CHAPTER V

RESULTS

Part I. Cell culture for propagation of virus

Vero cells; a continuous cell line of African green monkey kidney, were subcultured, the number of the passages of 215 - 354 were used in this study. Cells were frozen in freezing media and kept at -70°C .

Part II. Herpes simplex virus

Isolation of Herpes Simplex Virus

One hundred nineteen specimens were obtained from the patients with suspected genital herpes; 79 (66.4%) specimens were from Bangkok and 40 (33.6%) specimens were from Chonburi. All of specimens were from 51 men and from 68 women that divided to 46 prostitutes and 22 house-wife contacts. The age of patients were between 14-62 years, 92 patients had prior history of genital herpes and 21 patients with vesicle and 98 patients with ulcer appearances. Twenty-two patients had prior history of antiviral drug uptake. Thirty-eight (31.9%) specimens were cultured positive for CPE. Twenty-eight (73.7%) were collected from Bangkok and 10 (26.3%) were collected from Chonburi. There were 3 patients with HIV infection, one of them was cultured positive for CPE and one of 119 specimens was bisexual orientation (Table 3).

No significant differences in viral isolations (α= .05) between place of collection (χ^2 = 0.56), sex (χ^2 = 2.15), female status (χ^2 = 0.5), age (χ^2 = 0.71) and prior history of antiviral drug uptake (χ^2 = 1.03), but were seen significantly different between prior history of genital herpes (χ^2 = 47.15) and genital herpes appearance (χ^2 = 47.15).

HSV identification

The development of ELISA technique for HSV-2 identification, the procedure was 100 µl of the polyclonal rabbit anti-HSV-2 was diluted 2 fold, beginning, 1 : 1000 to 1 : 8000 for coating the wells of microtiter plate overnight at 4°C, then the plate was washed 3 times with PBS-T and 150 µl of blocking solution was added for one hour at 37°C to protect the nonspecific binding. The plate was washed 3 times with PBS-T and added 100 µl of HSV-2, strain 186 which was 2 fold diluted with diluent starting, $5x10^2$ to $1x10^5$ PFU/ml, for one hour at 37°C. Then, the plate was washed 3 times with PBS-T and added 100 µl of 1 : 200 of peroxidase - conjugate rabbit anti-HSV-2 was added and incubated at 37°C for one hour. The plate was washed 3 times with PBS-T and 100 µl of substrate was added for one hour at 37°C and the reaction was stopped with 50 µl of 4N H_2SO_4. OD at 492 nm was recorded. It was found that the appropriate dilution of rabbit anti-HSV-2 for coating was 1 : 4000 (Table 4 and Figure 13). When the rabbit anti-HSV-2, 1 : 4000 was coated to the wells and the reaction was done with conjugate which was diluted 2 fold, beginning, 1 : 200 to 1 : 1000. The optimal conjugate dilution was 1 : 200 and the optimal viral concentration was 1×10^4 PFU/ml (Figure 14). Therefore, in this study, 100 µl of the rabbit anti-HSV-2, 1 : 4000 for coating, the viral concentration $1 x10^4$ PFU/ml, and conjugate dilution 1 : 200 were used for HSV identification of 38 HSV isolates. Results of the HSV-2 identification by ELISA were showed in Table 6. The average OD of reagent control (no virus) was 0.117. The cut off value that was 2 times of control OD, was 0.234. The range of HSV-2 isolate OD was 0.634 - 1.200.

The result in comparison of HSV plaque size between HSV-1 strain KOS, HSV-2 strain 186 and HSV isolates showed all plaques of HSV isolates were bigger than plaque of HSV-1 strain KOS and the plaque size of isolates were similar to that of HSV-2, strain 186.

Therefore, all of viral isolates were identified to be HSV-2.

Titration of virus

The thirty-eight HSV-2 isolates from primary culture were titrated and virus concentration as shown in Table 7 were used in this study. The range of HSV-2 isolates concentration was $1.2x10^6$ - $4.2x10^7$ PFU/ml.

Part III. Study of antiviral activity of acyclovir against HSV strain 186.

Antiviral activity (ED_{50}) of acyclovir against HSV strain 186 by pre-treatment, post-treatment and inactivation were 0.56, 0.72 and 0.5 μl/ml, respectively (Table 8). There was significant difference among treatments. Table 9 showed the statistic using ANOVA which compared the mean of dual treatments. Pre-treatment / post-treatment ($t_{.05}$= 0.16) and post-treatment/ inactivation ($t_{.05}$ = 0.22) were significantly different. No significant difference between inactivation and pre-treatment ($t_{.05}$ = 0.06) and the least concentration of ACV (0.5 μg/ml) in activation was observed. So, this treatment was used to study antiviral activity of acyclovir and medicinal plant extracts against HSV-2 strain 186 and HSV-2 isolates.

Part IV. Study of antiviral activity of acyclovir against HSV-2 isolates.

Thirty-eight HSV-2 isolates showed the ED_{50} of ACV in range 0.38 - 0.87 μg/ml, the mean ± SD was 0.585 ± 0.1 μg/ml (Table 10). The ED_{50} of ACV against 38 HSV-2 isolates and HSV-2 strain 186 by inactivation activity were found significantly different ($t = 5.31$, $P < .05$).

Part V. Study of antiviral activity of medicinal plant extracts against HSV-2 strain 186 and HSV-2 isolates

Thirty-five of medicinal plant extracts from *Cerbera odollam* Gaertn., *Clausena excavata* Burm.F., *Coleus amboinicus* Lour., *Phyla nodiflora* (L.) Greene, and *Thevetia peruviana* Schum were used. All of extracts were tested for cytotoxicity assay in Vero cell, the results were indicated in Table 11. The cytotoxic concentration of almost extracts were >50 μg/ml, except, fraction 5(F5) of *Cerbera odollam* Gaertn., fraction 2 (F2) and fraction 5 (F5) of *Coleus amboinicus* Lour. were >10 μg/ml. While, fraction 2 (F2) of *Clausena excavata* Burm.F., and fraction 2 (F2) and fraction 5 (F5) of *Thevetia peruviana* Schum. were >20 μg/ml.

Table 12 indicated the ED_{50} of medicinal plant extracts against HSV-2 strain 186, the extracts from F1, F2, F4, and F5 elicited active antiviral activity against HSV-2 strain 186, therefore, these fraction-extracts were used to study antiviral activity against HSV-2 isolates by inactivation-activity.

Table 13 showed the range and mean $\pm$ SD of ED_{50} of medicinal plant extracts against all HSV-2 isolates (the raw data showed in Appendix III). The most active extracts of *Cerbera odollam* Gaertn., *Clausena excavata* Burm.F., *Coleus amboinicus* Lour., *Phyla nodiflora* (L.) Greene, and *Thevetia peruviana* Schum. were F5 (4.99$\pm$0.91 μg/ml), F2 (8.97$\pm$0.78 μg/ml), F2 (3.57$\pm$0.77 μg/ml), F4 (20.90$\pm$3.14 μg/ml) and F2 (3.04$\pm$0.70 μg/ml), respectively. There were significant differences between groups of fraction in the same plant, likewise it was seen significantly different between groups of plant in the same fraction.

Results of the tests of significance for ED_{50} between groups of fraction in the same plant were shown in Table 14, the mean ED_{50} of fractions in 5 plants were significantly different and the mean could be ranked by Dancan test with significant

level .05 into 3 subsets, except *P. nodiflora* divided into 2 subsets. The tests of significance for ED_{50} between groups of plant in the same fraction were indicated in Table 15. All fractions were significantly different and the mean could be ranked by Dancan test with significant level .05 into 4 subsets .

Part VI. Study of antiviral activity of inhibiting viral entry into cell, growth inhibition of viral replication in the cell and direct viral demolishment of medicinal plant extracts against HSV-2 strain 186 and HSV-2 isolates

ACV 0.5 μg/ml, F1 extracts from 5 plants 50 μg/ml were used as drug control and medicinal plant extracts. HSV-2 strain 186 and 6 HSV-2 isolates chose by simple random sampling were used in this study . Six isolates were A6, A8, A13, A47, BC4 (isolate No. 3, 4, 5, 19, 27 in Appendix III) from Bangkok and C8 (isolate No. 33 in Appendix III) from Chonburi. Results of antiviral activity of ACV and F1 medicinal plant extracts against HSV-2 strain 186 and HSV-2 isolates were shown in Figure 15. It was found that the pre - treatment and post - treatment activity could not reduce the percent of plaque forming of HSV-2 strain 186 and 6 HSV-2 isolates. In spite of increasing exposure time of medicinal plant extracts in the reaction up to 3 hours, the extracts showed no more antiviral activity. On the other hand, the percent of plaque forming were reduced by ACV and all of 5 plants in inactivation.

Table 3. Patients and lesion characteristics

Characteristic	Total (N=119)	CPE + (N=38)	CPE - (N=81)	p value
Place of collection				
Bangkok	79 (66.4)	28 (73.7)	51 (63.0)	p>.05
Chonburi	40 (33.6)	10 (26.3)	30 (37.0)	
Sex				
male	51 (42.9)	20 (52.6)	31 (36.3)	p>.05
female	68 (57.1)	18 (47.4)	50 (61.7)	
Female status				
prostitute	46 (38.7)	11 (28.9)	35 (43.2)	p>.05
house-wife contact	22 (18.5)	7 (18.4)	15 (18.5)	
Age				
14-29	70 (58.8)	24 (36.2)	46 (56.8)	p>.05
30-49	46 (38.7)	14 (36.8)	32 (39.5)	
≥50	3 (2.5)	0 (0)	3 (3.7)	
Prior history of genital herpes	92 (77.3)	35 (92.1)	57 (70.4)	* p<.05
No history of genital herpes	27 (22.7)	3 (7.9)	24 (29.6)	
Genital herpes appearance				
vesicle	21 (17.6)	20 (52.6)	1 (1.2)	* p<.05
ulcer	98 (82.4)	18 (47.4)	80 (98.8)	
Prior history of antiviral drug uptake	22 (18.5)	5 (13.2)	17 (21.0)	p>.05
No history of antiviral drug uptake	97 (81.50	33 (86.8)	64 (79.0)	
HIV status				
infected	3 (2.5)	1 (2.6)	2 (2.5)	
not infected	104 (87.4)	37 (97.4)	67 (82.7)	
unknown	12 (10.1)	0 (0)	12 (14.8)	
Sexual orientation				
bisexual	1 (0.8)	0 (0)	1 (1.2)	
heterosexual	118 (99.2)	38 (100)	80 (98.8)	

Value are no. (%), p≤.05 = significance

Table 4. Titration of dilution of rabbit anti-HSV-2 serum against viral concentration. (Conjugate dilution 1: 200)

Viral concentration (PFU/ml)	O.D.ELISA of rabbit anti-HSV-2 serum dilution coated plate			
	1:1000	1:2000	1:4000	1:8000
$5x10^2$	0.137	0.153	0.161	0.152
$1x10^3$	0.191	0.215	0.259	0.225
$5x10^3$	0.459	0.589	0.674	0.555
$1x10^4$	0.662	0.818	0.942	0.769
$5x10^4$	1.413	1.738	1.925	1.512
$1x10^5$	1.481	1.845	1.992	1.622

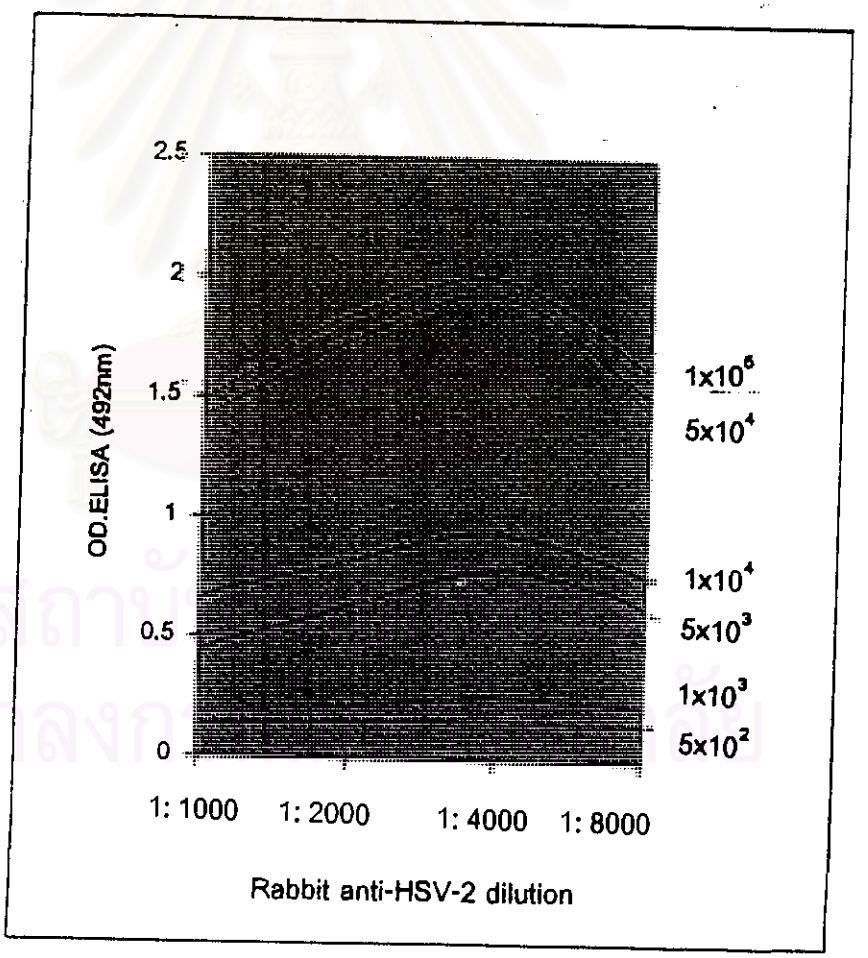

Figure 13. Titration curves of dilution of rabbit anti-HSV serum against viral concentration. (Conjugate dilution 1 : 200)

Table 5. Titration of dilution of conjugate rabbit anti-HSV serum against viral concentration. (Rabbit anti-HSV-2 dilution 1: 4000)

Viral concentration (PFU/ml)	O.D.ELISA of conjugate dilution			
	1:200	1:400	1:800	1:1000
$5x10^2$	0.135	0.115	0.121	0.126
$1x10^3$	0.391	0.248	0.182	0.141
$5x10^3$	0.612	1.369	0.224	0.119
$1x10^4$	1.604	0.916	0.571	0.199
$5x10^4$	2.162	1.222	0.766	0.242
$1x10^5$	3.001	2.043	1.301	0.347

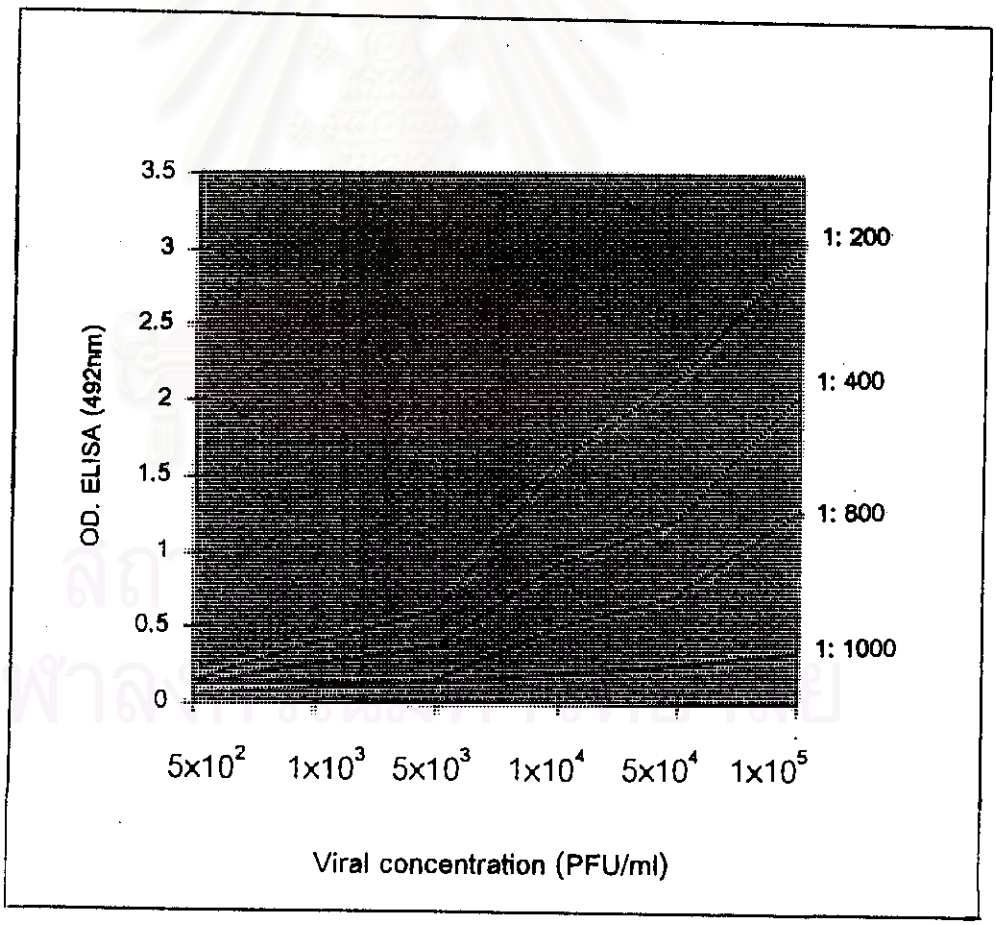

Figure 14. Titration of dilution of conjugate rabbit anti-HSV serum against viral concentration. (Rabbit anti-HSV-2 dilution 1: 4000)

Table 6. OD ELISA (492 nm) of HSV-2 identification.

Isolate No.	OD ELISA	Results	Isolate No.	OD ELISA	Results
reagent 1	0.118	blank	MEM	0.126	- control
reagent 2	0.112	blank	MEM	0.134	- control
reagent 3	0.121	blank	HSV-2(188)	0.614	+ control
1.	0.766	+	20	1.030	+
2	0.944	+	21	0.871	+
3	0.987	+	22	0.858	+
4	0.654	+	23	0.871	+
5	0.887	+	24	0.916	+
6	0.675	+	25	0.785	+
7	0.897	+	26	1.124	+
8	1.010	+	27	0.634	+
9	0.753	+	28	0.671	+
10	1.102	+	29	1.120	+
11	0.875	+	30	0.854	+
12	1.120	+	31	0.985	+
13	0.992	+	32	0.785	+
14	0.895	+	33	0.992	+
15	0.852	+	34	0.884	+
16	0.987	+	35	1.200	+
17	0.843	+	36	1.109	+
18	0.658	+	37	1.192	+
19	0.753	+	38	0.952	+

Cut Off Value (COV) = $\frac{0.118 + 0.112 + 0.121}{3} \times 2 = 0.234$

Negative control = 0.130

Table 7. Viral titration of HSV-2 strain 186 and HSV-2 isolates

Isolate No.	concentration (PFU/ml)	Isolate No.	Concentration (PFU/ml)
1.	$6x10^6$	21	$1.8x10^6$
2	$5.2x10^6$	22	$2.4x10^6$
3	$8x10^6$	23	$2.6x10^6$
4	$6.2x10^6$	24	$3.2x10^6$
5	$1x10^7$	25	$1x10^7$
6	$5x10^6$	26	$2.6x10^6$
7	$8.0x10^6$	27	$3.2x10^6$
8	$1.8x10^6$	28	$3.6x10^6$
9	$1.2x10^6$	29	$4.2x10^7$
10	$1.24x10^6$	30	$1.2x10^7$
11	$3.2x10^6$	31	$4.0x10^6$
12	$3.7x10^6$	32	$1.2x10^6$
13	$3.2x10^6$	33	$2.4x10^6$
14	$4.2x10^6$	34	$2.6x10^6$
15	$1.2x10^6$	35	$1.2x10^6$
16	$1x10^7$	36	$5.0x10^6$
17	$3.2x10^6$	37	$2.6x10^6$
18	$4.0x10^6$	38	$3.9x10^6$
19	$2.4x10^6$	HSV-2(186)	$5.0x10^6$
20	$2.6x10^6$	Range	$1.2x10^6$-$4.2x10^7$

The average value from duplicated experiments of primary culture were done.

Table 8. Antiviral activity of ACV against HSV-2 strain 186

Experiments	ED_{50} of ACV (μg/ml)			p value
	pre-treatment	post-treatment	inactivation	
1	0.52	0.65	0.5	p<.05
2	0.55	0.70	0.5	
3	0.60	0.80	0.5	
total	1.67	2.15	1.5	
mean	0.56	0.72	0.5	

$p < .05$ = significance

Table 9. ANOVA table of antiviral activity of ACV against HSV-2 strain 186

source	degree of freedom(df)	sum of square (ss)	mean square MS = ss/df	VR
among group	2	0.08	0.04	25
within group	6	0.01	0.0016	
total	8	0.09		p<.05

Compare mean with Least Significant Difference (LSD)

LSD $t_{.05}$ = 0.0976

Pre-treatment / Post-treatment = [0.56-0.72] = 0.16, p<.05

Post-treatment / Inactivation = [0.72-0.50] = 0.22, p<.05

Inactivation / Pre-treatment = [0.50-0.56] = 0.06, p>.05

Table 10. ED_{50} of ACV against HSV-2 isolates

Isolate No.	µg/ml[a]	Isolate No.	µg/ml[a]
1.	0.630	21	0.595
2	0.595	22	0.670
3	0.565	23	0.620
4	0.450	24	0.485
5	0.625	25	0.600
6	0.375	26	0.530
7	0.625	27	0.430
8	0.460	28	0.610
9	0.550	29	0.400
10	0.655	30	0.640
11	0.720	31	0.567
12	0.690	32	0.550
13	0.730	33	0.525
14	0.530	34	0.870
15	0.605	35	0.545
16	0.610	36	0.645
17	0.595	37	0.550
18	0.470	38	0.750
19	0.660	sum	22.227
20	0.505	mean±SD	0.585±0.1

[a] The average value from duplicated experiments were done

Table 11. Cytotoxic concentration of medicinal plant extracts on Vero cell

Plants	Cytotoxic concentration of extracts (μg/ml)[a]						
	F1	F2	F3	F4	F5	F6	F7
Cerbera odollam Gaertn.	>50	>50	>50	>50	>10	>50	>50
Clausena excavata Burm.F.	>50	>20	>50	>50	>50	>50	>50
Coleus amboinicus Lour.	>50	>10	>50	>50	>10	>50	>50
Phyla nodiflora (L.) Greene.	>50	>50	>50	>50	>50	>50	>50
Thevetia peruviana Schum.	>50	>20	>50	>50	>20	>50	>50

[a] The average value from duplicated experiments were done.

F1 = methanol extract F5 = aqueous- hexane extract

F2 = chloroform extract F6 = butanol extract

F3 = aqueous- methanol extract F7 = aqueous-butanol extract

F4 = hexane extract

Table 12. ED_{50} of medicinal plant extracts against HSV-2 strain 186

Plants	ED_{50} of extracts (μg/ml)[a]						
	F1	F2	F3	F4	F5	F6	F7
Cerbera odollam Gaertn.	7.50	10.25	≥50	7.25	4.25	≥50	≥50
Clausena excavata Burm.F.	11.25	9.25	≥50	23.25	19.75	≥50	≥50
Coleus amboinicus Lour.	20.88	3.25	≥50	12.75	4.00	≥50	≥50
Phyla nodiflora (L.) Greene.	23.75	22.5	≥50	16.25	21.25	≥50	≥50
Thevetia peruviana Schum.	10.25	3.25	≥50	10.25	12.63	≥50	≥50

[a] The average value from duplicated experiments were done.

F1 = methanol extract F5 = aqueous-hexane extract

F2 = chloroform extract F6 = butanol extract

F3 = aqueous-methanol extract F7 = aqueous-butanol extract

F4 = hexane extract

Table 13. ED_{50} of medicinal plant extracts against HSV-2 isolates (N=38)

Fractions		*C. odollam* (μg/ml)	*C. excavata* (μg/ml)	*C. amboinicus* (μg/ml)	*P.nodiflora* (μg/ml)	*T.peruviana* (μg/ml)	p value
F1	range	7.50-15.75	11.00-18.25	7.50-15.75	16.25-26.10	8.25-13.25	
	X±SD	10.30±1.87	13.78±2.05	18.64±1.95	22.00±2.36	10.64±1.47	p<.05
F2	range	8.63-12.70	7.50-11.00	2.13-5.12	21.50-35.00	2.13-5.00	
	X±SD	10.57±0.92	8.97±0.78	3.57±0.77	27.26±3.90	3.04±0.70	p<.05
F4	range	6.25-9.25	19.25-29.80	9.75-16.25	16.25-27.10	9.50-14.75	
	X±SD	7.44±0.76	22.98±2.93	12.93±1.86	20.90±3.14	12.17±1.10	p<.05
F5	range	3.25-6.63	17.00-27.50	2.50-5.25	13.25-27.13	10.00-13.75	
	X±SD	4.99±0.91	20.61±2.46	3.60±0.56	21.14±3.01	12.63±0.88	p<.05
p value		p<.05					

Table 14. Tests of significance for ED_{50} of medicinal plant extracts between groups of fraction in the same plant by ANOVA (Randomized Complete Block Design).

C. odollam

Source	df	SS	MS	F
Treatment	3	791.75	263.92	302.16
Block(isolate)	37	116.30	3.14	3.60
Residual	111	96.94	.87	
Total	151	1004.99		
Dancan test with significant (α .05)				
Fraction	F5	F4	F1	F2
Mean	4.99[a]	7.44[b]	10.34[c]	10.57[c]

C. excavata

Source	df	SS	MS	F
Treatment	3	4612.12	1537.37	267.85
Block(isolate)	37	287.96	7.78	1.36
Residual	111	648.58	5.74	
Total	151	5534.19		
Dancan test with significant (α .05)				
Fraction	F2	F1	F5	F4
Mean	8.97[a]	13.78[b]	20.27[c]	22.97[c]

C. amboinicus

Source	df	SS	MS	F
Treatment	3	6280.72	2093.57	1103.77
Block(isolate)	37	91.21	2.47	1.30
Residual	111	210.54	1.90	
Total	151	6582.47		
Dancan test with significant (α .05)				
Fraction	F2	F5	F4	F1
Mean	3.57[a]	3.60[a]	12.93[b]	18.64[c]

Table 14. (Continued)

P. nodiflora

Source	df	SS	MS	F
Treatment	3	1021.58	340.53	36.18
Block(isolate)	37	423.86	11.46	1.22
Residual	111	1044.86	9.41	
Total	151	2490.30		
Dancan test with significant (α .05)				
Fraction	F4	F5	F1	F2
Mean	20.90[a]	21.14[a]	22.00[a]	27.26[b]

T. peruviana

Source	df	SS	MS	F
Treatment	3	2276.88	758.96	1112.73
Block(isolate)	37	96.23	2.60	3.81
Residual	111	75.71	.68	
Total	151	2448.82		
Dancan test with significant (α .05)				
Fraction	F2	F1	F4	F5
Mean	3.04[a]	10.63[b]	12.17[c]	12.63[c]

The same superscript shows no significantly different

F1 = methanol extract

F2 = chloroform extract

F4 = hexane extract

F5 = aqueous-hexane extract

Table 15. Tests of significance for ED_{50} of medicinal plant extracts between groups of plant in the same fraction by ANOVA (Randomized Complete Block Design).

Fraction 1 (F1)

Source	df	SS	MS	F
Treatment	4	3987.82	996.95	305.55
Block(isolate)	37	229.18	6.19	1.90
Residual	148	482.89	3.26	
Total	189	4699.89		

Dancan test with significant (α .05)

Plant	*C.odollam*	*T.peruviana*	*C.excavata*	*C.amboinicus*	*P.nodiflora*
Mean	10.30^{a}	10.83^{b}	13.78^{c}	18.64^{d}	22.00^{d}

Fraction 2 (F2)

Source	df	SS	MS	F
Treatment	4	14698.95	3674.24	1116.18
Block(isolate)	37	167.69	4.53	1.38
Residual	148	486.32	3.29	
Total	189	15350.96		

Dancan test with significant (α .05)

Plant	*T.peruviana*	*C.amboinicus*	*C.excavata*	*C.odollam*	*P.nodiflora*
Mean	3.04^{a}	3.57^{a}	8.97^{b}	10.57^{c}	27.28^{d}

Fraction 4(F4)

Source	df	SS	MS	F
Treatment	4	6358.35	1589.59	345.71
Block(isolate)	37	197.66	5.34	1.16
Residual	148	680.52	4.60	
Total	189	7236.53		

Dancan test with significant (α .05)

Plant	*C.odollam*	*T.peruviana*	*C.amboinicus*	*P.nodiflora*	*C.excavata*
Mean	7.44^{a}	12.17^{b}	12.93^{b}	20.90^{c}	22.97^{d}

Table 15. (Continued)

Fraction 5(F5)

Source	df	SS	MS	F
Treatment	4	10606.08	2651.52	876.37
Block(isolate)	37	163.36	4.96	1.64
Residual	148	447.79	3.03	
Total	189	11237.22		

Dancan test with significant (α .05)

Plant	*C.amboinicus*	*C.odoliam*	*T.peruviana*	*C.excavata*	*P.nodiflora*
Mean	3.60^{a}	4.99^{b}	12.63^{c}	20.81^{d}	21.14^{d}

The same superscript shows no significant difference

F1 = methanol extract

F2 = chloroform extract

F4 = hexane extract

F5 = aqueous-hexane extract

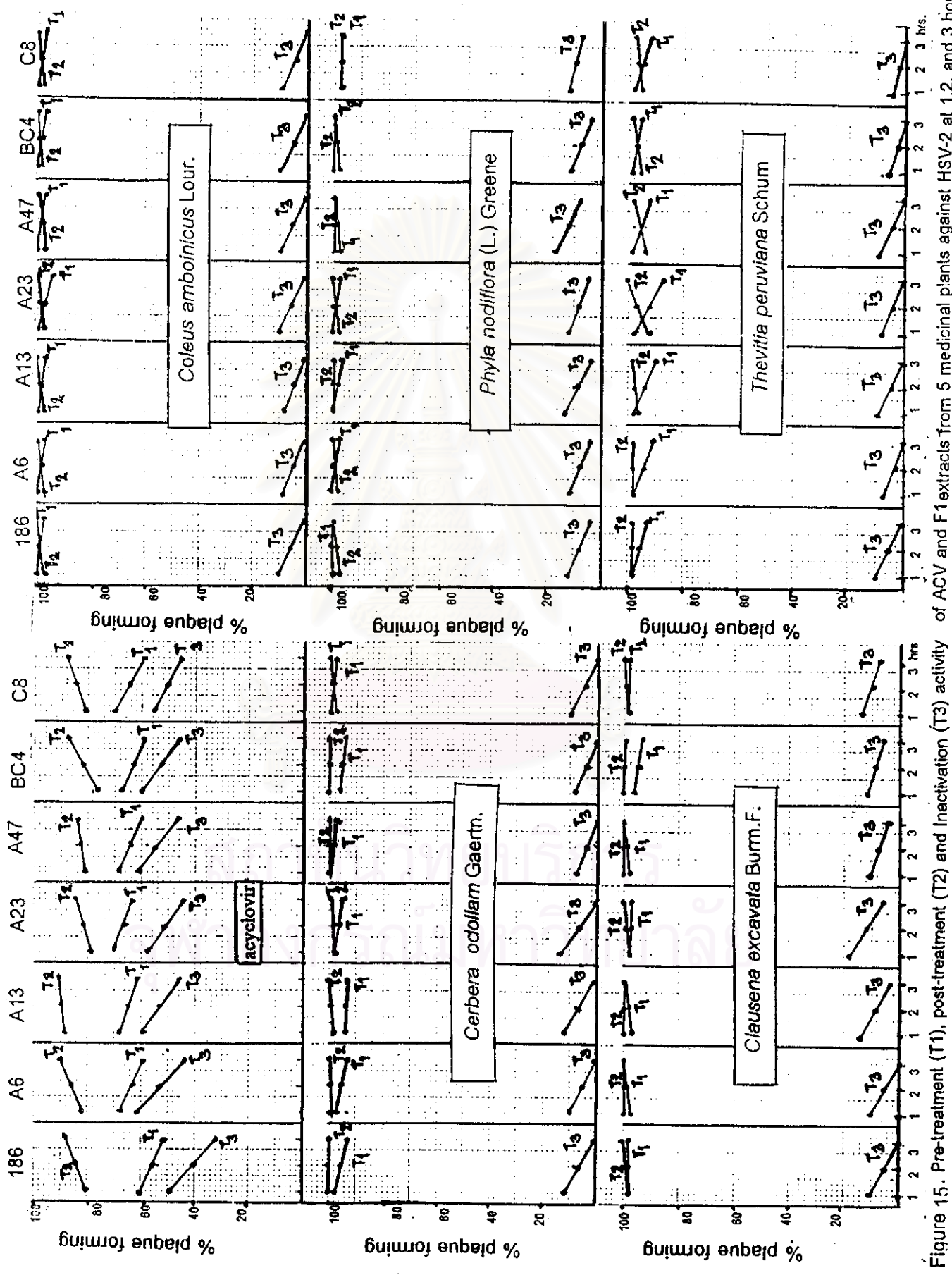

Figure 15. Pre-treatment (T1), post-treatment (T2) and Inactivation (T3) activity of ACV and F1 extracts from 5 medicinal plants against HSV-2 at 1,2, and 3 hours.