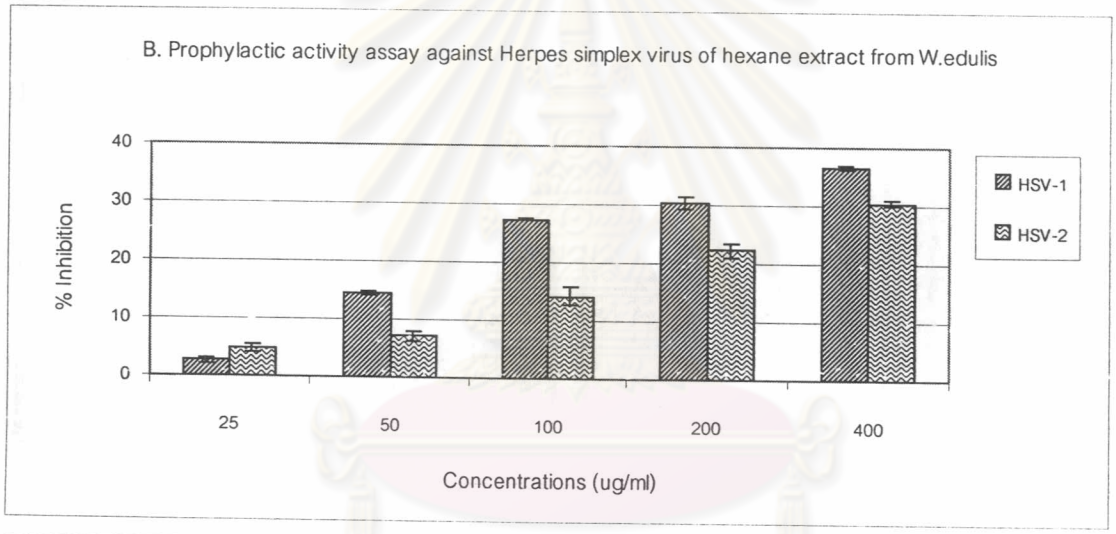


Figure 6 Antiviral acitvity of hexane extract from *Ipomoea maxima* against herpes simplex virus. A. Inactivation assay, B. Prophylactic activity assay, C. Plaque reduction assay. bar represented SD from three independent experiments





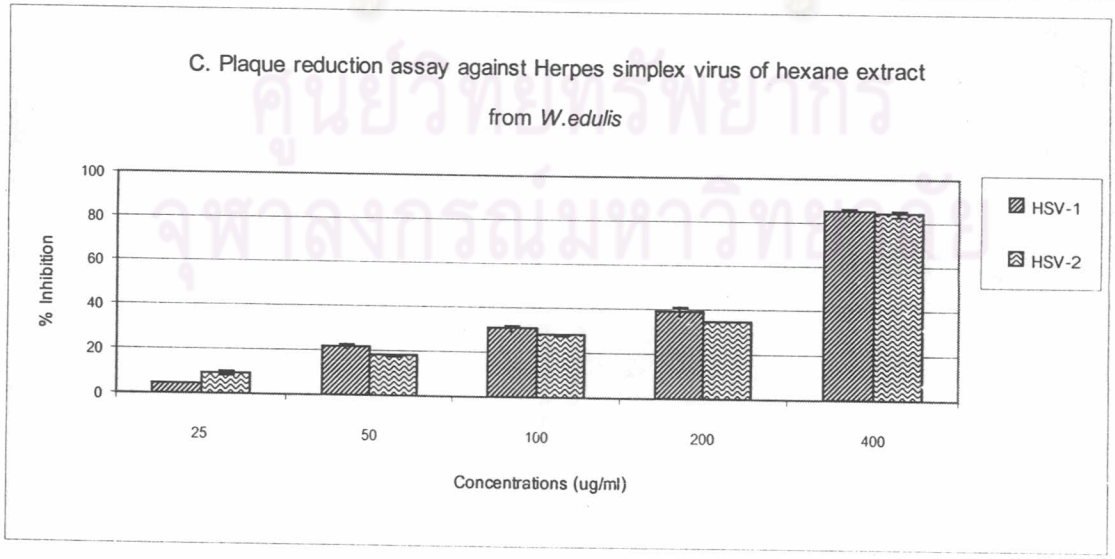


Figure 7 Antiviral acitvity of hexane extract from *Willughbeia edulis* against herpes simplex virus. A. Inactivation assay, B. Prophylactic activity assay, C. Plaque eduction assay. bar represented SD from three independent experiments

Table 9. Antiviral activity of acyclovir against herpes simplex virus

Acyclovir	[a]EC_{50}(mean±SD)	
	HSV-1	HSV-2
Inactivation assay	0.082±0.002	0.45±0.002
Prophylactic activity assay	0.103±0.004	0.58±0.005
Plaque reduction assay	0.160±0.002	0.69±0.01

[a] EC_{50} was deterined by three independent experiments

4. Preliminary test for anti-HSV-1 and 2 activities of plant extracts

The most active fraction of each plant, F4 /*G. pentaphylla*, F3/ *I. maxima* and F4 / *W. edulis* were selected for the further experiments for preliminary mechanism of antiviral activity in post binding assay and penetration inhibition assay and virus yield inhibition assay.

The effect of these plant extracts on adsorption were determined by post binding assay. The virus was adsorbed to Vero cells for 2 h at 4°C, unbound virus was removed and washed with various concentrations of extracts using PBS as negative control and citrate buffer as positive control. The number of viruses that bound to cells was assayed by plaque assay and the inhibition of HSV binding to host cells of the extracts was determined compared to PBS control. . Figure 8-10 indicated the effect of these three plant extracts on post binding assay .The increase in concentration-dependent manner(r=0.95-0.99) in percent inhibition of plaque forming against both HSV-1 and HSV-2 were observed. . It was showed that the extract of *G. pentaphylla*, added to cell cultures after the initial viral binding period at 4 °C, was able to inhibit the infectivity of HSV-1 and HSV-2 stably attached to Vero cells. The maximum of inhibition in plaque forming were observed at 55% for F4 of *G. pentaphylla*, 70% for F3 of *I. maxima*, and 55% for F4 of *W. edulis*. The effects of extracts in post binding assay in HSV-1 and HSV-2 exhibited at the same extent. Treatment of HSV-1 and HSV-2 stably attached to cells with a low pH citrate buffer (positive control) for 1 min at 4°C reduced to 100 ± 0.004 % of control values the amount of virus which had penetrated into cells after the shift to 37°C

Virus penetration into cells is also one of the indicator of antiviral targets. Thus, in order to investigate the effects of the extracts on penetration of HSV, a penetration inhibition assay was performed . As described in the part of method, the virus was adsorbed for 2h at 4°C to Vero cell and the unbound virus was removed. The penetration of virus was allowed by the shift of infected cells to 37°C at various times of incubation of 0, 15, 30, and 60 min. After the specified incubation, cells were treated

with various concentrations of extract and the cells were incubated further for 48 h. The amount of virus which had penetrate into cells were evaluated as the number of plaque forming unit (PFU) and the inhibition of penetration was demonstrated compared to PBS control. The results were demonstrated in figure 11-13 The inhibitory effect was more pronounced when the 1-min treatment period with extract was made 15, 30 min and 60 min after the temperature shift at 37°C. The effect of these plant extracts also was concentration dependent(r=0.88-0.97) and time dependent(r=0.92-0.99) as indicated by higher inhibition in plaque forming of virus compared to the lower concentration. The maximum inhibition in plaque forming were exhibited at o min of penetration of HSV-1 and HSV-2 into the cells were 60% for all three plant extracts F4 /*G. pentaphylla*, F3 / *I. maxima*, and F4 /*W. edulis*.

Effects on virus-induced cytopathic effect were performed by adding the extracts to Vero cells infected with HSV and incubated in the various concentration of the extracts and the virus was titrated by plaque assay at the incubation time at 37°C for 1, 8, 24, 48, and 72 h. The result of virus yield inhibition assay was showed in figure 14-16, It was found that the percent inhibition of plaque forming was maximum at 72 h incubation and was concentration-dependent(r=0.81-0.97) and time-dependent(r=0.87-0.99) for all 3 plant extracts. There were no differences in the effect in virus yield inhibition assay of plant extracts against HSV-1 and HSV-2. The 50% inhibitory concentration (IC_{50}) in virus inhibition yield assay were showed in Table 9. The IC_{50} against HSV-1(HSV-2) were 275.79 ±9.15 (296.67±8.16), 126.75±3.32 (116.00±33.27) and 296.74±5.46 (269.51±6.25) for F4/*G. pentaphylla*, F3 /*I. maxima*, and F4 /*W. edulis* at 72 h incubation. The effect of ACV in post binding assay , penetration inhibition assay and virus yield inhibition assay were demonstrated in figure 17 and table 11-12. Acyclovir could inhibit the binding of virus to cells and inhibied virus penetration into the cells. Virus yield inhibition assay indicated the replication of virus in the cells was inhibited.

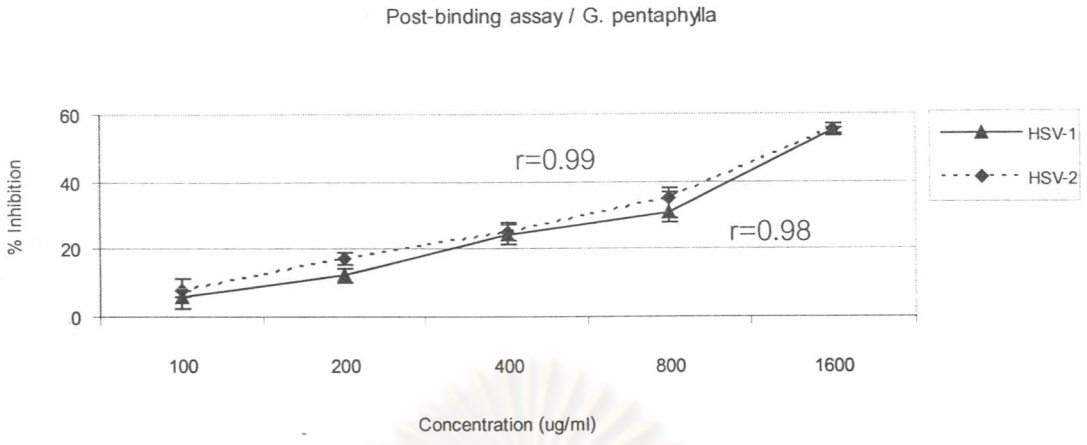


Figure 8 Post binding assay of hexane extract from *Glycosmis pentaphylla* against herpes simplex virus type 1 and type 2.

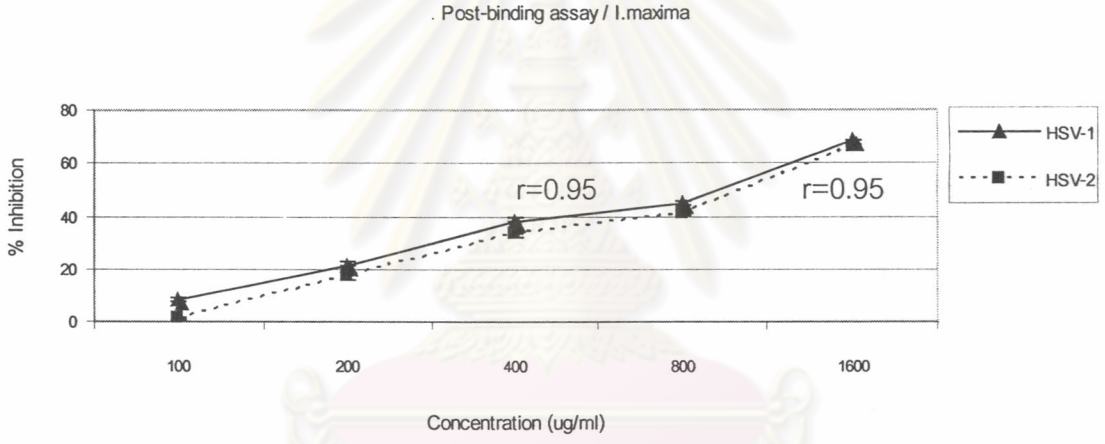


Figure 9 Post binding assay of aqueous extract from *Ipomoea maxima* against herpes simplex virus type 1 and type 2.

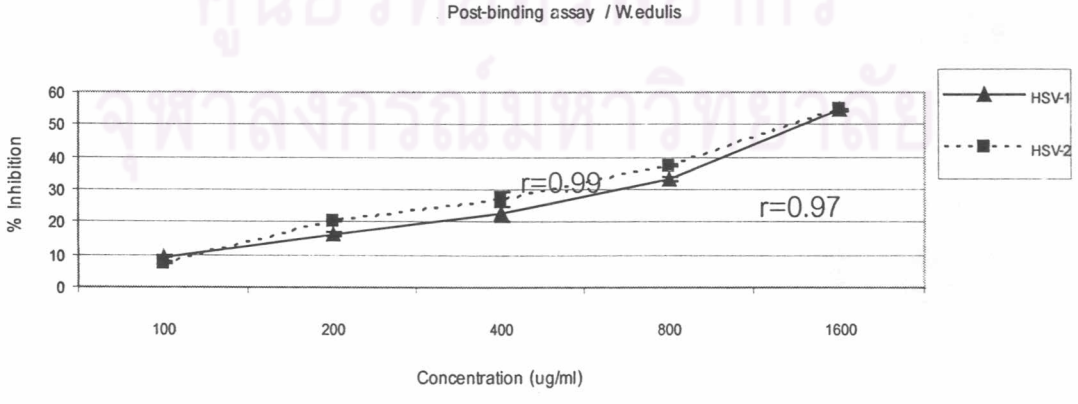
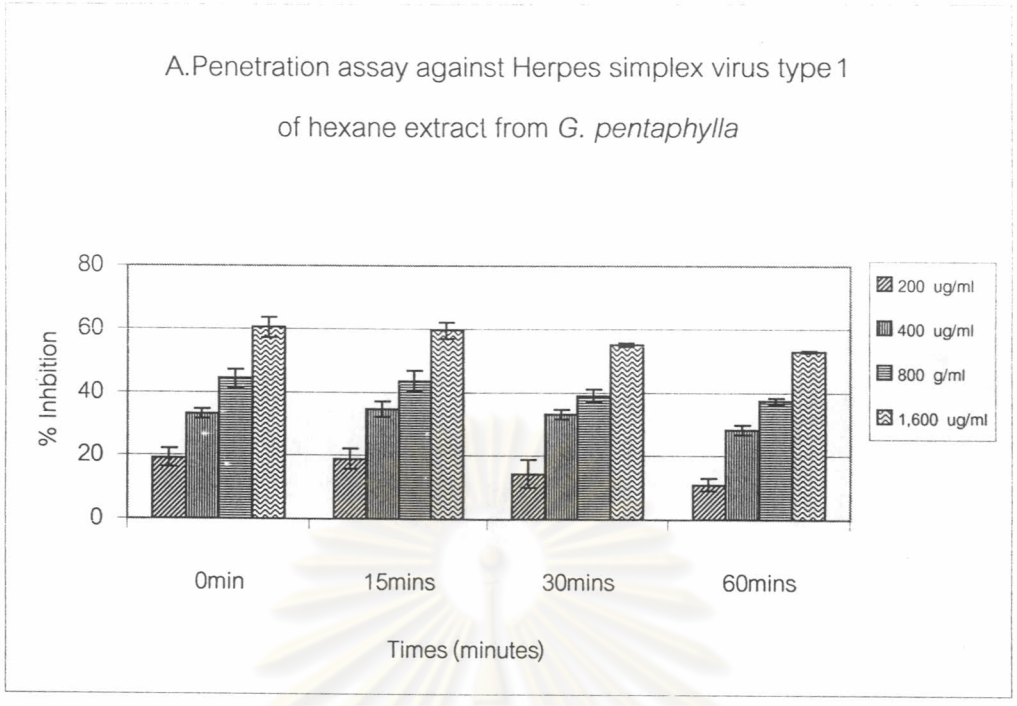


Figure 10 Post binding assay of hexane extract from *Willughbeia edulis* against herpes simplex virus type 1 and type 2.



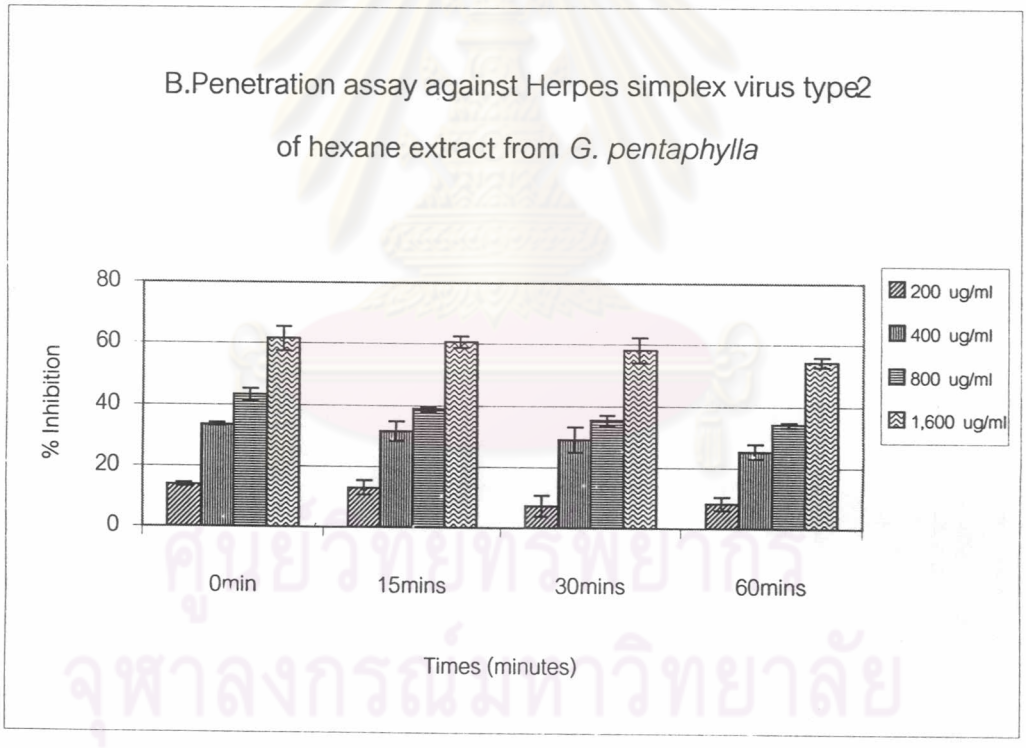
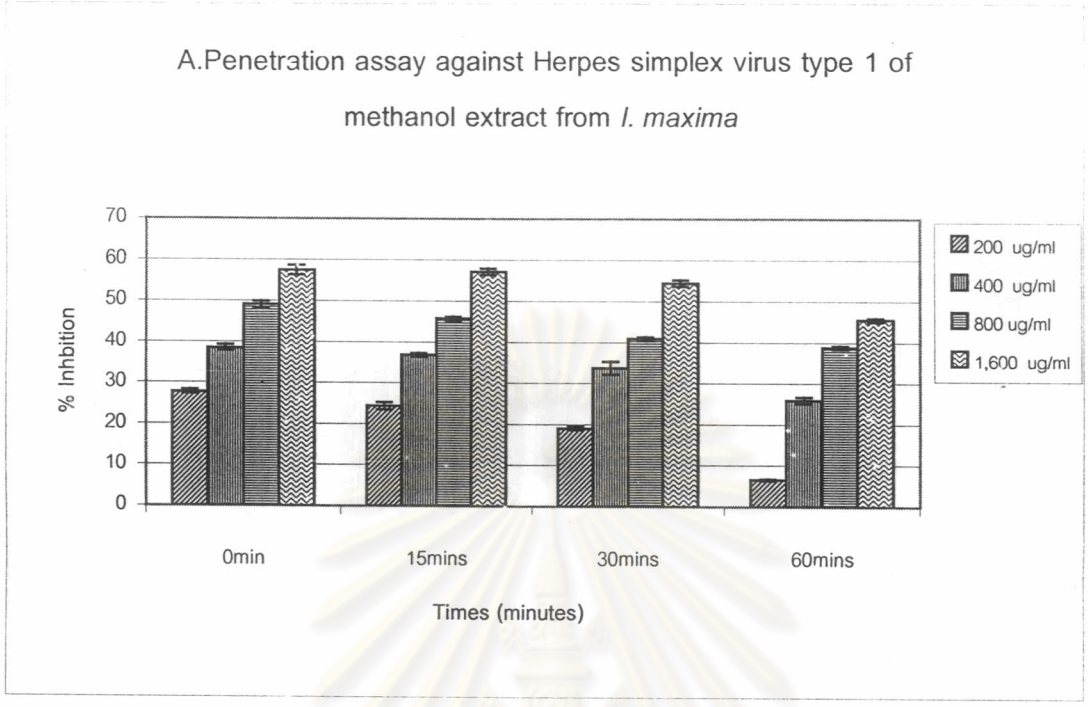


Figure 11 Penetration assay against herpes simplex virus type 1 (A) and type 2 (B) of hexane extract from *Glycosmis pentaphylla*, bar represented SD from three independent experiments.



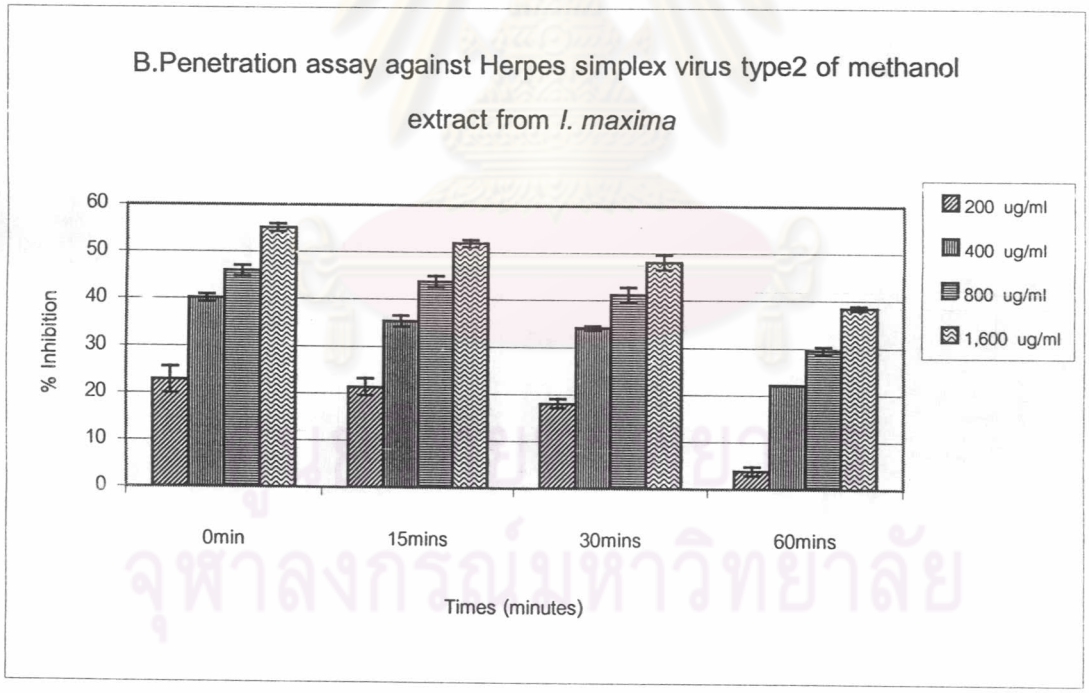
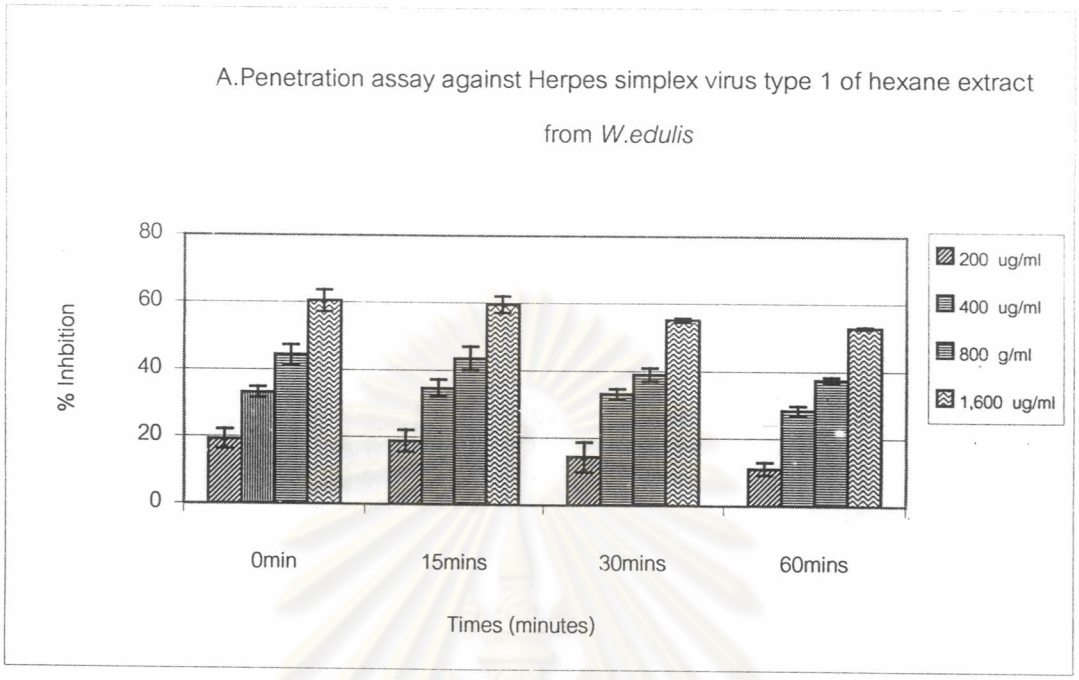


Figure 12 Penetration assay against Herpes simplex virus type 1 (A) and type 2 (B) of methanol extract from *Ipomoea maxima*, bar represented SD from three independent experiments.



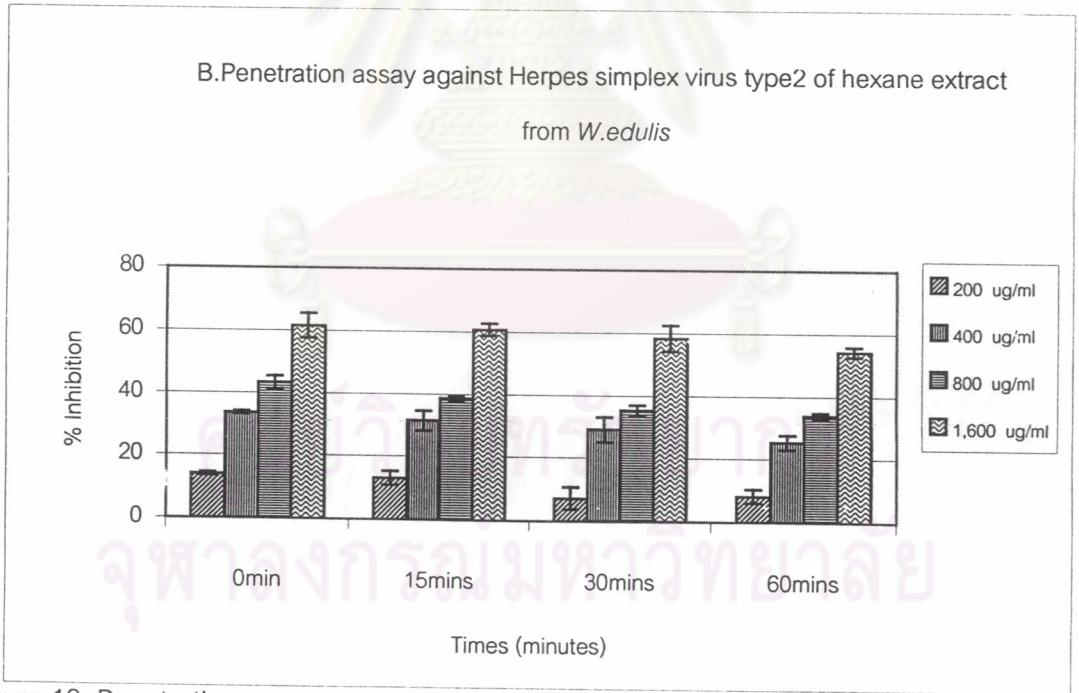
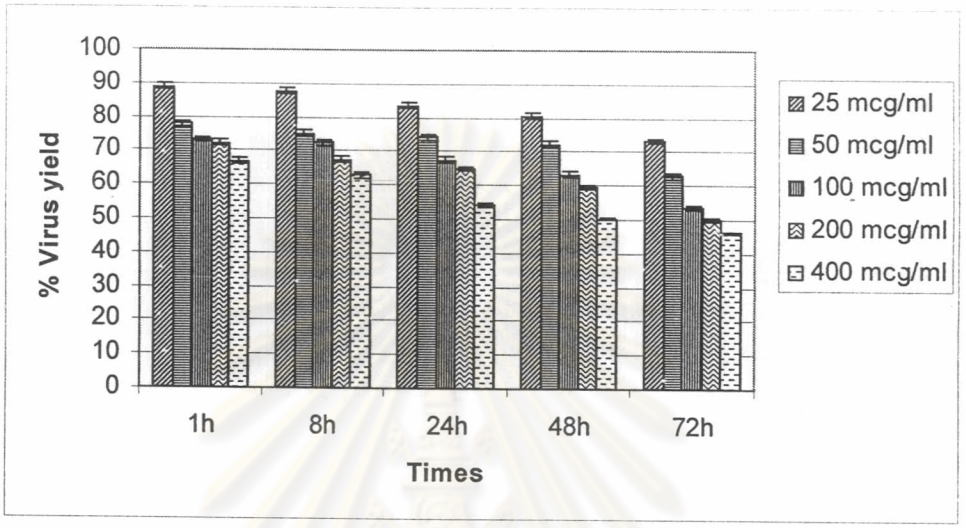


Figure 13 Penetration assay against Herpes simplex virus type 1 (A) and type 2 (B) of hexane extract from *Willughbeia .edulis*, bar represented SD from three independent experiments.

A



B

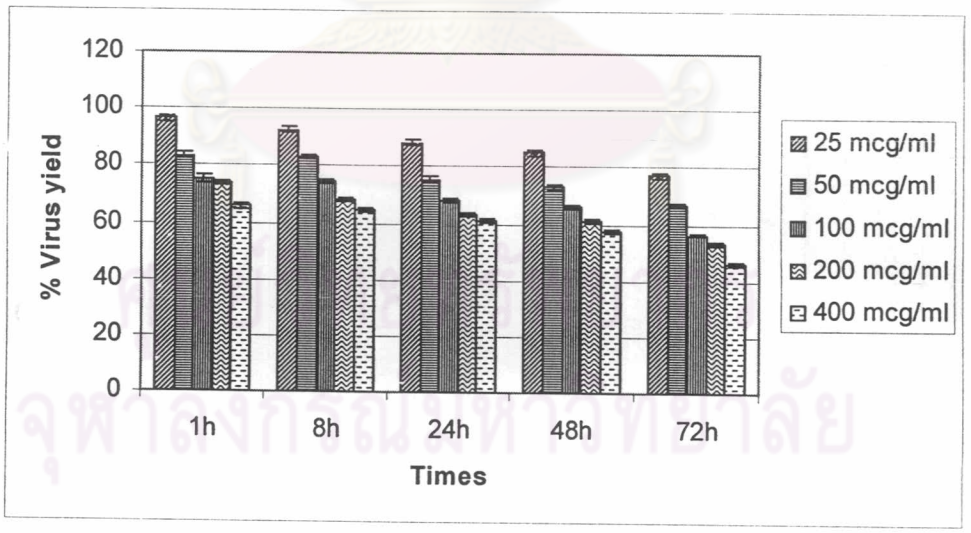
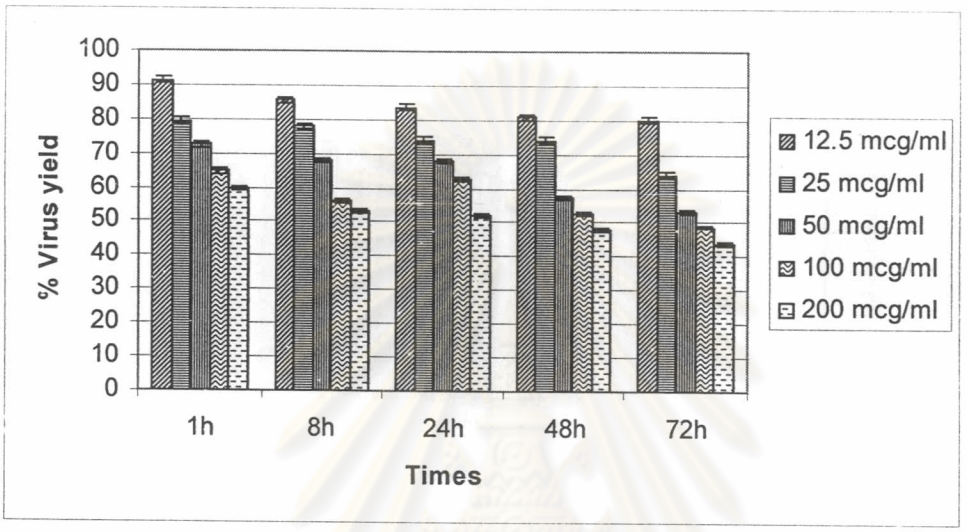


Figure 14 Virus yield inhibition assay against HSV-1(A) and HSV-2(B) of hexane extract from *Glycosmis pentaphylla*., bar represented SD from three independent experiments

A



B

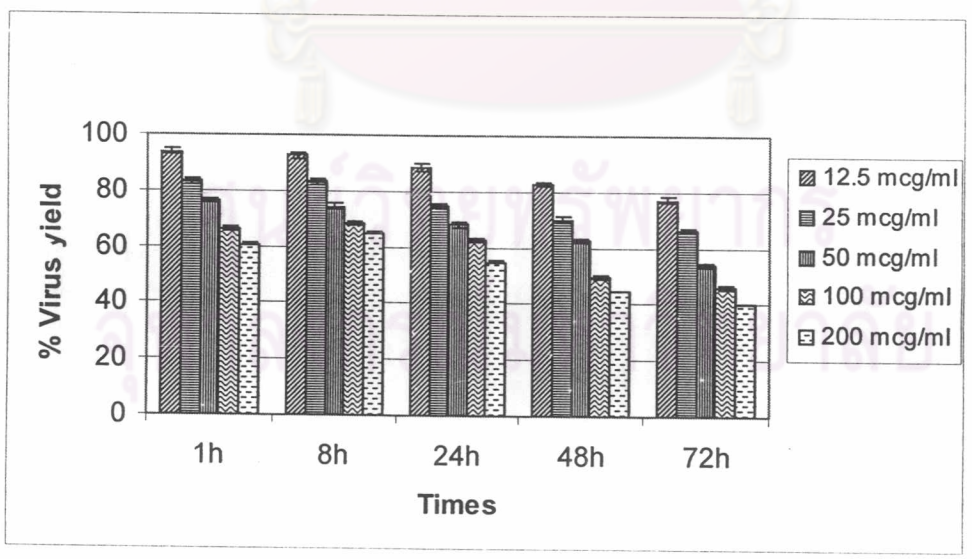
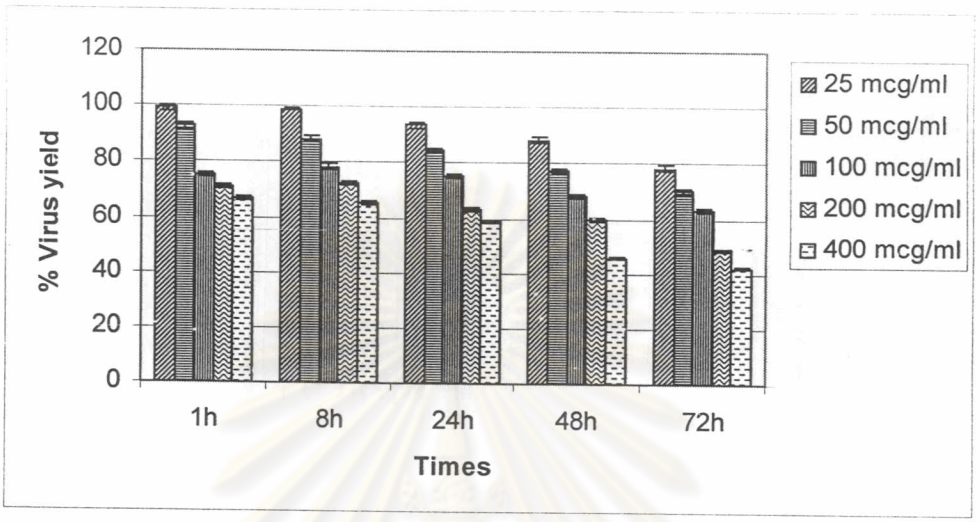


Figure 15 Virus yield inhibition assay against HSV-1(A) and HSV-2(B) of methanol extract from *Ipomoea maxima*, bar represented SD from three independent experiments.

A



B

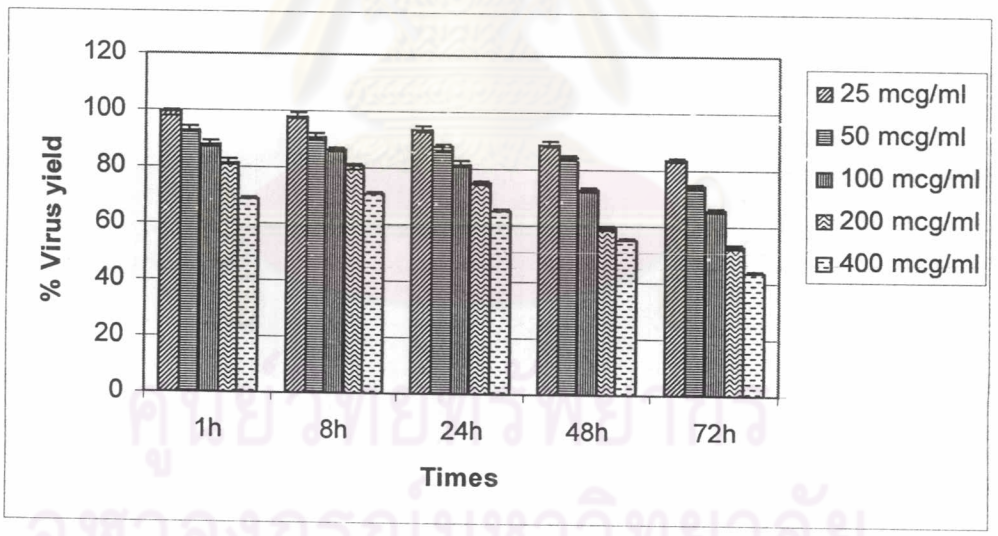


Figure 16 Virus yield inhibition assay against HSV-1 and HSV-2 of hexane extract from *Willughbeia edulis*, bar represented SD from three independent experiments.

Table 10. Inhibitory concentration (IC_{50}) of medicinal plants in virus yield inhibition assay.

Extracts	[a]Times(h)	[b]IC_{50}(mean±SD) ug/ml	
		HSV-1	HSV-2
F4/ *G.pentaphylla*	1	741.54±12.29	601.62±8.67
	8	597.1 ±10.32	560.28±8.64
	24	436.53±7.69	520.85±9.80
	48	372.48±7.74	466.54±9.63
	72	275.79±9.15	296.67±8.16
F3/ *I.maxima*	1	242.09±3.77	241.04±3.60
	8	200.28±3.66	231.07±3.57
	24	191.45±3.38	210.86±3.76
	48	156.33±3.38	142.92±3.14
	72	126.75±3.32	116.00±3.27
F4/ *W.edulis*	1	558.95±7.22	728.27±8.85
	8	557.58±7.00	691.74±8.67
	24	448.10±6.32	591.42±8.13
	48	329.96±5.31	400.39±6.38
	72	296.74±5.45	269.51±6.25

[a]. Incubation time of the extracts in infected Vero cell.

[b] IC_{50} was deterined by three independent experiments.

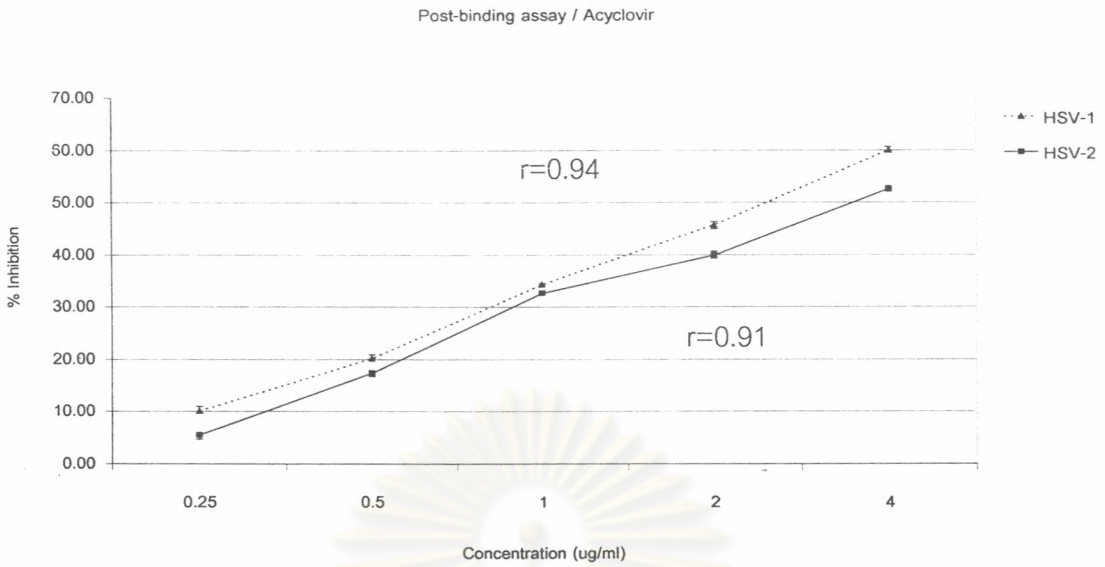


Figure17. Post-binding assay of acyclovir against herpes simplex virus, bar represented SD from three independent experiment.

Table 11. Penetration assay of acyclovir against herpes simplex virus

Acyclovir	Times[a] (Min)	[b]EC_{50}(mean±SD)ug/ml	
		HSV1	HSV2
Penetration assay	0	2.88±0.04	3.36±0.13
	15	3.15±0.01	3.64±0.12
	30	3.57±0.03	3.97±0.01
	60	4.55±0.07	5.04±0.03

[a] the time of viral penetration performed by shifting the cell to 37°C.

[b]EC_{50} was deterined by three independent experiments.

Table 12. Virus yield inhibition assay of acyclovir against herpes simplex virus

Acyclovir	Times[a] (h)	[b]EC_{50}(mean±SD) ug/ml	
		HSV1	HSV2
Virus yield inhibition assay	1	0.62±0.01	0.83±0.12
	8	0.51±0.01	0.69±0.46
	24	0.47±0.01	0.59±0.04
	48	0.38±0.12	0.45±0.01
	72	0.30±0.04	0.33±0.06

[a]Incubation time of ACV in infected vero cell.

[b] EC_{50} was deterined by three independent experiments.