CHAPTER 4

RESULTS AND DISCUSSION

1. Medicinal plant

Different parts of twenty-three medicinal plants used in this study including *Andrographis paniculata* (Burm.f.) Wall. ex Nees., *Cissus quadrangularis* L., *Coscinium fenestratum* (Gaertn.) Colebr., *Croton oblongifolius* Roxb, *Croton roxburghii* N.P.Balakr., *Derris scandens* (Roxb.) Benth., *Eclipta prostrata* (L.) L., *Glycyrrhiza glabra* L., *Gynostemma pentaphyllum* (Thunb.) Makino., *Houttuynia cordata* Thunb., *Leptochloa chinensis* (L.) Nees, *Momordica charantia* Linn., *Phyllanthus amarus* Schum.&Thonn., *Pluchea indica* (Linn.) Less., *Pseuderatherum platiferum* (Wall.) Radlk. ex. Lindau., *Rhinacanthus nasutus* (Linn.) Kurz, *Schefflera leucantha* R. Vig., *Senna alata* (Linn.) Roxb., *Sphenodesme* sp., *Stemona tuberosa* Lour., *Stephania venosa* (Blume) Spreng., *Thunbergia laurifolia* Lindl., *Tinospora crispa* (Linn.) Miers ex Hook.f. & Thomson and *Zingiber montanum* (Koenig) Linkex Dietr. These plants were selected based on their information that they were commonly used in Thai folklore medicine to treat different infectious diseases.

2. Extraction of medicinal plants

After extraction, percentage yield of aqueous and ethanolic extracts of medicinal plants were ranged from 2.25 to 22.40% (Figure 43). The highest percentage yield of aqueous extract was obtained from *S. tuberosa* extract, which showed percentage yield of 15.54% whereas *Sphenodesme* sp. showed the lowest

yield of 2.25%. Furthermore, ethanolic extract of *C. roxburghii* showed the highest percentage yield of 22.40%, while *P. platiferum* had the lowest yield of 2.87%. Moreover, comparison of percentage yield between aqueous and ethanolic extract of each medicinal plant extract was demonstrated in Figure 43. Crude extracts were reconstituted by dimethyl sulfoxide (DMSO) to prepare stock solution at concentration of 100 mg/ml. Appearance of crude extracts showed various color from light brown, yellow-brown, red-brown, deep brown, black-brown, deep green and green-brown (Table 4).

Water and 95% ethanol were used as solvent for extraction because these solvents were able to solubilize compounds depending on their polarity. Moreover, water is universal solvent, which can extract the polar constituents of medicinal plants while ethanol is unpolar characteristic more than water that can extract unpolar compound. Thus, the solvent was able to extract medicinal plants constituents such as active, inactive or inert compounds (Osonwa *et al*., 2012; Tiwari *et al*., 2011).

Moreover, the periods of extraction procedure and temperature were also important in extraction procedure because some compounds were sensitive to chemical degradation (Sasidharan *et al*., 2011). Additionally, different extraction step also affected quantity and secondary metabolite composition of extracts. Geographical location of plants, collection period, drying methods and storage condition were also influence on the extract (Das *et al*., 2010b; Odey *et al*., 2012).

Table 4 Crude medicinal plant extracts after reconstitution in DMSO

Medicinal plants	Color appearance	
	Aqueous extract	**Ethanolic extract**
Andrographis paniculata	deep green	deep green
Cissus quadrangularis	deep brown	green-brown
Coscinium fenestratum	yellow-brown	deep brown
Croton roxburghii	deep brown	deep brown
Derris scandens	deep brown	light brown
Eclipta prostrata	deep brown	green-brown
Glycyrrhiza glabra	red-brown	yellow-brown
Gynostemma pentaphyllum	deep brown	deep green
Houttuynia cordata	black-brown	yellow-brown
Leptochloa chinensis	light brown	deep brown
Momordica charantia	deep brown	deep green
Phyllanthus amarus	deep brown	deep green
Pluchea indica	deep brown	deep green
Pseuderatherum platiferum	deep brown	deep brown
Rhinacanthus nasutus	green-brown	deep green
Schefflera leucantha	yellow-brown	green-brown
Senna alata	deep brown	green-brown
Sphenodesme sp.	deep brown	deep brown
Stemona tuberosa	deep brown	light brown

Table 4 (continued)

Medicinal plants	Color appearance	
	Aqueous extract	**Ethanolic extract**
Stephania venosa	deep brown	deep brown
Thunbergia laurifolia	deep brown	deep green
Tinospora crispa	deep brown	green-brown
Zingiber montanum	light brown	yellow-brown

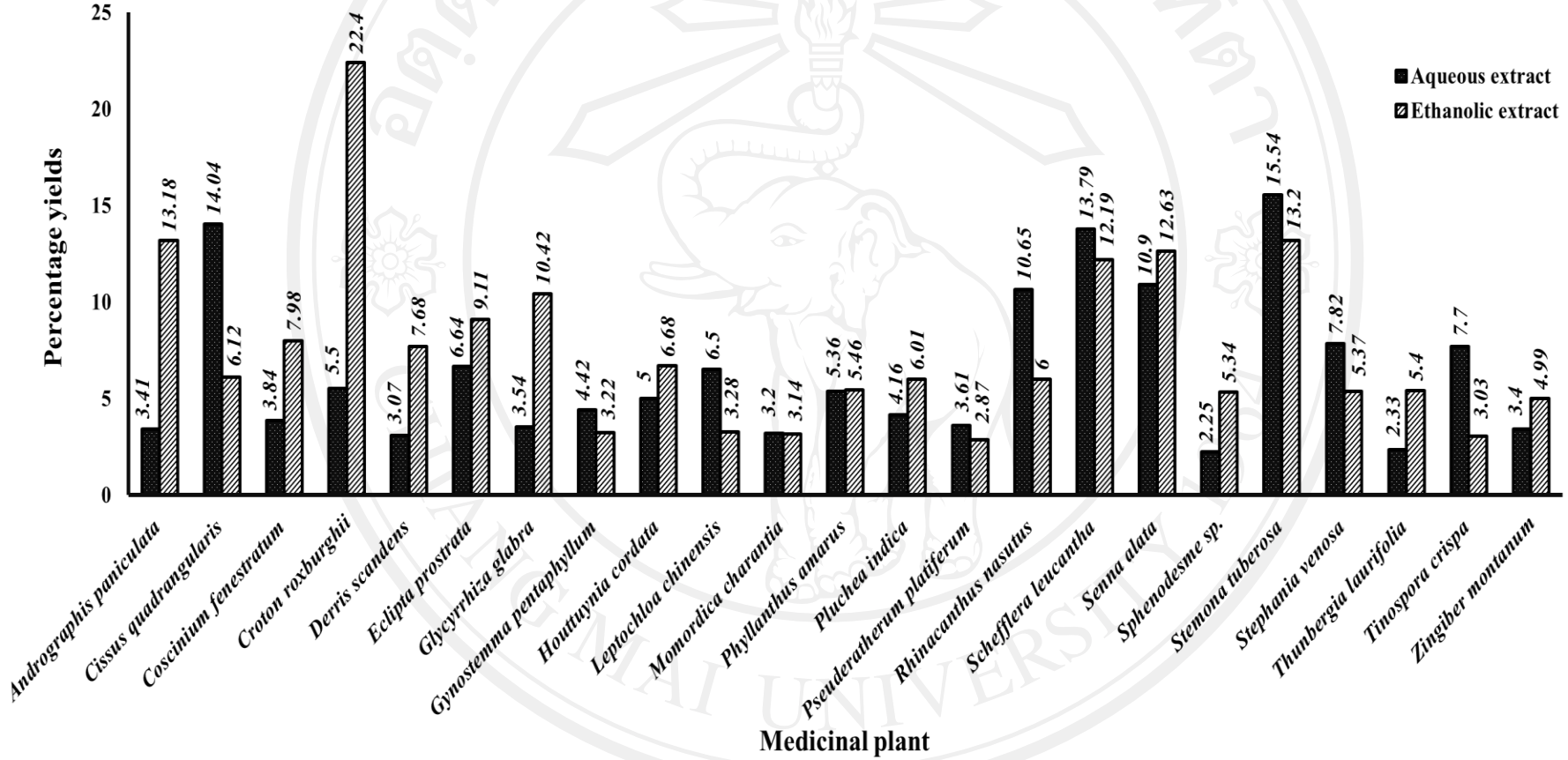

Figure 43 Comparison of percentage yield of aqueous and ethanolic extracts of medicinal plants

3. Cytotoxicity of medicinal plant extract by cell viability assay

Determination of cytotoxicity is an important section for safety margin of antiviral agent before evaluation of the efficacy of medicinal plant or tested compound against HSV infection.

In order to assess cytotoxicity of medicinal plant extracts, cell viability assay was performed. After exposure of plant extract to Vero cells for 3 days, the attached cells were stained by crystal violet. Cytotoxicity doses of both aqueous and ethanolic extracts were expressed as cytotoxic dose, 50% (CD_{50}), which was the concentration of the extract that caused cell detachment from the wells by 50% as compared to the cell control. The CD_{50} value was determined from dose-response curve and calculated according to modified protocol as previously described (Reed and Muench, 1938).

DMSO was used to reconstitute medicinal plant extracts. Cytotoxicity of DMSO on Vero cell was also evaluated by cell viability assay. The result showed CD_{50} value of DMSO on Vero cell was 4.47±0.00%. Thus, percentage of DMSO that used to reconstitute plant extract was not affect or interfere the results.

The similar result was documented by Georges *et al* (2002). The report showed that concentration of DMSO that lowers than 10% was not induced the apical membrane permeability in Caco2/TC7 cells. DMSO was used as solvent to reconstitute the compound (Spruance *et al*., 1983). Moreover, ACV was used as positive drug control to protect Vero cell from HSV infection. Hence, investigation of ACV on their toxicity on Vero cell was also demonstrated by cell viability assay. CD_{50} value of ACV was 1,004.69±0.00 µg/ml.

The result showed that, medicinal plant extracts were expressed in the different level of cytotoxicity on Vero cells. CD_{50} value obtained for these extracts were ranging from 20.10 to 5,677.00 µg/ml. Aqueous extract of *S. alata* exerted the highest toxicity on Vero cells, which observed by the lowest concentration of the extract that caused cells death with CD_{50} value of 179.50±12.37 µg/ml whereas *S. tuberosa* extract showed the lowest toxicity with CD_{50} was 5,677.00±0.00 µg/ml (Table 5). On the other hand, ethanolic extract of *A. paniculata* exhibited high cytotoxicity in Vero cells with CD_{50} value of 20.10±0.00 µg/ml while *Spenodesme* sp. showed the lowest toxicity with CD_{50} value of was 4,019.00±0.00 µg/ml (Table 6).

Additionally, cytotoxicity on Vero cells of ethanolic extracts was more toxic than aqueous extract as observed from CD_{50} values of ethanolic extract that were lower than aqueous extract. Hence, the highest non-toxic doses of aqueous and ethanolic extracts on Vero cells ranging from 3.90-4,000.00 µg/ml. These highest non-toxic doses of plant extracts that used for evaluation of antiviral activities were showed in Table 5 and 6.

The toxicity on the cells correlated with the reduction of cell, morphology change of cells that were different from untreated cell control when observed under an inverted microscope. Thus, cytotoxic effects of medicinal plant extracts lead to cells death as well as more subtle effects on cell that may not be deleterious such as granules production in cytoplasm, which affect cell morphology. Similar observation also reported that toxic of extracts revealed changes of cell rounding, appearance of cytoplasmic inclusions and loss of cell monolayer confluence (Ojo *et al.*, 2009; Suárez *et al.*, 2010).

The cytotoxicity may result from the different chemical compound in various plant extracts that could lead to the different effects to cell culture. Moreover, nonpolar compounds can pass through the cell membrane to cause cell toxicity whereas polar compound are able to damage only the cell surface (Wirotesangthong and Rattanakiat, 2006). Potential drugs should not interfere activity and metabolism of host cell while selective on HSV mechanism. The drug should not affect cell shape, morphology and cellular change (Ananil *et al*., 2000; Klawikkan *et al*., 2011; Soni *et al.*, 2002).

Table 5 Cytotoxicity doses, 50% (CD_{50}) and highest non-toxic concentrations of the aqueous extracts used in this present study

Medicinal plants	**CD_{50} (µg/ml)***	**The highest non-toxic concentration used (µg/ml)**
Andrographis paniculata	2,845.30±0.00	1,000
Cissus quadrangularis	2,845.30±11.75	1,000
Coscinium fenestratum	358.20±19.80	125
Croton roxburghii	2,845.30±0.00	2,000
Derris scandens	714.70±94.83	500
Eclipta prostrata	2,845.30±0.00	2,000
Glycyrrhiza glabra	1,426.00±0.00	500
Gynostemma pentaphyllum	1,426.00±0.00	250
Houttuynia cordata	2,845.30±0.00	1,000
Leptochloa chinensis	1,268.00±91.22	1,000
Momordica charantia	714.70±46.59	500
Phyllanthus amarus	358.20±33.06	250
Pluchea indica	2,845.30±11.75	500
Pseuderatherum platiferum	2,845.30±11.75	250
Rhinacanthus nasutus	1,268.00±0.00	1,000
Schefflera leucantha	714.70±0.00	500
Senna alata	179.50±12.37	62.5
Sphenodesme sp.	2,865.60±0.00	2,000

Table 5 (continued)

Medicinal plants	CD_{50} (µg/ml) *	The highest non-toxic concentration used (µg/ml)
Stemona tuberosa	5,677.00±0.00	2,000
Stephania venosa	1,426.00±60.81	500
Thunbergia laurifolia	2,521.00±0.00	5,00
Tinospora crispa	5,048.00±0.00	2,000
Zingiber montanum	2,845.30±11.75	2,000

*****The data table are presented as mean ± standard deviation (SD) of duplicate experiments.

Table 6 Cytotoxicity doses, 50% (CD_{50}) and highest non-toxic concentrations of the ethanolic extracts used in this present study

Medicinal plants	**CD_{50} (µg/ml)**	**The highest non-toxic Concentrations used (µg/ml)**
Andrographis paniculata	20.10±0.00	15.6
Cissus quadrangularis	2,845.30±11.75	500
Coscinium fenestratum	201.90±51.07	62.5
Croton roxburghii	714.70±46.59	500
Derris scandens	90.00±28.82	31.3
Eclipta prostrata	3,582.00±0.00	1,000
Glycyrrhiza glabra	2,845.20±11.75	2,000
Gynostemma pentaphyllum	1,271.00±0.00	125
Houttuynia cordata	1,426.00±0.00	500
Leptochloa chinensis	358.20±33.06	62.5
Momordica charantia	201.90±20.72	62.5
Phyllanthus amarus	358.30±0.00	125
Pluchea indica	637.00±37.36	125
Pseuderatherum platiferum	714.70±0.00	500
Rhinacanthus nasutus	50.40±0.00	3.9
Schefflera leucantha	1,426.00±49.65	500
Senna alata	358.20±33.06	250
Sphenodesme sp.	4,019.00±0.00	4,000
Stemona tuberosa	1,426.00±0.00	250

Table 6 (continued)

Medicinal plants	**CD_{50} (µg/ml)**	**The highest non-toxic Concentrations used (µg/ml)**
Stephania venosa	179.50±0.00	62.5
Thunbergia laurifolia	3,185.00±0.00	1,000
Tinospora crispa	714.70±56.37	500
Zingiber montanum	90.00±0.00	31.3

* The data in table are presented as mean ± standard deviation (SD) of duplicate experiments.

4. Plaque titration assay of virus

HSV-1 strain F and HSV-2 strain G were obtained from Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiang Mai University. Quantitation of standard HSV-1F and HSV-2G were investigated by plaque titration assay. The result showed that the titers of HSV-1F and HSV-2G were $2.25 \times 10^6 \pm 0.42$ and $5.7 \times 10^7 \pm 0.27$ PFU/ml, respectively (Table 7).

5. Inhibitory effect of acyclovir (ACV) against HSV-1F and HSV-2G infection

Activity of ACV on HSV infection was evaluated by plaque reduction assay. ACV was serially two-fold diluted before adding to confluence of infected Vero cell and incubated for 36-48 hours. After incubation period, the supernatant was aspirated and cell were washed twice with PBS (1X) before staining with 0.1% crystal violet in 1% ethanol. Efficacy of ACV on HSV infection was expressed as 50% effective dose (ED_{50}).

The result showed that, ACV could inhibit HSV-1F and HSV-2G with ED_{50} values of 1.50 and 3.13 µg/ml, respectively (Table 7). Thus, ACV at ED_{50} concentration, which was the dose that can inhibit HSV infection by 50% was used as drug positive control in antiviral assay.

Table 7 Percentage of ACV inhibition on HSV-1F and HSV-2G infection

Concentration (µg/ml)	Inhibition (% ± SD*)	
	HSV-1F	HSV-2G
0.00	0.00±0.00 [a]	0.00±0.00 [a]
0.39	34.58±0.29 [b]	21.55±0.28 [b]
0.78	43.02±0.44 [bc]	33.10±0.55 [bc]
1.56	51.57±0.32 [cd]	44.41±0.28 [cd]
3.13	69.80±0.38 [de]	50.00±0.71 [de]
6.25	74.57±0.41 [ef]	61.00±1.09 [ef]
12.50	84.00±0.57 [fg]	75.25±0.74 [fg]
25.00	95.10±0.03 [gh]	82.50±0.23 [gh]
50.00	100.00±0.00 [h]	97.00±0.00 [h]
100.00	100.00±0.00 [h]	100.00±0.00 [h]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

6. Determination of activity of medicinal plant extracts against HSV-1F and HSV-2G infection by plaque reduction assay

The efficacy of twenty-three selected medicinal plant extracts was tested against HSV-1F and HSV-2G infection on Vero cell by plaque reduction assay.

Plaque reduction assay was used for detection of antiviral activity of medicinal plant extract on cell culture. The presence of clear areas or plaques was developed from the area of the infected cells that were killed and showed cytopathic effect. One plaque is represented infected area of cells caused by one viral infectious unit. The number of plaques from control and extract treated groups were used for calculation of the percentage of plaque inhibition. The extracts were considered active if they could reduce number of plaques more than 50% compared to control (Wirotesangthong and Rattanakiat, 2006).

The result showed that, aqueous and ethanolic extracts of *C. roxburghii* and *Sphenodesme* sp. demonstrated strong inhibitory effect on HSV-1F and HSV-2G by 100% (Table 8 and 9). Moreover, aqueous extract of *E. prostrata, H. cordada, L. chinensis, M. charantia, P. indica, R. nasutus, S. leucantha, S. tuberosa, T. crispa* and *Z. cassumunar* inhibited HSV-1F more than 50% (Table 8). Additionally, aqueous extract of *A. paniculata, E. prostrata, H. cordada, P. amarus, P. indica, R. nasutus, S. leucantha, S. tuberosa, T. crispa* and *Z. montanum* inhibited HSV-2G more than 50% (Table 8). On the other hand, ethanolic extract of *C. quadrangularis, C. fenestratum, E. prostrata, G. glabra, G. pentanphyllum, H. cordada, R. nasutus, S. leucantha, S. tuberosa, T. laurifolia* and *T. crispa* inhibited plaque formation of HSV-1F more than 50% (Table 9). Additionally, ethanolic extract of *A. paniculata, C. quadrangularis, E. prostrata, G. glabra, G. pentaphyllum, H. cordada, P. amarus,*

R. nasutus and *T. crispa* could inhibit HSV-2G more than 50% (Table 9). The result observed that these ethanolic extracts had potent inhibitory effect on HSV-1F and HSV-2G more than aqueous extract (Table 8 and 9).

The results showed that all medicinal plant extracts had different inhibitory effect on HSV infection, although some extracts revealed inhibition only medium or low activity against HSV but at least it could be the source of potential antiviral drugs (Rajbhandari *et al.,* 2001). Some of medicinal plant extracts did not exert antiviral activity because the high concentration of medicinal plant extracts, which was limited in range of Vero cell tolerance (Mohamed *et al*., 2010). Moreover, the part of plants used and the type of extraction might result in the efficacy against HSV activity. Plant species, time of collection, storage conditions and distribution of phytochemical constituents might lead the different level of inhibitory activity and specificity against the virus (Mohamed *et al.,* 2010).

Viral plaques were resulted from virus replication within the infected cell and virus progenies in infected neighboring cells (Yoosook *et al.*, 1999). Although the extract could inhibit HSV less than 50%, but the plaque size were reduced more than viral plaque control. It was found that many medicinal plants extracts both aqueous and ethanolic extracts were able to reduce plaque sizes of HSV-1F or HSV-2G comparing to viral plaque control. Thus, the result revealed that these medicinal plant extracts could reduce the transmission or expansion of lateral cell-to-cell spread of infection to the neighboring cells or interfered with the production of infectious virions (Lückemeyer *et al*., 2012; Yoosook *et al*., 1999; Yucharoen *et al*., 2011). Furthermore, it was possible that antiviral agent from medicinal extract have various

actions depending on treatment schedule and ability of virus to spread via syncytia (Nikomtat *et al.,* 2011b).

The similar result on inhibitory effect of several human viruses by medicinal plant extracts has been demonstrated by Chiang *et al.* (2003b). The study demonstrated that hot water extract of seeds of *Pisum sativum* inhibited adenoviruses (ADV) including black seed of *Nigella sativa* could block infection of Laryngotracheitis virus (Zaher *et al.,* 2008). Verma *et al.* (2008) reported that *Swertia chirata* had anti-viral activity against HSV-1.

Table 8 Percentage of inhibition of aqueous extracts against HSV-1F and HSV-2G and concentrations of the extracts that used in this study

Medicinal plant aqueous extract	**Concentrations (µg/ml)**	**Inhibition (%)***	
		HSV-1F	**HSV-2G**
Andrographis paniculata	1,000	48.30±0.31	57.40±2.16
Cissus quadrangularis	1,000	3.60±0.84	0±0.00
Coscinium fenestratum	125	44.30±1.45	16.70±0.35
Croton roxburghii	2,000	100.00±0.00	100.00±0.00
Derris scandens	500	32.00±2.53	32.10±0.76
Eclipta prostrata	2,000	85.80±0.76	95.20±1.34
Glycyrrhiza glabra	500	35.40±3.06	23.40±0.33
Gynostemma pentaphyllum	250	39.30±2.12	10.30±1.31
Houttuynia cordata	1,000	62.80±3.12	82.30±0.84
Leptochloa chinensis	1,000	60.20±1.95	42.50±3.16
Momordica charantia	500	63.60±0.41	26.90±0.66
Phyllanthus amarus	250	42.90±0.43	61.40±2.03
Pluchea indica	500	60.50±2.53	77.40±1.32
Pseuderatherum platiferum	1,000	23.00±1.85	13.00±3.32
Rhinacanthus nasutus	1,000	92.30±1.39	98.50±3.56
Schefflera leucantha	62.5	24.70±2.64	28.30±1.42
Senna alata	2,000	94.50±2.93	95.60±2.35
Sphenodesma sp.	2,000	100.00±0.00	100.00±0.00
Stemona tuberosa	2,000	97.90±1.09	54.60±4.64

Table 8 (continued)

Medicinal plant aqueous extract	Concentrations (µg/ml)	Inhibition (%)*	
		HSV-1F	HSV-2G
Stephania venosa	500	26.30±3.69	22.10±3.11
Thunbergia laurifolia	500	49.00 ±1.43	36.20±3.93
Tinospora crispa	2,000	62.80±0.03	76.00±1.49
Zingiber montanum	2,000	69.70±2.55	74.50±2.11

* The data in table are presented as mean ± standard deviation (SD) of duplicate experiments.

Table 9 Percentage of inhibition of ethanolic extracts against HSV-1F and HSV-2G and concentrations of the extracts that used in this study

Medicinal plant ethanolic extract	**Concentrations (µg/ml)**	**Inhibition (%)***	
		HSV-1F	**HSV-2G**
Andrographis paniculata	15.6	42.10±0.00	65.10±1.64
Cissus quadrangularis	500	63.30±0.59	51.40±1.83
Coscinium fenestratum	62.5	52.90±1.41	36.70±1.37
Croton roxburghii	500	100.00±0.00	100.00±0.00
Derris scandens	31.3	32.00±1.71	27.10±3.18
Eclipta prostrata	1,000	56.60±4.21	73.10±1.52
Glycyrrhiza glabra	2,000	69.70±0.32	86.90±2.51
Gynostemma pentaphyllum	125	62.90±0.43	100.00±0.00
Houttuynia cordata	500	92.90±0.51	91.10±0.93
Leptochloa chinensis	62.5	21.90±0.32	14.00±2.33
Momordica charantia	62.5	40.00±5.13	34.70±4.02
Phyllanthus amarus	125	33.30±0.24	71.40±2.21
Pluchea indica	125	32.50±3.24	49.00±2.46
Pseuderatherum platiferum	500	25.90±1.54	19.50±1.90
Rhinacanthus nasutus	3.9	56.40±1.54	69.70±3.17
Schefflera leucantha	250	36.40±1.43	27.30±3.74
Senna alata	500	68.20±2.93	41.20±4.14
Sphenodesme sp.	4,000	100.00±0.00	100.00±0.00
Stemona tuberosa	250	77.90±4.64	42.90±3.30

Table 9 (continued)

Medicinal plant ethanolic extract	Concentrations (µg/ml)	Inhibition (%)*	
		HSV-1F	HSV-2G
Stephania venosa	62.5	37.90±0.52	33.30±3.34
Thunbergia laurifolia	1,000	50.00±0.03	23.30±0.62
Tinospora crispa	500	79.60±0.61	59.70±0.76
Zingiber montanum	31.3	15.00±1.45	12.00±0.31

* The data in table were presented as mean ± standard deviation (SD) of duplicate experiments.

From our results, five medicinal plants; *E. prostrata, H. cordata, R. nasutus*, *Sphenodesme* sp. and *S. tuberosa* that showed high anti-HSV activity were selected to further investigation on the other mode of action against HSV infection (Table 10).

Table 10 Medicinal plant extracts and concentration used in the study

Crude medicinal plant extract	**Given name**		**Concentration (µg/ml)**	
	Aqueous	**Ethanolic**	**Aqueous**	**Ethanolic**
E. prostrata	EPA	EPE	2,000	1,000
H. cordata	HCA	HCE	1,000	500
R. nasutus	RNA	RNE	1,000	3.90
Sphenodesme sp.	SPA	SPE	2,000	4,000
S. tuberosa	STA	STE	2,000	250

7. Mechanism of medicinal plant extracts on inhibition of HSV infection

There are many *in vitro* experiments to determine antiviral activity of medicinal plant extracts on cell culture. In this present study, the efficacy of medicinal plant extracts on various stages of HSV-1F and HSV-2G multiplication cycles was elucidated. HSV was treated with medicinal plant extracts before, during and after viral attachment and virus titers were investigated using plaque reduction assay. Inhibition of viral particles and viral replication were also determined. Effect on viral DNA and protein synthesis was also demonstrated.

7.1 Effect of medicinal plant extracts on HSV when treatment the extracts before viral attachment

Inhibition effect of medicinal plant extracts on HSV infection was determined when treatment was performed before viral attachment to Vero cell. Vero cells were pretreated with various non-toxic concentrations of medicinal plant extracts before HSV infection to demonstrate prophylactic activity of medicinal plant extracts on cell surface receptor. Plaque reduction assay was used to determine anti-HSV activity of aqueous extracts; *E. prostrata* (EPA), *H. cordata* (HCA), *R. nasutus* (RNA), *Sphenodesme* sp. (SPA), *S. tuberosa* (STA) and ethanolic extracts; *E. prostrata* (EPE), *H. cordata* (HCE), *R. nasutus* (RNE), *Sphenodesme* sp. (SPE) and *S. tuberosa* (STE). Moreover, ACV has been used as positive antiviral control.

Efficacy of these medicinal plant extracts on HSV infection was expressed and concentration of compound, which reduced number of plaques by 50% with respect to virus control was determined as ED_{50} (Robin *et al*., 2002). Low ED_{50} exerted the high safety margin of medicinal plant extracts (Tolo *et al.*, 2006).

ED_{50} value of the extract was evaluated from graphic plot, which was determined by linear regression equation, Y = mx + C; where Y was the amount of inhibition plaques formation, X was the concentration of extract, while m and c were the constant values. In addition, TI value was calculated from the ratio of CD_{50} and ED_{50} value. This value showed the therapeutic potential of medicinal plant extracts since the high TI value reflected high therapeutic potential effect of the medicinal plant extracts.

The results revealed that plaques formation of HSV-1F and HSV-2G on Vero cells was inhibited by all of aqueous and ethanolic of selected medicinal plant extracts with effective doses at 50% (ED_{50}) values ranging from 3.23-1,559.18 μg/ml and therapeutic indices (TI) ranged from 2.63-41.30 (Table 11). Moreover, the result found that ethanolic extracts; STE, RNE, SPE, EPE and EPA could protect the cell from HSV-1F infection with ED_{50} values of 34.53±0.21, 3.23±0.42, 665.37±7.23, 798.52±10.69 and 1,117.53±6.59, respectively and showed TI values of 41.30±0.25, 15.76±2.04, 6.04±0.07, 4.49±0.06 and 2.63±0.28, respectively. Moreover, SPE, SPA, EPE and STA extracts inhibited HSV-2G with ED_{50} values of 264.70±2.39, 304.27±3.22, 876.18±3.44 and 1,559.18±5.12 and TI values of 10.83±0.10, 9.42±0.10, 4.09±0.02 and 3.64±0.01, respectively.

From our result observed that when applied the medicinal plant extracts before adding viral attachment, EPE and SPE showed strongest protection of the cell from infection of both HSV-1F and HSV-2G as observed by TI values. EPA, RNE and STE were able to inhibit only HSV-1F while STA and SPA were able inhibit only HSV-2G when the extracted were applied to the cell before adding

virus. EPE and SPE showed the highest activity against HSV-1F and HSV-2G infection, respectively.

Thus, anti-HSV activities of medicinal plant extracts on HSV-1F activity when treatment before viral attachment were ranked from highest to lowest activities in the following order; STE>RNE>SPE>EPE>EPA, respectively. On the other hand, the highest inhibition when treatment before HSV-2G attachment was observed from extracts of SPE>SPA>EPE>STA, respectively. All medicinal plant extracts showed inhibitory effects on HSV-1F higher than HSV-2G except STA, SPA and SPE extracts (Table 11). Some medicinal extracts; HCA, RNA, STA, SPA and HCE have little effect on HSV-1F. EPA, HCA, RNA, HCE, RNE and STE could protect the cells from HSV-2G infection less than 50%, thus, ED_{50} of these medicinal plant extracts could not be calculated. Inhibition effect against HSV infection by aqueous and ethanolic extract of these selected medicinal plants was presented in Table 11.

The concepts for prophylaxis of infectious disease were defined as compounds that could interact with viral outer surface proteins that were responsible for specific attachment of the pathogen to the host cell membrane (Gescher *et al*., 2011). Thus, inhibition of this process may prevent or decrease the severity of infection (Spear *et al.,* 1972). These findings could support that the compounds in medicinal plant extracts had prophylaxis effect against HSV infections, which might block viral entry or penetration process of viral particle into host cell culture (Mazzanti *et al.,* 2008). The extract might be also interfere with specific interaction between viral particle and host cell receptor, and lead to the conformational change and impair the ability of HSV fusion of viral particles with target on cell surface

membrane. This process could reduce viral infectivity to the cell (Greco *et al*., 2007; Reichling *et al.,* 2009). Similar result on inhibition of viral entry to cell culture was found from the study of Palamara *et al.* (1995), which reported that *Cordia salicifolian* could inhibit HSV-1 on Hela cell and *Aloe vera* extract could block HSV-2 when treatment before infection of virus on Vero cells (Zandi *et al.,* 2007).

Table 11 Inhibition effect of HSV infection by aqueous and ethanolic of plant extracts on before viral attachment

Medicinal plant	HSV-1F		HSV-2G	
	ED±SD (µg/ml)*	TI±SD*	ED±SD (µg/ml)*	TI±SD
Aqueous extract				
E. prostrata (EPA)	1,117.53±6.59 [a]	2.63±0.28 [a]	0 [a]	0 [a]
H. cordata (HCA)	0 [a]	0 [a]	0 [a]	0 [a]
R. nasutus (RNA)	0 [a]	0 [a]	0 [a]	0 [a]
Sphenodesme sp. (SPA)	0 [a]	0 [a]	304.27±3.22 [a]	9.42±0.10 [b]
S. tuberosa (STA)	0 [a]	0 [a]	1,559.18±5.12 [a]	3.64±0.01 [a]
Ethanolic extract				
E. prostrata (EPE)	798.52±10.69	4.49±0.06	876.18±3.44 [a]	4.09±0.02 [a]
H. cordata (HCE)	0 [a]	0 [a]	0 [a]	0 [a]
R. nasutus (RNE)	3.23±0.42 [a]	15.76±2.04 [a]	0 [a]	0 [a]
Sphenodesme sp. (SPE)	665.37±7.23 [a]	6.04±0.07 [a]	264.70±2.39 [a]	10.83±0.10 [b]
S. tuberosa (STE)	34.53±0.21 [a]	41.30±0.25 [a]	0 [a]	0 [a]

$TI = CD_{50}/ED_{50}$

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

7.2 Effect of medicinal plant extracts on HSV when treatment the extracts during viral attachment

The inhibitory effect of medicinal plant extracts during viral attachment to cell culture was evaluated. Various non-toxic concentrations of medicinal plant extracts and HSV were applied together on monolayer of Vero cells. Plaque reduction assays were also performed to determine the efficacy of medicinal plant extracts on inhibition of HSV attachment and infection.

The results showed that HSV-1F was inhibited by SPE, EPA, SPA, STE, EPE, HCA and HCE extracts with ED_{50} values of 90.17±7.62, 107.17±0.96, 195.03±2.69, 132.68±1.58, 529.58±0.97, 810.15±0.14 and 890.08±7.36, respectively and TI values of 44.73±3.78, 26.55±0.24, 14.70±0.20, 10.75±0.13, 6.67±0.01, 3.51±0.00 and 1.60±0.01, respectively. Moreover, SPE, SPA, EPE, EPA, HCA, RNA, HCE and STA extracts also inhibited HSV-2G attachment with ED_{50} values of 36.35±0.52, 70.57±2.62, 110.88±8.29, 177.85±12.68, 635.62±15.88, 305.17±1.32, 382.35±2.41 and 1,600.85±5.49, respectively and TI values of 110.59±1.57, 41.07±1.18, 30.85±0.24, 16.04±1.14, 4.48±0.11, 4.16±0.02, 3.73±0.02 and 3.55±0.01, respectively (Table 12).

Therefore, EPA, HCA, SPA, EPE, HCE and SPE extracts showed inhibitory effect against both types of HSV-1F and HSV-2G when treatment during viral attachment. RNA and STA could inhibit only HSV-1F whereas STE could inhibit only HSV-2G (Table 12). The highest anti-HSV-1F and HSV-2G were observed by highest TI value of SPA and SPE. However, some extracts; RNA, STA and RNE inhibited HSV-1F less than 50% and STE also inhibited HSV-2G less than 50%, thus ED_{50} values of these extracts did not calculated (Table 12). This obtained result

indicated that these aqueous and ethanolic plants extracts had different antiviral activity on both types of HSV as shown in Table 12. Additionally, smaller plaque size was observed after treatment HSV with HCE when compared to plaque sized of viral control.

From the results could suggest that various types of compounds in medicinal plant extracts might have inhibitory effect against HSV infection by blocking or direct interfering and/or competitive binding of during viral particles to specifically cellular receptor on host cell surface. This process might occur by interference the initial attachment of virion to cells that mediated independently by interactions of either viral envelope glycoprotein C (gC) or B (gB) with heparan sulphate proteoglycan residues on the surface of the target cells (Gescher *et al*., 2011; Greco *et al*., 2007; Tal-Singer *et al*., 1995).

The extract might interfere fusion process between virion envelope and cell surface receptor that required for penetration (Manservigi *et al*., 1977). Moreover, the extract might interfere with gD and oligomers of gH and gL from interaction with cell receptor (Rajcáni and Vojvodová, 1998; Roizman and Spear, 1996).

In addition, compounds in the extracts might affect their antiviral activity by binding directly on HSV particle to form a complex with virus and prevented the virus from being adsorbed to their binding sites on specific cell receptor. Interference of plant extract on viral structure such as viral attachment proteins, which was necessary for viral adsorption on specific receptor on cell culture during the initial step of viral entry might be occurred (Barakat *et al*., 2010). Besides, the efficacy of medicinal plant extracts might affect HSV particle and destroy viral particles directly.

Anti-interaction agents between viral outer surface proteins with specific attachment to host cell membrane led to specific inhibition during the first process towards infection and might prevent or decrease the severity of infection (Gescher *et al*., 2011). Moreover, plant compounds might be developed specifically against adhesion proteins or receptor molecules as potential molecular targets (Gescher *et al*., 2011). Other studies on inhibitory efficacy of plant extract during viral attachment showed that aqueous extract of *Carissa edulis* inhibited HSV and HSV resistant strain during viral attachment (Tolo *et al*., 2006).

.

Table 12 Inhibition effect of HSV infection by aqueous and ethanolic of plant extracts when treatment during viral attachment

Medicinal plant	HSV-1F		HSV-2G	
	ED±SD (µg/ml)*	TI±SD*	ED±SD (µg/ml)*	TI±SD*
Aqueous extract				
E. prostrata (EPA)	107.17±0.96 [a]	26.55±0.24 [ab]	177.85±12.68	16.04±1.14 [ab]
H. cordata (HCA)	810.15±0.14 [a]	3.51±0.00 [a]	635.62±15.88 [a]	4.48±0.11 [a]
R. nasutus (RNA)	0 [a]	0 [a]	305.17±1.32 [a]	4.16±0.02 [a]
Sphenodesme sp. (SPA)	195.03±2.69 [a]	14.7±0.20 [b]	70.57±2.62 [a]	41.07±1.18 [b]
S. tuberosa (STA)	0 [a]	0 [a]	1,600.85±5.49 [a]	3.55±0.01 [a]
Ethanolic extract				
E. prostrata (EPE)	529.58±0.97 [a]	6.76±0.01 [ab]	110.88±8.29 [a]	30.85±0.24 [ab]
H. cordata (HCE)	890.08±7.36 [a]	1.60±0.01 [a]	382.35±2.41 [a]	3.73±0.02 [a]
R. nasutus (RNE)	0 [a]	0 [a]	0 [a]	0 [a]
Sphenodesme sp. (SPE)	90.17±7.62 [a]	44.73±3.78 [b]	36.35±0.52 [a]	110.59±1.57 [b]
S. tuberosa (STE)	132.68±1.58 [a]	10.75±0.13 [a]	0 [a]	0 [a]

$TI = CD_{50}/ED_{50}$

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

7.3 Effect of medicinal plant extracts on HSV when treatment after viral attachment

The inhibitory effects on HSV infection by medicinal plant extracts was also investigated when treatment after viral attachment to cell culture. After viral adsorption for 1 hour, each concentration of medicinal plant extracts was applied onto the infected cells. This step demonstrated antiviral replication activity, which may interfere the steps of virus uncoating, intracellular localization, replication, transcription, translation, virion assembly and release from the cells (Hundson *et al*., 2000).

Inhibition effects of aqueous and ethanolic extracts on HSV-1F and HSV-2G when treatments after viral attachment were demonstrated using plaque reduction assay. The result showed that, SPE, RNE, EPE, SPA, STA, STE, EPA, HCE and HCA extracts inhibited plaque formation of HSV-1F with ED_{50} values of 103.71±7.14, 1.34±0.05, 323.29±1.83, 266.97±6.60, 981.14±3.54, 246.29±6.09, 992.88±1.27, 534.39±2.56 and 1,275.27±4.65, respectively and TI values of 38.85±2.67, 37.78±1.40, 11.08±0.06, 10.74±0.27, 5.79±0.02, 5.79±0.14, 2.87±0.00, 2.67±0.01 and 2.23±0.01, respectively (Table 13). SPE, EPA, STA, EPE, SPA, RNA and HCA extracts had the effective anti-HSV-2G activity when treatment after viral attachment with ED_{50} values of 90.41±0.86, 317.62±10.64, 785.56±3.07, 498.05±1.44, 558.88±2.45, 697.19±11.50 and 1,801.74±5.13, respectively and TI values of 44.46±0.42, 8.96±0.30, 7.23±0.03, 7.19±0.02, 5.13±0.02, 1.82±0.02 and 1.58±0.01, respectively (Table 13). Interestingly, all of extracts were able to inhibit HSV-1F except RNA while HCE, RNE and STE extracts did not have any effect on HSV-2G when applied the extract after viral attachment. SPE extract exerted the

most potent inhibitory effect on both HSV-1F and HSV-2G activity as observed from TI values (Table 13). The plaque sizes after treatment HSV-1F and HSV-2G with EPA, EPE, RNA, RNE, HCE, STA, SPA and SPE were smaller than plaque size of viral controls. Moreover, comparison of inhibitory effect of the plant extracts against HSV infection when treatment after viral attachment was presented in Table 13.

The anti-HSV activities obtained from these selected plants were pronounced when applied after viral attachment. These extracts might express their efficacy against viral replication by interfering of viral multiplication cycle of HSV infection. The medicinal plant extracts might inhibit viral production and prevent immediate early (α)-genes transcription including viral gC and gD expressions and viral DNA synthesis (Kiani *et al*., 2010).

The similar researches on antiviral activity of medicinal plant extract were reported that extract of various plant species could inhibit HSV after viral attachment such as fraction of *Mallotus peltatus* showed strong inhibit HSV-1 and HSV-2 (Bag *et al*., 2012) as well as *Clinacanthus nutans* and *C. siamernsis* extract (Kunsorn *et al.*, 2013). Hot water extract of *H. cordata* could inhibit HSV-1 and HSV-2 and had effect against HSV-2 more than HSV-1 (Chiang *et al*., 2003a). *Hypericum mysorense* and *H. hookerianum* (Vijayan *et al*., 2004), *Psidium incanum, Phyllanthus niruri* and *Limonium brasiliense* showed efficacy to inhibit HSV-1 infection (Faral-Tello *et al.*, 2012). Pure compound of *Carissa edulis,* lupeol (Tolo *et al.*, 2010) and methanolic extract of *Eucalyptus globulus* also showed strong inhibition of HSV-1 when treatment after viral attachment (Davood *et al.*, 2012). Additionally, HSV-2 was blocked by *E. tirucalli, E. cotinifolia, E. cestrifolia, E. tirucalli* and *H. cordata* extracts (Betancur-Galvis *et al.*, 2002; Chen *et al*., 2011).

Table 13 Inhibition effect of HSV infection by aqueous and ethanolic of plant extracts when treatment after viral attachment

Medicinal plant	HSV-1F		HSV-2G	
	ED±SD (µg/ml)*	TI±SD*	ED±SD (µg/ml)*	TI±SD*
Aqueous extract				
E. prostrata (EPA)	992.88±1.27 [a]	2.87±0.00 [a]	317.62±10.64 [a]	8.96±0.30 [a]
H. cordata (HCA)	1,275.27±4.65 [a]	2.23±0.01 [a]	1,801.74±5.13 [a]	1.58±0.01 [a]
R. nasutus (RNA)	0 [a]	0 [a]	697.19±11.50 [a]	1.82±0.02 [a]
Sphenodesme sp. (SPA)	266.97±6.60 [a]	10.74±0.27 [a]	558.88±2.45 [a]	5.13±0.02 [a]
S. tuberosa (STA)	981.14±3.54 [a]	5.79±0.02 [a]	785.56±3.07 [a]	7.23±0.03 [a]
Ethanolic extract				
E. prostrata (EPE)	323.29±1.83 [a]	11.08±0.06 [a]	498.05±1.44 [a]	7.19±0.02 [a]
H. cordata (HCE)	534.39±2.56 [a]	2.67±0.01 [a]	0 [a]	0 [a]
R. nasutus (RNE)	1.34±0.05 [a]	37.78±1.40 [a]	0 [a]	0 [a]
Sphenodesme sp. (SPE)	103.71±7.14 [a]	38.85±2.67 [a]	90.41±0.86 [a]	44.46±0.42 [a]
S. tuberosa (STE)	246.29±6.09 [a]	5.79±0.14 [a]	0 [a]	0 [a]

$TI = CD_{50}/ED_{50}$

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

7.4 Inactivation kinetics

In this study, virus was incubated with the test compounds and the mixture was added to the cells. The mixtures of virus and plant extract were incubated at room temperature for 20 minutes interval up to 240 minutes. Moreover, concentrations of the ethanolic plant extract that showed strong inactivation of HSV particle were decreased and incubation period of inactivation was performed at 5, 10 and 15 minutes. Virucidal effect of plant extracts was determined from viral residual after treatment with the extracts, which was shown by a reduction of amount of plaques compared with untreated viral control (Lückemeyer *et al*., 2012). In this study, direct inactivation of HSV-1F and HSV-2G particles was shown after treatment with medicinal plants; *E. prostrata, H. cordata, R. nasutus, Sphenodesme* sp. and *S. tuberosa* extracts.

Log amount of HSV-1F was drastically reduced at 240 minutes after incubation with the extracts. Aqueous extract of EPA showed significantly highest inhibition of HSV-1F reduction of virus titter following by SPA, HCA, RNA and STA by 1.98±0.52, 1.71±0.57, 1.52±0.04, 1.49±0.06 and 1.35±0.51 PFU/ml, respectively (Table 14). The antiviral activities of extracts on viral particles were increased by the time. Moreover, ethanolic extract of EPE and SPE were significantly shown highest inhibition of HSV-1F titer at 240 minutes by 5.96±0.03 PFU/ml. However, HCE also reduced log HSV-1F titer by 5.96±0.03 PFU/ml. In addition, RNE and STE inhibited HSV-1F titer by reduction of log virus titer by 1.97±0.06 and 1.19±0.51 PFU/ml, respectively (Table 15).

Effect of these medicinal plant extract on HSV-2G was also demonstrated. At 240 minutes of inactivation, HSV-2G was inactivated by significantly reduction of

log virus titer (PFU/ml) by 1.89±0.62 when treatment with aqueous extract of SPA, followed by EPA, HCA, STA and RNA by 1.49±0.19, 1.46±0.12, 1.11±0.01 and 0.98±0.06 PFU/ml, respectively (Table 16). Inactivation of HSV-2G after treatment with aqueous extract of these medicinal plants was increased by the time. Moreover, ethanolic extract of EPE, HCE and SPE revealed virucidal effect against HSV-2G with showed significantly inhibition of HSV-2G by reduction of viral titer by 5.38±0.00 PFU/ml. RNE and STE also inhibited HSV-2G and reduction of log virus titer by 1.82±0.03 and 1.78±0.43 PFU/ml, respectively (Table 17).

Table 14 Direct inactivation of HSV-1F by aqueous extracts

Medicinal plant extracts	Concentration (µg/ml)	Reduction of log virus titer (PFU/ml) compared to virus control (Mins) ± SD*							
		20	40	60	80	100	120	180	240
E. prostrata (EPA)	2,000	0.18±0.01 [b]	0.28±0.01 [b]	0.53±0.01 [b]	0.71±0.02 [b]	0.97±0.20 [b]	1.56±0.27 [b]	1.77±0.64 [b]	1.98±0.52 [b]
H. cordata (HCA)	1,000	0.12±0.03 [a]	0.23±0.05 [a]	0.36±0.04 [a]	0.82±0.02 [a]	0.96±0.19 [a]	1.13±0.22 [a]	1.14±0.16 [a]	1.52±0.04 [a]
R. nasutus (RNA)	1,000	0.10±0.01 [a]	0.16±0.01 [a]	0.25±0.01 [a]	0.34±0.06 [a]	0.44±0.02 [a]	0.79±0.01 [a]	1.22±0.25 [a]	1.49±0.06 [a]
Spenodesme sp. (SPA)	2,000	0.16±0.01 [a]	0.42±0.00 [a]	0.51±0.16 [a]	0.58±0.31 [a]	0.61±0.04 [a]	0.91±0.05 [a]	1.42±0.24 [a]	1.71±0.57 [a]
S. tuberosa (STA)	2,000	0.17±0.07 [a]	0.23±0.01 [a]	0.37±0.01 [a]	0.41±0.02 [a]	0.62±0.13 [a]	0.71±0.13 [a]	1.12±0.42 [a]	1.35±0.51 [a]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

Table 15 Direct inactivation of HSV-1F by ethanolic extracts

Medicinal plant extracts	Concentration (µg/ml)	Reduction of log virus titer (PFU/ml) compared to virus control (Mins) ± SD*							
		20	40	60	80	100	120	180	240
E. prostrata (EPE)	1,000	6.70±0.00 [c]	6.57±0.00 [c]	6.48±0.00 [c]	6.36±0.00 [c]	6.32±0.00 [c]	6.25±0.00 [c]	6.03±0.00 [c]	5.96±0.03 [c]
H. cordata (HCE)	500	3.01±0.03 [b]	4.01±0.04 [b]	4.23±0.03 [b]	4.42±0.01 [b]	4.97±0.06 [b]	6.25±0.00 [b]	6.03±0.00 [b]	5.96±0.03 [b]
R. nasutus (RNE)	3.90	0.00±0.03 [a]	0.00±0.02 [a]	0.40±0.01 [a]	0.74±0.38 [a]	1.27±0.00 [a]	1.21±0.01 [a]	1.69±0.15 [a]	1.97±0.06 [a]
Sphenodesme sp. (SPE)	4,000	6.70±0.00 [c]	6.57±0.00 [a]	6.48±0.00 [a]	6.36±0.00 [a]	6.32±0.00 [a]	6.25±0.00 [a]	6.03±0.00 [a]	5.96±0.03 [a]
S. tuberosa (STE)	250	0.14±0.02 [a]	0.21±0.02 [a]	0.25±0.02 [a]	0.32±0.05 [a]	0.29±0.23 [a]	0.63±0.17 [a]	1.01±0.22 [a]	1.19±0.51 [a]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

Table 16 Direct inactivation of HSV-2G by aqueous extracts

Medicinal plant extracts	Concentration (µg/ml)	Reduction of log virus titer (PFU/ml) compared to virus control (Mins) ± SD*							
		20	40	60	80	100	120	180	240
E. prostrata (EPA)	2,000	0.21±0.04 [c]	0.28±0.10 [c]	0.57±0.03 [c]	0.68±0.04 [c]	0.82±0.01 [c]	1.31±0.03 [c]	1.36±0.06 [c]	1.49±0.19 [c]
H. cordata (HCA)	1,000	0.25±0.06 [cd]	0.68±0.00 [cd]	0.84±0.03 [cd]	0.93±0.02 [cd]	1.04±0.01 [cd]	1.12±0.16 [cd]	1.31±0.52 [cd]	1.46±0.12 [cd]
R. nasutus (RNA)	1,000	0.00±0.02 [a]	0.00±0.09 [a]	0.17±0.22 [a]	0.14±0.02 [a]	0.25±0.02 [a]	0.54±0.09 [a]	0.82±0.09 [a]	0.98±0.06 [a]
Sphenodesme sp. (SPA)	2,000	0.28±0.01 [d]	0.89±0.03 [d]	0.97±0.06 [d]	0.74±0.26 [d]	1.18±0.74 [d]	1.44±0.09 [d]	1.55±0.06 [d]	1.89±0.62 [d]
S.tuberosa (STA)	2,000	0.15±0.06 [b]	0.17±0.02 [b]	0.23±0.01 [b]	0.37±0.03 [b]	0.48±0.11 [b]	0.67±0.01 [b]	1.09±0.02 [b]	1.11±0.01 [b]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

Table 17 Direct inactivation of HSV-2G by ethanolic extracts

Medicinal plant extracts	Concentration (µg/ml)[a]	Reduction of log virus titer (PFU/ml) compared to virus control (Mins) ± SD*							
		20	40	60	80	100	120	180	240
E. prostrata (EPE)	1,000	2.88±0.48 [b]	3.21±0.03 [b]	3.48±0.07 [b]	3.95±0.17 [b]	4.50±0.37 [b]	5.60±0.00 [b]	5.42±0.00 [b]	5.38±0.00 [b]
H. cordata (HCE)	500	5.59±0.00 [c]	5.95±0.00 [c]	5.79±0.00 [c]	5.69±0.00 [c]	5.66±0.00 [c]	5.60±0.00 [c]	5.42±0.00 [c]	5.38±0.00 [c]
R. nasutus (RNE)	3.90	0.00±0.08 [a]	0.12±0.01 [a]	0.35±0.00 [a]	0.43±0.13 [a]	0.53±0.03 [a]	0.95±0.12 [a]	1.48±0.04 [a]	1.82±0.03 [a]
Sphenodesme sp. (SPE)	4,000	6.30±0.00 [c]	5.95±0.00 [c]	5.79±0.00 [c]	5.69±0.00 [c]	5.66±0.00 [c]	5.60±0.00 [c]	5.42±0.00 [c]	5.38±0.00 [c]
S. tuberosa (STE)	250	0.04±0.00 [a]	0.16±0.02 [a]	0.45±0.06 [a]	0.53±0.05 [a]	0.76±0.04 [a]	0.90±0.01 [a]	1.35±0.22 [a]	1.78±0.43 [a]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

Interestingly, HSV-1F and HSV-2G were completely inactivated after treatment with ethanolic extracts of EPE, HCE and SPE at concentration of 1,000, 500, and 4,000 μg/ml, respectively as shown in Table 15 and 17. Thus, these extracts had strong inhibitory effect on viral particles so it was selected to further investigate on viral particle when concentration of extracts and incubation period of virus and extracts were reduced. Dose-dependent effect was observed when the concentration of extract was increased, plaque inhibition was also increased. The result showed that maximum reduction of plaque number was observed after treatment with EPE extract at concentration of 1,000 μg/ml with significantly reduction of log HSV-1F titer by 6.70±0.00 PFU/ml at 20 minutes. SPE extract at concentrations of 4,000 μg/ml also inhibited HSV-1F viral titer by 6.70±0.00 PFU/ml (Table 18). Furthermore, EPE extract at concentration of 125, 250 and 500 μg/ml were reduced HSV-1F titer by 1.01±0.09, 1.31±0.06 and 3.20±0.50, PFU/ml, respectively. On the other hand, HCE extract at concentration of 250, 500 and 1,000 μg/ml could reduce HSV-1F titer by 0.91±0.18, 1.89±0.00 and 3.01±0.03 PFU/ml, respectively. Moreover, SPE extract at concentration of 500, 1,000 and 2,000 μg/ml could reduce viral titer by 1.40±0.13, 1.50±0.17 and 2.88±0.81 PFU/ml, respectively (Table 18). The ability of extract on directly inactivation of HSV was significantly increased by the time.

Inactivation kinetic of HSV-2G particles was also significantly observed after treatment with SPE extract at concentration of 500, 1,000, 2,000 and 4,000 μg/ml. The viral titers were reduced by 1.21±0.38, 4.36±0.13 6.30±0.00 and 6.30±0.00 PFU/ml, respectively. EPE extract at concentrations of 125, 250, 500 and 1,000 μg/ml inhibited viral titer by 0.67±0.11, 1.51±0.02, 2.49±0.05 and 2.88±0.48 PFU/ml, respectively. Moreover, HCE extract at concentration of 250, 500 and

1,000 μg/ml inhibited viral titer by 0.86±0.11, 2.38±0.05 and 5.59±1.20 PFU/ml, respectively (Table 19). The obtained result found that all medicinal plants were able to reduce viral titer (Table 14-19).

The results demonstrated that, viral infection was reduced with the time in all experiments. Thus, HSV particles were inactivated after treatment with extracts by time and dose-dependent manner. Thus, both aqueous and ethanolic extracts of plant extracts were effective on viral particles with different range of inhibition on each type of virus. Therefore, the direct inactivation could be at least one of the modes of the inhibitory effect (Serkedjieva and Ivancheva, 1998). However, the result showed that ability of ethanolic extract on inactivation of HSV-1F and HSV-2G particle was better than aqueous extract. Thus, these results showed that these selected plants showed strongly inhibition of HSV-1F and HSV-2G particles and promising plants could be used for treatment of HSV infection.

This result suggested the bioactive compounds could be isolated by ethanol and were able to inhibit HSV particle better than aqueous compound. Furthermore, storage time of the extract, steps of extraction might also affect inactivation activity of the extract. Moreover, suitable solvent should be selected after consideration of low price and extraction potency.

In addition, virucidal effect of the extracts on HSV particle was not caused by DMSO that was used to dissolve the extracts. The results from the present study showed that medicinal plants had anti-HSV potential.

Virucidal effect of viral particles by extract was occurred by inactivation of the virion, either by disruption of viral particle or by interfering with its ability to initiate a replication cycle (Hundson *et al*., 2000). Moreover, compounds of

medicinal plant extracts might block or interfere or destroy virion envelope structure. Subsequently HSV particles were inactivated directly by masking viral structures or viral receptors on viral envelope, which was necessary for adsorption or entry into the host cell. In addition, envelope viral protein might be degraded by the extract (Lückemeyer *et al*., 2012; Mazzanti *et al*., 2008; Reichling *et al*., 2009; Yoosook *et al.,* 1999). Thus, it could indicate that the plant extracts might prevent re-infections with the newly produced viruses and also inhibited extracellular virus (Yarmolinsky *et al.,* 2010).

Similar results of other extracts on inactivation of viral particles were shown by reduction of virus titers. Ethanolic extract of *Salvadora Persical* could inhibit cell free virus by virucidal effect (Taha, 2008). *Bidens pilosa* extract could directly inactivate HSV-1 and HSV-2 (Nakama *et al.*, 2012). The similar result was observed by the report of Cheng and Xu (2006) that HSV-1 and HSV-2 was inhibited by polysaccharide of *Prunella vulagaris*. Moreover, inactivation kinetic of isoborneol on HSV-1F was observed by 4 log10 within 30 minutes of incubation periods (Armaka *et al*., 1999).

Table 18 Direct inactivation of HSV-1F by ethanolic extracts

Medicinal plant extracts	Concentration (µg/ml)	Reduction of log virus titer (PFU/ml) compared to virus control (Mins) ± SD*		
		5	10	15
E. prostrata	1,000	6.80±0.00 ^{e}	6.78±0.00 ^{e}	6.73±0.00 ^{e}
(EPE)	500	2.00±0.04 ^{c}	2.43±0.04 ^{c}	2.84±0.05 ^{c}
	250	1.02±0.11 ^{b}	1.28±0.06 ^{b}	0.95±0.09 ^{b}
	125	0.93±0.08 ^{ab}	0.93±0.18 ^{ab}	0.83±0.11 ^{ab}
H. cordata	1,000	2.18±0.02 ^{c}	2.76±0.73 ^{c}	2.76±0.09 ^{c}
(HCE)	500	1.22±0.18 ^{b}	1.40±0.13 ^{b}	1.46±0.13 ^{b}
	250	0.22±0.06 ^{a}	0.36±0.03 ^{a}	0.42±0.01 ^{a}
Sphenodesme sp.	4,000	5.78±1.41 ^{d}	5.73±0.07 ^{d}	6.73±0.00 ^{d}
(SPE)	2,000	1.53±0.01 ^{c}	2.33±0.04 ^{c}	2.17±0.01 ^{c}
	1,000	1.39±0.01 ^{b}	1.04±0.01 ^{b}	1.44±0.19 ^{b}
	500	0.81±0.06 ^{b}	1.29±0.06 ^{b}	1.29±0.29 ^{b}

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

Table 19 Direct inactivation of HSV-2G by ethanolic extract

Medicinal plant extracts	Concentration (µg/ml)	Reduction of log virus titer (PFU/ml) compared to virus control (Mins) ± SD*		
		5	10	15
E. prostrata	1,000	0.54±0.42^{ab}	0.74±0.06^{ab}	2.05±0.04^{ab}
(EPE)	500	0.56±0.04^{ab}	0.71±0.08^{ab}	1.17±0.13^{ab}
	250	0.50±0.04^{ab}	0.71±0.00^{ab}	1.16±0.1^{ab}
	125	0.30±0.03^{a}	0.54±0.08^{a}	0.61±0.04^{a}
H. cordata	1,000	2.61±0.01^{d}	5.74±0.04^{d}	5.49±0.11^{d}
(HCE)	500	1.57±0.13^{b}	1.96±0.01^{b}	2.32±0.27^{b}
	250	0.15±0.01^{a}	0.25±0.01^{a}	0.46±0.18^{a}
Sphenodesme sp.	4,000	6.45±0.81^{e}	6.39±0.00^{e}	6.34±0.00^{e}
(SPE)	2,000	6.45±0.81^{e}	6.39±0.00^{e}	6.34±0.00^{e}
	1,000	2.43±0.01^{c}	2.86±0.09^{c}	4. 91±0.21^{c}
	500	0.58±0.06^{ab}	0.60±0.01^{ab}	0.56±0.08^{ab}

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

7.5 Effect of plant extracts on HSV multiplication

The effect of medicinal plant extracts on viral multiplication was performed in this study. Efficacy of medicinal plant extracts on viral multiplication was carried out by determination of viral titer at different time after viral multiplication by yield reduction assay. ACV was used as positive control at ED_{50} value of 1.5 and 3.1 µg/ml for HSV-1F and HSV-2G, respectively.

Inhibitory effects of medicinal plant extracts on viral multiplication were observed. Extracts that inhibited virus by reduction more than 1 log virus yield reduction were considered to have antiviral activity (Yoosook *et al*., 1999). At 30 hours after treatment the infected cell with medicinal plant extracts, HSV-1F yield was inhibited as observed by reduction of log virus titer (PFU/ml). The result showed that HSV-1F yields were reduced after treatment with aqueous extract of EPA, SPA, STA, HCA and RNA by 2.82±0.09, 2.65±0.08, 1.71±0.70, 1.48±0.10 and 0.98±0.01 log PFU/ml, respectively when compared to untreated viral control (Figure 44). Whereas, HSV-2G yield were reduced after treatment with EPA, SPA, HCA, RNA and STA by 2.21±0.22, 2.07±0.21, 1.52±0.34, 1.13±0.21 and 0.47±0.10 log PFU/ml, respectively (Figure 46). HSV-1F and HSV-2G yield were reduced by 2.87±0.83 and 2.67±0.81 when treatment with ACV at ED_{50} values. Reductions of HSV-1F after treatment with ethanolic extract of SPE, EPE, HCE, RNE and STE were 5.34±0.04, 5.14±0.04, 5.10±0.23, 3.07±0.28 and 1.23±0.00 log PFU/ml, respectively, while yields of HSV-2G were reduced after treatment with SPE, EPE, HCE, RNE and STE by 5.60±0.04, 5.27±0.04, 5.01±0.73, 3.52±0.34 and 1.10±0.13 log PFU/ml, respectively (Figure 45 and 47).

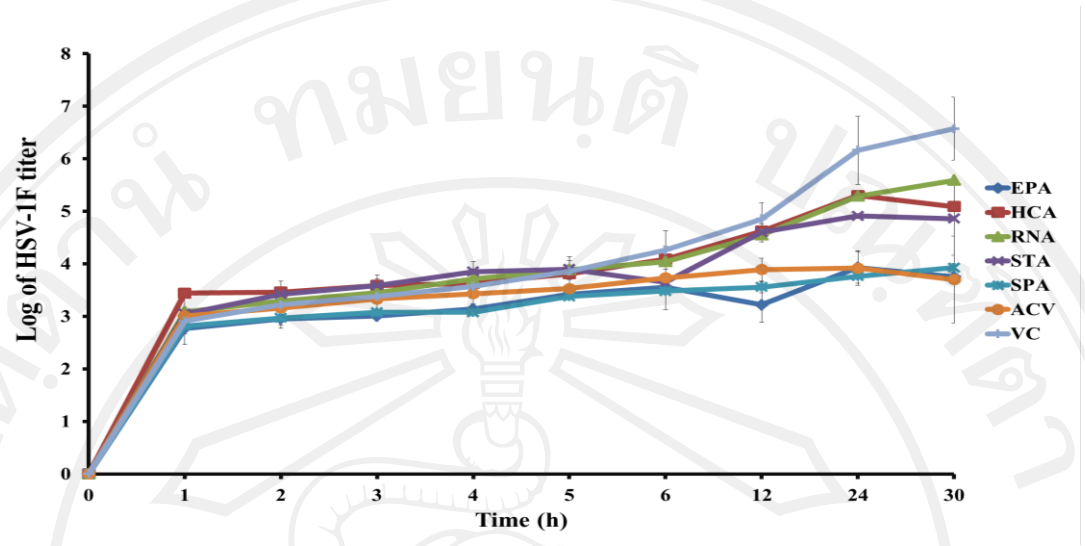

Figure 44 Log of HSV-1F titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with aqueous extract of *E. prostrata* (EPA), *H. cordata* (HCA), *R. nasutus* (RNA), *Sphenodesme* sp. (SPA) and *S. tuberosa* (STA) compared to antiviral agent, ACV 1.5 µg/ ml and viral control (VC).

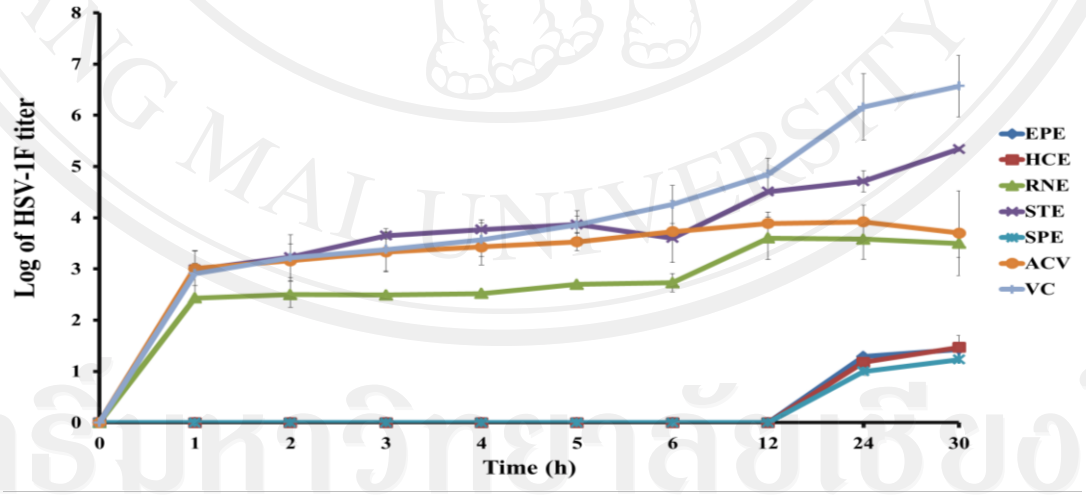

Figure 45 Log of HSV-1F titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethnolic extract of *E. prostrata* (EPE), *H. cordata* (HCE), *R. nasutus* (RNE), *Sphenodesme* sp. (SPE) and *S. tuberosa* (STE) compared to antiviral agent, ACV 1.5 µg/ ml and viral control (VC).

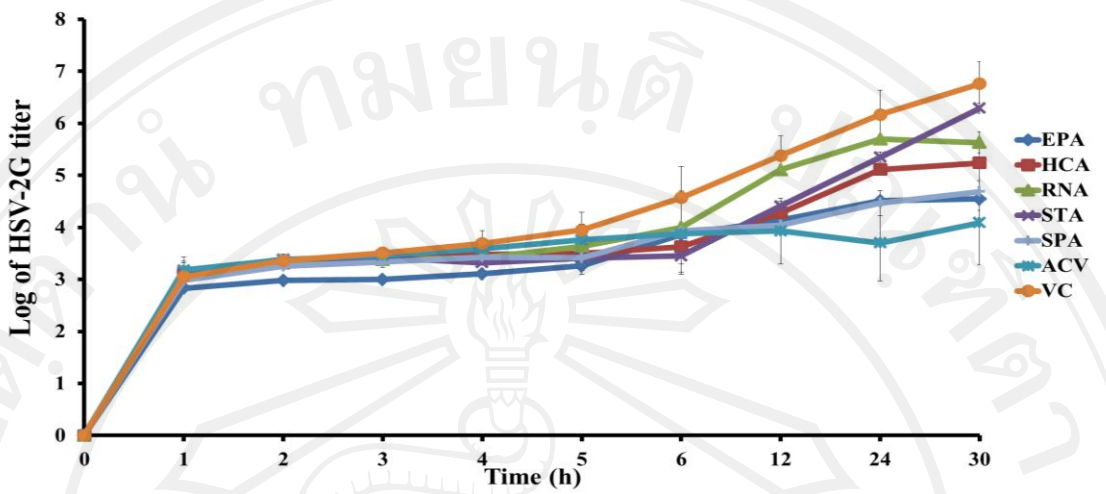

Figure 46 Log of HSV-2G titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with aqueous extract of *E. prostrata* (EPA), *H. cordata* (HCA), *R. nasutus* (RNA), *Sphenodesme* sp. (SPA) and *S. tuberosa* (STA) compared to antiviral agent, ACV 3.1 µg/ ml and viral control (VC).

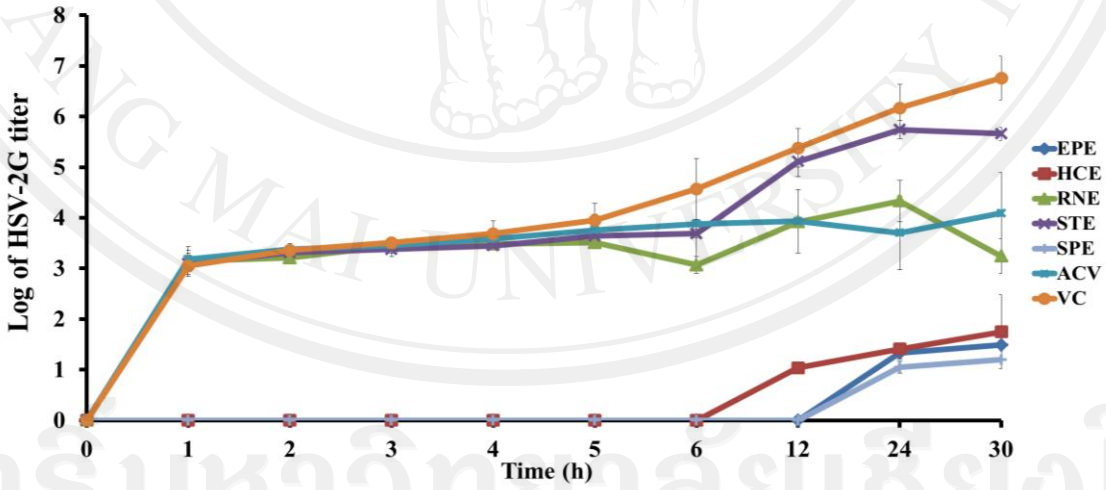

Figure 47 Log of HSV-2G titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *E. prostrata* (EPE), *H. cordata* (HCE), *R. nasutus* (RNE), *Sphenodesme* sp. (SPE) and *S. tuberosa* (STE) compared to antiviral agent, ACV 3.1 µg/ ml and viral control (VC).

Interestingly, EPE, HCE and SPE extracts showed strong inhibition in early stage of viral multiplication within 6 hour after HSV infection. SPE extract at concentration of 1,000, 2,000 and 4,000 µg/ml could reduce HSV-1F yield by 3.24±0.27, 4.68±0.08 and 5.34±0.04 log PFU/ml, respectively (Figure 50), while EPE extract at concentration of 250, 500 and 1,000 µg/ml could inhibit HSV-1F yield by 3.25±0.01, 4.27±0.04 and 5.14±0.04 log PFU/ml (Figure 48). Reduction of HSV-1F yield were 3.52±0.30, 4.84±1.23 and 5.10±0.23 log PFU/ml after treatment with HCE extract at concentration of 250, 500 and 1,000 µg/ml (Figure 49). Yield reductions of HSV-2G were 3.75±0.01, 4.89±0.02 and 5.56±0.04 log PFU/ml after treatment with SPE extract at concentration of 1,000, 2,000 and 4,000 µg/ml, respectively (Figure 53). HSV-2G yield were reduced after treatment with EPE extract at concentration of 250, 500 and 1,000 µg/ml by 3.39±0.42, 4.83±0.02 and 5.27±0.04 log PFU/ml, respectively (Figure 51), while yields of HSV-2G were reduced by 3.46±0.26, 4.72±1.23 and 5.01±0.73 log PFU/ml after treatment with HCE extract at concentration of 250, 500 and 1,000 µg/ml, respectively (Figure 52).

The results indicated that ethanolic extracts showed anti-HSV activity against HSV multiplication stronger than aqueous extracts. SPE extract exhibited significantly strongest ability to inhibit viral replication against both types of virus.

Within 4 hours after virus infection, the viral genes that are essential for HSV multiplication cycle are expressed as the immediate early (α) genes and transcribed to protein that activates transcription of early β genes. At 4-8 hours after viral infection, the viruses are attached, penetrated and viral DNA are entered into nucleus. The β genes and essential proteins promote viral DNA replication. Moreover, the late γ gene products are expressed after 12 hours of infection following

translation into viral structural proteins (Nikomtat, 2010). It suggested that some medicinal plant extracts revealed the inhibitory action at early and late stages of replication since the extracts revealed completely inhibited HSV multiplication in early hour and late stage of infection.

Inhibition of viral progeny production was observed by the time. The early infection showed the reduction of viral titer more than late infection when observed by reduction of viral titer by plaque reduction assay. Our result could suggest that medicinal plant extracts might inhibit HSV replication by interfering with the initial stages of virus replication, viral growth and release of new virus (Serkedjieva and Ivancheva, 1998). Moreover, compounds in plants might influence antiviral activity by blocking HSV replication, which affect different processes such as attachment, penetration and fusion step that required specific interaction between the viral glycoprotein and the receptors site on host cell surface (Roizman *et al.*, 2007; Taylor *et al.*, 2002). Although, some medicinal plant extracts might lost their anti-viral activity since the extract showed less efficacy to reduce viral titer when treated for long time (Nikomtat *et al.*, 2011a).

The extracts could inactivate progeny virus from the first round of multiplication and prevent development of CPE (Hudson *et al*., 2000). The result also showed that these medicinal plants extract exerted their inhibitory activity against HSV multiplication more efficient than ACV at concentration of ED_{50} value. Thus, the antiviral agent of medicinal plants extracts was developed as for alternative treatment of HSV infection, which least side effect and had different mode of action from ACV.

Moreover, mechanisms of anti-HSV action are important together with the safety of products. Inhibition of HSV should be selectively targeted on its particle or its multiplication cycle inside the host cell. However, the inhibition viral growth step was more efficient than inhibition of viral particle. Therefore, antiviral multiplication agent could be utilized in both primary and recurrent infection to reduce the viral shedding and prevent the viral infection (Greco *et al*., 2007).

Many publications reported about antiviral multiplication activity by medicinal plant extracts. Blackberry (Danaher *et al.,* 2011), *Minthostachys verticillata* (Sabini *et al.,* 2010), *Peganum harmala* (Kiani *et al.,* 2010) and *H. cordata* (Chou *et al*., 2009) extracts reveled anti HSV-1 replication. *Echium amoenum* extract could inhibit HSV-1 replication within 2 hours (Farahani, 2013) as well as ethanolic extract of *Salvadora Persica* (Taha, 2008) that also demonstrated anti-HSV-1 replication cycle. Furthermore, Park *et al* (2005) reported that methanolic extract of *Symphyocladia latiuscula* and their fraction could inhibit resistant and wild type strain of HSV-1, and could block viral replication (Bedows *et al*., 1986). The similar results were also found by water extract of *H. cordata* that was able to block HSV-1 and HSV-2 replication by inhibition of NF-κB (Chen *et al*., 2011). Methanolic and acetonic extract of apple pomace (Suárez *et al.,* 2010) could inhibit HSV replication. Therefore, both HSV-1 and HSV-2 replication was also inhibited by aqueous extract of *Pongamia pinnata* (Elanchezhiyan *et al*., 1993) and putranjivain A from *Euphorbia jolkini* (Cheng *et al*., 2004). Crude hot glycerine extract of Aloe vera gel (Zandi *et al.,* 2007) and lemon balm extract (Mazzanti *et al*., 2008) could inhibit HSV-2 replication.

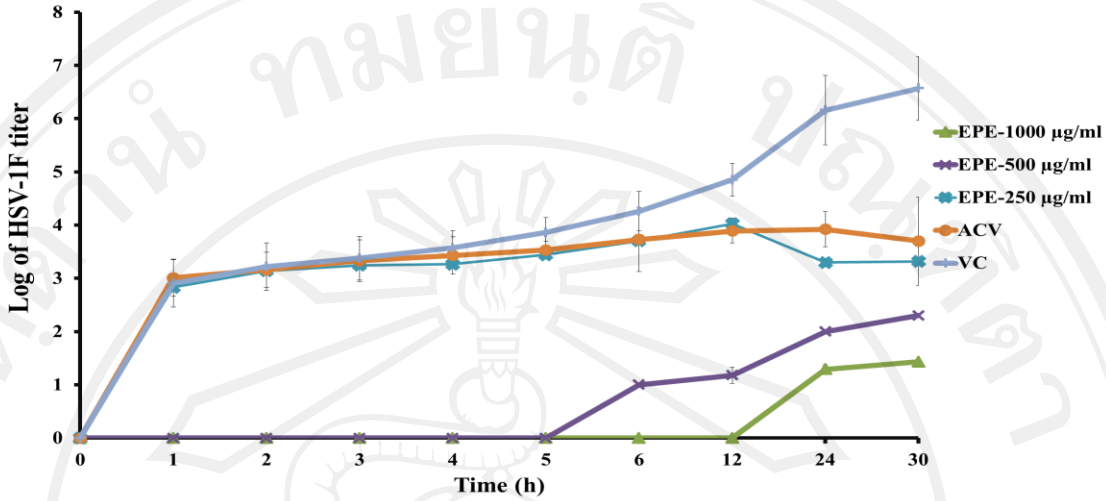

Figure 48 Log of HSV-1F titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *E. prostrata* (EPE) at concentration of 250, 500 and 1,000 µg/ ml compared to antiviral agent, ACV 1.5 µg/ ml and viral control (VC).

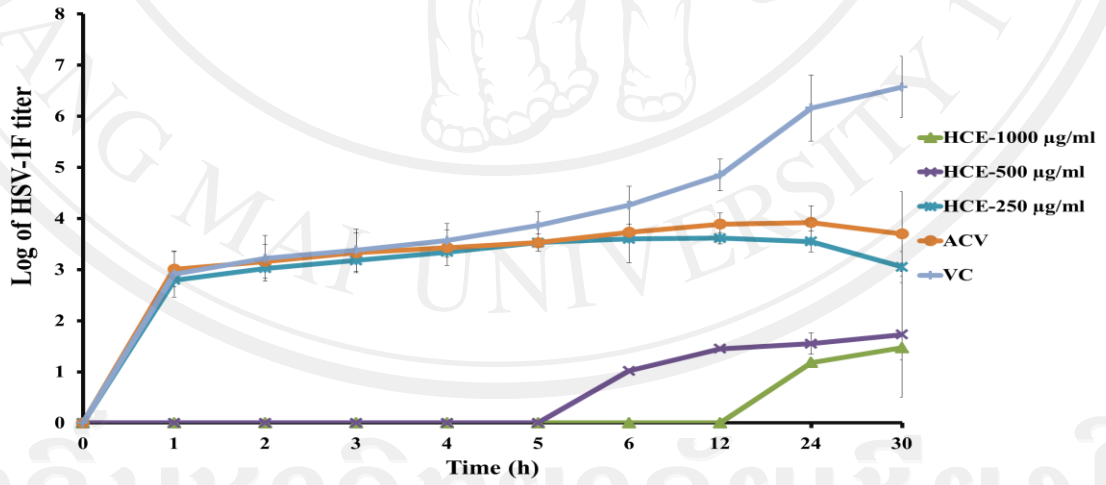

Figure 49 Log of HSV-1F titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *H. cordata* (HE) at concentration of 250, 500 and 1,000 µg/ ml compared to antiviral agent, ACV 1.5 µg/ ml and viral control (VC).

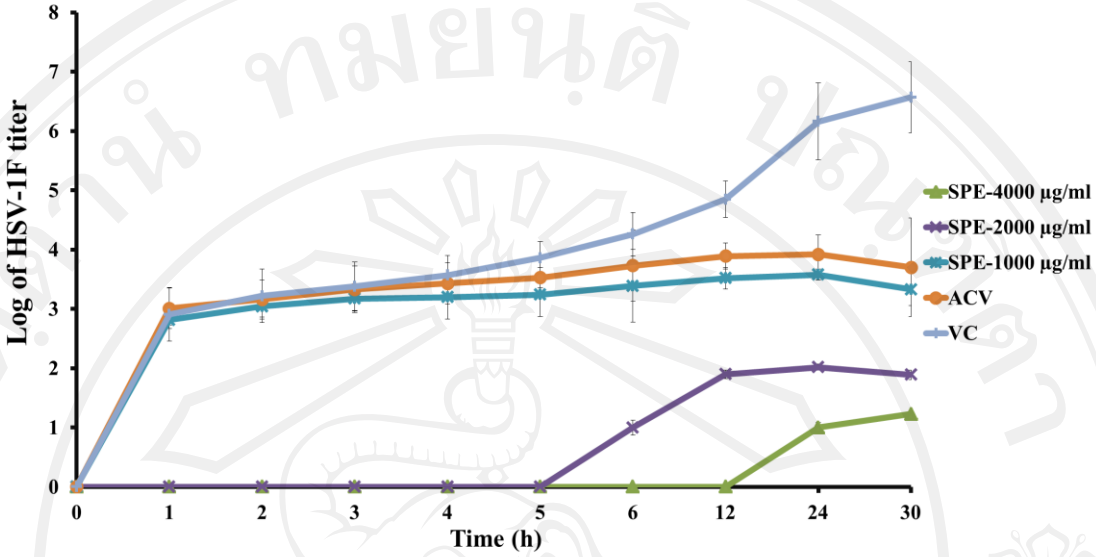

Figure 50 Log of HSV-1F titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *Sphenodesme* sp. (SPE) at concentration of 1,000, 2,000 and 4,000 µg/ ml compared to antiviral agent, ACV 1.5 µg/ ml and viral control (VC).

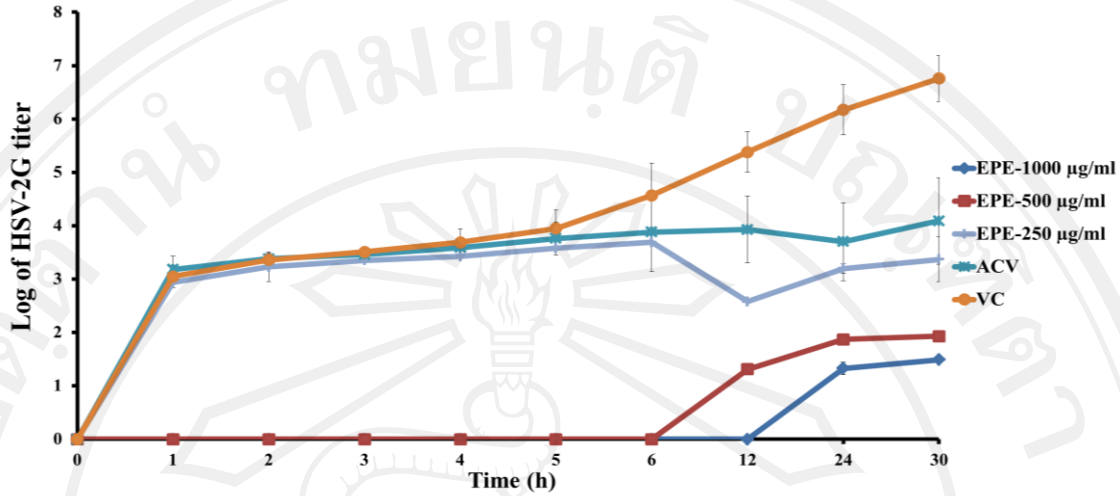

Figure 51 Log of HSV-2G titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *E. prostrata* (EPE) at concentration of 250, 500 and 1,000 µg/ ml compared to antiviral agent, ACV 3.1 µg/ ml and viral control (VC).

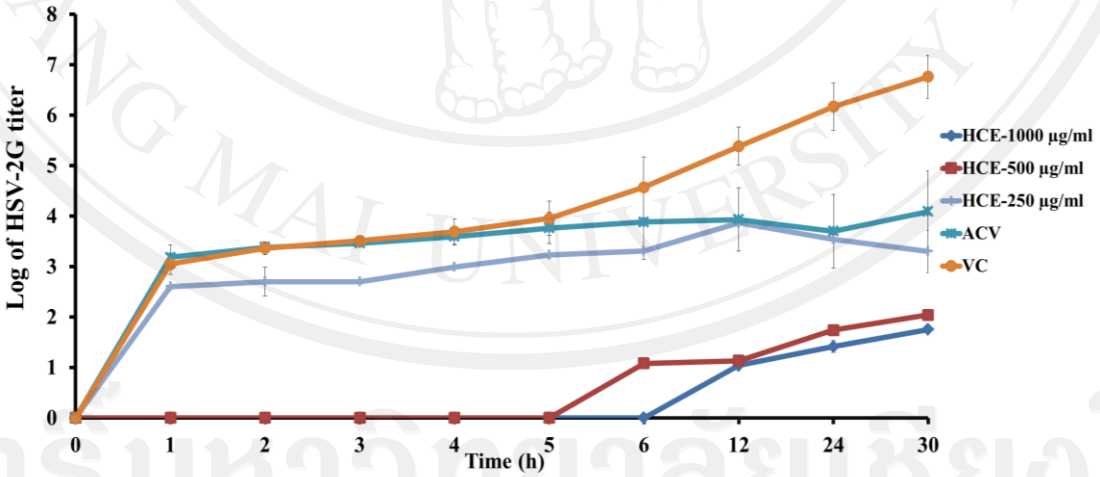

Figure 52 Log of HSV-2G titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *H. cordata* (HCE) at concentration of 250, 500 and 1,000 µg/ ml compared to antiviral agent, ACV 3.1 µg/ ml and viral control (VC).

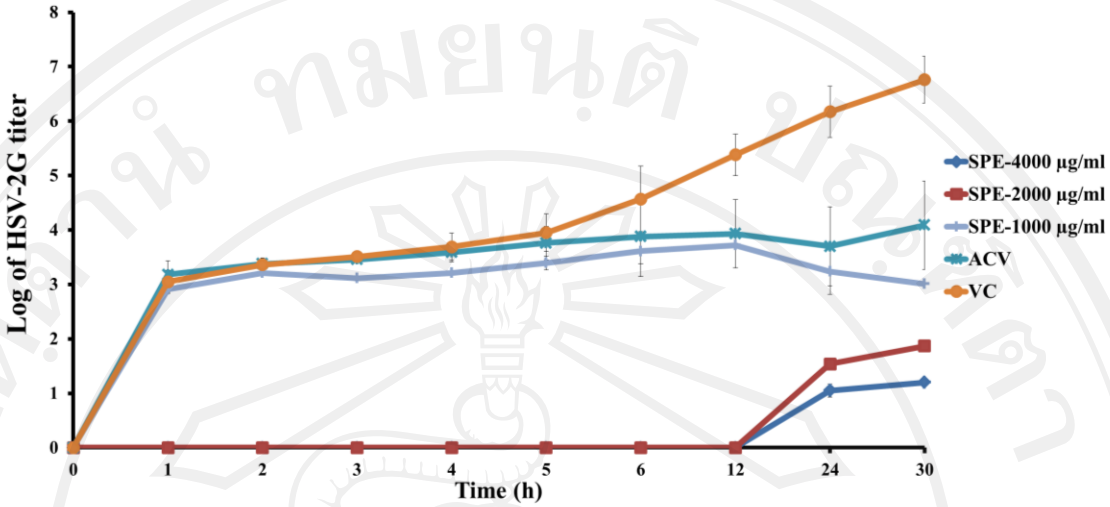

Figure 53 Log of HSV-2G titer at 0, 1, 2, 3, 4, 5, 6, 12, 24 and 30 hours after treatment with ethanolic extract of *Sphenodesme* sp. (SPE) at concentration of 1,000, 2,000 and 4,000 µg/ ml compared to antiviral agent, ACV 3.1 µg/ ml and viral control (VC).

7.6 Effect of medicinal plant extracts on viral DNA

Viral DNA was determined in order to elucidate the effect medicinal plant extracts on viral HSV-1F and HSV-2G DNA synthesis.

The results revealed that percentages of HSV-1F DNA remaining after treatment with aqueous extracts of *R. nasutus, S. tuberosa, E. prostrata, H. cordata and Sphenodesme* sp. were 88.84±4.49, 80.95±3.94, 75.13±11.58, 58.81±7.43 and 36.14±6.26, respectively. Percentages of HSV-1F DNA remaining after treatment with ethanolic extract of *R. nasutus, S. tuberosa, E. prostrata, H. cordata* and *Sphenodesme* sp. were 49.91±7.31, 39.28±4.34, 27.05±8.79, 13.64±2.78 and 8.65±0.11, respectively (Table 20 and Figure 54). Thus, ethanolic extract of *Sphenodesme* sp. showed highest inhibition of HSV-1F DNA synthesis from infected cell when compared to other extracts, which observed by lowest DNA remaining. On the other hand, aqueous extract of *S. tuberosa* had the lowest inhibitory effect on HSV-1F DNA synthesis which showed the highest DNA remaining (Table 20 and Figure 54).

Inhibitory effect of medicinal plant extract on HSV-2G DNA synthesis was also evaluated. The result showed that DNA remaining of HSV-2G after treatment with aqueous extract of *S. tuberosa, R. nasutus, H. cordata E. prostrata* and *Sphenodesme* sp. demonstrated percentage DNA remaining by 75.73±3.10, 66.92±5.92, 54.48±4.56, 33.69±4.60 and 23.19±5.11, respectively (Table 21 and Figure 55). Moreover, ethanolic extract of *R. nasutus S. tuberosa, H. cordata, E. prostrata* and *Sphenodesme* sp. revealed percentage of HSV-2G DNA remaining by 50.31±4.70, 46.66±9.73, 28.39±0.85, 19.16±2.08 and 6.21±2.60, respectively

(Table 21 and Figure 55). Comparison of inhibitory effect of each extract on HSV-1F and HSV-2G synthesis was representing in Figure 54-55.

The obtained result showed that viral DNA was decreased after treatment with various medicinal plants extracts at maximum concentration compared to untreated viral control. This result suggested that extract might interfere viral DNA synthesis or interfere viral DNA replication directly or interfere essential enzymes for viral DNA synthesis. Thus, it might reflect inhibition of viral protein expression (Nikomtat *et al.*, 2011b; Nikomtat, 2010). The extracts might interfere HSV DNA synthesis, which corresponding to the stage of alpha, beta and late gene expression (Roizman *et al.*, 2007; Taylor *et al*., 2002). Inhibition or interference of some viral essential gene expressions might occur. For examples, U_L30 and U_L42 gene products might affect, which encoded a DNA polymerase processivity factor that essential for DNA replication (Crute *et al*., 1989; Gottlieb and Challberg, 1994; Morello *et al*., 2011). U_L5, U_L8 and U_L52 gene products were necessary for helicase-primase complex of protein production and function for DNA replication (Constantin *et al*., 1999; Dudex *et al*., 2011; Klinedinst *et al*., 1994; Lokonis *et al*., 1997; Sherman *et al*., 1992). Moreover, it might affect U_L 38 (VP 19C) which was essential for capsid assembly and DNA maturation (Flanagan *et al.*, 1991). Additionally, the extracts might inhibit or interfere U_L17, U_L15, U_L25, U_L28 (ICP 18.5) and U_L33, which required for processing and packaging of DNA into capsid (Abbotts *et al.*, 2000; Higgs *et al*., 2008; Thurlow *et al*., 2005). U_L29 (ICP8) is a major DNA-binding protein that is essential for viral DNA replication (Boehmer and Lehman, 1993; Da costa *et al*., 2000).

Table 20 Percentage of HSV-1F DNA remaining after treatment with plant extracts

Medicinal plant	Mean DNA remaining (% ± SD*)	
	Aqueous	Ethanolic
E. prostrata	75.13±11.58 [bc]	27.05±8.79 [bc]
H. cordata	58.81±7.43 [ab]	13.64±2.78 [ab]
R. nasutus	88.84±4.49 [c]	49.91±7.31 [c]
Sphenodesme sp.	36.14±6.26 [a]	8.65±0.11 [a]
S. tuberosa	80.95±3.94 [c]	39.28±4.34 [c]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

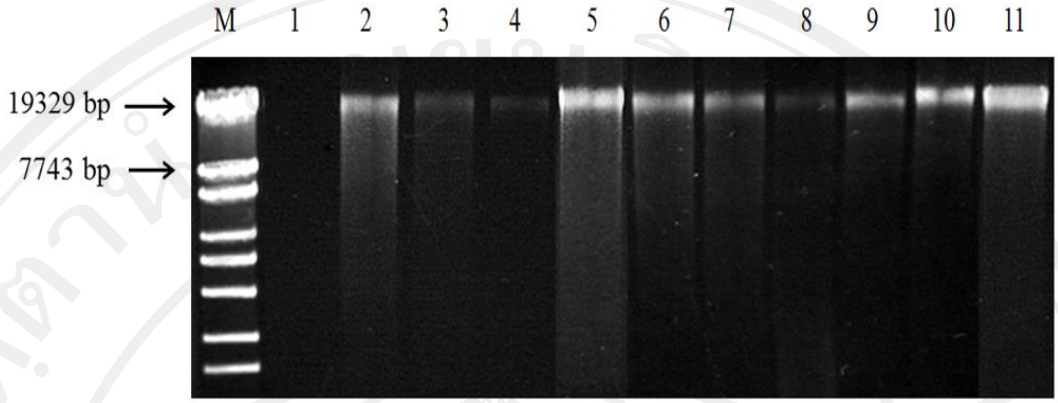

Figure 54 Inhibitory effect of crude medicinal plant extracts at non-toxic concentration on genomic DNA of HSV-1F synthesis in Vero cells after treatment in the presence of ethanolic extract of *Sphenodesme* sp. (1), aqueous extract of *E. prostrata* (2), aqueous extract of *Sphenodesme* sp. (3), ethanolic extract of *E. prostrata* (4), aqueous extract of *R. nasutus* (5), aqueous extract of *H. cordata* (6), ethanolic extract of *S. tuberosa* (7), ethanolic extract of *H. cordata* (8), ethanolic extract of *R. nasutus* (9), aqueous extract of *S. tuberosa* (10) and untreated viral control (11).

Table 21 Percentage of HSV-2G DNA remaining after treatment with plant extracts

Medicinal plant	Mean DNA remaining (% ± SD*)	
	Aqueous	Ethanolic
E. prostrata	33.69±4.6 [ab]	19.16±2.08 [ab]
H. cordata	54.48±4.56 [b]	28.39±0.85 [b]
R. nasutus	66.92±5.92 [c]	50.31±4.7 [c]
Sphenodesme sp.	23.19±5.11 [a]	6.21±2.60 [a]
S. tuberosa	75.73±3.1 [c]	46.66±9.73 [c]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

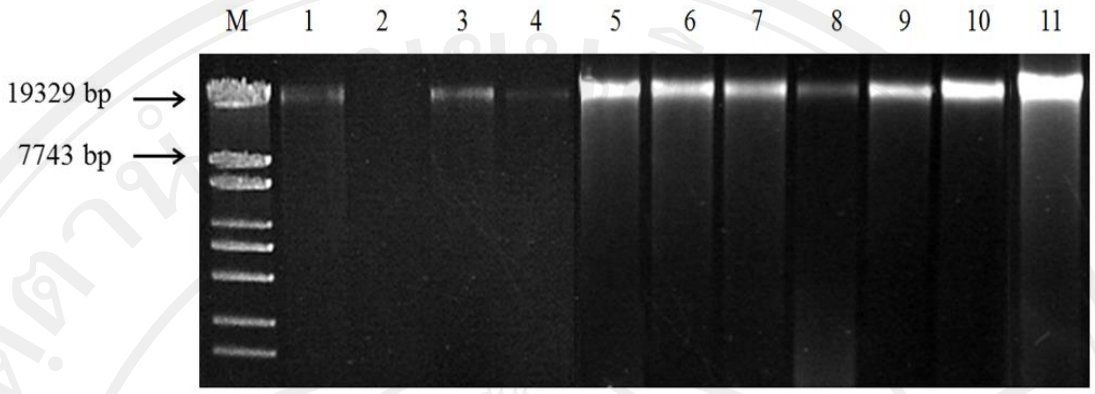

Figure 55 Inhibitory effect of crude medicinal plant extracts at non-toxic concentration on genomic DNA of HSV-2G synthesis in Vero cells after treatment in the presence of ethanolic extract of *Sphenodesme* sp. (1), aqueous extract of *E. prostrata* (2), aqueous extract of *Sphenodesme* sp. (3), ethanolic extract of *E. prostrata* (4), aqueous extract of *R. nasutus* (5), aqueous extract of *H. cordata* (6), ethanolic extract of *S. tuberosa* (7), ethanolic extract of *H. cordata* (8), ethanolic extract of *R. nasutus* (9), aqueous extract of *S. tuberosa* (10) and untreated viral control (11).

7.7 Effect of medicinal plant extracts on viral protein synthesis

Medicinal plant extracts at maximum non-toxic concentration has been examined for its efficacy on inhibition of HSV protein synthesis in the presence or absence of extracts. Effect of plant extracts on HSV protein expression were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The whole HSV proteins were electrically transferred to nitrocellulose membrane sheet and specified detection with goat anti-herpes virus type 1 and 2 conjugated with horseradish peroxidase enzyme using semi-dry blotting analysis. The level of viral protein remaining in the presence and absence of plant extracts were analyzed by Gene Tools Match (USA) to calculate percentage of inhibition of plant extracts on viral protein synthesis compared to untreated viral protein.

The result showed that medicinal plants extracts affected viral protein at various molecular weights as shown in Table 22-23 and Figure 56-60. Interestingly, both aqueous and ethanolic extract of *E. prostrata* and *Sphenodesme* sp. completely inhibited HSV-1F protein synthesis at molecular weight of 135 kDa compared to the protein of viral control and protein marker. Ethanolic extract of RNE also completely inhibited this 135 kDa HSV protein by 100%, while STA, HCE, HCA, RNA and STE were also inhibited HSV-1F protein synthesis at 135 kDa by 97.35±1.53, 97.34±2.02, 16.58±2.40, 10.5±6.31% and 6.40±5.39%, respectively. Interestingly, the results assumed that aqueous and ethanolic extract of *E. prostrata* and *Sphenodesme* sp. showed completely inhibition on approximately 75 kDa of HSV-1F protein synthesis. Moreover, RNE and STA also inhibited of HSV-1F protein at approximately 75 kDa by 100% followed by HCE, HCA, STE and RNA, which 75 kDa protein was reduced

by 96.50±1.14, 36.28±4.84, 27.76±2.60 and 3.62±6.27%, respectively. All of medicinal plant extracts were able to completely reduce HSV-1F protein at approximately 48 kDa except HCA that could reduce the protein by 29.60±3.13%. Furthermore, these medicinal plants extracts also reduced HSV-1F protein synthesis at approximately 45 kDa. The result showed that aqueous and ethanolic extracts of *E. prostrata* and *Sphenodesme* sp. revealed the same result that could inhibit the 45 kDa HSV protein by 100%. RNE also completely inhibited this 45 kDa HSV protein by 100%, while HCE, STA, STE, RNA and HCA could reduce protein synthesis at 45 kDa by 94.40±2.70, 70.56±8.17, 48.02±4.10, 48.00±2.57 and 34.16±1.41, respectively (Table 22 and Figure 56-60).

Moreover, approximately 75 kDa proteins of HSV-2G were also remarkably completely reduced after treatment with the aqueous and ethanolic extracts of *E. prostrata*, *S. tuberosa* and *Sphenodesme* sp. compared to untreated viral control. Additionally, HCE and RNE were reduced 75 kDa protein of HSV-2G by 100%, while HCA and RNA was reduced the 75 kDa protein by 22.21±8.68 and 14.35±4.68, respectively. Aqueous and ethanolic extracts of *E. prostrata* and *Sphenodesme* sp. completely reduced approximately 48 kDa protein as well as ethanolic extract of *H. cordata*, *R. nasutus* and *S. tuberosa* also inhibited the 48 kDa protein by 100%. Moreover, STA, RNA and HCA were reduced the 48 kDa protein by 43.93±4.87, 43.29±1.59 and 17.86±8.17%, respectively. Furthermore, inhibition effect of ethanolic extract of *E. prostrata* and *Sphenodesme* sp. could observe that HSV-2G protein at molecular weights of 45 kDa was completely inhibited by 100%. HSV-2G protein at approximately 45 kDa also reduced by 97.15±4.03, 94.43±9.64, 87.09±4.48, 75.64±2.83, 70.07±4.31, 43.71±2.33 and 38.44±2.78% after treatment

with SPA, EPA, HCE, STE, RNE, STA and RNA, respectively compared to infected viral control (Table 23 and Figure 56-60). Interestingly, after 22 hours post infection the infected cells were observed under an inverted microscope. It was found that, the infected cells were changed in shape, size and cell membrane. Moreover, the infected cells were fused to form giant cells when compared to uninfected cell control. Therefore, the important potential target of anti-HSV agent should be focused on the viral proteins that involved HSV replication (Greco *et al*., 2007; Whitley and Roizman, 2001).

From the result observed that medicinal plant extracts had potent activities to reduce HSV protein synthesis at approximately 135, 75 48 and 45 kDa. These protein were likely to be proteins generated form ICP8 (Lehtinen, 1986; Ruyechan, 1983), U_L6 (Patel *et al*., 1996), VP24 (Stevenson *et al*., 1997) and VP21 (Person *et al*., 1993), respectively. ICP8 is major single-stranded DNA-binding proteins required for viral DNA replication and essential for stimulates late gene transcription (Zhou and Knipe, 2002). Moreover, ICP8 also acts as DNA helicase/primase complex protein, which stimulates the primer synthesis and ATPase activity of helicase primase (Hamatake *et al*., 1997). Besides, U_L6 protein is important protein required for cleavage viral DNA before packaging into capsid (Higgs *et al*., 2008; White *et al*., 2003) and essential for replication of viral DNA and DNA maturation (Ogasawara *et al*., 2001; Patel *et al*., 1996). Furthermore, U_L26 act as protease to cleaved themselves to release minor scaffold proteins, VP24 and VP21 that are essential for capsid maturation (Sheaffer *et al*., 2000; Thomsen *et al*., 1995).

Table 22 The percentage of HSV-1F viral protein inhibition by aqueous and ethanolic extracts of medicinal plant extract compared to viral control

Medicinal plant extracts	Solvent	Inhibition (% ± SD*)			
		135 kDa	75 kDa	48 kDa	45 kDa
E. prostrata (EPA)	Aqueous	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]
E. prostrata (EPE)	Ethanolic	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]
H. cordata (HCA)	Aqueous	16.58±2.40 [a]	36.28±4.84 [a]	29.60±3.13 [a]	34.16±1.41 [a]
H. cordata (HCE)	Ethanolic	97.34±2.02 [b]	96.50±1.14 [b]	100±0.00 [b]	94.40±2.70 [b]
R. nasutus (RNA)	Aqueous	10.50±6.31 [a]	3.62±6.27 [a]	100±0.00 [a]	48.00±2.57 [a]
R. nasutus (RNE)	Ethanolic	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]
Sphenodesme sp. (SPA)	Aqueous	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]
Sphenodesme sp. (SPE)	Ethanolic	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]	100±0.00 [b]
S. tuberosa (STA)	Aqueous	97.35±1.53 [b]	100±0.00 [b]	100±0.00 [b]	70.56±8.17 [b]
S. tuberosa (STE)	Ethanolic	6.40±5.39 [a]	27.76±2.60 [a]	100±0.00 [a]	48.02±4.10 [a]

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

Table 23 The percentage of HSV-2G viral protein inhibition by aqueous and ethanolic extracts of medicinal plant extract compared to viral control

Plants	Solvent	Inhibition (% ± SD*)		
		75 kDa	48 kDa	45 kDa
E. prostrata (EPA)	Aqueous	100±0.00 ^{c}	100±0.00 ^{c}	94.43±9.64 ^{c}
E. prostrata (EPE)	Ethanolic	100±0.00 ^{c}	100±0.00 ^{c}	100±0.00 ^{c}
H. cordata (HCA)	Aqueous	22.21±8.68 ^{a}	17.86±8.17 ^{a}	14.95±6.54 ^{a}
H. cordata (HCE)	Ethanolic	100±0.00 ^{bc}	100±0.00 ^{bc}	87.09±4.48 ^{bc}
R. nasutus (RNA)	Aqueous	14.35±4.68 ^{a}	43.29±1.59 ^{a}	38.44±2.78 ^{a}
R. nasutus (RNE)	Ethanolic	100±0.00 ^{bc}	100±0.00 ^{bc}	70.07±4.31 ^{bc}
Sphenodesme sp. (SPA)	Aqueous	100±0.00 ^{c}	100±0.00 ^{c}	97.15±4.03 ^{c}
Sphenodesme sp. (SPE)	Ethanolic	100±0.00 ^{c}	100±0.00 ^{c}	100±0.00 ^{c}
S. tuberosa (STA)	Aqueous	100±0.00 ^{b}	43.93±4.87 ^{b}	43.71±2.33 ^{b}
S. tuberosa (STE)	Ethanolic	100±0.00 ^{bc}	100±0.00 ^{bc}	75.64±2.83 ^{bc}

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

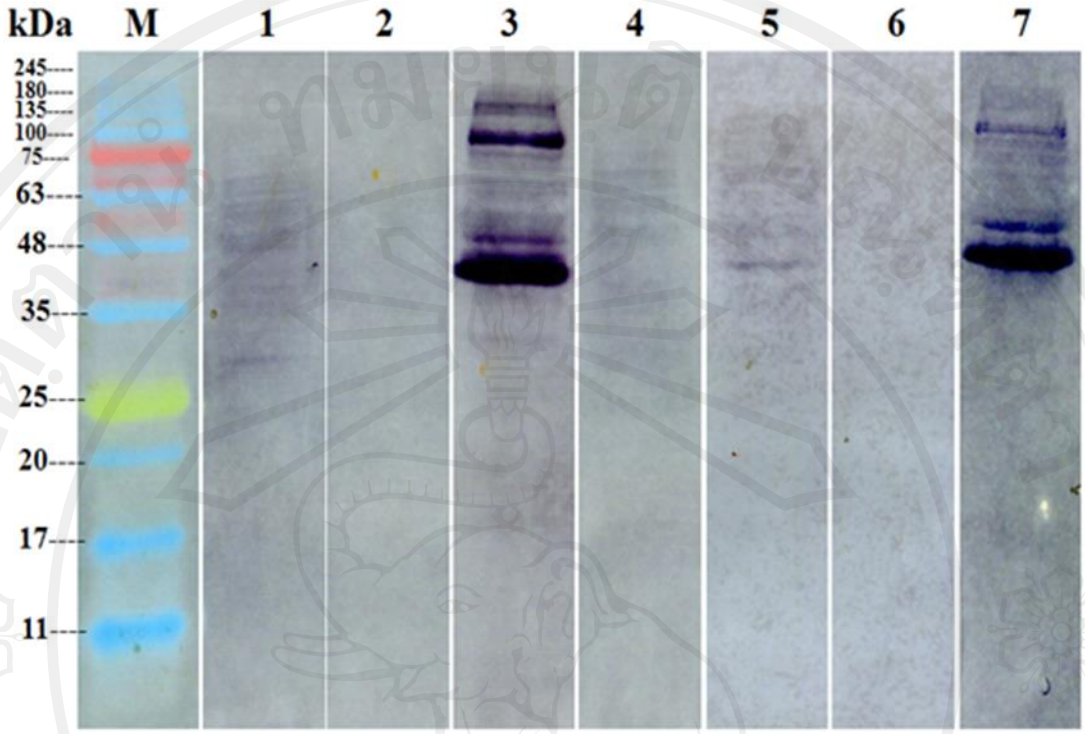

Figure 56 Effect of *E. prostrata* extracts on HSV-1F protein synthesis in Vero cells detected by Western blotting analysis in the present of aqueous extract (lane 1), ethanolic extract (lane 2), absence of extract (lane 3), uninfected Vero cells (lane 4) and HSV-2G protein synthesis in the presence of aqueous extract (lane 5), ethanolic extract (lane 6) and the absence of extract (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV.

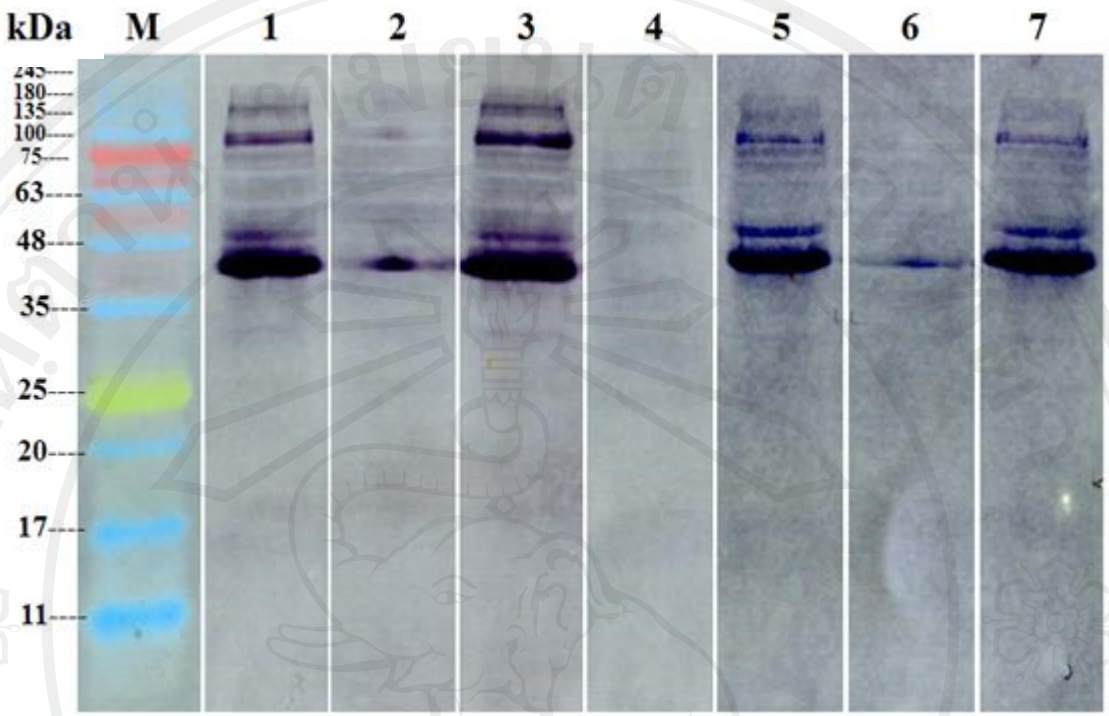

Figure 57 Effect of *H. cordata* extracts on HSV-1F protein synthesis in Vero cells detected by Western blotting analysis in the present of aqueous extract (lane 1), ethanolic extract (lane 2), absence of extract (lane 3), uninfected Vero cells (lane 4) and HSV-2G protein synthesis in the presence of aqueous extract (lane 5), ethanolic extract (lane 6) and the absence of extract (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV.

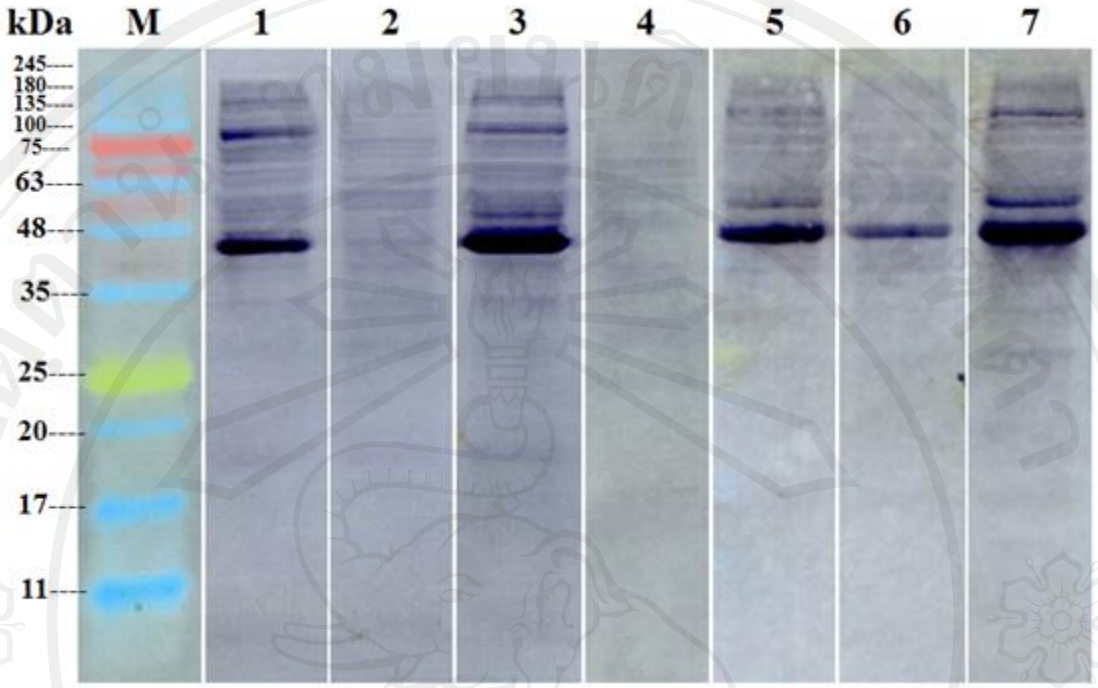

Figure 58 Effect of *R. nasutus* extracts on HSV-1F protein synthesis in Vero cells detected by Western blotting analysis in the present of aqueous extract (lane 1), ethanolic extract (lane 2), absence of extract (lane 3), uninfected Vero cells (lane 4) and HSV-2G protein synthesis in the presence of aqueous extract (lane 5), ethanolic extract (lane 6) and the absence of extract (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV.

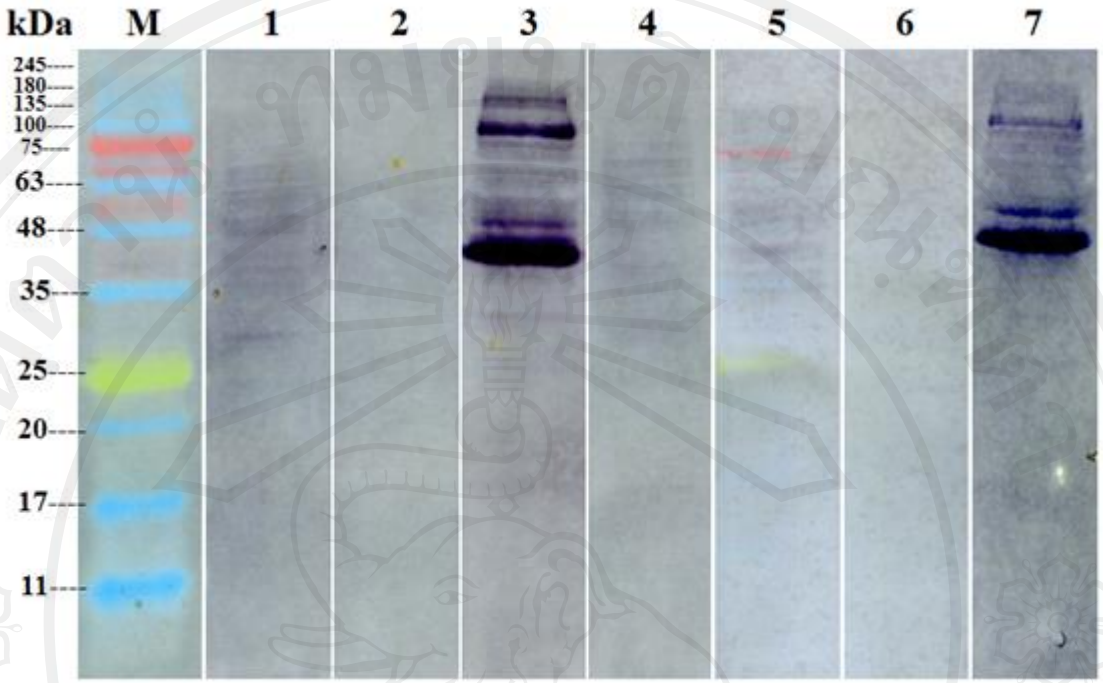

Figure 59 Effect of *Sphenodesme* sp. extracts on HSV-1F protein synthesis in Vero cells detected by Western blotting analysis in the present of aqueous extract (lane 1), ethanolic extract (lane 2), absence of extract (lane 3), uninfected Vero cells (lane 4) and HSV-2G protein synthesis in the presence of aqueous extract (lane 5), ethanolic extract (lane 6) and the absence of extract (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV.

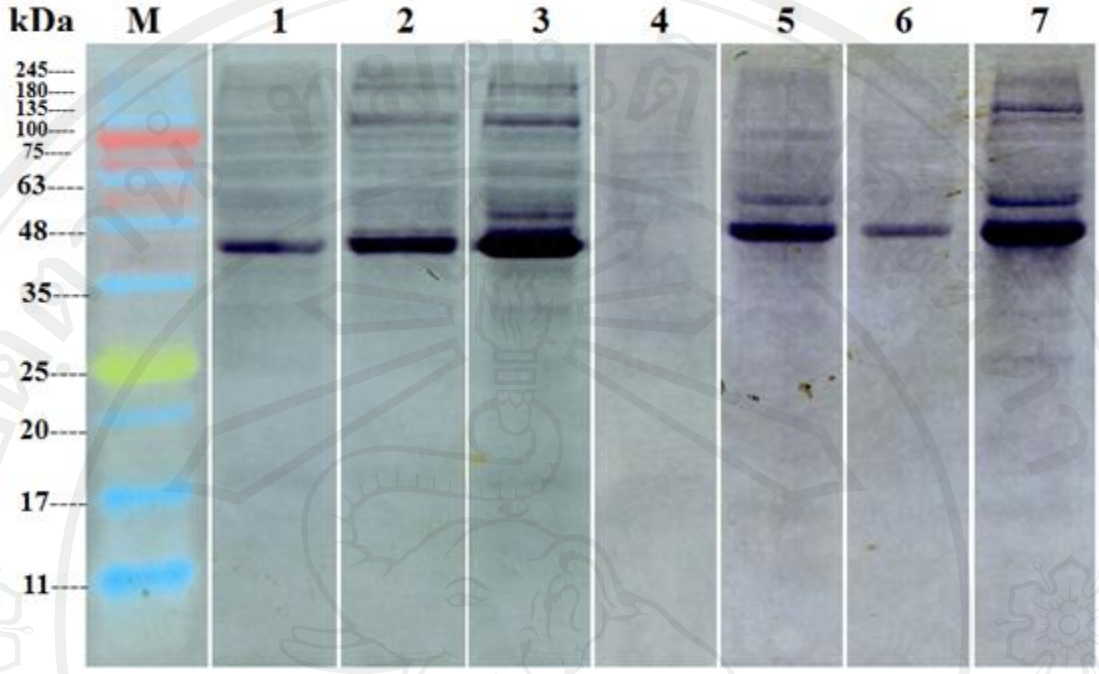

Figure 60 Effect of *S. tuberosa* extracts on HSV-1F protein synthesis in Vero cells detected by Western blotting analysis in the present of aqueous extract (lane 1), ethanolic extract (lane 2), absence of extract (lane 3), uninfected Vero cells (lane 4) and HSV-2G protein synthesis in the presence of aqueous extract (lane 5), ethanolic extract (lane 6) and the absence of extract (lane 7) after detection by horseradish peroxidase-conjugated IgG against HSV.

8. Separation and partial purification of medicinal plant extracts

Extraction methodology is procedure that used to separate the medicinally active compound in plant materials by selective solvents in standard extraction method. In addition, extraction process should be efficient, simple, rapid and inexpensive.

The potential inhibitory effects of medicinal plant extracts against HSV-1F and HSV-2G infection were investigated to clarify their antiviral activity. The ethanolic extracts of *E. prostrata* and *Sphenodesme* sp. showed anti-HSV activity better than *H. cordata, R. nasutus* and *S. tuberosa* extracts. Hence, ethanolic extracts of *E. prostrata* and *Sphenodesme* sp. were selected to be further fractionated to determine their composition of bioactive compounds that affected HSV infection by plaque reduction assay. Phytochemical groups were also evaluated by phytochemical screening. Furthermore, the fraction that showed the highest anti-HSV activity was analyzed to determine the extract pattern on TLC and selected the similar fraction to further isolated by column chromatography. The anti-HSV efficacy of isolated fraction against HSV infection was compared with the crude extracts.

Dried powder of *E. prostrata* and *Sphenodesme* sp. was extracted by ethanol, 95% with the ratio of plant powder and solvent of 1:10. The plant powder was macerated twice at room temperature for three days before filtration and evaporation under reduced pressure to obtain crude ethanolic extract. Yields of crude ethanolic extract of *E. prostrata* and *Sphenodesme* sp. were 147.0 and 186.0 g, which showed percentage yield of 2.94 and 4.13%, respectively.

8.1 Separation of *E. prostrata*

8.1.1 Separation of *E. prostrata* extracts by partition technique

Crude ethanolic extract of *E. protrata* was selected to separate by partition technique and eluted with four different solvent to separate the constituent of plant extracts bases on their polarity from low to high polarity to give four partial fractions of hexane, ethyl acetate, n-butanol and aqueous fraction (Figure 61). Moreover, all of partition fractions were evaporated to eliminate the solvents and each fraction was weight, and percentage yield recovered was calculated. Then, the dried fraction was reconstituted by DMSO before investigation for their toxicity and anti-HSV activity.

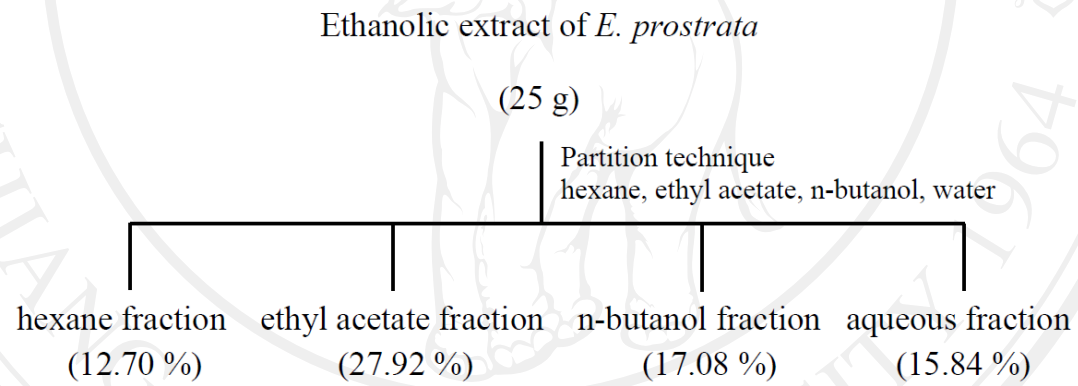

Figure 61 Separation of *E. prostrata* extract by partition technique

The result revealed that percentage yield of *E. prostrata* recovered by partition method ranked from 12.70-27.92%. Ethyl acetate fraction of *E. prostrata* gave the highest percentage yield followed by n-butanol, aqueous and hexane fraction by 27.92, 17.08, 15.84 and 12.70%, respectively (Table 24).

In addition, the partition fractions of *E. prostrata* that received from various solvent exerted various color appearance of yellow-brown and deep brown (Table 24).

Table 24 Percentage yield of fractions of *E. protrata* by partition technique

Plant	Solvent	Yield (w/w, %)	Appearance
E. prostrata	Hexane	12.70	deep brown
	ethyl acetate	27.92	deep brown
	n-butanol	17.08	yellow-brown
	Aqueous	15.84	yellow-brown

8.1.2 Cytotoxicity of partial purified fraction isolated from ethanolic extract of *E. prostrata* on Vero cell

Cell cytotoxicity of *E. prostrata* fraction was determined on Vero cell. The CD_{50} was calculated according to modified protocol of Reed and Muench (1938). Partition fractions of *E. prostata* extract showed the toxicity with CD_{50} value of 447.74±0.00 µg/ml (Table 25). On basis of these obtained results on cell toxicity assay, antiviral activity of plant fractions against HSV infection was performed by plaque reduction assay using the highest non-toxic concentration of the fractions (Table 25). The difference in toxicity of each fraction on Vero cell might result from the different compound in each fraction that obtained after extraction by various types of solvent (Samarth *et al*., 2008).

Table 25 The cytotoxicity dose, 50% (CD_{50}) and the highest of non-toxic concentrations of partial purified fraction isolated from ethanolic extract of *E. prostrata*

Plants	Fractions	CD_{50} (µg/ml)*	The highest non-toxic concentrations (µg/ml)
E. prostrata	hexane	447.74±0.00	156.25
	ethyl acetate	447.74±0.00	156.25
	n-butanol	447.74±0.00	78.13
	aqueous	447.74±0.00	156.25

Note: Cell viability was observed and CD_{50} value was calculated (Reed and Muench, 1938)

* The data in table are presented as mean ± standard deviation (SD) of duplicate independent experiments.

8.1.3 Antiviral activity of partial purified fractions isolated from *E. prostrata* by plaque reduction assay

Antiviral activity of partition fractions of *E. prostata*, which prepared from four solvents; hexane, ethyl acetate, n-butanol and water was investigated. The fractions were subsequently subjected to confirm their efficacy against HSV-1F and HSV-2G infection by plaque reduction assay. It was demonstrated that hexane fraction of *E. prostrata* extract had inhibitory effect against HSV-1F infection more than aqueous extract with ED_{50} values of 65.69±6.22 and 141.52±0.77 µg/ml, respectively and TI values were 6.85±0.65 and 3.16±0.02, respectively. Other

fractions did not affect viral infection. Moreover, n-butanol, ethyl acetate, hexane and aqueous fractions exerted ED_{50} values of 63.66±4.38, 68.97±0.20, 139.95±0.49 and 150.47±2.89 µg/ml, respectively and TI values of 7.05±0.49, 6.49±0.02, 3.20±0.01 and 2.98±0.06, respectively when treatment after HSV-2G attachment to cell culture (Table 26). The higher TI value reflected the higher therapeutic potential of the extracts.

This fraction might have the highest amount of bioactive compounds that could inhibit HSV infection. Furthermore, anti-HSV activity by these fractions might be resulted from different types of extracts with had diversity of structures or chemical constituents. Moreover, extraction procedures that used various types of solvents from non-polar to polar solvent might affect HSV infection (Das *et al*., 2010b).

Table 26 Inhibition of HSV by partial purified fractions of *E. prostrata* extract

Viruses	Fractions	Inhibition (% ± SD*)	ED ± SD (µg/ml)*	TI ± SD*
HSV-1F	hexane	66.36±1.29 [a]	65.69±6.22 [ab]	6.85±0.65 [a]
	ethyl acetate	40.34±0.81 [a]	0 [a]	0 [a]
	n-butanol	37.88±0.71 [a]	0 [a]	0 [a]
	aqueous	51.89±6.67 [a]	141.52±0.77 [c]	3.16±0.02 [a]
HSV-2G	hexane	54.40±1.99 [a]	139.95±0.49 [ab]	3.20±0.01 [a]
	ethyl acetate	69.08±2.79 [a]	68.97±0.20 [a]	6.49±0.02 [a]
	n-butanol	55.62±1.45 [a]	63.66±4.38 [a]	7.05±0.49 [a]
	aqueous	50.81±7.89 [a]	150.47±2.89 [c]	2.98±0.06 [a]

$TI = CD_{50}/ED_{50}$

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

8.1.4 Column chromatography of *E. prostrata*

Previous investigation on anti-HSV infection activity of fractions that isolated from *E. prostrata* showed that hexane fraction of *E. prostata* had highest inhibition of both types of HSV infection. Thus, *E. prostrata* was selected to further study their active compound against HSV infection by column chromatography using celite as stationary phase and eluted with suitable solvent; hexane, hexane:ethyl acetate, ethyl acetate and 95% ethanol, consecutively. Each sub-fraction was collected at volume of 25 ml and similar patterns of sub fraction were combined together in accordance with the information on the basis of their TLC pattern after visualization with ultraviolet light at 254 and 366 nm. After that, the solvent of sub-fraction was removed by evaporation and reconstituted by DMSO as a stock concentration.

The obtained results showed that after crude ethanolic extract of *E. prostata* was separated by various solvent and column chromatography using celite as stationary phase, twelve major fractions, K101-K112 fractions were obtained. These fractions showed the different percentage yield ranging from 0.4913-42.4237 (Table 27). Percentage yield of K111 fraction was recovered the most by 42.4247%, while the lowest percentage yield was obtained from K102 by 0.4913%.

Table 27 Percentage yield of each fraction isolated from *E. prostrata* by column chromatography using celite as stationary phase

Fractions	Yield (w/w, %)
K101	23.9454
K102	0.4913
K103	0.6273
K104	2.7048
K105	2.0850
K106	0.4978
K107	0.8487
K108	6.4044
K109	0.9974
K110	0.6434
K111	42.4237
K112	7.0649

8.1.5 TLC screening of *E. prostrata* after separation by column chromatography

Thin layer chromatography (TLC) is the chromatography technique that widely used to identify separated components. The technique is the simplest and cheapest to detect natural product compound on the basis of retention factor (Rf) values and color of spots. All fractions were analyzed on TLC pattern after the small spot of sample was applied on the plat, and then dipped in a solvent or solvent mixture system as mobile phase. Different compounds in the sample mixture moved at different rates. Visualization of chromatogram under UV light at 254 nm for conjugated double bond while 366 nm shows orange-yellow bands for flavonoids and blue fluorescent bands for phenolic acids (Males and Medic-Saric, 2001).

After fractionation of hexane fraction by column chromatography, 12 major fractions were obtained when the fractions that had the same TLC profile were combined. The TLC pattern of these fractions was further observed their pattern compared to hexane fraction, which showed highest anti-HSV activity.

TLC studies of these fractions showed the best separation using hexane: ethyl acetate (90:10) as a mobile phase. It was found that chemical patterns on TLC plate of K101, K102, K103 and K104 fractions were similar to hexane fraction in terms of characteristics pattern of Rf value, color appearance and number of spots when exposed under UV light at 254 and 366 nm (Figure 62). Hence, four fractions; K101-104 were selected to further evaluated their cytotoxicity and efficacy against HSV infection by plaque reduction assay.

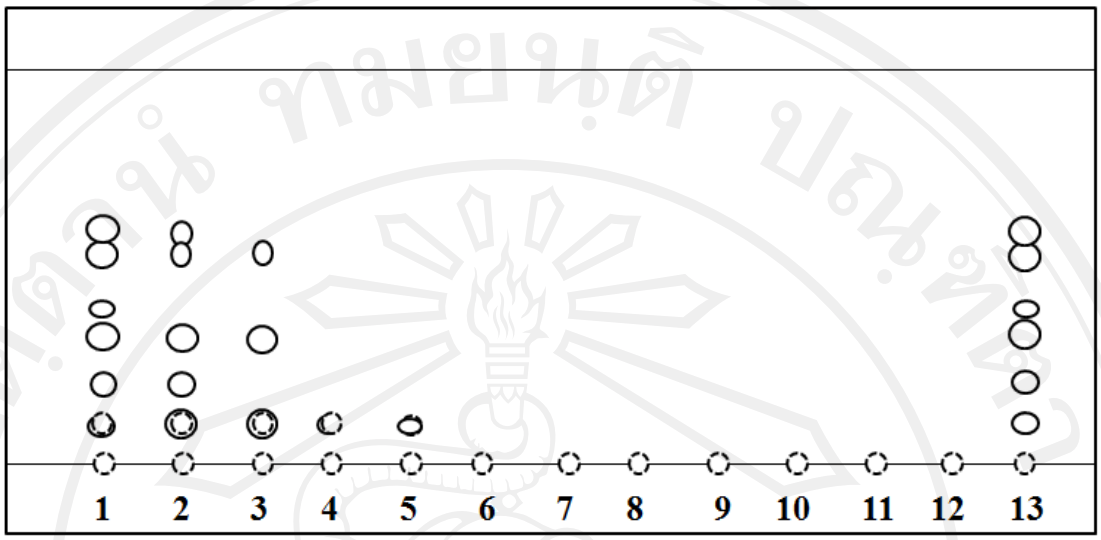

Figure 62 Chromatogram of the partial purified fractions of *E. prostrata* by TLC analysis using hexane: ethyl acetate (90:10) as mobile phase when detected under wavelength at 254 and 366 nm; K101 (1), K102 (2), K103 (3), K104 (4), K105 (5), K106 (6), K107 (7), K108 (8), K109 (9), K110 (10), K111 (11), K112 (12) and hexane fraction (13).

8.1.6 Cytotoxicity of *E. prostrata* fractions isolated from column chromatography on Vero cell

This study was carried out to determine the efficacy of fractions separated from *E. prostata* extract by column chromatography using celite as stationary phase. Four reconstituted fractions, K101-K104 fractions were selected to investigate their cytotoxicity on Vero cell. The concentration that did not affect viability of cell culture was evaluated and expressed as CD_{50} before investigating of antiviral activity by plaque reduction assay. The concentrations that were lower than CD_{50} values were used to determine the efficacy of these fractions against HSV infection.

After reconstitution of *E. prostrata* fractions by DMSO, the deep green color was shown. Cytotoxicity of several fractions isolated from *E. prostrata* extract was examined and exhibited cytotoxicity by dose dependent manner (Table 28). The result showed that, four fractions had the toxicity on Vero cells with CD_{50} values ranging from 63.40-199.54 µg/ml. Besides, the highest toxicity of four fractions isolated from *E. prostrata* was shown from K103 and K104 fractions followed by K102 and K101. High CD_{50} value reflected low toxicity of plant fractions on Vero cell (Table 28). In addition, non-toxic concentrations at 40-80 µg/ml of each fraction were used to further investigate antiviral activity against HSV infection by plaque reduction assay (Table 28).

Table 28 The cytotoxicity dose, 50% (CD_{50}) and non-toxic concentrations of *E. prostrata* fractions

Fractions	**CD_{50} (µg/ml)***	**The highest non-toxic concentrations (µg/ml)**
K101	199.54±0.00	80
K102	100.01±21.14	40
K103	63.40±0.00	40
K104	63.40±0.00	40

* The data in table are given as mean ± standard deviation (SD) of duplicate experiments.

8.1.7 Antiviral activity by *E. prostrata* fraction isolated from column chromatography

Antiviral activity against HSV infection by non-toxic concentration of four fractions; K101, K102, K103 and K104 isolated from *E. prostrata* by column chromatography was investigated by plaque reduction assay.

The result showed that HSV-1F was sensitive to K102 fraction with ED_{50} value of 35.32±0.30 µg/ml and TI value of 2.83±0.02 followed by K103 fraction with ED_{50} value of 35.36±1.26 µg/ml and TI value of 1.79±0.06. K101 and K104 were weakly effective against HSV-1F infection, which could inhibit HSV infection less than 50%. On the other hand, HSV-2G infected cell were treated with four fractions after viral attachment, only K101 fraction was able to inhibit HSV-2G with ED_{50} value of 76.13±0.65 µg/ml and TI value of 2.62±0.02 (Table 29).

From our result showed that both types of HSV-1F and HSV-2G were affected differently by four fractions of *E. prostrata* extracts. It was suggested that the main compound in each fraction may affect the inhibitory effect against HSV infection. However, the efficacy against HSV infection from these fractions was decreased when compared to their crude ethanolic extract. These may be resulted from various beneficial bioactive compounds in crude extract that had the synergistic action between each compound that had action against HSV infection greater than their individual effects (Yucharoen *et al*., 2012).

The result also confirmed that all phytochemical compounds in this crude plant extract had the effective antiviral agent better than pure compound alone as shown by ED_{50} and TI values. Thus, further fractionation of fraction was not performed.

Table 29 Antiviral activity of fractions of *E. prostrata* extract

Viruses	Fractions	Inhibition (% ± SD*)	ED±SD (µg/ml)*	TI±SD*
HSV-1F	K101	47.80±4.86 [a]	0 [a]	0 [a]
	K102	64.66±0.40 [a]	35.32±0.30 [a]	2.83±0.02 [a]
	K103	56.55±1.83 [a]	35.36±1.26 [a]	1.79±0.06 [a]
	K104	30.53±1.49 [a]	0 [a]	0 [a]
HSV-2G	K101	52.62±1.95 [a]	76.13±0.65 [a]	2.62±0.02 [a]
	K102	44.74±1.99 [a]	0 [a]	0 [a]
	K103	22.78±3.03 [a]	0 [a]	0 [a]
	K104	5.42±0.59 [a]	0 [a]	0 [a]

TI = CD_{50}/ED_{50}

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

8.2 Separation of *Sphenodesme* sp.

8.2.1 Separation of *Sphenodesme* sp. extracts by partition technique

Crude ethanolic extract of *Sphenodesme* sp. was separated by partition technique to give four partial fractions of hexane, ethyl acetate, n-butanol and aqueous fraction (Figure 63).

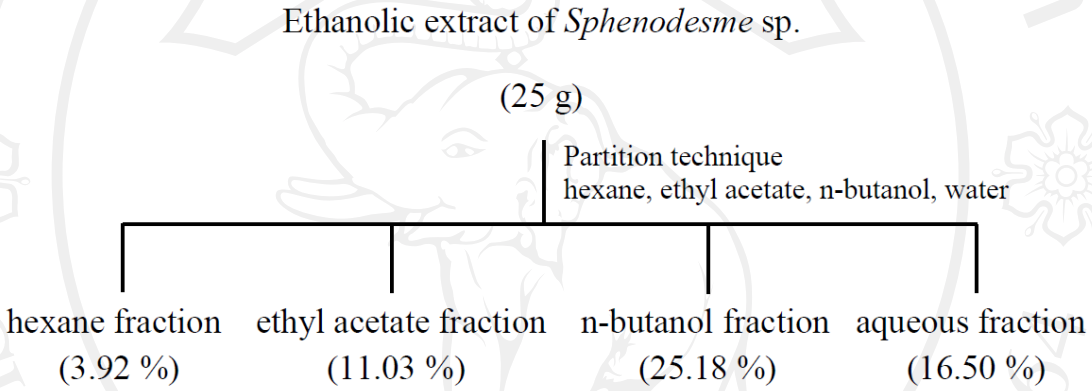

Figure 63 Separation of *Sphenodesme* sp. extract by partition technique

The result revealed that, percentage yield recovered from *Sphenodesme* sp. after partition ranged from 3.92-25.18%. It was shown that n-butanol, aqueous, ethyl acetate and hexane fraction separated from *Sphenodesme* sp. extract showed percentage yield by 25.18, 16.50, 11.03 and 3.92%, respectively (Table 30). The partition fractions of *Sphenodesme* sp. that received from various solvent exerted various color appearance of yellow-brown and deep brown (Table 30).

Table 30 Percentage yield of fractions of *Sphenodesme* sp. by partition technique

Plant	Solvent	Yield (w/w, %)	Appearance
Sphenodesme sp.	Hexane	3.92	yellow-brown
	ethyl acetate	11.03	deep brown
	n-butanol	25.18	deep brown
	Aqueous	16.50	deep brown

8.2.2 Cytotoxicity of partial purified fraction isolated from ethanolic extract of *Sphenodesme* sp. on Vero cell

Cell cytotoxicity of *Sphenodesme* sp. fractions was determined on Vero cell. Partition fractions of *Sphenodesme* sp. extracts showed the toxicity ranged from 447.74-1,782.50 µg/ml. The result showed that aqueous fraction of *Sphenodesme* sp. presented the lowest toxicity on Vero cell with CD_{50} value of 1,782.50±0.00 µg/ml whereas, hexane, ethyl acetate and n-butanol fractions showed CD_{50} value of 447.74±0.00 µg/ml (Table 31).

Table 31 The cytotoxicity dose, 50% (CD_{50}) and the highest of non-toxic concentrations of partial purified fraction isolated from ethanolic extract of *Sphenodesme* sp.

Plant	Fractions	CD_{50}(µg/ml)*	The highest non-toxic concentrations (µg/ml)
Sphenodesme sp.	hexane	447.74±0.00	78.13
	ethyl acetate	447.74±0.00	156.25
	n-butanol	447.74±0.00	156.25
	aqueous	1782.50±0.00	1,250.00

* The data in table are given as mean ± standard deviation (SD) of duplicate experiments.

8.2.3 Antiviral activity of partial purified fractions isolated from *Sphenodesme* sp. by plaque reduction assay

Four fractions isolated from ethanolic extract of *Sphenodesme* sp.; hexane, ethyl acetate, n-butanol and aqueous were subsequently subjected to test their efficacy against HSV-1F and HSV-2G infection by plaque reduction assay. The result showed that aqueous fraction isolated from *Sphenodesme* sp. gave the highest inhibitory effect on HSV-1F infection compared to other fractions with ED_{50} values of 189.43±19.35 µg/ml and TI value of 9.46±0.97, while ethyl acetate fraction had ED_{50} value of 64.49±4.55 µg/ml and TI value of 6.96±0.49. However, hexane and n-butanol could not protect Vero cell on HSV-1F infection. Interestingly, HSV-2G was inhibited by n-butanol, aqueous, ethyl acetate fraction with ED_{50} values of 41.02±8.60, 92.05±1.87

and 217.26±1.45 μg/ml and TI values of 11.16±2.34, 8.21±0.06 and 4.87±0.10, respectively (Table 32). The result could suggest that each partition fraction had the different anti-HSV activity. Aqueous fraction isolated from *Sphenodesme* sp. extract revealed strongly inhibition on both types of HSV by 100%.

Table 32 Inhibition of HSV by partial purification of *Sphenodesme* sp. extract

Viruses	Fractions	Inhibition (% ± SD*)	ED±SD* (μg/ml)	TI±SD
HSV-1F	hexane	47.54±3.49 [a]	0 [a]	0
	ethyl acetate	89.40±0.71 [ab]	64.49±4.55 [b]	6.96±0.49
	n-butanol	49.39±4.31 [ab]	0 [a]	0
	aqueous	100±0.00 [c]	189.43±19.35 [c]	9.46±0.97
HSV-2G	hexane	44.35±10.26 [a]	0 [a]	0
	ethyl acetate	82.59±1.67 [ab]	92.05±1.87 [b]	4.87±0.10
	n-butanol	74.26±5.37 [ab]	41.02±8.60 [a]	11.16±2.34
	aqueous	100±0.00 [c]	217.26±1.45 [c]	8.21±0.06

TI = CD_{50}/ED_{50}

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

8.2.4 Isolation of bioactive fraction from ethanolic extract of *Sphenodesme* sp. by column chromatography

Effect on viral infection of the bioactive fraction isolated from ethanolic extract of *Sphenodesme* sp. by column chromatography was performed in this study. Ethanolic extract of *Sphenodesme* sp. was separated into four fractions; hexane, ethyl acetate, n-butanol and aqueous fractions. These fractions were tested on anti-HSV activity by plaque reduction assay and showed that aqueous fraction had strongest anti-HSV type 1 and type 2 activities. Thus, active compound against HSV infection may be soluble in high polar solvent, so the high polar solvent system was used.

Active ingredients of ethanolic extract of *Sphenodesme* sp. were separated on column chromatography. Then, various solvent as mobile phase from low to high polar was subsequently added to elute the mixture by starting with hexane:ethyl acetate, ethyl acetate, ethyl acetate:ethanol and 95% ethanol, consecutively. 25 ml of each fraction was collected and fraction that had similar Rf patterns was combined together after exanimated by thin layer chromatography under ultraviolet light at 254 and 366 nm, and anisaldehyde sulfuric acid spraying reagent. Then, the solvent was removed by evaporated.

The results showed that after the ethanolic extract of *Sphenodesme* sp. was eluted with various solvent by a column chromatography using celite as stationary phase. Ten major fractions, P101, P102, P103, P104, P105, P106, P107, P108, P109 and P110 were collected. Each fraction showed the different percentage yield recovered, which ranged from 0.09-18.04% after extraction. Percentage yields of P106 fractions could recover the most with 18.04% while the lowest percentage yield

recovered was P105 with 0.09% (Table 33). Weight and percentage yield recovered of fractions isolated from *Sphenodesme* sp. was represented in Table 33.

Table 33 Percentage yield recovered of each fraction isolated from *Sphenodesme* sp. extracts by column chromatography

Fractions	Yield (w/w, %)
P101	4.46
P102	0.85
P103	0.42
P104	0.60
P105	0.09
P106	18.04
P107	17.20
P108	5.11
P109	3.53
P110	1.14

8.2.5 TLC screening of partition technique

Ten major fractions were further visualized under UV light compared with aqueous fraction from partition method that showed the highest efficacy against both types of HSV infection. The result showed that TLC studies of *Sphenodesme* sp. fractions showed the best separation using by $CHCl_3$: MeOH (80:20) as mobile phase. Chromatogram of the obtained fraction was shown in Figure 64. Hence, the fractions; P101, P104, P105, P106, P107, P108, P109 and P110 were selected based on their presented of chromatogram, which were similar to aqueous fraction from partition technique to further study on anti-HSV activity against HSV infection.

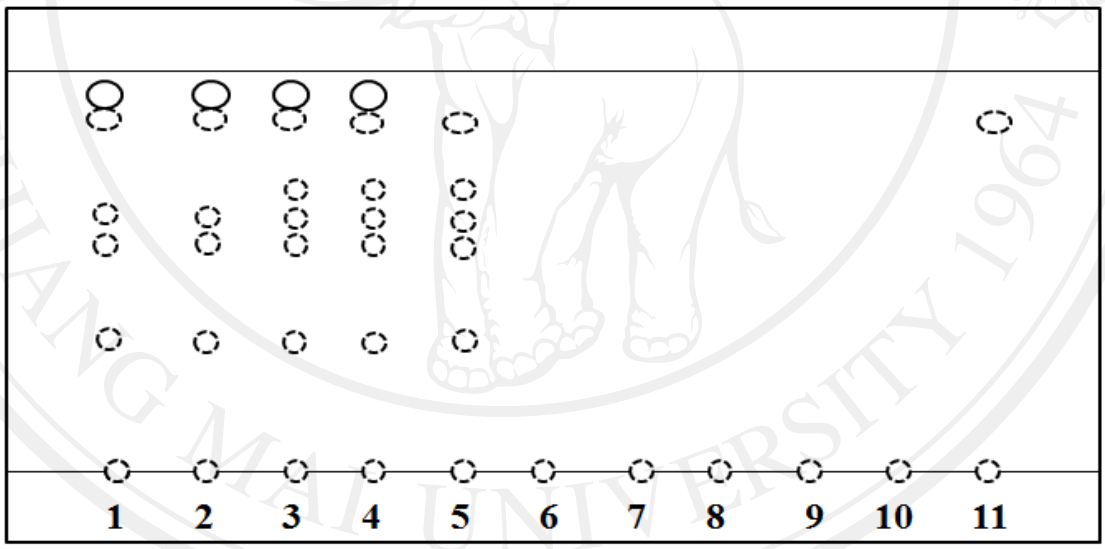

Figure 64 Chromatogram of the partial purified fractions of ethanolic extract of *Sphenodesme* sp. by TLC analysis using chloroform : methanol (80:20) as mobile phase and detected under short wavelength (254 nm) and long wavelength (366 nm) UV light; P101 (1), P102 (2), P103 (3), P104 (4), P105 (5), P106 (6), P107 (7), P108 (8), P109 (9), P110 (10) and ethyl acetate fraction (11).

8.2.6 Cytotoxicity of *Sphenodesme* sp. fractions isolated from column chromatography on Vero cell by cell cytotoxicity

Cytotoxicity of each fraction was evaluated on Vero cell. The result showed that, CD_{50} of fractions on Vero cell were ranged from 44.77-224.40 μg/ml. The color of ten fractions after reconstituted with DMSO showed the different colors; yellow, brown mixed with yellow and deep brown (Table 34).

Interestingly, P109 fraction showed the highest cytotoxicity on cell culture with CD_{50} value of 44.77±3.22 μg/ml followed by P106, which showed CD_{50} value of 50.35±3.22 μg/ml. Moreover, P101 and P104 fractions showed the same CD_{50} values of 63.36±0.00 μg/ml, while P110 and P108 fractions had CD_{50} values of 126.48±0.00 and 200.46±13.82 μg/ml, respectively. Additionally, P105 and P107 fractions showed the same CD_{50} values of 224.40±0.00 μg/ml (Table 34). Fractions that showed the highest cytotoxicity on Vero cell was P109 while P105 and P107 fractions demonstrated the lowest toxicity, respectively based on their CD_{50} value. Therefore, non-toxic concentrations of these fractions on Vero cell, which used for evaluation of anti-HSV activity were ranged from 40-160 μg/ml.

Table 34 The cytotoxicity dose, 50% (CD_{50}), non-toxic concentrations and color of *Sphenodesme* sp. fractions

Fractions	CD_{50} (µg/ml)*	The highest non-toxic concentrations (µg/ml)	Color of extracts
P101	63.36±0.00	40	yellow
P104	63.36±7.51	40	yellow-brown
P105	224.40±0.00	160	brown
P106	50.35±3.22	40	deep brown
P107	224.40±0.00	160	deep brown
P108	200.46±13.82	120	deep brown
P109	44.77±3.22	40	deep brown
P110	126.48±0.00	80	deep brown

* The data in table are given as mean ± standard deviation (SD) of duplicate experiments.

8.2.7 Antiviral activity by *Sphenodesme* sp. fractions isolated from column chromatography

In order to evaluate anti-HSV activity, these *Sphenodesme* sp. fractions were assayed using the highest non-toxic concentration which was not produced any morphological change in cell. In this present study, efficacy against HSV infection by P101, P104, P105, P106, P107, P108, P109 and P110 fractions were determined by plaque reduction assay.

The result showed that P107, P108, P110, P106, P101 and P104 showed the percentage of HSV-1F inhibition more than 50%, with ED_{50} values of 38.54±0.19, 59.08±1.72, 53.61±0.40, 29.82±2.19, 37.74±1.58 and 38.45±0.25, μg/ml, respectively and TI values of 5.82±0.03, 3.4±0.10, 2.36±0.02, 1.69±0.12, 1.65±0.12 and 1.65±0.01, respectively (Table 35). In contrast, P105 and P109 fractions had the weak inhibitory potential on HSV-1F infection by less than 50%.

Interestingly, it was found that P107, P106, P108, P110 and P109 fractions exhibited inhibitory effect on HSV-2G plaque formation with ED_{50} values of 58.38±0.15, 14.81±1.49, 69.17±0.47, 50.41±3.40 and 28.20±0.76 μg/ml and TI values of 3.84±0.01, 3.42±0.35, 2.90±0.02, 2.52±0.17 and 1.59±0.04, respectively when treatment after viral attachment to cell culture (Table 35). However, P101, P104 and P105 fractions showed little effect on HSV-2G infection, which could inhibit viral infection less than 50% when treatment after viral attachment.

Interestingly, among selected eight fractions isolated from *Sphenodesme* sp. by column chromatography, P107 displayed strongest activity against HSV-1F and HSV-2G infection as observed by highest TI value. Moreover, P107 fraction also displayed less cytotoxicity compared with another fraction.

Hence, the P107 fraction was further purified on column chromatography using Sephadex as stationary phase to investigate phytochemical constituents and bioactive compounds.

Table 35 Inhibition of HSV infection by *Sphenodesme* sp. fraction isolated from column chromatography using celite as stationary phase

Viruses	Fractions	% inhibition ± SD*	ED±SD (µg/ml)*	TI±SD*
HSV-1F	P101	51.27±2.55 ^{ab}	37.74±1.58 ^{a}	1.65±0.12 ^{ab}
	P104	53.61±2.16 ^{ab}	38.45±0.25 ^{a}	1.65±0.01 ^{ab}
	P105	26.39±4.22 ^{a}	0 ^{a}	0 ^{a}
	P106	60.87±1.53 ^{bc}	29.82±2.19 ^{a}	1.69±0.12 ^{ab}
	P107	100±0.00 ^{c}	38.54±0.19 ^{a}	5.82±0.03 ^{b}
	P108	82.70±0.90 ^{bc}	59.08±1.72 ^{a}	3.40±0.10 ^{ab}
	P109	33.58±2.26 ^{ab}	0 ^{a}	0 ^{ab}
	P110	75.00±2.89 ^{bc}	53.61±0.40 ^{a}	2.36±0.02 ^{ab}
HSV-2G	P101	43.86±2.35 ^{ab}	0 ^{a}	0 ^{ab}
	P104	38.22±0.57 ^{ab}	0 ^{a}	0 ^{ab}
	P105	24.62±1.72 ^{A}	0 ^{a}	0 ^{a}
	P106	86.32±0.52 ^{bc}	14.81±1.49 ^{a}	3.42±0.35 ^{ab}
	P107	100.00±0.00 ^{c}	58.38±0.15 ^{a}	3.84±0.01 ^{b}
	P108	79.98±2.00 ^{bc}	69.17±0.47 ^{a}	2.90±0.02 ^{ab}
	P109	60.95±0.33 ^{ab}	28.20±0.76 ^{a}	1.59±0.04 ^{ab}
	P110	95.86±3.26 ^{bc}	50.41±3.40 ^{a}	2.52±0.17 ^{ab}

TI = CD_{50}/ED_{50}

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

8.2.8 Isolation of P107 fraction from ethanolic extract of *Sphenodesme* sp. by column chromatography

The active P107 fraction isolated from dry column chromatography using celite as stationary phase had the highest antiviral activity against both types of HSV. Thus, this fraction was further fractionated by reverse phase column chromatography using Sephadex™ LH 20 as stationary phase. Then, 0.20 g of P107 fraction was separated and subsequently eluted with gradient solvent systems as followed, 95% ethanol, 75% ethanol: 25% H_2O, 50% ethanol:50% H_2O, 25% ethanol:75% H_2O, 25% acetone:75% H_2O, 50% acetone: 50% H_2O, 75% acetone: 25% H_2O and acetone, consecutively. Each sub fraction was collected based on color of band appearance and the solvent was removed by evaporator to obtain fraction powder.

The result showed that after P107 fraction was separated by gradient solvent system, six fractions; P107-01, P107-22, P107-41, P107-57, P107-71 and P107-97 were obtained. Each fraction was collected based on band appearance with showed different color such as yellow and red. P107-41 and P107-71 fractions revealed the highest percentage yield recovered by 32.90% followed by P107-22, P107-01 and P107-97, which showed the yields of 22.20, 14.15 and 2.70%, respectively. However, small amount of fraction P107-57 was obtained after separation, so percentage yield could not calculated (Table 36).

Fraction isolated from P107; P107-01, P107-41 and P107-71 were selected to further evaluate for anti-HSV activity based on their information of color appearance. P107-22 showed no-color with was not interesting to study while P107-57 and P107-97 fractions showed small amount, which was not enough to further

study. P107-01, P107-41 and P107-71 fractions were selected to study and renamed to P107-01, P107-02 and P107-03, respectively.

Table 36 Fractions and percentage yield of P107 recovered after separation by wet column chromatography

Fraction name	Sub-faction	Color appearance	Yield (w/w, %)
P107-01	1-21	yellow	14.15
P107-22	22-40	colorless	22.20
P107-41	41-58	red	32.90
P107-57	59-70	colorless	< 1
P107-71	71-96	red	32.90
P107-97	97-α	colorless	2.70

8.2.9 Cytotoxicity of *Sphenodesme* sp. fractions isolated from column chromatography on Vero cell

Cytotoxicity on Vero cell of three fractions, P107-01, P107-02 and P107-03 were demonstrated by cell viability assay. Cytotoxicity doses, 50% (CD_{50}) and the highest non-toxic concentration that used in this experiment were exhibited in Table 37. The result showed that P107-01 had the lowest toxicity with CD_{50} value of 1,262.00±0.00 µg/ml followed by P107-03 and P107-02 with CD_{50} values of 316.25±0.00 and 50.35±0.00 µg/ml. Moreover, the maximum concentrations of P107-01, P107-02 and P107-03 fractions used on further study were 900, 50 and 250 µg/ml, respectively (Table 37).

Table 37 The cytotoxicity dose, 50% (CD_{50}) and the highest of non-toxic concentrations of each fraction isolated from P107 fraction

Fractions	CD_{50} (µg/ml)*	The highest of non-toxic concentrations (µg/ml)
P107-01	1,262.00±0.00	900
P107-02	50.35±0.00	50
P107-03	316.25±0.00	250

* The data in table are presented as mean ±standard deviation (SD) of duplicate experiments.

8.2.10 Antiviral activity by fractions isolated from P107 by column chromatography

Three major fractions; P107-01, P107-02 and P107-03 that obtained from isolation of P107 fraction by celite column chromatography was investigated for the antiviral efficacy on HSV infection by plaque reduction assays.

P107-03, P107-01 and P107-02 fractions had antiviral activity against HSV-1F with ED_{50} values of 29.58±1.63, 404.97±20.00 and 36.41±0.06 μg/ml, respectively and TI values of 10.71±0.59, 3.12±0.15 and 1.38±0.02, respectively. Moreover, HSV-2G infection was inhibited by P107-03 with ED_{50} and TI values of 28.40±0.12 μg/ml and 11.14±0.05, respectively following by P107-01 and P107-02 fractions with showed ED_{50} values of 416.90±12.23 and 28.56±0.42 μg/ml and TI values of 3.03±0.09 and 1.76±0.03, respectively (Table 38).

Thus, after fractionation, P107-03 fraction displayed a potent active fraction that completely inhibited HSV-1F and HSV-2G infection by 100%. Although, P107-03 had potent anti-HSV activity than other fraction but it was not further separated because the small amount of fraction. However, P107-03 was further evaluated for phytochemical constituent.

Table 38 Anti-HSV activity of fractions isolated from *Sphenodesme* sp. extract by column chromatography

Viruses	Fractions	% inhibition ± SD*	ED±SD (µg/ml)*	TI±SD*
HSV-1F	P107-01	86.37±3.99 [ab]	404.97±20.00 [b]	3.12±0.15 [b]
	P107-02	62.61±0.91 [a]	36.41±0.06 [a]	1.38±0.02 [a]
	P107-03	100±0.00 [c]	29.58±1.63 [a]	10.71±0.59 [a]
HSV-2G	P107-01	86.50±2.58 [ab]	416.90±12.23 [b]	3.03±0.09 [b]
	P107-02	73.34±3.15 [a]	28.56±0.42 [a]	1.76±0.03 [a]
	P107-03	100±0.00 [c]	28.40±0.12 [a]	11.14±0.05 [a]

$TI = CD_{50}/ED_{50}$

*****The data are presented as mean ± standard deviation (SD) of triplicate experiments. The statistical analysis (SPSS statistic 17.0) conducted by comparing between the groups in each column using randomized complete blocks (RCB) and Post hoc Tukey's b test as well as different alphabets in each group within each column showed significantly different value ($P<0.05$).

9. Preliminary determination of phytochemical groups

Crude ethanolic extract of *E. prosstrata* and *Sphenodesme* sp. were selected to further elucidate the major phytochemical constituents profile by phytochemical analysis. Fractions, K101 isolated from *E. prostrata* and fraction P107-03 isolated from *Sphenodesme* sp. were also evaluated their phytochemical constituent. Phytochemical analysis was performed by standard methods for identification of biological activity; alkaloids, antraquinone glycoside, cardiac glycosides, cumarin, flavonoid, phenolic compounds, saponins and tannin.

Phytochemicals of crude ethanolic extract and bioactive K101 fraction of *E. prostata* showed many groups of chemical constituents, which composed of tannin, flavonoid and phenolic compound (Table 39).

Major constituent compound in *E. prostrata* was also investigated by other studies. Khanna and Kannabiran (2007), and Pandey *et al*. (2012) demonstrated that tannin was found in *E. prostrata* extract. Moreover, *E. prostrata* was also composed of flavonoids (Dhandapani, 2007; Khanna and Kannabiran, 2007; Kodithala *et al.*, 2012; Lee *et al*., 2008). Phenolic compound was also found as a major phytochemical constituent of *E. prostrata* (Borkataky *et al*., 2013; Chitravadivu *et al*., 2009; Malla *et al*., 2013). Furthermore, other compounds such as coumastanes, polypepetides, flavonoids, steroids, triterpenes, polyacetylenes, alkaloids and saponins were found in *E. alba* (Kumari *et al*., 2006; Zhi-Hua *et al*., 2010).

Phytochemical screening of crude ethanolic extract of *Sphenodesme* sp. showed the presence of tannin, cardiac glycoside and phenolic compound. These results were in accordance with P107-03 fraction separated by column

chromatography, which showed the presence of tannin and phenolic compound (Table 39).

The efficacy against HSV-1F and HSV-2G infection by P107-03 fraction isolated from *Sphenodesme* sp. extracts and K101 fraction isolated from *E. prostrata* were less than crude extract that observed by TI value. This result suggested that action of these medicinal plants on antiviral activity may result from many groups of phytochemical compounds in plant extract that might act together and affected different mode of antiviral action by additive or synergistic action of many compounds (Yucharoen *et al*., 2012). In addition, this result indicated that plant had several secondary products that were present in combination. Therefore, actions of each plant were unique to a particular plant species or group (Briskin, 2000; Joshi *et al*., 2011). On the other hand, the solvent that used to extract the phytochemical compound should be suitable for dissolving the phytochemical compounds and should not be easy or difficult to volatile (Mohamed *et al*., 2010). Furthermore, active constituents that were present in medicinal plant depended on geographical distribution, season of collection and also ecological condition at collection site (Rajbhandari *et al*., 2001).

However, phytochemicals in plant extract have different mechanism of action on microorganism. Tannin was demonstrated antiviral mechanism, which affected viral adsorption, attachment and prevent HSV entry into host cell. Moreover, tannin has been found inhibition cell protein synthesis and also could inhibit penetration process (Gescher *et al*., 2011). Flavonoids constituents in plant conferred antibacterial and antifungal properties. Furthermore, flavonoids also acted in inhibition during viral adsorption, viral binding and blocking viral entry process into

the host cell (Chattopadhyay and Naik, 2007). In addition, flavonoid affected viral enzyme that necessary for viral replication and it could form complex with extracellular protein (Kitamura *et al*., 1998). Amentoflavone isolated from *Rhus succedanea* and catechin from Orange and Grape could inhibit HSV. Alkaloids are used as analgesics, stimulants, anaesthetic, hallucinogens and antibacterial agents. Alkaloid compound extracted from *Stephania cephanantha* demonstrated potent on HSV-1 infection (Nawawi *et al*., 1999).

Furthermore, phenolics isolated from *Centella asiatica, Plantago major, Aloe brabadensis* and *Mangifera indica* showed potent on HSV infection. Saponin isolated from *Maesa lanceolata, Bupleurum nigidum* as well as tannin isolated from *Terminalia arjuna, Geum japonicum* and *Limonium sinens* also showed inhibitory effect on HSV infection. Terpenes or sterols isolated from *Ocimum basillicum, Ocimum basilicum, Syzygium claviflorum, Cassia javanica, Euphorbia segetalis* and *Rhus javanica* demonstrated anti-HSV activities (Chattopadhyay and Naik, 2007).

Table 39 Phytochemical groups of crude ethanolic extract and fraction of medicinal plant extracts

Phytochemical groups	Test	Extracts			
		E. prostrata	**K101**	***Sphenodesme* sp.**	**P107**
1. Alkaloid	Dragendroff's reagent	-	-	-	-
	Wagner's reagent	-	-	-	-
	Hager's reagent	-	-	-	-
	Mayer's reagent	-	-	-	-
2. Antroquinone glycoside	Borntrager's test	-	-	-	-
3. Cardiac glycoside	Lieberman – Burchard test	-	-	-	-
	Keller – kiliani test	-	-	-	-
4. Cumarin	NaOH – paper	-	-	-	-

(-) = negative reaction

(+) = positive reaction

Table 39 (continued)

Phytochemical groups	Test	Extracts			
		E. prostrata	**K101**	***Sphenodesme* sp.**	**P107**
5. Flavonoid	Shinoda's test	+	+	-	-
6. Phenolic	Iron (III) chloride	+	+	+	+
7. Saponin	Froth test	-	-	-	-
8. Tannin	Gelatin solution, 1%	+	+	+	+
	$FeCl_3$, 1%	+	+	+	+
	Formaldehyde - HCl	+	+	+	+
	Vanillin – HCl	+	+	+	+
	Lime water	+	+	+	+
	Lead acetate solution	+	+	+	+

(-) = negative reaction

(+) = positive reaction

9.1 Chromatographic fingerprint of ethanolic extracts of *Sphenodesme* sp. and P107-03

From the experiment, ethanolic extract of *Sphenodesme* sp. and P107-03 fraction had the highest anti-HSV activity. These extracts were determined their chromatographic fingerprints by GC-MS and HPLC.

9.1.1 Chromatographic fingerprint by gas chromatography/Mass spectrometry assay (GC-MS)

The ethanolic extract of *Sphenodesme* sp. was subjected to Gas chromatography/Mass spectrometry (GC/MS) for determination of their aromatic compounds. The samples were sent to Central Laboratory (Thailand) Co., Ltd.; Thailand. All their results were compared to Wiley version 7.0 library databases, which chromatographed by capillary GC and filtrate with MS using selectivity for bases (SB) column (150 x 4.6 mm), formate buffer 10mM, pH 4 : acetonitrile (60:40).

It was found that, the major constituents in ethanolic extract of *Sphenodesme* sp. composed of 1,2-benzenediol, methoxy phenol, isoeugenol, phenyl propanol, propanamide, lidocaine and ester of palmitic acid (Table 40; Figure 65).

Table 40 Major constituents of crude ethanolic extract of *Sphenodesme* sp. determined by GC-MS scan

Extract	Retention time (min)	Compounds	Peak area / g
Ethanolic	15.23	1,2-Benzenediol	1.54×10^{11}
	17.45	methoxy phenol	6.51×10^{10}
	17.54	isoeugenol	3.99×10^{10}
	17.68	phenyl propanol	1.48×10^{11}
	19.69	propanamide	6.21×10^{10}
	23.92	lidocaine	5.77×10^{10}
	24.68	ester of palmitic acid	5.44×10^{10}

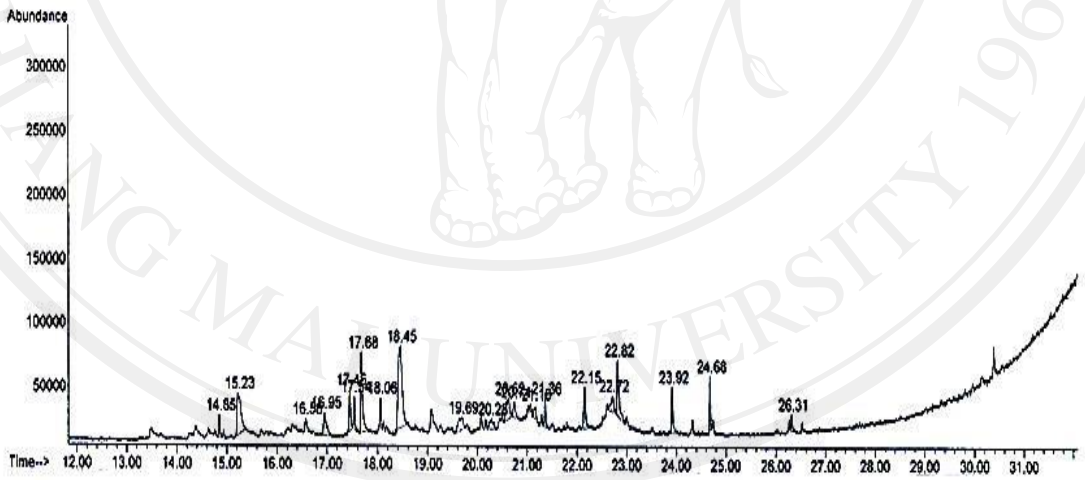

Figure 65 Chromatogram of ethanolic extract of *Sphenodesme* sp. using GC-MS scan

9.1.2 Chromatographic fingerprint of plant extracts by High performance liquid chromatography (HPLC)

HPLC analysis had 2 data images, which showed the chemical profile or chromatogram and spectrum (UV spectrum) of compounds in the extract. Furthermore, P107-03 isolated from ethanolic extract of *Sphenodesme* sp. by column chromatography was observed their constituent using HPLC analysis after detection by UV absorbance at 254, 263 and 366 nm, respectively.

It was found that phytochemical profiles of P107-03 fraction showed 23 dominant peaks (Retention time, Rt: 2.480-136.305 mins) when detected by UV spectrum at 254 nm and showed HPLC chromatogram as demonstrated in Figure 66. After detection by UV spectrum at 263 nm, the fraction showed 2 dominant peaks (Rt: 43.377-46.611 mins) (Figure 67). In addition, this fraction was showed 15 dominant peaks (Rt: 3.024-119.256 mins) when detected by UV spectrum at 366 nm and showed HPLC chromatogram as demonstrated in Figure 68.

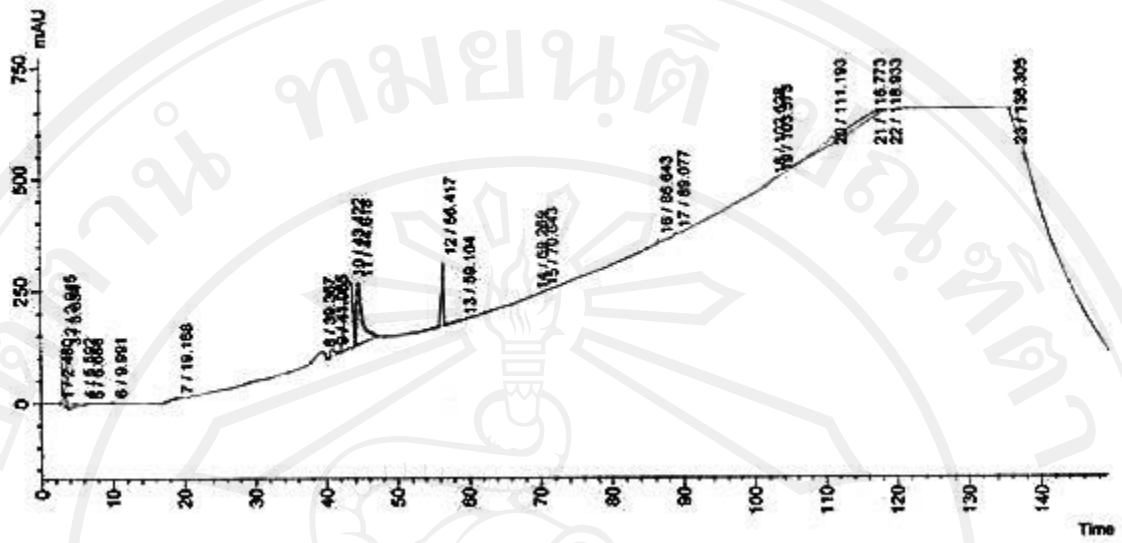

Figure 66 HPLC-chromatograms of P107-03 fraction (254 nm)

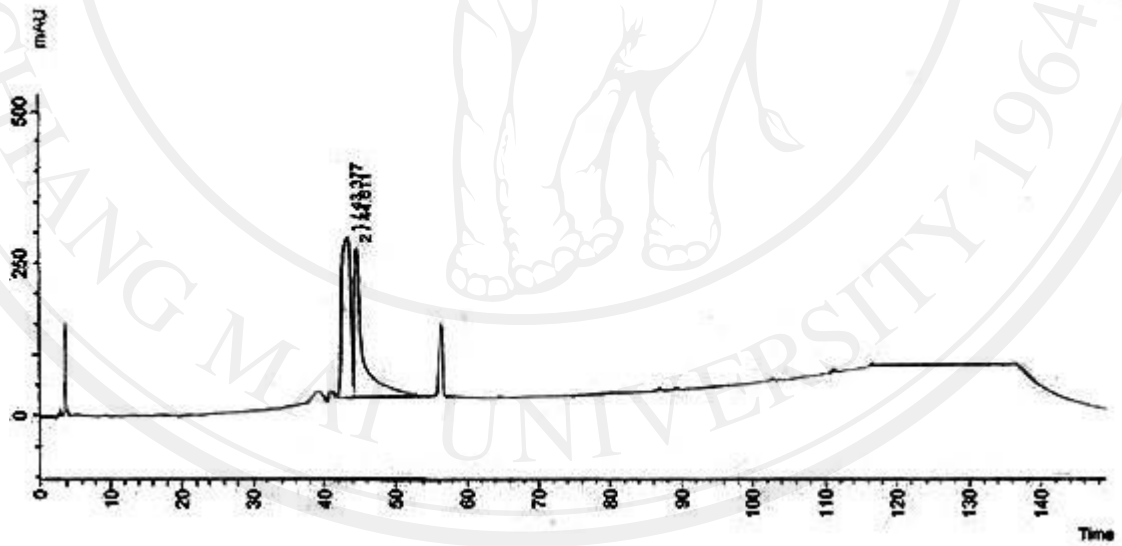

Figure 67 HPLC-chromatograms of P107-03 fraction (263 nm)

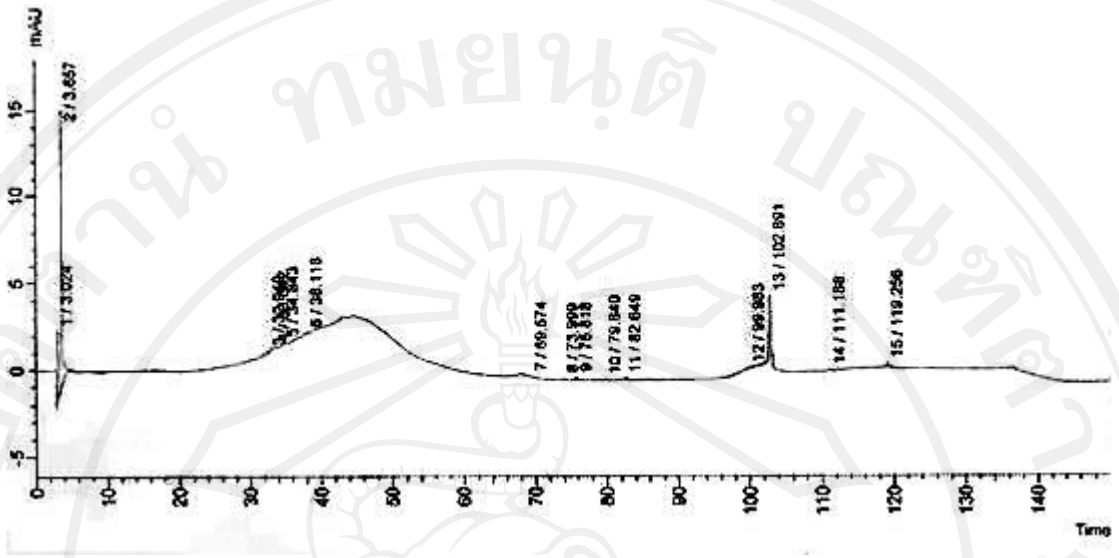

Figure 68 HPLC-chromatograms of P107-03 fraction (366 nm)

10. Development of gel product for treatment of HSV infection

The previous results showed that ethanolic extract of *Sphenodesme* sp. revealed the highest antiviral efficacy against HSV infection. Thus, ethanolic extract from *Sphenodesme* sp. was selected to develop antiviral gel product to treat HSV infection.

The gel product was evaluated for their efficacy against HSV infection by plaque reducing assay. Additionally, the herbal gel was also studied their stability and physical properties including pH, viscosity, color and smell. Moreover, the efficacy of this herbal gel product was also evaluated after heating-cooling and storage at different temperature for 7 months. Skin irritation of herbal gel product was also tested on skin area of volunteers.

The purpose of stability test was to ensure that the herbal gel product could maintain its physical properties and antiviral activities when storage at appropriate conditions throughout their shelf life.

10.1 Herbal gel product preparation

Recently, the natural products have become a major demand for use in day life. Gel product was widely used in pharmacological application as carrier for vehicles contained the efficacy various agents or benefit medicament in order to delivery active substances due to its easily spreadable and penetration through mucosal surfaces before absorption to percutaneous (Das *et al*., 2010a; Fathy *et al*., 2010; Shalaby and Shukr, 2011; Shivhare *et al.*, 2009). Additionally, our result found that ethanolic extract of *Sphenodesme* sp. showed the most effective anti-HSV activity

on various stages of HSV multiplication cycle. Hence, herbal gel product containing ethanolic extract of *Sphenodesme* sp. was produced.

The herbal gel product was produced by mixed the gel components that composed of carbopol 940, methyl paraben, propylene glycol, triethanolamine, tween 80 and crude ethanolic of *Sphenodesme* sp. at concentration of 4000 µg/ml. The herbal gel showed red mixed with brown color and translucent with pH value of 7.0 (modified from Chaliewchalad, 2008; Sangtongdee *et al*., 2006). This herbal gel was recondition of the quantity of each component to give the suitable physical characteristic for the herbal gel product contained ethanolic extract of *Sphenodesme* sp. In addition, the gel base did not show anti-HSV activity (Chaliewchalad, 2008).

Carbopol is a gel-forming, which could deform the gel structure, and has bioadhesive properties. Furthermore, this compound also used as thickening agent and also used in pharmaceutical products for drug delivery system (Aminabhavi *et al*., 2004; Asasutjarit *et al.,* 2005; Bonacucina *et al.*, 2004; Shalaby and Shukr, 2011). Additionally, methyl parabens are non-toxic substance that used as oral and parenteral compound and completely absorbed through the skin. It was used as preservatives, antiseptics and antimicrobial agents against fungi and bacteria (Boukarim *et al.,* 2009; Soni *et al.,* 2002). Triethanolamine was used as buffer agent, emulsifier and surfactant. Moreover, tween 80 was surfactants with low toxicity and acts as stabilizer or emulsifier, and also enhances drug absorption (Simões *et al*., 2005; Zhang *et al.*, 2003). In addition, propylene glycol had low toxicity with non-irritation to the skin.

10.2 Cytotoxicity of herbal gel product

Cytotoxicity of herbal gel product containing ethanolic extract of *Sphenodesme* sp. was determined on Vero cell by cell viability assay and CD_{50} values was calculated according to modified protocol of Reed and Muench (1938).

The result on cytotoxic analysis showed that CD_{50} value of the gel product was 5,035.35 µg/ml while ethanolic extract of *Sphenodesme* sp. showed CD_{50} value of 4,019.00 µg/ml. This result could suggest that combination between plant extract and gel base might reduce the toxicity of extract.

10.3 Stability test of herbal gel product

Herbal gel product containing ethanolic extract of *Sphenodesme* sp. at concentration of 4,000 µg/ml was evaluated for their stability. Stability study is carried out to determine the quality of active substance under environmental stress. Moreover, stability test was demonstrated to ensure the quality and safety of herbal gel product throughout the storage conditions (Bajai *et al*., 2012; Daberte *et al.*, 2011; Vipul and Devesh, 2012).

Stability study was observed after the *Sphenodesme* sp. herbal gel was storage at different condition for short-term and long-term period. Stability test of herbal gel was investigated on short-term storage by heating-cooling cycle while long-term condition was observed by storage the herbal gel at 4, 25 and 45ºC for 7 months. The gel was protected from the light during the storage time.

10.3.1 Short-term determination of herbal gel stability after heating-cooling cycle

Stability of chemical and phytochemical constituents of pharmaceutical products was important (Daberte *et al*., 2011). Stability of herbal gel was analyses by heating and cooling. Herbal gel product was placed at 4ºC for 48 hours then the product was placed at 45ºC for 48 hours for 1 cycle. The cycle was repeated for 6 times, which took time for 24 days. After that, physical properties including pH, viscosity, forming of emulsion, color and smell were observed compared to control of the gel product. In addition, the herbal gel was further investigated for antiviral activity against HSV infection by plaque reduction assay.

The result showed that after heating-cooling cycle, the layer of gel was not separated and retained color of red-brown. Furthermore, this herbal gel also retained smell while the viscosity was a little decreased after heating-cooling for 5 cycles. Moreover, pH was increased from 7 to 8 when heating-cooling for 4 cycles (Table 41, Figure 69).

The herbal gel containing ethanolic extract of *Sphenodesme* sp. was further tested for anti-HSV activity by plaque reduction assay. The result showed that after heating-cooling cycle, the herbal gel also retained the antiviral efficacy against HSV infection by 100% (Figure 70 and 71).

From our result could suggest that the herbal gel changed their physical properties after storage at 4ºC for 48 hours and 45ºC for 48 hours for 6 cycles. In addition, the herbal gel also maintained its stability on antiviral activity against HSV infection.

(a) (b)

Figure 69 Herbal gel product (a) and herbal gel after 6 cycles of heating-cooling cycle (b)

Table 41 Characteristics of herbal gel containing ethanolic extract of *Sphenodesme* sp. at concentration of 4,000 µg/ml

Gel	No. of cycles*	Separation	Color	Smell	Viscosity	pH
Primary gel	0	-	red-brown	+++	+++	7.0
Heating-	1	-	red-brown	+++	+++	7.0
cooling	2	-	red-brown	+++	+++	7.0
	3	-	red-brown	+++	+++	7.0
	4	-	red-brown	+++	+++	8.0
	5	-	red-brown	+++	++	8.0
	6	-	red-brown	+++	++	8.0

* One cycle is the gel containing ethanolic extract of *Sphenodesme* sp.was incubated at 25°C for 48 hours and incubated at 45°C for 48 hours

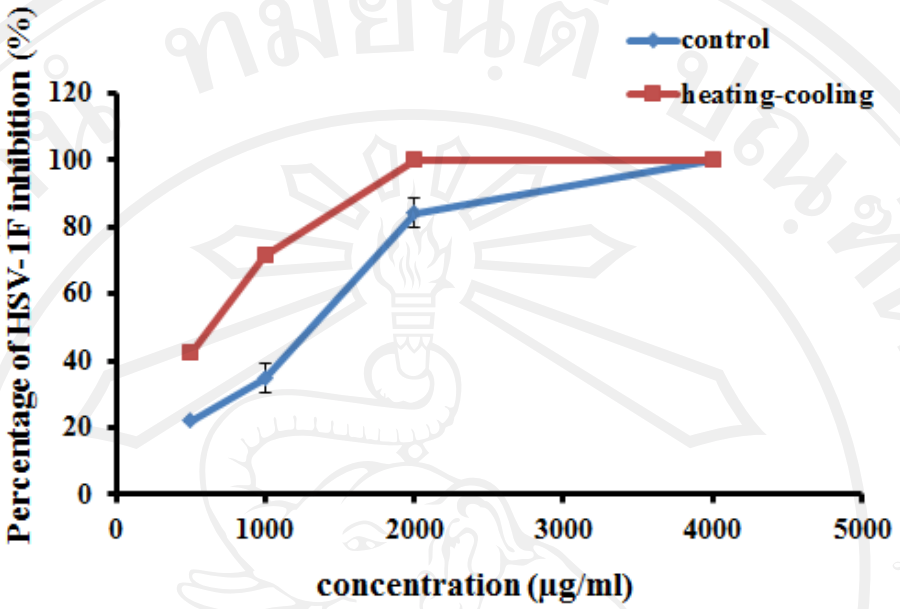

Figure 70 Inhibitory effect of herbal gel containing ethanolic extract of *Sphenodesme* sp. against HSV-1F after heating-cooling

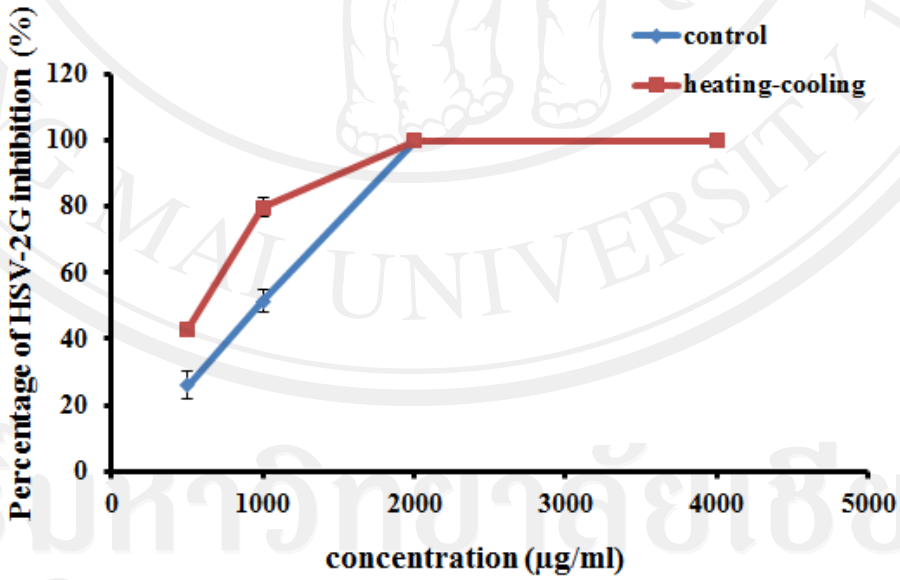

Figure 71 Inhibitory effect of herbal gel containing ethanolic extract of *Sphenodesme* sp. against HSV-2G after heating-cooling

10.3.2 Long-term determination of herbal gel stability

Stability study of herbal gel product was performed in order to determine effect of storage condition on antiviral efficacy against HSV. After gel product was produced, long-term stability study was determined effect of temperature and incubation period on physical properties of the herbal gel product such as pH, viscosity, smell, color and emulsion forming compared to herbal gel control. Furthermore, the herbal gel also investigated for the efficacy against HSV infection by plaque reduction assay.

Stability of herbal gel product when kept at different temperature was observed. Physical properties of herbal gel product after storage for 7 months revealed that gel layer was not separated when storage at 4ºC. Moreover, color of herbal gel was changed from red-brown into yellow-brown (Figure 74). Smell and viscosity of the herbal gel product were decreased upon the longer time of storage. Furthermore, the pH was changed from 7 to 8 in 3 months (Table 42).

Inhibition effect of herbal gel product against HSV infection by plaque reduction assay was observed. The result showed that, the gel product after storage at 4ºC retained their efficacy to inhibit HSV-1F and HSV-2G infection by 100% when testing every month (Figure 72 and 73).

Table 42 Characteristics of gel containing ethanolic extract of *Sphenodesme* sp. at concentration of 4,000 µg/ml after storage at 4°C for 7 months

Test	Month	Separation	Color	Smell	Viscosity	pH
Primary gel	0	-	red-brown	+++	+++	7
4°C	1	-	red-brown	+++	+++	7
	2	-	yellow-brown	+++	+++	7
	3	-	yellow-brown	+++	++	8
	4	-	yellow-brown	++	++	8
	5	-	yellow-brown	++	++	8
	6	-	yellow-brown	++	++	8
	7	-	yellow-brown	+	+	8

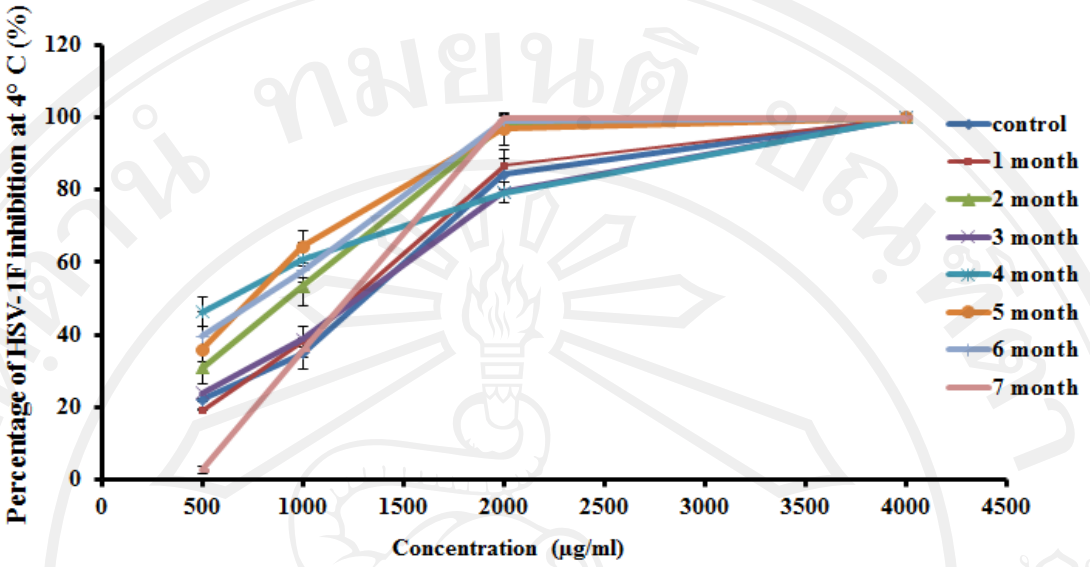

Figure 72 Stability test of gel containing ethanol extract of *Sphenodesme* sp. at various concentrations (500-4,000 µg/ml) against HSV-1F after storage at 4ºC for 7 months

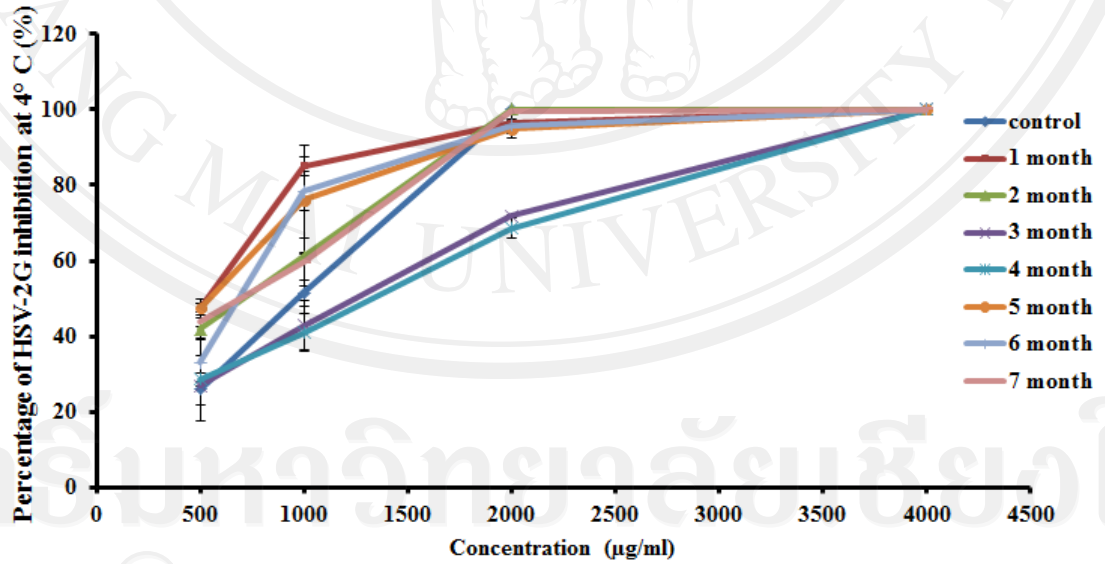

Figure 73 Stability test of gel containing ethanol extract of *Sphenodesme* sp. at various concentrations (500-4,000 µg/ml) against HSV-2G after storage at 4ºC for 7 months

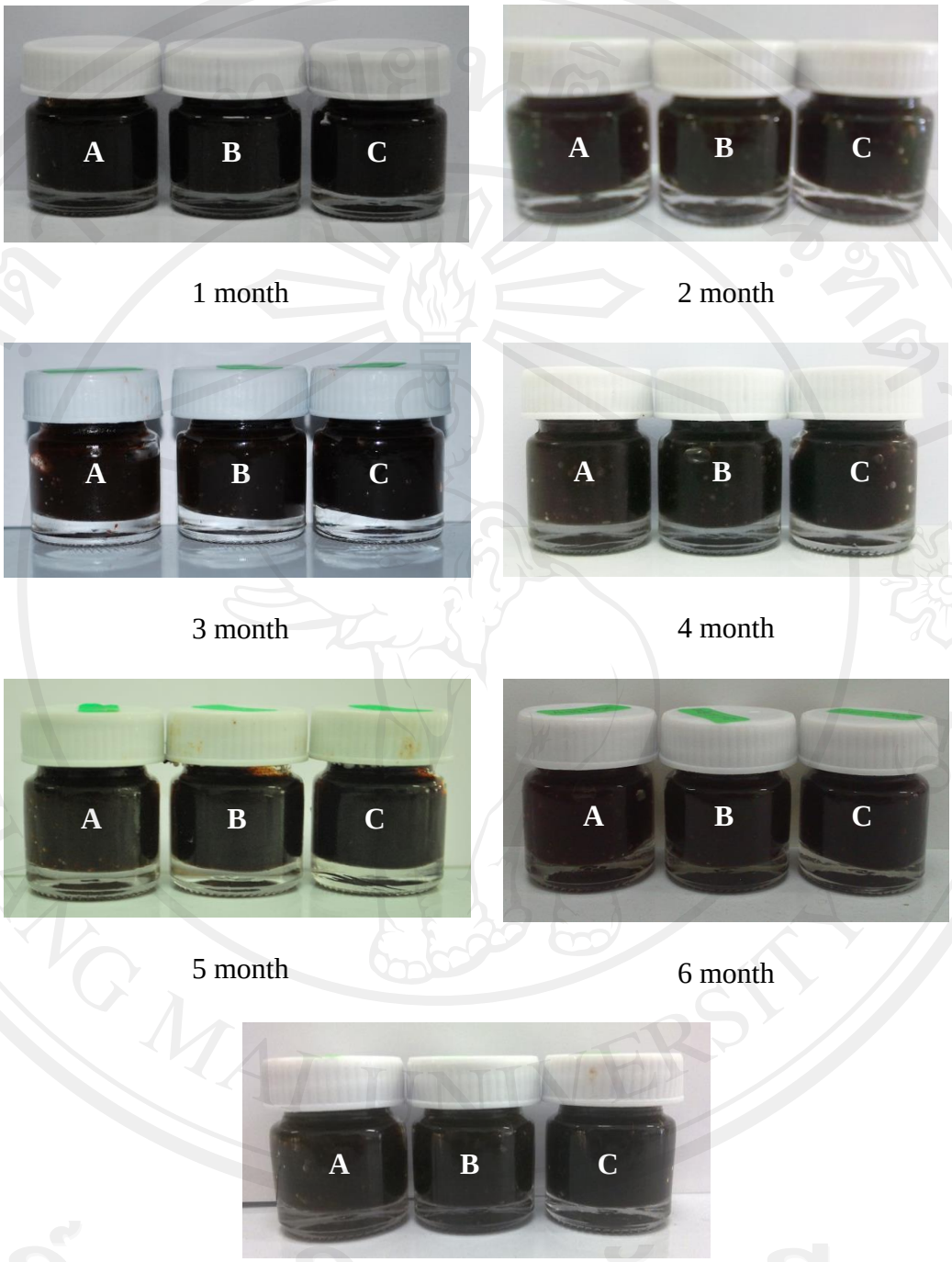

Figure 74 Herbal gel contained ethanolic extract of *Sphenodesme* sp. after storage for 7 months (A= 4°C, B=25°C and C=45°C)

Stability of herbal gel when storage at 25ºC was investigated in this study. The result showed that the layer of herbal gel was not separated. However, color of herbal gel was changed from red-brown into yellow-brown after storage for 2 months (Figure 74). The smell of herbal gel from constituents in the gel was decreased after 5 months of storage. Viscosity was also decreased after 3 months of storage. In addition, pH was increased from 7 to 8 within 3 months. This same result was obtained when the gel was storage at 4ºC (Table 43).

Antiviral activity against HSV infection by herbal gel containing ethanolic extract of *Sphenodesme* sp. at concentration of 4,000 µg/ml when storage at 25ºC was shown. The herbal gel retained potent inhibitory effect on both types of HSV infection by 100% when determined every month by plaque reduction assay (Figure 75 and 76).

Table 43 Characteristics of gel containing ethanolic extract of *Sphenodesme* sp. at concentration of 4,000 µg/ml after storage at 25°C for 7 months

Test	Month	Separation	Color	Smell	Viscosity	pH
Primary gel	0	-	red-brown	+++	+++	7
25°C	1	-	red-brown	+++	+++	7
	2	-	yellow-brown	+++	+++	7
	3	-	yellow-brown	+++	++	8
	4	-	yellow-brown	+++	++	8
	5	-	yellow-brown	++	++	8
	6	-	yellow-brown	++	++	8
	7	-	yellow-brown	+	+	8

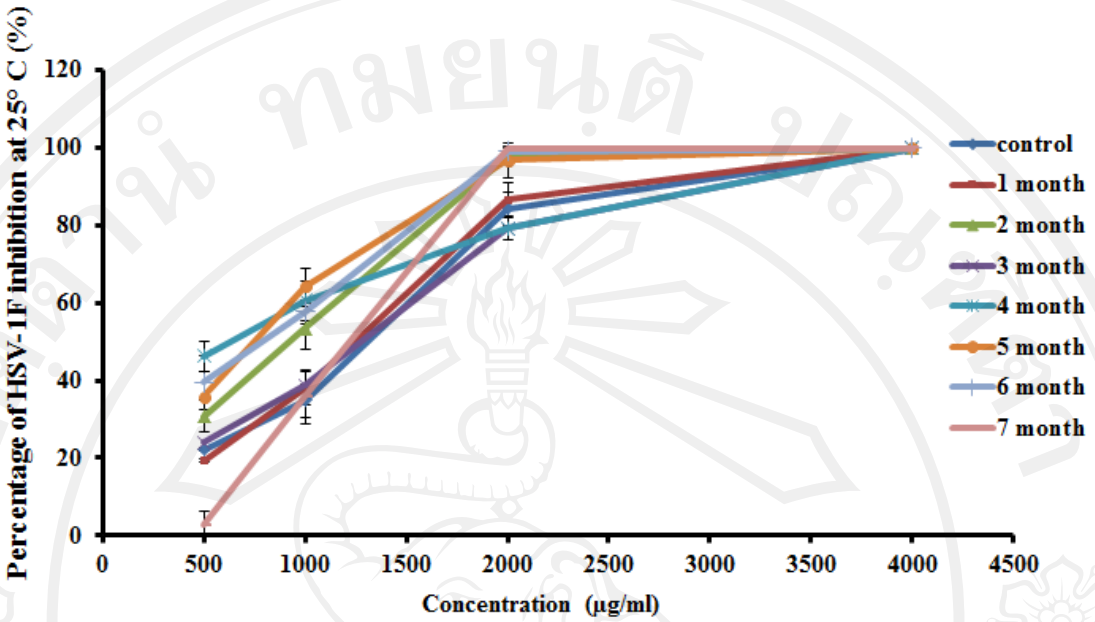

Figure 75 Stability test of gel containing ethanolic extract of *Sphenodesme* sp. at various concentrations (500-4,000 µg/ml) against HSV-1F after storage at 25ºC for 7 months

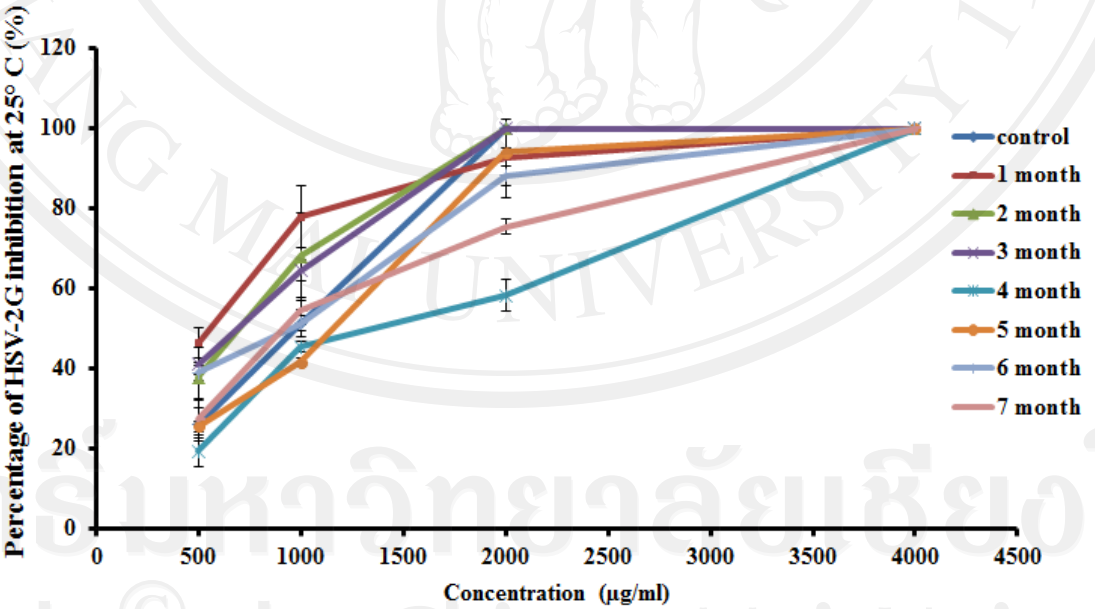

Figure 76 Stability test of gel containing ethanolic extract of *Sphenodesme* sp. at various concentrations (500-4,000 µg/ml) against HSV-2G after storage at 25ºC for 7 months

Stability test of herbal gel product when storage at 45ºC for 7 months was observed. The physical properties of the herbal gel revealed that the gel layer was not separated. Besides, the color of herbal gel was changed from red-brown after storage for 1 month and became yellow-brown within 4 months (Figure 74). Moreover, the smell of constituents in gel was decreased in 4 months while the viscosity was little decreased in 3 months and viscosity was lost in 6 months. Variations of pH was observed when storage at 45ºC since pH was increased to 8 in 3 months, which similar to the pH of herbal gel when storage at 4 and 25ºC (Table 44).

Plaque reduction assay was used to confirm the efficacy of herbal gel against HSV infection. After the herbal gel was kept at 45ºC for 7 months, it was found that the herbal gel also retained their inhibitory efficacy on both types of HSV by 100%, which was similar to the antiviral activity when storage at 4 and 25ºC (Figure 77 and 78).

Stability of herbal gel product was changed by time under the influence of factors such as temperature that affected pH, viscosity and color of the herbal gel. From the results, it was found that the herbal gel changed their physical properties when storage at different condition at least 7 months. In addition, herbal gel was also retained the potent inhibitory effect on HSV infection during 7 months of storage by 100%. Thus, the development of this herbal gel should be performed as an anti-HSV agent for treatment of HSV infection disease. Although, the physical properties of herbal gel product was changed when kept at various condition. However, it still retained their antiviral effect against both typea of HSV infection. Hence, this preliminary study on herbal gel product contained ethanolic extract of *Sphenodesme* sp. should be further developing the stability of this herbal gel.

Table 44 Characteristics of gel containing ethanol extract of *Sphenodesme* sp. at concentration of 4,000 µg/ml after storage at 45°C for 7 months

Test	Month	Separation	Color	Smell	Viscosity	pH
Primary gel	0	-	red-brown	+++	+++	7
45°C	1	-	red-brown	+++	+++	7
	2	-	red-brown	+++	+++	7
	3	-	red-brown	+++	++	8
	4	-	yellow-brown	++	+	8
	5	-	yellow-brown	++	+	8
	6	-	red-brown	+	-	8
	7	-	yellow-brown	+	-	8

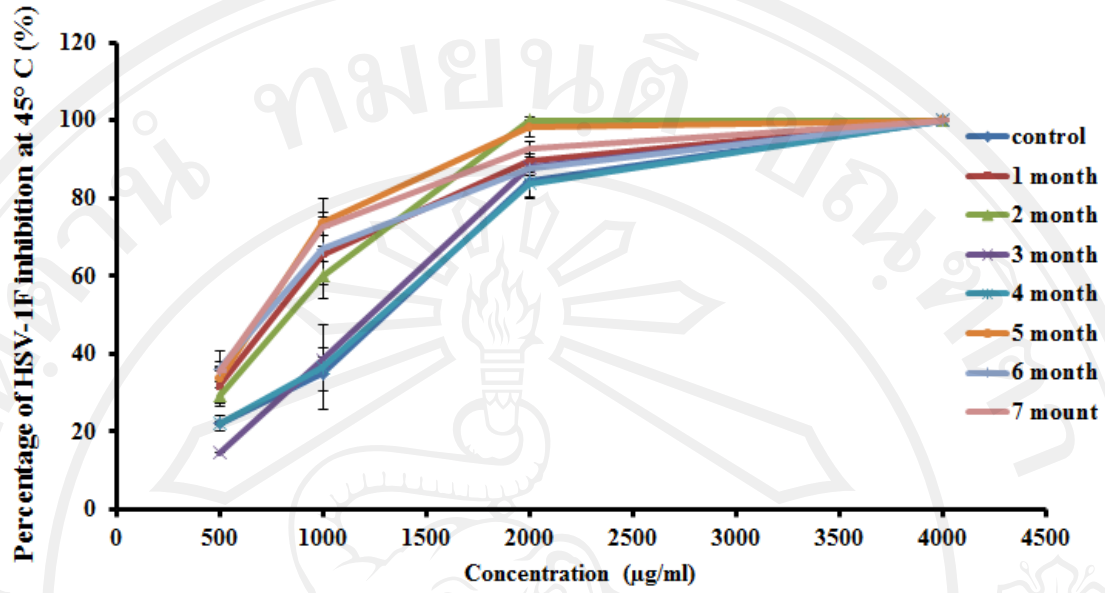

Figure 77 Stability test of gel containing ethanolic extract of *Sphenodesme* sp. at various concentrations (500-4,000 µg/ml) against HSV-1F after storage at 45ºC for 7 months

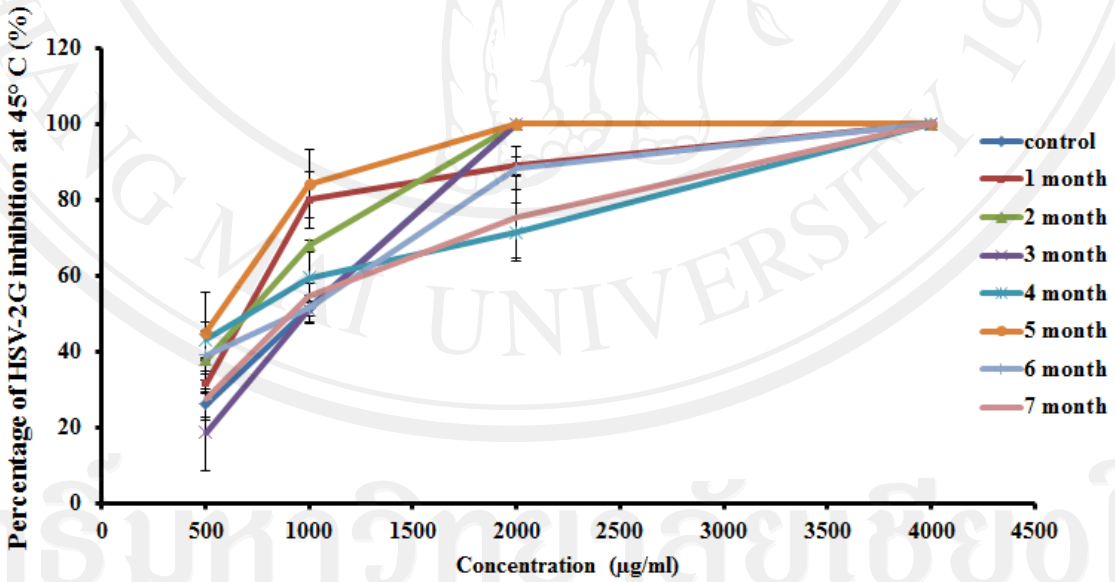

Figure 78 Stability test of gel containing ethanolic extract of *Sphenodesme* sp. at various concentrations (500-4,000 µg/ml) against HSV-2G after storage at 45ºC for 7 months

10.3.2 Study on irritation of herbal gel product (modified from Sarnjai, 2010)

Herbal gel product containing ethanolic extract of *Sphenodesme* sp. at concentration of 4,000 μg/ml was also tested for their irritation effect on volunteer's skin. Skin irritation is common adverse effects in humans depended on many factors such as concentration of plant extracts, duration, the frequency of exposure, the site of exposed skin, rate of penetration and intrinsic toxic potential of the substrate (More *et al*., 2013). The irritation effect of herbal gel on body organs of volunteers at the site of contact was divided into four categories; acute (24 hours), subacute (14-28 days) subchronic (90 days), and chronic toxicity (Shi *et al*., 2013). Acute irritation within 24 hours was selected to evaluate in this present study. Thus, herbal gel product was applied on skin of human volunteers to test irritation on the applied surface.

Ten healthy volunteers were 5 men and 5 women with age ranged from 20-28 years were tested for skin irritation. The herbal gel was applied once on a surface area of 1 x 1 cm^2 on lateral arms of volunteers. The tested skin was thereafter washed off by tab water after 24 hours. Then, skin irritation was observed at the tested sites for erythema and edema at 24 hours after sample application. After herbal gel product was applied on skin area for 24 hours, skin irritation test was calculated as irritation index of the test skin. Irritation was not observed on the skin of all volunteer, thus irritation index = 0 (Table 45 and Figure 79).

Taken together, this herbal gel product was suitable for use as anti-HSV agent (Figure 80). However, the efficacy of the herbal gel to treat HSV infection should be further studied in laboratory animal. The use of this herbal gel product will be an alternative choice instead of using imported synthetic drugs. Moreover the cost of drug usage against HSV infection will be reduced.

Table 45 Irritation test of gel containing *Sphenodesme* sp. extracts in volunteers

Volunteers	Ages (years)	Irritation index*
1	23	0
2	23	0
3	25	0
4	24	0
5	24	0
6	27	0
7	26	0
8	28	0
9	26	0
10	20	0

*** Irritation index (Aroonrerk and Kamkaen, 2009)**

Index	Evaluation
0.00	No irritation
0.04-0.99	Irritation barely perceptible
1.00-1.99	Slight irritation
2.00-2.99	Mild irritation
3.00-5.99	Moderate irritation
6.00-8.00	Severe irritation

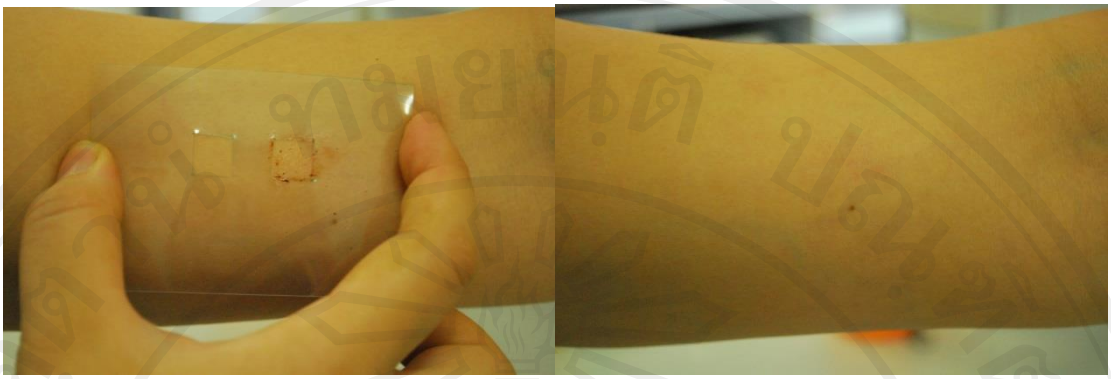

Figure 79 Irritation test of herbal gel product on tested skin of volunteer

Figure 80 Herbal gel product containing ethanolic extract of *Sphenodesme* sp.